Follicle stimulating hormone is an autocrine regulator of the ovarian cancer metastatic niche through Notch signaling

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Abstract

The association between upregulated Notch and FSH signaling and ovarian cancer is well documented. However, their signaling functions have been predominantly probed independently, and in the context of primary tumor tissues. The aim of this study was to investigate the interactive effects of FSH and Notch signaling on the formation and maintenance of disseminated metastatic ovarian cancer cells. We found that FSH upregulated Notch signaling in the ovarian cancer cells by increasing the expression of Notch receptors and ligands. The proliferation rate of the ovarian cancer cells was also increased in the presence of FSH. Further, high levels of FSH were detected in the ascites of patients with serous ovarian adenocarcinoma. The spheroids from the ascites of the patients, as well as, the spheroids from the ovarian cancer cell lines, expressed FSHβ subunit mRNAs and secreted the hormone into the medium. In contrast, primary ovarian tumor tissues and cell line monolayers expressed very low levels of FSHβ. Ovarian cancer cells cultured as spheroids exhibited higher expression of the FSH receptor (FSHR) and Notch downstream genes

than countterpart monolayers. The Single chain Fragment variables (ScFvs) specific for the Negatively Regulatory Region (NRR) of Notch, and antibodies against the extracellular domain of FSHR, in combination, significantly inhibited spheroid formation and cell proliferation *in vitro*. In conclusion, this study demonstrates the autocrine regulation of ovarian cancer spheroidogenesis through FSH-driven Notch signaling. Further, the Notch and FSHR together are the potential immunotherapeutic targets for the treatment of the ovarian cancer metastasis.

Introduction

Ovarian cancer is the leading cause of death among the gynecological malignancies [1]. One of the many risk factors recognized for the ovarian cancer is the excessive exposure of the normal ovarian surface epithelium to gonadotropins - Follicle stimulating hormone (FSH) and Luteinizing hormone (LH) during menopause, ovulation or infertility therapy [2-4]. The gonadotropins are heterodimeric glycoproteins comprising a common α subunit associated non-covalently with the hormone-specific β subunit, secreted by the anterior pituitary and are essential for follicular development in the ovary [5]. In addition, FSH stimulates growth of the ovarian cancer cells [6, 7] while inhibiting apoptosis [8, 9] and high levels of FSH in the ascites of the ovarian cancer patients are inversely correlated to their survival [10].

Notch signaling plays role in a wide spectrum of cell fate decisions. There are four Notch receptors (Notch1- 4) and four ligands (Jagged1 and 2, Delta 1 and 4) known in mammals, and alterations in the Notch signaling pathway are associated with different cancers and are potential therapeutic targets [11, 12]. There is a direct link between aberrant Notch signaling and ovarian

cancer progression [13]. Interestingly, FSH upregulates Notch1 receptor in ovarian cancer cells [14].

With the progression of the ovarian cancer, the cancer cells detach from the primary tumor, either as single cells or cellular aggregates termed as spheroids, which either remain in the ascites and contribute to the recurrence of the disease or attach to the peritoneum for the development of secondary tumors [15]. The spheroids have been shown to be less susceptible to chemotherapy than the single cells and disruption of the spheroids resensitizes the ovarian tumor cells to chemotherapy with platinum-based drugs [16, 17].

In this study, the link between FSH and Notch pathways has been investigated in more details using three different ovarian cancer cell lines. We demonstrate that FSH upregulates Notch signaling in these cell lines. Further, we demonstrate higher levels of FSH in the ascites of the ovarian cancer patients and trace the origin of this high FSH to the spheroids obtained from the patients.

Materials and Methods

Ovarian cancer cell lines

The ovarian cancer cell lines OVCAR-3, SKOV-3 and OVCAR-4 were authenticated by Short Tandem Repeat (STR) analysis. The OVCAR-3 cells were maintained in RPMI medium (Sigma) supplemented with 15% Fetal Bovine Serum (FBS) (GIBCO). The SKOV-3 cells were maintained in McCoys medium (Sigma) with 10% FBS. The OVCAR-4 cells were maintained in DMEM medium (Sigma) supplemented with 10% FBS. All media were supplemented with penicillin and streptomycin at the prescribed concentrations.

Hormone and Antibodies

The iodination grade-purified hormones and cAMP antiserum were obtained from the National Hormone and Peptide Program, NIDDK, National Institutes of Health. Polyclonal antibodies against FSH receptor (FSHR) extracellular domain (RF5) [18] and Notch3 receptor Negative regulatory region (NRR) were raised in the rabbits using the immunization protocol well established in the laboratory [19]. The Single chain variable fragments (ScFv) against the Notch3 NRR were isolated from the yeast display library using the standardized protocol [20]. The interesting ScFv clone (ScFv42) was expressed in *E. coli. Bl21* and purified by His^6 tag affinity chromatography and used for further experiments.

FSH Receptor Binding assay

The binding of FSH to the receptors present on the ovarian cancer cell lines was analyzed by radio receptor assay. Radioiodination of FSH was carried out using the IODO-GEN method[21]. The specific binding of ^{125}I-FSH to the receptor preparations from the ovarian cancer cell lines was demonstrated as described earlier [22].

In Vitro cAMP Measurement

Approximately 1×10^5 OVCAR-3 cells/well were plated in a 48-well plate and 24 hours later were incubated with fresh medium containing 1mM phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) for 30 minutes at 37 °C in a CO_2 incubator (100µl) and then incubated with varying concentrations of FSH for 15 minutes at 37 °C (100µl). At the end of the incubation, the cells were lysed with the addition of 200µl of 0.2 N HCl, and the total cAMP produced was determined by cAMP Radio-immuno assay (RIA) as described earlier [18].

Flow cytometry-based assays for the detection of FSH receptor and Notch receptors

The presence of FSH and Notch receptors on the ovarian cancer cell lines was demonstrated by flow cytometry-based assay. The ovarian cancer cells were detached from the tissue culture flasks using 0.5mM Ethylenediaminetetraacetic acid (EDTA) in Dulbecco's phosphate buffer saline (DPBS) and resuspended in a medium containing 10% FBS. Approximately $1X10^5$ cells were incubated with the primary antibody (Notch3 NRR a/s or RF5 a/s for FSHR) at 1:500 dilution for 1 hour at 4ºC, followed by washing thrice with DPBS and resuspension in 100µl of the medium with 10% FBS and 1:1000 dilution of anti-rabbit secondary antibody- fluorophore conjugated (Invitrogen) for 45 minutes at 4ºC. At the end of the incubation, the cells were washed and resuspended in DPBS and analyzed using the Becton Dickinson Accuri C6 and the median fluorescence values were analyzed.

Luciferase reporter assay for the determining the Notch signaling activity

The Notch signaling in the ovarian cancer cell lines was determined as described earlier [23]. Briefly, the ovarian cancer cell lines were seeded (5 X 10^4 cells/well) in 24 well plates and transfected with 790ng of 12XCSL (CBF1/Su(H)/Lag-1) reporter plasmid and 10ng of pGL3 basic or 800ng of pGL3 control along with 1ng of pRL-Tk using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. The transfected cells were incubated with increasing concentrations of FSH in the presence or absence of RF5 a/s, Notch inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester) (5µM) (Sigma) or ScFv42 (10µg/ml) and the Luciferase reporter activity was estimated using the Dual-luciferase assay kit (Promega) in TD200 Luminometer following the manufacturer's protocol.

Ovarian cancer cell line proliferation

The proliferation of the ovarian cancer cell lines in the presence of FSH or FSHR and Notch antagonists was determined using the BrdU (5-bromo-2'-deoxyuridine) incorporation assay. Briefly, 5×10^3 cells were seeded per well in a 96-well plate. The cells were synchronized by incubating in medium without FBS overnight. Next day, the cells were treated with FSH or FSHR antibodies and Notch antagonists in the respective media supplemented with FBS. The cells were labeled with BrdU for 12 hours and its incorporation was determined as per the manufacturer's recommendation (Calbiochem).

Expression analysis by Real time PCR

The total RNA from the ovarian tumors or spheroids obtained from the patients' peritoneal tap, or from the ovarian cancer cell lines was isolated by Trizol based method. Briefly, the cells were washed with DPBS and lysed in Tri Reagent (Sigma). RNA was extracted according to the manufacturer's recommended protocol. cDNA was synthesized from 1 µg of total RNA using the cDNA preparation kit (Thermo Scientific) using the random primers. The transcript levels of the interested genes were analyzed using the Syber-Green (Thermo Scientific) in Eppendorf Realplex thermocycler. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or Ribosomal protein L35 (RPL35) were used as the internal controls. The ΔΔCt method was used to calculate the fold difference in transcript levels of different genes on various treatments. The sequences of the primers used in the analysis listed in the supplementary Table 1.

Estimation of FSH in patients' ascites

The FSH levels in the ascites and sera of the patients as well as the hormone secreted by the ovarian cancer cell lines were determined by Radio-immuno assay (RIA) using the reagents supplied by the National Hormone Pituitary Agency (NHPP), USA.

Spheroid formation by different ovarian cancer cell lines

The cellular aggregates of the ovarian cancer cell lines were developed by culturing the cells under low attachment conditions. Briefly, the cells (~10^4/well) were seeded in 96 well low attachment plates (NUNC sphera) and allowed to form aggregates for 24 to 96 hours. The aggregates were treated with RF5 a/s and/or ScFv42 at the different time points. At regular intervals, images of the aggregates were captured using the Zeiss microscope at 60X zoom. Effect of different treatments on cellular aggregation or disaggregation was determined by analyzing the area of aggregates using a script developed in MATLAB R 15a (100 pixels in the image was equivalent to 150µm) fixing the ellipticity constant at 0.1.

Statistical analyses

The statistical analyses were performed using the Graphpad Prism 5.0 software. The p-value of < 0.05 was considered statistically significant. The experiments were repeated three times.

Results

FSH and Notch signaling in the ovarian cancer cell lines

Presence of the functional FSH receptor (FSHR) and Notch receptors in the ovarian cancer cell lines was demonstrated using different approaches. Presence of FSHR in these cells was demonstrated by determining specific binding of ^{125}I-FSH to the membranes obtained from OVCAR-3, SKOV-3 and OVCAR-4 cell lines. The membrane preparations were incubated with ^{125}I FSH in the presence of increasing concentrations of unlabeled FSH, binding was determined, and the binding data obtained were converted into Scatchard plots. As shown in Figure 1A, the

dissociation constants of the OVCAR-3 and SKOV-3 were $1.7x10^{-9}$M and $1.9x10^{-9}$M respectively, while that of the OVCAR-4 was $2.8x10^{-9}$M. The B_{max} for the receptor was highest in the OVCAR-3 cells (Figure 1A).

Presence of FSHR on the ovarian cancer cells was also confirmed by flow cytometry using the antiserum raised against FSHR (RF5 a/s) [18]. The cells were incubated with RF5 a/s and its binding was determined using anti-Rabbit IgG-FITC. As shown in the supplementary Figure 1A, all the three ovarian cancer cell lines exhibited binding of the a/s indicating the presence of FSH receptors on their cell surfaces with the OVCAR-3 cell line displaying the highest antibody binding, which agrees with the Scatchard analysis.

