

# Follicle-Stimulating Hormone Receptor as a Target in the Redirected T-cell Therapy for Cancer

Katarzyna Urbanska<sup>1</sup>, Caitlin Stashwick<sup>1</sup>, Mathilde Poussin<sup>1</sup>, and Daniel J. Powell Jr<sup>1,2</sup>

## Abstract

Adoptive transfer of T cells engineered to express chimeric immunoreceptors is an effective strategy to treat hematologic cancers; however, the use of this type of therapy for solid cancers, such as ovarian cancer, remains challenging because a safe and effective immunotherapeutic target has not yet been identified. Here, we constructed and evaluated a novel redirected T-cell-based immunotherapy targeting human follicle-stimulating hormone receptor (FSHR), a highly conserved molecule in vertebrate animals with expression limited to gonadal tissues, ovarian cancer, and cancer-associated vasculature. Receptor ligand-based anti-FSHR immunoreceptors were constructed that contained small binding fragments from the ligand for FSHR, FSH, fused to T-cell transmem-

brane and T-cell signaling domains. Human T cells transduced to express anti-FSHR immunoreceptors were specifically immunoreactive against FSHR-expressing human and mouse ovarian cancer cell lines in an MHC-nonrestricted manner and mediated effective lysis of FSHR-expressing tumor cells, but not FSHR-deficient targets, *in vitro*. Similarly, the outgrowth of human ovarian cancer xenografts in immunodeficient mice was significantly inhibited by the adoptive transfer of FSHR-redirected T cells. Our experimental observations show that FSHR is a promising immunotherapeutic target for ovarian cancer and support further exploration of FSHR-targeted immune therapy approaches for patients with cancer. *Cancer Immunol Res*; 3(10); 1130–7. ©2015 AACR.

## Introduction

The follicle-stimulating hormone receptor (FSHR) is a G-protein-coupled receptor that is primarily expressed in the granulosa cells of the ovary and the Sertoli cells of the testis of adults (1, 2). Although the role of FSHR expression in the development and progression of ovarian cancer is not fully understood, one hypothesis currently being tested is that gonadotrophin signals, including those transmitted through FSHR, may be involved in the transformation and progression of normal ovarian surface epithelium (OSE) to neoplastic OSE. Indeed, ovarian cancer is more common in conditions in which gonadotropin levels are elevated, such as in postmenopausal women (3). FSHR mRNA can be detected in ovarian cancer (4), although protein detection by IHC has been challenging and requires further investigation. *In vitro* results from overexpressing cell lines suggest that the protein may play a role as a stimulator of cancer cell proliferation (5), and treatment of some ovarian cancer cells with FSH, the ligand

for FSHR, can result in growth stimulation in a dose- and time-dependent manner *in vitro* (6), which suggests that FSHR-targeted therapy could be effective in ovarian cancer. Furthermore, recent biodistribution studies from Radu and colleagues show ubiquitous ectopic expression of FSHR in the tumor-associated blood vessels of various tumor types, including prostate adenocarcinoma, urothelial carcinoma, renal cell carcinoma, and ovarian cancer, without detectable expression in surrounding healthy vasculature (7, 8), although this finding has yet to be confirmed by others. The biologic significance of its expression in tumor blood vessels remains unknown, but the FSHR expression level correlates with the response to antiangiogenic therapies and suggests that anti-FSHR therapy could be used broadly as a tumor vasculature disruption agent across solid malignancies. Thus, FSHR expression by both ovarian cancer cells and its tumor-associated vasculature (4, 9) may provide a unique opportunity to deliver synergistic effects from a single, anti-FSHR therapy, thus making FSHR targeting particularly attractive in ovarian cancer.

T-cell targeting of antigens on the cancer cell surface can now be achieved through the genetic modification of primary T cells to express a chimeric antigen receptor (CAR). Adoptive immunotherapy using CAR T cells specific for CD19 has been shown to induce durable remissions in hematologic malignancies (10); however, targeting of solid tumors has not yet achieved parallel success. Multiple factors may limit the efficacy and safety of this form of therapy in solid tumors, one of which is the risk associated with on-target, off-tumor toxicity. For example, CD19 CAR therapy induces profound B-cell aplasia through coordinate elimination of healthy and malignant CD19<sup>+</sup> B lymphocytes (10), and transfer of ERBB2 CAR T cells following lymphodepleting chemotherapy was associated with rapid-onset respiratory arrest and death, presumably via

<sup>1</sup>Department of Obstetrics and Gynecology, Ovarian Cancer Research Center, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania. <sup>2</sup>Department of Pathology and Laboratory Medicine, Abramson Cancer Center, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania.

**Note:** Supplementary data for this article are available at Cancer Immunology Research Online (<http://cancerimmunolres.aacrjournals.org/>).

**Corresponding Author:** Daniel J. Powell Jr, University of Pennsylvania, 3400 Civic Center Boulevard, Building 421, Smilow CTR, Room 08-103, Philadelphia, PA 19104. Phone: 215-573-4783; Fax: 215-573-7627; E-mail: [poda@mail.med.upenn.edu](mailto:poda@mail.med.upenn.edu)

**doi:** 10.1158/2326-6066.CIR-15-0047

©2015 American Association for Cancer Research.

cytokine release by CAR T cells triggered by the recognition of low levels of ERBB2 on lung epithelial cells (11). The potential for on-target, off-tumor toxicity from CAR T-cell therapy has limited more widespread translation of this promising therapy to other malignancies. Still, the targeting of *bona fide* cancer/testis antigens, such as NY-ESO-1, with expression restricted to the ovary, male germ cells in the testis, and malignant cells but not somatic tissue, has paved the path for safe and effective gene-modified T-cell therapy (12). In the absence of tumor-restricted antigens, other antigens that are expressed by tumor cells and cells of healthy organs with nonessential function for patient survival, such as reproductive organs, may be attractive targets for CAR-based immunotherapy. FSHR is a member of this class of antigens. The restricted expression pattern of abundant FSHR in ovarian cancer (2, 4, 13), tumor vessels (7, 8), and gonadal tissues (1), relative to that found in adult somatic tissues (14), lowers the risk for on-target, off-tumor toxicity and makes FSHR an appealing target for T-cell-based immunotherapy.

