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**TITLE:** Development of Novel Molecularly Targeted Therapy to Secreted Frizzled-Related Protein 2 for Breast Cancer

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**14. ABSTRACT** Most antiangiogenic drugs evaluated in breast cancer clinical trials inhibit angiogenesis by targeting the VEGF pathway. VEGF is a driver of tumor angiogenesis in breast cancer, however modest or negative phase III clinical results suggest further targets, pathways, or factors play a significant role. Furstenburger et al. evaluated VEGF expression in primary breast cancers from patients and adjacent normal breast tissue and found no increase in VEGF levels. We hypothesized that pro-angiogenesis factors other than VEGF are drivers of human breast cancer angiogenesis. To identify these pro-angiogenesis factors, we developed a novel method of immuno-laser capture microdissection coupled with RNA amplification and genome-wide gene expression to profile tumor vasculature cells from human breast tumors with comparison to normal breast samples. In our analysis we identified that secreted frizzle-related protein 2 (SFRP2) mRNA levels were increased more than 6-fold in breast cancer endothelium compared to normal vessels from benign breast tissue, and as shown by immunohistochemistry 85% of breast tumors showed intense staining for SFRP2 in the neovasculature. Importantly, SFRP2 was highly expressed in the vasculature of luminal, Her2/neu, and basal tumors. Interestingly, VEGF was expressed at the same level in both tumor and benign endothelium, suggesting again that VEGF might not be a major driver of breast tumor angiogenesis. We subsequently showed that SFRP2 induces angiogenesis in vitro and in vivo, and that antagonism of SFRP2 with a monoclonal antibody inhibits triple negative breast carcinoma and angiosarcoma growth in mice. We further identified that the angiogenic activity of SFRP2 is mediated by activating the non-canonical Wnt calcineurin/ nuclear factor of activated T-cells c3 (NFATc3) pathway. NFAT is a transcription factor that plays a critical role in mediating angiogenic responses. We have generated a humanized SFRP2 mAb that is nonimmunogenic and efficacious against triple negative breast cancer.

**15. SUBJECT TERMS**
WNT, breast cancer, metaplastic breast cancer, angiogenesis, NFAT, immunotherapy

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1. **INTRODUCTION:** The purpose of this research is to establish a novel therapy for metastatic breast cancer and determine its mechanism of action. We have humanized a monoclonal antibody to secreted frizzled related protein 2 (SFRP2) and will determine the pharmacokinetic, pharmacodynamic, efficacy and toxicity profile in breast cancer. We will determine mechanism of action in the Wnt-signaling pathway with molecular imaging *in vivo* and will establish if there is increased efficacy in combination with other therapies.

*2.* **KEYWORDS:** WNT, breast cancer, metaplastic breast cancer, angiogenesis, NFAT, immunotherapy.

3. **ACCOMPLISHMENTS: What were the major goals of the project?**

**Major Task 1:** Determine PK, and PD of the hSFRP2 mAb in immunocompetent BALB/CJ mice. (Months 1-6) **This task is complete (DEMORE and BROOME/ ROSENZWEIG)**

**Major Task 2:** Determine the efficacy of hSFRP2 mAb in triple negative 4T1 breast cancer cells in BALB/CJ mice, human triple negative cells in nude mice (MDA-MB-231 and HS578t), and a triple negative PDX breast cancer (TM00089) *in vivo*. Will test multiple doses in 4T1 and MDA-MB-231 (2, 4 ,10 and 20 mg/kg) (6-15 months) **This task is 90% completed. (DEMORE and BROOME/ ROSENZWEIG)**

**Major Task 3:** Determine the effects of the hSFRP2 mAb on host and tumor immune system in 4T1 cells in BALB/CJ mice. (Months 12-18) **This task is 100% complete, modified for another tumor type since 4T1 was not sensitive to hSFRP2 mAb (DEMORE)**

**Major Task 4:** Show that hSFRP2 mAb reduces NFAT-dependent transcriptional activity in 4T1 and HS578t cells *in vivo* using molecular imaging (Months 18-26) **Not started**

**Major Task 4:** Show whether the hSFRP2 mAb is additive or synergistic with agents that have a a similar mechanism of action: Drugs that inhibit NFAT activation (bevacizumab) and drugs that inhibit PD-1. Show whether the hSFRP2 mAb is additive or synergistic with agents that have a different mechanism of action: SFRP2 has no effect on proliferation(24), we will evaluate for synergy with anti-mitotic drugs. (months 24-36). **This task is 25% complete (DEMORE)**

**Major Task 5:** Show whether the hSFRP2 mAb is effective in doxorubicin-resistant breast cancer. (Months 24-36) We are in the process of culturing MDA-MB-231 cells with doxorubicin to create a dox-resistant cell line. **This task is 75% complete (DeMore)**

**What was accomplished under these goals?**

1) Major activities were

a.) Determine the pharmacodynamics of the hSFRP2 mAb in tumor bearing mice and non-tumor b earing mice

c). Determine the effect of hSFRP2 mAb on T-cells in vitro

d) Determine the effect of hSFRP2 mAb on T-cells in vivo

D) Develop a doxorubicin-resistant breast cancer cell line.

2) Specific objectives:

A) Determine the Pharmacodynamics of the hSFRP2 mAb in mice.

B) Determine the efficacy of the hSFRP2 mAb in triple negative breast cancer in vitro and in vivo.

C) Show that the hSFRP2 mAb activates NFATc3 on T-cells, and effects on downstream cytokines

D) Evaluate whether the SFRP2mAb is efficacious in breast cancer cell line resistant to doxorubicin.

3) Significant results. Note: In the section below we also tested the antibody on osteosarcoma GEMM cells. This data is included, although DOD funding was NOT USED for this experiment, and this was done under a separate IACUC protocol.

*Major Task 1) Biodistribution of hSFRP2 mAb in vivo.* The PK study is published (Garcia et al. Annals of Surg Onc). The following results for biodistribution will be submitted for publication this month. We assessed the uptake and clearance patterns of fluorophore-conjugated SFRP2 (**Fig. 1**) compared to that of fluorophore-conjugated IgG (**Fig. 2**) after tail vein injection into breast cancer model mice and control mice, i.e., naïve mice containing no tumor. We first acquired a longitudinal (in time) series of images at the following time points: just prior to injection of the antibodies (to

obtain a baseline signal with no fluorophore signal); immediately after the injection of the antibodies; and then 24, 48, 72, and 96 hours after the injection of antibodies.

Prior to injecting the fluorophore-tagged antibodies, we imaged the fluorophore-conjugated antibodies in solution in a non-fluorescent vial to quantify the total injected fluorophore and to measure a clean fluorescence spectra. For the analysis of the *in vivo* fluorescence imaging, we first determined the component spectra for the fluorophore-tagged antibodies and for the mouse prior to antibody injection. We used spectral decomposition to decompose each image at each longitudinal time point into its component images and then recorded image-intensity-scaled fluorophore component images for each mouse at each longitudinal time point. As seen **in Fig. 1** (top panels), fluorophore-conjugated SFRP2 accumulated within the orthotopically implanted tumor within 24 hours post injection, with minimal uptake in normal organ of the control mouse. Signal intensity was retained over the 96 hours period. As expected, there was some minor uptake into the liver. Unbound SFRP2 (**Fig. 1** (bottom panels)) was quickly excreted through the urinary bladder over a 72 hours period. In naïve control mice, SFRP2 rapidly cleared through the urinary bladder over a 72 hours period.

