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Matrix metalloproteinases and their inhibitors in human cumulus and granulosa cells as biomarkers for oocyte quality estimation

Alice Luddi, Ph.D.,[a] Martina Gori, Ph.D.,[a] Camilla Marrocco, B.Sc.,[a] Angela Capaldo, Ph.D.,[a] Valentina Pavone, B.Sc.,[b] Laura Bianchi, Ph.D.,[b] Letizia Boschi, B.Sc.,[a] Giuseppe Morgante, M.D.,[a,c] Paola Piomboni, Ph.D.,[a,c] and Vincenzo de Leo, M.D.[a,c]

[a] Department of Molecular and Developmental Medicine and [b] Department of Life Science, University of Siena; and [c] Center for Diagnosis and Treatment of Couple Sterility, S. Maria alle Scotte Hospital, Siena, Italy

Objective: To study the molecular profile of metalloproteinases and their tissue inhibitors in granulosa and cumulus cells in a subset of fertile and infertile women.
Design: Molecular study with granulosa and cumulus cells.
Setting: University hospital.
Patient(s): Forty-four women undergoing assisted reproductive techniques for female infertility factor, with partners having a normal spermiogram and 15 normally fertile women with male partner affected by severe oligoasthenoteratozoospermia or nonobstructive azoospermia.
Intervention(s): In vitro fertilization.
Main Outcome Measurement(s): We investigated gene expression level of metalloproteinases (*MMP2*, *MMP9*, *MMP11*) and their tissue inhibitors (*TIMP1*, *TIMP2*) by means of quantitative reverse-transcription polymerase chain reaction, protein quantification by means of Western blot, and localization by means of immunofluorescence.
Result(s): We firstly validated *HPRT1* as the most reliable housekeeping gene enabling correct gene expression analysis in both granulosa and cumulus cells. Gene expression, Western blot, and immunofluorescence analysis of MMP2, MMP9, and MMP11 and their tissue inhibitors TIMP1 and TIMP2 demonstrated that these enzymes are finely tuned in these cells. MMP9 is specifically expressed only in granulosa, whereas MMP2 is more expressed in cumulus and granulosa cells in cases of reduced ovarian response and decreased fertilization rate.
Conclusion(s): This study sheds light on MMP and TIMP expression in granulosa and cumulus cells, and it may help in understanding the fine regulation of oocyte maturation inside the follicle. Although further studies are needed to fully understand the molecular mechanisms involved in these processes, our findings may be useful in the identification of biomarkers of oocyte maturation, competence acquiring, and fertilization. (Fertil Steril® 2018;109:930–9. ©2018 by American Society for Reproductive Medicine.)
Key Words: In vitro fertilization, granulosa cells, cumulus cells, qRT-PCR, oocyte quality

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In mammals, reproduction is a finely regulated and highly specialized process. In the female, the reproductive cycle begins with oocyte growth inside the ovarian follicle, which provides for and influences the oocyte quality. Indeed, the oocyte development is the result of complex and dynamic developmental relationship processes within the antral follicle (1), characterized by the presence of specialized cells lining the antrum filled with the follicular fluid (FF). It has been previously reported that the FF presents with a huge protein complexity and a very wide dynamic range of proteins involved in numerous different pathways that may affect follicle growth, oocyte maturation, and competence acquiring (2).

The human FF results, prevalently, from capillary diffusion as well as from granulosa, cumulus, and theca cell secretions; granulosa cells (GCs)

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Reprint requests: Paola Piomboni, Ph.D., Department of Molecular and Developmental Medicine, University of Siena, Via Aldo Moro 2, 53100 Siena, Italy (E-mail: paola.piomboni@unisi.it).

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line the follicle wall and cumulus cells (CCs) maintain direct contact with the developing oocyte (3). This close proximity allows a cross-talk to be established between oocyte and CCs leading to proper CC differentiation by directly affecting gene expression and protein synthesis (4, 5). This closed metabolic cooperation has a significant influence also in oocyte maturation and fertilization (1). Therefore, the characterization of the mechanisms regulating oocyte differentiation within the ovarian follicle and the definition of the somatic cells' role in this delicate process are crucial for the development of more effective protocols for ovarian stimulation and in vitro fertilization (IVF). In addition, it would help to improve pregnancy outcome with the use of IVF and reduce the multiple-birth rate by allowing the transfer of single embryos.

