Molecular and Cellular Endocrinology 366 (2013) 68–80

Contents lists available at SciVerse ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

The glycan structure in recombinant human FSH affects endocrine activity and global gene expression in human granulosa cells

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ARTICLE INFO

Article history:
Received 28 August 2012
Received in revised form 28 October 2012
Accepted 27 November 2012
Available online 20 December 2012

Keywords:
FSH
Glycosylation variant
Steroid
Inhibin
Gene expression
Human granulosa cell

ABSTRACT

The aim of this study was to analyse the biological response to different recombinant human FSH (rhFSH) glycosylation variants on the endocrine activity and gene expression at whole-genome scale in human granulosa-like tumor cell line, KGN. The effects of differences in rhFSH sialylation and oligosaccharide complexity were determined on steroid hormone and inhibin production. A microarray approach was used to explore gene expression patterns induced by rhFSH glycosylation variants. Set enrichment analysis revealed that hormone sialylation and oligosaccharide complexity in rhFSH differentially affected the expression of genes involved in essential biological processes and molecular functions of KGN cells. The relevance of rhFSH oligosaccharide structure on steroidogenesis was confirmed assessing gene expression by real time-PCR. The results demonstrate that FSH oligosaccharide structure affects expression of genes encoding proteins, growth factors and hormones essential for granulosa cells function.

1. Introduction

The growth of ovarian follicles is a complex process regulated by gonadotrophins, steroids, and growth factors (Richards et al., 2002). FSH plays an essential role during ovarian folliculogenesis and its actions have important implications in fertility since female mice deficient in FSH β-subunit or FSH receptor, are infertile (Abel et al., 2000; Kumar et al., 1997). This gonadotrophin not only regulates granulosa cell proliferation and oestradiol production, but also prevents granulosa cell apoptosis and follicular atresia (Chun et al., 1996; Robker et al., 1998).

Like other glycoprotein hormones, FSH consists of a family of glycosylation variants which differ from each other in their oligosaccharide structure including completion of branch synthesis, degree of branching and sialic acid content (Ulloa-Aguirre et al., 1999). Several experimental models have been used to demonstrate the relevance of the FSH oligosaccharides in the regulation of ovarian function. It has been shown that the *in vitro* bioactivity of less sialylated FSH was higher than that exhibited by the more sialylated counterparts in terms of oestradiol production (Zambrano et al., 1996). The biological effect of FSH microheterogeneity has also been studied on follicular growth, antral formation and oestradiol secretion; these studies have demonstrated that FSH glycosylation variants have complementary and specific actions on the developing follicles, and that a specific balance of glycoforms is required for optimal follicle development (Barrios-de-Tomassi et al., 2006; Vitt et al., 1998). Further evidence was obtained by the use of a number of human FSH (hFSH) glycosylation variants isolated from different sources (recombinant, pituitary extracts, urine, etc.). The results clearly demonstrated a close relationship between the biological responses and the oligosaccharide structure present in the FSH molecule (Ulloa-Aguirre et al., 2003).

Molecular biology has strongly contributed to the understanding of how hormones regulate the expression of specific genes in endocrine-dependent cells. FSH has been shown to modify the expression of steroidogenic enzymes including aromatase and cholesterol side chain cleavage (P450scc) as well as genes controlling

Abbreviations: rhFSH, recombinant human FSH; rhFSH-AC, more acidic/sialylated rhFSH charge analogues; rhFSH-BA, less acidic/sialylated rhFSH charge analogues; rhFSH-CO, rhFSH glycoforms bearing complex∗ triantennary and bisecting oligosaccharides; rhFSH-HY, rhFSH glycoforms bearing high mannose/hybrid-type oligosaccharides.

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http://dx.doi.org/10.1016/j.mce.2012.11.021

lipid biosynthesis, differentiation marker genes and inhibin/activin subunits (Eimerl et al., 2002; Fitzpatrick et al., 1991; Liu et al., 2009; Mayo et al., 1994).

More recently developed techniques have made it possible to study large-scale changes in gene expression under a variety of experimental conditions. The microarray approach has been used to analyse the gene expression profile of granulosa cells isolated from women undergoing controlled ovarian hyperstimulation with human recombinant or urinary FSH. The expression of a large number of genes, with functional diversity such as signal transduction, biosynthesis of cholesterol and steroids, gluconeogenesis and regulators of differentiation was differentially affected by the type of the FSH variant used (Brannian et al., 2010; Grøndahl et al., 2009). The discrepancy observed in gene expression may be attributed to the FSH carbohydrate moiety; particularly, since recombinant and urinary FSH differ in their carbohydrate structures (Hård et al., 1990; Lambert et al., 1998).

No information is yet available about the impact that different hFSH glycosylation variants may have on the granulosa cell global gene expression. In view of the relevance that the presence and structure of FSH oligosaccharides may have on the biological action of the hormone, the aim of the present study was to investigate the effects of sialylation and oligosaccharide complexity of rhFSH on the endocrine activity and on the expression of genes involved in the regulation of granulosa cell function.

2. Materials and methods

2.1. Hormones and reagents

Recombinant human FSH (rhFSH) was purchased from the National Hormone & Peptide Program of the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK)-NIH (Torrance, CA, USA). Progesterone (P_4), androstenedione (Δ_4A), oestradiol (E_2), 25-hydroxycholesterol (25-OH-Chol) and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Dulbecco Modified Eagle Medium/Ham F-12 medium (DMEM:F12, 1:1 vol/vol), fungizone (250 μg/mL), penicillin–streptomycin (10 mU/mL–10 mg/mL) and fetal bovine serum (FBS, certified) were obtained from Gibco® by Life Technologies Corporation (Carlsbad, CA, USA). Oestradiol [6,7-^{3}H(N)] (^{3}H-oestradiol) and progesterone [1,2,6,7-^{3}H(N)] (^{3}H-progesterone) were obtained from PerkinElmer NEN® (Boston, MA, USA). Transcriptor First Strand cDNA Synthesis system, TaqMan Master reaction, TaqMan probes and capillaries were obtained from Roche Applied Science (Indianapolis, IN, USA). All other chemicals were of reagent grade from standard commercial sources.

2.2. Isolation of rhFSH glycosylation variants

All glycosylation variants were isolated from rhFSH according to either sialylation degree or complexity of oligosaccharides using preparative isoelectric focusing and lectin affinity chromatography, respectively as previously described (Bedecarrás et al., 1998; Creus et al., 1996).

2.2.1. Preparative isoelectric focusing

Preparative isoelectric focusing (IEF) was used to isolate rhFSH charge analogues according to their sialylation degree using a Rotofor system (Rotofor Preparative Cell, Bio Rad Laboratories Inc., CA, USA) as previously described (Bedecarras et al., 1998). Twenty fractions, from a 3–10 pH gradient, were harvested and their pH was determined. The rhFSH recovery range was 70–85%. Based on the variations observed in the proportion of serum FSH isolated at pH intervals 2.56–4.00 and at pH > 5.00, associated with changes in ovarian activity (Velasquez et al., 2006; Loreti et al., 2009), two rhFSH preparations were selected to explore the biological effect of their sialylation degree. Fractions from pH 2.56 to 4.00 were combined and concentrated to obtain a more acidic/sialylated charge analogues mix (rhFSH-AC); fractions at pH > 5.00 were combined to obtain a less acidic/sialylated preparation (rhFSH-BA).

2.2.2. Concanavalin A chromatography

Concanavalin A chromatography (ConA) was used to isolate three groups of rhFSH glycosylation variants according to the complexity of their oligosaccharides: (i): unbound: rhFSH glycoforms bearing complex, triantennary and bisecting oligosaccharides, (ii) weakly bound: rhFSH glycoforms bearing biantennary carbohydrate chains and (iii) firmly bound: rhFSH glycoforms bearing high mannose/hybrid-type oligosaccharides. The technique described by Creus et al. (1996) was employed. The range of recovered rhFSH was 75–90%. Two rhFSH preparations: rhFSH-CO (rhFSH glycoforms bearing complex) and rhFSH-HY (rhFSH glycoforms bearing high mannose/hybrid-type oligosaccharides) were used to explore the biological effect of their oligosaccharide complexity on granulosa cell function. They were selected based on the previously observed variations in the relative proportion of these two types of glycoforms when determined under different physiological conditions in normal women (Creus et al., 1996; Velasquez et al., 2006; Loreti et al., 2009).

2.3. Human granulosa-like tumor cell line, KGN

The human granulosa-like tumor cell line, KGN, was provided by Dr. Mashatoshi Nomura, Department of Medicine and Bioregulatory Science, Graduate School of Medical Science, Kyushu University, Japan. KGN cells maintained physiological characteristics of ovarian granulosa cells, including the expression of functional FSH receptor and steroidogenic activities (Nishi et al., 2001). KGN cells were maintained in a DMEM/F12 medium supplemented with FCS (10% vol/vol), fungizone (2.5 μg/mL) and penicillin–streptomycin (50 U/mL; 50 μg/mL, respectively) at 37 °C in an incubator with 95% O_2 and 5% CO_2.

