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Identification of genes targeted by FSH and oocytes in porcine granulosa cells

E.J.C. Verbraak[a], E.M. van 't Veld[a], M. Groot Koerkamp[b], B.A.J. Roelen[c], T. van Haeften[a], W. Stoorvogel[a], C. Zijlstra[a,*]

[a] *Department of Biochemistry and Cell Biology, Utrecht University, Yalelaan 2, 3584 CM Utrecht, the Netherlands*
[b] *Department of Physiological Chemistry, University Medical Centre Utrecht, Heidelberglaan 100, 3584 CX Utrecht, the Netherlands*
[c] *Department of Farm Animal Health, Utrecht University, Yalelaan 104, 3584 CN, Utrecht, the Netherlands*

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Abstract

In the mammalian ovarian follicle maturing oocytes are nurtured and supported by surrounding somatic cells, the mural granulosa cells and the cumulus cells. These cells are regulated by follicle-stimulating hormone (FSH), originating from the pituitary, and paracrine factors derived from the oocyte. To gain insight into the mechanisms involved in the regulation of granulosa cell function, this study aimed to identify genes in mural granulosa cells that are regulated by FSH and oocyte secreted factors using the pig as a model organism. Mural granulosa cells were collected from 3–6 mm follicles from sow ovaries and cultured in serum free medium in the presence or absence of FSH and/or isolated cumulus oocyte complexes (COCs). FSH significantly increased both the metabolic activity and progesterone production of granulosa cells, while the presence of COCs reversed these FSH effects. Expression levels of mRNA in the absence/presence of FSH and COCs were analyzed on porcine specific microarrays representing 11,300 genes. Both previously identified and novel FSH target genes as well as some oocyte affected genes were found. Expression of inhibitor of DNA binding protein 2 and 3, *ID2* and *ID3*, was decreased by FSH but increased by COCs, as validated by quantitative PCR. These proteins function as dominant negative basic helix loop helix (bHLH) transcription factors and since all regulated genes contain the consensus E-box sequence that can bind bHLH factors, our data suggest that FSH and COCs may regulate granulosa cell function by tuning the activity of bHLH factors, through ID2 and ID3.

Keywords: Ovary; Granulosa cell; FSH; Oocyte secreted factors; Gene expression; Oocyte quality

1. Introduction

The development of mammalian ovarian follicles is regulated by endocrine factors originating from the pituitary, i.e. follicle-stimulating hormone (FSH) and luteinizing hormone (LH), as well as by paracrine oocyte secreted factors (OSFs) [1]. The relevant somatic cells that are directly influenced by these factors are the follicular granulosa cells. Early in folliculogenesis, only one population of granulosa cells (GCs) is present which, upon formation of the antrum, differentiates into mural GCs and cumulus cells. Mural GCs line the follicular wall and antrum and are primarily involved in steroidogenesis [2]. Cumulus cells are closely associated with the oocyte, together forming the cumulus-oocyte-complex (COC), and regulate oocyte growth, development and nuclear maturation through direct cell contact [1,3–7].

* Corresponding author: Tel: + 31 (0) 30-253 3500; fax: + 31 (0) 30-253 5492.
E-mail address: c.zijlstra@uu.nl (C. Zijlstra).

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For the initiation of folliculogenesis and development up to the secondary follicle stage, FSH and LH are not required [8,9], but follicles do become dependent on FSH during late preantral stages [10,11], coinciding with the initiation of FSH receptor mRNA expression in GCs [12]. Release of FSH by the pituitary is under control of the hypothalamus derived gonadotrophin-releasing hormone, and FSH receptors are located exclusively on granulosa cells of developing follicles. Physiological responses to FSH are accomplished through activation of multiple signaling cascades that regulate the expression of select target genes [13–16]. FSH action includes the induction of GC proliferation [17,18], stimulation of GC oestradiol production by increasing aromatase expression [19] and induction of LH receptor expression on both GCs and theca cells [20,21].

