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14. ABSTRACT
The goal of this proposal is to examine the impact of interleukin-28 in promoting the resistance of prostate cancer cells in bone. In the third year of this award we have made significant progress and have published our work Nature Communications (IF=15). The publication represents completed Aim 1&4. Aim 2&3 are nearing completion.

15. SUBJECT TERMS
Prostate Cancer, Bone Metastasis, Interleukin-28, Apoptosis Resistance, STAT Signaling, Osteoblasts, Mesenchymal Stem Cell, MSC, Osteoblast, Osteoclast.

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1. Introduction

This year in the United States alone, prostate cancer will claim the lives of over 26,000 men. The reason for the demise of these patients is that their disease has spread/metastasized from the prostate to secondary sites and has become resistant to therapy. Castrate resistant prostate cancer (CRPC) typically presents as metastatic disease (mCRPC) in the skeleton. Studies have shown that 90% of men that succumb to the disease, have evidence of bone metastasis. In the skeleton, prostate cancer cells manipulate the normal cells of the bone to generate lesions that have areas of extensive bone destruction caused by cells known as osteoclasts and bone formation caused by cells known as osteoblasts. These bony metastases are very painful and greatly impact the patient's quality of life. Clinically, androgen deprivation therapy (enzalutamide, abiraterone), chemotherapy (docetxel, cabazitaxel), and radiation therapy (radium-223/Xofigo) have increased overall survival. Unfortunately, it is only a matter of time before the disease becomes castrate and/or chemoresistant to these therapies and progresses. Given the number of men dealing with bone metastases, understanding how resistance arises and identifying new therapies that extend overall survival are an urgent and unmet clinical need. Our group has been investigating castrate resistant bone metastatic prostate cancer and emerging work has revealed a number of new findings. ***Our preliminary findings:*** Mescenchymal stromal/stem cells (MSCs) reside in the bone marrow and in response to prostate cancer derived factors can become osteoblasts and contribute to bone formation. We observed that reciprocally, MSCs can promote the evolution of mCRPC cell populations that have enhanced resistance to cell death. Furthermore, the MSC educated prostate cancer cells were also significantly more resistant to the chemotherapy, docetaxel. We have found that and MSC secreted factor, interleukin-28 (IL-28) can promote prostate cancer cell death by binding to its receptor IL-28R. The IL-28R receptor typically stimulates the activity of targets known as STAT1 and STAT3. We observed that the MSC educated prostate cancer cells have reduced STAT1 activity and elevated STAT3 activity. STAT3 has been shown to be active in human cases of bone metastatic prostate cancer. Here at Moffitt we have developed a novel inhibitor that blocks STAT3 activity, S3I-201. Our early results show that MSC educated prostate cancer cells are sensitive to this inhibitor *in vitro* and an expected outcome is that these cells will also be sensitive to STAT3 inhibition in pre-clinical mouse models of bone metastatic prostate cancer. We also expect that blocking STAT3 will make the resistant prostate cancer cells more sensitive to docetaxel chemotherapy.

2. Keywords

Prostate Cancer, Bone Metastasis, Interleukin-28, Apoptosis Resistance, STAT Signaling, Osteoblasts, Mesenchymal Stem Cell, MSC, Osteoblast, Osteoclast.

3. Accomplishments

Aim 1. Do MSC-educated prostate cancer cells have a growth advantage or impact bone disease in vivo compared to MSC naïve prostate cancer cells? The intratibial growth of naïve and MSC educated prostate cancer cells (PAIII and DU145) in the presence or absence of mCherry labeled MSCs will be measured via bioluminescence imaging. Relative luminescence units (RLUs) will be used as pre-clinical endpoints to generate survival curves. Bone pathophysiology changes will be analyzed via μCT and histomorphometry. Cancer cell growth, MSC content, and stromal responses will be determined histochemically.

Progress. This Aim has been completed and have identifies that MSC educated prostate cancer cells grow significantly faster than their parental counterparts (**Fig. 1**). The results of our studies have been published in *Nature Communications* this year.

