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Ovarian Transcriptomes as a Tool for a Global Approach of Genes Modulated by Gonadotropic Hormones in Human Ovarian Granulosa Cells

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Follicle-stimulating hormone (FSH) is a key stimulant for the development of the ovarian follicle, while luteinizing hormone (LH) plays a major role in triggering ovulation and luteinization. Both FSH and LH are glycoprotein hormones that share the same alpha subunit but bind to specific seven transmembrane-domain G coupled receptors located on the cell membrane of the granulosa cells, which comprise the main somatic population of the ovarian follicle. These hormone–receptor complexes may trigger different signaling cascades, but the entire repertoire of genes modulated by these hormones is far from being understood, in particular on the transcriptional level. The development of DNA microarrays technique, using the entire genome profile of some mammalian species, allows a global approach and screening of multiple signal transduction pathways. This method opened new insights into the cellular and molecular events that control ovulation and desensitization of the corpus luteum to hyperstimulation by gonadotropic hormones. In addition, this technique permitted the discovery of novel members of the EGF family, such as epiregulin and amphiregulin, that were found to be expressed in the gonadotropin-stimulated cells and were discovered to play a crucial role in the mechanism of ovulation. However, because of the pitfalls in interpreting the data other approaches, for example, Northern blots and RT-PCR must be conducted in parallel to verify the validity of the data.

Key Words: DNA microarray; global approach; ovulation; EGF-like factors; ovarian follicle cells; specific FSH and LH responsive genes.

Introduction

Granulosa cells are a vital component in regulating ovarian function (reviewed in ref. *1*). FSH regulates follicular growth by co-stimulating with estrogen and various growth factors the development of granulosa cells, which become in each estrus/ menstrus cycle the main bulk of population of the somatic follicular cells surrounding the oocyte (reviewed in refs. *2* and *3*). These cells nurse the oocyte and control the induction of its maturation via intracellular gap junctions *(4)*. These junctions communicate between the oocyte and the granulosa cells as well as among the granulosa cells themselves, allowing substances of MW<1000 to pass through 60 Å channels *(4)*.

Among these substances is cAMP. This cyclic nucleotide is formed by conversion of ATP catalyzed by the enzyme adenylyl cyclase, leading to a pronounced formation of cAMP *(4)*. The latter catalyzes the formation of progesterone, which serves also as a precursor for estradiol by conversion first of estradiol to andronstendione, which takes place in the theca interna cell layers (reviewed in refs. *1, 2, 3,* and *5–7*).

The receptors to FSH are distributed all around the granulosa cells, while the receptors to LH are mainly confined to the outer layers of granulosa cells, bordering the inner side of the theca cells, thus providing a domino effect by movement of cAMP to the inner layers of the granulosa cells, reaching the entire population of the granulosa cells, including the cumulus cells as well as the oocyte at the time of ovulation *(8)*. All those effects are regulated by a timely release of FSH and LH from the anterior hypophysis and stimulating the seven-transmembrane-domain G coupled gonadotropin receptors, which are located on the surface of the granulosa cells *(9)*.

Recently, the development of a new technique, the DNA microarray, and the knowledge of the entire genome of mammals including human, mouse, cow, and rat made it possible to analyze global expression and modulation of gene expression following stimulation by drugs, hormones, growth factors, etc. *(10,11)*. Because major effects of gonadotropins are controlling ovarian function, we decided to explore the gene modulation in two experimental systems: (1) Cells obtained from IVF program after they regain response to gonadotropins in primary culture *(12–15)*, and (2) immortalized rat granulosa cell lines established in our laboratory. These lines respond well to gonadotropin stimulation *(5,14,16,17)*.

Surprisingly, we have discovered by using DNA microarray upon RNA isolated from the cells, modulation of num-

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erous genes not reported earlier to be affected by gonadotropic hormones, both in human granulose cells and in gonadotropin-responsive immortalized granulosa cells *(10,13–15)*.