The functional nature of FSHR was further confirmed by determining the response of the OVCAR-3 cells to FSH *in vitro*. The cells ($5X10^4$) were incubated with increasing concentrations of FSH for 15 minutes at 37^oC and cAMP formed was determined by RIA. As shown in the Figure 1B, FSH produced a dose-dependent increase in cAMP. In the next experiment, the OVCAR-3 cells were incubated with FSH in presence and absence of the RF5 a/s, and cAMP produced was determined. As shown in Figure 1B, the response to the hormone was significantly inhibited in the presence of RF5 a/s indicating the antagonistic nature of the a/s [18].

The presence of Notch receptors in the ovarian cancer cell lines was demonstrated by flow cytometry, using the a/s raised against the Negative regulatory region (NRR) of Notch3 receptor that has full cross reactivity with the Notch1 receptor (Supplementary Figure 1B). As shown in the supplementary Figure 1C, all the three ovarian cancer cell lines exhibited binding of the NRR a/s, indicating the presence of Notch receptors on their cell surface. The Notch signaling in the

OVCAR-3 cells, as determined by 12X CSL reporter, was stimulated by Dll4 (see below, Figure 2C) and inhibited by DAPT (Figure 1C).

Effect of FSH on Notch signaling in the ovarian cancer cell lines

The possible effect of FSH on Notch signaling in the ovarian cancer cell lines was investigated by incubating the cell lines with increasing concentrations of FSH for 48 hours and determining Notch signaling in cells expressing a 12XCSL Luciferase reporter. As shown in the Figure 2A, in OVCAR-3 cells, there was an FSH dose-dependent increase in Luciferase activity indicating enhanced Notch signaling, which was further confirmed by the increased transcript levels of the Notch target genes Hes1, Hes5 and Heyl (Figure 2B). Similar results were obtained for SKOV-3 and OVCAR-4 (Figure 3A and B). The stimulatory effect of FSH on Notch signaling was completely inhibited by the RF5 a/s, which by itself had no effect on Notch signaling. The hormone stimulated Notch signaling was also inhibited by DAPT (Figure 2A).

FSH also upregulated the expression of Notch1 and Notch3, as well as, the Notch ligands Dll1 and Dll4 in OVCAR-3 cells as determined by RT-PCR (Figure 2B). In addition, increase in the Notch receptors and Dll4 was also demonstrated by flow cytometry (supplementary Figure 2). In the case of OVCAR-4 cell line, the upregulation was observed predominantly of Notch3, as well as, the three ligands, Jag1, Dll1 and Dll4 (Figure 3B). Thus, increased Notch signaling in the ovarian cancer cell lines in response to FSH was a result of an increased expression of both Notch receptors and ligands.

Dll4 present on the surface of the stromal cells in the ovarian cancer microenvironment has been shown to be involved in the progression of the ovarian tumor [24] and as shown above, FSH upregulates Dll4 expression. Therefore, effect of exogenous Dll4 [23] together with FSH on the

Notch signaling was determined. The OVCAR-3 cells were incubated with FSH (100mIU/ml) and the immobilized Dll4, individually and in combination, and the Notch signaling was determined. As shown in the Figure 2C, both ligands stimulated Notch signaling in an additive manner.

Effect of RF5 a/s and Notch inhibitory ScFv42 on Notch signaling was investigated in the same experiment. The ScFv42 is a Notch3 NRR specific ScFv and can inhibit the basal and FSH stimulated Notch signaling in a dose dependent manner (Supplementary Figure 3). As shown in Figure 2C, the RF5 a/s inhibited FSH-stimulated Notch signaling while the ScFv42 inhibited basal, Dll4-, FSH- and FSH+Dll4-stimulated Notch signaling. A combination of RF5 a/s and ScFv42 inhibited Notch signaling to the same extent as the ScFv42 alone.

Effect of FSH and Notch ligand on the proliferation of ovarian cancer cell lines

The role of Notch signaling in the proliferation of ovarian cancer cell line is very well documented while as shown above, FSH upregulates Notch signaling in the ovarian cancer cell lines. Therefore, the effect of FSH on the proliferation of OVCAR-3 cells was determined. The OVCAR-3 cells were incubated with increasing concentrations of the hormone for 48 hours and the BrdU incorporation was analyzed. As shown in Figure 4A, FSH stimulated OVCAR-3 cell proliferation in a dose-dependent manner.

Next, the effects of FSH and Notch ligand, individually and in combination, on the proliferation of OVCAR-3 cell line was examined. The cells were incubated with FSH and the immobilized Dll4 and the rate of proliferation was determined. As shown in Figure 4B, both ligands stimulated the cell proliferation in an additive manner. This increase in proliferation was inhibited by RF5

a/s, as well as, ScFv42. Further, the FSH- and Dll4-stimulated proliferation was inhibited by both RF5 a/s, as well as, the Notch3 NRR-specific ScFv even below the basal level.

FSH levels in Ovarian cancer patients' ascites

To understand the role of FSH and Notch signaling in the ovarian cancer progression, FSH levels in the ascites of the cancer patients were estimated. Ascites were obtained by peritoneal tap from 26 ovarian cancer patients while the serum samples were obtained from seven patients and FSH levels were determined by radioimmunoassay (RIA). FSH was detected in ascites of all the patients ranging from 15mIU/ml – 208mIU/ml. The serum could be the possible source of FSH in the ascites. Average serum FSH level in post-menopausal Indian women (age 54 $\pm$ 8) are 62 $\pm$ 30 and premenopausal women are 25.8$\pm$10 mIU/ml [25, 26]. As shown in table 1 the ascitic FSH levels in twelve patients (# 2, 3, 6, 10, 11, 12, 13, 14, 17, 18, 23, 25) were more than 62 mIU/ml and the seven patients who had low FSH in their ascites, either did not have malignant cells detected in their ascites (# 1, 8, 9, 15) or were undergoing chemotherapy (#, 20, 21, 24). Of the seven patients, whose serum samples were available for analysis, three patients (# 23, 25, 26) showed high FSH levels in both sera as well as ascitic fluids (> 100mIU/ml). However, in the case of remaining 4 patients, there was no correlation between serum and ascitic FSH levels.

To identify the source of the hormone in the ascites, ovarian tumors from the 6 ovarian cancer patients (Patient # 27-32) and multicellular spheroids from the ascitic fluid of 8 patients (Patient # 2, 3, 5, 10, 11, 13, 14 and 18) were obtained. The total RNA was isolated and subjected to RT-PCR analysis for FSHβ, FSHα and FSHR transcripts. As shown in Table 1, the primary ovarian patients tumor's samples exhibited high Ct values of FSH α and β suggesting very low hormone message in the primary tumors whereas the FSHR transcript levels were very high as indicated

by the low Ct values in most of the samples (Patient # 27, 28, 29,30 and 31). The other possibility of FSH being expressed by the metastatic tumor epithelium represented by spheroids in the ascitic fluids was next explored. Multicellular spheroids were isolated from the cell mass obtained from the ascites from patients and as shown in the Table 1, the spheroids obtained from the patients 2, 3 ,10, 11, 13, 14 and 18 exhibited high levels of FSHβ message with lower cycle threshold (Ct) values compared to those obtained from the patient 5, an ovarian cancer patient with the recurrent disease. The FSHβ transcript levels were consistent with the ascitic hormone levels. Interestingly, there was not much difference in the expression of α subunit as depicted by the Ct values, whereas there was a significantly high expression of FSH receptor expression in the spheroids of the ovarian cancer patients (2, 3, 10, 11, 13, 14 and 18) compared to the patient 5 (Table 1).

Ovarian cancer cell lines spheroids

To simulate the conditions of in vivo spheroid formation, the ovarian cancer cell lines were cultured under the low attachment conditions that facilitated the cancer cells to aggregate and form compact spheroidal aggregates [27]. As shown in the Figure 5A, spheroid-like aggregates of the OVCAR-3 cells were observed after 48 hours of culture. The ovarian cancer cell lines, SKOV-3 and OVCAR-4 formed aggregates of different geometries under these conditions (Figure 5B and 5C). Expression of FSH by the spheroids/aggregates was demonstrated by determining the hormone levels in the culture supernatants collected at different time points and by RT-PCR for FSHβ transcript levels. As shown in Figure 5D and 5E, there was an increase in FSHβ transcripts under non-attachment conditions with corresponding increase in secreted FSH by OVCAR-3 and SKOV-3 cells, while there was neither any increase in the FSHβ transcripts

nor in secreted FSH in the medium when the cells were grown as monolayer cells. The OVCAR-4 cells did not show any increase in FSHβ transcripts, as well as, the secreted hormone (Figure 5F).

Effect of FSH and Notch inhibitors on OVCAR-3 spheroids

It has been demonstrated that the formation of ovarian cancer cell line spheroids mimics the ovarian tumor metastatic condition and these spheroids exhibit chemo resistance as they increase in size [28]. Higher chemo resistance has also been correlated with increased Notch signaling [29, 30]. Therefore, the expression of Notch receptors, ligands and downstream genes was investigated under the conditions that facilitated spheroid formation. As shown in the Figure 6A, there was an increase in Notch1 and Notch3, Jag2 and Dll4 and the downstream genes Hes1 and Heyl as well as FSH receptor on spheroid formation. Thus, an up-regulation of Notch signaling correlates with the increase in FSHβ and FSHR expression in ovarian cancer spheroids. Further, as shown in the Figure 7A, the mean size of the aggregates formed was additively and significantly less in the presence of RF5 a/s and ScFv42, and this effect was significantly enhanced in presence of both inhibitors.

In the next experiment, the effect of both these inhibitors on the preformed spheroids was investigated. The OVCAR-3 cells were grown under the low attachment conditions for 48 hours to form the aggregates and then either RF5 a/s or ScFv42 individually, or in combination were added. As shown in Figure 7B, there was a decrease in the mean aggregate size when both FSH and Notch signaling were inhibited.

Discussion

The etiology of ovarian cancer and carcinomatosis is not very well understood. Exposure to elevated levels of gonadotropin during cycles, post menopause or treatments for infertility and IVF have been implicated as the risk factors for the ovarian cancer [2]. FSH upregulated the genes involved in cellular proliferation and metabolism, while downregulating the apoptotic genes in the ovarian cancer cell lines [31, 32]. Interestingly, ovarian teratomas have been reported in FSHR "Gain-of-function" transgenic animals [33, 34] and the FSH receptor has also been considered as an effective immunotherapeutic target [35, 36], clearly establishing the link between the hormone and ovarian cancer.

The link between FSH and Notch was indicated by increase in Notch1 expression by the hormone in SKOV-3 cells [14]. The TCGA database indicates an amplification of all Notch receptors, particularly Notch3, highlighting the involvement of Notch in the ovarian cancer. Both FSH and Notch have been shown to be involved in ovarian tumor angiogenesis, epithelial to mesenchymal transition, resistance to chemotherapy and cancer stem cell activity [2, 13].