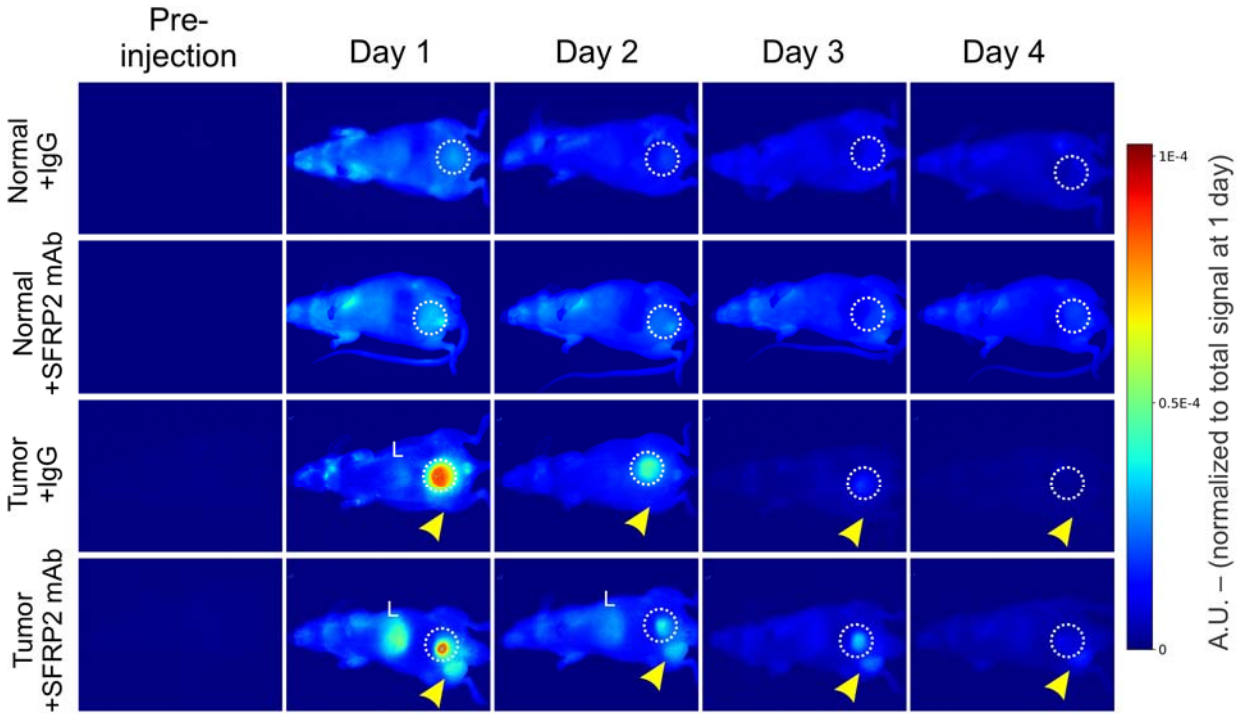



**Fig. 1**. Longitudinal live fluorescent imaging of mice orthotopically implanted with MDA-MB-231 cells (top two panels) or without tumors (bottom panels) injected with fluorophore-labeled SFRP2 mAb over a period of 96 hours. Representative imaging hotmaps analyzed on the same scale of the mice are shown (N=3 for each). In mice with tumor (bottom two panels), SFRP2 mAb is taken up in the tumor and liver and unbound mAb is excreted through the urinary bladder. Mice without tumors do not accumulate SFRP2 mAb in the tumor or the organs and the mAb is excreted through the bladder. Yellow arrows point to site of tumor, circles are around urinary bladder.

Upon completion of the longitudinal *in vivo* fluorescence imaging, we injected each mouse a second time with fluorescing antibodies. Seventy-two hours after the second injection, we euthanized the mice and harvested the organs and tumor. We acquired additional fluorescence images of the harvested organs from each mouse. For the analysis of the organ and tumor images, we used the component spectra for the fluorophore-tagged antibodies and determined the component spectra for each organ from a mouse that had not been injected with the fluorophore-tagged antibodies. We used spectral decomposition to decompose each image into its component images and then recorded image-intensity-scaled fluorophore component images for each organ and tumor **(Fig. 2).**

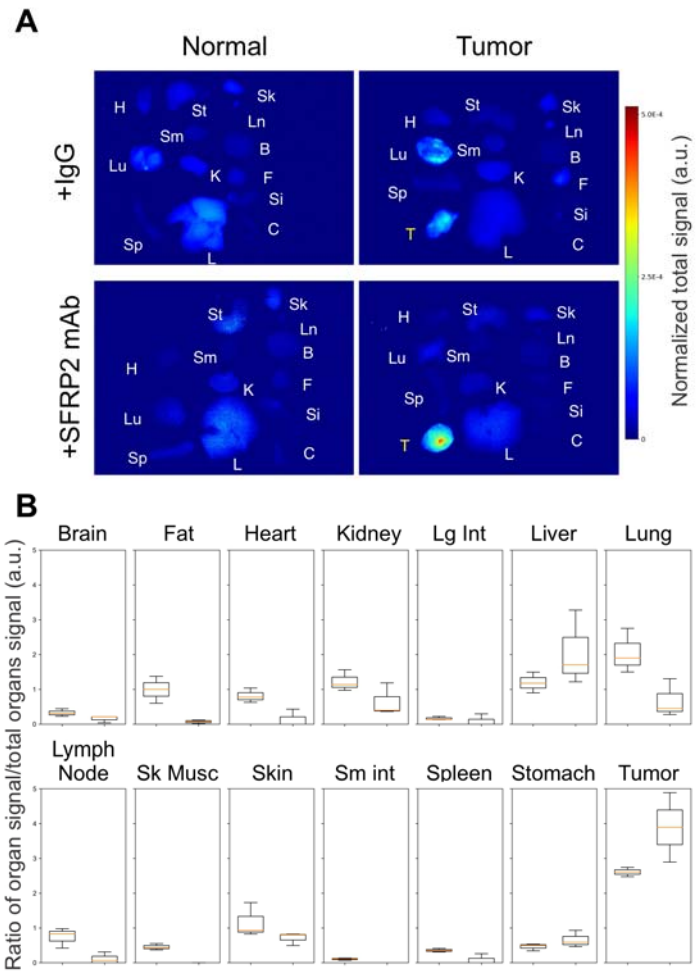



**Fig. 2.** Fluorescent imaging of organs from mice treated with IgG control and hSFRP2 mAb. When compared to IgG1-control, hSFRP2 had significantly lower fluorescence in multiple non-specific organs, which included fat ($p=0.015$), heart ($p=0.028$), lung ($p=0.048$), lymph node ($p=0.033$), skeletal muscle ($p<0.01$), and small intestine ($p<0.001$).