2.4. Cell culture

KGN cells were seeded onto plastic 24-well plates (Nunc, Roskilde, Denmark); initial plating density was 3 μg DNA/well. They were incubated in DMEM/F12 medium containing FCS (1% vol/vol) in the absence or presence of Δ_4A (100nM), 25-OH-Chol (25 μM), native rhFSH and its glycosylation variants: rhFSH-AC, rhFSH-BA, rhFSH-CO and rhFSH-HY (concentration range: 5–500 ng/mL). Steroids were dissolved in absolute ethanol before addition to the culture medium (final ethanol concentration < 0.1%). KGN cells were cultured for 24, 48 or 72 h to determine the optimal experimental condition to evaluate steroid and inhibin/activin production and gene expression, and to measure extracellular cAMP. Following incubation, KGN cells were stored at −80 °C until DNA determination or RNA extraction and conditioned media were stored at −20 °C until hormone measurements were carried out. Total DNA was determined by the method of Labarca and Paigen (1980).

2.5. Hormone measurements

A double-antibody RIA was used to measure immunoactive FSH in pituitary extracts as well as in eluates from the preparative isoelectric focusing and Concanavalin A affinity chromatography procedures. The hFSH RIA was performed using reagents provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Hormone and Pituitary Program (NHPP)

(Bethesda, MD, USA), as previously reported (Creus et al., 2001). The reference preparation LER-907 (1 mg LER-907-53 IU 2nd International Reference Preparation HMG) (NIDDK) was used to construct the standard curves. The polyclonal human (rabbit) antibody anti-hFSH-6 was used as antiserum.

Simultaneous curve fitting of the dose–response curves obtained in the RIA of the glycoforms revealed no significant differences among the slopes generated by the standard (LER-907) and the different rhFSH glycosylation variants, suggesting that the glycoforms were equally recognized by the antibody. rhFSH is expressed in terms of hFSH-2 standard (NIDDK). To minimise the effects of interassay variations as well as to determine the degree of parallelism between the unknown samples and the FSH standards, all isoform preparations were analyzed at multiple dose levels in the same assay run. The intra- and interassay coefficients of variation were less than 9% and 12%, respectively. The sensitivity of the assay was 4.5 ng of LER-907/tube.

Oestradiol was determined by RIA as previously described (Escobar et al., 1976). Intra- and interassay coefficients of variation were 8% and 15%, respectively. Progesterone levels were determined by RIA, as previously described (Irusta et al., 2003). The steroids antibodies were kindly provided by Dr. G.D. Niswender (Animal Reproduction and Biotechnology Lab, Department of Physiology, Colorado State University, Fort Collins, CO, USA). Intra- and interassay coefficients of variation were 8.0% and 14.2%, respectively.

Dimeric inhibin A and B, inhibin α-subunit (Pro-αC) and total activin A levels in the culture media were measured using specific two-site enzyme-linked immunoabsorbent assays (ELISA) (Oxford Bio-Innovation Ltd., Oxon, UK) for each peptide, as previously described (Groome et al., 1994, 1995, 1996; Knight et al., 1996). Recombinant human inhibin A and B, activin A (Genentech, San Francisco, CA, USA) and a partially purified (>75% purity) Pro-αC preparation were used as standards. The assay sensitivity was 7 pg/mL for inhibin A, 15 pg/mL for inhibin B, 2 pg/mL for Pro-αC and 78 pg/mL for activin A. Intra- and interassay coefficients of variation were less than 10% for all assays.

2.6. cAMP measurement

Cells were cultured in the presence of 0.1 mM IBMX (specific inhibitor of phosphodiesterase activity) with or without native rhFSH (dose: 20 ng/mL) for 24 h. The extracellular content of cAMP was determined by radioimmunoassay as previously described (Brignoni et al., 1993).

2.7. RNA isolation

Total cellular RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. Extracted RNA was stored at −70 °C until microarray or qRT-PCR analysis was carried out. The total RNA was checked for clearly visible 18S and 28S RNA bands and the amount and quality of RNA were estimated spectrophotometrically at 260/280 nm (A260/280 ratio of >1.8).

2.8. DNA microarray analysis

In order to analyze the gene expression pattern in KGN cells, total RNA from each sample was treated according to the Affymetrix 100 ng Total RNA Labeling Protocol (Affymetrix, Santa Clara, CA, USA). Briefly, the total RNA was reverse transcribed into cDNA twice. Single-stranded DNA was fragmented and labeled with biotinylated nucleotides and the samples (two biological replicates in each treatment group) were hybridized to the GeneChip® Human Gene 1.0 ST Array (Affymetrix). The array represents 28,869 well-annotated genes with 764,885 distinct probes.

All the arrays were processed as follows. First, Expression Console® 1.1 RMA-SKETCH algorithm (default parameters) was applied at gen level to obtain the summarized and normalized expression intensity. Only those probesets that, according to the manufacturer annotation codify for "main" (i.e. no control nor cross-hybridization) and have an EntreZ database identifier associated (GeneID accession number), were included for gene expression analysis. Second, gene expression analysis was carried out using linear models through "limma" BioConductor package (Smith, 2005).

Only pair wise biological meaningful contrasts were evaluated and genes were selected by p-value and $\log_2$ (fold change) thresholds. The threshold was selected so that it would provide enough information from an ontology analysis point of view as described by Fresno et al. (2012). Then, for each contrast, visual heatmap of selected differentially expressed genes (DEGs) was accomplished to confirm experimental design. All contrasts were able to accurately cluster the chips of each experimental condition.

2.9. Gene set enrichment analysis

Set Enrichment Analysis (SEA) (DAVID Bioinformatics Resources) (Huang et al., 2007, 2009) was carried out in order to relate DEGs with biologically relevant functions in KGN cells, whose expression was significantly affected by rhFSH glycosylation variants when compared to basal. A modification of the Multi Reference Contrast Method was used (Fresno et al., 2012). Thus, the desired visual Gene Ontology rhFSH glycosylation variants contrast was obtained, where variant specific pathways/terms were easily highlighted in each of the main Gene Ontology graphs (Biological Process, Molecular Function or Cellular Process).

2.10. Real-time PCR (RT-PCR)

Total RNA (1 μg) was reverse transcribed using the Transcriptor First Strand cDNA Synthesis kit with oligo $(dT)_{18}$ primers according to the manufacturer's protocols. Primers and probes for qRT-PCR amplifications were designed by the Universal Probe-Library Assay Design Center from Roche, (http://qpcr2.probefinder.com/organism.jsp); sequences are shown in Table 1. Real-time PCR assays

Table 1
Oligonucleotides and probes used for real-time PCR analysis.

Gene	NCBI reference sequence	Primers (5′→3′)		Amplicon (bp)	Probe number
		Forward	Reverse		
STAR	NM_000349.2	ggcatccttagcaaccaaga	actttgtccccattgtcctg	62	11
CYP11A1	NM_000781.2	gacccataggagtcctgttgaa	ttcttggtggcctctgga	94	79
HSD3B2	NM_000198.2	cttggacaaggccttcagac	tcaagtacagtcagcttggtcct	78	50
CYP19A1	NM_000103.3	caaacccaatgaatttactcttga	caaacccaatgaatttactcttga	111	76
HSD17B8	NM_014234.3	cgtaggaaaggtggggaac	tgggtcagcccaatcact	72	89
INHA	NM_002191.2	gtctcccaagccatcctttt	tggcagctgacttgtcctc	61	5
INHBA	NM_002192.2	ctcggagatcatcacgtttg	ccttggaaatctcgaagtgc	68	72
GAPDH	NM_002046.3	agccacatcgctcagacac	gcccaatacgaccaaatcc	66	60

were performed under identical conditions using 1 μL of cDNA, and normalization was achieved in all cases by comparing the gene of interest against the housekeeping gene coding for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), whose expression levels were unaffected by the hormonal treatment. Amplifications were carried out using the LightCycler® 2.0 (Roche Applied Science, Mannheim, Alemania), according to the following protocol: activation of FastStart Taq DNA polymerase and DNA denaturation at 95 °C for 10 min, proceeded by 45 amplification cycles consisting of 10 s at 95 °C, 30 s at 60 °C, and 1 s at 72 °C.

2.11. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Data were log transformed prior to statistical analysis when appropriate. Statistical analysis was performed using either Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test for multiple comparisons. The significance was established when the two-tailed *p*-value was $\leqslant 0.05$. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Determination of experimental conditions

The endocrine activity of KGN cells was assessed in terms of steroids, monomeric and dimeric inhibin, and activin A production. As shown in Fig. 1 (panel A), KGN cells produced oestradiol (195.2 ± 8.5 pg/μg DNA) only when incubated in the presence of androstenedione. Progesterone was produced under basal conditions and the addition of 25-hydroxycholesterol, previously identified as the best steroidogenic substrate for progesterone production in ovarian cells, had no effect: 219.0 ± 11.8 vs 220.6 ± 21.1 pg/μg DNA (25-OH-Chol vs basal, NS).