The present study is the first to demonstrate active FSH signaling in the ovarian cancer cell lines OVCAR-3, SKOV-3 and OVCAR-4. All three cell lines express FSHR with affinities in the nanomolar range with the OVCAR-3 cell line showing highest FSHR density and response to the hormone with an increase in cAMP production. Presence of FSHR in all three cell lines was confirmed by binding of RF5 a/s raised against the extracellular domain of FSHR. These a/s inhibited binding of the hormone to the receptor (unpublished data) and completely inhibited increase in cAMP in response to the hormone indicating the antagonistic nature of this a/s.

FSH increased Notch signaling in a dose-dependent manner, which was attributed to the upregulation of Notch1 and Notch3 and Notch ligands Dll1 and Dll4. Interestingly, Dll4 is also reported to be present on the stromal cells of the ovarian cancer [24]. When both Dll4 and FSH were present together, there was an additive increase in the Notch signaling. FSH increased proliferation of the ovarian cancer cell lines, which is in agreement with previous reports [6, 37]. This increase in proliferation was mediated by increased Notch signaling as it was inhibited by Notch3 NRR specific ScFv. The proliferation was brought even below the basal rates by the combination of Notch3 NRR ScFv and FSHR a/s.

The progression of ovarian cancer frequently leads to accumulation of ascites in the peritoneal cavity of the patients which provides a complex mixture of soluble factors and cellular components creating a pro-inflammatory and tumor-promoting microenvironment for the cells [38]. FSH is probably one such factor and its levels in the ascites of twelve ovarian cancer patients out of 26 patients were significantly high. Earlier reports [39] also indicated the serum/tumor fluid FSH ratios to be low in the ovarian cancer patients suggesting that the origin of FSH in the ascites may not be the serum, which could be higher in the post-menopausal status of many of the patients [10]. The present study is however the first to posit that spheroids obtained from the ascites are the source of FSH. We observed that OVCAR-3 and SKOV-3 cells expressed FSHβ mRNA and secreted FSH into the medium only when cultured as spheroids, and not when grown as monolayers. However, the OVCAR-4 cells, when cultured as aggregates neither expressed FSHβ message nor secreted the hormone. Expression of the α subunit mRNA was observed in the cancer cell lines cultured both as monolayers and as spheroids, as well as, the spheroids obtained from the patients. In contrast, FSHβ expression is seen only when the cells are organized into the spheroidal geometries. In support of our cell line-based studies, the expression of FSHβ (and even

FSHα) was observed to be very low in cells from primary ovarian patient tumor samples. Despite low ligand expression, the FSHR expression was high. This suggests that the FSH secreted by ascitic spheroids might not just impact the morphogenesis of the latter, but also turn on FSH signaling in the FSHR expressing primary tumor driving its proliferation. This feedback loop however, is dependent upon the establishment of the metastatic niche, and might be perhaps one of the earliest examples of how the metastatic niche feeds back to alter the behavior of the primary tumor niche of a cancer. It is known that FSHβ expression is the rate limiting step in the secretion of the hormone from the pituitary [40] and once the β subunit expression is turned on, the heterodimeric hormone is secreted into the environment.

Turning 'on' of FSHβ expression in the spheroids is very intriguing and has not been reported earlier. Spheroids have been known to show altered epigenetic regulation with a general increase in histone acetylation [41]. However, the effect on FSHβ expression appears to be specific as neither the expression of LHβ is upregulated nor was there any secretion of LH under these conditions. Therefore, whether such a spheroid-specific expression and secretion of FSH is prognosticative of a metastatic, niche-specific secretome needs to be investigated further.

It is evident that FSH expressed by the ovarian cancer spheroids upregulates Notch signaling, which together with the stromal Dll4 leads to increased proliferation of the tumor cells. It was also interesting that the spheroidal cells also exhibited higher expression of FSHR (supplementary Figure 9) probably making them extremely sensitive to the increasing concentrations of the hormone. These data suggest that there is an autocrine regulation of FSH-FSHR signaling that plays an important role in the ovarian cancer progression.

The ovarian cancer cell spheroids are known to be associated strongly with the recurrence of the ovarian cancer, as they show resistance to chemo- and radio-therapies with larger spheroids being

more resistant to the therapies [17]. Under these conditions, both FSH and Notch pathways are interesting immunotherapeutic targets. The Notch NRR ScFv42, individually, and in combination with FSHR a/s significantly inhibited the formation of bigger OVCAR-3 spheroids. The antibodies also decreased the average size of the pre-formed spheroids. These two antibodies inhibited ovarian cancer cell proliferation further, suggesting the potential therapeutic potential of these antibodies. Our observation that the FSHR antibody can perturb spheroidal morphogenesis individually but inhibit proliferation of monolayers only in an FSH-added condition suggests a morphology-specific higher activity of FSH signaling and its integration with Notch transduction. The mechanistic investigations of such signaling crosstalk will be explored in future endeavors.

In conclusion, in this study we demonstrate a clear link between FSH and Notch in the ovarian cancer progression and show that these are the potential targets for future therapeutic efforts.

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Authors' contributions: SG, RB[1] and RRD designed the study and wrote the manuscript. SG carried out the execution of the project. SKS, SNS, RB[2], AV and RG were involved in identifying and characterizing the ovarian cancer patients. RB[1]: Ramray Bhat; RB[2]: Rahul Bhagat

Ethics approval and consent to participate:

The studies described here were carried out as per the guidelines of the Institutional review board and in agreement with the ethical guidelines of the Kidwai Cancer Institute and the Indian Institute of Science (IISc) after obtaining the informed consent from the patients.

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Figure 1. Characterization of FSH signaling in Ovarian cancer cell lines A. The presence of FSHR on the ovarian cancer cell lines OVCAR-3, SKOV-3 and OVCAR-4 cell was investigated by determining the binding of ^{125}I-FSH to their membranes (10µg each) in the presence of increasing concentrations of cold FSH. The binding data were converted into a Scatchard plot and the inset table shows the K_d and B_{max} of hormone in the three cell lines. **B.** The activity of FSH receptor was investigated by analyzing the levels of cAMP in the presence of hormone by radio-immunoassay (RIA). The OVCAR-3 cells were incubated with increasing concentrations of FSH or **C.** with 100mIU/ml FSH in the presence and absence of 5µg/ml of RF5 a/s and cAMP levels

were determined. Significance is calculated by comparing the treated with basal using non-parametric unpaired T test where ns represents P> 0.05, * represents P≤ 0.05, and *** represents P≤ 0.001. Error bars represent mean ± Standard Deviation (S.D.) and n is the number of repeats. **D.** The Ovarian cancer cells were transfected with 12XCSL luciferase reporter plasmid and incubated with 5µM DAPT for determining the Notch signaling activity. The reporter activity was analyzed after 24 hours of incubation by dual luciferase assay. RLU, Relative Luciferase Unit.

Figure 2. Effect of FSH on Notch signaling. A. The OVCAR-3 cells were transfected with 12XCSL luciferase reporter plasmid and incubated with increasing concentrations of FSH or 100mIU/ml of FSH in the presence of 5µg/ml FSHR antagonist RF5 a/s and 5µM DAPT in different combinations for 48 hours. The reporter activity was measured by dual luciferase assay after 48 hours of incubation. **B**. The OVCAR-3 cells were incubated with 100mIU/ml of FSH for 48 hours and real time PCR was performed for indicated Notch receptors, ligands and target genes. The fold change in the expression of receptor and ligands was calculated with respect to cells cultured without hormone after normalizing with GAPDH expression. **C.** OVCAR-3 transfected with 12XCSL luciferase reporter plasmid were cultured on pre-coated Dll4-Fc. 100mIU/ml of FSH, or 5µg/ml FSHR antagonist RF5 a/s, or 10µg/ml ScFv42, or in different combinations were added to the cells for 48 hours and the effect on the Notch signaling was measured by dual luciferase assay. RLU is Relative luciferase unit. Significance is calculated by comparison of treated and basal values using non-parametric unpaired T test where ns represents P> 0.05, * represents P≤ 0.05, ** represents P≤ 0.01 and *** represents P≤ 0.001 compared to basal values. Error bars represent mean ± SD and n is the number of repeats.

Figure 3. Effect of FSH on Notch signaling A. SKOV-3, and **B.**OVCAR-4 cells were transfected with 12XCSL luciferase reporter plasmid and incubated with increasing concentrations of FSH.

The effect on the Notch signaling was measured by dual luciferase assay after 48 hours. RLU, Relative luciferase unit. Error bars represent mean ± SD. n is the number of repeats. **C.** SKOV-3 and **D.** OVCAR-4 cells were incubated with 100mIU/ml of FSH for 48 hours and real time PCR was performed for indicated Notch receptors, ligands and target genes. The fold change in the transcript levels of Notch genes was calculated with respect to the levels in cells cultured without hormone after normalizing with GAPDH expression. Error bars represent mean ± SD. n is the number of repeats.

Figure 4. Effect of FSH on proliferation of Ovarian cancer cells. A. The OVCAR-3 cells were synchronized by culturing them in serum free medium overnight and incubated with increasing FSH concentrations. The proliferation rate was investigated by analyzing the BrdU incorporation in the cells after 48 hours. **B**. OVCAR-3 cells (synchronized as above) were cultured on pre-coated Dll4-Fc and incubated with 100mIU/ml of FSH, or 5µg/ml FSHR antagonist RF5 a/s, or 10µg/ml ScFv42, or in different combinations for 48 hours. Proliferation rate was investigated by BrdU incorporation and significance was calculated by unpaired T test compared to the basal levels where ns represents $P > 0.05$, * represents $P \leq 0.05$, ** represents $P \leq 0.01$, *** represents $P \leq 0.001$ compared to basal. n is the number of repeats. Error bars represent mean ± SD.

Figure 5. Expression of FSH in Ovarian cancer cell lines spheroids. Photomicrographs images of cellular aggregates of **A.** OVCAR-3, **B**. SKOV-3 and **C.** OVCAR-4 cells cultured in low attachment conditions. **D.** OVCAR-3, **E**. SKOV-3 and **F.** OVCAR-4 cultured in low attachment conditions or as monolayer. FSH level in the conditioned media of aggregates/ spheroids or monolayers cultured for different time periods were analyzed by RIA. Real time-PCR was performed to assay the transcript levels of FSHβ in cellular aggregates. Fold change was calculated with respect to cells cultured as monolayers after normalizing with GAPDH

expression. Significance is calculated by comparison of treated and basal values using non-parametric unpaired T test. n is the number of repeats. Error bars represent mean ± SD.

Figure 6. Status of Notch and FSH signaling genes in OVCAR-3 spheroids. The OVCAR-3 cells were cultured in low attachment conditions or as monolayer and harvested after 48 hours. Real time PCR was performed to assay the transcript levels of indicated Notch receptors, ligands targets and **B**. FSHR and α subunit. The fold change in the expression level of genes was calculated with respect to OVCAR-3 cell cultured as monolayer after normalizing with GAPDH expression. Error bars represent mean ± SD, n is the number of repeats.

