Molecular Human Reproduction, Vol.18, No.7 pp. 362–371, 2012
Advanced Access publication on February 20, 2012 doi:10.1093/molehr/gas008

MHR Basic science of reproductive medicine

ORIGINAL RESEARCH

Ovarian reserve status in young women is associated with altered gene expression in membrana granulosa cells

Christine C. Skiadas[1], Shenghua Duan[2], Mick Correll[2], Renee Rubio[2], Nilay Karaca[1], Elizabeth S. Ginsburg[1], John Quackenbush[2,3], and Catherine Racowsky[1,*]

[1]Department of Obstetrics, Gynecology, and Reproductive Biology, Brigham and Women's Hospital and Harvard Medical School, 75 Francis Street, ASB 1+3, Rm 082, Boston, MA 02115, USA [2]Center for Cancer Computational Biology, Department of Biostatistics and Computational Biology, Dana Farber Cancer Center, Boston, MA 02115, USA [3]Department of Biostatistics, Harvard School of Public Health, Boston, MA 02115, USA

*Correspondence address. Tel: +1-617-732-5455; Fax: +1-617-975-0825; E-mail: cracowsky@partners.org

Submitted on June 22, 2010; resubmitted on February 2, 2012; accepted on February 9, 2012

ABSTRACT: Diminished ovarian reserve (DOR) is a challenging diagnosis of infertility, as there are currently no tests to predict who may become affected with this condition, or at what age. We designed the present study to compare the gene expression profile of membrana granulosa cells from young women affected with DOR with those from egg donors of similar age and to determine if distinct genetic patterns could be identified to provide insight into the etiology of DOR. Young women with DOR were identified based on FSH level in conjunction with poor follicular development during an IVF cycle ($n = 13$). Egg donors with normal ovarian reserve (NOR) comprised the control group ($n = 13$). Granulosa cells were collected following retrieval, RNA was extracted and microarray analysis was conducted to evaluate genetic differences between the groups. Confirmatory studies were undertaken with quantitative RT–PCR (qRT–PCR). Multiple significant differences in gene expression were observed between the DOR patients and egg donors. Two genes linked with ovarian function, anti-Mullerian hormone (AMH) and luteinizing hormone receptor (LHCGR), were further analyzed with qRT–PCR in all patients. The average expression of AMH was significantly higher in egg donors (adjusted P-value = 0.01), and the average expression of LHCGR was significantly higher in DOR patients (adjusted P-value = 0.005). Expression levels for four additional genes, progesterone receptor membrane component 2 (PGRMC2), prostaglandin E receptor 3 (subtype EP3) (PTGER3), steroidogenic acute regulatory protein (StAR), and StAR-related lipid transfer domain containing 4 (StarD4), were validated in a group consisting of five NOR and five DOR patients. We conclude that gene expression analysis has substantial potential to determine which young women may be affected with DOR. More importantly, our analysis suggests that DOR patients fall into two distinct subgroups based on gene expression profiles, indicating that different mechanisms may be involved during development of this pathology.

Key words: granulosa cells / oocyte quality / diminished ovarian reserve / IVF / microarray analysis

Introduction

Infertility is a prevalent condition in the USA with more than 2 million affected couples (Chandra *et al.*, 2005). Although infertility treatments have evolved dramatically over the past 30 years, the ability to diagnose the exact causes of infertility for many patients has yet to be identified. One of the least well-characterized etiologies of infertility is that of diminished ovarian reserve (DOR). In 2009 the condition of DOR was the second most common diagnosis among infertility patients undergoing IVF in the USA (15%; SART-CORS, 2009). Although it is expected that ovarian response declines with age, young women can be affected with DOR as well; it is unknown whether this represents an acceleration of physiologic ovarian aging, or a distinct pathology. Aside from standard fertility treatment, there is no targeted therapy for DOR, and until the condition is present, there are no current screening tests to predict who may develop DOR at an early age.

In the mature follicle, the oocyte is surrounded by, and in direct communication with, the cumulus granulosa cells which, in turn, are connected to the membrana granulosa cell layers to comprise

© The Author 2012. Published by Oxford University Press on behalf of the European Society of Human Reproduction and Embryology. All rights reserved.
For Permissions, please email: journals.permissions@oup.com

Downloaded from https://academic.oup.com/molehr/article/18/7/362/1057377 by guest on 20 August 2022

a heterologous gap junction network. Indeed, bidirectional communication between the oocyte and granulosa compartments is an essential feature of normal follicular growth and oocyte development (Gilchrist *et al.*, 2004), as well as the differentiation and pre-ovulatory expansion of the cumulus cells. Due to this unique relationship, investigation of the molecular and cellular physiology of the membrana and cumulus cells may provide great insight into the developmental competence and gene expression of the oocyte.

There is evidence for a link between genetic perturbations and ovarian function in several known conditions, including age of menopause (de Bruin *et al.*, 2001), premature ovarian failure (Fassnacht *et al.*, 2006) and waiting time to pregnancy (Christensen *et al.*, 2003). In addition, women with DOR demonstrate clear differences with respect to hormonal levels throughout the cycle compared with age-matched controls with normal ovarian reserve (NOR) suggesting that granulosa cells are a potential target that can be used to identify patients with DOR. Moreover, studies in mice (Yan *et al.*, 2001) and sheep (Hanrahan *et al.*, 2004) have shown changes in ovulation and fertilization rates, as a result of differences in gene expression. These collective observations suggest impaired granulosa cell function in women with DOR (Pal *et al.*, 2010).

Previous studies investigating granulosa cell gene expression as a function of ovarian reserve have been limited due to the small numbers of patients assessed. The largest such study included nine women with NOR and nine women with DOR (Chin *et al.*, 2002). However, this analysis included women of all age groups, thereby introducing age as a potential confounder in the analyses; previous work has shown that age affects gene expression in oocytes (Steuerwald *et al.*, 2007). Regardless, this was the first report of microarray analysis revealing differential gene expression in granulosa cells with respect to ovarian reserve status, warranting additional studies with increased sample size and restricting the age of the comparison groups. Oocyte quality is expected to decline with age; therefore conducting an analysis of young women would elucidate factors that are related exclusively to the pathologic condition of DOR, as opposed to genes that could be involved in normal aging.

Our objective for the present study was to evaluate differences in gene expression of membrana granulosa cells in young women (≤35 y) within two distinct groups: egg donors with no known fertility problems and young women with DOR. We undertook broad screening using microarray analysis to identify candidate genes or patterns associated with DOR and then performed confirmatory studies using quantitative RT–PCR (qRT–PCR).

Materials and Methods

The study was approved by the Partners' Institutional Review Board for use of discarded materials.