KGN cells were cultured for 24, 48 and 72 h to evaluate inhibin/activin production under basal conditions. Pro-αC was present in the culture medium after 24 h; a significant and time-dependent increase was observed thereafter (1.7 ± 0.4 vs 3.7 ± 0.4 vs 6.3 ± 0.3 pg/μg DNA; 24 vs 48 vs 72 h, $p < 0.05$). At 24 h of culture, inhibin A levels were undetectable while at 48 h a small amount was detected and a significant rise was observed at 72 h (0.9 ± 0.3 vs 3.9 ± 0.4 pg/μg DNA, 48 vs 72 h, $p < 0.01$) (Fig. 1, panel B). Activin A was detected after 24 h of culture (236.1 ± 23.3 pg/μg DNA) and remained without changes for longer periods of time. Inhibin B was not detected under any of the experimental conditions studied.

Accordingly, we investigated the effects of rhFSH glycosylation variants on peptide production in 72 h cultures.

To confirm the responsiveness of KGN cells to native rhFSH stimulus, cAMP concentrations were evaluated in the culture media. Detectable levels of cAMP were observed under basal conditions which significantly increased after stimulation with native rhFSH (7.43 ± 0.01 vs 3.76 ± 0.19 pmol/mL, rhFSH vs basal, $p < 0.01$). These results indicated the presence of a functional FSH receptor in these cells as previously reported by Nishi et al. (2001).

KGN cells were stimulated with increasing doses of native rhFSH (5–500 ng/mL) (Fig. 1, panel C). Maximal response in terms of Pro-αC and progesterone production was achieved at rhFSH doses of 50 and 100 ng/mL, respectively. Analysis of dose–response curves indicated that ED_{50} for Pro-αC was 23.3 ± 0.2 ng/mL and 47.5 ± 0.1 ng/mL for progesterone.

Based on these results, the selected doses of rhFSH glycosylation variants chosen to assess differential biological effects on KGN cells were 20 and 50 ng/mL.

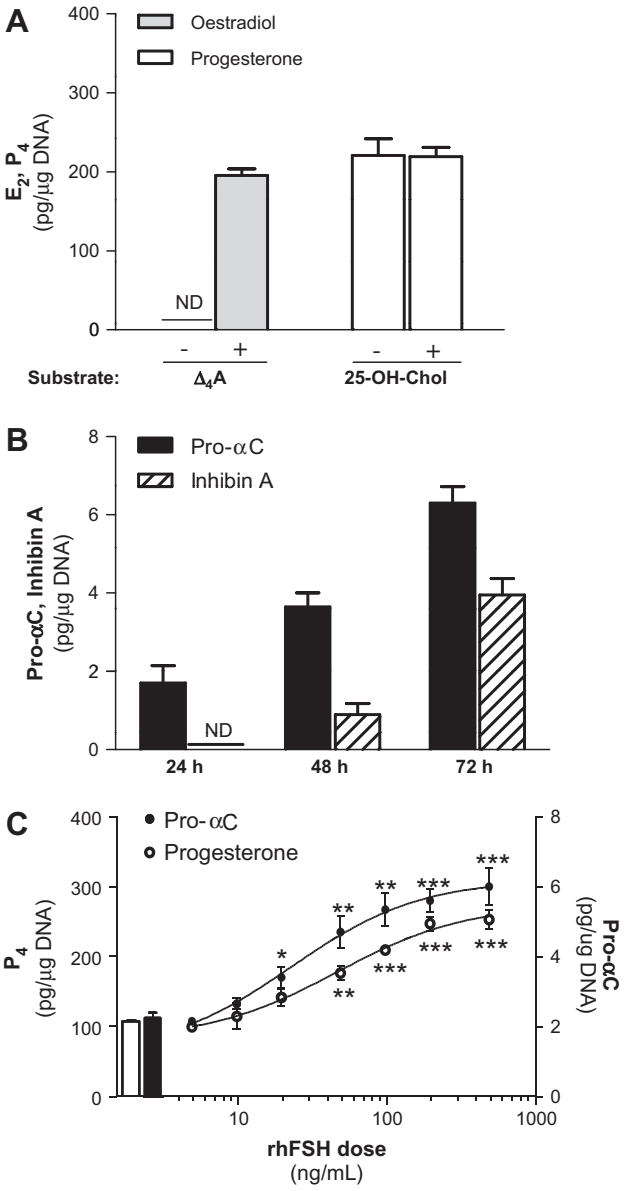

Fig. 1. Experimental conditions to assess steroid and inhibin production by human granulosa-like cell line, KGN. Cells were incubated in the absence or presence of steroidogenic substrates: androstenedione (Δ_4A, 100nM) and 25-hydroxycholesterol (25-OH-Chol, 25 μM) (panel A). KGN cells were cultured for 24, 48 or 72 h to determine the optimal experimental condition for evaluating inhibin/activin production (panel B). KGN cells were cultured for 24 and 72 h with increasing doses of native rhFSH (5–500 ng/mL) to evaluate progesterone and Pro-αC production, respectively (panel C). The native rhFSH dose is expressed in terms of hFSH-2 standard (NIDDKNHPP). Inhibins and steroids were determined in the conditioned media. The results are expressed as mean ± SEM of triplicate incubations of three independent experiments.

3.2. Effect of rhFSH glycosylation variants on steroids and inhibin production

3.2.1. Effect of rhFSH sialylation

KGN cells were incubated in the presence of more or less acidic/sialylated rhFSH charge analogues. The differential effects on steroid and inhibin production were evaluated depending on the degree of sialylation and the dose (20 and 50 ng/mL) of hormone.

The results showed that less sialylated rhFSH charge analogues were more biopotent than the more acidic counterparts in stimulating oestradiol production under the 50 ng/mL dose: 1.3 ± 0.02

vs 1.1 ± 0.01 (rhFSH-BA vs rhFSH-AC respectively, $p < 0.001$). No effect on oestradiol production was observed when the dose of 20 ng/mL was used. Progesterone production was only stimulated by the less sialylated gonadotrophin preparation; this effect was observed at the two doses studied ($p < 0.05$ when compared to basal) (Fig. 2, panels A and B).

Pro-αC production was stimulated by both more and less sialylated rhFSH charge analogues ($p < 0.001$ when compared to basal). However, cells exposed to the less sialylated counterparts, responded to the 20 and 50 ng/mL doses with a more marked increment in Pro-αC secretion when compared to the more acidic charge analogues (i.e.: 20 ng/mL dose: 2.7 ± 0.1 vs 1.7 ± 0.1-fold stimulation over basal, $p < 0.001$) (Fig. 2, panel C).

The more acidic rhFSH preparation was unable to stimulate inhibin A production. Conversely, the less sialylated rhFSH preparation induced a similar stimulatory effect ($p < 0.001$ when compared to basal) when 20 or 50 ng/mL of rhFSH charge analogues were used (20 ng/mL: 2.5 ± 0.2 and 50 ng/mL: 2.6 ± 0.2-fold stimulation over basal, NS); (Fig. 2, panel D).

3.2.2. Effect of rhFSH glycan branching

As shown in Fig. 2A–D, a differential effect on steroid and inhibin production was observed depending on the oligosaccharide complexity of the gonadotrophin preparation to which the cells were exposed.

At the dose of 20 ng/mL, rhFSH bearing fully processed carbohydrates was unable to exert any effect on oestradiol, progesterone or inhibin A production; however, a 50 ng/mL dose significantly increased progesterone (1.7 ± 0.1-fold stimulation over basal, $p < 0.01$ when compared to basal) and Pro-αC production (2.0 ± 0.1-fold stimulation over basal, $p < 0.001$ when compared to basal) was observed.

In contrast, KGN cells responded to 20 ng/mL rhFSH bearing high mannose/hybrid-type oligosaccharides with a significant increment in the production of oestradiol, progesterone and inhibin A ($p < 0.05$ when compared to basal). Progesterone levels were further increased when a 50 ng/mL was used (20 ng/mL: 2.2 ± 0.1 and 50 ng/mL: 3.0 ± 0.1-fold stimulation over basal, $p < 0.001$).

Pro-αC production was stimulated by both rhFSH glycoforms bearing fully processed carbohydrates and those bearing high mannose/hybrid-type oligosaccharides at the 20 ng/mL dose ($p < 0.001$ when compared to basal); the latter rhFSH preparation was able to induce a more marked increment (rhFSH-HY: 3.8 ± 0.2 vs rhFSH-CO: 1.5 ± 0.1, $p < 0.001$). Proα-C levels were further increased when a 50 ng/mL dose of rhFSH glycoforms bearing fully processed carbohydrates was used (20 ng/mL: 1.5 ± 0.1 and 50 ng/mL: 2.0 ± 0.1-fold stimulation over basal, $p < 0.05$).