Figure 7. Effect of FSH and Notch antagonist (RF5 a/s and ScFv42 respectively) on the aggregation of OVCAR-3 spheroids. A. The OVCAR-3 cells were cultured in low attachment conditions in the presence of RF5 a/s (5µg/ml) and ScFv42 (10µg/ml) individually and in combination for 48 hours and the images were taken (5 fields/well) before and after the treatment. The area of the spheroids in each image was analyzed in MATLAB. The distribution of area of the 100 OVCAR-3 spheroids cultured in the presence and absence of antagonists is plotted. Each symbol represents the area of single spheroid. **B.** OVCAR-3 spheroids cultured for 48 hours were incubated with either RF5 a/s (5µg/ml), and/ or ScFv42 (10µg/ml) and the microscopic images were taken at regular intervals as above. The area of the spheroids in each image was analyzed in MATLAB. Distribution of area of the 100 spheroids was measured at 48 hours (before the addition of antagonists) and at 96 hours (i.e. two days after addition). Each symbol represents one spheroid and the solid symbols represents the spheroids incubated with different antagonists. Significance is calculated by comparison of treated and basal values using ANNOVA. Error bars represent mean± SEM. n represents the number of repeats and N is the number of spheroids

analyzed. ns represents P> 0.05, * represents P≤ 0.05, ** represents P≤ 0.01 and *** represents P≤ 0.001 compared to non-treated.

Table 1. FSH in patients' ascites and serum. Ascites and serum were obtained from the Ovarian cancer patients and the levels of FSH in all the samples were determined by Radioimmuno assay (RIA) in a single experiment. Total RNA was isolated from the primary tumors and spheroids obtained from the ascites of ovarian cancer patients and cDNA was prepared from 1 µg of total RNA. The transcript levels of FSHβ subunit, α subunit, FSHR and RPL35 were examined by real time PCR. The Cycle threshold values (Ct) are represented as the mean of three experimental repeats (n).

Supplementary Figure 1. FSHR on Ovarian cancer cell lines. The presence of FSHR on the ovarian cancer cell lines OVCAR-3, SKOV-3, and OVCAR-4 was analyzed by flow cytometry. The ovarian cancer cell lines were incubated with RF5 a/s (1:500 dilution) or Normal rabbit a/s (NRS) and analyzed for binding using FITC-conjugated anti-rabbit secondary antibody. The histograms are representive of three independent experiments. **Notch receptors on Ovarian cancer cell lines. B.** HEK293 cells and Notch1 and Notch3 overexpressing HEK293 cells were incubated with Notch3 NRR a/s (1:500 dilution) followed by anti-rabbit FITC and binding was analyzed by flow cytometry. **C.** The presence of Notch receptor on the Ovarian cancer cell lines OVCAR-3, SKOV-3, and OVCAR-4 was analyzed by flow cytometry. The Ovarian cancer cell lines were incubated with Notch3 NRR a/s (1:500 dilution) or Normal rabbit a/s (NRS) and analyzed for binding by anti-rabbit secondary antibody conjugated with FITC. The histograms are represented of three independent experiments (MFI, Median fluorescent intensity, FITC, Fluorescein Isothiocyanate).

Supplementary Figure 2. Effect of FSH on Notch receptors and ligands. The OVCAR-3 cells were harvested with EDTA after incubation with 100mIU/ml of FSH for 48 hours and labelled with **A.** Notch3 NRR a/s and **B.** Dll4 antibody for analysis by flow cytometry. The antibody bound was determined by anti-rabbit Alexa 647 and anti-mouse Alexa 488 respectively. Binding of a/s was analyzed by comparing the median fluorescent intensity in the presence and absence of hormone. The histograms are representive of three independent experiments. Quantitative analysis of the same is represented below. Significance is calculated by comparison of treated and basal values using non-parametric unpaired T test. Error bars represent mean $\pm$ SD. n is the number of repeats.

Supplementary Figure 3. Effect of anti Notch3 ScFv on Notch signaling OVCAR-3 cells were transfected with luciferase plasmid and incubated with increasing concentrations of ScFv42 (Notch3 NRR antagonist) in the presence or absence of 100mIU/ml FSH. The reporter activity was measured by dual luciferase assay after 48 hours of incubation. RLU, Relative luciferase unit. n is the number of repeats.

Supplementary Figure 4. Expression of FSHβ in Ovarian cancer TCGA data was explored for the expression of FSHβ and α subunit and FSHR in individual ovarian cancer stage. The expression is plotted as transcripts per million. N is the number of samples [42].

Supplementary Table 1. List of real time primers.

Supplementary Table 2. Details of the patients from whom ascites and serum samples were obtained.

Figure 1.

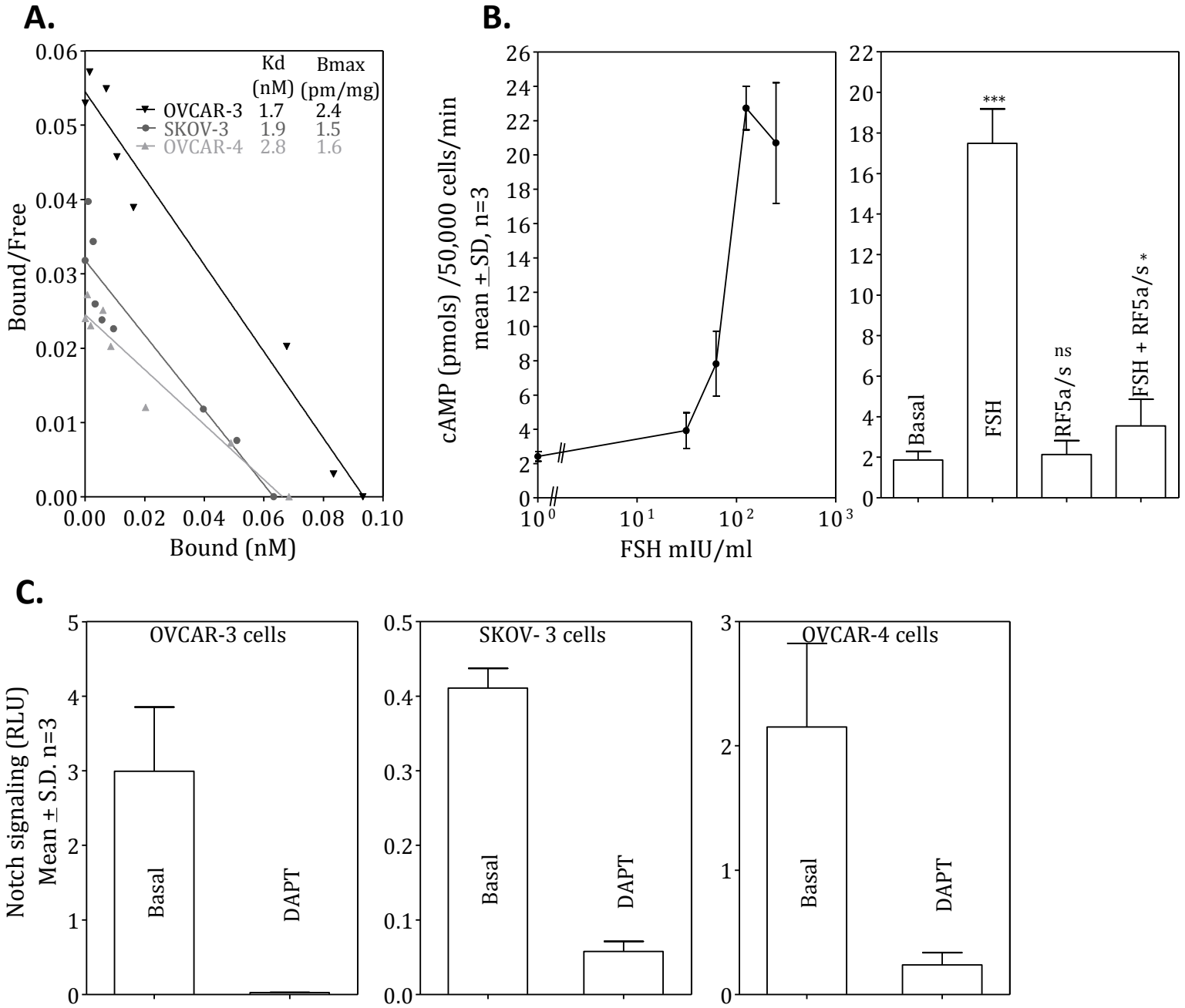

A.
Kd (nM)
Bmax (pm/mg)
OVCAR-3 1.7 2.4
SKOV-3 1.9 1.5
OVCAR-4 2.8 1.6
Bound/Free
Bound (nM)
B.
cAMP (pmols) /50,000 cells/min mean ± SD, n=3
FSH mIU/ml
***
ns
*
Basal
FSH
RF5a/s
FSH + RF5a/s
C.
OVCAR-3 cells
SKOV- 3 cells
OVCAR-4 cells
Notch signaling (RLU) Mean ± S.D. n=3
Basal
DAPT

Figure 2.

