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Follicle-stimulating hormone receptor as a target in the redirected T-cell therapy for cancer

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Abstract

Adoptive transfer of T-cells engineered to express chimeric immunoreceptors is an effective strategy to treat hematological cancers, however, such 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, follicle stimulating hormone (FSH), fused to T-cell transmembrane 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 a MHC-non-restricted 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-redirectioned T-cells. Our experimental observations show for the first time that FSHR is a promising immunotherapeutic target for ovarian cancer and supports further exploration of FSHR-targeted immune therapy approaches for cancer patients.

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

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Authorship

Contribution: K.U. and D.J.P. designed the experiments and wrote the manuscript. K.U. performed experiments and analyzed data. M.P. performed in vivo assays. C.S. performed ELISA and assisted in vector construction.

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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 where gonadotropin levels are elevated, such as in post-menopausal 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 biological significance of its expression in tumor blood vessels remains unknown, but FSHR expression level correlates with the response to anti-angiogenic therapies and suggests that anti-FSHR therapy could be utilized 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 utilizing CAR T-cells specific for CD19 has been shown to induce durable remissions in hematological 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 being 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+ B lymphocytes (10), and transfer of ERBB2 CAR T-cells following lymphodepleting chemotherapy was associated with rapid-onset respiratory arrest and death presumably via 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 (14,15) and gonadal tissues (1), relative to that found in adult somatic tissues (16), 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 the 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 (IRs), specific for human and mouse FSHR, were created utilizing peptides derived from the natural FSHR ligand, FSH, for redirected T cell activity. Herein, we report the results of in vitro testing of FSHR-redirectioned 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 future development of FSHR-targeted immunotherapies.

Materials and Methods

Anti-FSHR immunoreceptor (FSHR-IR) construction

Segments of FSH peptides including flanked 3'-Bam-H1, 5'-Nhe-1 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 α 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 (17). 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 48h post-transfection.

Lymphocytes

Primary human CD4⁺ and CD8⁺ T-cells isolated from healthy volunteer donors were purchased from the Human Immunology Core at University of Pennsylvania, activated and transduced with lentiviral vectors as described (18).

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 ATCC. The murine mesothelioma cell line, AE17, and 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 (18).

Flow cytometric analysis

APC-Cy7 Mouse Anti-Human-CD3; FITC-anti-human-CD4; APC-anti-human-CD8; PE-human-CD45; 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. 7AAD (Biolegend) was used to assess viability. For *in vivo* T-cell quantification, 50 μ 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 per manufacturer's instructions. Flow cytometry data were analyzed using FlowJo software.

FSHR - PCR

Total RNA was extracted from 5×10^6 viable tumor cells using RNeasy Mini kit (Qiagen). RNA quantity and quality were verified using a NanoDrop 2000 spectrophotometer (Thermo). cDNA was generated from 1 μ 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'-GCTCATCTAGTTGGGTTCCATT-3' (GeneID: 2492), mouse-FSHR 5'-GGGATCTGGATGTCATCACT-3' and 5'-GGAGAACACATCTGCCTCTA-3' (GeneID: 14309).

Cytokine release assays and intracellular cytokine staining (CBA)

Cytokine release assays were performed by co-culture of 1×10^5 FSHR-IR T-cells with FSHR-expressing CaOV3 and FSHR-negative 293T cells, or mouse FSHR-expressing ID8, as described previously (18). After 16h, co-culture supernatants were assayed for presence of cytokines using an ELISA Kit (Biolegend) and Cytokine Bead Array (BD Biosciences) according to manufacturer's instructions.

Cytotoxicity

fLuc-transduced targets were plated at 1×10^4 /well in triplicate. T-cells were added at the indicated effector:target (E:T) ratios. Co-cultures were incubated overnight in phenol-free CM. 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/ γ -chain $^{-/-}$ (NSG) mice were bred, treated, and maintained under pathogen-free conditions in-house under University of Pennsylvania IACUC-approved protocols. Six to twelve week old female mice were purchased from the University of Pennsylvania Stem Cell and Xenograft Core and 5×10^6 CaOV3-fLuc tumor cells were inoculated subcutaneously (5 mice/group). Twenty and 25 days later, mice were injected intravenously with 6×10^6 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 greatest longitudinal diameter and width is greatest transverse diameter.

Statistical analysis

Student's *t*-test was used to evaluate differences in T-cells specific cytotoxicity and cytokine secretion. GraphPad Prism 4.0 (GraphPad Software) was used for the statistical calculations. $P < 0.05$ was considered significant.