3.3. Microarray and set enrichment analysis of gene expression data

We next investigated the effects of rhFSH glycosylation variants on global gene expression in granulosa cells.

For this purpose a microarray approach was used to explore gene expression patterns by comparing untreated KGN cells with those stimulated with the following four rhFSH glycosylation variants: rhFSH-AC, rhFSH-BA, rhFSH-CO and rhFSH-HY during 24 h. The list of genes whose expressions were significantly affected by the different rhFSH preparations when compared to basal conditions are provided in http://200.45.112.41/bdmg/?page_id = 333.

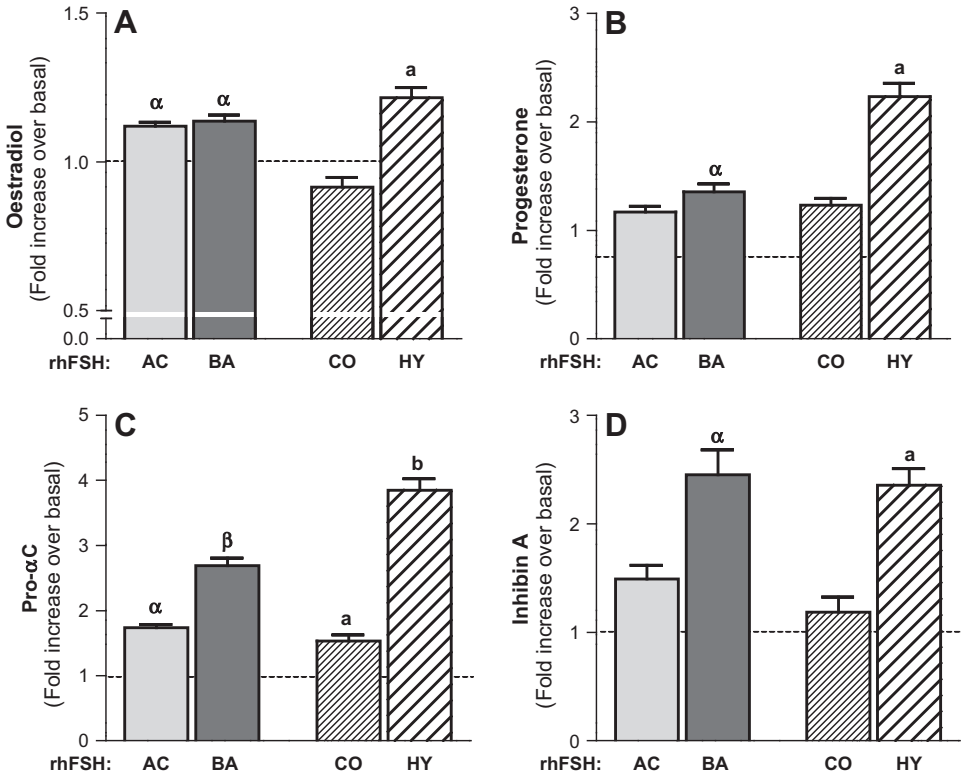

Fig. 2. Effect of rhFSH glycosylation variants on peptide and steroids production by human granulosa-like cell line, KGN. Cells were cultured with or without 20 ng/mL of the more acidic/sialylated charge analogues, rhFSH-AC, the less acidic/sialylated charge analogues, rhFSH-BA, the glycoforms bearing complex (triantennary and bisecting) oligosaccharides, rhFSH-CO, or the glycoforms bearing high mannose and hybrid-type oligosaccharides, rhFSH-HY. The dose of rhFSH glycosylation variants is expressed in terms of hFSH-2 standard (NIDDKNHPP). Steroids and inhibins were determined in the conditioned media after 24 and 72 h of culture, respectively. Results are presented as fold stimulation over basal production. Values are mean ± SEM of triplicate incubations of three independent experiments. Different letters indicate significant differences between treatments ($p < 0.05$).

Fig. 3 (panel A) depicts a representative example of a heatmap showing gene expression changes induced by rhFSH-BA and rhFSH-HY when compared to that obtained under basal conditions. As depicted, microarray analysis of mRNAs from cells treated with the rhFSH preparations, represented by Venn diagrams, showed that the carbohydrate moiety of rhFSH affected differentially the expression of FSH targeted granulosa cell genes. However, the expression of a small proportion of genes differed from basal in the presence of either rhFSH-AC or the rhFSH-BA variants. A similar result was obtained when the effect of the oligosaccharide complexity of rhFSH glycosylation variants, rhFSH-CO or rhFSH-HY, was analyzed.

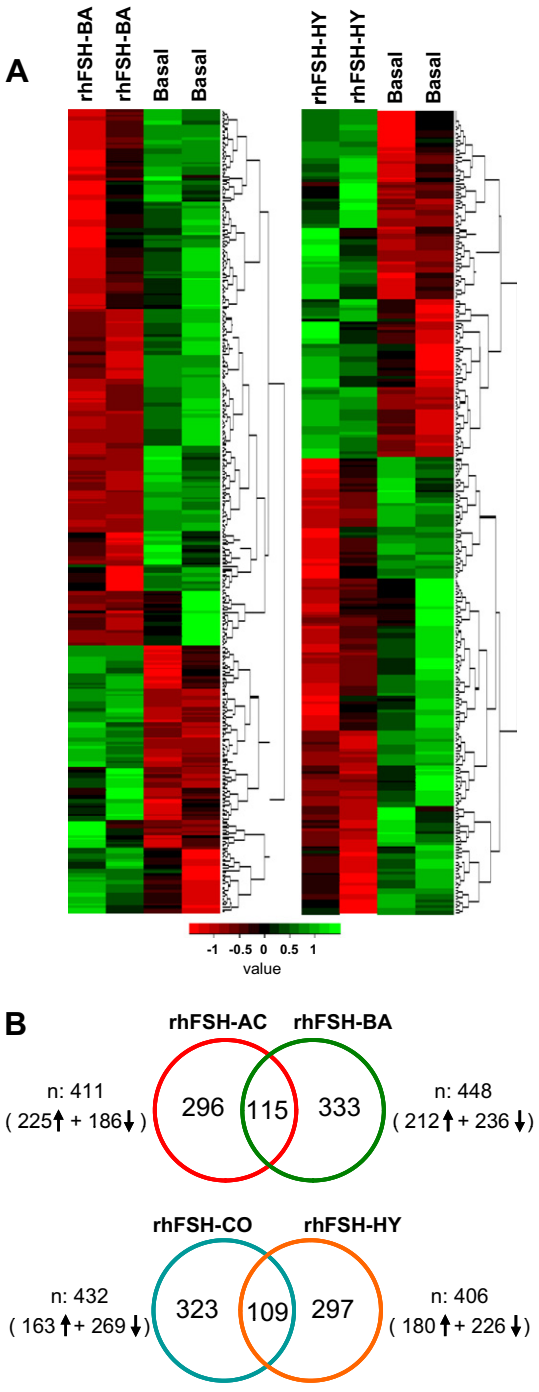

Fig. 3. Microarray analysis of genes regulated by rhFSH glycosylation variants. Heat maps represent differentially expressed genes when compared the response induced by less sialylated rhFSH charge analogues (rhFSH-BA) and the glycoforms bearing high mannose and hybrid-type oligosaccharides (rhFSH-HY). All conditions were compared with control incubations (panel A). Each column represents a sample and each row on the horizontal axis represents a gene. The colors indicate the relative expression levels for each gene. Venn diagrams depict differences and overlap in gene expression profiles in response to rhFSH charge analogues (rhFSH-AC and rhFSH-BA) and rhFSH glycoforms (rhFSH-CO and rhFSH-HY) (panel B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Overall, microarray analysis showed that incubations in the presence of more acidic rhFSH charge analogues (rhFSH-AC) affected the expression of 411 genes ($p < 0.05$). Amongst them, 225 were up-regulated and 186 were down-regulated, whereas the less sialylated rhFSH charge analogues (rhFSH-BA) modified the expression of 448 genes ($p < 0.05$), 212 were up-regulated and 236 down-regulated. Both rhFSH charge analogues, rhFSH-AC and rhFSH-BA, affected the expression of 115 genes. Interestingly, in some genes the magnitude of the change induced by rhFSH-AC or rhFSH-BA was significant ($p < 0.05$, data not shown).

In analyzing the effect of oligosaccharide complexity of rhFSH glycosylation variants on the KGN cells gene expression profile, it was found that expression of 432 genes ($p < 0.05$) was affected by rhFSH glycosylation variants bearing fully processed carbohydrates (rhFSH-CO); 163 of them were up-regulated and 269 were down-regulated. rhFSH glycosylation variants bearing high mannose/hybrid-type oligosaccharides (rhFSH-HY) modified the expression of 406 genes ($p < 0.05$), 180 of them were up-regulated and 226 were down-regulated. Both rhFSH-CO and rhFSH-HY, affected the expression of 109 genes although the magnitude of the change induced on the expression level of some of these genes was statistically different when rhFSH-CO or rhFSH-HY was used ($p < 0.05$, data not shown) (Fig. 3, panel B).