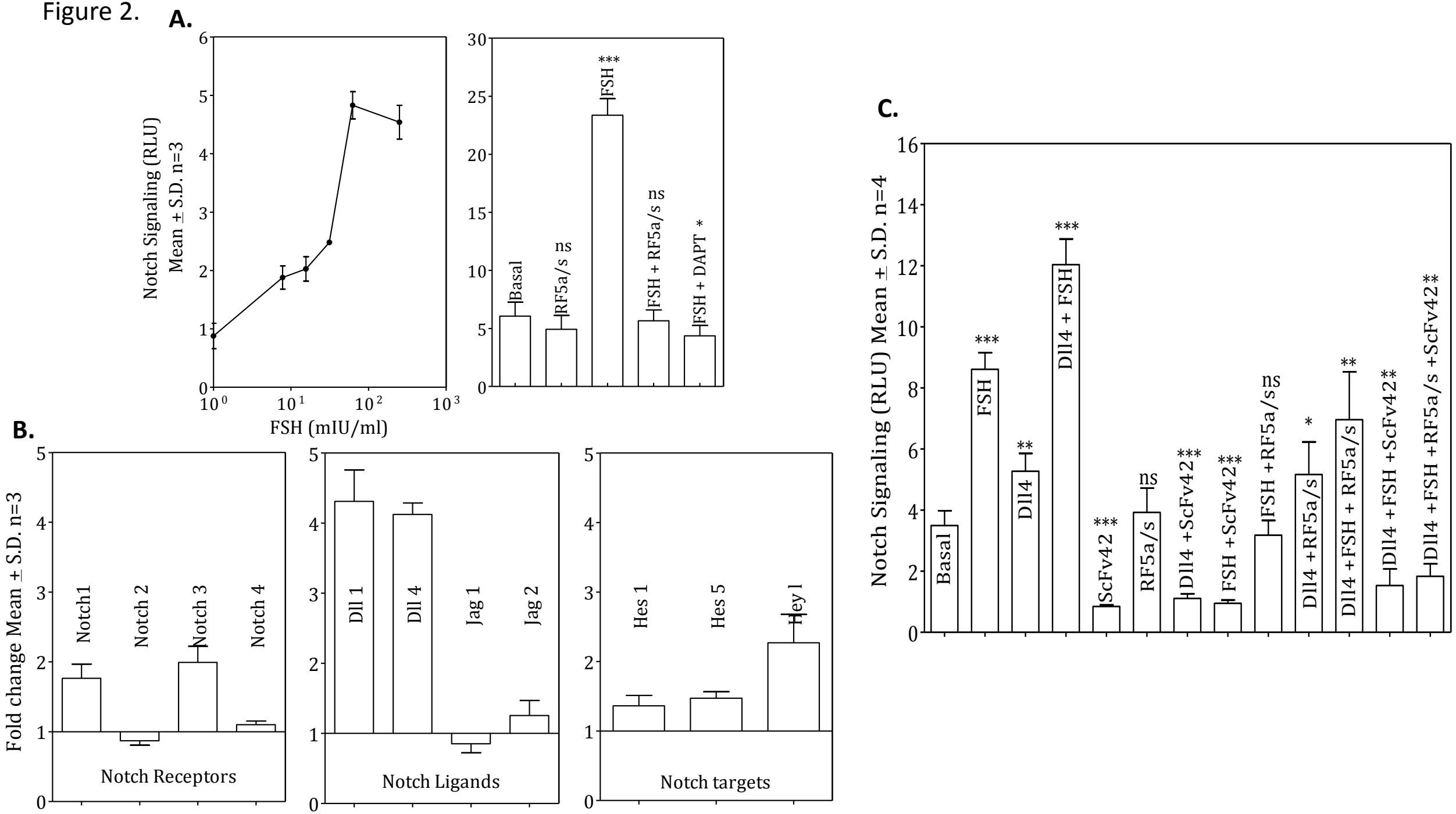

A.
Notch Signaling (RLU)
Mean ± S.D. n=3
FSH (mIU/ml)
Basal
RF5a/s
FSH
FSH + RF5a/s
FSH + DAPT
ns
***
ns
*
B.
Fold change Mean ± S.D. n=3
Notch1
Notch 2
Notch 3
Notch 4
Notch Receptors
Dll 1
Dll 4
Jag 1
Jag 2
Notch Ligands
Hes 1
Hes 5
Hey 1
Notch targets
C.
Notch Signaling (RLU) Mean ± S.D. n=4
Basal
FSH
Dll4
Dll4 + FSH
ScFv42
RF5a/s
Dll4 +ScFv42
FSH +ScFv42
FSH +RF5a/s
Dll4 +RF5a/s
Dll4 +FSH + RF5a/s
Dll4 +FSH +ScFv42
Dll4 +FSH +RF5a/s +ScFv42

Figure 3.

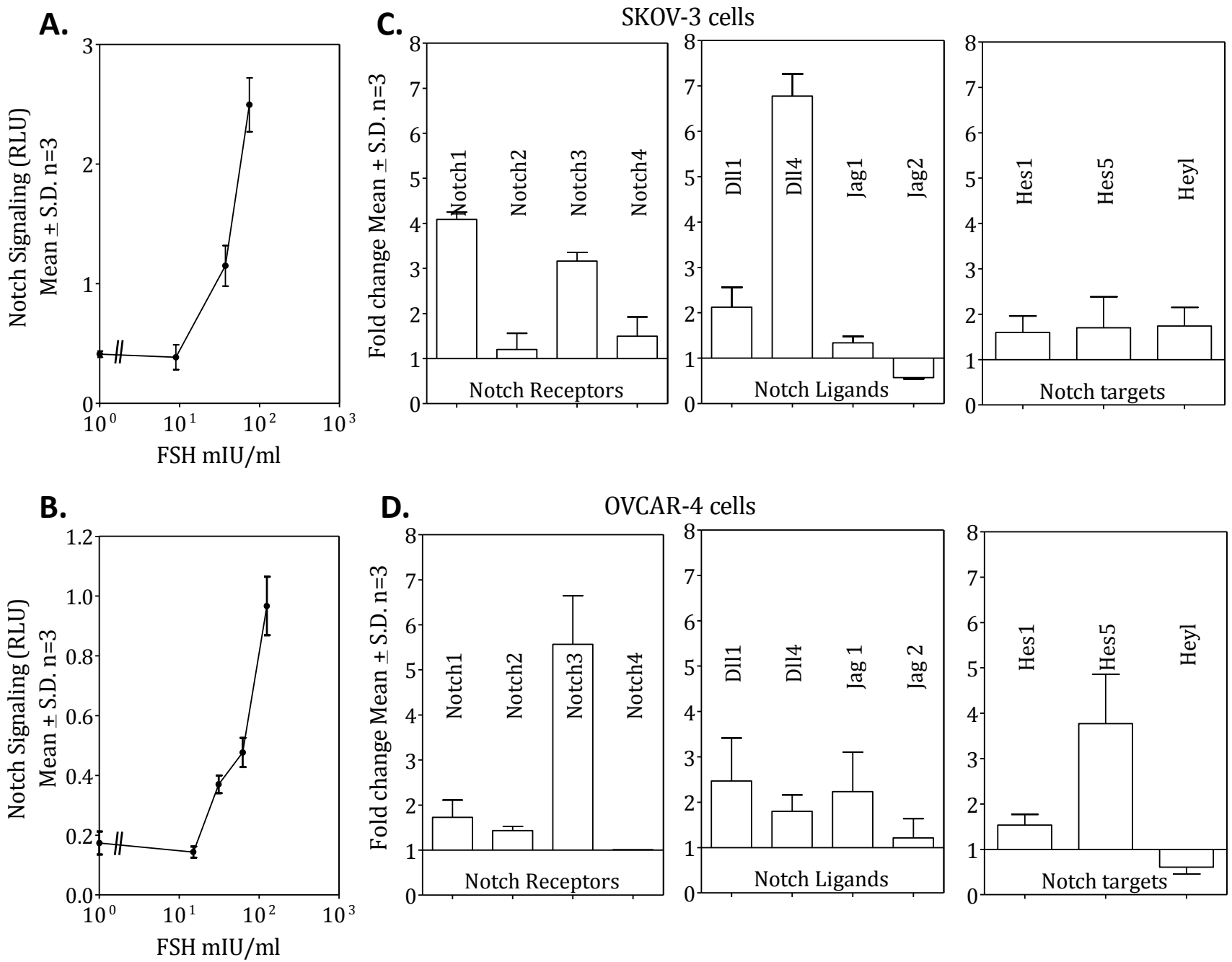

A.
Notch Signaling (RLU)
Mean ± S.D. n=3
FSH mIU/ml
B.
Notch Signaling (RLU)
Mean ± S.D. n=3
FSH mIU/ml
C.
SKOV-3 cells
Fold change Mean ± S.D. n=3
Notch1
Notch2
Notch3
Notch4
Notch Receptors
Dll1
Dll4
Jag1
Jag2
Notch Ligands
Hes1
Hes5
Heyl
Notch targets
D.
OVCAR-4 cells
Fold change Mean ± S.D. n=3
Notch1
Notch2
Notch3
Notch4
Notch Receptors
Dll1
Dll4
Jag 1
Jag 2
Notch Ligands
Hes1
Hes5
Heyl
Notch targets

Figure 4.

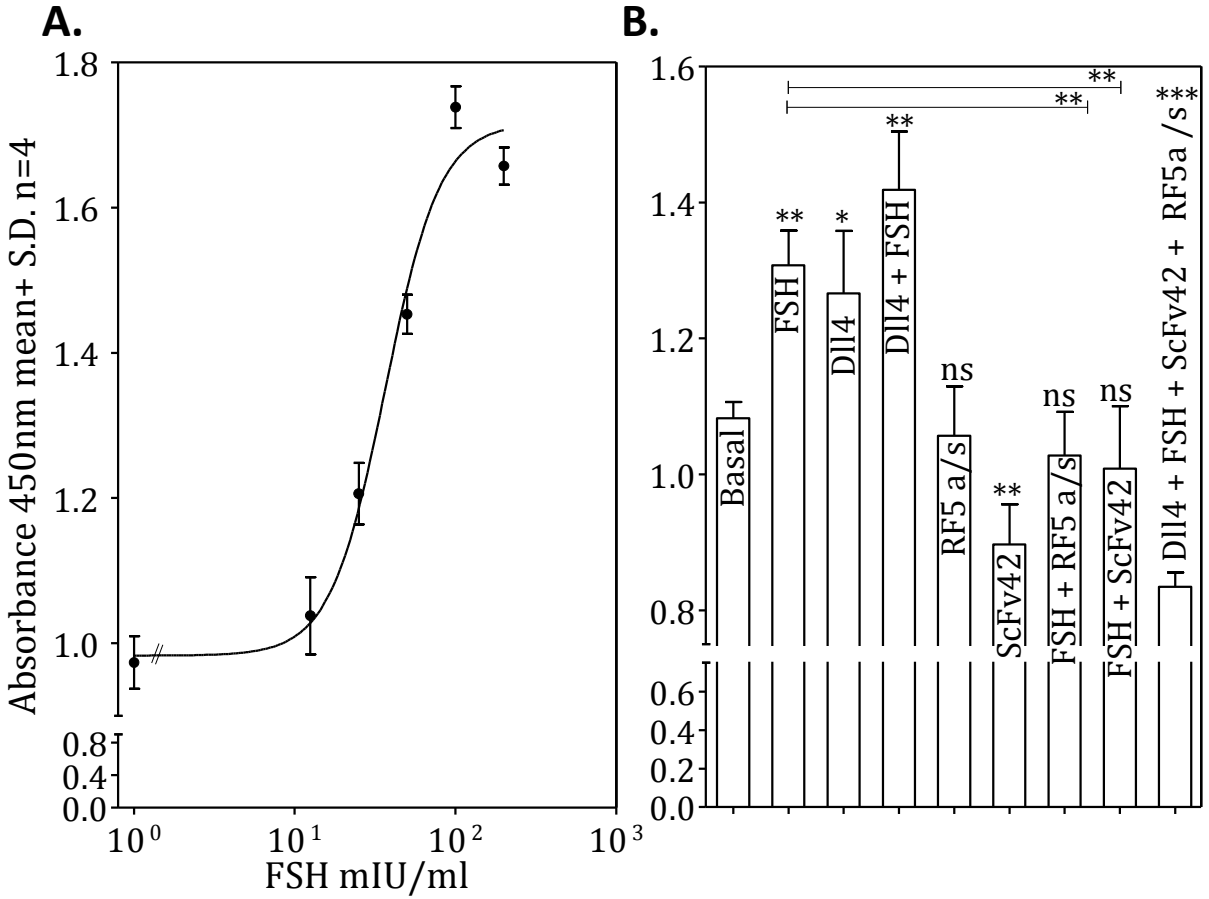
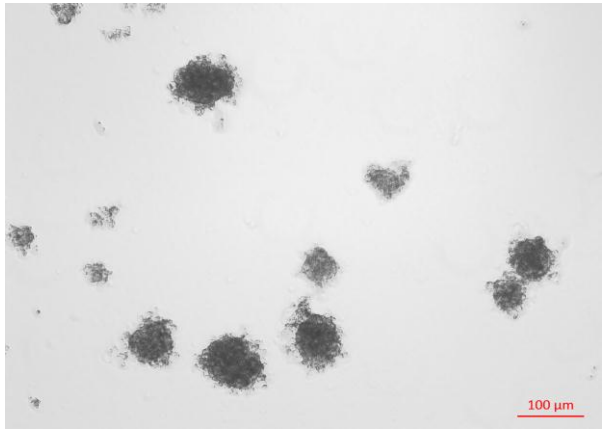
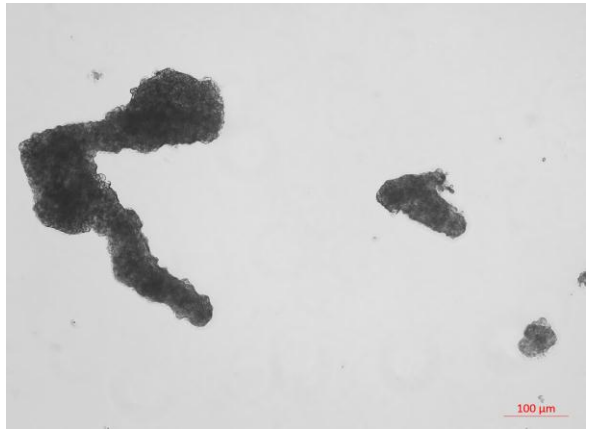

A.
B.
Absorbance 450nm mean+ S.D. n=4
FSH mIU/ml
Basal
FSH
Dll4
Dll4 + FSH
RF5 a/s
ScFv42
FSH + RF5 a/s
FSH + ScFv42
Dll4 + FSH + ScFv42 + RF5a /s
ns

Figure 5.