In this review we discuss the novel information obtained recently in our and others laboratories using various DNA microarrays and RNA from granulosa cells, or the intact follicle where the granulosa cells consist of the main bulk of the somatic population of the mature follicle *(10,11,13–15)*. On the other hand we discuss in detail the potential and pitfalls that may occur using the DNA microarray as a principle technique to follow gene activity *(15)*. We also discuss how one can overcome possible obstacles in interpretation of the results.

Isolation of RNA from the Biological Sample

One of the considerations in choosing the biological system for isolation of RNA for hybridization on DNA microarray is the homogeneous RNA derived from a homogeneous population of cells. Complex tissue with various cell types or cells at a different stage of differentiation may yield confusing results, because both the basal activity and activity of genes affected by a specific factor may be different, and, thus, blur the profile of gene activity in different cell types or at different degrees of maturation. We have chosen for our studies granulosa cells from an IVF program, because they are highly luteinized, i.e., in their final stage of differentiation *(12,18,19)*. Another potential source of RNA can be a monoclonal cell line derived from one cell as we utilized rat FSH-responsive cell line established in our laboratory *(5,14)*.

These two systems proved to yield a very sharp profile of gene activity revealing novel genes that were not reported earlier to be modulated by gonadotropins such as epiregulin (Ep) and amphiregulin (Ar), membrane interacting protein (RGS16), phospholipase A1 (PS-PLA1), chemokine CX3C, granzymes, adenylate cyclase 7 and 9, and phosphodiesterases (PDNHD) *(13–15)*.

On the other hand, some typical genes reside in the ovary such as those coding for steroidogenic factors or steroidogenic enzymes [e.g., aromatase, steroidogenic acute regulatory protein (StAR), cytochrome p450 side cleavage (p450scc) enzyme], and were also modulated as expected *(11,13–15)*. Those observations were helpful to verify the validity of the DNA microarray method.

Several laboratories used intact follicles or intact ovary or isolated granulosa derived from preovulatory follicles, as a source of RNA for hybridization on DNA microarrays *(6,20–22)*. The richest profile of gene activity was obtained from isolated preovulatory granulosa cells and showed several novel genes as well as well-known genes expressed in the ovary. Among them 20 ovulation-related genes revealed in mice *(11)*, some of them in line with our observations on human granulosa cells as will be discussed later. They could also confirm upregulation of adrenodoxin, StAR, and 3α-hydroxy steroid dehydrogenase (3α-HSD). Conclusions: the more homogeneous the RNA, the sharper the profiles of gene activity that may be obtained.

Properties of DNA Microarray as a Matter of Choice

Obviously no specific DNA microarrays of specific brands will be discussed, just the principles of arrays used by the different research groups will be dealt with. In DNA microarrays, oligonucleotides resembling specific sequences of the authentic DNA sequence are attached to a solid phase and, when interacting with specific RNAs (isolated and labeled), then a signal is generated indicating the RNA contains a sequence complementary to the specific DNA sequence. In parallel, scrambled sequences are placed as a controls. Signal intensities are normalized allowing interarray comparisons. Normalized data sets are filtered and subjected to computational analysis *(23,24)*.

Repeating the experiment several times (three to five times) on identical arrays will increase the reliability of the data *(15, 22,25)* and differentially regulated transcripts should then be validated and confirmed using independent methods *(23,24)*.

In different DNA microarrays different sequences of the same gene of specific length (normally between two dozens to70 nucleotide) will be used.

Data from different microarrays cannot be coupled together in a trivial manner because different sequences of the same gene may present different affinities to the corresponding RNA sequences of the samples (unpublished), and the same applies to different generations of DNA array of the same company. Therefore, fold difference in gene activities between nontreated and gonadotropin-treated cells may vary from one chip to another, suggesting the need to verify the results with complementary methods such as quantitative RT-PCR *(15,26)*.

