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*FELLOWSHIP FINAL REPORT*

# Pleotropism of gonadotropin action

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REPORT INFO

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ABSTRACT

Evidence exists that the gonadotropins LH and FSH can substitute to each other under certain circumstances, in addition to the fact that they can act together in granulosa cells. The aim of this study is to investigate how the two human gonadotropins influence each other in granulosa cells expressing both receptors, or by co-culturing cells expressing either the LHCGR or the FSHR (as a model granulosa/theca interaction). Plasmids encoding the c-myc-tagged-LHCGR and the FLAG-tagged FSHR under the control of an inducible coumermycin-responsive or doxycycline-responsive promoter, respectively were produced. These plasmids were used to permanently transfect human granulosa cell-derived KGN cells and HEK293 cells. The following cell lines were obtained and partially characterized: #1 c-myc-tagged-LHCGR-KGN; #2 FLAG-tagged FSHR_HEK293; #3 FLAG-tagged FSHR-KGN; #4 Double, c-myc-tagged-LHCGR and FLAG-tagged FSHR-KGN. After induction of receptor expression, the cell lines #1 and #2 and #3 responded to hCG and FSH stimulation, respectively by producing cAMP. Receptor expression was demonstrated by RT-PCR and flow cytometry. The characterization of the cell line #4 is ongoing. These cell lines are now available for the study of cell signaling and steroid synthesis, as well as in silico modeling, to gain insight into the dynamics of the intertwined cell response to FSH and LH in granulosa cells. These experiments will continue in parallel in both laboratories involved. Our cell lines represent new, very valuable instruments for the study of molecular pharmacology of FSH and LH, in order to improve infertility treatment, (multi)follicular growth for assisted reproduction, ovulation and spermatogenesis.

## 1- Introduction

The gonadotropins LH and FSH are necessary to ensure steroidogenesis, gametogenesis and reproduction. In primates, a third gonadotropin, hCG, acts during pregnancy via the same receptor for LH, the LHCGR, to stimulate progesterone production by the corpus luteum and maintain pregnancy. The main action of LH is to produce testosterone (in both sexes), of hCG to produce progesterone (in pregnancy) and of FSH to produce gametes. In addition, gonadotropins are growth and differentiation factors, modulating cell proliferation, survival and apoptosis. The interplay between the two gonadotropins FSH and LH is complex and several *in vitro* and *in vivo* models have demonstrated that, under certain circumstances, the lack of one of the two can, at least in part, be compensated for by the other, especially in the murine species and especially in the case of spermatogenesis (1). LH and FSH work together to support follicular growth and the receptors for the two hormones are co-

expressed on the same granulosa cells at late maturation stages. Since both hormones stimulate essentially the same signal transduction pathways, the question arises why are they both necessary and how can the cell differentiate between the two when expressing both receptors. In addition, what is the mechanism by which one gonadotropin can take over the action of the other in certain circumstances is unknown. Clinical examples thereof are the maintenance of spermatogenesis in the absence of FSH (2), but also by FSH activity alone (3), or multiple follicular growth and maturation in cycles of assisted reproduction (ART) in which only FSH is used after profound pituitary down regulation by GnRH analogs. The picture is further complicated by the fact that, clinically, hCG is traditionally used to provide LH action in therapy, while we now know that LH and hCG exert different action on the same receptor (4, 5). In addition, in human granulosa cells, FSH potentiates the hCG-dependent steroidogenic and pro-apoptotic signals while enhancing the potency of LH-dependent proliferative and anti-apoptotic signals exerted through pERK1/2 and pAKT, resulting in higher cell viability compared to FSH and hCG co-treatment (6).

In the growing follicle of the pre-antral, antral and terminal stages, the action of FSH predominates to promote granulosa cell proliferation, until the FSH-induced expression of the LHCGR reaches a defined level of expression. Thereafter, the expression level of the FSHR drops and cells of the follicle continue its growth process, which culminates with ovulation. This study intends to investigate the molecular mechanism of LH/hCG and FSH, action alone and in combination, in a homologous cell system. In particular, in this project, we produced some novel human granulosa cell-derived, KGN cell lines conditionally expressing the LHCGR or/and the FSHR

## 2- Materials and Methods

The experimental procedures carried out during the fellowship at the host lab included: 1) Construction of plasmids encoding the c-myc-tagged-LHCGR and the FLAG-tagged FSHR under the control of an inducible coumermycin-responsive or doxycycline-responsive promoter; 2) Production of human granulosa cell-derived KGN cells and HEK293 cells permanently expressing the inducible c-myc-tagged-LHCGR and the FLAG-tagged FSHR; 3) partial characterization of cell lines obtained.

