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FELLOWSHIP FINAL REPORT

Research Fellowship in Antibody fragments targeting ovarian GPCRs to control reproduction

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ABSTRACT

In this study, we tackled the challenge of developing modulatory antibodies against G protein-coupled receptors, with a specific focus on the follicle-stimulating hormone receptor (FSHR), a pivotal regulator of reproduction. Leveraging variable domains of heavy chain-only antibodies (VHHs), we constructed two immune VHH libraries and implemented multiplexed phage display techniques. Our methodology integrated Multiplexed Phage Display, High-Throughput Sequencing, and Functional Assays to identify modulatory VHHs targeting FSHR. Following library construction, next-generation sequencing identified 34 clusters of specifically enriched sequences. These sequences underwent functional assessment in a primary screen based on a cAMP response element (CRE)-dependent reporter gene assay. Impressively, 23 VHHs displayed either negative or positive modulation of FSH-induced responses, indicating a high success rate for the multiplexed strategy. Subsequently, we focused on the largest identified cluster, PRC1, which exhibited positive modulation of FSH action. We provided evidence that PRC1 specifically binds to human FSHR and the FSHR/FSH complex, enhancing FSH-induced cAMP production and Gs recruitment. In conclusion, our study showcases an improved selection strategy that effectively identifies functionally active VHHs and can be adapted to target other challenging membrane receptors. Notably, this investigation led to the discovery of PRC1, the first potential positive modulator VHH reported for the human FSHR.

1- Introduction

The follicle-stimulating hormone receptor (FSHR), a class-A G protein-coupled receptor (GPCR), plays crucial roles in spermatogenesis in testicular Sertoli cells and follicle maturation and estrogen synthesis in ovarian granulosa cells. While recombinant FSH is commonly used in assisted reproductive technologies, its associated side effects and links to diseases have sparked interest in developing alternative pharmacological agents targeting FSHR signaling.

GPCRs are the largest family of receptors and prime drug targets. Agonists typically bind to orthosteric sites on the extracellular portion of receptors, inducing structural changes and facilitating coupling to transducers like G proteins and arrestins. Allosteric ligands, which bind to distinct sites from the orthosteric site, can modulate receptor activity positively (PAMs) or negatively (NAMs), maintaining physiological regulation.

VHHs, derived from heavy chain-only antibodies found in camelids, are promising tools for targeting challenging receptors like GPCRs due to their small size and unique structure. However, selecting high-affinity/selectivity VHHs for specific pharmacological profiles remains challenging.

In this study, we employed various screening methods to generate a panel of VHHs potentially modulating FSHR activity. Additionally, we characterized one VHH as a potential PAM for the cAMP pathway downstream from FSHR. This integrated approach offers insights into developing novel pharmacological agents for modulating FSHR signaling.

2- Experimental details

Ligands and Materials

Recombinant FSH was diluted in mQ H2O to concentrations suitable for cell stimulation. Ninety-six-well white plates were utilized for the experiments. Coelenterazine-H was procured from Interchim. The anti-hen egg lysozyme (HEL) VHH was chosen to design the NR control VHH-Hinge-Fc. Proteoliposomes were obtained from Synthelis.

Cell Culture and Transfection

HEK293A cells were cultured in DMEM medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin. Transient transfections of HEK293A cells were performed in suspension in 96-well plates using Metafectene Pro following the manufacturer's protocol. ExpiCHO cells were cultured in ExpiCHO Expression Medium at 37°C, 8% CO2, and 120 rpm shaking. CHO cells were cultured in complete Ham's F-12K medium supplemented with 10% (v/v) fetal bovine serum and 0.1 mg/mL streptomycin. Transfection of cells was carried out using jetOPTIMUS® according to the manufacturer's protocol.

Llama Libraries

The VHH libraries used in this study originated from two separate llama immunizations. The first involved injecting HEK293 cell membranes expressing FSHR, while the second library was generated from immunizing a llama with cDNA encoding hFSHR, hLHCGR, or hTSHR.