In this study, we developed a strategy for treatment of human ovarian cancer via the redirection of primary human T cells against FSHR and validated for the first time the suitability of FSHR as a target of T-cell-based immunotherapy. Anti-FSHR immunoreceptors (FSHR-IR), specific for human and mouse FSHR, were created using peptides derived from the natural FSHR ligand, FSH, for redirected T-cell activity. Herein, we report the results of *in vitro* testing of FSHR-redirected T cells for recognition and reactivity against human FSHR-expressing ovarian cancer cells, and the *in vivo* efficacy of anti-FSHR-IR T-cell therapy in a xenograft model of human ovarian cancer. This study serves as a foundation for the future development of FSHR-targeted immunotherapies.

## Materials and Methods

### Anti-FSHR-IR construction

Amino acid sequences encoding FSH peptides, including flanked 3'-*Bam*HI and 5'-*Nhe*I restriction sites, were synthesized. DNA products were digested with *Bam*HI and *Nhe*I enzymes and ligated into pELNS, a third-generation self-inactivating lentiviral expression vector, containing human CD8alpha-hinge and transmembrane region fused to CD3z or CD28-CD3z signaling endodomains, under an EF-1 $\alpha$  promoter. Resulting constructs were designated anti-FSHR IR-z and anti-FSHR-28z, respectively.

### Recombinant lentivirus production

High-titer replication-defective lentiviral vectors were produced and concentrated as described previously (15). Briefly, 293T cells were transfected with pVSV-G (VSV glycoprotein expression plasmid), pRSV.REV (Rev expression plasmid), pMDLg/p.RRE (Gag/Pol expression plasmid), and pELNS transfer plasmid using Lipofectamine 2000 (Invitrogen). The viral supernatant was harvested at 24 and 48 hours after transfection.

### Lymphocytes

Primary human CD4<sup>+</sup> and CD8<sup>+</sup> T cells isolated from healthy volunteer donors were purchased from the Human Immunology Core at University of Pennsylvania (Philadelphia, PA), activated, and transduced with lentiviral vectors as described previously (16).

### Cell lines

The immortalized normal fetal renal 293T cell line and human cell lines used in immune-based assays, including CaOV3, CaOV434, SKOV3, and OVCAR3, were purchased from and maintained according to the ATCC. The murine mesothelioma cell line AE17 and the murine ovarian cancer cell line ID8 were provided by Steven M. Albelda and George Coukos at the University of Pennsylvania, respectively. Cells were transfected with firefly luciferase as described previously (16).

### Flow-cytometric analysis

APC-Cy7 mouse anti-human-CD3, FITC-anti-human-CD4, APC-anti-human-CD8, PE-human-CD45, and APC-human-CD69 antibodies were purchased from Biolegend. FSHR expression was detected using clone6266717 (R&D Systems). T-cell transduction was measured by GFP transgene expression. 7-Aminoactinomycin D (AAD; Biolegend) was used to assess viability. For *in vivo* T-cell quantification, 50  $\mu$ L blood was obtained from mice via retro-orbital bleeding and labeled for human CD45, CD3, and CD8. Cell numbers were quantified using BD TruCount tubes according to the manufacturer's instructions. Flow cytometry data were analyzed using FlowJo software.

### FSHR-PCR

Total RNA was extracted from  $5 \times 10^6$  viable tumor cells using an RNeasy Mini kit (Qiagen). RNA quantity and quality were verified using a NanoDrop 2000 spectrophotometer (Thermo). cDNA was generated from 1  $\mu$ g total RNA using the High-Capacity-RNA-to-cDNA kit (Applied Biosystems). The human *FSHR* was PCR amplified using the following primers: 5'-CTCACCAAGCTTCGAGTCATCCAA-3' and 5'-GCTCATCTAGTTGGGTCCATT-3' (Gene ID: 2492), mouse FSHR 5'-GGGATCTGGATGTCATCACT-3' and 5'-GGAGAACACATCTGCCTCTA-3' (Gene ID: 14309).

### Cytokine release assays and intracellular cytokine staining (CBA)

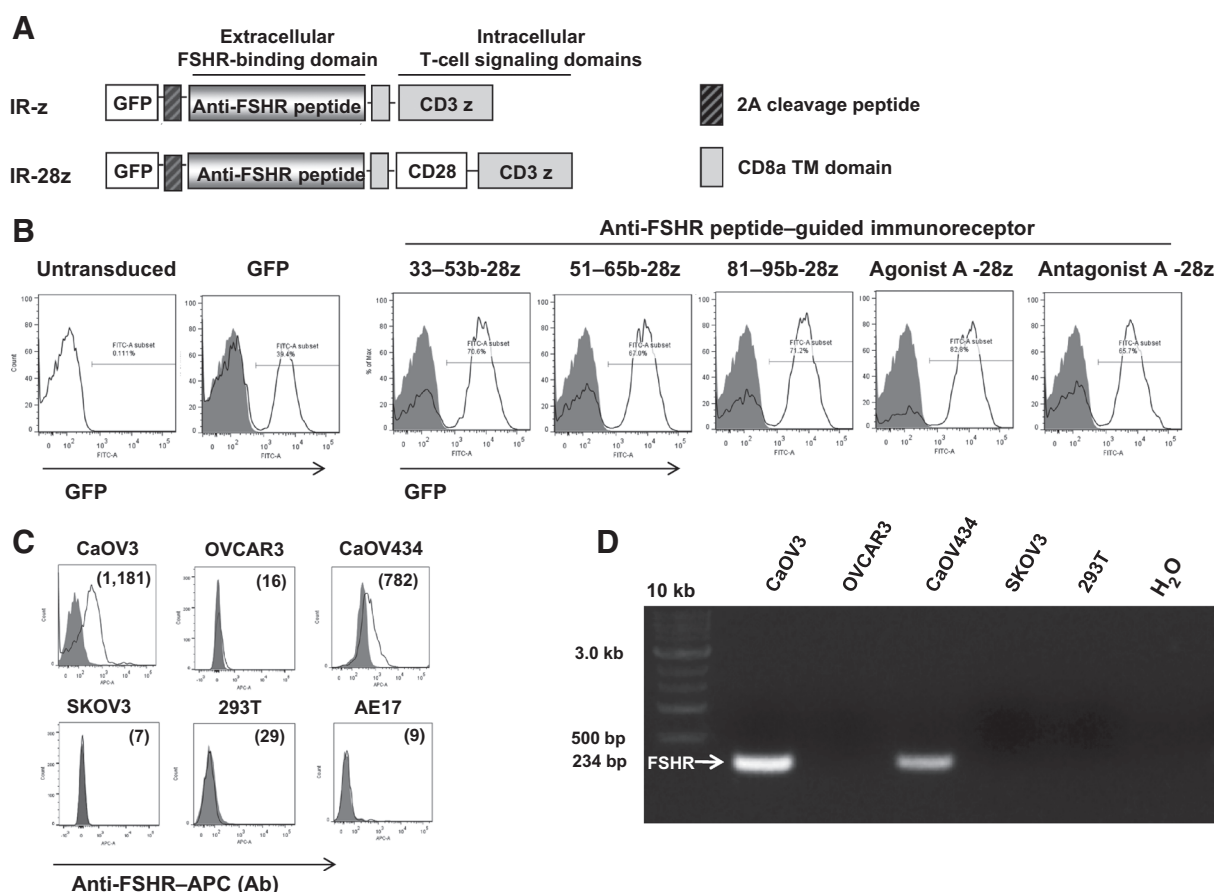
Cytokine release assays were performed by coculture of  $1 \times 10^5$  FSHR-IR T cells with FSHR-expressing CaOV3 and FSHR<sup>-</sup> 293T cells, or mouse FSHR-expressing ID8, as described previously (16). After 16 hours, coculture supernatants were assayed for the presence of cytokines using an ELISA kit (Biolegend) and Cytokine Bead Array (BD Biosciences) according to the manufacturer's instructions.