### *2.1 Plasmids*

The human FSHR, human LHCG or Luciferase (positive transfection control) mRNA were cloned into *EcoRI-XhoI* restriction sites of the pSBtet expression system. The empty vector was used as negative control. The plasmids were amplified in DH5α *E. Coli* (New England BioLabs, ref C2527H).

The vector includes: a response element activated by the Tet-sensitive transactivator (activated by doxycycline), the TRE minimal promoter, the cDNA of interest (FSHR or LHCG), green fluorescent protein (GFP), Puromycin resistance, Ampicillin resistance. Control plasmids contain, downstream of the inducible promoter, either luciferase or no cDNA (negative control).

The FSHR and LHCGR cDNA are N-terminally tagged with FLAG or *c-myc*, respectively, to allow immune-recognition (in flow cytometry). Four vectors were obtained, encoding the c-myc-tagged-LHCGR and the FLAG-tagged FSHR under the control of an inducible coumermycin-responsive or doxycycline-responsive promoter, respectively.

### *2.2 Cell lines*

Cell lines were obtained starting from the KGN cells (21), a steroidogenic human granulosa cell line in which the endogenous FSHR is not expressed, and from HEK293 cells (human embryonic kidney derivation). The HEK293 WT3, obtained previously and permanently expressing the human FLAG-FSHR was used as positive control.

Cells were cultured in DMEM (Eurobio ref CM1DME60-01) containing $NaHCO_3$ and

glucose, 1% Glutamine (Eurobio, ref CSTGLU01-0U), 1% of antibiotics Penicillin and Streptomycin (Gibco, ref 15140-122) 10,000 U / mL, and 10% of SVF (Eurobio, ref: CVFSVF06-01). Transfected cells were cultured in SVF without tetracycline, called "tet-free" (Ozyme, ref: 631106) in order not to induce the Tet system unintentionally.

After initial validation, 80% confluent cells were transfected using Metafectene Pro (Biontex Laboratories, GmbH, Germany). Stable clones were selected by addition of puromycin (InvivoGen, ref: QLL-39-01A, 10mg/mL) or by coumarin (Promega, Madison, USA). Back-up clones were frozen in liquid nitrogen in a mixture of 10% DMSO (Sigma Life Science, ref: D2650) with 90% FCS.

Gene expression of FSHR or LHCGR in pSB-tet was induced by Doxycycline (Sigma-Aldrich, ref: 10592-13 -9 D3447, 2 µg/mL).

*2.3 Flow cytometry*

Flow cytometry was used to detect the expressed receptors at the cell surface in the obtained cell lines. The following antibodies were used:

Anti MYC-PE: dilution 1/200 (Abcam, ref : ab72468) ; Anti FLAG-PE : dilution 1/200 (MACS Milteni Biotec, ref : 130-101-577) ; Anti MYC-APC : dilution 1/200 (RD systems, ref : IC 36964) ; Anti HIS-APC : dilution 1/100 (Abcam, ref : ab72579). Fixed cells were incubated with the antibody for 1 hr at 4°C in the dark, washed, resuspended in PBS and analysed in a MACSQuant 10 (MACS Miltenyl Biotec).

*2.4 RT-PCR*

Clones grown in 6-well plates were lyzed in 200 µl of TRI reagent and total RNA was extracted following the Direct-Zol protocol according to the manufacturer's instructions (Direct-zol RNA miniprep, Zymo Research Corp., Irvine, USA). After reverse transcription with Protoscript II reverse transcriptase cDNAs were amplified by 35 cycles with annealing temperature of 59°C for 3sec, using OneTaq DNA polymerase. All enzymes were purchased at New England Biolabs, Evry, France.