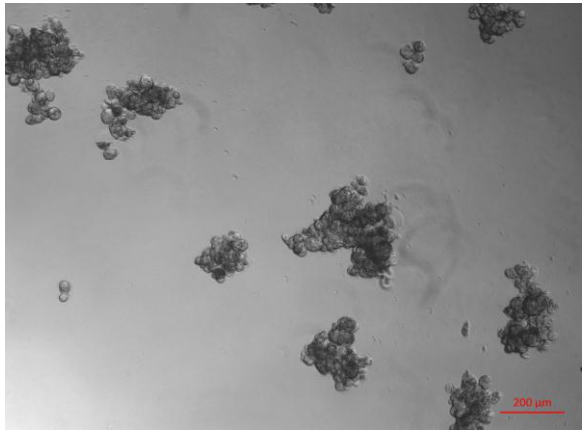
A. OVCAR-3 cells



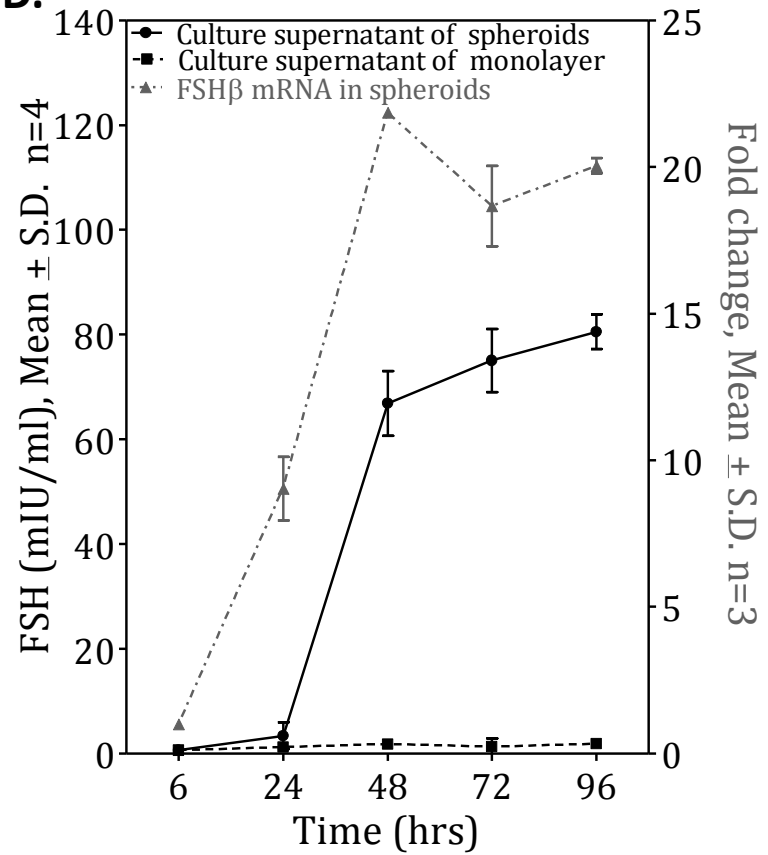
B. SKOV-3 cells



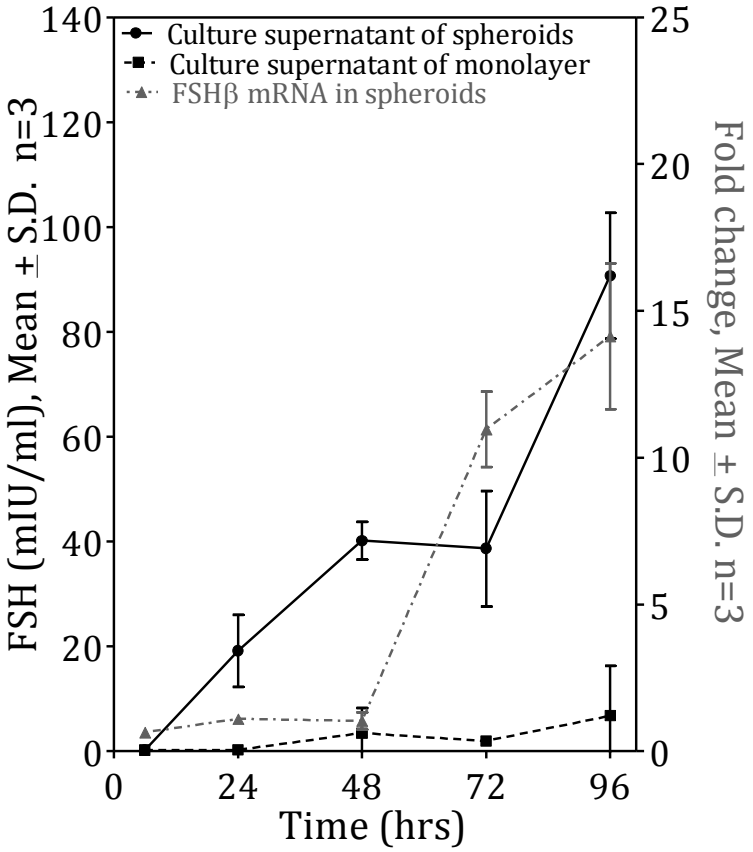
C. OVCAR-4 cells



D.



E.



F.

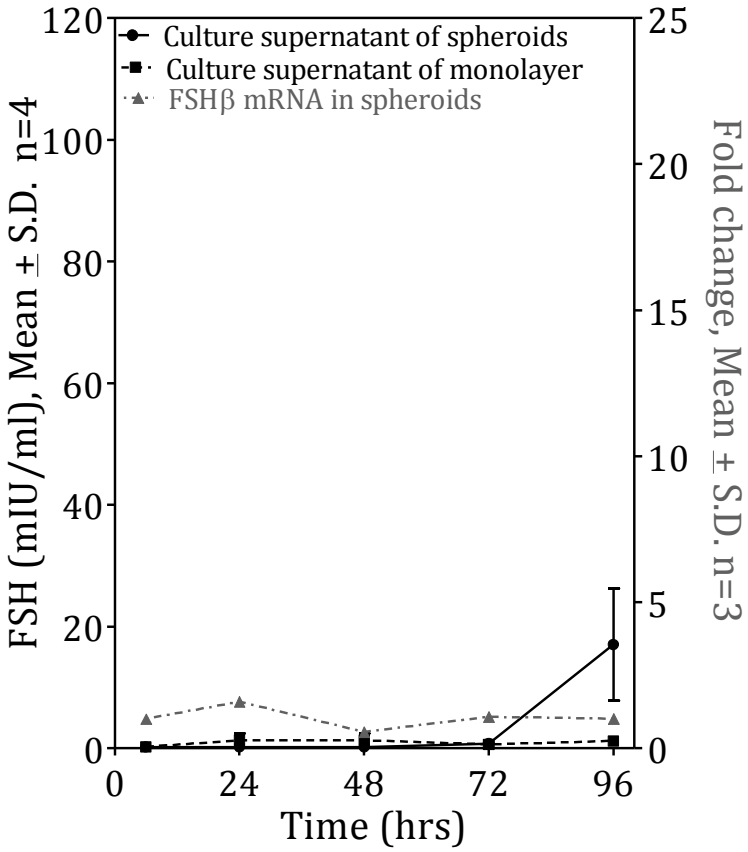


Figure 6.

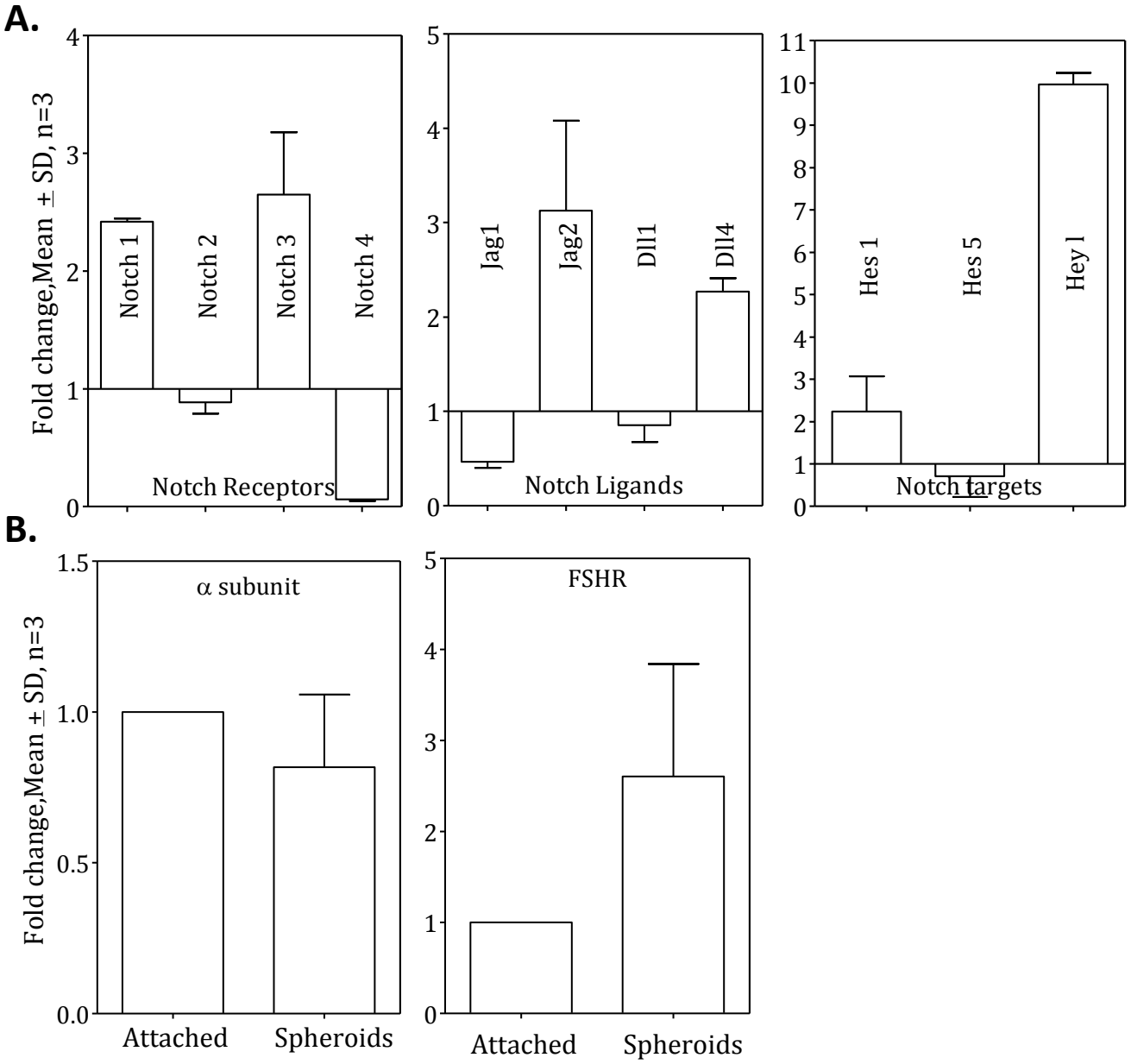

A.
Fold change,Mean ± SD, n=3
Notch 1
Notch 2
Notch 3
Notch 4
Notch Receptors
Jag1
Jag2
Dll1
Dll4
Notch Ligands
Hes 1
Hes 5
Hey l
Notch targets
B.
Fold change,Mean ± SD, n=3
α subunit
FSHR
Attached
Spheroids
Attached
Spheroids

Figure 7.