Global Approach to the Modulation of Gene Activity in Human Granulosa Cells

When mRNA of human granulosa cells obtained from IVF patients that were maintained in monolayers for 7 d and subsequently were stimulated with LH, FSH, or forskolin was analyzed on DNA microarray, modulation of activity of a high number of genes was recorded in each treatment, with considerable overlap among the three treatments *(10)*. The reason for maintaining the cells for 7 d in hormone-free medium was to release the cells from desensitization to the previous in vivo stimulation with high dose of gonadotropins *(12)*.

Among LH and FSH upregulated genes, 76% were overlapping. Seventy five percent of LH responsive and 77% of FSH responsive genes were modulated by forskolin, which elevates nonspecifically intracellular cAMP levels. These data suggest that 25% of LH responsive genes and 23% of

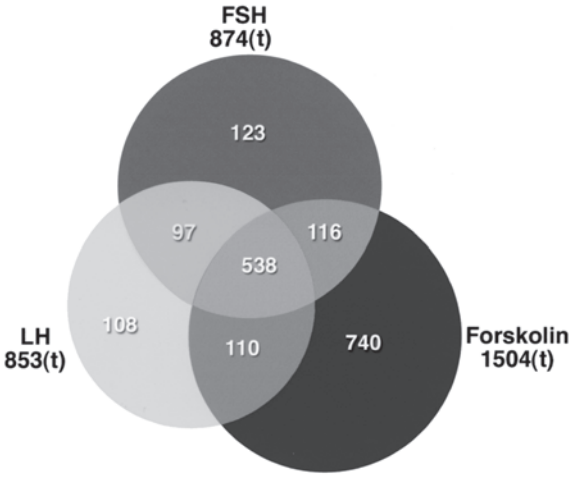

Fig. 1. Modulation of gene expression in human granulosa cells. Human granulosa cells obtained from IVF patient were cultured for 7 d as monolayers and subsequently were stimulated with 1 IU/mL of hLH or 1 IU/mL hFSH or 50 μ*M* of forskolin for 24 h. Gene activity was analyzed on U95 microarrays (Affymetrix) using mRNA isolated from each group of treated cells. The area of each circle is proportional to the number of genes modulated in each treatment. The number of total genes, which are regulated in each treatment, are indicated (t), as well as the number of specific genes for each treatment. The number of genes, which are modulated by more than one treatment, are indicated in the overlapping areas.

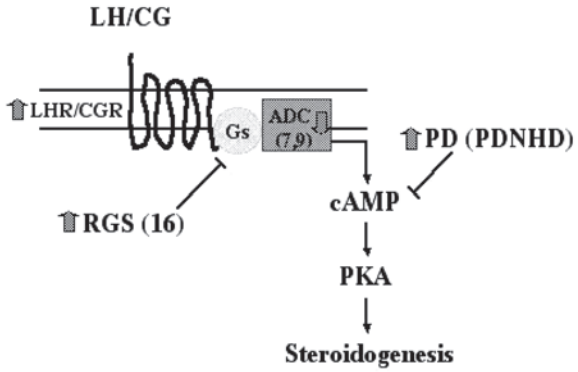

Fig. 2. Possible mechanism in attenuation of gonadotropins/cAMP response and steroidogenesis in luteinized human granulosa cells. Arrows resemble direction of signaling. Wide arrows indicate changes in gene activity modulated by LH. LH, luteinizing hormone; CG, chorionic gonadotropin; LHR, LH receptor; CGR, CG receptor; Gs, G coupled stimulatory; ADC, adenylate cyclase; PD, phosphodiesterase; PDNHD, phosphodiesterase 4D, cAMP-specific; RGS, regulators of G protein signaling.

FSH responsive genes are modulated in a cAMP independent manner (Fig. 1 and ref. *10*).

Ovulatory Response

There are representative examples where DNA microarray analysis could provide useful information concerning ovarian physiology, in most cases, this information could be confirmed by other workers performing gene activities or by independent methods such as RT-PCR and Western blots.