Phage Display

Phage display experiments were conducted on various substrates, including proteoliposomes, whole cells expressing FSHR, recombinant hFSHR ectodomain fused to maltose-binding protein, an N-terminal peptide from hFSHR, recombinant hFSHR ectodomain, or cell membrane expressing hFSHR with appropriate negative controls. DNA from amplified phages corresponding to each panning experiment was amplified by PCR using VHH-selective primers and sequenced by Next Generation Sequencing (NGS). The sequences were then grouped into clusters of identical sequences exclusive to the phage display conditions with FSHR. The VHH sequences were reformatted

by adding Hinge-Fc from mouse IgG2a containing the LALAPG mutations to suppress effector functions, along with a FLAG tag and an IL2 signal sequence. The constructs were codon-optimized for expression in ExpiCHO cells and cloned into the mammalian expression vector pTwist CMV BG WPRENeo using the NucleoSpin Plasmid Miniprep kit.

NGS Analysis by Germinal Lineage

NGS sequences were processed using a custom SnakeMake pipeline, involving trimming, fusion, translation, and annotations (FRs and CDRs). The reading frame was determined using a conserved protein label in FR1, followed by translation and extraction of complementarity-determining regions (CDRs) to generate the VHH "signature". Seqcollapse program was used to obtain non-redundant sequences and a file describing sequence numbers for each selection round. Seq2kmermat encoded sequences as kmer vectors, and a UMAP was created from the resulting matrix using the R package Uwot.

In Silico Prediction of Epitopes

MAbTope method, ranking semirigid docking poses with an AI-based scoring function, predicted antibody/antigen epitope and paratope pairs. Epitope comparison involved counting residues predicted as epitopes with high, medium, or very high probability, and antibodies were considered in competition if this ratio exceeded 0.3. CDR sequences were delimited using Chotia's numbering, and secondary structures were predicted. Antibody CDRs were encoded and compared, with a similarity higher than 50 indicating a 0.95 probability of sharing the same epitope. FSHR ECD (PDB ID 8I2H) was used as the antigen.

Autologous Reporter Gene Assay

HEK293A cells were transfected with human FSHR, candidate VHH-Hinge-Fc, and pSomLuc plasmid expressing firefly luciferase reporter gene under CRE control. After 48 hours, cells were stimulated with FSH for 6 hours, then treated with BrightGlo Substrate. Luminescence was measured in a Mithras LB 943 plate reader, and values were expressed in relative luciferase activity units (RLU).

Transfection and Culture

ExpiCHO cells were transfected with plasmids corresponding to VHH-Hinge-Fc and cultured at 37°C, 8% CO2, and 120 rpm shaking for 16 hours. Following transfection, ExpiCHO Enhancer and ExpiCHO Feed were added. The culture was then maintained for five days at 32°C, 5% CO2, and 120 rpm shaking.

Medium Collection and Purification

After 11 to 12 days of transfection, the medium containing VHH-Hinge-Fc was collected and centrifuged. The supernatants were stored at -20°C until further use. For larger-scale production, ExpiCHO cells were transfected in 30 mL cultures. After ten days, the supernatant was dialyzed and VHH-Hinge-Fc was purified using UNOsphere SUPrA Affinity Chromatography Media.

SDS-PAGE Analysis

Protein samples were boiled and separated by SDS-PAGE on polyacrylamide gels, then stained with Coomassie blue.

BRET Assays

Cellular responses to VHH-Hinge-Fc were evaluated in HEK293 cells transfected with plasmids coding for human FSHR and EPAC sensors. BRET signals were recorded upon stimulation with FSH or VHH-Hinge-Fc.

Flow Cytometry

Binding of VHH-Hinge-Fc to FSHR was assessed in CHO cells transfected with various receptors. Cells were stained with VHH-Hinge-Fc and analyzed using flow cytometry.

Binding Validation with BLI

FSHR ECD-Hinge-Fc was biotinylated and immobilized on streptavidin-coated biosensors. Interaction with PRC1-Hinge-Fc was evaluated using BLI.

NanoBiT FSH Binding Assay

Competition between PRC1-Hinge-Fc and FSH binding to FSHR was assessed using a luminescence assay based on split luciferase fused to the proteins of interest.

Statistical Analysis

Data from experiments were analyzed using GraphPad Prism 6 software, and statistical significance was determined by two-way ANOVA. Throughout the process, rigorous experimental procedures were followed to ensure the accurate production and assessment of VHH-Hinge-Fc.

3- Results and discussion

Please, refer to the following article for pictures, references and further information: Zehnaker et al. Int J Mol Sci. 2023;24(21):15961.