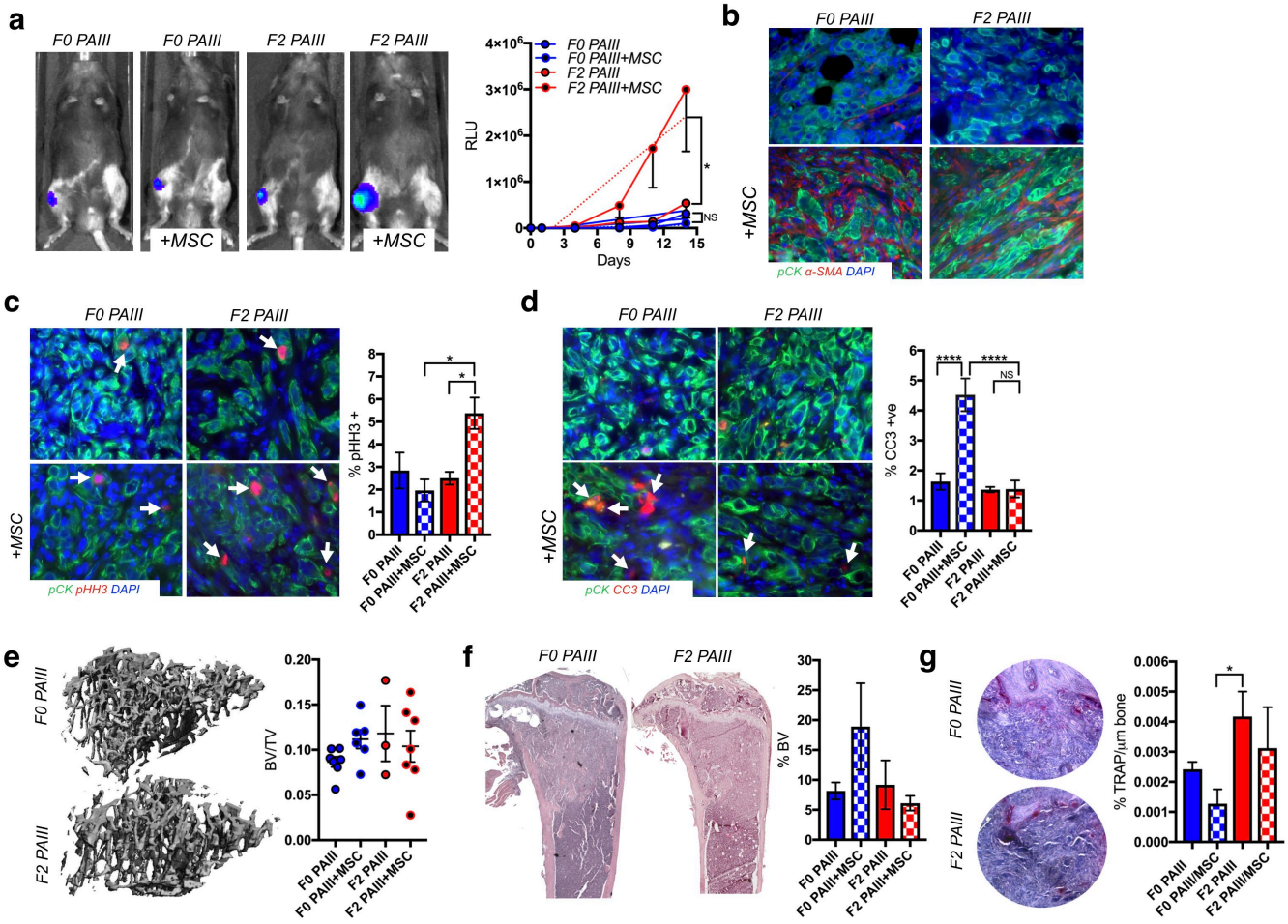

Figure 1. MSC selected prostate cancer cell growth is promoted rather than suppressed by the presence of MSCs. **a,** Parental (F0 PAIII) and MSC selected (F2 PAIII) growth over time in the presence (1:1 ratio) or absence of MSCs (n≥8/group). Representative images of bioluminescence in each group are shown at day 11 time point. Graphs illustrate collected RLUs over time for each group. **b,** Representative images of smooth muscle actin staining (α-SMA; red) in tissues derived from the F0 and F2 groups in the presence or absence of MSCs. Pan-cytokeratin (pCK; green) was used to localize prostate cancer cells. Dashed box in merge represents area of magnification. **c, d,** *Ex vivo* analyses from study endpoint of proliferative and apoptotic indices using phospohistone H3 (pHH3; red arrows; b) and cleaved caspase 3 (CC3; red, arrows, b) respectively. Pan-cytokeratin (green) was used to identify prostate cancer cells. **e,** μCT scan analysis of cancer-induced bone destruction. Representative μCT images of the trabecular bone are shown for the F0 and F2 PAIII group. The trabecular bone volume was calculated as a ratio to total volume analyzed (BV/TV). **f,** Trabecular bone volume (BV) was measured via histomorphometry on non-sequential H&E multiple sections derived from each group and calculated as a percentage of total volume. Representative gross H&E images are illustrated from the F0 and F2 groups. **g,** The number of osteoclasts (TRAcP positive; red, multi-nucleated; arrows) per μm of bone was calculated in non-sequential sections derived from each group. Asterisks denotes statistical significance (*p≤0.05, ****p≤0.0001) while NS denotes not significant.