One of the most dramatic physiological processes in the ovary is the ovulatory response to the gonadotropin surge (reviewed in ref. *11*). Twenty ovulatory-specific genes have been identified in rat preovulatory follicles following single dose of LH or its homolog hCG. These include 5-aminolevulinate synthase; early growth response protein-1; gamma-glutamylcysteine synthetase; cyclooxygenase-2; epiregulin; pituitary adenylate cyclase-activating polypeptide; tumor necrosis factor-stimulated gene-6; regulator of G protein–signaling protein-2; adrenodoxin; steroidogenic acute regulatory protein; 3 alpha-hydroxysteroid dehydrogenase; CD63, a disintegrin and metalloproteinase with thrombospondin motifs; tissue inhibitor of metalloproteinase-1; carbonyl reductase, a G protein–coupled receptor; pancreatitis-associated protein-III; glutathione S-transferase; and metallothionein-1 *(11)*. Modulation of six of these genes by ovulatory dose of LH/CG was detected in granulosa cells isolated from IVF patients, using Affymetrix DNA microarrays technique. These include epiregulin (Ep), disintegrin-like metalloproteinase with thrombospondin (ADAMTS1), steroidogenic acute regulating protein (StAR), corlonyl reductase 1, glutathione-S-transferase, and metallothionein 1 *(13–15)*. This would suggest that such an approach, using DNA microarray, could reveal specific genes involved in the ovulatory dose of LH/CG in different mammalian species and compare them.

The Corpus Luteum

It is well known that the mammalian corpus luteum produces high levels of progesterone while becoming refractory to LH/CG stimulation, although receptors to LH/CG are present *(27)*. In order to get insight into this enigma, granulosa lutein cells in primary cultures were recently exposed to LH stimulation, RNA was isolated and hybridized on Affymetrix DNA microarray, and transcript activity was inspected *(15)*. A dramatic changes in gene transcripts (transcriptomes) coding for signaling enzymes was observed following LH stimulation. This included the downregulation of adenylyl cyclase 7 and 9, elevation of cAMP-dependent phosphodiesterase, and the upregulation of a negative regulator of G protein signaling (RGS16) that may negate gonadotropin signaling via guanine nucleotide–binding proteins. Thus, luteinized cells, despite increased gene transcripts to LH/CG receptors, respond inefficiently to gonadotropin stimulation of signal transduction owing to possible changes in gene transcript in critical points of the cyclic AMP signaling cascade (Fig. 2).

EGF-like Factors

The mechanism of triggering ovulation is not completely understood. The complexity arises from the fact that the re-

ceptors to luteinizing hormone are present in the outer layers of the ovarian follicle, the membrana granulosa, while the cumulus–oocyte complexes are devoid of LH receptors *(5)*. Therefore, there must be a remote control mechanism using a second messenger, which could reach the cumulus cells and the oocyte. One possibility is that cAMP, which rises on LH stimulation, can pass from one cell to another through gap junctions *(4,28)*. Because gap junctions are distributed among granulosa cells, cumulus cells, and the oocyte, the transfer of small molecules like cAMP occurs through these junctions and reaches the oocytes. Subsequently, closing these gap junction gates may trigger the egg to resume its meiotic process *(28)*. It was recently found that LH triggers the cascade of MAPK resulting in phosphorylation of ERK1 and ERK2 *(29,30)*. It was suggested recently that phosphorylation of connexin 43, a major building block of the junctional complex, could affect the closure of the gap junction channels, which would lead to initiation of the ovulatory response *(28)*. However, this hypothesis does not exclude the existence of second or third messenger molecules that can control the LH-dependent ovulatory response.

Using the global approach of DNA microarrays to analyze mRNA from human granulosa lutein cells, it was found that LH can induce dramatically activation of transcriptomes coding for members of the EGFs family, epiregulin and amphi-regulin *(26,31,32)*. Independently, it was demonstrated that epiregulin and amphiregulin can induce mucification of the cumulus cells and resumption of meiosis in mice oocytes *(33)*. It was also demonstrated that epiregulin transcription was elevated by ovulatory dose of LH/CG in intact female mice *(33)*. Taken together, these data demonstrate that DNA microarray technology can help in a better understanding of the regulation of ovulation.