Patient selection

To eliminate potential confounding caused by ovarian aging, all patients were ≤35 years. Moreover, as polycystic ovarian syndrome may be associated with differences in ovarian gene expression (Diao *et al.*, 2004; Jansen *et al.*, 2004; Wood *et al.*, 2007); patients diagnosed with this condition were excluded from the study. As there is no consistent and agreed upon definition of DOR, patients were selected for our two groups (NOR and DOR) using the common strategy of standard pretreatment hormonal assessment (Day 3 FSH and estradiol levels) combined with ovarian response (based on number of follicles on the day of the ovulatory hCG trigger). While a Day 3 FSH cutoff of ≥10 mIU/ml has been used by some to classify DOR, we chose to identify the cutoff specific to our own population of women ≤35 years. Based on our finding that FSH < 8 mIU/ml provided the most accurate inflection point for predicting embryo quality and live birth in this age group, we chose this FSH level as a guide to stratify patients. However, one patient with a Day 3 FSH of 7 mIU/ml was included in the DOR group because her Day 10 FSH was 14 mIU/ml and she met the follicular criteria (≤7 follicles of ≥12 mm diameter at the time of ovulatory trigger). There was a narrow margin of follicular number for women with DOR, given that it is our usual protocol to cancel cycles with <4 follicles or with an E2 ≤ 500. Therefore we collected follicular samples from women who had between four and seven follicles at the time of hCG trigger (with one exception of a patient with three follicles who was allowed to proceed to retrieval). The NOR group comprised oocyte donors with no known fertility problems who had a Day 3 FSH level <8.0 mIU/ml, a Day 3 estradiol level ≤50 pg/ml, and who had ≥10 follicles of ≥12 mm diameter at the time of ovulatory trigger (see Table I for definitions of groups). Sample collection was initiated in August 2008 and was continued through July 2009.

IVF procedures and membrana granulosa cell isolation

Ovarian stimulation protocols were as previously described (Skiadas *et al.*, 2006, 2008). Patients who met inclusion criteria were identified on the day of ovulatory trigger, and the laboratory was notified. Oocyte retrieval was performed 36 h after the administration of hCG to promote the final maturation stage of the oocyte. Patients were given a single dose of IV doxycycline (100 mg), or alternative IV antibiotic in the setting of doxycycline allergy, and IV general anesthesia was administered. Patients were positioned in dorsal lithotomy and the vagina was rinsed with sterile saline. Transvaginal ultrasound guidance was used to visualize both ovaries and follicles were serially punctured using a 17 gauge needle (Cook, Bloomington, IN, USA) for all visualized follicles. Gentle suction was applied using a rocket pump (Cook) to collect follicular fluid. The fluid was collected in warmed test tubes and passed off immediately to the waiting embryology team, and oocytes were identified and placed in separate dishes. All dishes were inspected twice for the presence of oocytes and then the remaining follicular fluid and culture media were handed off to a member of the study team (C.C.S., C.R. and N.K.) to collect the granulosa cells. Our standard IVF protocols were used for the clinical handling of all oocytes and embryos. Methods for fertilization check, embryo culture and embryo transfer were as described previously (Skiadas *et al.*, 2006, 2008). Prior to cell collection, a dedicated bench in the IVF laboratory was cleaned with 75% ethanol and RNase away (VWR international,

Table I Inclusion criteria for study patient subgroups.

	Age (years)	Day 3 FSH (mIU/ml)	Day 3 E2 (pg/ml)	Number of follicles on day of hCG trigger
NOR ($n = 13$ samples)	≤35	<8.0	≤50	≥10
DOR ($n = 15$ samples)	≤35	≥8.0	No limit	≤7

Downloaded from https://academic.oup.com/molehr/article/18/7/362/1057377 by guest on 20 August 2022

West Chester, PA, USA) to remove any possible contaminating RNA, and to reduce the likelihood of degrading RNase from extraneous sources. Within 30 min of completion of oocyte pick-up, the membrana granulosa cells were harvested from the plates by a study team member by visual inspection using a dissecting microscope. Cells were sequentially washed, placed into RNALater (Ambion, Austin, TX, USA) and then stored at 4°C for up to 48 h before being moved to −70°C for long-term storage prior to RNA extraction.

RNA extraction

RNA extraction was performed as follows: The thawed samples were centrifuged at 8000*g* for 5 min to pellet the cells and the supernatant was removed. When the cellular concentration was high, an equal volume of phosphate buffered saline (0.5 ml) was added to the samples, as recommended by Ambion, the manufacturer of RNALater, prior to centrifugation. Cells were disrupted using 700 μl Trizol reagent (Invitrogen, Carlsbad, CA, USA), and 140 μl chloroform and 1 μl glycogen (Invitrogen) were added to complete RNA separation from the protein and interphase layers. The aqueous RNA layer was then removed and purified using the Qiagen miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA amounts were measured via a Nano-Drop spectrophotometer to calculate total RNA per sample.

Microarray analysis

Prior to microarray analysis, all samples were monitored with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) to ensure high-quality RNA (RNA integrity ≥8). HumanRef-8 v2.0 Expression BeadChips (Illumina, San Diego, CA, USA) BeadStation 500GX (Illumina) were used to evaluate mRNA gene expression differences between NOR and DOR groups. Samples were prepared for microarray analyses by diluting the RNA to a standard concentration of ∼150 ng of RNA in 10 μl of RNase free water (range 131–244 ng of total RNA for the 28 samples), followed by transcription *in vitro*, and by cRNA labeling with biotin using the TotalPrep RNA Amplification kit (Applied Biosystems/Ambion, Austin, TX, USA) recommended by Illumina. Illumina microarray chips were hybridized at the Children's Hospital Boston Microarray Core Facility. Full details of the cell collection and RNA isolation were as previously described.

Quantitative PCR and calculation of gene expression

Microarray analysis was performed to identify significant differences in gene expression between the DOR and NOR groups; the data are available through ArrayExpress (Accession #E-MTAB-391). Individual genes were then researched to identify associations with known biologic functions of the ovary. Two genes, which immediately were recognizable as associated with ovarian function and possibly ovarian reserve status, were anti-Mullerian hormone (AMH) and the luteinizing hormone receptor (LHCGR). Differences in the expression of these two genes were confirmed using qRT–PCR. TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA) were used to convert the RNA to cDNA and qRT–PCR was then conducted using commercially available primers. The primer used for LHCGR was Taqman Gene Expression Assay, Assay ID: Hs00174885_m1 and the primer used for AMH was Taqman Gene Expression Assay, Assay ID: Hs00174915_m1 (Applied Biosystems). For each test sample, an endogenous primer control (18S ribosomal RNA; Applied Biosystems) was run in parallel with test samples. Prior to calculating relative expression for each gene, the results were normalized against the relative quantity of endogenous 18S RNA. The expression data were analyzed using the comparative Ct method (Schmittgen and Livak, 2008).

To further validate our array results, we profiled four additional genes, progesterone receptor membrane component 2 (PGRMC2), prostaglandin E receptor 3 (subtype EP3) (PTGER3), steroidogenic acute regulatory protein (StAR) and StAR-related lipid transfer domain containing 4 (StarD4), in five patients from each group; each assay was replicated four times. The primers from Applied Biosystems were, respectively, PGRMC2 Gene expression assays: Assay ID: Hs00175051_m1; PTGER3 Assay ID: Hs00168755_m1*; StAR Gene expression assays: Assay ID: Hs00264912_m1; and StARD4 Gene expression assays: Assay ID: Hs00287823_m1. We also attempted to validate expression of PRLH (Assay ID: Hs00175080_m1*), but the assay failed in nearly all assays.