*Major Task 2) Determine the efficacy of hSFRP2 mAb in triple negative 4T1 breast cancer cells in BALB/CJ mice, human triple negative cells in nude mice (MDA-MB-231 and HS578t), and a triple negative PDX breast cancer (TM00089) in vivo.* The efficacy of hSFRP2 mAb in HS578t has been published (Garia et al, Annals of Surg Onc). Twenty six mice were injected with 5 million MDA-MB-231 cells in a 100 ul suspension of 50% HBSS and 50% basement membrane phenol red-free HC Matrigel in the right mammary fat pad. Tumor size was measured every three days with calipers and volumes calculated using the formula $(L \times W^2)/2$. Treatment was initiated once tumors approached 50-100 $mm^3$ in volume at day 19. Mice were randomly distributed into either the IgG1 control treatment group (n=11) or the hSFRP2 mAb treatment group (n=11). In accordance with previous MTD and PK studies, hSFRP2 mAb treatment was administered iv every 3 days and IgG1 was administered iv weekly. Both treatments were delivered via tail vein injections at 4 mg/kg iv until control tumors reached the end point of 2cm in diameter. Animal weights were recorded weekly. Treatment concluded at 78 days when control tumors reached a maximum dimension of 2 cm. Over the course of treatment, one IgG1-control treated mouse expired early due to ascites. This mouse had no primary tumor on autopsy and was excluded from the study. At the conclusion of the study, one mouse from each treatment group was also excluded due to no tumor present on autopsy. The mean tumor volume at the end of the experiment was 2,998 $mm^3$ (n=9, 95% CI 2,619-3,376 $mm^3$) in the IgG1 control group and 1,159 $mm^3$ (n=10, 95% CI 800-1,519 $mm^3$) in the hSFRP2 group. The hSFRP2 mAb-treated group had a 61% reduction in tumor volume, which was a significant reduction in tumor volume compared to the IgG1 control group ($p<0.001$, Figure 3A). There was no significant weight loss, hair loss, or lethargy in any of the mice over the course of treatment (Figure 3B). This suggests that the treatment had no toxic effect on the animals. For 4T1 cells we had a difficult time getting consistent results in the controls for tumor metastases so results were in conclusive.

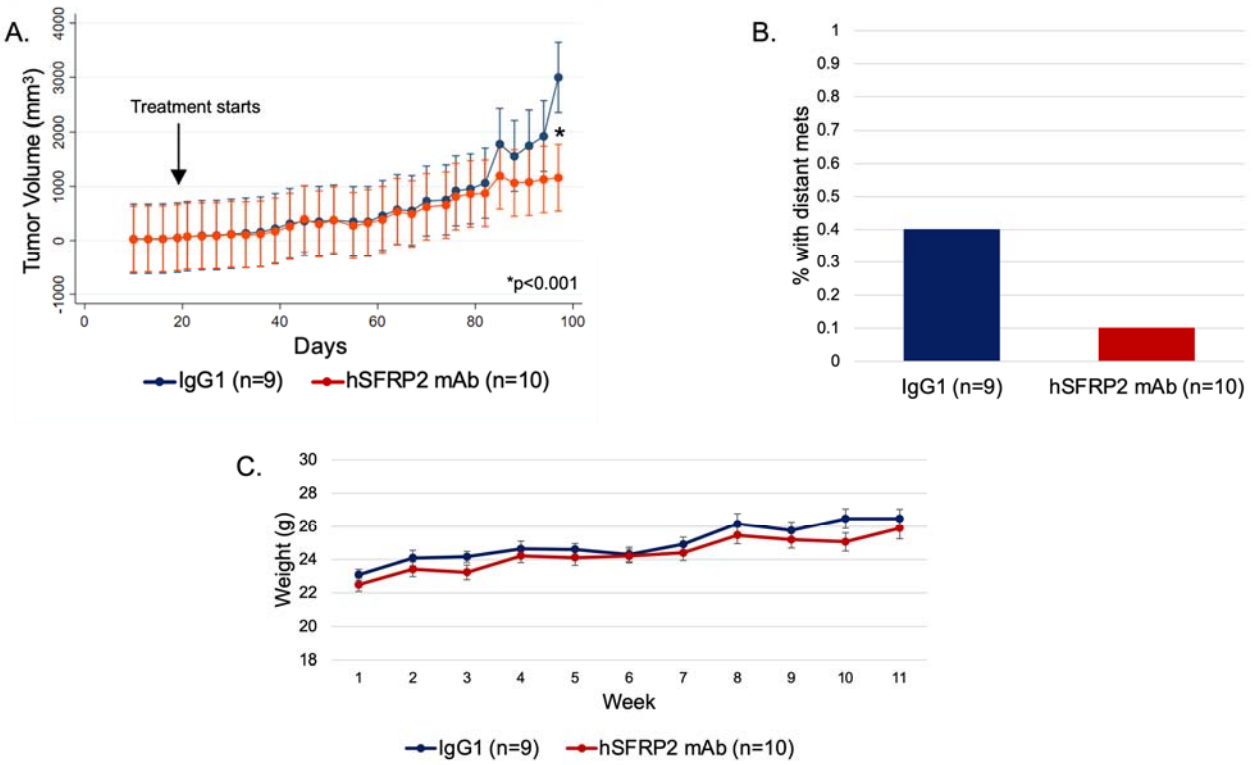



**Fig. 3:** hSFRP2 mAb inhibits tumor growth *in vivo*. (A) MDA-MB-231 cells were injected in the mammary fat pad of nude mice. Treatment of either hSFRP2 mAb 4 i.v. mg/kg (n=10) or IgG1 control i.v. 4 mg/kg (n=9) was started 19 days after tumor injection and continued until day 97. Tumors were measured every three days with volumes calculated by the formula $LxW^2/2$. At day 97, there was a significant reduction in tumor volume in the hSFRP2 mAb treated group (*p<0.001). (B) At autopsy, four IgG1 control-treated mice (40%) had distant metastases and one hSFRP2-treated mouse had a distant metastasis (10%). (C) Weights were measured weekly in all mice. There was no significant difference in weights between the two treatment groups throughout treatment.

*KPB GEMM triple negative cells*: We obtained the KPB GEMM triple negative breast cancer cell line from Dr. Chuck Perou from UNC. We chose this cell line because it grows in immunocompromised mice, so we can use it to study combination therapy with immunotherapy. Also, Dr. Perou's data shows that this cell line is particularly resistant to immunotherapy, therefore if we see synergy with immunotherapy this would be a very significant finding. Our first studies were to characterize whether the KPB cell line has SFRP2, FZD5, and PD-L1 protein. For comparison, we tested against two osteosarcoma GEMM cell lines (RF420 and RF577). Cells were lysed, protein was extracted and subjected to Western blot with antibodies to SFRP2, FZD5, PD-L1, and actin. This shows that the KPB breast cancer cell line expresses PDL-1, SFRP2 and FZD5 (**Fig. 4**)

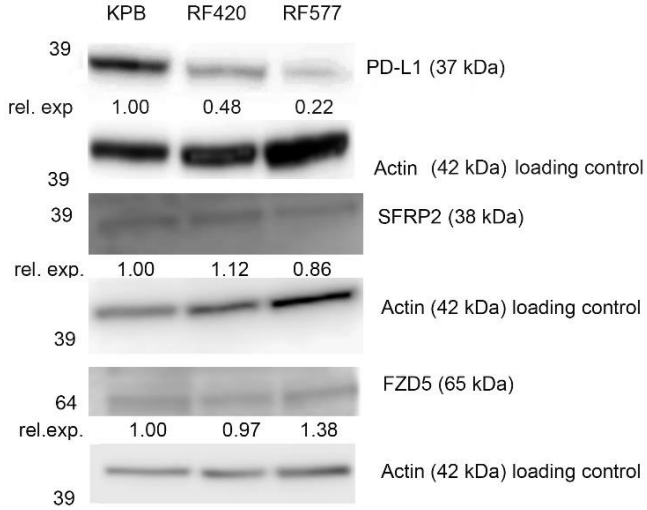



**Fig. 4**. Western blot using cell lysates from KPB breast cancer cells, and RF420 and RF577 osteosarcoma cells. KPB has higher protein levels of PD-L1, with RF577 having the lowest level. All three cell types have SFRP2 and FZD5 protein.