### Cytotoxicity

fluc-transduced targets were plated at  $1 \times 10^4$  per well in triplicate. T cells were added at the indicated effector:target (E:T) ratios. Cocultures were incubated overnight in phenol-free conditioned medium. The Extended-Glow Bioluminescent Reporter Gene Assay (Applied Biosystems) was used to measure residual luciferase activity from remaining targets, and lysis was calculated as follows: Percent lysis =  $100 - [(average\ signal\ from\ T-cell-treated\ wells)/(average\ signal\ from\ untreated\ target\ wells) \times 100]$ .

### Xenograft model of OvCa

NOD/SCID/ $\gamma$ -chain<sup>-/-</sup> (NSG) mice were bred, treated, and maintained under pathogen-free conditions in-house under the University of Pennsylvania IACUC-approved protocols. Six- to 12-week-old female mice were purchased from the University of Pennsylvania Stem Cell and Xenograft Core, and  $5 \times 10^6$  CaOV3-fluc tumor cells were inoculated s.c. (5 mice per group).



**Figure 1.** Anti-FSHR-IR construction and expression in primary human T cells. A, schematic of lentiviral anti-FSHR-IR vectors containing an anti-FSHR peptide linked to intracellular signaling domains from CD3-z (IR-z) alone and in tandem with CD28 (IR-28z). Anti-FSHR constructs also encode GFP separated by a viral T2A (2A) ribosomal skip peptide. B, transgene expression in primary human T cells. Expression of anti-FSHR-IR constructs in primary human T cells was measured by GFP. TM, transmembrane domain; UNT, untransduced T cells. C, FSHR expression detected by flow cytometry using rabbit anti-FSHR-APC IgG (open histogram) and isotype control (gray). Specific mean fluorescence intensity (MFI) is shown on each plot. D, expression of human *FSHR* in ovarian cancer cell lines and control 293T cells determined by RT-PCR using FSHR-specific PCR primers. Controls included 293T cells and no input (H<sub>2</sub>O). RT-PCR using FSHR primers based on human sequence (NM\_0) amplified the predicted 234-bp product from cDNA templates. Five-fold less PCR-amplified cDNA from CaOV434 cells was loaded relative to other lanes to avoid spillover.

Twenty and 25 days later, mice were injected i.v. with  $6 \times 10^6$  T cells. Tumor growth was assessed by weekly caliper measurements. Tumor volume was calculated using the following formula:  $V = 1/2 (\text{length} \times \text{width}^2)$ , where length is the greatest longitudinal diameter and width is the greatest transverse diameter.

**Statistical analysis**

The Student *t* test was used to evaluate differences in T-cell-specific cytotoxicity and cytokine secretion. GraphPad Prism 4.0 (GraphPad Software) was used for the statistical calculations.  $P < 0.05$  was considered statistically significant.

**Table 1.** FSH peptides used for anti-FSHR-IR construction

Construct name <sup>a</sup>	FSH-derived peptides	Sequence	Predicted affinity	Predicted cross-reactivity
Anti-FSHR 33-53β-IR	β-Chain 33-53aa	YTRDLVYKDPARPKIQKTCTF	10 <sup>-5</sup> mol/L	Human/mouse
Anti-FSHR 51-65β-IR	β-Chain 51-65aa	CTFKELVYETVRVPGC	10 <sup>-4</sup> mol/L	Human
Anti-FSHR 81-95β-IR	β-Chain 81-95aa	QCHCGKCDSDTCT	10 <sup>-5</sup> mol/L	Human/mouse
Anti-FSHR antagonist A-IR	β-Chain (87-94aa) + α-chain (25-42aa)	CDSDSTDCILQCMGCCFSRAYPTPLR	10 <sup>-8</sup> mol/L	Human/mouse
Anti-FSHR agonist A-IR	β-Chain (87-94aa) + α-chain (25-42aa)	CDSDSTDCILQCMGCCFSRAYPTPLRWAGYCYCYTRD	10 <sup>-7</sup> mol/L	Human/mouse
	+b (27-45aa)	VKDPARP		
Anti-FSHR a+b-IR	FSH (α-chain + β-chain)	GenBank Gene IDs: 1081 (FSH, α subunit) + 2488 (FSH, β subunit)	NA	Human/mouse

Abbreviation: NA, not available.

<sup>a</sup>Final chimeric IR constructs were engineered to encode for either an intracellular CD3z (-z) or a tandem CD3z and CD28 (-28z) domain.

