The Observation of FSH’s Cellular internalization

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Abstract
Follicle stimulating hormone (FSH) is a kind of glycoprotein gonadotropin, and plays an important role in the diagnosis and treatment of infertility. Follicle stimulating hormone receptor (FSHR) is a kind of G protein coupled receptor (GPCR), found in the ovary and testes, and its activation is required for the hormonal operation during the breeding period. In this study, an experimental model of FSHR mediated FSH into cell membrane, which exhibited a phenomenon of fluorescent localized on cell surfaces internalized into cell interior, was established to verify biological activity of FSH.

Keywords
FSH Receptor; Target binding; Cellular internalization

1. Introduction

Human follicle stimulating hormone (hFSH), synthesized and secreted by the pituitary gland, is a kind of glycoprotein gonadotropin[1]. It is heterologous dimers glycoprotein that composed of FSHα subunit and FSHβ subunit[2]. FSHα and FSHβ subunits are both involved in receptor
binding and signal mediating. Separating FSHα subunit and FSHβ subunit will cause the FSH to lose biological activity[3].

The glycosylation of protein directly affects the biological activity of FSH. Studies found that, although FSH can be better binding with the FSH receptor after removing the sugar chain, the biological activity of FSH was completely lost[4-5]. The biological activity of FSH is not only related to the protein structure, but also related to the content of the sialic acid and its isoelectric point. The study showed that, as decreased content of sialic acid the isoelectric point was increased and the capacity of FSH binding receptor was enhanced. At the same time, the biological activity of FSH in vitro was enhanced[6].

The role of FSH in male and female is not the same. In female body, the main function of FSH is to stimulate the development of the follicle, ovulation and endometrial growth. Because FSH is a macromolecular protein, it requires receptor follicle stimulating hormone receptor (FSHR) to enter into the cell membrane. FSH receptor FSHR is presented in the granulosa cells of the follicle[7]. In male body, FSH can stimulate sperm production and promote the mature of sperm by the synergy of Luteinizing Hormone (LH) and androgen, to promote the mature of sperm[8]. The combination of FSH and FSHR through the G protein coupling mechanism, would produce two different effects: one is the activation of aromatase; the other is to induce the formation of LH receptors[9].

According to the role of FSH played in the human body, it is mainly used in clinical treatment of infertility. With the development of gene engineering, domestic and foreign scholars have conducted a lot of researches on recombinant human Follicle-stimulating hormone (rhFSH). Currently, the mainly products on the market are rhFSH produced by genetic engineering. The aim of genetic engineering expressed recombinant human follicle stimulating hormone protein is to explore its medicinal value and to use in clinical research in the future.

In this study, the positive biological activity of FSH standard sample was verified by cell experiment in vitro. FSH protein is a kind of Glycosylated protein, the biological activity of FSH protein is possessed after glycosylation modification, and its biological function is determined by the structure of FSH protein. Only if there be a suitable protein structure, the protein would have a corresponding biological function. FSH is a macromolecular protein, its signal transmission is dependent on specific receptor. FSH needs to be bound to the receptor at least in a region of the receptor binding domain and the resulting effect. The target protein that mediates FSH into the cell is FSHR.

In this work, labeled FSHR protein was expressed on the cell membrane
and added FSH protein standard sample. If the FSHR protein is able to identify FSH, it can be mediated into the cell membrane. In this way, fluorescent tagged FSHR proteins enter the cell from the cell membrane, and form the phenomenon of intrinsic fluorescence. The cellular internalization of FSH standard sample can provide reference for the expression of rhFSH protein's activity detection.

2. Targeted binding receptor

2.1. The construction of eukaryotic expression vector

Using gene recombination technology to design primers and FSHR gene sequence was amplified by PCR and connected to the pSNAPf-ADRβ3 vector by double enzyme digestion to construct the expression vector. Then, transformation of the pSNAPf-ADRβ2-FSHR expression vectors into expression host strain DH-5α, extracted pSNAPf-ADRβ2-FSHR recombinant plasmid and identified by restriction enzyme digestion and then sequenced, by this way the pSNAPf-ADRβ2-FSHR expression vector was successfully constructed.

2.2. The stable expression of vector in cells

Transfected the pSNAPf-ADRβ2-FSHR expression vector into HEK-293 cells, cultured for 24 hours in carbon dioxide incubator, digest the cells and antibiotic screening in 24-well plate. After screening for a period of time, the clone cells were obtained. The clone cells can express fusion protein of SNAP-tag protein and FSH protein.

2.3. Cell staining

Digest the cells that express fusion protein stably and inoculate in 96-well plate. Start SNAP-Surface 549 staining until the density of HEK-293 cells grew to 80% (SNAP-Surface 549 needs to be diluted in DEME complete medium. After dyeing, can be recycled to continue to use.), put 96-well plate in carbon dioxide to incubator cells for a moment. Remove stain and wash the cells with PBS three times to remove the remaining stain (Notice: Wash the cells slowly, its better not to lose cells.) Observed the stained cells under the microscope. Photo recorded.

2.4. rhFSH targeted combination FSHR

After SNAP-Surface 549 staining, the red fluorescence of the cell membrane surface can be clearly observed by fluorescence microscopy, and there is none in the cells. It indicated that the fusion protein was on the cell membrane. At this time, add the purified rhFSH target protein, and put 96-well plate in carbon dioxide to incubator cells for
a moment. Observation under fluorescence microscope. The red fluorescence on the cells membrane is internalized into the cells. Photo recorded.

3. Results
3.1. Comparison of transfection and non transfection of HEK-293T cells

FSHR protein was expressed on the surface of cell membrane of HEK-293 cells transfected with pSNAPf-ADRβ2-FSHR vector. After staining, the cell membrane formed a layer of red fluorescence. No red fluorescence was observed in the cell membrane of the non transfected HEK-293 cells. Its comparison chart as shown below:

Figure 1. HEK-293 without vector

Figure 2. HEK-293 transfected with pSNAPf-ADRβ2-FSHR vector
3.3. Results of FSH and FSHR targeted binging

The FSH protein standard was added to the HEK-293 cells after staining. Incubated for a period of time in incubator, the fluorescence of the HEK-293 cells membrane became dark and irregularly, and red fluorescence appeared in cell interior.

According to the addition of different FSH concentrations, as well as the level of protein activity and incubation time, the degree of internalization was incongruent. Higher protein activity and concentration of added, relatively higher degree of intrinsic fluorescence and more cell fluorescence moved from the membrane to the cell interior.

Its comparison chart is shown below:

![Figure 3. HEK-293 without adding FSH](image1)

![Figure 4. HEK-293 adding FSH](image2)
4. Discussion

In this study, the establishment of cell fluorescence system was used to make the target protein targeted binding receptor and to verify the biological activity of FSH. External target protein targeted binding receptor FSHR proved that FSH has the functional domain that can be combined with the target protein, and FSHR can mediate rhFSH entry into cells to play the biological function of FSH.
Target protein targeted binding receptor is to directly observe whether the FSH can be combined with its receptor FSHR. And the method is fast and convenient. But it needs to be noticed in the operation false positive results are likely to result from the regulation of the microscope and the treated of cells. Thus, it requires careful observation and analysis, in order to grasp the intrinsic phenomenon of fluorescence.

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