Next, we evaluated whether KPB cells are sensitive to the hSFRP2 mAb in vitro. KPB cells were seeded at 15,000 cells / well in 96 well plate and allowed to settle overnight. The next day cells were treated with 10uM IgG control or 10uM hSFRP2 mAb. After 1 hour of treatment the cells were labeled using reagents from the PromoCell Apoptotic/Necrotic cells detection kit (#PK-CA707-30017), according the manufactures protocol. Images were acquired using the EVOS FLc digital imaging system and counted using ImageJ. N=12 per group. This showed an increase in apoptosis in hSFRP2 mAb treated cells (n=12, IgG= 4.93% ± 0.19%, hsFRP2 (mAb) = 43.54%± 0.16%, p<0.0001, **Fig. 5**)

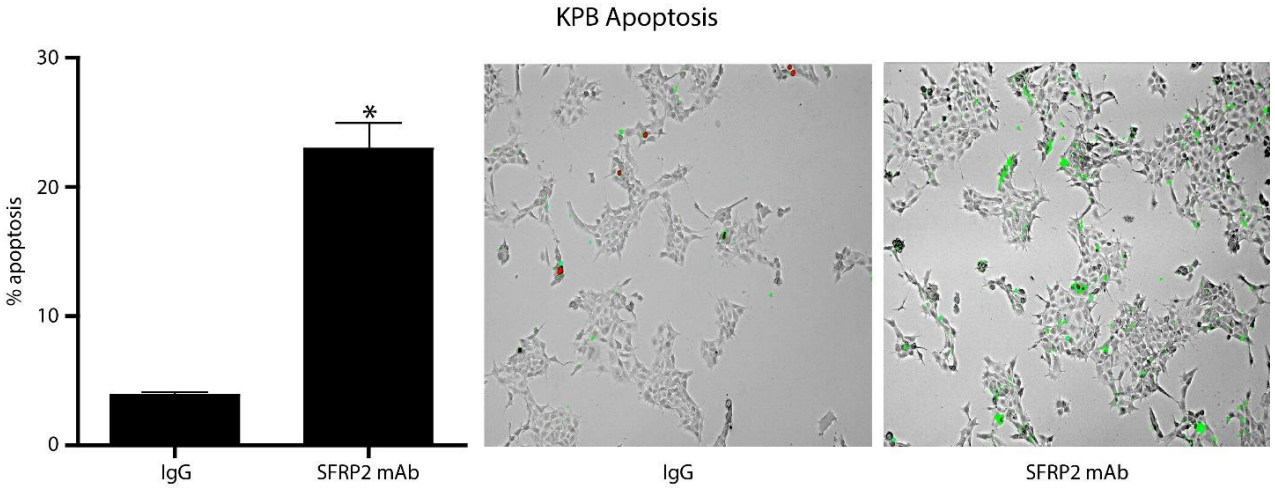
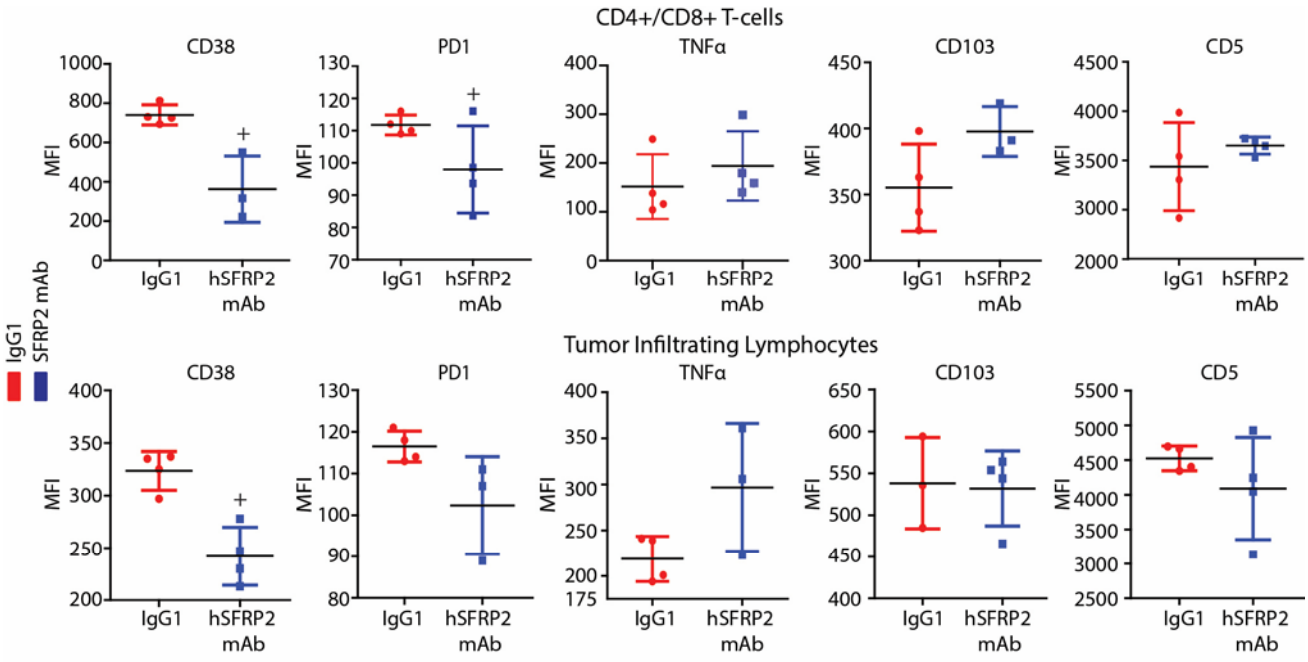



**Fig. 5**. hSFRP2 mAb increases apoptosis in vitro in KPB triple negative breast cancer cells *in vitro*. N=12 per group, p<0.0001).

*Major task 3)* Determine the effects of the hSFRP2 mAb on host and tumor immune system in 4T1 cells in BALB/CJ mice. Since we could not get the 4T1 model to work consistently, we looked at the effect of the hSFRP2 mAb on the immune system in another tumor model in an immune competent mouse.

C57BL6 mice were innuculated with RF420 cells and IGG control or hSFRP2 mAb treatment began on day 7 for 21 days. Mice were sacrificed and tumor infiltrating lymphocytes were extracted. Spleen were collected and CD4+ and CD8+ T-cells were isolated. Flow cytometry showed a reduction in CD38 in TILS and T-cells, and a reduction in PD-1 in TILs from mice treated with hSFRP2 mAb (FIG. 6). The reduction in CD38 was confirmed in T-cells by Western blot (Fig. 7).




| | IgG | CD38 mAb | p |
|---|---|---|---|
| Spleen | 728 (63) | 316 (328) | **0.03** |
| Lung | 330 (25) | 239 (41) | **0.02** |

| | IgG | PD1 mAb | p |
|---|---|---|---|
| Spleen | 111 (5) | 96 (19) | 0.19 |
| Lung | 116 (6) | 107 (22) | **0.03** |

| | IgG | TNF-α mAb | p |
|---|---|---|---|
| Spleen | 127 (84) | 169 (90) | 0.15 |
| Lung | 200 (43) | 306 (138 | 0.16 |

| | IgG | CD103 mAb | p |
|---|---|---|---|
| Spleen | 350 (51) | 391 (36) | 0.16 |
| Lung | 536 (109) | 549 (54) | 1.00 |

| | IgG | CD5 mAb | p |
|---|---|---|---|
| Spleen | 3424 (652) | 3675 (124) | 0.39 |
| Lung | 4534 (305) | 4148 (993) | 0.25 |

**Fig. 6. hSFRP2 mAb reduces T-cell CD38 and PD-1 protein in vivo.** Splenic CD4⁺ and CD8⁺ T-cells (*top*) and tumor infiltrating lymphocytes (TILs; *bottom*) isolated from RF420 tumor-bearing mice treated with IgG1 control (*red*) or hSFRP2 mAb (*blue*) for 21 days were stained for the following immune markers labeled with fluorochromes: CD38, PD-1, TNF-α, CD103, and CD5. Dot plot graphs show Mean fluorescent intensity (MFI) analyzed by FACS for each of the immune makers of interest within the CD4⁺/CD8⁺ and TIL populations. For IgG1 and hSFRP2 mAb treatments, the number of independent samples (n) was 4. CD38 was significantly decreased with hSFRP2 mAb in both splenocytes (+p<0.05) and TILs (+p<0.05). PD-1 was significantly decreased in CD8⁺ TILs treated with hSFRP2 mAb (+p<0.05). Bottom tables: Median MFIs (interquartile range) for each immune marker.