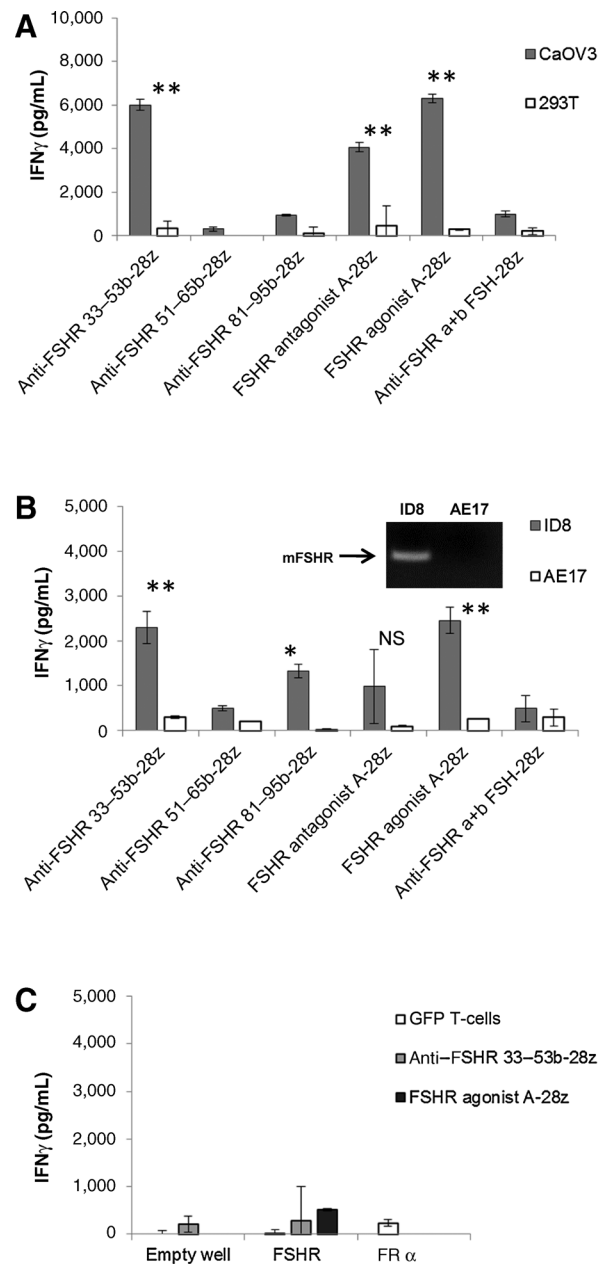
## Results and Discussion

### Generation of the anti-FSHR-IR

The chimeric immunoreceptor was constructed for the redirection of primary human T cells against FSHR-expressing targets. FSH, the natural ligand for FSHR, was selected as the binding moiety for the immunoreceptor. FSH is a heterodimeric glycoprotein gonadotropin, consisting of  $\alpha$  and  $\beta$  subunits, and several peptides derived from either the FSH beta subunit alone or mixed  $\beta/\alpha$  subunits are reported to specifically bind to FSHR, as high affinity FSHR agonists or antagonists (16). On the basis of their predicted binding affinity for FSHR, we selected multiple anti-FSHR peptides for the construction of a panel of chimeric immunoreceptors shown in Table 1. Some FSHR-binding sites have been reported, including amino acid fragments 33–53, 51–65, and 81–95 of the FSH $\beta$ -chain (17–19). In particular, FSH $\beta$  33–53 peptide appears to be functional when covalently attached to nanoparticles, providing high-selectivity nanoparticle delivery to FSHR-expressing ovarian tumors (20). FSH peptides were cloned into previously validated lentiviral constructs with the intracellular CD3z domain alone or with the CD28 costimulatory signaling domain in tandem (16) and referred to as anti-FSHR-IR-z and anti-FSHR-IR-28z (Fig. 1A). We reproducibly achieved a high transduction efficiency of primary human T cells, as measured by coexpression of GFP (65%–80%; Fig. 1B). Transduction efficiencies of all anti-FSHR-IR constructs were essentially identical.

