Molecular characterization of the human ovulatory cascade— Lesson from the IVF/IVM model

Gil M. Yerushalmi · Ettie Maman · Yuval Yung ·
Alon Kedem · Ariel Hourvitz

Received: 8 May 2011 / Accepted: 24 May 2011 / Published online: 11 June 2011
© Springer Science+Business Media, LLC 2011

Abstract

Aims Ovarian follicular development and ovulation in mammals is a complex and highly regulated process. Most advances in the understanding of the ovulatory process have come from animal models. However, translational research in humans is of crucial importance for improving fertility treatment and control.

Methods IVM/IVF procedures allow us to obtain follicular fluid and granulosa cells (GC) from follicles in different developmental stages with and without hCG priming.

Results Using the cells and fluids obtained in IVM/IVF procedures allowed us to characterize human ovulatory gene expression during antral folliculogenesis and ovulation, examine gene expression in luteinized and non-luteinized GC in vivo and in vitro and to use cumulus GC genes as biomarkers for oocyte and embryo maturity and competence.

Conclusion Biological material obtained during IVM/IVF procedures is an important tool to study the human ovulatory cascade and can serve to improve IVM techniques and fertility treatment and control.

Keywords IVM · IVF · Model · Ovulation · Ovulatory genes

Introduction

Ovarian follicular development and ovulation in mammals is a highly regulated process. This process involves the selection of a dominant follicle, reactivation of oocyte meiosis, rupture of the follicle wall, cumulus expansion and tissue remodeling to form the corpus luteum. These processes are fundamental to successful establishment of pregnancy, but importantly also impact on the developmental potential of resultant embryos. Genes involved in this processes, have been the subject of growing interest [1, 2].

The LH surge, derived from the pituitary, is the single trigger, initiating the cascade of events, leading to ovulation. Elucidation of these fascinating processes and understanding the molecular regulation of the human ovulatory cascade is of critical importance.

The main events resulting from the LH surge are cumulus expansion, oocyte maturation, follicular rupture, and luteinization, all of which are essential for proper reproduction (Scheme 1).

In response to the LH surge, various changes in gene expression and follicular structure occur in the theca, granulosa, cumulus and oocyte compartments of the ovarian follicle. Systemic and local inputs co-ordinate with signals from the oocyte, Thus ovulation is under complex control facilitating synchronization of oocyte maturation with its release and permitting the selection of oocytes with full developmental competence [3].

Traditionally, animal models have served for the study of human biological processes [4]. Animal models have several important advantages including availability, low variability, genetic homogeneity, use in time-course experiments and transgenic and knock-out models. In fact, it will be reasonable to say that research on animals has taught us nearly all we know about cell biology [4, 5].

Capsule We show how biological material obtained during IVM/IVF procedures is used for translational research from animal models to the human ovulatory cascade.

G. M. Yerushalmi (✉) · E. Maman · Y. Yung · A. Kedem ·
A. Hourvitz
IVF Unit, Department of Obstetrics and Gynecology, Chaim
Sheba Medical Centre, Tel-Hashomer, Affiliated to the Sackler
School of Medicine, Tel-Aviv University,
Tel-Aviv, Israel
e-mail: gil.yerushalmi@gmail.com

Scheme 1 Ovulation pathways following the LH surge

However, animal models have also several disadvantages including the variability from humans and the ethical controversies regarding animal research and their welfare [4, 6]. Yet, most scientists agree that the need for models organism will only diminish once most of the fundamental mechanisms of biology have been solved to allow the greater use of both human tissue cultures and in-silico methods for drug discovery [4].

Most of the recent advances in understanding of the molecular basis of folliculogenesis and ovulation have come from rodent models [7]. Also a considerable contribution came from studies in domestic animal and primate species. However, which species are most appropriate model for understanding critical genes and processes in human ovulation is currently unclear.