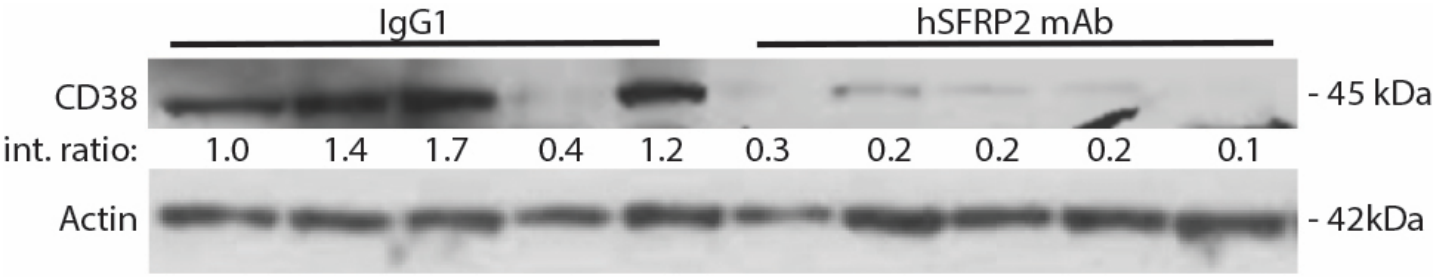



**Fig 7. Treatment with hSFRP2 mAb in mice with metastatic osteosarcoma decreases CD38 protein levels in splenocytes.** Splenocytes isolated from mice with RF577 OS lung metastases treated with IgG1 control (n=5) vs. hSFRP2 mAb (n=5) were lysed and prepared for Western blot analysis probing for CD38. *Int. ratio*: intensity ratios listed below CD38 Western blot were calculated after normalization to both actin and reference sample (indicated by 1.00). Overall, CD38 protein levels were reduced in splenocytes from mice treated with hSFRP2 mAb by 82%, compared to the control group (p=0.004).

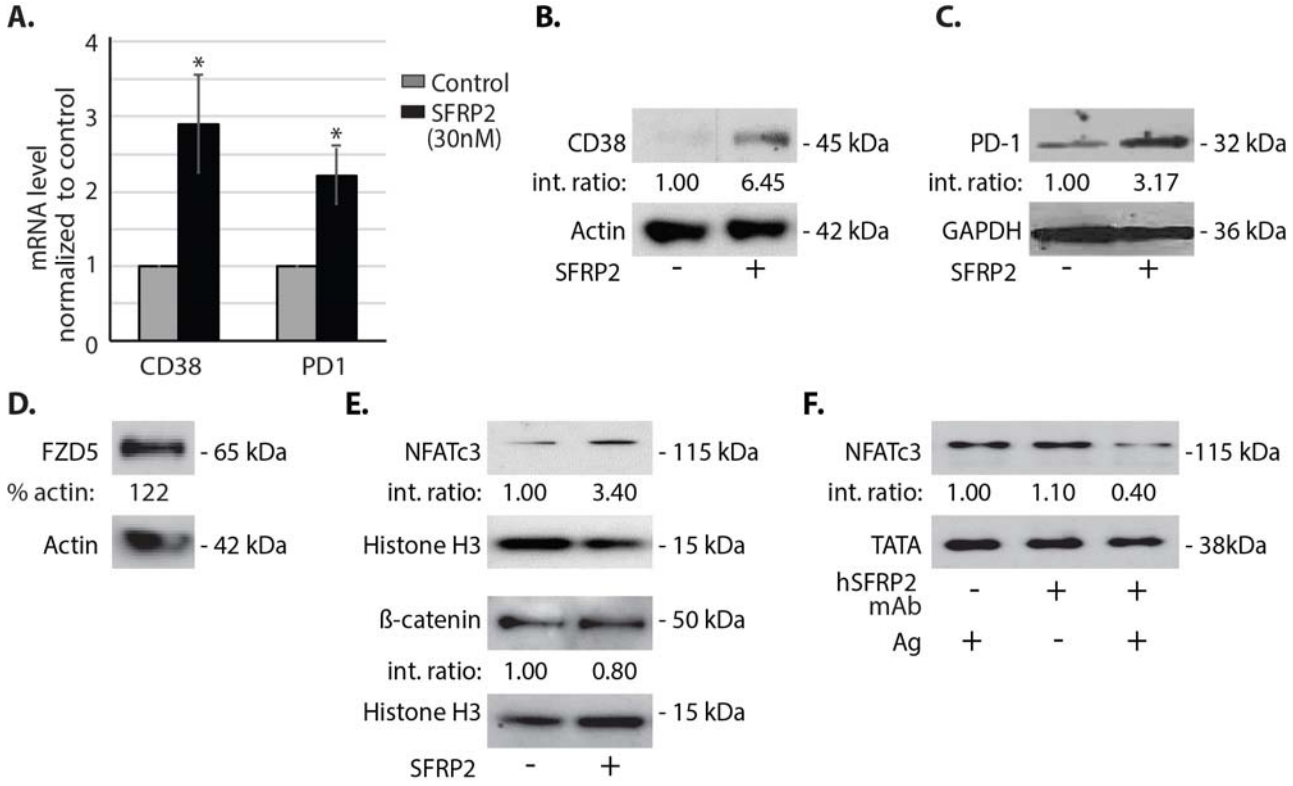



**Figure 8. SFRP2 increases CD38 and PD-1 mRNA and protein levels in T-cells.** (**A**) Splenic T-cells cultured in IL2-containing medium were treated with or without SFRP2 (30nM) for 1h and the mRNA levels for CD38 and PD-1 were measured by qRT-PCR (n=8). GAPDH was used as an internal control. (**B-F**) All T-cells were treated with IL-2. Samples were probed using Western blot with antibodies to the indicated protein markers. Actin was the loading control for cytoplasmic fraction; Histone H3 and TATA were loading controls for nuclear fractions. (**B, C, E, F**) *Int. ratio*: intensity ratios listed below Western blots for B-F were calculated after normalization to both loading control and reference samples (indicated by 1.00). (**B, C**) Cytoplasmic fractions of wild type splenic T-cells were isolated and protein levels of CD38 (**B**) and PD-1 (**C**) were increased in SFRP2-treated T-cells, compared to untreated cells. (**D**) Western blot probing for FZD5 protein in mouse splenic T-cells. % actin: total band intensity was normalized to actin. (**E**) Splenic T-cells were untreated or treated with recombinant SFRP2 protein for 1h. The nuclear fraction was isolated from the T-cells and nuclear NFATc3 and β-catenin protein levels were measured. Nuclear fractions demonstrated increased NFATc3 protein levels, but no change in β-catenin levels with SFRP2 treatment. (**F**) Splenic T-cells were activated with antigen gp100 (0.87μM) with or without 10μM of hSFRP2 mAb treatment. Nuclear fractions demonstrated decreased NFATc3 protein levels in hSFRP2 mAb-treated cells activated by antigen.