Statistical and bioinformatics analysis

After the scanning of the microarray, the BeadStudio software generated a raw data matrix. Raw data, after background subtraction, were normalized to remove sample and batch effects using the Bioconductor Lumi package (Supplementary data, Fig. S1) and reported as $\log_2$-transformed expression values. Normalized gene values were loaded into MeV (http://www.tm4.org/mev; Saeed *et al.*, 2003, 2006) for data filtering and analysis, including average-linkage hierarchical clustering using a Pearson correlation coefficient distance measure.

The Bioconductor Limma package was used to build a statistical linear model comparing NOR and DOR samples; significant genes were identified with a false discovery rate cutoff of <0.05. MsigDB (Broad Institute, http://www.broadinstitute.org/gsea/msigdb/index.jsp) was used to interpret the gene signature function pathways using the canonical pathway module, which includes well-characterized metabolic and cell-signaling pathways coming from published sources and the KEGG database. The 100 genes with the largest variance across samples were selected for exploratory data analysis. These were used in average-linkage hierarchical clustering with a Pearson correlation coefficient distance measure to identify co-expressed genes and to explore the structure of the patients groups based on gene expression profiles.

To determine the 40 genes with the highest likelihood of a direct relationship with ovarian reserve status, we used a combination of microarray *P*-values, research into known biologic function, and Gene Set Enrichment Analysis.

Results

Over the time course of 1 year of collecting samples in which a total of 1448 retrievals were performed in our program, only 21 NOR and 18 DOR patients met our patient selection criteria and had their cells collected for analysis. Of these 39 samples, 28 (13 NOR and 15 DOR samples) passed the strict specifications from the Bioanalyzer Analysis (RNA integrity ≥8, as described in the Materials and Methods section) to proceed with microarray analysis; with a final sample size of $n = 28$. Of note, two patients in the DOR group were represented twice as they underwent two cycles during the study period—the final numbers of contributing patients were 13 to the DOR samples and 13 to those for NOR. Patient characteristics are shown in Table II.

When the DOR patients were compared with the NOR egg donors, significant differences in gene expression were identified and these were linked to alterations in metabolic and signaling pathways. There were two pathways where there was significant overlap of number of genes in the pathway with differences in our microarray data: HSA04060 Cytokine–Cytokine receptor interaction, where 12

Downloaded from https://academic.oup.com/molehr/article/18/7/362/1057377 by guest on 20 August 2022

Table II Demographics for study cycles.

	NOR cycles ($n = 13$)	DOR cycles ($n = 15$)	*P*-value
Age	26.5 ± 2.6	34.1 ± 1.0	<0.001
Day 3 FSH	5.6 ± 1.7	11.5 ± 6.0	0.002
Total number of follicles	15.8 ± 4.1	5.7 ± 1.3	<0.001
Total number of eggs	19.3 ± 7.0	5.3 ± 2.5	<0.001
% MII/total number of eggs	85.2 ± 13.1	86.4 ± 22.1	0.858
Average LH (IU)	40.4 ± 145.6	1950 ± 1559.9	<0.001
Average FSH (IU)	2850.4 ± 1778.7	5465 ± 2426.8	<0.001
%2 pn/MII	72.7 ± 13.8	73.1 ± 31.1	0.959
Average number of embryos	12.5 ± 6.7	3.3 ± 1.7	<0.001
Number of embryos with eight or more cells	5.6 ± 4.9	1.5 ± 1.4	0.012
Average embryo cell number	6.9 ± 1.24	7.2 ± 1.1	0.560
Average fragmentation score	1.7 + 0.4	1.3 + 0.7	0.125
Average number of embryos transferred	1.9 ± 0.6	2.1 ± 0.8	0.400
Positive hCG (%)	9/13 (69.2)	5/15 (33.3)	0.13
Ongoing pregnancy (%)	5/13 (38.5)	4/15 (26.7)	0.68
Implantation rate[a] (%)	8/24 (33.33)	6/31 (19.4)	0.35
Average number of embryos frozen	5.9 ± 5.7	0.4 ± 1.1	0.005

Student's *t*-test was used to compare continuous variables and Fisher's exact test was used to compare categorical variables, given the small numbers. *P*-values are two-tailed.

[a]In the Egg Donor Group, one recipient had no embryos transferred as she experienced bleeding prior to transfer; in the DOR group, one patient did not undergo transfer as she had a failed fertilization.

genes overlapped, as well as HSA MAPK Signaling pathway, where 11 genes overlapped. In addition, we saw significant overlap among the pathway identified as Breast Cancer Estrogen Signaling pathway (six genes), as well as a smaller number of gene overlap in other pathways, but where there was putative biologic significance, including: HSA04020 Calcium signaling pathway, HSA 04810 Regulation of Actin Cytoskeleton, HSA04210 Apoptosis, Cholesterol biosynthesis, HSA04912 GnRH signaling pathway, HSA04350 TGF Beta Signaling pathway. Using both the pathway analysis, and the *P*-values of the microarray data, additional research was conducted on the top 100 candidate genes, until a list of the 40 genes was identified for further study based either on previous implication in ovarian function or assignment to pathways involved in hormonal synthesis (Supplementary data, Table SI).

From this list two genes were selected with putative biological significance; AMH and LHCGR. The average expression of AMH was significantly higher in NOR egg donors (adjusted *P*-value = 0.01), and the average expression of LHCGR was significantly higher in DOR patients (adjusted *P*-value = 0.005; Table III). These results were confirmed with qRT–PCR; the expression of AMH was 2.02-fold higher in the NOR group when compared with DOR patients and LHCGR was expressed at a 2.19-fold higher in DOR patients relative to NOR egg donors (Fig. 1).

As expected, hierarchical clustering separated the patients into distinct NOR and DOR groups confirming our hypothesis that there are substantial difference in expression profiles between groups (although one DOR patient, 112108-2, clustered with the NOR controls, and this patient conceived twins after the transfer of two embryos). However, the DOR patients separated into two distinct groups (Fig. 2) which did not differ significantly for any measured clinical variable (Table IV), but, when we compared gene expression levels between them, we found distinct differences in two genes, small proline-rich protein 2C (SPRR2C) and small proline-rich protein 2B (SPRR2B), that had previously been associated with families of genes related to embryo quality, ovulation cycle and female pregnancy. One of the DOR groups had a significantly higher level of expression of both SPRR2B and SPRR2C than the other group. In addition, this expression level was much higher than that seen in the egg donor group as well.

Further confirmation of our microarray data with qRT–PCR on four additional genes, PGRMC2, PTGER3, Star and StarD4, confirmed

Table III Fold change and *P*-values from Microarray analysis.