In the human, like in the mouse, LH and FSH were shown in vitro and in clinical practice to orchestrate the ovulatory cascade. Only few studies in human revealed common mechanisms with animal studies. For example, FSH was shown to induce LHR in mural granulosa cells. [8, 9], and hCG to induce amphiregulin expression and progesterone synthesis [9, 10]. However, it appears that there are differences in a variety of molecular mechanisms between human and animal models. For example, SULT1E1 was found to be expressed in the rodent ovary and highly regulated during folliculogenesis. Moreover, knock-out mice suffered from impaired ovulation [11]. However, this gene was not found to be expressed in primate and human granulosa cells (personal communication). Another gene, sFRP4 was shown in the mouse to be up-regulated by hCG [12–14] and presumably to inhibit β -catenine, an inhibitor of the LH pathway [13]. We found

that sFRP4 in human is down-regulated by hCG both in vitro and in vivo, while sFRP5 is induced by hCG and may replace the role of sFRP4 as LH mediated β -catenine suppression in the human ovary [15].

Thus, data obtained from animal studies must be validated in human experiments. However, there are relatively few publications on the molecular mechanism of human ovulatory process which is probably due to ethical and practical difficulties in obtaining relevant samples.

For example, the process of cumulus expansion in ovulation is extensively studied and well characterized in mammals, especially murine models. However, the data on this important process in humans is scarce and mainly observational (Table 1).

Table 1 Summary of available data in humans of genes involved in the process of cumulus expansion in mammals

Gene name	Expression In human GC	Documented Regulation in human GC	Documented Functional role in human GC	Reference
GDF-9	+	+	–	[16, 17]
COX-2	+	+	+	[18]
HAS-2	+	–	–	[19]
TSG-6	+	+	–	[20]
PTX3	+	+	–	[21]
IL-6	+	–	–	[22]
Gp130/IL-6st	+	–	–	[22]
CD44	+	–	–	[23]

Therefore, characterization of the human ovulatory cascade should be performed, based on the data accumulated from mammals' studies. Information from such research is of crucial importance for improving fertility treatment and control.

Our aim is to explore the human folliculogenesis and the preovulatory follicular events. Our model, described in this article is based on follicular fluids and granulosa cells obtained during ART treatments.

Materials and methods

IVF Protocol

GCs from large follicles were obtained at the time of oocyte retrieval for in vitro fertilization (IVF) patients. Briefly, normo-ovulatory patients (<37 years old) undergoing IVF due to male or tubal factors were selected. Treatment protocols included the "long protocol" or "antagonist protocol" described elsewhere [24, 25]. When the leading follicles reached 18 mm in diameter, the patients received chorionic gonadotropin (hCG) (Ovitrelle 250 mcg, Merck Serono). Oocyte retrieval was performed by transvaginal ultrasound-guided needle aspiration.

IVM protocols

In the hormonal-stimulated protocol, normo-ovulatory patients underwent an IVM cycle according to accepted IVM protocols [26]. Briefly, a baseline evaluation that included a hormonal profile and an ultrasound scan was performed on day 2–3 of the menstrual cycle. On day three, 150 IU/day recombinant FSH was added for 3 days. A second evaluation was performed on day 6 of the menstrual cycle. An injection of 10,000 IU hCG (Pregnyl; Organon, Oss, Holland) was administered SC when the endometrial thickness was 5 mm and the leading follicle was at least 12 mm. Transvaginal oocyte retrieval was scheduled 36–38 hr after hCG injection. During oocyte aspiration, the follicles were measured and the follicular fluid (FF) was separated into two groups of small (4–8 mm) and large (10–14 mm) follicles.

For the non-stimulated protocol, normo-ovulatory patients who were unable to receive hormonal treatment underwent an IVM cycle without any hormonal supplementation [26]. Similar baseline evaluation that included a hormonal profile and an ultrasound scan was performed on day 2–3 of the menstrual cycle. A second evaluation was performed on day 6 of the menstrual cycle. Transvaginal oocyte retrieval was scheduled when the endometrial thickness was 5 mm and the leading follicle was at least 12 mm. The FF collection was the same as for the stimulated protocol.