*hSFRP2 mAb inhibits CD38 in T cells and restores NAD+.* Since expression of CD38 was regulated by SFRP2, we evaluated whether hSFRP2 mAb treatment of T-cells *in vitro* inhibits CD38 and also restores NAD+ levels in TGFβ-exposed T-cells. TGFβ is a cytokine present in the tumor microenvironment that increases CD38 from T-cells. Treatment of T-cells with TGFβ resulted in an increase in SFRP2 by Western blot which was reversed with the hSFRP2 mAb (**Fig. 9A**). FACS analysis showed a statistically significant increase in CD38 + cells with the addition of TCR/TGFβ, which was significantly inhibited by the hSFRP2 mAb (**Fig. 9B**, N=3, $p<0.001$), Along with this there was a decrease in NAD+ concentration with TGFβ, which was increased with hSFRP2 treatment (**Fig. 9C**, N=3, $p=0.02$). PD-1 s a checkpoint inhibitor known to be regulated by NFAT. We treated T-cells with TGF-β, which resulted in and increase in the number PD-1 positive CD8 and CD4 cells, which was inhibited with the addition of the hsFRP2 mAb (**Fig. 9D, E**).

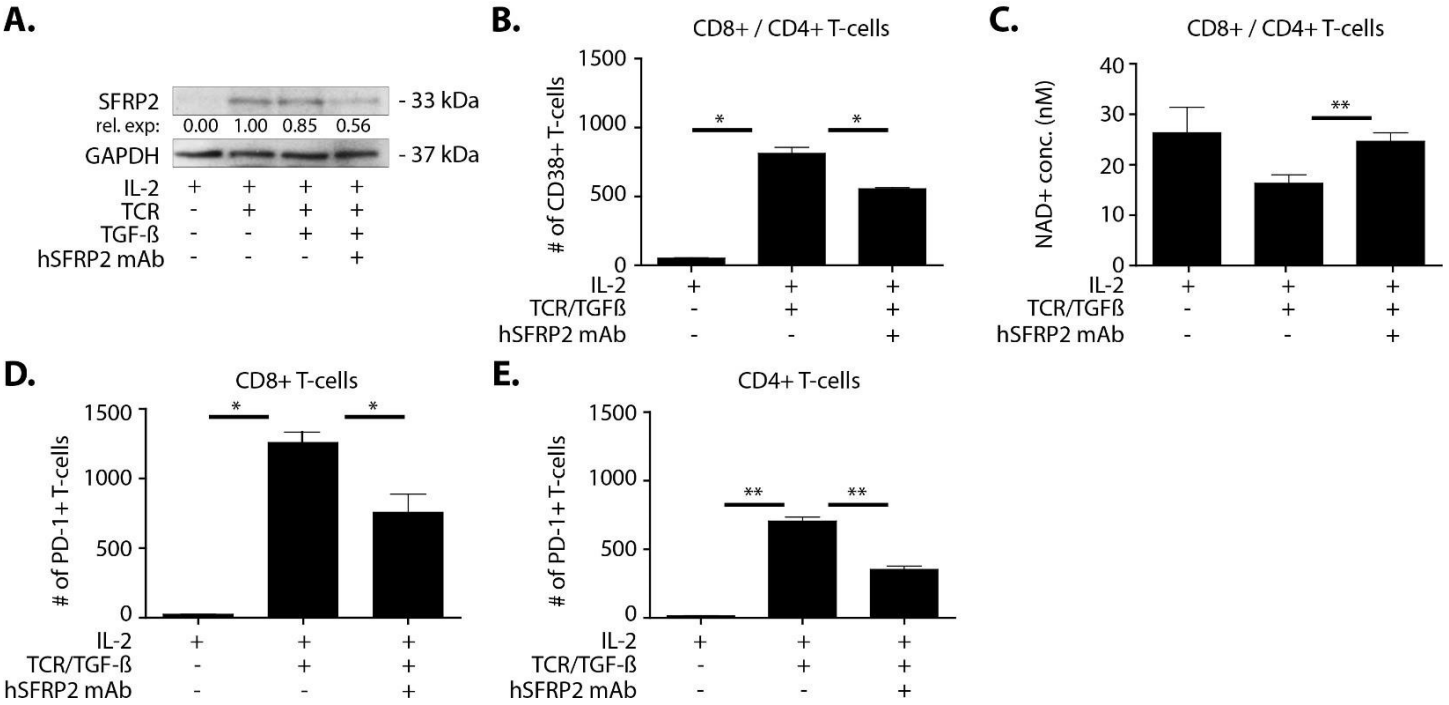



**Fig. 9**: Quantification of CD38, PD-1 and NAD+ splenic T-cells in response to treatment with hSFPR2 mAb. A) T cells were treated with IL-2, IL-2 + TCR, IL-2 + TCR/TGFβ, or the combination plus hSFRP2 mAb (10μM). Cell lysates were prepared for western blot and probed for SFPR2. B,C) Mouse splenocytes were treated with IL-2 (6,000 u/well), with or without TCR/TGFβ (5ng/ml) for 3 days, and with or without hSFRP2 mAb (10μM) for 48 hours (n=3 per group). CD4 and CD8+ T-cells were then isolated. B) CD38+ cells were quantified by FACS analysis C) NAD+ levels were measured. D-E) CD8+ T-cells were analyzed by flow cytometry for differential PD-1 expression. * $p < 0.001$, ** $p<0.05$.

*Major task 4)* While we were working to identify and characterize an alternate murine triple negative cell line that we could use to study synergy with immunotherapy, we evaluated whether there is synergy of the hSFRP2 mAb with immunotherapy in another tumor cell line that we are working on in the lab. This study was conducted under a separate IACUC protocol and not using DOD funds, however we are including this data as it will guide our similar experiments that we will be conducting this year with the KPB triple negative cell line. The purpose of this study was to analyze the efficacy of a humanized SFPR2 mAb against osteosarcoma tumor models as a monotherapy and in combination with PD-1 inhibition and examine the role of SFRP2 in immune signaling. Our *in vivo* experiments utilized immunocompetent C57/BL6 mice injected with osteosarcoma cell line RF577 via tail vein allowing lung metastases to develop for seven days prior to commencement of treatment. Our first experiment was with hSFRP2 mAb as monotherapy on established tumors, with IgG control (4mg/kg iv q3days) or hSFRP2 mAb (4 mg/kg iv q 3 days) started on day 7 and continued for 21 days. Mice were then euthanized, and lungs and spleens were collected. Surface macro metastases were counted. This showed a statistically significant reduction in the number of surface metastases (**Fig. 10A**, $p<0.05$). Splenic T cells and tumor infiltrating lymphocytes were collected and subjected to flow cytometry comparing activation markers CD38, CD103 and CD5 between control and hSFRP2 treated mice, which showed a significant decrease in splenic T-cell and tumor infiltrating lymphocyte CD38, but not CD103 or CD5 (**Fig.10B**). A portion of lungs were fixed in formalin and embedded in paraffin, and immunohistochemistry with antibody to CD31 was performed to quantify changes in tumor angiogenesis. This showed a reduction in microvascular density in hSFRP2 mAb treated tumors (**Fig. 10C**, $p<0.001$).).