Gene symbol	Entrez Gene ID	Log fold change	Average expression		*P*-value	Adjusted *P*-value
			NOR	DOR		
AMH	Anti-Mullerian hormone	0.405242	7.372056	6.966814	<0.001	0.01
LHCGR	Luteinizing hormone/choriogonadotropin receptor	−0.721502	9.176257	9.897759	<0.001	0.005
PGRMC2	Progesterone receptor membrane component 2	−0.367801	11.516736	11.884537	<0.001	0.001
PRLH	Prolactin-releasing hormone	−0.049408	6.525164	6.574572	<0.001	0.005
PTGER3	Prostaglandin E receptor 3 (subtype EP3)	−0.072022	6.657942	6.729964	<0.001	0.008
STAR	Steroidogenic acute regulatory protein	−0.924572	10.076599	11.001171	<0.001	0.005
STARD4	StAR-related lipid transfer (START) domain containing 4	−0.160879	6.568660	6.729538	<0.001	0.004

Downloaded from https://academic.oup.com/molehr/article/18/7/362/1057377 by guest on 20 August 2022

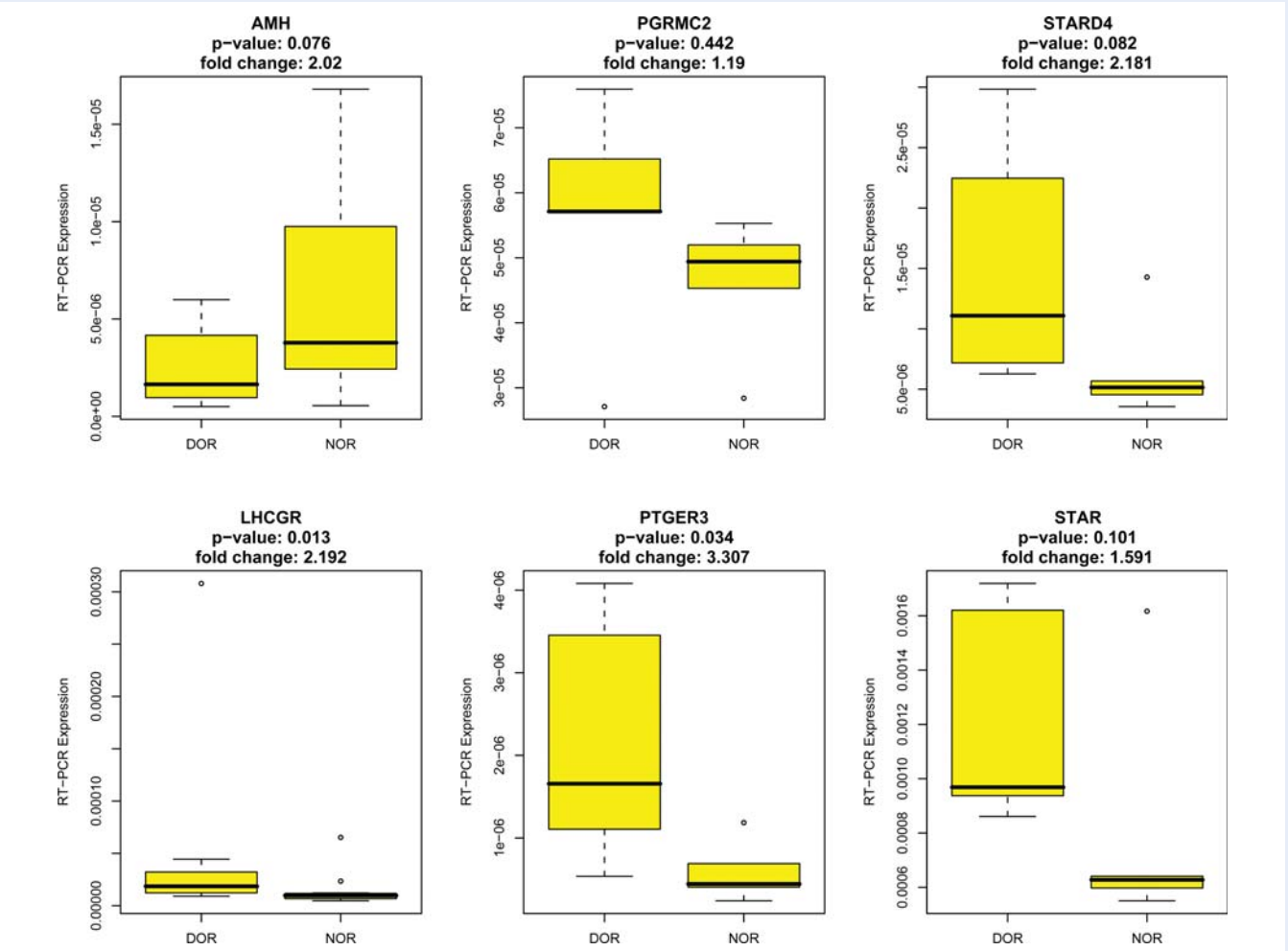


Figure 1 Box plots representing qRT−PCR gene expression levels for showing significant differences for AMH (2.02-fold, $P = 0.08$) and LHCGR (2.19-fold, $P = 0.01$) between DOR and NOR patients, respectively. The four additional genes are included for confirmation purposes only and were not able to be run against all samples. Fold changes for PGRMC2 (1.19-fold), PTGER3 (3.31-fold), StAR (1.59-fold) and StarD4 (2.18-fold) all were similar in both direction and magnitude to that found in the microarray.

similar directionality and magnitude of difference between the groups for all four of the genes (data included in Fig. 1). A fifth gene, PRLH, did not amplify in the majority of samples and was excluded this from further analysis.