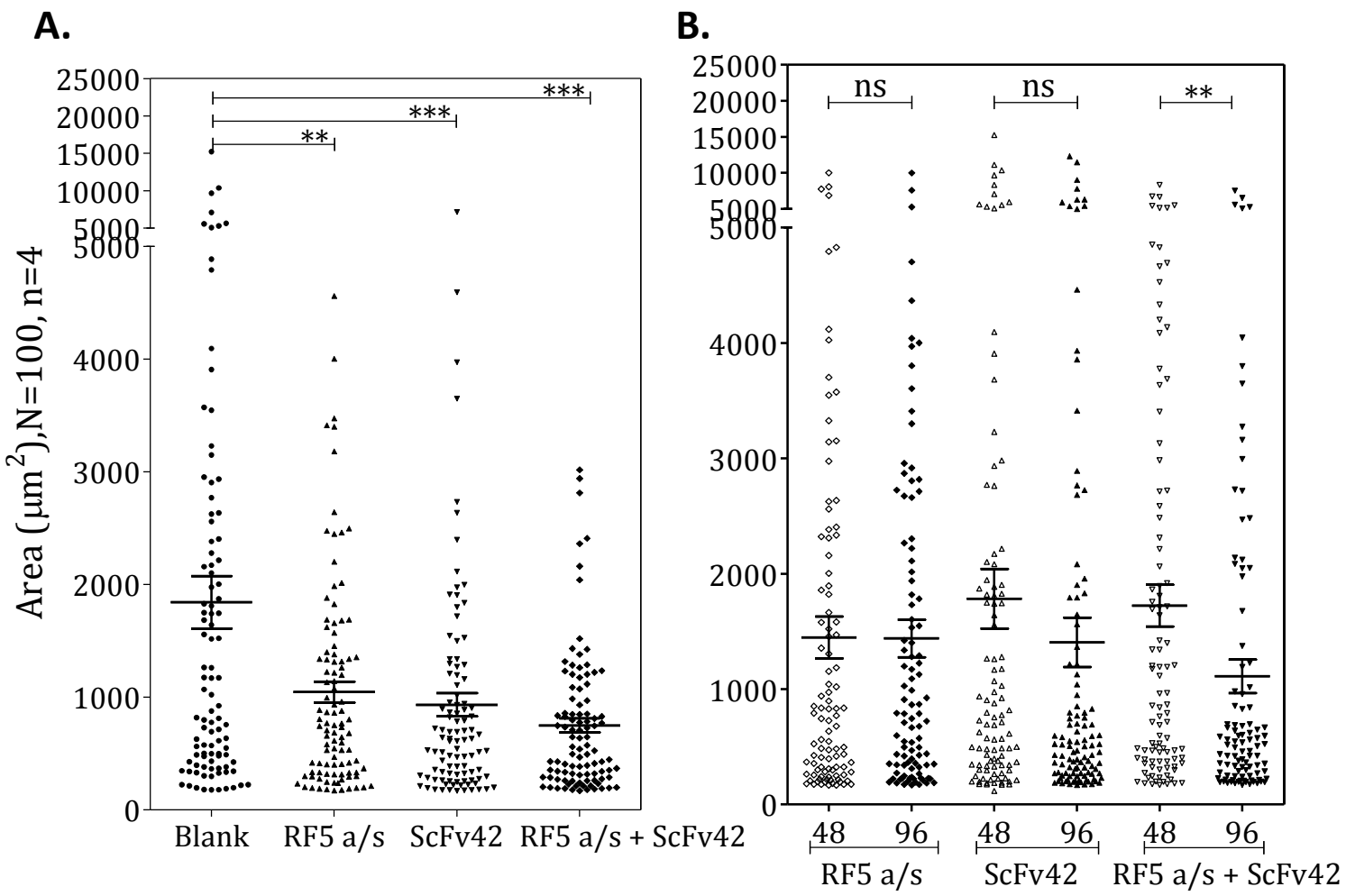

A.
B.
Area (μm2),N=100, n=4
25000
20000
15000
10000
5000
4000
3000
2000
1000
0
**
***
***
Blank
RF5 a/s
ScFv42
RF5 a/s + ScFv42
ns
ns
**
48
96
48
96
48
96
RF5 a/s
ScFv42
RF5 a/s + ScFv42

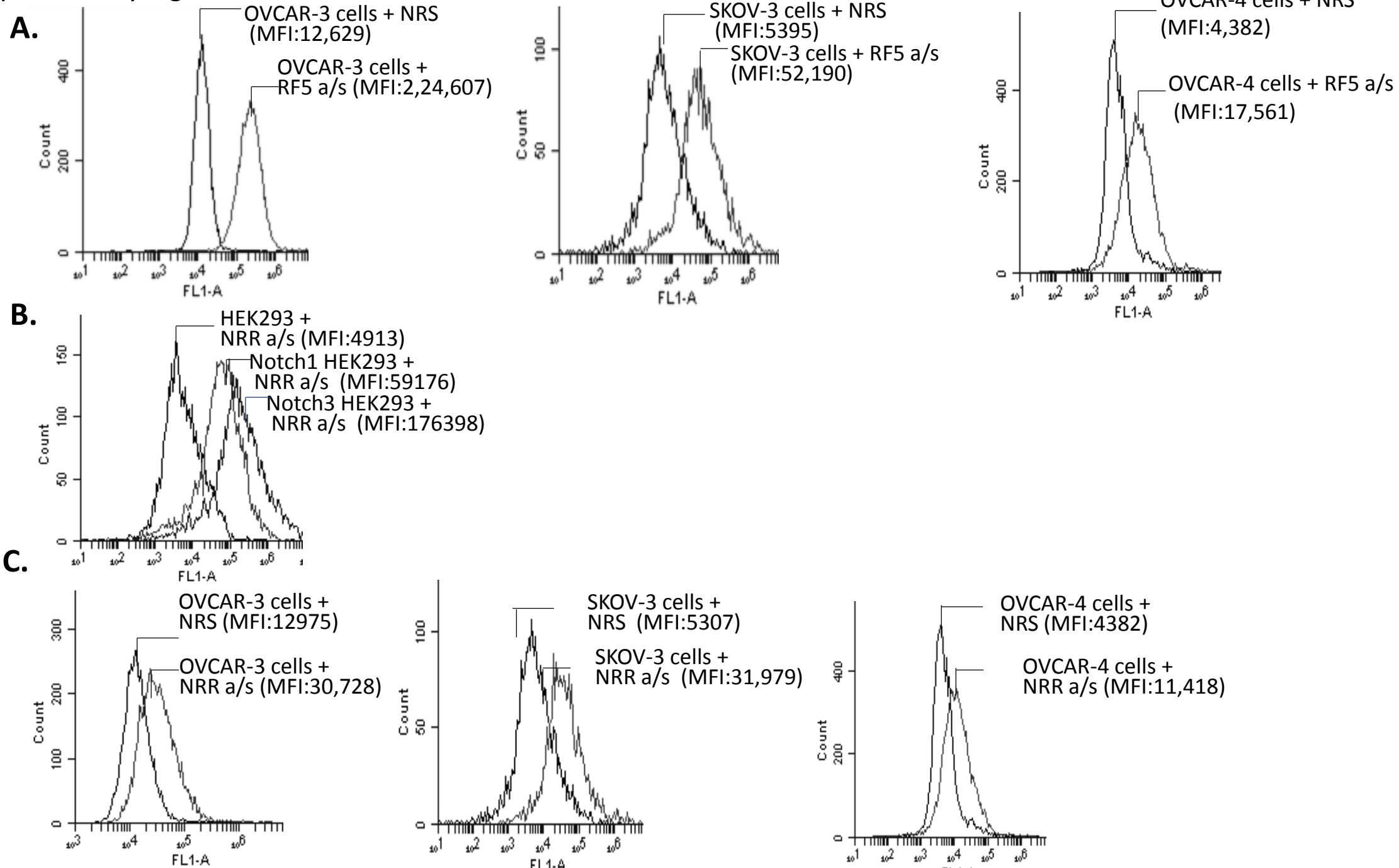
Table 1

Pt #	Age (yrs)	Ascitic FSH (mIU/ml)	Spheroids FSHβ Ct (mean, n=3)	FSHα Ct (mean, n=3)	FSHR Ct (mean, n=3)	RPL35 Ct (mean, n=3)
1	NA	22.2	NA	NA	NA	NA
2	70	71.04	28.53	24.17	24.51	24.96
3	53	124.14	28.02	24.14	24.33	25.1
4	58	45.2	NA	NA	NA	NA
5	50	38	32.25	24.69	31.31	24.86
6	72	102.8	NA	NA	NA	NA
7	49	36.6	NA	NA	NA	NA
8	40	34.2	NA	NA	NA	NA
9	56	13.5	NA	NA	NA	NA
10	42	136.32	26.9	25.1	24.67	23.96
11	50	134.24	27.16	24.4	23.6	25.66
12	48	98.24	NA	NA	NA	NA
13	45	90.96	27.12	24.83	26.15	25.11
14	55	96.32	26.64	25.82	26.11	24.15
15	68	15	NA	NA	NA	NA
16	NA	40.9	NA	NA	NA	NA
17	NA	102.77	NA	NA	NA	NA
18	54	104.85	27.78	24.51	29.06	25.71
19	53	35.01	NA	NA	NA	NA