The study was approved by the local Institutional Review Board (IRB) committee, and written informed consent was obtained from each patient.

Mural and cumulus GC extraction and purification

Mural GCs were collected from FF under a microscope, carefully avoiding blood clots, and re-suspended in IVF medium or phosphate buffered solution (PBS). After allowing the cells to settle by gravity for few minutes, the top medium was aspirated, and this step was repeated 2–3 times until the medium and the pellet became clear. The cells were centrifuged at 200 g for 5 min at room temperature, and the resulting pellets were resuspended in red blood cell lysing buffer (Sigma-Aldrich, St. Louis, MO). After a 1-min incubation at 37°C, the mix was diluted with 12 ml PBS and the cells were centrifuged at 200 g for 5 min at room temperature, after which the resulting pellets were subjected to RNA purification or cell culture.

Cumulus GCs were obtained during oocyte denudation for the intracytoplasmic sperm injection (ICSI) procedure. Briefly, after oocyte retrieval, the cumulus cells were removed from the cumulus oocyte complex (COC) by hyaluronidase. The cells were centrifuged at 200 g for 5 min at room temperature and the resulting pellets were subjected to RNA purification or cell culture.

GCs in culture

Preovulatory GCs that had been obtained from follicles during the IVF procedure as described earlier were plated into culture at a density of 50×10^3 cells/well of 24-well plates in basic medium (10% fetal calf serum; Invitrogen, Carlsbad, CA) in Dulbecco's modified Eagle's medium (DMEM) with 1% penicillin/streptomycin. After 48 hrs, the medium was replaced with fresh basic medium with or without 1 U/ml hCG (Ovitrelle, Merck Serono, Geneva Switzerland). The medium was collected for hormonal measurements at different times, and the cells were collected and subjected to total RNA purification, reverse transcription and quantitative real-time PCR (qRT-PCR).

RNA extraction from GCs

Total RNA was extracted from GCs by the Mini RNA Isolation I Kit (Zymo Research Corp., CA), according to manufacturer's instructions. Total RNA (100 ng) from each sample was used for cDNA synthesis using High capacity RT-PCR Kit (Applied Biosystems, Carlsbad, CA) according to manufacturer's instructions: 37°C for 120 min followed by 85°C for 5 min with random hexamer primers (25 ng/ μ l final concentration) in a 10 μ l total volume reaction.

For PCR, aliquots of the cDNA (10%) ReadyMix RedTaq PCR reactive mix (Sigma-Aldrich, St. Louis, MO), and specific primers were mixed to amplify the selected gene, and actin served as an internal control. Amplification was performed using DNA Engine (Bio-Rad, Hercules, CA) with 50°C as annealing temperature and 25 cycles for all primer pairs.

To carry out qRT-PCR, Real time PCR reaction mix contained cDNA, Fast SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA) and specific primers for sFRP4 and β -actin (endogenous control) in a total volume of 10 μ l. Reaction was performed in a StepOne plus realtime PCR apparatus (Applied Biosystems) with specific primers. Cycling parameters were 20 sec at 95°C, and 40 cycles at 95°C for 3 sec and at 60°C for 30 sec. A melting curve analysis was performed at the end of each run to ensure a single amplicon. Analysis of qRT-PCR results was carried out using StepOne software (Applied Biosystems). Relative gene expression was calculated using the Delta delta C (T) method. When an experiment was run on more than one 96 well plate, combination of data was possible using Data assist software (Applied Biosystems). Gene expression levels were calculated relative to the β -actin level in the same sample.

Results and discussion

Up to date, luteinized GC obtained from OPU during IVF serve as the main source of biological material for the study of human ovulation. However, these cells cannot serve as an optimal model to study pre-ovulatory non-luteinized events. Therefore, we take the advantage of IVM procedure to obtain non-luteinized cells and biological material from earlier stages of folliculogenesis.