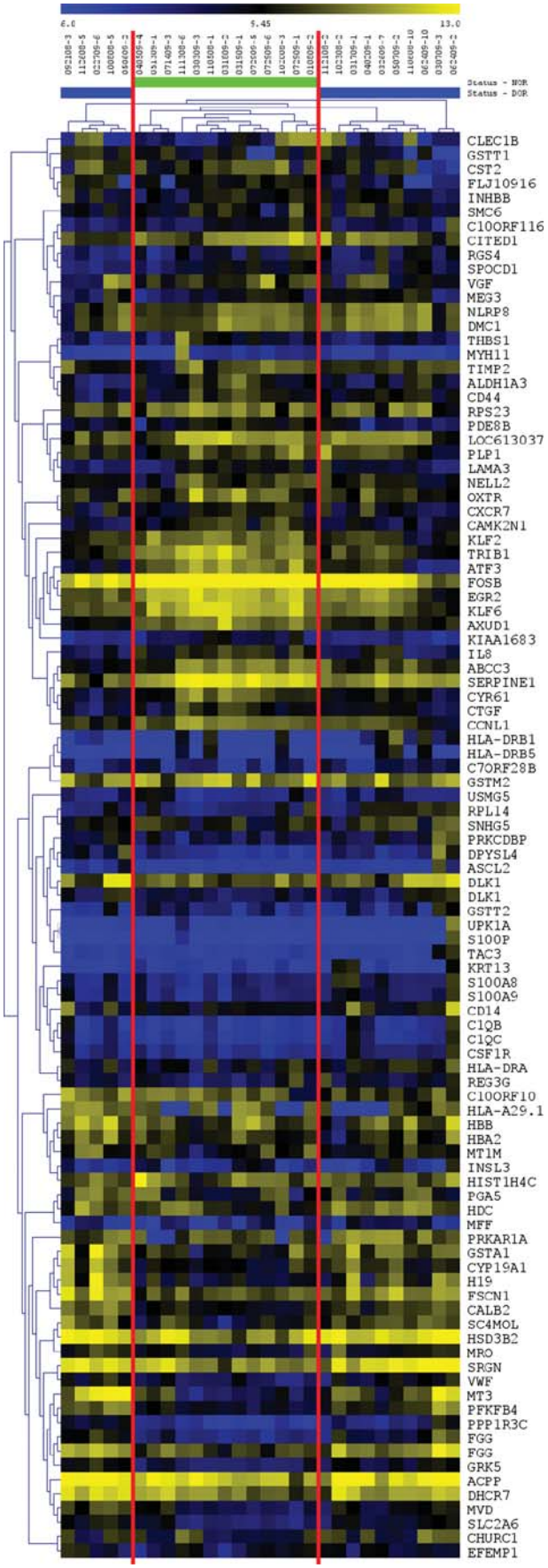
Discussion

The present investigation was undertaken as a pilot study to determine if there significant differences in granulosa cell gene expression exist with respect to ovarian reserve status in young women. Although this is a pilot study with only 26 patients contributing 28 samples for analysis, this represents the largest series of microarray analysis performed to date for exploring expression profiles relative to ovarian reserve status, and the first to restrict the age of women to $\leq$35.

Statistical analysis of gene expression differences between the DOR and NOR patients, followed by functional meta-analysis allowed us to identify a list of 40 potential genes for further study (Supplementary data, Table SI). Of these, the two genes of greatest interest were AMH and LHCGR, both of which are associated with ovarian function and ovarian reserve status; the differences in expression of these genes between our two patient groups were confirmed using qRT−PCR. We also performed confirmatory PCR on an additional four genes (PGRMC2, StAR, StARD4 and PTGER3) that were selected based on significant differences between the patient groups. The overall small quantities of total RNA from the samples limited us to comparing the expression of these genes in five patients from each group. The collective qRT−PCR data validated our microarray analyses, allowing us to conclude that the gene expression profile of membrana granulosa cells differs between DOR and NOR patients.

AMH, also known as Mullerian inhibiting substance, known to be involved in sexual differentiation of the genital tract of fetuses, was first discovered in adult granulosa cells in 1984 (Vigier *et al.*, 1984), with the gene cloned in 1986 (Picard *et al.*, 1986). Studies in both rats (Baarends *et al.*, 1995) and humans (Pellatt *et al.*, 2007) have shown that the highest production of AMH occurs in small follicles with decreasing (or absent) expression in large antral follicles. These observations are consistent with the finding that AMH levels are not influenced by a GnRH agonist stimulation test (van Rooij *et al.*,

Downloaded from https://academic.oup.com/molehr/article/18/7/362/1057377 by guest on 20 August 2022



2002), or when FSH or LH are added to medium in which granulosa cells are cultured (Pellatt *et al.*, 2007). Thus, levels of AMH are held relatively stable throughout the menstrual cycle. In addition, AMH levels have been linked with ovarian reserve status, with higher levels associated with normal ovarian reserve. Our observation that AMH RNA expression was higher in the NOR group when compared with the DOR group, is consistent with previous reports that serum levels of AMH are higher in women with NOR, and also are correlated with both antral follicle count and number of oocytes retrieved during stimulation (van Rooij *et al.*, 2002). To our knowledge, the current findings are the first to show such variant AMH gene expression at the tissue level based on ovarian reserve status.

We also found significantly lower expression of the LHCGR in the NOR group compared with the DOR patients (Fig. 1). Although this difference may be linked to ovarian reserve status, it may be confounded by differences in ovarian stimulation regimen used between the two groups. LH receptor is a member of the G-protein coupled receptor super-family and is known to be induced by FSH on granulosa cells (Strauss and Barbieri, 2004). Given the differences in stimulation regimens (necessitated by the expected differences in ovarian response of our NOR versus DOR patients), it is possible that the increased expression of LH receptor in the DOR group was due to the higher amounts of FSH to which these patients were exposed. However, DOR patients also were receiving a higher amount of exogenous LH (in the form of human menopausal gonadotrophins)—which has been shown to down-regulate LH receptor expression by inhibiting synthesis and mechanisms of degradation (Schwall and Erickson, 1984). Nevertheless, granulosa cells lose LH/hCG receptors after the endogenous LH surge (Jaaskelainen *et al.*, 1980), or after the administration of exogenous LH (Richards *et al.*, 1976; Rao *et al.*, 1977) which all patients in both groups did receive in the form of i.m. hCG injection ~36 h prior to oocyte retrieval.

One potential way in which the up-regulation of LHCGR in DOR patients could be linked with ovarian reserve status is through premature luteinization. Premature luteinization has been associated with DOR and occurs when progesterone levels begin to rise earlier in the cycle than anticipated (usually measured on day of hCG trigger). Women with signs of premature luteinization have had higher levels of Day 3 FSH, have required higher doses of stimulation medication and were noted to have decreased pregnancy rates (Hofmann *et al.*, 1995; Younis *et al.*, 1998, 2001). The mechanisms underlying these observations have not been determined, but it is possible that as the ovary undergoes 'aging' the LH receptor is up-regulated and may account for some of the clinical findings of premature luteinization. Although premature luteinization was not a clinical marker in our study population, future research should target the downstream pathways that result from activation of LHCGR, including progesterone synthesis and the map kinase pathways (Riezel *et al.*, 2010), which have been shown to be important in both steroidogenesis

Figure 2 Unsupervised average-linkage hierarchical clustering using a Pearson correlation coefficient distance metros and the 100 most variable genes across samples clearly distinguish NOR (green bar) and DOR patients (blue bar), but also separates the DOR patients into distinct subgroups.

Downloaded from https://academic.oup.com/molehr/article/18/7/362/1057377 by guest on 20 August 2022

Table IV Clinical patient demographics for two identified groups of DOR patients.

	DOR group 1 ($n = 5$)	DOR group 2 ($n = 10$)	*P*-value
Age (years)	34.4 ± 0.4	34.0 ± 1.2	0.369
Day 3 FSH (mIU/ml)	11.3 ± 2.1	11.6 ± 7.3	0.928
Number of follicles	6.0 ± 1.0	5.5 ± 1.43	0.449
Number of eggs	6.6 ± 3.1	4.7 ± 2.0	0.265
% MII/total number of eggs	82.7 ± 28.9	88.3 ± 19.3	0.705
Number of LH (IU)	1860 ± 1780	1995 ± 1540	0.889
Number of FSH (IU)	6210 ± 3139	5092 ± 2079	0.499
%2 pn/MII	71.7 ± 18.3	73.8 ± 36.9	0.882
Number of embryos	3.4 ± 0.89	3.3 ± 2.06	0.898
Number of embryos with eight or more cells	1.6 ± 0.6	1.7 ± 1.7	0.917
Number of cell on Day 3	7.0 ± 0.9	7.3 ± 1.2	0.613
Fragmentation score	1.5 + 0.6	13 + 0.8	0.582
Number of embryos transferred	2.4 ± 0.6	1.9 ± 0.9	0.201
Positive hCG (%)	3/5 (60.0)	2/9 (22.0)[a]	0.266
Ongoing pregnancy (%)	2/5 (40.0)	2/9 (22.0)[a]	0.580
Implantation rate (%)	3/12 (25.0)	3/19 (15.8)	0.653
Number of embryos frozen	0.0 ± 0.0	0.6 ± 1.4	0.193

Values are means ± STD.
Student's *t*-test was used to compare continuous variables and Fisher's exact test was used to compare categorical variables, given the small numbers. *P*-values are two-tailed.
[a]In the DOR group 2, one patient did not undergo transfer as she had a failed fertilization.

and oocyte maturation (Tajima *et al.*, 2003; Fan and Sun, 2004; Sun *et al.*, 2009).