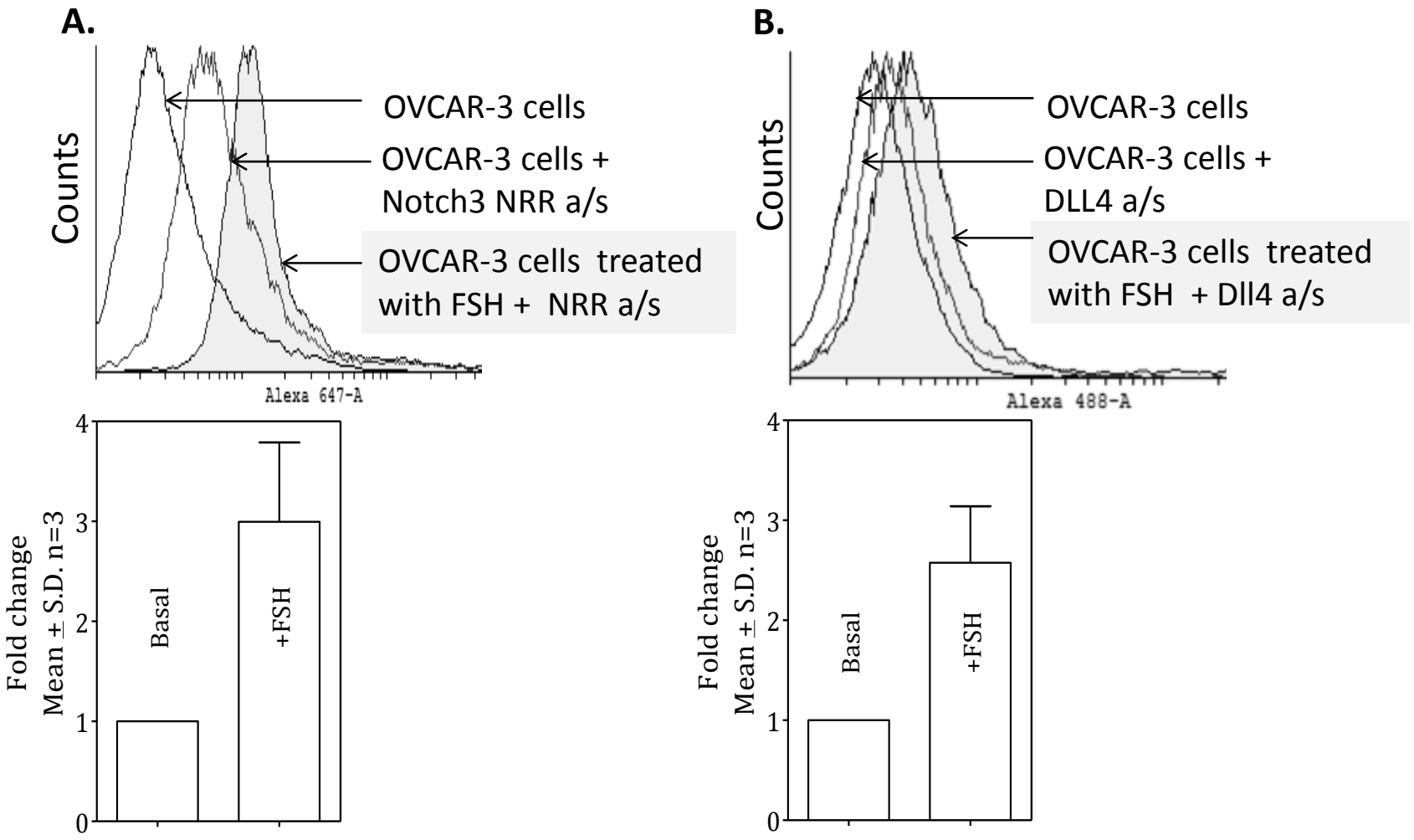
Pt #	Age (yrs)	Ascitic FSH (mIU/ml)	Serum FSH (mIU/ml)
20	55	23.41	24.44
21	43	24	84.55
22	64	40.83	Nil
23	58	208.32	122.19
24	46	14.76	5.85
25	55	115.44	131.43
26	73	59.71	147.26

Pt #	Tumor FSHβ Ct (mean, n=3)	FSHα Ct (mean, n=3)	FSHR Ct (mean, n=3)	RPL35 Ct (mean, n=3)
27	29.12	29.72	24.81	26.76
28	28.38	29.43	23.88	25.78
29	28.64	29.46	21.83	23.41
30	29.27	30.28	26.62	26.45
31	28.97	30.08	26.35	25.96
32	29.98	30.08	28.67	25.87

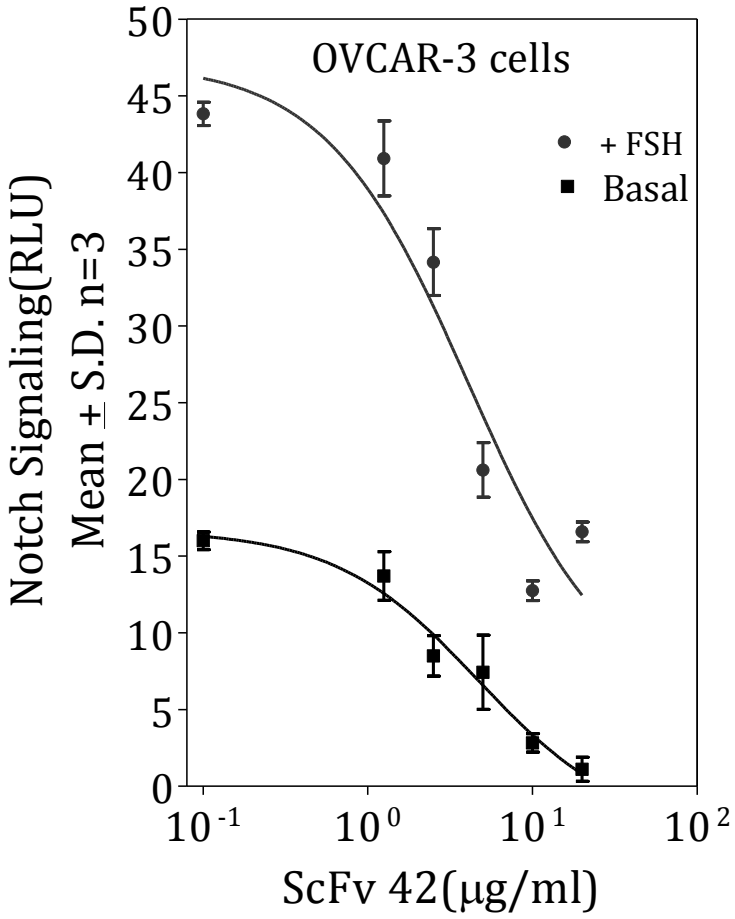
Supplementary Figure 1.



Supplementary Figure 2.

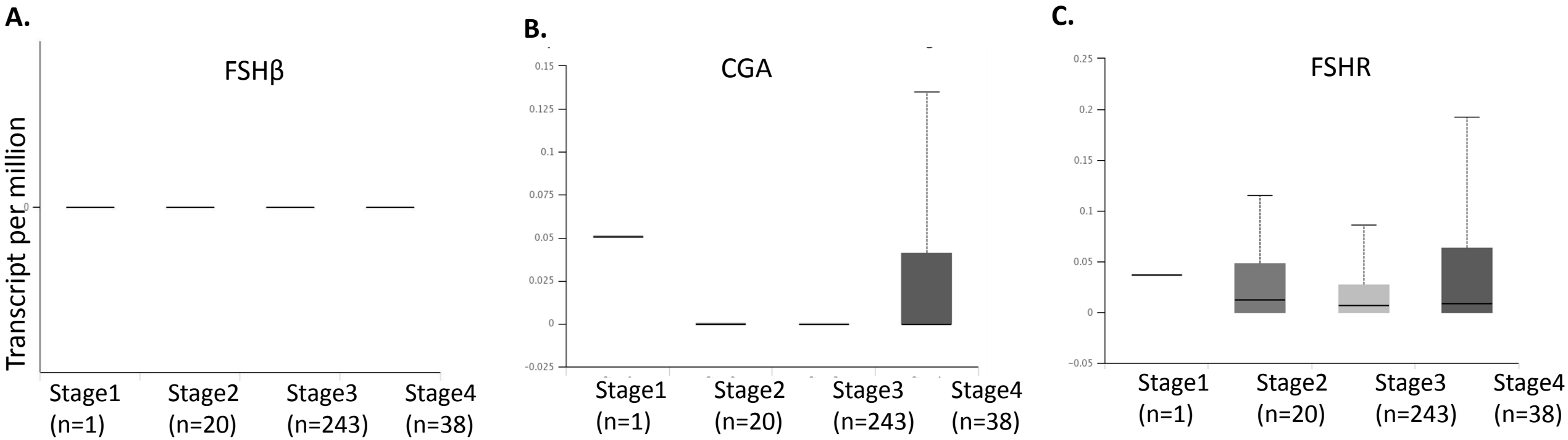


Supplementary Figure 3.



Supplementary Figure 4.

TCGA Samples



Supplementary Table 1

Notch1	FP 5’ GTCAACGCCGTAGATGACC 3’
	RP 5’ TTGTTAGCCCCGTTCTTCAG 3’
Notch2	FP 5’ ATGACTGCCCTAACCACAGG 3’
	RP 5’ TGCAGTCATCTCCACTCCAG 3’
Notch3	FP 5’ GGGAAAAAGGCAATAGGC 3’
	RP 5’ GGAGGGAGAAGCCAAGTC 3’
Notch4	FP 5’ AACTCCTCCCCAGGAATCTG 3’
	RP 5’ CCTCCATCCAGCAGAGGTT 3’
Jag1	FP 5’ TCACGGGAAGTGCAAGAGTC 3’
	RP 5’ GTTTCAACAGTAGGCCCCCTC 3’
Jag2	FP 5’ CCCACCTGTGCCCTTGACA 3’
	RP 5’ GGCTTCCCCTTCACAGTCGTTGA 3’
Dll1	FP 5’ GCAGCTCTTCACCCTGTTCT 3’
	RP 5’ GGTGCAGGAGAAGTCGTTCA 3’
Dll4	FP 5’ ACGAATGCATCCCCCACAAT 3’
	RP 5’ GACAGGTGCAGGTGTAGCTT 3’
Hes1	FP 5’ TTCCCTCCGGACTCTAAACA 3’
	RP 5’ CAAACATCTTTGGCATCACA 3’
Hes5	FP 5’ GCCGTTTTAGGACAATCAGG 3’
	RP 5’ GAGTTCGGCCTTCACAAAAG 3’
Heyl	FP 5’ AGAATCCCTAGTGGGGCTGT 3’
	RP 5’ GGCTTGGGGATAGAAACACA 3’
FSHR	FP 5' AACACCCATCCAAGGAATGG 3'
	RP 5' GGGCTAAATGACTTAGAGGGACAA 3'
FSHβ	FP 5’ ATACAAGGAATCTGCATGGTGAG 3’
	RP 5’ TGGTGTGCTGGCTACTGCTAC 3’
FSHα	FP 5’ TCCCACTCCACTAAGGTCCAA 3’
	RP 5’ CCCCATTACTGTGACCCTGTT 3’
GAPDH	FP 5’ GAGAAGGCTGGGGCTCATTT 3’
	RP 5’ AGTGATGGCATGGACTGTGG 3’
RPL35	FP 5’ TCTAAGATCCGAGTCGTCCG 3’
	RP 5’ GCATGGCACGTGTCTTCTTA 3’