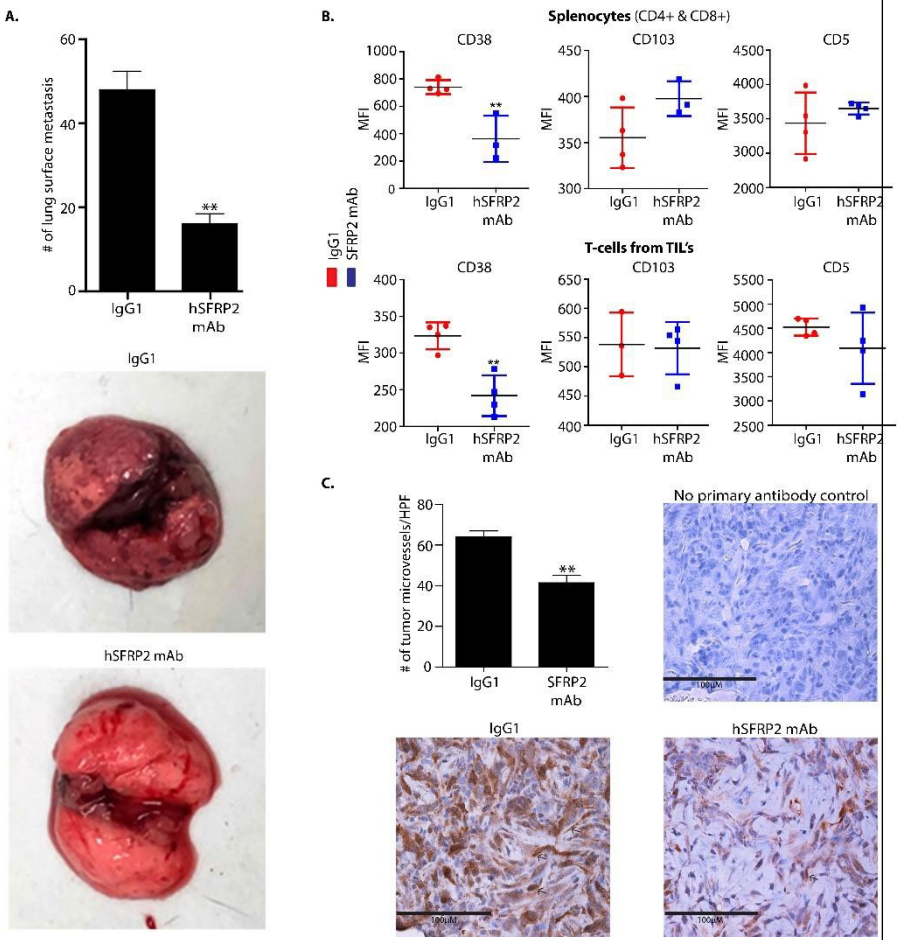



**Fig. 10**. *In vivo* monotherapy with hSFPR2 mAb in RF420 lung metastasis model. Osteosarcoma RF420 cells were injected intravenously in C57BL6 mice. Treatments with an IgG1 control or hSFRP2 mAb (4 mg/kg every 3 days), starting 10 days after the injection of tumor cells. Three weeks later, the animals were euthanized, their lungs were resected, and surface nodules were counted. A) *Top left* - Quantification of lung metastases in IgG1 treated control mice versus hSFRP2 mAb treated mice. (n=10). *Bottom left* - Representative lungs with tumor metastases. B) T-cells isolated from spleens of mice from in vivo experiment. Cells were stained for immune markers to CD38, CD103, or CD-5 which were labeled with a fluorochrome and mean fluorescent intensity (MFI) was analyzed by FACS. Dot blot graphs showing the measurements of fluorescence obtained from T-cells isolated from 4 different spleens for each treatment (N=4). Only CD38 was statistically different with hSFRP2 mAb in both splenocytes and TILs. C) Tumors from in vivo experiment were removed at necropsy stained with CD31 at a dilution of 1:200. ** p≤0.001.

Next, we evaluated whether the hSFRP2 mAb is synergistic with PD-1 mAb. C57BL6 mice were injected with RF420 cells via tail vein, and treatments started on day 10. Treatments were an IgG1 control (n=12), mouse anti-PD-1 (n=10), hSFRP2 mAb (n=12), and a combination of both anti-PD-1 and hSFRP2 mAb (n=11). Lungs were resected after 21 days of treatment, and serum was collected. Surface metastases were counted in all groups. This showed no effect of the PD-1 mAb as monotherapy on tumor metastases. The hSFRP2 mAb was efficacious as monotherapy at inhibited tumor growth, with an additive effect in combination with the PD-1 mAb (**Fig. 11A**). There were no signs of toxicity with any of the treatment groups, and no weight loss (**Fig. 11B**). Serum was collected and tested for SFRP2 protein by ELISA comparing mice with no tumor and mice with tumor in the IgG group. This showed and elevation in serum SFRP2 in mice with tumor (**Fig. 7C**).

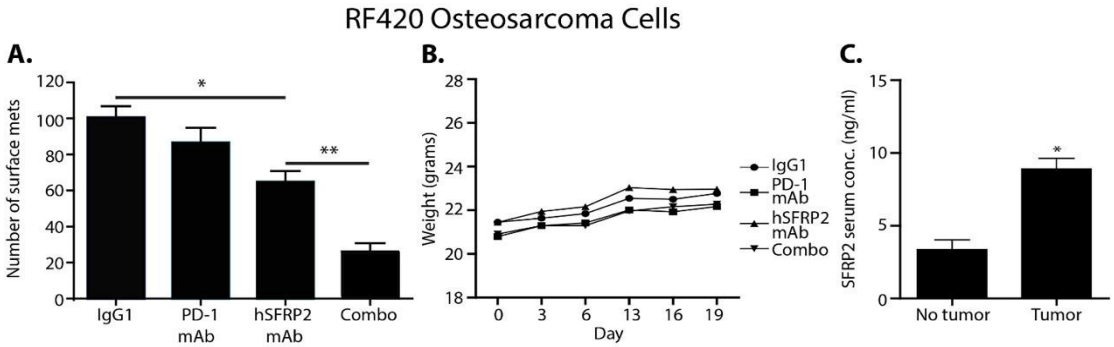
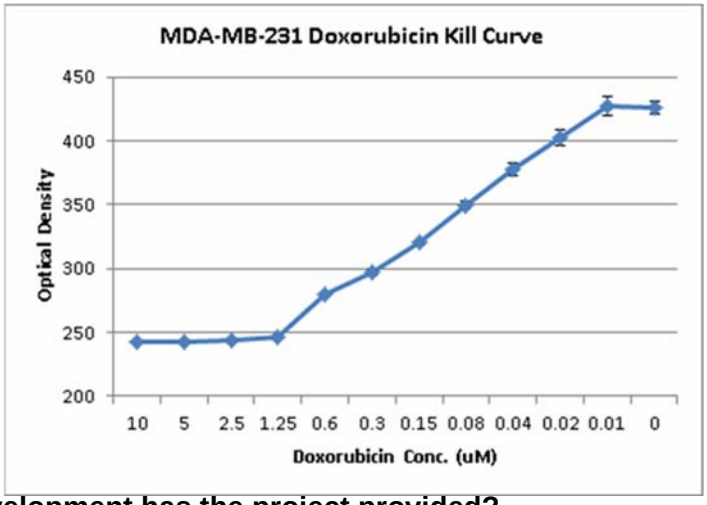