Differentially expressed genes fed Set Enrichment Analysis using DAVID Bioinformatics Resources based on the controlled vocabulary of the Gene Ontology Consortium (GO), with the MRMC technique. This analysis was carried out in order to relate genes, whose expression was significantly affected by rhFSH glycosylation variants when compared to basal, with biologically relevant functions in KGN cells. Results were focused on Biological Processes and Molecular Functions since the Cellular Component category did not reveal enriched term differences under the studied experimental conditions (data not shown). In order to achieve reliable SEA analysis, the number of differentially expressed genes feeding DAVID should be large enough to provide informative enriched terms.

Set enrichment analysis showed that hormone sialylation and its oligosaccharide complexity differentially modulated the expression of genes involved in the regulation of KGN cells function. Enriched gene ontology terms affected by more acidic rhFSH charge analogues (rhFSH-AC) are depicted in Suppl. Fig. 1, panels A and B. Those found at the Biological Process category (BP) were cellular homeostasis, nucleosome assembly and iron ion transport (Suppl. Fig. 1, panel A). The enriched gene ontology terms at the Molecular Function category (MF) were steroid dehydrogenase activity, glucose transmembrane activity, calcium channel activity, steroid and nucleic acid binding and ligand-dependent nuclear receptor activity (Suppl. Fig. 1, panel B).

When the genes affected by the less sialylated rhFSH charge analogues (rhFSH-BA) were analyzed, the enriched gene ontology terms at the Biological Process category were regulation of cell differentiation, regulation of secretion, gamete generation and reproductive process (Suppl. Fig. 2, panel A). The enriched gene ontology terms at the Molecular Function category were growth factor binding, calcium ion binding, nitric oxid synthase regulator activity, serine-type endopeptidase inhibitor activity and protein tyrosine kinase activity (Suppl. Fig. 2, panel B).

When the genes affected by rhFSH glycoforms bearing complex oligosaccharides (rhFSH-CO) were analyzed, the enriched gene

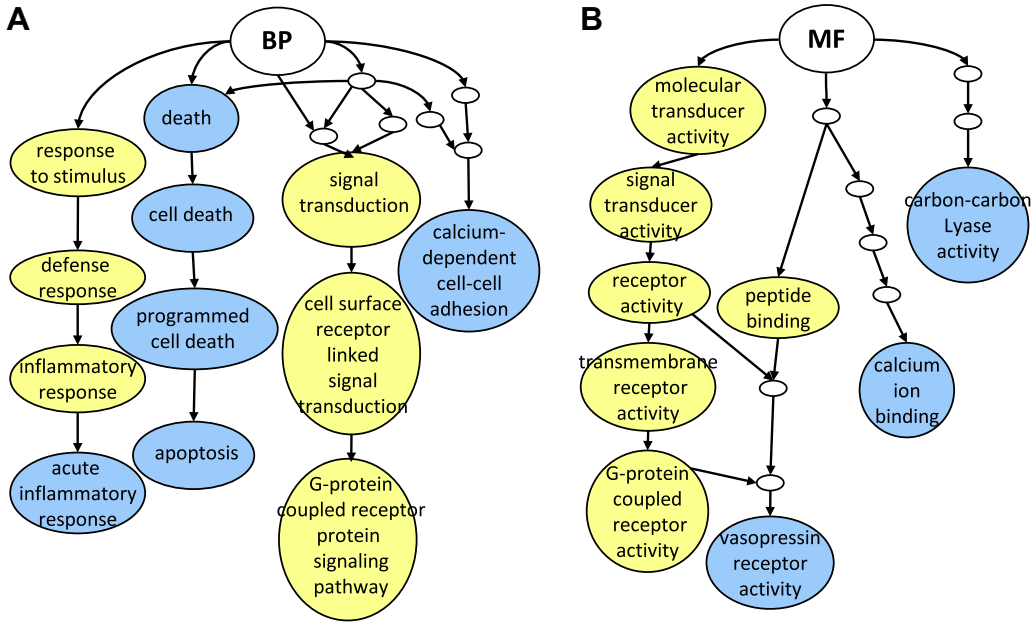

Fig. 4. Gene enrichment analysis. Enriched gene ontology terms associated to Biological Process (BP) (panel A) and Molecular Function (MF) (panel B) categories of differentially expressed genes affected by rhFSH-CO glycoforms. Enriched nodes are represented in blue and common enriched branches among the studied experimental conditions are displayed in yellow. Analysis was carried out using DAVID Bioinformatics Resources. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ontology terms at the Biological Process category were apoptosis, acute inflammatory response and calcium-dependent cell–cell adhesion process (Fig. 4, panel A). The enriched gene ontology terms at the Molecular Function category were carbon–carbon lyase activity, calcium ion binding and vasopressin receptor activity (Fig. 4, panel B). rhFSH glycoforms with hybrid type oligosaccharides (rhFSH-HY) affected the expression of genes related to Biological Processes such as steroid biosynthesis, the response to oestrogen stimulus, intra-S DNA damage checkpoint, ovarian follicle development and cholesterol metabolism (Fig. 5, panel A). The enriched gene ontology terms at the Molecular Function category were ATPase binding, interleukin-7 receptor binding and oxygen binding (Fig. 5, panel B).

Interestingly enough, common enriched branches with an ended node related to either G-protein coupled receptor protein signaling pathway (Biological Process category) or G-protein coupled receptor activity (Molecular Function category) were present in all the experimental conditions studied (Figs. 4 and 5, Suppl. Figs. 1 and 2).

Tables 2–5 show the genes assigned to each specific term (enriched ended nodes) regarding Biological Process and Molecular Function categories.

3.4. Effect of rhFSH glycosylation variants on gene expression by real time-PCR

As a result of the gene set enrichment analysis (Section 3.3) particular Biological Processes, represented by ended nodes, were associated with specific rhFSH glycosylation variants. As mentioned, the steroid biosynthesis process was one of the enriched terms associated with rhFSH glycoforms bearing high mannose/hybrid-type oligosaccharides (rhFSH-HY). Therefore, the genes coding for STAR, HSD3B2, CYP19A1 and HSD17B were selected to evaluate the effects of rhFSH glycosylation variants by real time-PCR.

STAR expression was stimulated by both, more and less sialylated rhFSH preparations ($p < 0.01$ when compared to basal); however, cells exposed to the less sialylated mix showed a more marked increment of its mRNA levels (rhFSH-BA vs rhFSH-AC, $p < 0.001$) (Fig. 6, panel A).

Regarding the effect of rhFSH glycosylation variants bearing different oligosaccharide complexity, only rhFSH-HY was able to stimulate *STAR* expression (rhFSH-HY vs basal and rhFSH-CO, $p < 0.01$).

The analysis of the effect of the rhFSH sialylation degree on the expression pattern of the *HSD3B2* gene showed a significant increase induced by both more and less sialylated rhFSH charge analogues (rhFSH-AC and rhFSH-BA vs basal, $p < 0.05$) (Fig. 6, panel C). However, more acidic counterparts exerted a more marked stimulatory effect on its mRNA levels (rhFSH-AC vs rhFSH-BA, $p < 0.001$).

Regarding the effect of rhFSH oligosaccharide complexity, only rhFSH-HY glycoforms were able to slightly stimulate *HSD3B2* gene expression (rhFSH-HY vs basal and rhFSH-CO, $p < 0.05$).

Considering that STAR as well as CYP11A1 and HSD3B2 are involved in progesterone synthesis, and STAR and *HSD3B2* gene expression was affected by the oligosaccharide structure present in the rhFSH molecule it was of interest to analyze the *CYP11A1* gene expression by RT-PCR. Fig. 6, panel B shows that rhFSH glycosylation variants exerted a similar effect on the expression pattern of the *CYP11A1* as that observed on the *STAR* gene. The expression was markedly stimulated by rhFSH-BA and rhFSH-HY ($p < 0.01$).

The expression of aromatase (*CYP19A1*) was up-regulated by all rhFSH preparations: more and less sialylated rhFSH charge analogues as well as rhFSH bearing complex carbohydrates and high mannose/hybrid-type oligosaccharides ($p < 0.05$ when compared to basal) (Fig. 7 panel A). However, cells exposed to the rhFSH mix bearing high mannose/hybrid-type oligosaccharides responded with a more marked increment in *CYP19A1* expression level (rhFSH-HY vs rhFSH-CO, $p < 0.001$).

The expression of the gene that encodes Type 8 17β-HSD (*HSD17B8*), the enzyme that converts oestradiol into oestrone, was only up-regulated by the more acidic rhFSH charge analogues ($p < 0.01$) (Fig. 7, panel B). rhFSH preparations with differences in their oligosaccharide complexity showed no effect on its expression level when compared to basal.