Metalloproteinases

A family of genes that was studied recently by DNA microarray, RT-PCR, and/or *in situ* hybridization is that of the anchored metalloproteinases and disintegrins termed ADAMs that can cleave and activate EGF-like factors *(34)*. Of special interest was ADAMTS-1, whose expression was changed during the ovulatory process *(35)*. The general view obtained is that during ovulation there is an increase in its expression in mice and monkeys. These studies used RT-PCR and *in situ* hybridization to further validate the array analysis *(35–38)*. On the other hand, in human granulose–lutein cells, the expression of ADAMTS-1 was down-reg-ulated by LH and Fk, which was confirmed by RT-PCR (unpublished). This apparent discrepancy could arise from different timing or the differences between in vivo and in vitro experiments, as well as differences in the mammalian species.

The Cytoskeleton

During early luteinization there are pronounced changes in the organization of cytoskeletal proteins in the rat, mice, and human granulosa cells. This includes mainly the actin cytoskeleton, but also the microtubules and associated proteins and intermediate filaments that undergo considerable changes *(2,39,40–43)*. In order to verify whether these changes are also associated with changes in gene expression, two sets of experiments were conducted. In one study, changes in mouse granulosa gene expression during early luteinization was conducted *(44)* and, in a second study, gonadotropin-induced gene regulation in human granulosa cells from IVF patients was conducted *(15)*. Among transcripts differentially regulated, there were clear and expected changes in genes involved in steroidogenesis as well as clusters of genes involved in modeling of the extracellular matrix, regulation of the cytoskeleton, and intra- and intercellular signaling *(36)*. In the other study, modulation of steroidogenic genes, cytoskeletal genes, and in genes coding for apoptotic signaling, protein kinases were observed *(15)*. In the first work, the changes in the expression of transcripts of vinculin, coffillin, tubulin, and tropomyosin, upon gonadotropin stimulation, were evident. In the human system changes in genes coding for vinculin alpha-actinin, tropomyosin, and tubulin were modulated. It should be noted that changes in tropomyosin were shown in earlier studies by Northern blot in rat and human granulosa cells upon LH/CG stimulation, as well as changes in intracellular levels of those proteins *(2,39–43)*. Thus, taking together these results support the notion that ovarian studies on genes activities were in several cases in line with Northern blot and RT-PCR activities. Correlation and similarities between different mammalian species were also evident. Moreover, the DNA array technique can screen numerous cytoskeleton and associated proteins, which may be a difficult task to obtain by RT-PCR, Northern blot, or *in situ* hybridization if carried separately on individual genes.

Kinases and Phosphatases

It was demonstrated that gonadotropic hormones could activate the MAPK cascade (phosphorylation of ERK1 and ERK2) during luteinization of the granulosa cells *(29,30)*. Thus, extensive modulation of signaling molecules during the luteinization process mediated by gonadotropins may be expected. Indeed, it was demonstrated recently that stimulation of human granulosa cells by LH, FSH, or Fk showed variations in a wide spectrum of related genes, of which the vast majority were not reported earlier to be mediated by gonadotropins in human granulosa cells. Among them mitogen-activated protein kinase 1, 5, and 7 (MAP2K1, MAP3K5, and MAP3K7) genes were upregulated by gonadotropins and Fk. By contrast, MAP2K3, MAPK4K4, MAPK6, and MAPK14 genes activities decreased. It is possible that activation of MAPK (phosphorylation of ERK1 and ERK2) may lead to changes in the expression of kinases involved in this cascade, although we do not know yet which of them are phosphorylated in response to gonadotropins and cAMP stimulation. An increase in protein kinase gene and the cAMP-