Aim 2. Is IL-28 the primary mechanism through which MSCs drive apoptotic resistant bone metastatic prostate cancer? Using IL-28Rα null (CRISPR) prostate cancer cell lines, we will identify whether MSC derived IL-28 is the primary molecular mechanism through which MSCs promote apoptosis resistance in prostate cancer cells *in vitro*. The impact of IL-28Rα ablation on the activity of downstream effectors such as STAT1 and STAT3 will also be determined. *In vivo*, we will address whether IL-28Rα impacts the progression of bone metastatic prostate cancer by comparing the growth rates, overall survival and bone pathophysiology of control or IL-28Rα null (PAIII and DU145) cell lines.

Progress. Aim 2 has also been more or less completed with the exception of performing the IL-28Rα null PAIII and DU145 cell line studies. CRISPR knockout has proved challenging and so we reverted to using shRNA and siRNA approaches. Subsequently, we have shown that the IL-28 receptor is critical in mediating the MSC induced apoptotic effect (**Fig. 2**). We spent much of our time examining the down stream signaling pathways in the parental and MSC educated cell lines focusing primarily on STAT1 and STAT3. Using immunoblot and STAT activity assays, we show demonstrate preferential STAT3 signaling in the PAIII/DU145 MSC educated cell lines compared to the parental counterparts while conversely STAT1 signaling is higher in the parental PAIII/DU145 cells compared to their MSC educated cell lines. We have also inoculated mice with control or shRNA-IL-28Rα PAIII cell lines and are currently collating data with respect to growth/MSC infiltration and cancer induced bone disease.

Aim 3. Can STAT3 inhibitors sensitize bone metastatic prostate cancer to chemotherapy? The efficacy of S3I-201 as single agent or in combination with docetaxel in limiting the viability of MSC naïve and educated prostate cancer cell (PAIII and DU145) growth *in vitro and in vivo* will be assessed. The effect of STAT3 inhibition on overall survival and bone pathophysiology will also be examined.