### FSHR-redirected T cells exhibit antigen-specific reactivity against tumor targets

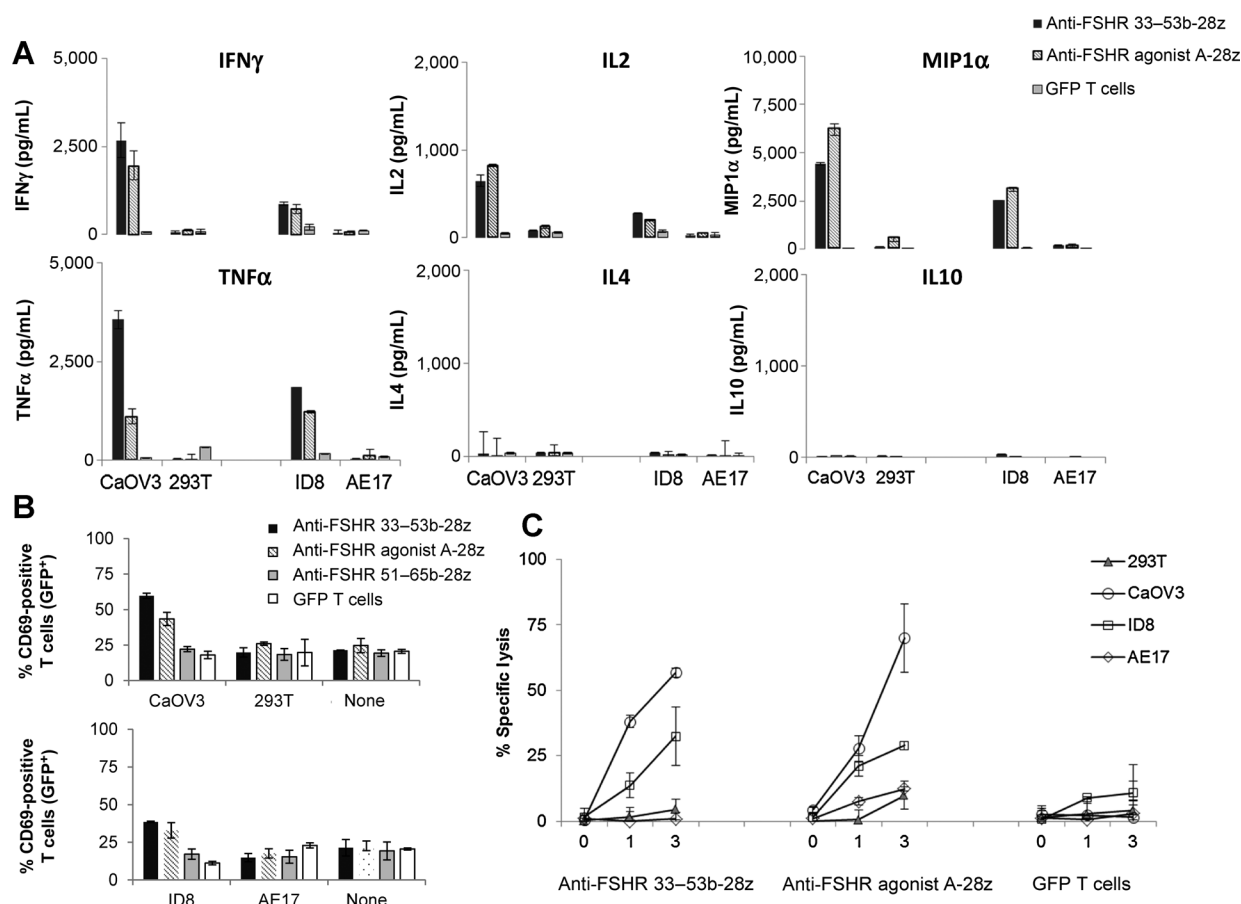
First, established human ovarian cancer cell lines were characterized for cell-surface FSHR expression. FSHR was detected on CaOV3 and CaOV434 cancer cells, whereas OVCAR3, SKOV3, and 293T (Fig. 1C), as well as OVCAR5 and OVCAR 8 (not shown), cancer cells lacked detectable FSHR expression. FSHR expression in CaOV3 and CaOV434 was confirmed by PCR using RT-PCR primers (Fig. 1D). The 18S was used as an internal control (Supplementary Fig. S1). To evaluate the FSHR-specific response of anti-FSHR-IR T cells, the panel of anti-FSHR-IR T cells was cocultured with FSHR<sup>+</sup> or FSHR<sup>-</sup> cells overnight, and supernatants were subsequently assayed for the secretion of the cytokine, IFN $\gamma$ . Anti-FSHR-IR-28z, comprised of extracellularly expressed peptide 33–53 $\beta$ , agonist A, and antagonist A, produced the highest levels of IFN $\gamma$  following coculture with FSHR-expressing CaOV3, but not against FSHR<sup>-</sup> 293T cells (Fig. 2A). Anti-FSHR 81–95 $\beta$  IR-28z and anti-FSHR  $\alpha$ + $\beta$  IR-28z ( $\alpha$ -chain +  $\beta$ -chain of FSH) produced much lower levels of IFN $\gamma$  against FSHR<sup>+</sup> targets, associated with predicted lower affinity of those constructs to FSHR. First-generation anti-FSHR-IR-z T cells also secreted IFN $\gamma$  in a similar pattern (data not shown). Similarly, when tested for immunoreactivity against the murine FSHR-expressing cell line ID8, anti-FSHR 33–53 $\beta$ -28z, anti-FSHR 81–95 $\beta$ -28z and anti-FSHR agonist A-28z IR T cells all exhibited moderate and specific immune recognition (Fig. 2B), consistent with the high (93%) homology between human and mouse FSHRs and known binding of human FSH, and FSH-derived peptides, to murine FSHR (21). This observation is of special interest, given the opportunity to address potential, but unpredicted, toxicity issues associated with targeting of FSHR in preclinical models. Notably, none of the anti-FSHR-IR constructs tested were capable of recognizing



**Figure 2.**

Anti-FSHR T cells recognize human and mouse ovarian cancer cells expressing FSHR but not cells without the FSHR expression. A, levels of IFN $\gamma$  in supernatants following overnight coculture of anti-FSHR-IR T cells with FSHR<sup>+</sup> and FSHR<sup>-</sup> CaOV3 or FSHR<sup>-</sup> 293T target cells detected by ELISA. Cocultures were established at 1:1 E:T ratio. B, IFN $\gamma$  in overnight cocultures of anti-FSHR-IR T cells with mouse FSHR<sup>+</sup> ID8 cells or FSHR<sup>-</sup> AE17 mesothelioma cell lines. C, lack of immune recognition of human recombinant FSHR protein by anti-FSHR-IR T cells. Results are presented as mean  $\pm$  SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (Student  $t$  test).

immobilized recombinant human FSHR protein commercially produced from *Escherichia coli* (Fig. 2C), consistent with previous reports demonstrating that multiple posttranslational modifications of both FSHR and FSH are required for proper folding and binding of the ligand to the receptor (22).



**Figure 3.** Anti-FSHR-IR T cells react against and lyse FSHR-expressing human and mouse cancer cells *in vitro*. A, cytokine secretion by anti-FSHR-redirection T cells and control GFP-transduced primary human T cells. IFN $\gamma$ , IL2, MIP1 $\alpha$ , TNF $\alpha$ , IL4, and IL10 secretion was detected by CBA 16 hours after tumor stimulation (data represent three independent experiments in triplicates). Results are presented as mean  $\pm$  SD. Values of  $P < 0.01$  and  $P < 0.05$  were considered statistically significant. We observed a specific and statistically significant production of cytokines; IFN $\gamma$ , IL2, MIP1 $\alpha$ , and TNF $\alpha$  by anti-FSHR 33-53b-28z and anti-FSHR agonist A-28z against FSHR $^{+}$  targets human CaOV3 and mouse ID8 cell lines when compared with cytokine levels in cocultures with FSHR $^{-}$  targets in human 293T and mouse AE17 cell lines. B, anti-FSHR $^{+}$  T cells upregulate surface CD69 expression upon 24-hour exposure to FSHR-expressing targets. The FSHR $^{+}$  IR T cells are identified by GFP expression. Graph, percentage of CD69-positive cells gated on the viable CD3 $^{+}$ /GFP-positive T-cell population. C, cytotoxicity of anti-FSHR T cells. Anti-FSHR 33-53 $\beta$ -28z or agonist A-28z IR T-cell killing of FSHR-expressing human CaOV3 and mouse ID8, or control 293T or AE17 cells, was assessed in a 16-hour luciferase-based killing assay. T cells were cocultured with target cells expressing firefly luciferase at E:T ratios of 0:1, 1:1, or 3:1. Residual luciferase signal was determined after 16 hours. The percentage of lysis was determined by luminescence in comparison with untreated target wells. Results, mean  $\pm$  SD. \*,  $P < 0.05$  was considered statistically significant.