Multiplexed Phage-Display and NGS Analysis

Two llamas (*Lama glama*) underwent immunization using different approaches: one received injections of HEK293 cell membranes overexpressing human FSHR, while the other was injected in the leg muscle with an expression vector encoding human FSHR complexed with a nanocarrier. This vector led to FSHR expression on muscle cell surfaces, triggering an immune response. Leukocytes isolated from blood samples had their RNAs purified, followed by reverse transcription and PCR amplification of VHH repertoires, which were then cloned into M13 phagemid vectors. These immune libraries were utilized for subsequent phage-display experiments.

To tackle the challenge of identifying VHHs capable of specifically modulating FSHR activity, we devised an original selection and screening strategy. In a total of 12 phage-display experiments, various forms of human FSHR were employed as follows:

I) Full-length human FSHR was transfected into either HEK293 or CHO cells (depending on the panning round), with living cells used in panning, and non-transfected cells as negative controls.

II) Membranes were prepared from transfected cells.

III) Empty (negative control) or human FSHR-positive proteoliposomes were generated using cell-free translation.

IV) The extracellular part of FSHR, with or without fusion with maltose-binding protein (MBP), was produced in E. coli, with MBP used as a negative control.

V) The N-terminal peptide (AIELRFVLTKLRVI) located in the FSHR ectodomain.

Phage fractions from each round of each panning modality underwent PCR amplification and NGS sequencing. The obtained sequences were aligned and grouped into clusters of identical sequences. Sequences enriched in one or more FSHR-positive modalities and absent in the negative controls were considered potential candidates. Applying these criteria, we identified 34 candidate clusters.

In anticipation of in vivo testing and to leverage the avidity associated with homodimers, the 34 VHHs were fused in frame with the Hinge-Fc of mouse IgG2a, resulting in VHH-Hinge-Fcs. These constructs were cloned into a mammalian expression vector. Notably, LALAPG mutations were introduced into the Hinge-Fcs to inhibit immune system activation, including complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC).

Autologously Expressed VHH-Hinge-Fc Assayed on CRE-Driven Luciferase Reporter Gene as a Primary Functional Screening

To establish a rapid and reliable functional assay in cells, we evaluated the ability of various co-transfected VHH-Hinge-Fcs fused to a signal peptide to influence CRE-dependent transcription in cells co-expressing FSHR and a CRE-sensitive luciferase reporter gene. The luminescence values are expressed as a percentage of the response obtained with the

negative control condition, which consisted of a non-relevant VHH-Hinge-Fc.

The VHH-Hinge-Fcs induced a range of effects on CRE-dependent transcription in the presence of three different FSH concentrations (0.3, 1.0, and 3.0 nM). Notably, similar results were observed across all three FSH concentrations. Specifically, considering the 0.3 nM concentration, 8 out of the 34 candidates exhibited a negative effect on transcription, while 15 others, including PRC1-Hinge-Fc, induced an increase in CRE-dependent transcription compared to the non-relevant control.

In the NGS analyses, the number of sequences per cluster was predictive of VHH affinity for FSHR. In this functional screen, the data suggest that 23 VHHs exert either negative or positive modulation on FSH-induced activity, indicating their likely binding to FSHR. Additionally, some of the nine VHH-Hinge-Fcs that did not exhibit any effect in the CRE–luciferase assay may still bind to FSHR without eliciting modulation on its FSH-induced signaling.

Therefore, the primary screening indicated that the multiplexed phage display strategy successfully identified binders to FSHR at a high rate, with a significant proportion demonstrating functional modulation of FSH-induced activity.

Clustering of VHH Sequences as a Mean to Assess Epitopic Diversity among Candidate VHHs

We sought to understand the extent to which the selected candidates were related to each other. It is generally assumed that nanobodies with similar VDJ sequences likely bind to the same epitope on the target. Therefore, we adapted the seqUMAP (uniform manifold approximation and projection) method to embed sequences into a two-dimensional (2D) space, such that closely related sequences are neighbors. SeqUMAP embeddings of the 34 selected candidates was depicted, overlaiding with different data for each variant. Interestingly, 32 out of the 34 candidates, including PRC1, were clustered together, while 2 candidates were clearly unrelated.

To further explore the potential epitopic diversity covered by the selected candidates, we carried out two unrelated yet complementary in silico analyses. First, we predicted the preferential binding regions on the FSHR ectodomain (ECD) for each candidate using the MAbTope method. Then, the overlaps between all the predicted binding regions were computed and plotted as a heat map in an overlap matrix. This analysis predicted at least three distinct classes of VHH, each potentially binding a distinct epitope, with PRC1 belonging to one of those clusters.