*2.5 measurement of cAMP*

Intracellular cAMP levels were measured using a homogeneous time-resolved fluorescence (HTRF ®) cAMP dynamic 2 assay kit (CisBio Bioassays, Bagnol sur Cèze, France), as described in (23). Forty-eight hours post-transfection cells were detached and seeded into white 384-well microplates with 5,000 cells/well in 5 µl of stimulation buffer (PBS 1×, 200 µM IBMX, 5 mM HEPES, 0.1% BSA). For their stimulation, 5 µl/well of the stimulation buffer containing or not different doses of hFSH and hCG as indicated were added. Then, cells were incubated for 30 min at 37°C and then lysed by addition of 10 µl/well of the supplied conjugate-lysis buffer containing d2-labeled cAMP and Europium cryptate-labeled anti-cAMP antibody, both reconstituted according to the manufacturer's instructions. Plates were incubated for 1 h in the dark at room temperature and time-resolved fluorescence signals were measured at 620 and 665 nm, respectively, 50 ms after excitation at 320 nm using a Mithras LB 943 plate reader.

*2.6 Statistical analysis*

Data were analyzed by GraphPad Prism 5.01 software (GraphPad Software Inc, San Diego, CA), essentially using non-parametric tests (Mann and Whitney, and Kruskal and Wallis). Signifiance was set at $p< 0.05$.

## 3- Results

To control the expression of the FSHR and LHCGR in KGN cells both temporally and quantitatively, we produced cell lines stably expressing either one or both receptors. After the construction and verification by sequencing, plasmids were amplified, purified and transiently expressed in KGN cells to validate the optimal experimental conditions. Inducibility was evaluated after transient transfection with the pSBTetLuc and pSBTet-myc-LHCGR plasmids. The highest dox-induced activity was observed after 24 or 48 hours, depending on the sensitivity of the assay. Subsequently, the appropriate concentration of puromycin for selection of the clones that have

integrated the plasmid was determined to be 2 µg/ml, by cell viability assay. After establishment of the optimal transfection conditions, several stable clones of KGN cells were obtained.

3.1 *Cell lines #1* : *c-myc-tagged-LHCGR-KGN*

At least two cell lines were positive for the inducible expression of LHCGR by RT-PCR, cytofluorimetry, cAMP and progesterone production. Further characterization is required.

*3.2 Cell lines #2: FLAG-tagged FSHR_HEK293*

Also in this case at least two cell lines were positive for the inducible expression of FSHR by RT-PCR, cytofluorimetry and cAMP production. Further characterization is required. These cell lines are not steroidogenic and can be used in case KGN cells do not support FSHR expression over time.

*3.2 Cell lines #3 FLAG-tagged FSHR-KGN*

At least two cell lines were positive for the inducible expression of FSHR by RT-PCR, cytofluorimetry, cAMP and progesterone production. Further characterization is required.

*3.3. Cell lines #4: Double, c-myc-tagged-LHCGR and FLAG-tagged FSHR-KGN*

KGN cells were simultaneously transfected with the pSBcou-FLAG-FSHR and pSBtet-myc-LHCGR, containing resistance gene to the Zeocin and Puromycin antibiotics, respectively. Clones have emerged very rapidly, in only a few days. Selected clones will be expanded and tested with the same functional assays as described for the other cell lines.

## 4- Discussion

The cell lines obtained will be instrumental to deepen our knowledge about gonadotropin function and molecular pharmacology. Gonadotropin action can be studied *in vitro* using cell lines or primary human granulosa-lutein cells (hGLC) obtained at oocyte pickup from ART programs. We have a long-lasting experience with such cells, which were demonstrated to be a good model to investigate gonadotropin interplay (4, 6, 16). We used the human granulosa cell line hGL5 to demonstrate how FSH and LH differently control proliferative or apoptotic signals. Using different experimental settings, we demonstrated that the expression of FSHR and LHCGR can be induced in hGL5 cells but that the FSHR-dependent cAMP/PKA pathway is constitutively silenced, possibly to protect cells from FSHR-cAMP-PKA-induced apoptosis. Also, we revealed previously unrecognized features intrinsic to the two structurally similar gonadotropin receptors, oppositely resulting in the regulation of life and death signals *in vitro* (20). Moreover, using transfected HEK293 cells and a steroidogenic, murine Leydig cell line (mLTC-1), it was shown that LH and hCG display biased agonism at the LHCGR and LH is partial agonist on β-arrestin recruitment and progesterone production but not on testosterone production in Leydig cells (21). These data were obtained in close collaboration between the fellow and the hosting institution.

