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Evaluation of a New Marker of the Ovarian Tumor Vasculature for Predicting Response to Treatment and as a Therapeutic Target

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The Follicle Stimulating Hormone receptor (FSHR) is a transmembrane cell surface receptor that binds FSH. The project is based on our observation that FSHR is highly expressed by the endothelial blood vessel cells in ovarian tumors. The experiments are aimed at characterizing FSHR expression levels in tumor sections using immunohistochemistry. The first goal is to determine if significant differences exist among the tumor types, and within the same tumor type, among tumor stages. The second goal is to evaluate the inhibitory effects on ovarian tumor angiogenesis and growth of an anti FSHR monoclonal antibody and of an antivascular agent. Using tumor tissue microarrays and a qualitative system of grading FSHR expression levels, we found that the main types of ovarian tumors and stages do not differ significantly in terms of vascular FSHR expression. A quantitative system was set-up for assessing FSHR expression, based on large tumor sections and quantitative determination of the density of FSHR-positive vessels at the interface between the tumor and the normal tissue. This more precise system offers better chances to detect differences among tumor types and stages. We found that FSHR-positive vessels are present in metastases of ovarian tumors to brain. We determined that the affinity of the anti FSHR monoclonal antibody 323 and/or its ability to suppress FSH/FSHR signaling is not sufficient to inhibit significantly tumor growth. Procedures to develop more efficient anti FSHR antibodies have been designed and are currently implemented. A cell line and a cell-ELISA type procedure, suitable for testing the agents targeted to FSHR in tumors, have been set-up.

Follicle Stimulating Hormone Receptor, vascular endothelial cells, tumor angiogenesis, ovarian cancer, mouse models;

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INTRODUCTION

The project is aimed at establishing the stage- and histotype-specific nature of FSHR expression and then investigating the feasibility of inhibiting ovarian tumor growth in mouse models using anti-tumor agents targeted to FSHR.

BODY

Aim 1. Correlate FSHR signaling with histology, survival and response to anti-angiogenic therapies

Task 1. Characterization of FSHR expression depending on the tumor type and disease stage

The experiments were aimed at characterizing FSHR expression levels in tumor sections using immunohistochemistry. The goal was to determine if significant differences exist among the tumor types, and within the same tumor type, among tumor stages. The relevance consists in the possibility that tumors could be categorized and their evolution predicted based on the FSHR expression levels. From a complementary perspective, the data could help determine which tumor types are most suitable for therapy using FSHR-targeted agents.

As specified in the research plan, we performed immunohistochemistry of tumor tissue microarrays (TMAs) of the main types of epithelial ovarian carcinomas, which account for 85 to 90 percent of all ovarian cancers. Analysis of the images revealed that not enough FSHR stained vessels are present in the analyzed samples to allow determination of statistically significant differences between tumor types and stages. The tissue samples collected for assembling TMAs consist in small areas of the tumors, of the order of 1 mm$^2$, because the general goal is to assemble on each microscope slide a large number of tumor samples. Moreover, the samples are preferentially collected from the central part of the tumors, to minimize the risk of collecting by error normal tissue adjacent to the tumor. We thought that these two factors could explain the low number of FSHR-positive vessels in the analyzed samples. In order to verify this assumption we performed immunohistochemistry on large tumor sections, of the order of 1-3 cm$^2$, which contained also normal tissue adjacent to the tumor. Analysis of the stained sections revealed that indeed areas of the order of 1 mm$^2$ located in the central parts of the tumors, of the kind most frequently collected to assemble microarrays, contained few blood vessels that were FSHR-positive. This observation explains the disappointing results mentioned previously. A high density of FSHR-positive vessels was however encountered at the border between the tumor and the normal tissue. We assessed that a band with a width of 5 mm, centered on the demarcation line between the tumor and the normal tissue, is optimal to evaluate the abundance of vascular FSHR staining.

We subsequently proceeded to determine, using this optimized evaluation, if differences can be detected among tumor types and stages. The staining abundance was graded, as described in the approved project, using a qualitative scale (0 (negative), +1 (mild-moderate), and +2 (strong)). FSHR-positive vessels were present in all samples analyzed. Subsequent statistical comparisons of the main types and grades did not reveal significant differences. We do not believe that this result means necessarily that there are no FSHR-related differences between the tumor types and stages. It is possible that the evaluation system that we used, although frequently used in clinical studies, was not powerful enough to detect significant differences in this particular case. A more precise evaluation system would to determine quantitatively the density of FSHR-positive blood vessels. This can be done by counting the number of FSHR-positive vessels per microscopic field, in multiple fields for each sample. Alternatively, the counting can be done on digital images taken from the sections. We tried both procedures and found out that they are too slow and difficult to implement, because counting has to be limited to the tissue band mentioned above. In order to overcome this difficulty we evaluated the possibility of acquiring images of whole sections using a Zeiss Axioplan 2IE microscope with a motorized stage, available in the shared Microscopy Facility of our Institution. The associated software stitches the tens or hundreds of images acquired from each section to generate a single image of the whole section. This image can the then inspected at any desired magnification, and precise information about the position of the visualized field within the section is immediately accessible. Unfortunately, many of our sections are larger than the maximal capacity of the microscope - approx. 100 images using a 20x objective, which corresponds to an area of approx. 6 x 4 mm$^2$. 