Next, we conducted a similarity analysis based on a method that integrates sequence similarities and elements of the 3D structure of the VHH. The similarity matrix was computed and represented as a heat map. Several clusters of similar VHHs were predicted, with PRC1 appearing to be unique.

We then assessed the congruence between the predictions made by these two independent in silico methods. A coherence matrix revealed a low rate of incoherencies, with PRC1 not being among them. Overall, these in silico analyses suggest that the multiplexed phage display strategy facilitates the exploration of epitopic diversity, which could be advantageous for generating drugs with pharmacological diversity.

Validation of PRC1-Hinge-Fc Activity Using ExpiCHO Extracts and cAMP BRET Assay

Given PRC1-Hinge-Fc's promising modulation of FSH-induced CRE luciferase activity and its representation within the largest cluster selected, we proceeded with further validation and characterization. To ensure rapid and cost-effective validation, we cloned the cDNA encoding PRC1-Hinge-Fc into a mammalian expression vector downstream from an IL-2 signal sequence for extracellular secretion. The construct was then transfected into ExpiCHO cells in small volumes.

To assess the impact of PRC1-Hinge-Fc on cAMP production, we employed a bioluminescence resonance energy transfer (BRET) assay using HEK293 cells expressing the FSHR and the cAMP BRET sensor (NLuc-Epac-vv). The assay was conducted in the presence or absence of FSH, with 10 µL of unpurified ExpiCHO supernatant containing PRC1-Hinge-Fc. A negative control comprising ExpiCHO cells transfected with an empty vector was also included.

Figure 4 illustrates the kinetic curves of cAMP production expressed as a percentage of the maximum response to FSH. At all tested FSH concentrations (0.3, 3, and 30 nM), PRC1-Hinge-Fc demonstrated a clear positive modulation compared to the effect of FSH alone at the same concentration. Importantly, PRC1-Hinge-Fc alone did not affect cAMP production, indicating its specificity in modulating FSH-induced signaling.

PRC1-Hinge-Fc Binding and Kinetic Rate Constants Determination

To further validate the action of PRC1-Hinge-Fc on FSHR and explore its pharmacological properties, we produced and purified it in ExpiCHO cells. A Western blot analysis of the purified PRC1 and PRC1-Hinge-Fc revealed two entities in the gel: one corresponding to the expected molecular weight of 45 kDa and another likely representing a cleavage product in the VHH or between the VHH and the Hinge. Importantly, PRC1-Hinge-Fc specifically detected the human FSHR.

Next, we conducted a competition assay to evaluate PRC1's binding to FSHR. The SmBiT subunit of split nano-luciferase fused in the N-terminus of human FSHR was transfected into HEK293 cells. Cells were then incubated with purified FSH-LgBiT and either PRC1 or an anti-HEL VHH used as a negative control. Similarly, a competition assay was performed with PRC1-Hinge-Fc and an anti-HEL-Hinge-Fc negative control. These assays demonstrated the competitive binding of PRC1 and PRC1-Hinge-Fc to FSHR.

Subsequently, a biolayer interferometry (BLI) assay was conducted to assess PRC1-Hinge-Fc's direct interaction with the FSHR ECD and determine the kinetic rate constants. Recombinant FSHR ECD bound to FSH was immobilized on streptavidin sensors, and increasing concentrations of PRC1-Hinge-Fc were exposed to measure association and dissociation. The association and dissociation curves were fitted with a 1:1 model, and the kinetic rate constants were determined. The estimated kon, koff, and KD of PRC1-Hinge-Fc were calculated to be 3.19 × 10^4 M^−1, 1.59 × 10^−3 M^−1, and 49.9 nM, respectively.

These findings demonstrate the ability of PRC1-Hinge-Fc to bind the FSH-occupied ECD of FSHR in an allosteric mode of action, providing further validation of its pharmacological properties.

PRC1-Hinge-Fc Binding Specificity and Binding Mode

To evaluate the binding specificity of PRC1-Hinge-Fc, we examined its interaction with the native form of human FSHR, its potential cross-reactivity with mouse FSHR, closely related human LHCGR, mouse LHR, and human TSHR. Flow cytometry analyses revealed that PRC1-Hinge-Fc binds specifically to the human FSHR, suggesting limited utility in rodent models for assessing in vivo or ex vivo efficacy. Additionally, we conducted a NanoBiT-based FSH binding assay, demonstrating that neither PRC1 nor PRC1-Hinge-Fc competes with FSH for binding at the receptor, consistent with an allosteric binding mode.