dependent regulatory type 1 alpha (PRKARIA) gene both by gonadotropins and cAMP were expected because gonadotropins and Fk significantly elevated intracellular cAMP in the rat ovary *(3)*. However, it has not yet been reported whether PRKARIA gene activity is elevated in the human ovary until it was revealed by DNA microarray study. Upregulation of serum glucocorticoid–regulated kinase (SGK) gene activity was shown to increase by LH and FSH using the DNA microarray technique and was demonstrated to be elevated by gonadotropins in the rat ovary *(45)*. Interestingly, DNA microarray analysis found no elevation of gene transcripts coding for SGK by Fk, suggesting for the first time that this gene is not modulated by cAMP *(15)*. According to the DNA microarray analysis gonadotropin stimulation leads to upregulation of the following transcripts for protein phosphatases: PPE14, PPP2R1A, PPP2R5B, PTP4A1, PTPRF, PTPRN, and PTPN21. By contrast, there was a reduction in the expression of transcripts including MKP-L, PPP2R5B, PPP3R1, and PTPN1 *(15)*. Because protein phosphatases can abrogate the activity of protein kinases *(9,29, 30)*, the net outcome of protein phosphorylation modulated by gonadotropins are still hard to predict. However, the use of DNA microarray may open the way in resolving complex signaling cascades, and the crosstalk between them not only on the post-transcriptional and post-translational levels, but also on the transcriptional level itself.

Specific LH and FSH Responsive Genes

The use of DNA microarrays can provide some insight to the possible preferential regulation of specific novel genes by LH or FSH in the same cells and whether cAMP could serve uniquely as a second messenger. For this purpose human granulosa cells obtained from the IVF program were challenged by LH, FSH, or Fk after a 7 d period of culturing in vitro in medium free of hormone for the purpose of desensitization to the gonadotropic hormones *(15)*. In significant cases the hormone-specific gene activation did not involve cAMP as second messenger (Table 1).

Genes preferentially activated by LH (and not FSH) via cAMP-independent manner are RAB11B, SMARCF1, SOX9, TGF beta, VEGF, RERE, and lipase protein. Amphiregulin and epiregulin were found to be activated by LH about 31-fold and 16-fold, respectively compared to FSH stimulation. Because both genes can be activated by Fk, it seems there could be some alternative pathway to activate the later two genes. RT-PCR of both amphiregulin and epiregulin could also confirm this notion *(26,31)*. SLL25A13 and RSN genes were found preferentially downregulated by LH and not by FSH with no significant effect by Fk. Genes that were found to be activated by FSH and not by LH and were not activated by Fk are BPES, TFCP2, ANGPT2, and EGR1 (Table 1).

Genes that were much more activated by FSH than by LH were RAB31, PGRMC1, claudin 3, FOXL2, JAG1, CD24 (downregulated), and EGR3 (downregulated). These are only preliminary results that have not yet been verified by RT-PCR. These exemplified the potential of the DNA microarray technique to screen genes of special interest on a large scale.

Concluding Remarks

The use of DNA microarray technique and RNA isolated from ovarian samples has the potential to reveal gene activities specific to the ovary that were not identified previously by other standard techniques like RT-PCT or Northern blot hybridization methods. This information can be obtained using commercially available microarrays. However, in order to reveal mutations in specific genes, tailored DNA arrays must be constructed. Other concerns are sensitivity of the method, quantitation, and comparing data obtained from different DNA chips to other alternative methods: according to the experiments conducted in our laboratory, a specific expression of a gene could be defined as "absent" on the DNA microarray, but can be demonstrated by RT-PCR. This was the case for EGF receptor (our unpublished observations). Therefore, the sensitivity of DNA microarray assay may be sometimes lower compared to conventional methods. As for quantitation, it seems that quantitative RT-PCR would give more accurate results than DNA microarray analysis. And, finally, comparison of data between different DNA chips, especially in using different oligonucleotides, can give the direction of changes upon specific stimulation rather than accurate magnitude of changes in gene expression.

A combined approach of DNA microarray and standard analyses are needed particularly for finding possible roles for gene transcripts that their function has not yet been defined.