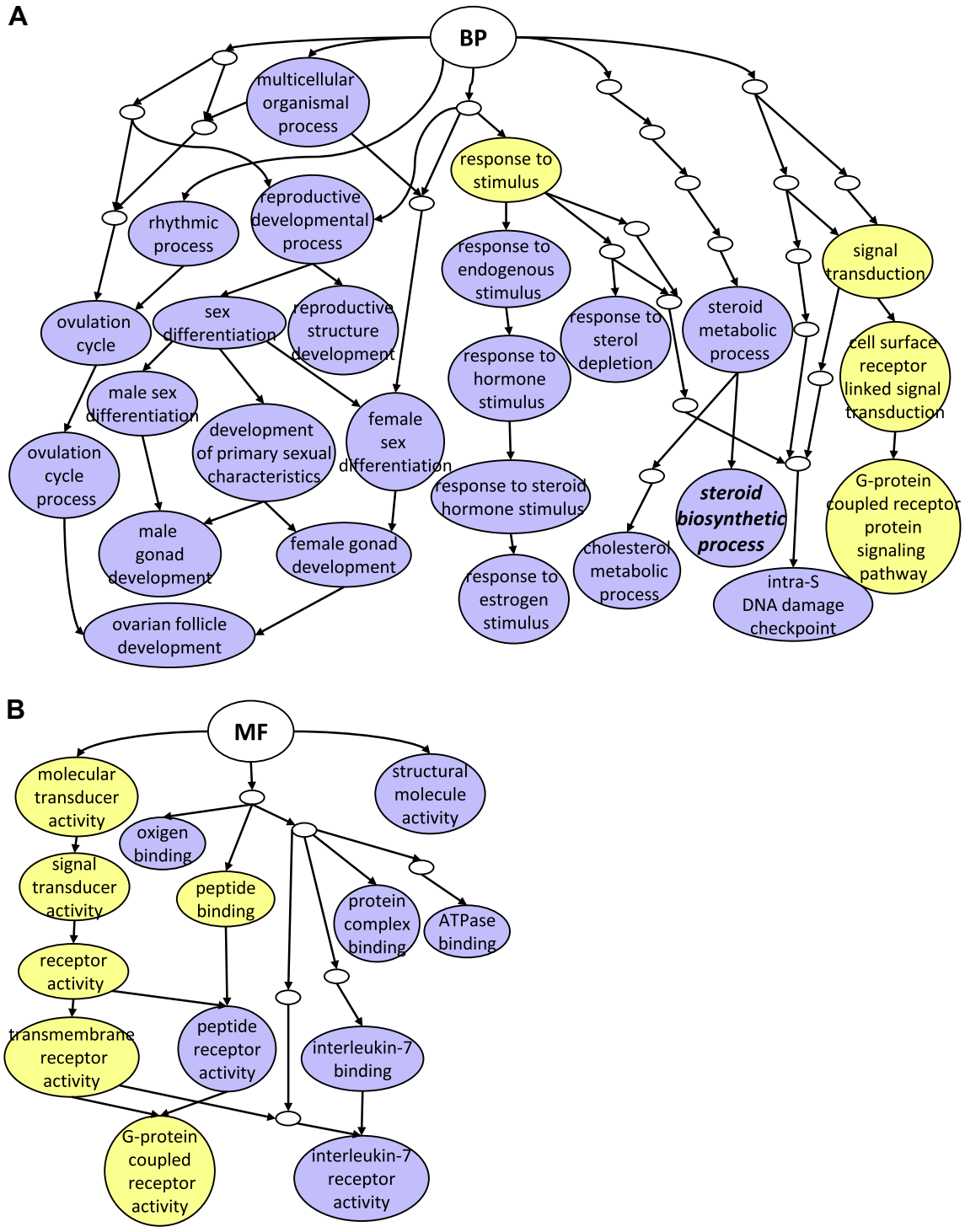

Fig. 5. Gene set enrichment analysis. Enriched gene ontology terms associated to Biological Process (BP) (panel A) and Molecular Function (MF) (panel B) categories of differentially expressed genes affected by rhFSH-HY glycoforms. Enriched nodes are represented in violet and common enriched branches among the studied experimental conditions are displayed in yellow. Analysis was carried out using DAVID Bioinformatics Resources. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

When the gene expression of inhibin/activin subunits was assessed, in coincidence with previous results obtained with activin A (Section 3.2), no changes in βA subunit (*INHBA*) expression were observed under all experimental conditions (Fig. 8, panel A).

In contrast, rhFSH oligosaccharide complexity was the only hormone characteristic that affected the α-inhibin subunit expression (*INHA*) in KGN cells (Fig. 8 panel B).

4. Discussion

To our knowledge, this is the first study evaluating the biological activity of the different rhFSH glycosylation variants in terms of steroids and peptides production as well as the gene expression at the whole-genome scale in human granulosa cells. It is worth noting that this study examined the effect of not only the degree of rhFSH sialylation but also its oligosaccharide complexity.

Table 2
Gene ontology analysis of differentially expressed genes in response to rhFSH-AC.

Biological process		Molecular function	
Gene symbol	Gene expression	Gene symbol	Gene expression
Cellular iron ion homeostasis		Ligand-dependent nuclear receptor activity	
Iron ion transport		Steroid binding	
LTF	Underexpressed	NR1H4	Underexpressed
CP	"	NR3C2	"
HFE	"	ESR1	"
TFRC	Overexpressed	COL11A2	Overexpressed
FTHL17	"	APOF	"
Nucleosome assembly		CALB1	"
HIST1H2BO	Underexpressed	Steroid dehydrogenase activity	
HIST1H2BE	Overexpressed	HSD3B1	Underexpressed
HIST1H2BH	"	HSD3B2	Overexpressed
HIST1H2BK	"	HSD17B2	"
HIST1H1B	"	Calcium channel activity	
G-protein coupled receptor protein		CATSPER4	Overexpressed
Signaling pathway		CACNG1	"
GNB3	Underexpressed	CATSPER3	"
TAS2R10	"	TRPV6	"
PPYR1	Overexpressed	SLC24A2	"
GABBR2	"	D-glucose transmembrane activity	
ADORA3	"	SLC2A4	Overexpressed
VIPR2	"	SLC2A2	"
CXCR3	"		

In our hands the granulosa-like tumor cell line, KGN, maintained the typical steroidogenic phenotype of native granulosa cells; thus, they were unable to synthesize oestrogens without the addition of an aromatizable substrate and conserved their capacity to respond to FSH by increasing cAMP levels, as previously reported (Nishi et al., 2001). In addition, increments in progesterone production after rhFSH treatment further supported this judgement. However, scarce information is available about KGN cells capacity to produce inhibins (Wu et al., 2005). This study showed that KGN cells were able to produce inhibin α-subunit, inhibin A and the homodimer activin A; this latter dimer was not affected by rhFSH.

This homologous cell assay confirmed and extended previous observations from our laboratory showing that both sialylation degree and complexity of FSH carbohydrates influence the biological response of target gonadal cells (Creus et al., 2001).

Previous studies have shown that the less sialylated FSH glycosylation variants isolated from pituitary extracts were more potent than the more acidic mix to increase oestradiol production in rat granulosa cells (Timossi et al., 2000; Zambrano et al., 1996). Our results showed that gonadotrophin sialylation degree differentially modulated steroid and inhibin production; a less acidic mix stimulated the secretion not only of oestradiol and progesterone but also of free inhibin α-subunit and inhibin A. Only the more acidic rhFSH glycosylation variants affected the production of oestradiol and the free inhibin α-subunit. Considering that changes in the sialylation degree of circulating FSH throughout the menstrual cycle have been reported (Padmanabhan et al., 1988; Zambrano et al., 1995), it can be speculated that the progressive loss of FSH sialic acid during the follicular phase may contribute to maintain low levels of steroids and selectively stimulate a particular dimeric form of inhibin.

Concerning the relevance of oligosaccharide complexity in the FSH molecule, rhFSH bearing fully processed carbohydrates, found in serum from fertile women (Loreti et al., 2009; Velasquez et al., 2006), was able to modulate Pro-αC and progesterone production. It must be taken into account that due to the complex oligosaccharide structure present in the FSH molecule, these glycosylation variants may be an appropriate substrate for the addition of sialic acid residues to their carbohydrate chains; in this way they may affect other endocrine parameters. On the other hand, the marked stimulation of all the studied parameters induced by the presence of partially processed oligosaccharides in the rhFSH molecule, independently of the dose used, suggested that this type of FSH glycoforms was unable to exert a more selective effect on endocrine products in granulosa cells.

Several studies have demonstrated that human FSH preparations, recombinant and urinary, differ in their *in vivo* and *in vitro* biological activities (Hård et al., 1990; Lambert et al., 1998; Timossi et al., 1998). More recently, it has been reported that these preparations also induce a different global gene expression profile in granulosa cells (Brannian et al., 2010; Grøndahl et al., 2009). Based on these findings and on the evidence obtained in the present study on granulosa cell endocrine activity, a microarray analysis

Table 3
Gene ontology analysis of differentially expressed genes in response to rhFSH-BA.