Results/Discussion

Generation of the anti-FSHR immunoreceptors

Chimeric immunoreceptor was constructed for the redirection of primary human T-cells against FSHR-expressing targets. Follicle-stimulating hormone (FSH), the natural ligand for FSHR, was selected as the binding moiety for the immunoreceptor. FSH is a heterodimeric glycoprotein gonadotropin, consisting of α and β subunits, and several peptides derived from either the FSH beta subunit alone or mixed β/α subunits are reported to specifically bind to FSHR, as high affinity FSHR agonists or antagonists (18). Based on 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 β chain (19–21). In particular, FSH β 33–53 peptide appears functional when covalently attached to nanoparticles, providing high selectivity nanoparticle delivery to FSHR-expressing ovarian tumors (22). FSH peptides were cloned into previously validated lentiviral constructs with intracellular CD3 ζ domain alone or with the CD28 costimulatory signaling domain in tandem (18), and referred to as anti-FSHR-IR-z and anti-FSHR-IR-28z (Figure 1A). We reproducibly achieved a high transduction efficiency of primary human T-cells, as measured by co-expression of GFP (65–80%; Figure 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 while OVCAR3, SKOV3 and 293T (Figure 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 utilizing RT-PCR primers (Figure 1D). The 18S was used as an internal control (Supplementary Figure 1). To evaluate the FSHR-specific response of anti-FSHR-IR T-cells, the panel of anti-FSHR-IR T-cells was co-cultured with FSHR-positive or FSHR-negative cells overnight, and supernatants were subsequently assayed for the secretion of the cytokine, IFN γ . Anti-FSHR-IR-28z comprised of extracellularly expressed peptide 33–53 β , agonist A and antagonist A produced the highest levels of IFN γ following a co-culture with FSHR-expressing CaOV3, but not against FSHR-negative 293T cells (Figure 2A). Anti-FSHR 81–95 β IR-28z and anti-FSHR a+b IR-28z (alpha chain + beta chain of FSH) produced much lower levels of IFN γ against FSHR $^{+}$ targets, associated with predicted lower affinity of those constructs to FSHR. First generation anti-FSHR IR-z T-cells also secreted IFN γ in a similar pattern (data not shown). Similarly, when tested for immunoreactivity against the murine FSHR-expressing cell line ID8, anti-FSHR 33–53 β -28z, anti-FSHR 81–95 β -28z and anti-FSHR agonist A-28z IR T-cells all exhibited moderate and specific immune-recognition (Figure 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 (23). 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 immobilized recombinant human FSHR protein commercially produced from *e. coli* (Figure 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 (24).

Anti-FSHR 33-53 β -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-IR T cells that encounter FSHR antigen release Th1-type cytokines other than IFN γ , as has been reported for conventional scFv-based CARs (18). As shown in Figure 3A, both anti-FSHR 33-53 β -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 utilizing CARs with a CD28 co-stimulatory domain (25,26). IFN γ 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 anti-human 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 impact CD69 level, confirming antigen specific reactivity against both mouse and human FSHR⁺ cell lines. Control GFP and anti-FSHR 51-65 β -28z T-cells did not respond to either cell line (Figure 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*

In order to evaluate the lytic proficiency of anti-FSHR-IR T-cells, we first engineered FSHR-positive and FSHR-negative target cell lines to constitutively express firefly luciferase. Following overnight co-culture of target cells with gene-engineered T-cells, percent of a specific lysis was calculated based upon residual luciferase signal. Both anti-FSHR 33-53 β -28z and anti-FSHR agonist A-28z IR T-cells showed dose-dependent lysis of FSHR⁺ targets (Figure 3C). As observed in IFN γ release assays, anti-FSHR-IR T-cells also exhibited specific lytic activity against the mouse FSHR⁺ ID8 cell line.