In vitro maturation of human oocytes (IVM) is an emerging technology with promising potential in specific indications. The main advantage of this technique is the fact that it does not require gonadotropin stimulation, and therefore the treatment is shorter, simpler, less expensive, and most importantly, safer, avoiding the risk of ovarian hyperstimulation syndrome (OHSS). The technique was found to be particularly suitable for women with PCOS and women who are at high risk of OHSS [27, 28].

During IVM process, cumulus-oocyte complexes (COCs) are obtained mostly at germinal vesicle (GV) stage and are matured in vitro by maturation medium into metaphase II (MII) stage [29]. Human oocyte can resume meiosis, reach metaphase II stage, and by intracytoplasmic sperm injection (ICSI) can be fertilized at high ratio [30]. However, the success rate for IVM is still lower compared to IVF procedure [31].

Using IVM-IVF procedures for human ovulatory research

IVM and IVF procedures allow us to obtain biological material from different stages of the follicular development in order to further characterize the human ovulatory cascade. This includes follicular fluid as well as granulosa cells—mural obtained from the follicular fluid and cumulus obtained during oocyte denudation. During IVM without hCG priming, non-luteinized cells can be obtained.

Gene characterization during different stages of antral folliculogenesis

Previously we generated a murine ovulatory-selective cDNA library [32]. In order to examine the presence of these genes and characterize their expression in follicles of different size in human GCs, we used the IVM procedure to obtain mural granulosa cells from small (4–8 mm) and large (10–14 mm) follicles and compare it to mural granulosa cells obtain from the large pre-ovulatory follicles of IVF. The results (Fig. 1) show the presence of several known ovulatory genes such as StAR, amphiregulin, epiregulin, LHR, cyp19A1 (aromatase) and EPHX1 but also genes that were not shown previously or were shown only recently by us in human ovary such as MAGOH, ELOVL5, sFRP4 [15] and ADAMTS1 [33].

This model system allowed the characterization of ovulatory gene expression in vivo during the different stages of antral folliculogenesis. For example, we found that the expression of sFRP4 was inversely correlated to follicular size: mural GCs obtained from small luteinized IVM follicles expressed higher levels of sFRP4 than mural

Fig. 1 Validation and characterization of gene expression in Human mural GCs. RT-PCR was employed on mRNA extracted from GCs purified from small and large follicles aspirated during OPU of IVM and large pre-ovulatory follicles aspirated during OPU of IVF. β -actin was used as control

GCs obtained from large luteinized IVM follicles, and that sFRP4 expression further decreased in mural GCs obtained from preovulatory luteinized follicles [15].

Ovulatory gene expression in luteinized versus non-luteinized GC in vivo

IVM protocols without hCG priming allows us to obtain GCs from non-luteinized follicles and compare them to cells from luteinized follicles from IVM. This allows us to study the effect of LH surge in vivo on selected genes. For example, we found that ADAMTS-1 levels in **non-luteinized** mural GCs were undetectable compared to the high expression that were present in **luteinized** mural GCs obtained from follicles of similar size (>10 mm). These results suggest that ADAMTS-1 is an hCG regulated gene in human mural GCs [33].

Ovulatory gene expression in luteinized versus non-luteinized GC in cell culture

Culture of granulosa cells obtained during ART procedures allows us to study ovulatory gene expression following treatment with various stimulants and inhibitors thus enabling us to further characterize ovulatory pathways in humans.

Luteinized GCs for culture are relatively easy to obtain during IVF treatment cycles and were used by many researchers in the past 20 years. However, there is a concern regarding the use of luteinized GCs in cell culture to study molecular ovulatory processes. Following the LH surge and luteinization there might be major differences in the cellular mechanisms and response to various stimuli of luteinized cells as compared to non-luteinized cells.

It was previously published that granulosa cells regain their responsiveness to gonadotropin stimulation following culture in hormone-free environment for 3–7 days [34] but there are only few published papers using non-luteinized GC [9] and regarding the use of luteinized GC compared to non-luteinized GC [8].