Oocyte quality estimation during IVF procedures is so far mainly based on unreliable morphologic criteria that are considered to be largely unsatisfactory. Although several supposed biomarkers have been identified (6–8), the outcome of IVF procedures remains poor owing to the limited improvements in clinical treatment strategy. In fact, despite the fundamental progresses of basic research, translational medicine still requires a great deal of effort to translate new discoveries into helpful clinical applications. The scientific community is therefore focused on discovering other molecular biomarkers to help in the selection of the best oocyte.

Based on their important relationship with the oocyte, investigation into the transcriptional profile of CCs and GCs represents an attractive method to noninvasively predict gamete and embryo quality. Although much still remains unknown or unclear, significant progress has been made in unraveling this complex system. Based on literature data derived from the proteomic analysis of human FF of women undergoing ovarian hyperstimulation for IVF, we functionally correlated all of the identified proteins (2, 9) by performing a pathway analysis. We presented evidence that a fine and tight control of enzymatic proteolysis regulates inflammatory reaction, wounding response, coagulation cascade, and extracellular matrix (ECM) remodeling into mature follicles (9). The network generated by our in silico analyses clearly indicated that gelatinases matrix metalloproteinase (MMP) 2 and MMP9 are the two main central hubs, with 95 and 84 distinct interconnections with other molecules, respectively, representing 40% of FF proteins entered in the net (9). The selective proteolytic activity of MMPs is finely balanced by endogenous tissue inhibitors of metalloproteinases (TIMPs) (10, 11). It has been demonstrated that TIMP1 is a specific inhibitor of MMP9 (12) and that TIMP2 regulates the activity of MMP2 in several tissues (13) and in particular in CCs (14).

These data suggest a deep involvement of MMPs and TIMPs both in ovarian follicle homeostasis and in oocyte maturation and competence acquiring. To determine the extent to which this may occur, gene expression analysis of key MMPs and TIMPs was carried out in GCs and CCs from women undergoing IVF procedures, and their expression correlated with IVF outcome parameters.

MATERIALS AND METHODS

Patient Selection

CCs and GCs were recovered from 63 white patients who underwent assisted reproduction techniques at the Center for Diagnosis and Treatment of Couple Sterility of the Obstetrics and Gynecology Unit, Siena University Hospital. All patients enrolled in this study provided informed consent to the use of organic waste material for research purposes. All experimental protocols, approved by institutional local Ethic Committees, were carried out in accordance with relevant guidelines and regulations.

Inclusion criteria for infertile women ($n = 44$) were: age >18 and <44 years, absence of chromosomal abnormalities, and IVF indication for endocrine, tubal, or unexplained infertility factor. Patients with hypothalamic hypogonadism, hyperprolactinemia, or endometriosis were excluded. Infertility due to male factor was also excluded.

Inclusion criteria for fertile women ($n = 15$) were: age >18 and <35 years, regular menstrual cycles every 25–32 days, and absence of chromosomal abnormalities, hypothalamic hypogonadism, hyperprolactinemia, endometriosis, hormonal disorders. A male infertility factor (nonobstructive azoospermia, severe oligoasthenoteratozoospermia) was diagnosed in each couple. Demographic characteristics of enrolled patients are summarized in Supplemental Table 1 (available online at www.fertstert.org).

Ovarian Hyperstimulation Protocol

Ovarian hyperstimulation was performed with the use of recombinant FSH. A GnRH antagonist was used to prevent a premature LH surge. According to our protocol, subcutaneous administration of recombinant FSH was started on day 2 or 3 of the menstrual cycle. The starting dose (225 IU/d) was increased up to 300 IU/d according to the individual response, as evaluated by means of ultrasound examination and E_2 and P serum levels, on alternate days starting from the 6th day of stimulation. Oocyte pick up was planned when at least one follicle reached 18 mm in diameter 34–36 hours after administration of recombinant human chorionic gonadotropin (hCG). FF samples were obtained after cumulus-oocyte complex (COC) recovery and used to isolate GCs.

Cumulus and Granulosa Cell Isolation

Human GCs were collected with the use of a 45% Pure Sperm gradient (Nidacon, Biocare Europe) according to a previously described procedure (15) and incubated at 37°C, with 5% CO_2, in Dulbecco Modified Eagle Medium (Invitrogen) containing 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin to allow adhesion, then the GCs were collected and stored at -80°C. To confirm enrichment in GCs and elimination of contaminating leukocytes, purified cells were tested for *CSF* gene expression. COCs were incubated in Continuous Single Culture Complete (CSCC) medium (Irvine, Biocare Europe) for ~2 hours before CC removal. COCs were exposed to Hyaluronidase Solution (Irvine, Biocare Europe) for 20 seconds, and then CCs were stripped from the oocyte with the use of a micropipette and the isolated oocytes transferred in 0.5 mL equilibrated CSCC

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