Pharmacological Profile of PRC1-Hinge-Fc

Quantitative assessment of PRC1-Hinge-Fc's pharmacological activity on three hallmarks of FSHR signaling - cAMP production, Gs recruitment, and arrestin recruitment - was conducted using dedicated BRET assays in living HEK293 cells transiently expressing the human FSHR.

First, we compared FSH-induced cAMP responses in the presence or absence of PRC1-Hinge-Fc at different hormone concentrations. Representative kinetics showed that PRC1-Hinge-Fc increased cAMP response, while a negative control had no effect. The whole dose response analysis revealed a significant shift in the EC50 and an increase in Emax in the presence of PRC1-Hinge-Fc, indicating its positive effect on cAMP production.

Next, a BRET assay was performed to assess the modulation of Gs recruitment to the FSHR. PRC1-Hinge-Fc increased mGs protein recruitment to the FSHR, with a slight shift in EC50 and no change in Emax, indicating its ability to modulate Gs coupling.

Finally, the effect of PRC1-Hinge-Fc on FSH-induced arrestin recruitment to the FSHR was evaluated using a BRET assay. Although a slight increase was observed in the presence of PRC1-Hinge-Fc, it did not reach statistical significance, and no significant shift was observed in the dose-response.

Discussion

These findings provide insight into the pharmacological profile of PRC1-Hinge-Fc, highlighting its positive modulation of cAMP production and Gs recruitment while showing limited effect on arrestin recruitment.

In this study, we introduced an improved method for selecting modulating VHHs against the FSHR, a challenging target belonging to the GPCR family, which is of great importance for therapeutic antibodies. Our method combines multiplexed phage display with NGS, aiming to increase the likelihood of obtaining VHHs capable of binding to the native form of the receptor. However, this strategy may generate a large number of clones for assessment.

To overcome this challenge and narrow down the candidates, we performed NGS analysis of all phage display conditions. Our findings revealed that randomly selecting a limited number of clones, as is typical, often leads to false positives while missing potentially interesting clones. Instead, analyzing millions of NGS sequences and applying stringent criteria significantly reduced the number of candidates. This approach resulted in the identification of hits at a higher rate compared to traditional VHH selection pipelines.

One limitation of relying on NGS for candidate selection is the need for gene synthesis before testing. However, we viewed this as an opportunity to reformat VHHs into VHH-Hinge-Fc and clone them into a mammalian expression vector with a signal sequence for secretion.

We hypothesized that reformatting VHHs into VHH-Hinge-Fc could offer several advantages: Firstly, VHH-Hinge-Fc, being dimeric, benefits from avidity, leading to better apparent affinity. Secondly, they exhibit extended pharmacokinetic properties in vivo due to Fc binding to FcRn, facilitating the transition from in vitro characterization to in vivo testing. Lastly, the ability of candidates to be secreted after transfection in mammalian cells was crucial for developing an autologous reporter gene assay, enabling fast, robust, and parallel functional assays in the initial screening process.

The autologous reporter gene assay provides a significant advantage by bypassing the laborious and expensive bioproduction step. Out of the 34 candidates selected from the NGS analysis, 23 VHHs demonstrated apparent modulatory effects on CRE-dependent transcriptional activity, indicating that the multiplexed strategy can facilitate the selection of candidates with pharmacological diversity. However, a potential drawback of this method is the variability in antibody concentration in media among candidates, which may hinder the identification of some interesting candidates. Nonetheless, our procedure enables the rapid detection of positive or negative modulators of receptor activity and the selection of candidates that can be efficiently produced and correctly secreted.

To further investigate the diversity of VHHs selected by our strategy, we employed three

distinct in silico methods to predict epitopic diversity. These analyses suggested that the multiplexed phage display strategy selects VHHs potentially presenting epitopic diversity, which could contribute to pharmacological diversity by targeting distinct epitopes on the receptor of interest.