Anti-FSHR 33-53 $\beta$ -28z and anti-FSHR agonist A-28z IR constructs were selected for further evaluation, based on their high immunoreactivity against FSHR $^{+}$  tumor cells. We first determined whether anti-FSHR-IRT cells that encounter FSHR antigen release T $_H$ 1-type cytokines other than IFN $\gamma$ , as has been reported for conventional scFv-based CARs (16). As shown in Fig. 3A, both anti-FSHR 33-53 $\beta$ -28z and anti-FSHR agonist A-28z IR T cells produce multiple proinflammatory cytokines, including TNF $\alpha$ , MIP1 $\alpha$ , and IL2, when stimulated with FSHR-expressing CaOV3 or ID8 cell lines. This is in line with results from previous studies using CARs with a CD28 costimulatory domain (23, 24). IFN $\gamma$  and TNF $\alpha$  production was significantly lower in response to ID8 cells, with almost negligible levels of IL2 detected. This may be due to a lower affinity of the anti-FSHR-IRs for mouse FSHR than human FSHR protein, or antigen density, as our antihuman antibody was unable to detect murine FSHR. Anti-FSHR-IR T

cells did, however, specifically upregulate cell-surface expression of CD69, an activation marker, when cocultured with FSHR $^{+}$  CaOV3 or ID8 cells. FSHR-deficient cell lines did not affect the CD69 level, confirming antigen-specific reactivity against both mouse and human FSHR $^{+}$  cell lines. Control GFP and anti-FSHR 51-65 $\beta$ -28z T cells did not respond to either cell line (Fig. 3B). Collectively, these data indicate that anti-FSHR-IRs can confer T cells with the capacity to specifically recognize human and mouse FSHR protein expressed on the tumor cell surface.

**Anti-FSHR-IR T cells mediate antigen-specific tumor cell killing *in vitro***

To evaluate the lytic proficiency of anti-FSHR-IR T cells, we first engineered FSHR $^{+}$  and FSHR $^{-}$  target cell lines to constitutively express firefly luciferase. Following overnight coculture of target cells with gene-engineered T cells, the percentage of

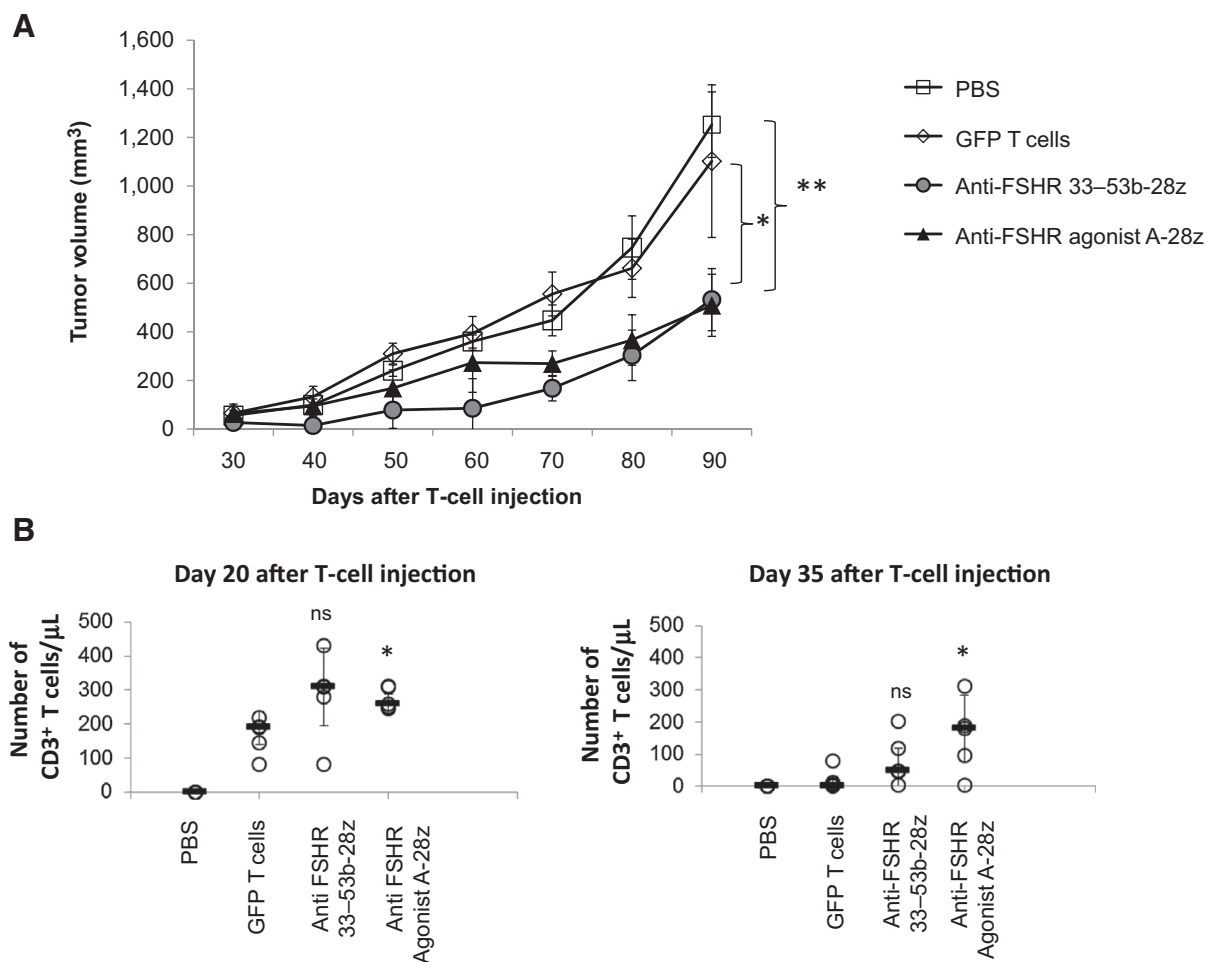
specific lysis was calculated on the basis of a residual luciferase signal. Both anti-FSHR 33–53 $\beta$ -28z and anti-FSHR agonist A-28z IR T cells showed dose-dependent lysis of FSHR<sup>+</sup> targets (Fig. 3C). As observed in IFN $\gamma$  release assays, anti-FSHR-IR T cells also exhibited specific lytic activity against the mouse FSHR<sup>+</sup> ID8 cell line.