Although AMH and LHCGR had the clearest links with ovarian function, we chose four additional genes based on biologic plausibility for differential expression between patients with DOR and NOR. PGRMC2 codes for progesterone receptor membrane component 2, and we saw an increased expression of PGRMC2 in patients with DOR compared with NOR. To our knowledge, there is little known about PGRMC2 with regards to ovarian responsiveness, but we know that progesterone plays a key role in health of the ovarian follicle and mediating the transition to the corpus luteum. As noted above, there are published reports of increased progesterone during controlled ovarian hyperstimulation, and premature luteinization as a marker for DOR (Younis *et al.*, 1998; Elnashar, 2010). It is not standard practice in our program to monitor progesterone levels during controlled ovarian hyperstimulation, therefore, we do not have serum progesterone levels to assess any greater likelihood for premature luteinization in the DOR group. Regardless, our data indicate that the role of progesterone receptor up-regulation and expression in patients with DOR should be evaluated with additional studies and that assessment of progesterone levels may be clinically useful in patient management.

We observed that StAR expression was increased in DOR patients in comparison with the NOR patients (Fig. 1). This is a surprising finding as StAR is one of the first key proteins involved in steroidogenesis, as it helps to facilitate transport of cholesterol from the outer to the inner mitochondrial membrane (Christenson and Strauss, 2001), and the absence of StAR results in congenital adrenal hypoplasia, and defects in all aspects of both adrenal and gonadal steroidogenesis. StAR has also been found to increase in response to gonadotrophins (Rimon *et al.*, 2004), and to be related to progesterone concentration within the follicle (Johnson *et al.*, 2002). Moreover, a previous study has shown decreased StAR production by cultured granulosa cells from DOR patients compared with those by cells from NOR patients (Phy *et al.*, 2002). Taken together, these collective findings indicate that further research is required to elucidate the role of StAR in DOR patients.

Consistent with StAR, we also observed up-regulation of the related protein, StARD4 in DOR patients. StARD4 is a member of the StAR-related lipid transfer (START) family (Wirtz, 1991), which consists of more than 25 different proteins (Christenson and Strauss, 2001). The exact mechanism of StARD4 has yet to be elucidated and homozygous knockout mice have not shown any deficits in reproductive behavior or fertility (Riegelhaupt *et al.*, 2010). It is possible that the up-regulation of StARD4 in our DOR group occurred in response to the increased StAR protein and that this is not independently related to DOR.

As with expression of StAR and StARD4, we observed an up-regulation of the PGTER3 gene in the DOR group compared with NOR patients. This receptor is one of four for prostaglandin E2 (PGE2). Within the female reproductive tract, prostaglandins have been linked to ovulatory function (Duffy, 2010), luteal hormone production (Vaananen *et al.*, 2001) and roles in uterine contractions (Kotani *et al.*, 2000). To our knowledge, there has been no direct association of PTGER3 with DOR, although, interestingly, mutations in this receptor have been linked to preterm birth (Ryckman *et al.*, 2010) and different subtypes of PTGER3 have been found throughout the uterus (Kotani *et al.*, 2000). Although a thorough discussion of prostaglandin mediated effects in the female reproductive tract is beyond the scope of this paper, our data do suggest that PGE2 and its receptor PTGER3 may have differential expression between DOR and NOR patients, thereby warranting future research.

One of the most interesting findings from our microarray analysis was the identification of two groups of DOR patients. Although clinically these groups did not appear significantly different, the fact that there was an underlying difference in granulosa cell gene expression of SPRR2B and SPRR2C, the genes coding for small proline-rich (SPRR) proteins 2b and 2c, raises the question of whether the clinical presentation of DOR is an end-result of several potential causal pathways.

SPRR2B and SPRR2C have been linked with reproductive function, including implantation and pregnancy rates, primarily with respect to differential expression within the uterus. A 2004 study by Hong *et al.* in ovariectomized mice found that SPRR2b and 2c proteins were strongly up-regulated by estrogen in the mouse uterus (Hong *et al.*, 2004). However, little is known about the function of these genes in the ovary despite the presence of members of this protein

Downloaded from https://academic.oup.com/molehr/article/18/7/362/1057377 by guest on 20 August 2022

family in the ovary (Cabral *et al.*, 2001). SPRR proteins are involved in controlling the toughness of the cornified cell envelope, which is a specialized structure beneath the plasma membrane of cells. These proteins are influenced by both environmental and extracellular factors, including aging (Garmyn *et al.*, 1992), UV exposure (Kartasova and van de Putte, 1988; Garmyn *et al.*, 1992) and malignancy (Abraham *et al.*, 1996) and may play a protective role against cellular damage. As DOR may be a premature type of ovarian aging, it is interesting to note the differences in the SPRR proteins between these two groups (as well as the differences between the DOR and egg donor patients). However, minimal work has been done on these proteins in the ovary to determine their function and possible protective role.

Despite the promising findings of this study, there are several limitations. First, the sample size of $n = 28$ (contributed from 26 patients) is smaller than would be ideal. However, given the rarity of the diagnosis of DOR in young women, as well as the cost of microarray analysis, we proceeded with this pilot study to generate hypotheses and to find additional targets for research. Clear differences were seen between groups, despite the limited number of patients. Secondly, despite our attempts to limit the age to women $\leq$35 year, the mean age of the groups remained significantly different, which could represent a confounding factor in our analysis.

Previous microarray data from failed-to-fertilize metaphase II (MII) oocytes stratified according to age ($<$32 years; 32–40 years; and $>$40 years), revealed that gene expression clustered according to age (Steuerwald *et al.*, 2007). Although we chose egg donors as our control group (in an effort to minimize any effect of infertility in the control group), another potential study design would be to choose age-matched women with isolated male-factor infertility as the control group to try to minimize any effect that age alone could have on granulosa cell gene expression status.

Further, the stimulation regimen differed significantly between the groups, which was a known limitation, based on our hypothesis that choosing the most clinically different groups would lead to the greatest difference in gene expression in this pilot study, and the inability to keep the stimulation standardized across groups; indeed, if the DOR patients received similar doses to the egg donors, their cycles would likely have resulted in a cancellation, instead of an egg retrieval. However, due to the differences in stimulation regimen, it is difficult to determine what role, if any, this played in the differences in gene expression of the granulosa cells observed. It is possible that the higher amounts of FSH (and LH) administered during the cycle are responsible for the difference in LHCGR expression. The only way to eliminate the potential confounding effect of stimulation regimens would be to do unstimulated cycle aspirations of single follicles which would be unlikely to yield sufficient RNA for analysis.