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This limitation could be circumvented recently, due to the acquisition of the Histology Core of our Institution of an Olympus slide scanner. This system can generate complete images of large sections, using a 20x or 40x objective.

In conclusion, we circumvented the initial difficulties and set up a quantitative method that allows precise determination of FSHR expression by the tumor vasculature. Using this method, we will compare as intended the various grades and stages of ovarian tumors, to determine if significant differences exist that can be used for diagnostic or clinical purposes. The same procedure is also adequate for performing with high confidence Task 2, namely investigating the correlation with antiangiogenic therapy.

In order to increase the clinical relevance of the measurements that will be done, we investigated the FSHR expression in metastatic ovarian tumors, which in most cases are responsible for the fatal outcome of the disease. We obtained so far metastases of ovarian tumors to brain. The immunohistochemistry experiments revealed that 50% of the analyzed cases had a high density of FSHR-positive vessels, while the remaining half presented moderate to weak FSHR expression.

**Task 3. Determine if the growth rate of ovarian primary tumors and metastases is correlated with the levels of circulating FSH.**

We stated in the approved project that while currently beyond the 2-year scope of the proposal, we will begin to collect the necessary samples/information to test this hypothesis. We used a set of 20 samples of serum stored at -80°C to verify the feasibility of measuring the FSH concentrations, using a FSH Hormone EIA kit from Cayman Chemical. The measurements yielded values in the expected range, and we are currently collecting serum samples from ovarian cancer patients.

**Aim 2. Define the effect of FSHR-targeted treatment on tumor growth and survival in a pre-clinical model of ovarian cancer**

**Task 1. To evaluate the effect of anti FSHR monoclonal antibody on ovarian tumor angiogenesis and growth**

**Subtask 1. To determine the optimal dose of antibody and frequency of injections in order to achieve the intended sustained intravascular concentration**

The anti FSHR 323 monoclonal antibody was labeled with 125-Iodine and injected into mice that carried tumors generated by human cancer cells. For the next five days the concentration of the antibody in the tumors was determined based on the data obtained using a microPET Concord system. The tumors were clearly distinguishable (Fig. 2). Images were analyzed using the ASIPro VM software, to quantitate the intensities of the signals. The curves in Fig. 3 indicate that the antibody concentration decreases gradually to approx. 50% of the peak values in approx. 2 days, and to 25% in approx. 4 days. The conclusion is that two injections per week would be sufficient to maintain a sustained concentration of the antibody and offers a good chance to detect an effect on tumors.

**Subtask 2. Testing the effects of an anti FSHR antibody on xenograft and orthotopic tumors.**

We injected the anti FSHR 323 monoclonal antibody in mice that carried SKOV3 tumors and monitored tumor growth. The antibody was injected 5 times at interval of 2-3 days. Control mice were injected with non-immune mouse IgG of the same class as the 323 antibody. Some of the tumors injected with the 323 antibody...
showed a transient reduction in growth, as illustrated in Fig. 4. However, overall the differences between the mice injected with the anti FSHR antibody and the control non-immune IgG were not statistically significant. The effect of the anti FSHR antibodies on the tumors is most likely proportional with the density of FSHR-positive blood vessels. Using mouse models that have a higher density of FSHR-positive blood vessels should increase the chance to detect consistent inhibition of tumor growth. For this reason put effort in finding mouse models that have high density of FSHR-positive vessels. We investigated xenograft tumor obtained by injecting A2780, OVCAR429 and OVCAR3 human ovarian tumor cells, and also allograft models, obtained by injecting the mouse ovarian tumor cell line Id8 [1]. We investigated also a transgenic mouse model, in which tumors form spontaneously, and therefore from this point of view the model is closer to the natural tumor formation. The transgenic model consists in mice that have two mutations, K-ras G12D and PTEN del-exon5 [2,3]. Among these models, we found FSHR-positive vessels only in the transgenic tumors [Fig. 5]. However the abundance was not higher than in the SKOV3 tumors. Taking into account that the xenograft models generate tumor in shorter time, the SKOV3-generated tumors remain the preferred model. We will continue to investigate other mouse models and conditions (number of cells injected, addition of matrigel, timing and doses of the IgG injections).