IVM protocols without hCG priming allows us to obtain GCs from non-luteinized follicles and compare them to cells from luteinized follicle in cell culture. Based on published data we established a culture protocol of luteinized GC for 48 h in culture media without gonadotropins.

We used non-luteinized mural GCs and luteinized GC in cell culture and examined the expression of several ovulatory genes following treatment with hCG and FSH. We show the expression of sFRP4 in cultured GC. We found that the expression pattern of sFRP4 in luteinized and non-luteinized cells in response to stimulus by hCG was similar, thus supporting the use of luteinized GCs as a model system to investigate ovulatory processes (Fig. 2).

Fig. 2 Effect of LH/hCG on sFRP4 gene expression in vitro in luteinized and non-luteinized GC. Mural GCs of IVM procedure with (luteinized, blue) or without (non-luteinized, red) hCG priming, were plated (50,000 cells per well of 24-well plates) in 10% FCS in DMEM with 1% penicillin/streptomycin. After three days, the medium was replaced with fresh medium with or without 1 U/ml hCG. At indicated times the cells were harvested and subjected to total RNA purification followed by reverse transcription and qRT-PCR. Primers used were sFRP4 and β -actin (loading control). sFRP4 expression was calculated relative to the β -actin level in the same sample. Results indicate that sFRP4 expression is reduced in response to hCG in both groups

Correlation of selected cumulus genes to oocyte maturation, fertilization capacity and pregnancy

Correlating gene expression in cumulus cells from individual COC to oocyte competence and fertilization outcome can usefully serve as a high throughput and non-invasive means for the assessment of oocyte quality, embryo competence and pregnancy outcome. This technology using quantitative RT-PCR can help to define the potential biomarkers correlated to a competent oocyte and also improve the selection of healthy embryos leading to pregnancy. Increasing number of papers have shown correlation of cumulus gene expression with oocyte maturation [35, 36], fertilization rate [35] and pregnancy outcome [36–38]. McKenzie et al. have shown that the expression levels of GDF9 targets in cumulus cells of individual oocytes has a correlation to embryo quality [35]. Assou et al. employed a robust multiple microarray approach to identify novel cumulus genes as biomarkers for embryo and pregnancy outcome [37]. They identified 45 genes as biomarkers and demonstrated a good correlation between the expression profile of these genes and pregnancy outcome without a relationship to the morphological grade of the embryos [37].

We have previously shown that ADAMTS-1 and sFRP4 can serve as a potential biomarker for oocyte quality. We found that sFRP4 expression in cumulus GC is inversely correlated to oocyte maturation, with the highest expression in the cumulus of GV oocytes compared to that of MI oocytes and the lowest expression in the cumulus of MII oocytes [15]. Also we found that ADAMTS-1 expression in cumulus GC is positively correlated to oocyte fertilization capacity [33].

In summary, in order to improve assisted reproductive technology (ART), there is an urgent need for basic and translational research of reproduction study in human. These studies should validate the relevance of the findings learned from the animal models. The introduction of IVM widens the possibilities of sample collection. Thus, using the combination of IVF-IVM to acquire biological material for research allows us to further characterize different aspects of the complex and essential process of ovulation.