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Table 1
Genes (Transcriptomes) Preferentially Modulated by LH or FSH in Human Granulosa Lutein Cells

			Fold change above control								
Gene	Accession	Abbreviation	LH			FSH			FK		
A. Preferentially modulated by LH											
"RAB11B, member RAS oncogene family"	X79780	RAB11B	P	55.7	I	A	24.2	NC	A	−1.3	MD
"SWI/SNF related, matrix associated, actin dependent regulator of chromati subfamily f. member 1"	AF231056.1	SMARCF1	P	36.7	I	A	10.6	NC	A	1.1	NC
amphiregulin (schwannoma-derived growth factor	NM_001657.1	AREG	P	31.4	I	P	13.7	I	P	45.6	I
epiregulin	NM_001432.1	EREG	P	16.8	I	P	7.3	I	P	17.6	I
"SRY (sex determining region Y)-box 9 (campomelic dysplasia, autosomal sex-reversal)"	AI382146	SOX9	P	13.9	I	A	4.6	NC	A	4.0	NC
vascular endothelial growth factor	AF091352.1	VEGF	P	11.9	I	P	7.3	NC	P	0.1	I
transforming growth factor-beta type III receptor	L07594	TGF-beta	P	10.9	I	P	1.7	NC	P	0.1	NC
arginine-glutamic acid dipeptide (RE) repeats	AF016005.1	RERE	P	7.0	I	A	3.5	NC	A	1.3	NC
lipase protein	AF225418.1	LP	P	5.3	I	P	2.0	NC	A	3.5	NC
"solute carrier family 25, member 13 (citrin)"	AC002540	SLC25A13	P	5.3	I	A	1.1	NC	A	3.2	NC
restin (Reed-Steinberg cell-expressed intermediate filament-associated prot	M97501.1	RSN	P	4.6	I	P	1.1	NC	P	1.0	NC
regulator of G-protein signalling 12	AF030111.1	RGS12	A	−13.9	D	A	−2.3	NC	A	−3.5	NC
B. Preferentially modulated by FSH											
claudin 3	NM_001306.1		A	14.44	I	P	22.1	I	A	6.25	NC
RAB31, member RAS oncogene family	BE789881	RAB31	P	9.0	I	P	16.0	I	M	−2.6	D
progesterone receptor membrane component 1	AL547946	PGRMC1	P	9.0	I	P	16.0	I	P	0.4	NC
transcription factor CP2	NM_005653.1	TFCP2	P	1.56	NC	P	7.3	I	A	1.44	NC
growth arrest and DNA-damage-inducible, gamma	NM_006705.2	GADD45G	P	2.4	I	P	5.8	I	P	0.6	MI
angiopoietin 2	AF187858.1	ANGPT2	P	1.56	NC	P	5.3	I	P	−0.16	D
forkhead transcription factor FOXL2 (BPES)	NM_023067.1	BPES	P	0.01	NC	P	0.6	MI	P	0.0	NC
jagged 1 (Alagille syndrome)	U77914.1	JAG1	P	−5.8	D	P	−27.6	D	P	−13.7	D
early growth response 3	X63741	EGR3	P	−4.4	D	P	−21.2	D	P	−4.4	D
CD24 antigen (small cell lung carcinoma cluster 4 antigen)	AA761181	CD24	P	−4.4	D	P	−8.4	D	P	−1.2	NC
early growth response 1	NM_001964.1	EGR1	P	−1.69	NC	P	−4.0	D	P	1.21	NC

Data obtained from U133 Affymetrix DNA chip are expressed as log (2) and were converted to antilog (2) to obtain the actual fold of chscale. LH, luteinizing hormone 1 IU/mL; FSH, follicle-stimulating hormone 1 IU/mL; FK, forskolin 50 μ*M*; P, present; fold of changes are significant $p < 0$. A, absent (below detection); changes are not significant $p < 0.05$. I, increase; MI, mild increase; D, decrease; MD, mild decrease; NO, no charge.

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