**Fig. 11**. *In vivo* metastatic osteosarcoma experiment with hSFRP2 mAb and anti-PD-1 mono and combination therapies performed with metastatic osteosarcoma RF420 cells C57/bl6 mice. RF420 cells were injected via tail vein into c57BL6 mice. Treatments were anti-PD-1 (200ug/100ul q3 days) IP (n=10), hSFPR2 (4mg/kg q3 days) IV (n=12), or a combination of both treatments (n=11) compared to IgG1 control (n=11). A) Lungs removed after three weeks of treatment were analyzed for metastatic disease and metastatic nodules were quantified for each treatment group. B) Animal weights were taken starting on the first day of treatment until the final day of treatment on day 19. C) Serum was taken from IgG1 control treated mice immediately following euthanasia tumors analyzed by ELISA for SFPR2 expression and compared with serum from normal C57/BL6 mice with no tumors injected (n=3).

cells were treated with doxorubicin (0.01uM to 10uM). Cells remained in treatment for 7 days. On day 7, cells were stained with Cyquant direct proliferation assay (ThermoFisher #C35011). The plate was read using the BioTek synergy2 plate reader at 480 nm wavelength**.** Using an on-line IC50 calculator, the IC50 was determined to be 0.12 µM. Cells have been serially cultured for 6 months starting in 0.002 µM, and are now in a concentration of .012 µM. An IC50 curve will be repeated, and an in vivo experiment will be performed this year comparing the sensitivity of wild-type MDA-MB-231 cells with dox-resistant MDA-MB-231 cells. As mentioned, we did have a problem with tumor take using the MDA-MB-231 cells previously, however, we have successfully used this cell line in the past and will increase the concentration of Matrigel in the next in vivo experiment, and will inject a larger number of cells.




4). Other achievements

**What opportunities for training and professional development has the project provided?**

Dr. Julie Seigel and Dr. Denise Garcia are surgical resident that each spent two years in the lab contributing to this research.

**How were the results disseminated to communities of interest?**

- This data was presented as an oral presentation March28,2019 at the Society of Surgical Oncology Annual Meeting in San Diego., and as an oral presentation at the American Pediatric Society September, 2019.
- This data was published: Denise Garcia, Patrick Nasarre, Ingrid V. Bonilla, Eleanor Hilliard, Yuri K. Peterson, Laura Spruill, Anne-Marie Broome, Elizabeth G. Hill, Jason T. Yustein, MD, Shikhar Mehrotra, Nancy Klauber-DeMore, Development of a Novel Humanized Monoclonal Antibody to Secreted Frizzled-Related Protein-2 that Inhibits Triple Negative Breast Cancer and Angiosarcoma Growth in vivo. Annals of Surgical Oncology, 2019 Dec;26(13):4782-4790.: PMID: 31515721

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

**What was the impact on the development of the principal discipline(s) of the project?**

Dr. DeMore has co-founded a start up company, Innova Therapeutics, to raise money for GMP manufacturing of hSFRP2 mAb, pre-clinical tox study, and phase 1 clinical trial

**What was the impact on other disciplines?**

- SFRP2 is expressed in other tumors, including sarcoma. Using non-DOD funds, we have conducted studies in parallel that show that the SFRP2 antibody is also effective in osteosarcoma in combination with immunotherapy. The mechanism and information obtained through the DOD grant will also impact patients with sarcoma.

**What was the impact on technology transfer?**

- We are using the data generated in this grant to talk with pharmaceutical companies and venture capital to attempt to raise funds for GMP manufacturing, pre-clinical tox studies, and Phase 1 trial. It is our hope that we will be successful at obtaining funds in the next reporting cycle.
- We have filed a patent on combination of hsFRP2 mAb and PD-1 inhbitor (see below)

- **What was the impact on society beyond science and technology?**
  - Nothing to report

**5. CHANGES/PROBLEMS:**

**Changes in approach and reasons for change**

- We had problems with cell lines initially planned for in this grant, so we moved toward the KPB GEMM triple negative cell line.

**Actual or anticipated problems or delays and actions or plans to resolve them**

- None

**Changes that had a significant impact on expenditures**

- Nothing to report

**Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

**Significant changes in use or care of human subjects**

None

**Significant changes in use or care of vertebrate animals.**

We added an additional cell line and obtained IACUC and ACURO approval

**Significant changes in use of biohazards and/or select agents**

None

*6.* **PRODUCTS:**

**Publications, conference papers, and presentations**

**Journal publications.**

- Denise Garcia, Patrick Nasarre, Ingrid V. Bonilla, Eleanor Hilliard, Yuri K. Peterson, Laura Spruill, Ann-Marie Broome, Elizabeth G. Hill, Jason T. Yustein, MD, Shikhar Mehrotra, Nancy Klauber-DeMore, Development of a Novel Humanized Monoclonal Antibody to Secreted Frizzled-Related Protein-2 that Inhibits Triple Negative Breast Cancer and Angiosarcoma Growth in vivo. Annals of Surgical Oncology, 2019 Dec;26(13):4782-4790.: PMID: 31515721 Federal support acknowledged.
- Nasarre, P.; Garcia, D.I.; Siegel, J.B.; Bonilla, I.V.; Mukherjee, R.; Hilliard, E.; Chakraborty, P.; Nasarre, C.; Yustein, J.T.; Lang, M.; et al. Overcoming PD-1 Inhibitor Resistance with a Monoclonal Antibody to Secreted Frizzled-Related Protein 2 in Metastatic Osteosarcoma. Cancers 2021, 13, 2696. https://doi.org/ 10.3390/cancers13112696. Federal support not acknowledged because DOD funds not used and this was on osteosarcoma
- The biodistribution manuscript will be submitted for publication this month

**Books or other non-periodical, one-time publications.** None

**Other publications, conference papers, and presentations.**

hSFRP2 mab Inhibits tumor growth, Society of Surgical Oncology Annual Meeting, March, 2019, San Diego, CA

**Website(s) or other Internet site(s)**

*None*

**Technologies or techniques**

*None*

**Inventions, patent applications, and/or licenses**

PHARMACEUTICAL COMBINATION FOR THE TREATMENT OF CANCER United States Patent Application No. 62/737,155 filed September 27, 2018, Inventor Dr. Nancy DeMore

**Other Products**

*None*

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

**What individuals have worked on the project?**

The individuals who have worked on the grant are unchanged from the original submission. These include: Dr. Nancy DeMore, Dr. Shikhar Merhotra, and Dr. Patrick Nasarre. Dr. Ann-Marie Broome left MUSC and was replaced by Dr. Steven Rosenzweig. Dr. Elizabeth Hill left MUSC and replaced by Dr. Rupak Mukherjee.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
*Dr. DeMore's effort on the DOD grant has not changed. However, she did receive to additional grants*

PI DeMore 4/1/19-3/31/20
Sponsor: Hollings Cancer Center Pre-Clinical and Clinical Concept Award
Calendar months 0.36
Total Budget:
Overlap: None

Title of Project: Window of Opportunity Trial using Curcumin for Breast Cancer
Goals: To evaluate whether there are changes in tumor proliferation and apoptosis in patients treated with curcumin in a window of opportunity trial
Co-PI DeMore 4/1/19-3/31/20
Sponsor: Hollings Cancer Center Pre-Clinical and Clinical Concept Award
Calendar months 0.36
Total Budget:
Title of Project: Adoptive Transfer of Tumor Infiltrating Lymphocytes for the Treatment of Breast Cancer
Goals: To obtain preliminary data of successful collection and expansion of human tumor infiltrating lymphocytes from human breast cancer
Overlap: None

**What other organizations were involved as partners?**
Nothing to report

**8. SPECIAL REPORTING REQUIREMENTS**
**COLLABORATIVE AWARDS:**
**QUAD CHARTS:**

*8.* **APPENDICES:**