Biological process		Molecular function	
Gene symbol	Gene expression	Gene symbol	Gene expression
Gamete generation		*Nitric-oxide synthase regulator activity*	
SPIN3	Underexpressed	ESR1	Underexpressed
KLF17	"	HSP90AA2	Overexpressed
WDR33	"	Calcium ion binding	
LMNA	"	CLCA1	Underexpressed
SBF1	"	FAT3	"
DNMT3L	"	PITPNM2	"
AGT	"	SRL	"
SLC26A10	"	PLA2G10	"
HSF2	Overexpressed	EFCAB5	"
DGCR14	"	CD97	"
TSNAX	"	TNNC2	"
GJA10	"	GALNT12	"
CATSPER4	"	DOC2B	Overexpressed
Positive regulation of cell differentiation		GRIN2B	"
IL20	Underexpressed	SYT9	"
ITPKB	"	EFCAB7	"
AGT	"	RCN2	"
TNFRSF12A	"	FAHD1	"
PLA2G10	"	CLGN	"
LYN	"	FSTL5	"
IL2RG	"	PCDHB17	"
TGFB3	Overexpressed	SCGN	"
HOXD3	"	F13A1	"
Regulation of secretion		CALB1	"
AGT	Underexpressed	CATSPER4	"
PLA2G10	"	FCN2	"
CIDEA	"	*Growth factor activity*	
OXTR	"	AGT	Underexpressed
RAB3C	"	GDF3	"
IL10	Overexpressed	C19orf10	"
GRIN2B	"	IL10	Overexpressed
TGFB3	"	GMFB	"
G-protein coupled receptor protein		TGFB3	"
Signaling pathway		FIGF	"
AGT	Underexpressed		
GPR151	"	*Protein tyrosine kinase activity*	
OR5D13	"	TYRO3	Underexpressed
OXTR	"	LTK	"
OR5T3	"	DDR1	"
OR4D11	"	LYN	"
TAAR6	"	CCL4	Overexpressed
GPR81	"	CLK4	"
GPRC5B	"	GUCY2F	"
RGS9	"	*Serine-type endopeptidase inhibitor activity*	
KNG1	"	AGT	Underexpressed
VN1R4	Overexpressed	SERPINA12	"
CGA	"	SPINK5L2	"
MRGPRX1	"	SERPINB7	Overexpressed
ADCY4	"	TFPI	"

Table 4
Gene ontology analysis of differentially expressed genes in response to rhFSH-CO.

Biological process		Molecular function	
Gene symbol	Gene expression	Gene symbol	Gene expression
Programmed cell death/apoptosis		*Calcium ion binding*	
IER3	Underexpressed	PLA2G10	Underexpressed
NIACR2	"	PRKCG	"
GPR65	"	PLGLA	"
RASSF5	"	GALNT13	"
ZC3H12A	"	MMP10	"
PDCL3	"	TGM5	"
TNFAIP3	"	CACNA1C	"
C17orf88	"	PCDHB1	"
SEMA6A	"	MMP12	"
MAPK7	"	PCDHB5	"
PLGLA	"	S100G	"
IL6	"	S100Z	"
ALDOC	"	FSTL5	Overexpressed
GZMH	"	S100A7	"
MTCH1	"	PCDHB2	"
AEN	"	CATSPER3	"
RASGRF1	"	EFCAB4B	"
NIACR1	Overexpressed	ITGB1BP2	"
Calcium-dependent cell–cell adhesion		*Vasopressin receptor activity*	
PCDHB5	Underexpressed	OXTR	Underexpressed
PCDHB13	"	AVPR2	"
PCDHB2	Overexpressed	*Carbon–carbon lyase activity*	
Cell surface receptor linked signal transduction		ALDOC	"
TGFB3	Overexpressed	PCK1	"
FIGF	"	PDXDC2	Overexpressed
OR2T1	"	HMGCLL1	"

Table 5
Gene ontology analysis of differentially expressed genes in response to rhFSH-HY.

Biological process		Molecular function	
Gene symbol	Gene expression	Gene symbol	Gene expression
Steroid biosynthetic process		*Lnterleukin-7 receptor activity*	
Cholesterol metabolic process		IL7R	underexpressed
MVK	Underexpressed	IL2RG	"
HMGCR	"	ATPase binding	
HSD17B8	"	S100A1	"
LDLR	"	LHCGR	"
INSIG1	"	ATP6V0A4	"
APOF	Overexpressed	*Protein complex binding*	
HSD3B2	"	UQCRH	"
CYP19A1	"	VCAM1	"
STAR	"	ACVRL1	"
Response to steroid hormone stimulus		LGALS3	"
GSTM3	Underexpressed	ICAM2	"
CRYAB	"	CCNE1	"
LDLR	"	CETN3	"
CCNE1	"	DOK3	"
OXTR	"	LYN	"
MMP13	Overexpressed	Oxygen binding	
Response to sterol depletion		HBG1	"
INSIG1	Underexpressed	HBG2	"
LYN	"	CYP3A7	"
Ovarian follicle development		CYP19A1	Overexpressed
FGF7	Underexpressed		
CCNE1	"		
LHCGR	"		
BCL2L1	"		
OXTR	"		
Intra-S DNA damage checkpoint			
TIPIN	Underexpressed		
NEK11	"		
Cellular sodium ion homeostasis			
NR3C2	Underexpressed		
C7	Overexpressed		

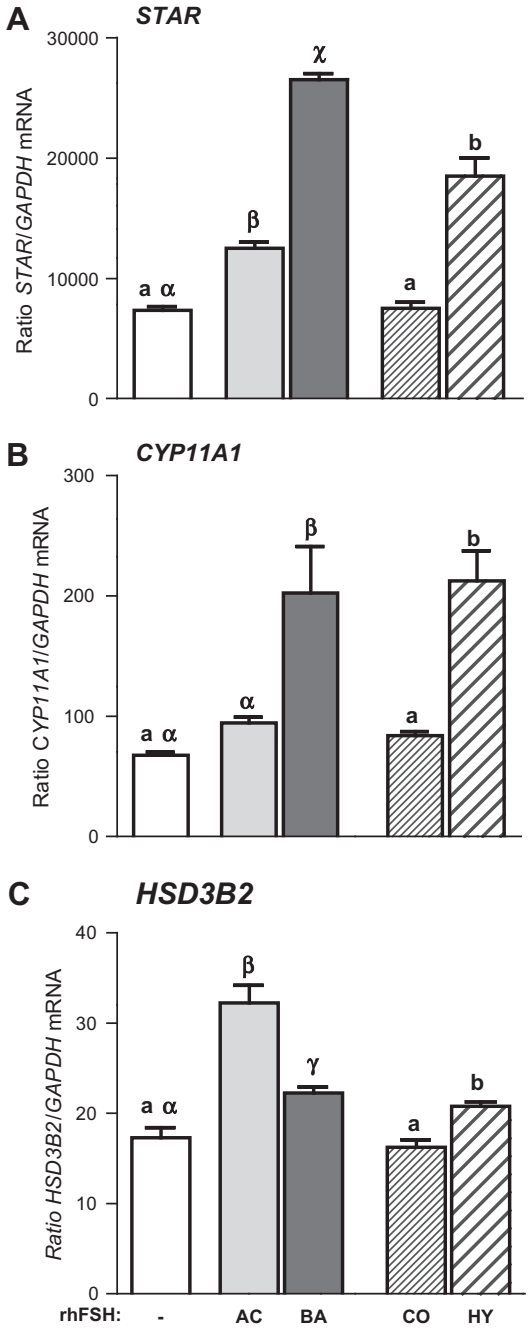

Fig. 6. Analysis of selected genes expression by real-time PCR. KGN cells were cultured with or without 20 ng/mL of rhFSH-AC; rhFSH-BA; rhFSH-CO and rhFSH-HY glycosylation variants during 24 h, mRNA levels were normalized against constitutively expressed *GAPDH* mRNA. Results are expressed as mean ± SEM of triplicate incubations of three independent experiments. Different letters indicate significant differences between treatments (rhFSH-AC and rhFSH-BA, greek letters; rhFSH-CO and rhFSH-HY, latin letters) ($p < 0.05$). *STAR*: steroidogenic acute regulatory protein, *CYP11A1*: cholesterol side-chain cleavage enzyme, *HSD3B2*: 3β-hydroxysteroid dehydrogenase type II, *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase.

was carried out to further evaluate the impact of FSH oligosaccharide structure on gene expression patterns in KGN cells.

Under the conditions used in this study, expression of the majority of granulosa cell genes depended on the oligosaccharides structure present in the rhFSH molecule, including those genes previously identified as FSH-dependent such as *CYP19A1* and *STAR* (Fitzpatrick et al., 1991; Kwintkiewicz et al., 2007; Sasson et al., 2003).