As an initial step towards validating the multiplexed phage display strategy, we focused on PRC1, which represented the largest sequence cluster. PRC1-Hinge-Fc exhibited a clear functional effect consistent with positive modulation of FSHR activity in the autologous reporter gene assay, which was later confirmed using crude ExpiCHO supernatant in a cAMP BRET assay. Further characterization of PRC1-Hinge-Fc revealed its specific binding to human FSHR with a KD of 49.9 nM, with no cross-reactivity observed with other glycoprotein hormone receptor family members. Importantly, PRC1-Hinge-Fc demonstrated an allosteric binding mode on the receptor, as evidenced by its ability to bind to the native FSHR without FSH and to the FSHR ECD/FSH complex, as well as its modulation of the Gs/cAMP pathway only in the presence of FSH. However, further pharmacological characterization and structural evidence are needed to confirm its positive allosteric modulation.

Based on the recently reported structure of the FSHR in inactive and active conformations, it is observed that the FSH–ECD undergoes a significant conformational change between the inactive and active states. In the inactive conformation, the FSH–ECD is parallel to the plasma membrane, whereas in the active conformation, it is perpendicular. This conformational change could potentially hinder FSH binding in the inactive state due to steric clashes with the plasma membrane. In this context, PRC1-Hinge-Fc may modify the equilibrium between inactive and active FSHR conformations, favoring the active state. This action could facilitate FSH binding and subsequent receptor activation. However, further structural studies, including epitope mapping and pharmacological characterizations, are necessary to validate this hypothesis.

Furthermore, our data indicate that PRC1-Hinge-Fc does not affect FSH-induced arrestin recruitment, suggesting that its positive allosteric modulation (PAM) effect may be biased. Additional investigations will be required to confirm this hypothesis. Previous studies have reported VHHs that bind to the FSHR without providing functional evidence, while others have shown inhibition of FSH-induced cAMP response. However, reports of VHHs with PAM activity against GPCRs are scarce, with examples mainly found for metabotropic receptors such as mGluR2 and mGluR4.

4- Conclusion

The FSHR plays a crucial role in reproductive control, and targeting it holds potential for infertility treatment, non-hormonal contraception, and ovarian cancer therapy. Moreover, the FSH/FSHR axis is implicated in various pathologies in post-menopausal women. Therefore, developing pharmacologically active agents that specifically target the FSHR to modulate their signaling properties is essential for fine-tuning downstream cellular events and physiological processes. Antibodies, particularly VHHs, offer promising prospects in this regard. The improved selection strategy presented in this study could accelerate the discovery of pharmacologically active VHHs not only for the FSHR but also for other challenging membrane targets.

5- Perspectives of future collaborations with the host laboratory

Prof. Livio Casarini has a 10-year collaboration with the hosting Research Group in the field of gonadotropins physiology and reproduction. This fellowship supported the present work, as well as those mentioned in the below point "*6- Articles published in the framework of the fellowship*".

During the year spent in the hosting Institution, Livio Casarini has written a research project

submitted for evaluation and funding the "Horizon EIC PathFinder Open 2024" call (Proposal title: *PCOS shutdown via simultaneous multi-molecular targeting by all-in-one nanobody fragments*; Proposal acronym: *SIMMONA*; Proposal ID: *101184870*; internal reference number: *SEP-211031298*). In this project, we proposed to develop a nanoantibody-based treatment for polycystic ovary syndrome. Collaborators of this proposal are: Livio Casarini (PI), Manuela Simoni, Antonio La Marca (University of Modena and Reggio Emilia, Modena, Italy) Eric Reiter, Pascale Crepieux, Frederic Jean-Alphonse (INRAE/CNRS, Tours, France), Crystele Racine, Nathalie Di Clemente (Sorbonne/INSERM, Paris, France) and Desmond Schofield (Evitria AG, Zurich, Switzerland).

Another project in collaboration with the hosting Group, started three years ago and still ongoing, is focused on the development of a nanoantibody-based contraceptive method. This project is funded by the Bill & Melinda Gates Foundation. Future project perspectives will focus on the characterization of extra-gonadal gonadotropin receptors in human tissues, by higly-specific nanoantibodies, as a controversial topic in the field. The two Groups are engaged since three years in the development and testing of several other therapeutic nanoantibodies targeting hormone receptors and one of these compounds is under characterization for possible patenting.

Finally, thanks to the support Le Studium, Livio Casarini and colleagues organized the international scientific meeting « *ICGR-V - 5th International Conference on Gonadotropins and Receptors* » (https://www.lestudium-ias.com/events/icgr-v-5th-international-conference-gonadotropins-and-receptors), where about forty World experts in the field gave lactures on gonadotropins physiology. In that occasion, the basis for future collaborations among this network where posed.

6- Articles published in the framework of the fellowship

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7- Acknowledgements

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