References

- Richards JS, Russell DL, Robker RL, Dajee M, Alliston TN. Molecular mechanisms of ovulation and luteinization. *Mol Cell Endocrinol*. 1998;145:47–54.
- Robker RL, Russell DL, Yoshioka S, Sharma SC, Lydon JP, O'Malley BW, et al. Ovulation: a multi-gene, multi-step process. *Steroids*. 2000;65:559–70.
- Richards JS, Pangas SA. The ovary: basic biology and clinical implications. *J Clin Invest*. 2010;120:963–72.
- Hunter P. The paradox of model organisms. The use of model organisms in research will continue despite their shortcomings. *EMBO Rep*. 2008;9:717–20.
- Lemon R, Dunnett SB. Surveying the literature from animal experiments. *BMJ*. 2005;330:977–8.
- Knight A. Systematic reviews of animal experiments demonstrate poor contributions toward human healthcare. *Rev Recent Clin Trials*. 2008;3:89–96.
- Russell DL, Robker RL. Molecular mechanisms of ovulation: coordination through the cumulus complex. *Hum Reprod Update*. 2007;13:289–312.
- Lindeberg M, Carlstrom K, Ritvos O, Hovatta O. Gonadotrophin stimulation of non-luteinized granulosa cells increases steroid production and the expression of enzymes involved in estrogen and progesterone synthesis. *Hum Reprod*. 2007;22:401–6.
- Negishi H, Ikeda C, Nagai Y, Satoh A, Kumasako Y, Makinoda S, et al. Regulation of amphiregulin, EGFR-like factor expression by hCG in cultured human granulosa cells. *Acta Obstet Gynecol Scand*. 2007;86:706–10.
- Ben-Ami I, Armon L, Freimann S, Strassburger D, Ron-El R, Amsterdam A. EGF-like growth factors as LH mediators in the human corpus luteum. *Hum Reprod*. 2009;24:176–84.
- Gershon E, Hourvitz A, Reikhav S, Maman E, Dekel N. Low expression of COX-2, reduced cumulus expansion, and impaired ovulation in SULT1E1-deficient mice. *FASEB J*. 2007;21:1893–901.
- Fan HY, Liu Z, Shimada M, Sterneck E, Johnson PF, Hedrick SM, et al. MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility. *Science*. 2009;324:938–41.
- Fan HY, O'Connor A, Shitanaka M, Shimada M, Liu Z, Richards JS. Beta-catenin (CTNNB1) promotes preovulatory follicular development but represses LH-mediated ovulation and luteinization. *Mol Endocrinol*. 2010;24:1529–42.
- Hsieh M, Mulders SM, Friis RR, Dharmarajan A, Richards JS. Expression and localization of secreted frizzled-related protein-4 in the rodent ovary: evidence for selective up-regulation in luteinized granulosa cells. *Endocrinology*. 2003;144:4597–606.
- Maman E, Yung Y, Cohen B, Konopnicki S, Dal Canto M, Fadini R et al (2011) Expression and regulation of sFRP family members in human granulosa cells. *Mol Hum Reprod*.
- Oron G, Fisch B, Ao A, Zhang XY, Farhi J, Ben-Haroush A, et al. Expression of growth-differentiating factor 9 and its type 1 receptor in human ovaries. *Reprod Biomed Online*. 2010;21:109–17.
- Shi FT, Cheung AP, Klausen C, Huang HF, Leung PC. Growth differentiation factor 9 reverses activin A suppression of steroidogenic acute regulatory protein expression and progesterone production in human granulosa-lutein cells. *J Clin Endocrinol Metab*. 2010;95:E172–80.
- Tsai EM, Chan TF, Chen YH, Hsu SC, Chuang CY, Lee JN. Mifepristone attenuates human chorionic gonadotropin-induced extracellular signal-regulated kinase 1/2 phosphorylation, cyclooxygenase-2, and prostaglandin E2 production in human granulosa luteal cells. *Fertil Steril*. 2008;89:1522–9.
- Dunning KR, Lane M, Brown HM, Yeo C, Robker RL, Russell DL. Altered composition of the cumulus-oocyte complex matrix during in vitro maturation of oocytes. *Hum Reprod*. 2007;22:2842–50.