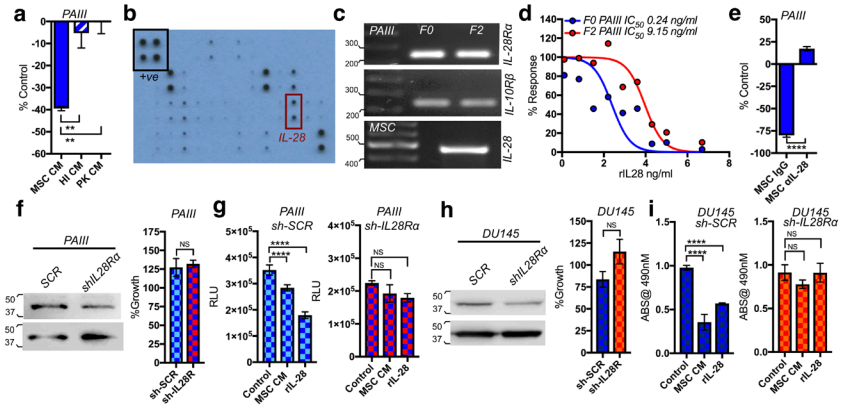

Figure 2. MSC-derived IL-28 directs PCa apoptosis. a, PAIII growth (F0) in response to treatment with MSC CM, heat-inactivated (HI) MSC CM, or proteinase-K (PK) treated MSC CM. b, Cytokine Array of MSC CM. Black box indicates positive control (+ve), red box indicates IL-28. c, RT-PCR analysis of PAIII (F0 and F2) of IL28Rα, IL-10Rβ and IL-28 expression. Molecular weights in base pairs are shown. d, Growth of PAIII (F0) in MSC CM immune-depleted of IL-28 (MSC αIL-28). IgG was used as negative control (MSC IgG). Growth is expressed as a percentage of non-treated cells. e, Treatment of PAIII F0 and F2 cell lines with the indicated concentrations of recombinant IL-28 (rIL-28) for 48 hr. f, Growth of IL-28Rα silenced (sh-IL28R) and scrambled control (sh-SCR) compared to parental PAIII cell lines. g, h, Control (sh-SCR) and IL-28Rα (sh-IL28R) PAIII and DU145 growth in MSC CM or rIL-28 as measured by luminescence assay and relative light unit (RLU) measurement or MTT assay. Asterisks denotes statistical significance (**p≤0.01, ****p≤0.0001)

Progress. We have shown that as a single agent, the STAT3 inhibitor, S3I-201 is effective inhibiting the growth of PAIII and DU145 but does not impact cancer induced bone disease. These results were included in our *Nature Communications* paper. Our next step will be to examine the impact of combined docetaxel treatment with S3I-201. We were set to begin these studies in March, 2020 but experiments were impacted by COVID-19. Subsequent to the reinitiation of wet-bench work, we have shown synergy between S3I-201 and docetaxel in the MSC educated PAIII cell lines (data not shown) and *in vivo* experiments are planned. We plan to finish these experiments during the no cost extension period.

Aim 4. What is the MSC content and pSTAT1/3 status in human bone metastatic cancer? MSC content in specimens and tissue microarrays of bone metastatic prostate cancer will be evaluated using immunofluorescent multispectral techniques (Vectra) to identify MSC CD73/CD90/CD105 markers. We will also examine the status of IL-28Rα and pSTAT1/3 in pan-cytokeratin positive prostate cancer cells.

Progress. We have have completed and published these studies in our *Nature Communications* publication this year. (**Fig. 3**).