#### FSHR-redirected primary human T cells suppress tumor growth *in vivo*

To assess the antitumor effect of anti-FSHR T cells *in vivo*, we established human ovarian cancer xenografts in NOD/SCID/IL2R $\gamma^{-/-}$  (NSG) mice by s.c. flank injection of the CaOV3 cancer cell line and then administered two i.v. injections of gene-engineered T cells at days 20 and 25, similar to our previous studies (15, 24). Consistent with their *in vitro* function, both anti-FSHR 33–53 $\beta$ -28z and anti-FSHR agonist A-28z IR T cells mediated the suppression of established tumor outgrowth that was statistically

superior to the control, GFP-engineered T-cell-treated group, which provided no benefit (Fig. 4A). Because objective clinical response to T-cell transfer therapy is often associated with the persistence of T cells after infusion (25), we measured peripheral blood for the continued persistence of engineered human T cells *in vivo* via TruCount bead-based counting (Fig. 4B). Consistent with the antitumor response, mice treated with anti-FSHR 33–53 $\beta$ -28z or anti-FSHR agonist A-28z IR T cells had increased peripheral blood CD3<sup>+</sup> T-cell counts compared with controls at 3 and 5 weeks after T-cell infusion, although only at the level of statistical significance in the anti-FSHR agonist A-28z IR T-cell group. No overt immune-related toxicity or immunopathology was observed.

In summary, we have successfully exploited a novel therapeutic antigen, the FSHR protein, expressed on the tumor cell surface, to deliver T-cell-based immune therapy to patients with confirmed expression of FSHR in their tumor. Given its highly restricted,



**Figure 4.**

Suppressed ovarian cancer growth in mice receiving anti-FSHR-IR T-cell treatment. A,  $5 \times 10^6$  luciferase-expressing CaOV3 cells were injected s.c. into NSG mice on day 0. A total of  $5 \times 10^6$  T cells were injected i.v. on days 20 and 25. Tumor growth was monitored by caliper measurement. Graphs, mean  $\pm$  SEM of 5 mice per experiment. *P* values were calculated compared with GFP T-cell- and PBS-treated control mice. \*, *P* < 0.05 was considered statistically significant. \*, *P* < 0.05; \*\*, *P* < 0.01. B, preferential expansion and survival of human T cells in peripheral blood of anti-FSHR-IR T-cell-treated mice, compared with control GFP T-cell and PBS-treated groups. Peripheral blood was collected 20 and 35 days after T-cell injection and absolute number of human CD3<sup>+</sup> T cells was quantified by flow cytometry using TruCount beads and reported as total cells per microliter of blood. Bar graphs, mean  $\pm$  SD for 5 mice per group. *P* values were determined compared with the control GFP T-cell-treated group. \*, *P* < 0.05.

abundant expression in cancer and gonadal tissues, the rationale for FSHR targeting in solid human malignancy is strong. FSHR may also serve as a target for cancer-associated vessel disruption (7, 8), although we were unable to detect FSHR protein in the blood vessels of human or mouse cancers by immunohistochemical analysis using commercially available antibodies. Possible risks associated with on-target/off-tissue toxicities appear limited. FSHR-deficient mice are vital but infertile with decreased size of ovaries and uterus (26), suggesting possible toxicities may be restricted to reproductive organs. However, in the scenario of a patient diagnosed with ovarian cancer, these organs are virtually nonessential and often surgically removed. In contrast, male FSHRKO mice exhibit reduced numbers of spermatocytes, which would suggest possible targeted toxicity against testis, an immunoprivileged organ (27). Our study shows that anti-FSHR IR T cells are capable of targeting and killing FSHR-expressing cancer cells, resulting in significant suppression of FSHR-expressing tumor outgrowth *in vivo*. Our findings warrant the examination of other molecules for FSHR targeting to create higher-affinity anti-FSHR chimeric immunoreceptors. Furthermore, the activity of peptide-based anti-FSHR-IRs may also be improved by optimization of other components of the immunoreceptor including modification of the hinge length (28), enhancement of affinity of peptide, or the use of scFv-based immunoreceptors, although such peptides and scFvs have not been described. Moving forward, it will be important to test this FSHR-targeted therapy in tumor models where FSHR is exclusively expressed by tumor blood vessels, compared with simultaneous expression on both tumor cells and its associated vasculature. This is of special interest given its potential for application not only in ovarian cancer but also across many solid cancer types.

## References

1. Simoni M, Gromoll J, Nieschlag E. The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. *Endocr Rev* 1997;18:739–73.
2. Zheng W, Magid MS, Kramer EE, Chen YT. Follicle-stimulating hormone receptor is expressed in human ovarian surface epithelium and fallopian tube. *Am J Pathol* 1996;148:47–53.
3. Riman T, Persson I, Nilsson S. Hormonal aspects of epithelial ovarian cancer: review of epidemiological evidence. *Clin Endocrinol* 1998;49:695–707.
4. Wang J, Lin L, Parkash V, Schwartz PE, Lauchlan SC, Zheng W. Quantitative analysis of follicle-stimulating hormone receptor in ovarian epithelial tumors: a novel approach to explain the field effect of ovarian cancer development in secondary müllerian systems. *Int J Cancer* 2003;103:328–34.
5. Choi JH, Choi KC, Auersperg N, Leung PC. Overexpression of follicle-stimulating hormone receptor activates oncogenic pathways in preneoplastic ovarian surface epithelial cells. *J Clin Endocrinol Metab* 2004;89:5508–16.
6. Parrott JA, Doraiswamy V, Kim G, Mosher R, Skinner MK. Expression and actions of both the follicle stimulating hormone receptor and the luteinizing hormone receptor in normal ovarian surface epithelium and ovarian cancer. *Mol Cell Endocrinol* 2001;172:213–22.
7. Gartrell BA, Tsao CK, Galsky MD. The follicle-stimulating hormone receptor: a novel target in genitourinary malignancies. *Urol Oncol* 2013;31:1403–7.
8. Radu A, Pichon C, Camparo P, Antoine M, Allory Y, Couvelard A, et al. Expression of follicle-stimulating hormone receptor in tumor blood vessels. *N Engl J Med* 2010;363:1621–30.
9. Zheng W, Lu JJ, Luo F, Zheng Y, Feng Y, Felix JC, et al. Ovarian epithelial tumor growth promotion by follicle-stimulating hormone