Despite these limitations, our microarray analysis confirmed clear, distinct patterns in genetic expression of granulosa cells between the NOR and DOR patients. Our findings not only provide insight into several potential genetic targets for future studies, but also reveal genetic differences between two divergent populations of DOR patients (based on gene expression), who were unable to be distinguished clinically. It is our hope that with additional research, a 'gene expression pattern' for ovarian reserve is identified to guide clinical treatment decisions, as well as to provide diagnostic insight into those patients whose infertility is currently unexplained through classic diagnostic paradigms.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

Authors' roles

C.C.S.: Responsible for Study design, collection of granulosa cell samples, extraction of RNA, preparation of samples for microarray analysis, PCR experiments and principle author of manuscript. S.D., M.C.: Responsible for statistical analysis of microarray and PCR data. R.R.: Instrumental in teaching CCS lab techniques for RNA extraction, PCR and preparation of microarray samples. In addition RR helped complete the additional PCR analysis when the reviewers asked for additional confirmatory data. N.K.: Responsible for collection of granulosa cell samples. E.S.G.: IVF laboratory director and involved in the design of group selection criteria based on ovarian reserve status. J.Q.: All RNA extraction and PCR analysis took place in the Quackenbush lab, and his direction was instrumental in determining the approach of microarray analysis to determine genetic differences between these two groups. His group has extensive experience in the computational biology approaches to interpreting microarray data. C.R.: Embryology lab director, extensive experience in clinical IVF and handling of granulosa cells. Determined the techniques applied for washing and extracting granulosa cells. Also involved in overall study design, granulosa cell collection and manuscript preparation.

Funding

This work was supported by The Endocrine Fellows' Foundation Grant; the Dana-Farber High-Tech Fund; and a grant from the NIH (R01-LM008795).

Conflict of interest

None declared.

References

Abraham JM, Wang S, Suzuki H, Jiang HY, Rosenblum-Vos LS, Yin J, Meltzer SJ. Esophagin cdna cloning and characterization: A tissue-specific member of the small proline-rich protein family that is not expressed in esophageal tumors. *Cell Growth Differ* 1996; **7**:855–860.

Baarends WM, Hoogerbrugge JW, Post M, Visser JA, De Rooij DG, Parvinen M, Themmen AP, Grootegoed JA. Anti-Mullerian hormone and anti-Mullerian hormone type ii receptor messenger ribonucleic acid expression during postnatal testis development and in the adult testis of the rat. *Endocrinology* 1995;**136**:5614–5622.

Cabral A, Voskamp P, Cleton-Jansen AM, South A, Nizetic D, Backendorf C. Structural organization and regulation of the small proline-rich family of cornified envelope precursors suggest a role in adaptive barrier function. *J Biol Chem* 2001;**276**:19231–19237.

Chandra A, Martinez G, Mosher W, Abma J, Jones J. Fertility, family planning, and reproductive health of U.S. Women: data from the 2002 national survey of family growth. *Vital Health Stat Ser* 2005; **23**:1–174.

Downloaded from https://academic.oup.com/molehr/article/18/7/362/1057377 by guest on 20 August 2022

Chin K-V, Seifer D, Feng B, Lin Y, Shih W-C. DNA microarray analysis of the expression profiles of luteinized granulosa cells as a function of ovarian reserve. *Fertil Steril* 2002;**77**:1214–1218.

Christensen K, Kohler HP, Basso O, Olsen J, Vaupel JW, Rodgers JL. The correlation of fecundability among twins: evidence of a genetic effect on fertility? *Epidemiology* 2003;**14**:60–64.

Christenson LK, Strauss JF 3rd. Steroidogenic acute regulatory protein: An update on its regulation and mechanism of action. *Arch Med Res* 2001; **32**:576–586.

de Bruin JP, Bovenhuis H, van Noord PA, Pearson PL, van Arendonk JA, te Velde ER, Kuurman WW, Dorland M. The role of genetic factors in age at natural menopause. *Hum Reprod* 2001;**16**:2014–2018.

Diao FY, Xu M, Hu Y, Li J, Xu Z, Lin M, Wang L, Zhou Y, Zhou Z, Liu J *et al.* The molecular characteristics of polycystic ovary syndrome (PCOS) ovary defined by human ovary cdna microarray. *J Mol Endocrinol* 2004;**33**:59–72.

Duffy DM. Prostaglandin dehydrogenase (PGDH) in granulosa cells of primate periovulatory follicles is regulated by the ovulatory gonadotropin surge via multiple g proteins. *Mol Cell Endocrinol* 2010;**333**:119–126.

Elnashar AM. Progesterone rise on the day of hcg administration (premature luteinization) in IVF: An overdue update. *J Assist Reprod Genet* 2010;**27**:149–155.

Fan HY, Sun QY. Involvement of mitogen-activated protein kinase cascade during oocyte maturation and fertilization in mammals. *Biol Reprod* 2004; **70**:535–547.

Fassnacht W, Mempel A, Strowitzki T, Vogt PH. Premature ovarian failure (POF) syndrome: towards the molecular clinical analysis of its genetic complexity. *Curr Med Chem* 2006;**13**:1397–1410.

Garmyn M, Yaar M, Boileau N, Backendorf C, Gilchrest BA. Effect of aging and habitual sun exposure on the genetic response of cultured human keratinocytes to solar-simulated irradiation. *J Invest Dermatol* 1992; **99**:743–748.

Gilchrist R, Ritter L, Armstrong D. Oocyte-somatic cell interactions during follicle development in mammals. *Anim Reprod Sci* 2004;**82–83**:431–446.

Hanrahan J, Gregan S, Mulsant P, Mullen M, Davis G, Powell R, Galloway S. Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in cambridge and belcare sheep (ovis aries). *Biol Reprod* 2004; **70**:900–909.

Hofmann GE, Scott RT Jr, Horowitz GM, Thie J, Navot D. Evaluation of the reproductive performance of women with elevated day 10 progesterone levels during ovarian reserve screening. *Fertil Steril* 1995; **63**:979–983.

Hong SH, Nah HY, Lee JY, Lee YJ, Lee JW, Gye MC, Kim CH, Kang BM, Kim MK. Estrogen regulates the expression of the small proline-rich 2 gene family in the mouse uterus. *Mol Cells* 2004; **17**:477–484.

Jaaskelainen K, Hyvonen T, Rajaniemi H. Human choriogonadotropin-induced desensitization of granulosa-cell adenylate cyclase to gonadotropins and loss of LH/hCG receptor. *Mol Cell Endocrinol* 1980;**20**:145–156.

Jansen E, Laven JS, Dommerholt HB, Polman J, van Rijt C, van den Hurk C, Westland J, Mosselman S, Fauser BC. Abnormal gene expression profiles in human ovaries from polycystic ovary syndrome patients. *Mol Endocrinol* 2004;**18**:3050–3063.

Johnson AL, Solovieva EV, Bridgham JT. Relationship between steroidogenic acute regulatory protein expression and progesterone production in hen granulosa cells during follicle development. *Biol Reprod* 2002;**67**:1313–1320.