The KGN cell line expressing both FSHR and LHR is expected to mimic the various physiological stages of granulosa cell growth and differentiation, which can now be studied *in vitro* by modulating the expression of either or both receptors in our inducible cell model. It is now well established that glycoprotein hormone receptors act as functional dimers on the cell surface (7), as most of the members of the G protein-coupled receptors (GPCRs) superfamily. GPCR dimerization was elegantly explored using mutant receptors, elucidating the modulatory role in agonist affinity, efficacy, and trafficking (7-12). Recently, it was shown that gonadotropin receptors can form heterodimers (13), resulting in cooperativity between FSHR and LHCGR (14). When naturally co-expressed in granulosa cells, they may form heterodimers and cooperate in activating signal transduction, as demonstrated *in vitro* (15). This finding suggests that FSH binding to its receptor may induce LH-like stimuli through activation of LHCGR by heterodimerization, modulating granulosa cell differentiation and follicle maturation. In light of these concepts, in a cell environment characterized by relatively low expression levels of gonadotropin receptor proteins, it

becomes reasonable that low amounts of FSH should be able to drive LH-like, proliferative activity through FSHR-LHCGR heterodimers. It should be considered that FSH displays about 100-fold lower potency than LH in inducing intracellular signaling (e.g. 50 nM *versus* 500 pM $EC_{50}$ calculated for cAMP (4, 16). These data suggest that FSHR is less sensible to hormone stimulation than LHCGR. However, in the early antral follicles, this difference is likely smoothened by the 100-fold higher expression level of FSHR than LHCGR gene (17), reasonably maintaining proper balance between each receptor-specific signaling. It is reasonable that relatively low amount of LHCGR may favor heterodimer formation with the largely available FSHR. This is interesting because, recently, bivalent ligands were developed, compounds able to bind and stabilize preexisting dimers without promoting ligand-induced dimerization (18). Bivalent ligands consist in two pharmacophores linked by a spacer sequence and are promising tool for detecting dimeric receptor structures *in vivo* (19). Understanding more in depth this complex interplay may lead to the development of novel therapeutic options, aiming at exerting the action of the two gonadotropins when and where it is needed and in the proper proportion between the two.

## 5- Conclusion

In conclusion, the main goal of the fellowship project was achieved and we obtained several different human granulosa cell-derived lines. So far, we know that 3 of them do not express FSHR or LHR in basal conditions but can be induced to express one or both of them under pharmacological induction. They are now ready to be employed to characterize the pleotropic action of LH and FSH. The double-inducible cell line has to be characterized further.

## 6- Perspectives of future collaborations with the host laboratory

The results of this work are only preliminary toward the completion of project, which will now continue in the two laboratories. We obtained a novel, very valuable and essential experimental instrument to address the following questions: Is it possible to recapitulate this differentiation process by controlling temporally and quantitatively the expression level of each gonadotropin receptor? May the action of the LHCGR substitute for the absence of FSHR and drive follicular growth? Conversely, in the absence of LHCGR, is forced FSHR expression able to drive the follicle to a progesterone-secreting luteinizing granulosa cell? What are the signaling pathways involved? In addition, physiologically, what happens when the LHCGR takes over from the FSHR? Are the effects only transcriptional or might the LHCGR heterodimerize with the FSHR to cause its retention in the cytosol and/or progressive loss of responsiveness?

Both the host and the fellow lab are now committed to carry on this research activity through exchanges of materials and researchers, as well as collaborative grant application in the near future and for the years.

## 7- Articles published in the framework of the fellowship

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## 8- Acknowledgements

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MS was on sabbatical leave from her position as full professor and Director of Unit of Endocrinology of the University Hospital of Modena, Italy. MS is grateful to her research and clinical assistants who took care of teaching and clinical activity in her absence.

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