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** K. Urbanska, C. Stashwick, D.J. Powell Jr  
**Development of methodology:** K. Urbanska, C. Stashwick, D.J. Powell Jr  
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** K. Urbanska, C. Stashwick, M. Poussin, D.J. Powell Jr  
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** K. Urbanska, M. Poussin, D.J. Powell Jr  
**Writing, review, and/or revision of the manuscript:** K. Urbanska, C. Stashwick, D.J. Powell Jr  
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** K. Urbanska, M. Poussin, D.J. Powell Jr  
**Study supervision:** K. Urbanska, D.J. Powell Jr

## Acknowledgments

Support for imaging and animal studies was provided by the Small Animal Imaging Facility and Stem Cell/Xenograft Core at the University of Pennsylvania, respectively. K. Urbanska is now employed by Janssen Research & Development, LLC, Oncology Discovery Research, Spring House, PA 19477.

## Grant Support

This work was supported by grants from the NIH (RO1-CA168900; D.J. Powell Jr) and a kind gift from the Bethesda Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 11, 2015; revised June 16, 2015; accepted June 16, 2015; published OnlineFirst June 25, 2015.

- and inhibition of the effect by luteinizing hormone. *Gynecol Oncol* 2000;76:80–8.
10. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med* 2014;371:1507–17.
  11. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther* 2010;18:843–51.
  12. Robbins PF, Morgan RA, Feldman SA, Yang JC, Sherry RM, Dudley ME, et al. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol* 2011;29:917–24.
  13. Syed V, Ulinski G, Mok SC, Yiu GK, Ho SM. Expression of gonadotropin receptor and growth responses to key reproductive hormones in normal and malignant human ovarian surface epithelial cells. *Cancer Res* 2001;61:6768–76.
  14. Robinson LJ, Tourkova I, Wang Y, Sharrow AC, Landau MS, Yaroslavskiy BB, et al. FSH-receptor isoforms and FSH-dependent gene transcription in human monocytes and osteoclasts. *Biochem Biophys Res Commun* 2010;394:12–7.
  15. Song D, Ye Q, Carpenito C, Poussin M, Wang LP, Ji C, et al. Improved T cell survival provided by CD137 costimulatory signaling to folate receptor-redirectioned T cells results in tumor localization and eradication. *Cancer Res* 2011;71:LB-156.
  16. Song DG, Ye Q, Poussin M, Harms GM, Figini M, Powell DJ Jr. CD27 costimulation augments the survival and antitumor activity of redirectioned human T cells *in vivo*. *Blood* 2012;119:696–706.

17. Santa Coloma TA, Dattatreymurthy B, Reichert LE Jr. A synthetic peptide corresponding to human FSH beta-subunit 33-53 binds to FSH receptor, stimulates basal estradiol biosynthesis, and is a partial antagonist of FSH. *Biochemistry* 1990;29:1194-200.
18. Lum LG, Rathore R, Cummings F, Colvin GA, Radie-Keane K, Maizel A, et al. Phase I/II study of treatment of stage IV breast cancer with OKT3 x trastuzumab-armed activated T cells. *Clin Breast Cancer* 2003;4:212-7.
19. Morbeck DE, Roche PC, Keutmann HT, McCormick DJ. A receptor binding site identified in the region 81-95 of the beta-subunit of human luteinizing hormone (LH) and chorionic gonadotropin (hCG). *Mol Cell Endocrinol* 1993;97:173-81.
20. Zhang XY, Chen J, Zheng YF, Gao XL, Kang Y, Liu JC, et al. Follicle-stimulating hormone peptide can facilitate paclitaxel nanoparticles to target ovarian carcinoma *in vivo*. *Cancer Res* 2009;69:6506-14.
21. Tena-Sempere M, Manna PR, Huhtaniemi I. Molecular cloning of the mouse follicle-stimulating hormone receptor complementary deoxyribonucleic acid: functional expression of alternatively spliced variants and receptor inactivation by a C566T transition in exon 7 of the coding sequence. *Biol Reprod* 1999;60:1515-27.
22. Jiang X, Liu H, Chen X, Chen PH, Fischer D, Sriraman V, et al. Structure of follicle-stimulating hormone in complex with the entire ectodomain of its receptor. *Proc Natl Acad Sci U S A* 2012;109:12491-6.
23. Carpenito C, Milone MC, Hassan R, Simonet JC, Lakhai M, Suhoski MM, et al. Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. *Proc Natl Acad Sci U S A* 2009;106:3360-5.
24. Lanitis E, Poussin M, Hagemann IS, Coukos G, Sandaltzopoulos R, Scholler N, et al. Redirected antitumor activity of primary human lymphocytes transduced with a fully human anti-mesothelin chimeric receptor. *Mol Ther* 2012;20:633-43.
25. Robbins PF, Dudley ME, Wunderlich J, El-Gamil M, Li YF, Zhou J, et al. Cutting edge: persistence of transferred lymphocyte clonotypes correlates with cancer regression in patients receiving cell transfer therapy. *J Immunol* 2004;173:7125-30.
26. Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, et al. Impairing follicle-stimulating hormone (FSH) signaling *in vivo*: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *Proc Natl Acad Sci U S A* 1998;95:13612-7.
27. Abel MH, Baker PJ, Charlton HM, Monteiro A, Verhoeven G, De Gendt K, et al. Spermatogenesis and sertoli cell activity in mice lacking sertoli cell receptors for follicle-stimulating hormone and androgen. *Endocrinology* 2008;149:3279-85.
28. Hudecek M, Sommermeyer D, Kosasih PL, Silva-Benedict A, Liu L, Rader C, et al. The nonsignaling extracellular spacer domain of chimeric antigen receptors is decisive for *in vivo* antitumor activity. *Cancer Immunol Res* 2015;3:125-35.