Furthermore, set enrichment analysis revealed the ability of rhFSH glycosylation variants to modulate the expression of genes

involved in biologically relevant functions in granulosa cells. The expression of only a low proportion (~20%) of genes was not affected by the sialylation degree and complexity of the oligosaccharides of our rhFSH preparations. This was the case for G-protein coupled receptor protein signaling pathway genes, whose expression was modulated by all rhFSH glycosylation variants. This result suggests that FSH, independently of its carbohydrate structure, ensures the granulosa cells response to different G-protein coupled receptors ligands.

In this study, it was clear that a number of genes involved in the regulation of important aspects of granulosa cell function were indeed under the regulation of FSH carbohydrate structure. In fact, rhFSH glycosylation variants bearing fully processed carbohydrates modulated the expression of genes associated to Biological Processes, such as homeostasis, cell differentiation and apoptosis. Moreover, those genes affected by glycoforms bearing incomplete oligosaccharides were related to other essential aspects of granulosa cell function, such as ovarian follicle development, ovulation, response to steroid hormone stimulus and, in particular, steroid biosynthesis.

Several genes whose expression was affected by the different rhFSH glycosylation variants have not yet been identified as FSH target genes. However, some information is available about the involvement of certain gene encoded proteins in the regulation of the ovarian function.

Less sialylated rhFSH affected the expression of genes modulating the regulation of cellular differentiation and gamete generation. For instance, the expression of *TGFB3*, one of the genes that appeared in the cellular differentiation category, was stimulated by these rhFSH glycosylation variants. It has been shown that this isoform is the more abundant in granulosa and theca cells during the development of antral follicles (Nilsson et al., 2003) and its effect on oestradiol production and aromatase expression has been recently reported (Liang et al., 2011). The transcription factor HSF2 is active during embryogenesis and spermatogenesis and has been described as a regulator of heat shock protein expression (Pirkkala et al., 2001). Disruption of this gene causes the production of abnormal eggs, a reduced number of ovarian follicles and hormone response defects (Kallio et al., 2002). In the present study, the expression of this gene was stimulated by less sialylated rhFSH charge analogues.

The complexity of rhFSH oligosaccharides affected, among others, the expression of genes associated with the inflammatory response. The presence of complex carbohydrate chains in the rhFSH molecule induced an inhibitory effect on the *IL-6* gene expression. It has been reported that the protein encoded by this gene modulates the cumulus cell-oocyte complex expansion and the expression of genes related to inflammatory processes as well as immune response (Liu et al., 2009). Furthermore, elevated levels of IL-6 in serum and follicular fluid have been found in infertile patients (Martínez et al., 2007; Pellicer et al., 1998).

These findings create an interesting opportunity for further studies looking at the selective control of follicle function by specific FSH glycosylation variants.

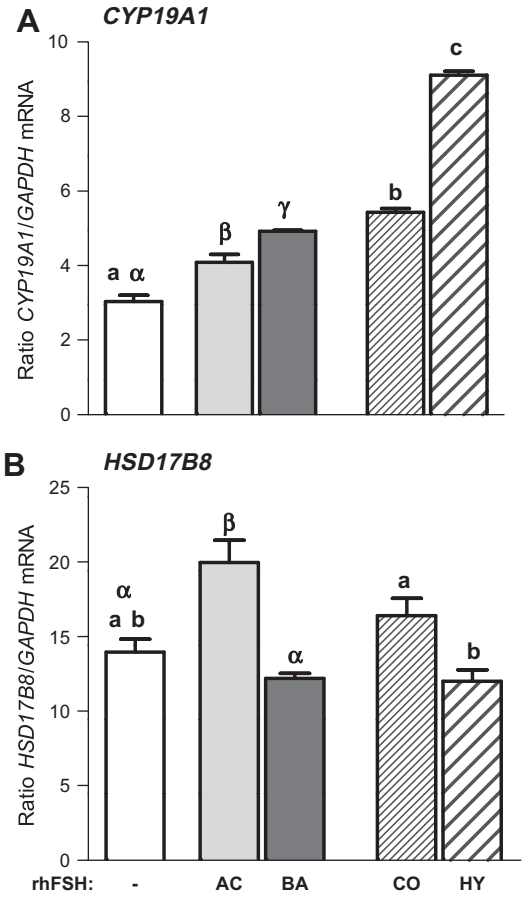

Fig. 7. Conditions were as those described under Fig. 6. Different letters indicate significant differences between treatments (rhFSH-AC and rhFSH-BA, greek letters; rhFSH-CO and rhFSH-HY, latin letters) ($p < 0.05$). *CYP19A1*: aromatase, *HSD17B8*: 17-β-hydroxysteroid-dehydrogenase 8, *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase.

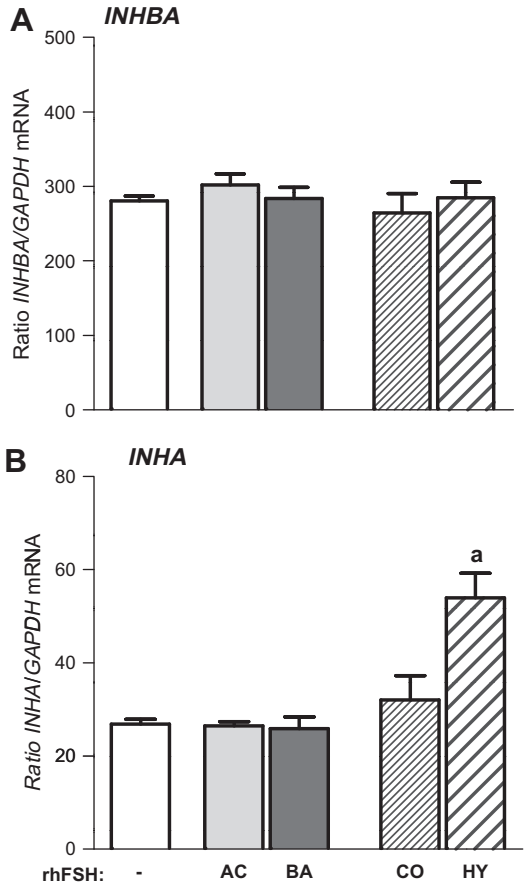

Fig. 8. Conditions were as those described under Fig. 6. Different letters indicate significant differences between treatments (rhFSH-AC and rhFSH-BA, greek letters; rhFSH-CO and rhFSH-HY, latin letters) ($p < 0.05$). *INHBA*: inhibin/activin βA-subunit, *INHA*: inhibin α-subunit, *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase.

We investigated the relevance of rhFSH carbohydrate structure on steroid biosynthesis in depth by evaluating the expression of several genes involved in ovarian steroidogenesis by real time PCR. The results demonstrated that the degree of sialylation and oligosaccharide complexity of FSH affected the expression of genes involved in the initial steroidogenic steps. Cholesterol transport to the inner mitochondrial membrane and its conversion to pregnenolone seemed to require the action of a particular mix of less sialylated rhFSH glycosylation variants bearing incomplete carbohydrate chains since they markedly increased the expression of *STAR* and *CYP11A1*. The next step in progesterone production, conversion of pregnenolone to progesterone, seemed to be more affected by rhFSH sialylation.

On the other hand, the expression of *CYP19A1* seemed to be more affected by the oligosaccharide complexity of rhFSH glycosylation variants.

It is worth noting that to our knowledge this is the first study providing evidence on rhFSH regulation of *HSD17B8* expression. The 17β-hydroxysteroid dehydrogenase isoenzyme, which selectively converts oestradiol to oestrone, has been proposed as a regulator of sex steroid concentration in gonadal tissue (Fomitcheva et al., 1998; Rotinen et al., 2009). Furthermore, based on its localization found in cumulus cells it has been speculated that this enzyme may affect oestrogen delivery to the oocyte (Fomitcheva et al., 1998). The results showed herein demonstrated that *HSD17B8* expression was affected by the degree of rhFSH sialylation, suggesting that sialic acid content in FSH may play a role in modulating oestrogen metabolism in granulosa cells.

In the present study, and in accordance with results obtained in terms of inhibin and activin A production in response to rhFSH, none of the rhFSH glycosylation variants affected βA-subunit expression. In contrast, inhibin α-subunit expression was clearly regulated by the different rhFSH glycosylation variants. Therefore, the rate of inhibin/activin subunit heterodimerization would be indirectly modulated by rhFSH oligosaccharide structure in this experimental model.

The results presented herein further support the concept that the specific glycan content in FSH molecular structure influences selectively the expression of genes needed for an adequate growth and function of the human ovarian follicle. Further studies are being conducted in order to establish the impact that this novel aspect of the hormone action may have on follicular development and oocyte quality.

Acknowledgements

The authors are very grateful to Dr. Mashatoshi Nomura for providing the human granulosa-like tumor cell line, KGN and Mercedes Astarloa and Eliana Pellizzari for the technical assistance. This work was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 5479), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT 2004 No. 25365) and Programa Latinoamericano de Investigación en Salud Sexual y Reproductiva (PLISSER, PLC/393/2008). S.C. and E.F. are established investigators from CONICET, Argentina.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mce.2012.11.021.

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