In comparison to our knowledge of FSH, much less is known about the control of the release of OSFs and their mode of action. OSFs have been reported to have opposite effects on GCs as compared to FSH; while FSH stimulates the steroid-metabolizing ability of GCs, OSFs decrease the overall steroidogenic capacity of both cumulus cells and mural GCs [22]. Furthermore, several studies have shown that OSFs play an important role in the differentiation of GCs into the mural and cumulus GC phenotypes and are involved in the maintenance of the follicular microenvironment [1,7,23]. Although experimental data suggest that cAMP plays a role in OSF-signaling [22], the precise mode of action of OSFs remains unclear.

Given the reverse effects of FSH and OSFs on GC function, the present study was undertaken to identify common target genes for FSH and OSFs. For that purpose, porcine GCs were used as a model and cultured in serum free medium in the presence or absence of FSH and/or intact COCs, with the latter serving as source of OSFs. The serum free culture system for porcine granulosa cells was previously established by Picton and colleagues [24]. In this system GCs maintain their oestradiol producing capacity, which is lost if the cells are cultured in the presence of serum.

2. Materials and methods

Ovaries from cyclic sows (*Sus scrofa*) were obtained immediately after slaughter at a slaughterhouse and transported to the laboratory at 27–33 °C within 2 h. Here, surrounding tissue was removed and ovaries were rinsed with running tap-water (27–33 °C) and subsequently submersed in phosphate buffered saline (PBS; Invitrogen, Breda, The Netherlands) supplemented with 100 U/mL penicillin (Invitrogen) and 100 μg/mL streptomycin (Invitrogen) at 37 °C.

2.1. Isolation and standard culture of mural GCs and COCs

Mural GCs were collected by aspirating follicular fluid from 3–6 mm healthy follicles [25] with a 26G needle. Collected cells were washed twice by centrifugation at 300× g for 5 min at room temperature with McCoy's 5A culture medium, containing L-glutamin (Invitrogen) and supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1% BSA (Fluka, Zwijndrecht, The Netherlands), 2.5 μg/mL transferrin (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 4 ng/mL sodium selenite (Sigma-Aldrich). Cumulus oocyte complexes (COCs) were removed from the cell suspension by filtration through a 70 μm cell strainer (Becton en Dickinson, Alphen a/d Rijn, The Netherlands), and discarded. The concentration of viable GCs in the sieved cell suspension was estimated by trypan blue exclusion and exceeded 80% in all experiments.

Isolated GCs were cultured for 48 h at 37 °C in a 5% CO2 atmosphere [24] in either 4-well plates (Nunc, Amsterdam, The Netherlands) or 24-well plates (Corning, Schiphol-Rijk, The Netherlands) at a seeding density of 1.4×10^6 cells/650 μL. For culture, the serum free culture medium described above was supplemented with 100 ng/mL testosterone (Fluka), as an oestradiol precursor, and 10 ng/mL insulin (Sigma-Aldrich). When indicated, Long R3- IGF-1 recombinant analogue (IGF-1) (Sigma-Aldrich) and/or porcine FSH (Sigma-Aldrich) were added to final concentrations of 100 ng/mL and 0.1 U/L, respectively. At the end of culture, media were collected for metabolic activity or steroid assays. All treatments within an experiment were carried out in duplicate wells and each experiment was replicated three times using cells isolated from independent batches of ovaries.

Intact COCs meant for culture were isolated from follicular fluid that was aspirated from 3–6 mm follicles with a 18G needle, as described by Schoevers and colleagues [26]. Briefly, follicular fluid contents were allowed to sediment by gravity and the sediment was washed with Tyrode's lactate-HEPES buffer [27] supplemented with 0.1% polyvinylpyrrolidone (PVP; Fluka). Individual COCs with at least two layers of compact cumulus cells and a dark and evenly pigmented ooplasm were selected and collected in wash medium. To evaluate whether oocytes resumed meiosis in the medium we used for GC culture, groups of 25