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

To assess the anti-tumor effect of anti-FSHR T-cells *in vivo*, we established human ovarian cancer xenografts in NOD/SCID/IL2R $\gamma^{-/-}$ (NSG) mice by subcutaneous flank injection of CaOV3 cancer cell line and then administered two intravenous injections of gene-engineered T-cells at days 20 and 25, similar to our previous studies (17,26). Consistent with their *in vitro* function, both anti-FSHR 33-53 β -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 (Figure 4A). Because objective clinical response to T-cell transfer therapy is often associated with the persistence of T-cells after infusion (27), we measured peripheral blood for the continued persistence of engineered human T-cells *in vivo* via TruCount bead-based counting (Figure 4B). Consistent with the anti-tumor response, mice treated with anti-FSHR 33-53 β -28z or anti-FSHR agonist A-28z IR T-cells had increased peripheral blood CD3⁺ T-cell counts compared to controls at three and five weeks post-T-cell infusion, though 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, 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 (28), suggesting possible toxicities may be restricted to reproductive organs. However, in the scenario of a patient diagnosed with ovarian cancer, these organs are virtually non-essential and often surgically removed. In contrast, male FSHRKO mice exhibit reduced numbers of spermatocytes, and would suggest possible targeted toxicity against testis, an immune privileged organ (29). 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. Further, 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 (30), 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 to simultaneous expression on both tumor cells and its associated vasculature. This is of a special interest given is potential for application in not only ovarian cancer but across many solid cancer types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Simoni M, Gromoll J, Nieschlag E. The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. *Endocrine reviews*. 1997; 18(6):739–73. [PubMed: 9408742]
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(1):47–53. [PubMed: 8546225]
3. Riman T, Persson I, Nilsson S. Hormonal aspects of epithelial ovarian cancer: review of epidemiological evidence. *Clinical endocrinology*. 1998; 49(6):695–707. [PubMed: 10209555]
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 mullerian systems. *Int J Cancer*. 2003; 103(3): 328–34. [PubMed: 12471615]
5. Choi JH, Choi KC, Auersperg N, Leung PC. Overexpression of follicle-stimulating hormone receptor activates oncogenic pathways in preneoplastic ovarian surface epithelial cells. *The Journal of clinical endocrinology and metabolism*. 2004; 89(11):5508–16. [PubMed: 15531506]
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. *Molecular and cellular endocrinology*. 2001; 172(1–2):213–22. [PubMed: 11165055]
7. Gartrell BA, Tsao CK, Galsky MD. The follicle-stimulating hormone receptor: a novel target in genitourinary malignancies. *Urol Oncol*. 2013; 31(8):1403–7. [PubMed: 22513137]
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(17):1621–30. [PubMed: 20961245]
9. Zheng W, Lu JJ, Luo F, Zheng Y, Feng Y, Felix JC, et al. Ovarian epithelial tumor growth promotion by follicle-stimulating hormone and inhibition of the effect by luteinizing hormone. *Gynecologic oncology*. 2000; 76(1):80–8. [PubMed: 10620446]
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(16):1507–17. [PubMed: 25317870]
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(4):843–51. [PubMed: 20179677]
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. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*. 2011; 29(7):917–24. [PubMed: 21282551]
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 research*. 2001; 61(18):6768–76. [PubMed: 11559549]
14. 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*. 363(17):1621–30. [PubMed: 20961245]
15. Gartrell BA, Tsao CK, Galsky MD. The follicle-stimulating hormone receptor: A novel target in genitourinary malignancies. *Urol Oncol*.
16. 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*. 394(1):12–7. [PubMed: 20171950]
17. 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- redirected T cells results in tumor localization and eradication. *Cancer Res*. 2011

18. Song DG, Ye Q, Poussin M, Harms GM, Figini M, Powell DJ Jr. CD27 costimulation augments the survival and antitumor activity of redirected human T cells in vivo. *Blood*. 2012; 119(3):696–706. [PubMed: 22117050]
19. Santa Coloma TA, Dattatreyaumurthy 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(5):1194–200. [PubMed: 2108722]
20. 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. *Clinical breast cancer*. 2003; 4(3):212–7. [PubMed: 14499016]
21. 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). *Molecular and cellular endocrinology*. 1993; 97(1–2):173–81. [PubMed: 8143901]
22. 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 research*. 2009; 69(16):6506–14. [PubMed: 19638590]
23. 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. *Biology of reproduction*. 1999; 60(6):1515–27. [PubMed: 10330114]
24. 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. *Proceedings of the National Academy of Sciences of the United States of America*. 2012; 109(31):12491–6. [PubMed: 22802634]
25. Carpenito C, Milone MC, Hassan R, Simonet JC, Lakhil 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
26. 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(3):633–43. [PubMed: 22127019]
27. 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(12):7125–30. [PubMed: 15585832]
28. 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. *Proceedings of the National Academy of Sciences of the United States of America*. 1998; 95(23):13612–7. [PubMed: 9811848]
29. 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(7):3279–85. [PubMed: 18403489]
30. 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(2):125–35. [PubMed: 25212991]

Figure 1. Anti-FSHR Immunoreceptor 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. GFP, green fluorescent protein; 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 (grey). 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 (H2O). RT-PCR using FSHR primers based on human sequence (NM_0) amplified the predicted 234bp product from cDNA templates. Fivefold less PCR-amplified cDNA from CaOV434 cells was loaded relative to other lanes to avoid spill over.