**Task 2. To use FSHR for targeting of antitumor agents in a mouse model**

**Subtask 1. To generate the antibody TF fusion proteins**

The results obtained with the antibody injections in mice that carried tumors suggested that the 323 monoclonal antibody does not have sufficient affinity for the receptor, and / or it is unable to block signaling induced by the endogenous FSH. This observation indicates that, before we generate fusion proteins targeted to FSHR, we have to identify a better ligand. The 323 antibody was generated more than 15 years ago [4]. Since then a large amount of information was published about the structure of FSHR, about functional domains that are responsible for binding FSH and for signaling, and about domains that have high antigenicity and could generate polyclonal antibodies. Based on careful analysis of all this information, we designed a procedure to generate new anti FSHR antibodies, which most likely will be more suitable for therapeutic purposes than the existing FSHR323 antibody. The immunizations are under way and we expect to analyze the hybridoma clones in next 2-3 months.
Subtask 2. Characterization of the fusion proteins in vitro

We solved so far two technical problems that are relevant for the successful implementation of the in vitro tests.

The first problem was to find an endothelial cell line that expresses FSHR. We tested three endothelial cell types: human placental endothelial cells in culture, a mouse tumor endothelial cell line, and immortalized human umbilical vein endothelial cells (HUVEC).

The first cell type was promising because we found that the endothelial cells in human placentas express FSHR. We obtained placental endothelial cells in culture from Dr. Carlos Escudero from the University of Bio-Bio from Chile [5,6]. We tested the FSHR expression by RT-PCR and by immunofluorescence. Both tests yielded negative results. The most probable explanation is that the cells lose the ability to express FSHR soon after being exposed to the in vitro culture conditions, as it happens for the VEGF receptors (Carlos Escudero, personal communication).

We tested also a mouse endothelial cell line of tumor origin, 2H-11 [7]. We expected that these cells would express FSHR based on their tumor origin. The results were however negative.

We found clear FSHR expression in an immortalized human umbilical vein endothelial cell line. This observation is in agreement with the fact that we found FSHR expression in human umbilical ECs in paraffin sections of umbilical cords. These cells will be used for the in vitro tests of the fusion proteins.

A second technical element that we perfected was a procedure to assess quantitatively the binding of anti FSHR antibodies to the target cells. The procedure consists in cell-ELISA.

The HUVECs and the cell ELISA procedure will be used to test in vitro the antitumor agents that will be generated.

KEY RESEARCH ACHIEVEMENTS
- The main types of ovarian tumors and stages have not been found to differ significantly in terms vascular FSHR expression, as assessed by using Tumor Microarrays and a qualitative system of grading FSHR expression levels;
- A quantitative system was set-up for assessing FSHR expression, based on large tumor sections and quantitative determination of the density of FSHR-positive vessels at the interface between the tumor and the normal tissue; this more precise system offers better chances to detect differences among tumor types and stages;
- FSHR-positive vessels are present in metastases of ovarian tumors to brain;
- The affinity of the anti FSHR monoclonal antibody 323 and / or its ability to suppress FSH/FSHR signaling is not sufficient to inhibit significantly tumor growth; procedures to develop more efficient anti FSHR antibodies have been designed and are currently implemented;
- a cell line and a cell-ELISA type procedure, suitable for testing the agents targeted to FSHR in tumors, have been set-up;

REPORTABLE OUTCOMES
A position of Postdoctoral Fellow was created in the Department of Developmental and Regenerative Biology of the Mount Sinai School of Medicine. The position was filled with a Postdoctoral Fellow that had four years of postdoctoral research experience.
CONCLUSIONS

The hypothesis that the expression levels of vascular FSHR in ovarian tumors depends on the tumor type and stage was tested. The conclusion was that the method used was not powerful enough to detect significant differences. The limitations of the method were clarified, and a new method was set-up, which is quantitative and takes into account the non-homogenous distribution of FSHR in the tumors. This method, which has much higher chances of success, will be used to address the question.

The experiments that tested the effect of a monoclonal anti FSHR antibody revealed that for consistent tumor inhibition new antibodies are necessary, which have higher affinity and ability to block FSH signaling. Procedures to obtain such antibodies have been designed and implemented.

REFERENCES

1. Son DS, Roby KF, Rozman KK, Terranova PF. Estradiol enhances and estriol inhibits the expression of CYP1A1 induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin in a mouse ovarian cancer cell line. Toxicology. 2002; 176(3):229-43

APPENDICES: N.A.