Kartasova T, van de Putte P. Isolation, characterization, and UV-stimulated expression of two families of genes encoding polypeptides of related structure in human epidermal keratinocytes. *Mol Cell Biol* 1988; **8**:2195–2203.

Kotani M, Tanaka I, Ogawa Y, Suganami T, Matsumoto T, Muro S, Yamamoto Y, Sugawara A, Yoshimasa Y, Sagawa N *et al.* Multiple signal transduction pathways through two prostaglandin E receptor EP3 subtype isoforms expressed in human uterus. *J Clin Endocrinol Metab* 2000;**85**:4315–4322.

Pal L, Zhang K, Zeitlian G, Santoro N. Characterizing the reproductive hormone milieu in infertile women with diminished ovarian reserve. *Fertil Steril* 2010;**93**:1074–1079.

Pellatt L, Hanna L, Brincat M, Galea R, Brain H, Whitehead S, Mason H. Granulosa cell production of anti-Mullerian hormone is increased in polycystic ovaries. *J Clin Endocrinol Metab* 2007;**92**:240–245.

Phy J, Stocco DM, Prien SD. Differences in steroidogenic acute regulatory protein expression from the luteinizing-granulosa cells of patients undergoing in vitro fertilization with embryo transfer: Implications for cycle outcomes. *Am J Obstet Gynecol* 2002;**186**:872–875.

Picard JY, Benarous R, Guerrier D, Josso N, Kahn A. Cloning and expression of cdna for anti-Mullerian hormone. *Proc Natl Acad Sci USA* 1986;**83**:5464–5468.

Rao MC, Richards JS, Midgley AR Jr, Reichert LE Jr. Regulation of gonadotropin receptors by luteinizing hormone in granulosa cells. *Endocrinology* 1977;**101**:512–523.

Richards JS, Ireland JJ, Rao MC, Bernath GA, Midgley AR Jr, Reichert LE Jr. Ovarian follicular development in the rat: Hormone receptor regulation by estradiol, follicle stimulating hormone and luteinizing hormone. *Endocrinology* 1976;**99**:1562–1570.

Riegelhaupt JJ, Waase MP, Garbarino J, Cruz DE, Breslow JL. Targeted disruption of steroidogenic acute regulatory protein d4 leads to modest weight reduction and minor alterations in lipid metabolism. *J Lipid Res* 2010;**51**:1134–1143.

Riezel Y, Elbaz J, Dekel N. Sustained lh activity of the egf receptor is an absolute requisite for lh-induced oocyte maturation and cumulus expansion. *Mol Endocrinol* 2010;**24**:402–411.

Rimon E, Sasson R, Dantes A, Land-Bracha A, Amsterdam A. Gonadotropin-induced gene regulation in human granulosa cells obtained from IVF patients: modulation of genes coding for growth factors and their receptors and genes involved in cancer and other diseases. *Int J Oncol* 2004;**24**:1325–1338.

Ryckman KK, Morken NH, White MJ, Velez DR, Menon R, Fortunato SJ, Magnus P, Williams SM, Jacobsson B. Maternal and fetal genetic associations of PTGER3 and PON1 with preterm birth. *PLoS One* 2010;**5**:e9040.

Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M *et al.* Tm4: a free, open-source system for microarray data management and analysis. *Biotechniques* 2003;**34**:374–378.

Saeed AI, Bhagabati NK, Braisted JC, Liang W, Sharov V, Howe EA, Li J, Thiagarajan M, White JA, Quackenbush J. Tm4 microarray software suite. *Methods Enzymol* 2006;**411**:134–193.

SART-CORS. www.Sart.Org. 2009.

Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative c(t) method. *Nat Protoc* 2008;**3**:1101–1108.

Schwall RH, Erickson GF. Inhibition of synthesis of luteinizing hormone (LH) receptors by a down-regulating dose of lh. *Endocrinology* 1984; **114**:1114–1123.

Skiadas C, Jackson K, Racowsky C. Early compaction on day 3 may be associated with increased implantation potential. *Fertil Steril* 2006; **86**:1386–1391.

Skiadas C, Missmer S, Benson C, Gee R, Racowsky C. Risk factors associated with pregnancies containing a monochorionic pair following assisted reproductive technologies. *Hum Reprod* 2008;**23**:1366–1371.

Downloaded from https://academic.oup.com/molehr/article/18/7/362/1057377 by guest on 20 August 2022

Steuerwald NM, Bermudez MG, Wells D, Munne S, Cohen J. Maternal age-related differential global expression profiles observed in human oocytes. *Reprod Biomed Online* 2007;**14**:700–708.

Strauss JF, Barbieri RL, (eds). Yen and Jaffe's reproductive endocrinology: physiology, pathophysiology and clinical management, 5th edn. Philadelphia, PA, 2004.

Sun QY, Miao YL, Schatten H. Towards a new understanding on the regulation of mammalian oocyte meiosis resumption. *Cell Cycle* 2009; **8**:2741–2747.

Tajima K, Dantes A, Yao Z, Sorokina K, Kotsuji F, Seger R, Amsterdam A. Down-regulation of steroidogenic response to gonadotropins in human and rat preovulatory granulosa cells involves mitogen-activated protein kinase activation and modulation of dax-1 and steroidogenic factor-1. *J Clin Endocrinol Metab* 2003;**88**:2288–2299.

Vaananen JE, Tong BL, Vaananen CC, Chan IH, Yuen BH, Leung PC. Interaction of prostaglandin f(2alpha) and prostaglandin e(2) on progesterone production in human granulosa-luteal cells. *Biol Signals Recept* 2001;**10**:380–388.

van Rooij IA, Broekmans FJ, te Velde ER, Fauser BC, Bancsi LF, de Jong FH, Themmen AP. Serum anti-Mullerian hormone levels: a novel measure of ovarian reserve. *Hum Reprod* 2002;**17**:3065–3071.

Vigier B, Picard JY, Tran D, Legeai L, Josso N. Production of anti-Mullerian hormone: another homology between sertoli and granulosa cells. *Endocrinology* 1984;**114**:1315–1320.

Wirtz KW. Phospholipid transfer proteins. *Annu Rev Biochem* 1991; **60**:73–99.

Wood JR, Dumesic DA, Abbott DH, Strauss JF 3rd. Molecular abnormalities in oocytes from women with polycystic ovary syndrome revealed by microarray analysis. *J Clin Endocrinol Metab* 2007; **92**:705–713.

Yan C, Wang P, DeMayo J, DeMayo F, Elvin J, Carino C, Prasad S, Skinner S, Dunbar B, Dube J *et al.* Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. *Mol Endocrinol* 2001;**15**:854–866.

Younis JS, Haddad S, Matilsky M, Ben-Ami M. Premature luteinization: Could it be an early manifestation of low ovarian reserve? *Fertil Steril* 1998;**69**:461–465.

Younis JS, Matilsky M, Radin O, Ben-Ami M. Increased progesterone/estradiol ratio in the late follicular phase could be related to low ovarian reserve in in vitro fertilization-embryo transfer cycles with a long gonadotropin-releasing hormone agonist. *Fertil Steril* 2001; **76**:294–299.

Downloaded from https://academic.oup.com/molehr/article/18/7/362/1057377 by guest on 20 August 2022