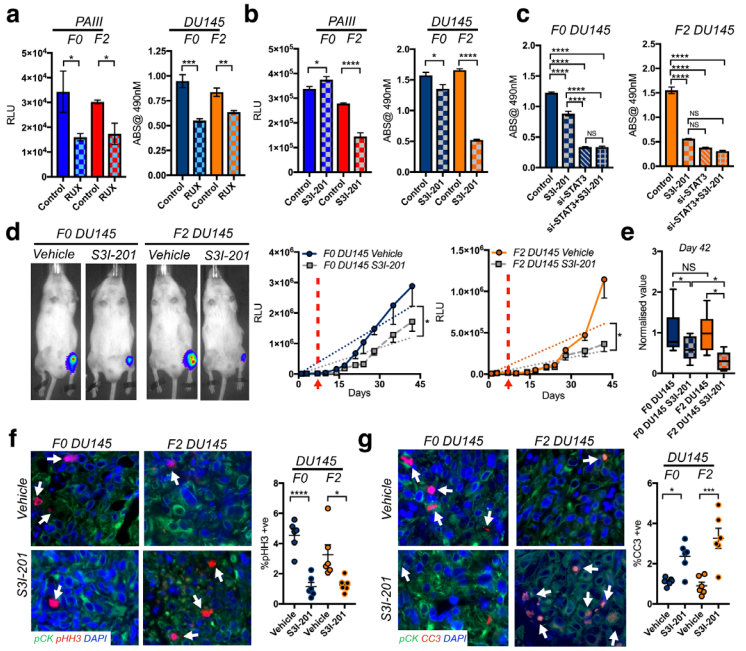

Figure 3. STAT3 inhibition impairs the growth of MSC-selected prostate cancer *in vitro* and *in vivo*. **a, b,** Parental (F0) and MSC-selected (F2) cell lines treated with vehicle control (Control) or the JAK2 inhibitor ruxolitinib (RUX)/STAT3 inhibitor (S3I-201) for 24 hr. **c,** F0 and F2 DU145 control (scr-siRNA) or STAT3 silenced (si-STAT3) cells treated with vehicle or S3I-201 for 24 hr. **d,** F0 and F2 DU145 growth over time in the presence or absence of STAT3 inhibitor, S3I-201 (n=10/group). Representative images of bioluminescence in each group are shown at day 35-time point. Arrow and dashed line represent time of treatment initiation. Graphs illustrate collected RLUs over time for each group. **e,** S3I-201 effect on F0 and F2 DU145 at day 42 normalized to respective controls. **f, g** *Ex vivo* analyses from study endpoint of proliferative and apoptotic indices using phospohistone H3 (pHH3; red arrows; f) and cleaved caspase 3 (CC3; red, arrows, g) respectively. Pan-cytokeratin (green) was used to identify prostate cancer cells. Asterisks denotes statistical significance (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001) while NS denotes not significant.

4. Impact.

Short-term impact: Studies in this proposal have determined how MSCs drive the evolution of more aggressive apoptosis resistant subpopulations of prostate cancer. Our high-impact studies have also shed light on novel molecular mechanisms that control prostate cancer cell survival namely, IL-28Rα activation and altered downstream STAT1/3 activity. Further, our results demonstrate that MSC educated prostate cancer cells are sensitive to STAT3 inhibition with small molecule inhibitors and provide rationale for targeting this pathway in the context of therapy resistant bone metastatic prostate cancer. As such we expect in the short term that our proposed studies will greatly impact the field's understanding of how cells of the bone microenvironment promote the progression of bone metastatic CRPC.

Long-term impact: Unraveling the mechanisms that contribute to disease resistance in patients with advanced bone metastatic prostate cancer will play a critical role in extending overall survival in this high-risk population. We expect that the results of our pre-clinical studies using STAT3 inhibitors will provide rationale for future clinical trials and/or the design of cancer specific targeted STAT3 inhibitors to offset potential adverse side effects. We are also excited by the prospect that STAT3 inhibition may resensitize chemotherapy resistant disease. The expected results could be of huge potential impact to advanced bone metastatic CRPC patients that have become refractory to chemotherapy.

5. Changes/Problems

We have encountered no difficulties in executing the proposed studies and have made no changes to the experimental approach. COVID-19, like many others, has impacted our progress and so we are requesting a no cost extension for this award in order to finish up the remaining experiments in Aim 3.

6. Products

Lynch CC. AACR Major Symposia. Bone Marrow Sensing of Distant Tumors: From Early Detection to Possible Therapy. "*MSCs drive the evolution of apoptotic resistant prostate cancer."* AACR, Atlanta, GA April 2, 2019

McGuire JJ, Frieling JS, Lo CH, Li T, Muhammad A, Lawrence HR, Lawrence NJ, Cook LM, **Lynch CC**. Mesenchymal stem cell-derived interleukin-28 drives the selection of apoptosis resistant bone metastatic prostate cancer. Nat Commun. 2021 Feb 1;12(1):723. PMID: 33526787

7. Participants & Other Collaborating Organizations

N/A

8. Special Reporting Requirements

N/A

9. Appendices

None