- Haouzi D, Assou S, Mahmoud K, Hedon B, De Vós J, Dewailly D, et al. LH/hCGR gene expression in human cumulus cells is linked to the expression of the extracellular matrix modifying gene TNFAIP6 and to serum estradiol levels on day of hCG administration. *Hum Reprod*. 2009;24:2868–78.
- Zhang X, Jafari N, Barnes RB, Confino E, Milad M, Kazer RR. Studies of gene expression in human cumulus cells indicate pentraxin 3 as a possible marker for oocyte quality. *Fertil Steril*. 2005;83 Suppl 1:1169–79.
- Keck C, Rajabi Z, Pfeifer K, Bettendorf H, Brandstetter T, Breckwoldt M. Expression of interleukin-6 and interleukin-6 receptors in human granulosa lutein cells. *Mol Hum Reprod*. 1998;4:1071–6.
- Ohta N, Saito H, Kuzumaki T, Takahashi T, Ito MM, Saito T, et al. Expression of CD44 in human cumulus and mural granulosa cells of individual patients in in-vitro fertilization programmes. *Mol Hum Reprod*. 1999;5:22–8.
- Elizur SE, Lerner-Geva L, Levron J, Shulman A, Bider D, Dor J. Factors predicting IVF treatment outcome: a multivariate analysis of 5310 cycles. *Reprod Biomed Online*. 2005;10:645–9.
- Hourvitz A, Lerner-Geva L, Elizur SE, Baum M, Levron J, David B, et al. Role of embryo quality in predicting early pregnancy loss following assisted reproductive technology. *Reprod Biomed Online*. 2006;13:504–9.
- Fadini R, Dal Canto MB, Mignini Renzini M, Brambillasca F, Comi R, Fumagalli D, et al. Effect of different gonadotrophin priming on IVM of oocytes from women with normal ovaries: a prospective randomized study. *Reprod Biomed Online*. 2009;19:343–51.
- Banwell KM, Thompson JG. In vitro maturation of Mammalian oocytes: outcomes and consequences. *Semin Reprod Med*. 2008;26:162–74.
- Hourvitz A, Maman E, Dor J. Oocytes in-vitro maturation—a new technique for reproductive endocrinologist practitioners. *Harefuah*. 2007;146:860–866, 909.
- Pincus G, Enzmann EV. The comparative behavior of mammalian eggs in vivo and in vitro: I. The activation of ovarian eggs. *J Exp Med*. 1935;62:665–75.
- Russell JB, Knezevich KM, Fabian KF, Dickson JA. Unstimulated immature oocyte retrieval: early versus midfollicular endometrial priming. *Fertil Steril*. 1997;67:616–20.
- Jurema MW, Nogueira D. In vitro maturation of human oocytes for assisted reproduction. *Fertil Steril*. 2006;86:1277–91.
- Hourvitz A, Gershon E, Hennebold JD, Elizur S, Maman E, Brendle C, et al. Ovulation-selective genes: the generation and characterization of an ovulatory-selective cDNA library. *J Endocrinol*. 2006;188:531–48.

33. Yung Y, Maman E, Konopnicki S, Cohen B, Brengauz M, Lojkin I, et al. ADAMTS-1: a new human ovulatory gene and a cumulus marker for fertilization capacity. *Mol Cell Endocrinol*. 2010;328:104–8.
34. Breckwoldt M, Selvaraj N, Aharoni D, Barash A, Segal I, Insler V, et al. Expression of Ad4-BP/cytochrome P450 side chain cleavage enzyme and induction of cell death in long-term cultures of human granulosa cells. *Mol Hum Reprod*. 1996;2:391–400.
35. 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;19:2869–74.
36. Feuerstein P, Cadoret V, Dalbies-Tran R, Guerif F, Bidault R, Royere D. Gene expression in human cumulus cells: one approach to oocyte competence. *Hum Reprod*. 2007;22:3069–77.
37. Assou S, Haouzi D, De Vès J, Hamamah S. Human cumulus cells as biomarkers for embryo and pregnancy outcomes. *Mol Hum Reprod*. 2010;16:531–8.
38. Hamel M, Dufort I, Robert C, Gravel C, Leveille MC, Leader A, et al. Identification of differentially expressed markers in human follicular cells associated with competent oocytes. *Hum Reprod*. 2008;23:1118–27.