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 γ in supernatants following overnight co-culture of anti-FSHR-IR T-cells with FSHR-positive CaOV3 or FSHR-negative 293T target cells detected by ELISA. Co-cultures were established at 1:1 E:T ratio. **B.** IFN γ in overnight co-cultures of anti-FSHR-IR T-cells with mouse FSHR-positive ID8 cells or FSHR-negative AE17 mesothelioma cell lines. **C.** Lack of immune recognition of human recombinant FSHR protein by anti-FSHR-IR T-cells. IFN γ , interferon gamma; E:T, Effector to Target. Results are presented as mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student's T-test).

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 redirected T-cells and control GFP transduced primary human T-cells. IFN γ , IL2, MIP1a, TNF α , IL4 and IL10 secretion was detected by CBA (Cytokine bead-based immunoassay) 16hr after following tumor stimulation (Data represents 3 independent experiments in triplicates). Results are presented as mean $\pm$ SD. Values of $P < 0.01$, $P < 0.05$ were considered statistically significant. We observed a specific and statistically significant production of cytokines; IFN γ , IL 2, MIP1a and TNF α by anti-FSHR 33-53b-28z and anti-FSHR agonist A-28z against FSHR positive targets human CaOV3 and mouse ID8 cell lines when compared to cytokine levels in co-cultures with FSHR negative targets human 293T and mouse AE17 cell lines. **B.** Anti-FSHR+ T-cells upregulate surface CD69 expression upon 24hr exposure to FSHR expressing targets. The FSHR+IR T-cells are identified by GFP expression. Graph represents the 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 β -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 16hr luciferase-based killing assay. T-cells were co-cultured with target cells expressing firefly luciferase at E:T ratios of 0:1, 1:1, or 3:1. Residual luciferase signal was determined after 16hrs. Percent lysis was determined by luminescence comparison to untreated target wells. Results are presented as mean $\pm$ SD. Values of $P < 0.05$ (*) were considered statistically significant.

Figure 4. Suppressed ovarian cancer growth in mice receiving Anti-FSHR-IR T-cell treatment
A. 5×10^6 luciferase-expressing CaOV3 cells were injected s.c. into NSG mice on d0. 5×10^6 T-cells were injected IV on days 20 and 25. Tumor growth was monitored by caliper measurement. Graphs represent mean $\pm$ SEM of five mice per experiment. P values were calculated compared to GFP-T-cells and PBS treated control mice. Values of $P < 0.05$ (*) were considered statistically significant. * indicates $P < 0.05$; ** indicates $P < 0.01$. **B.** Preferential expansion and survival of human T-cells in peripheral blood of anti-FSHR-IR T-cell-treated mice, compared to 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+ T-cells was quantified by flow cytometry using TruCount beads and reported as total cells/uL of blood. Bar graphs represent mean $\pm$ SD for five mice per group. P values were determined compared to control GFP T-cell treated group. * indicates $P < 0.05$.

Table 1
Follicle stimulating hormone peptides used for anti-FHSR immune receptor construction

Construct name*	FSH-derived peptides	Sequence	Predicted affinity	Predicted cross-reactivity
anti-FSHR 33-53p-IR	β chain 33-53aa	YTRDLVYKDPARPKIQKTCIF	10 ⁻⁵ M	Human/Mouse
anti-FSHR 51-65p-IR	β chain 51-65aa	CTFKELVYETVRVPGC	10 ⁻⁴ M	Human
anti-FSHR 81-95p-IR	β chain 81-95aa	QCHCGKCDSDSTDCT	10 ⁻⁵ M	Human/Mouse
anti-FSHR antagonist A-IR	β chain (87-94aa)+α chain (25-42aa)	CDSDDTDCILQCMGCCFSRAYPTPLR	10 ⁻⁸ M	Human/Mouse
anti-FSHR agonist A-IR	β Chain (87-94aa)+α chain (25-42aa)+b(27-45aa)	CDSDDTDCILQCMGCCFSRAYPTPLRWCAGYCYCYT RD VKDPPARP	10 ⁻⁷ M	Human/Mouse
anti-FSHR a+b-IR	FSH (α chain + β chain)	GenBank Gene IDs: 1081 (follicle stimulating hormone, alpha subunit) + 2488 (follicle stimulating hormone, beta subunit)	NA	Human/Mouse

* Final chimeric immune receptor constructs were engineered to encode for either an intracellular CD3z (-z) or a tandem CD3z and CD28 (-28z) domain.