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Identifying the Mechanism(s) Responsible for the Translational Regulation of the Stress
Signaling Kinase MKK4

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14. ABSTRACT
Since our last progress report we have focused on developing the cell lines and *in vivo* approaches that are needed to complete the work proposed in Aim 2. We have also revised our SOW accordingly to reflect these and other changes required to best achieve our outcomes. As described in the last report, we were particularly interested in the potential use of the CWR22Rv1 prostate cancer cell line, which recapitulates key aspects of clinical disease. In the same light, we extended our studies into the C4-2B cell line, which has also been reported to be bone-seeking and potentially metastasis-forming. Given that our goal is to test the effect of MKK4 levels on metastatic colonization, it was imperative that we first focus our efforts on: 1) confirming the reported metastatic ability of both the CWR22Rv1 and C4-2B cell lines, and 2) optimizing the conditions and approach for quantitative evaluation of the effect of modulating MKK4 protein levels on bone metastasis formation after intracardiac injection.

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INTRODUCTION: Since our last progress report we have focused on developing the cell lines and *in vivo* approaches that are needed to complete the work proposed in Aim 2. We have also revised our SOW accordingly to reflect these and other changes required to best achieve our outcomes. As described in the last report, we were particularly interested in the potential use of the CWR22Rv1 prostate cancer cell line, which recapitulates key aspects of clinical disease. In the same light, we extended our studies into the C4-2B cell line, which has also been reported to be bone-seeking and potentially metastasis-forming. Given that our goal is to test the effect of MKK4 levels on metastatic colonization, it was imperative that we first focus our efforts on: 1) confirming the reported metastatic ability of both the CWR22Rv1 and C4-2B cell lines, and 2) optimizing the conditions and approach for quantitative evaluation of the effect of modulating MKK4 protein levels on bone metastasis formation after intracardiac injection.

BODY: Our overall progress on the Research-Specific Tasks specified in our SOW Modifications (06 – 13 – 13) is delineated below. (Please Note: Subtasks in light gray font were completed prior to YR3, however, we included them as they were modified in our modified SOW.)

Aim 1: To test the hypothesis that translational repression of MKK4 is mediated by specific miRNAs (Overall Goal: Preparation of reagents and tools needed to the *in vivo* functional effect of modulating MKK4 protein levels proposed in Aim 2)

☐Major Task 1: Do miRNAs play a role in translational repression of MKK4 in prostate cancer cell lines?

Subtask 1a: Studies to validate the differential expression of candidate miRNAs in prostate cancer cells lines with low MKK4 levels *Cell Lines Used:* PC3, LNCaP, C4-2B, DuPro, Du145, LAPC4, CWR22Rv1, CWR-R1, VCAP
Status: Completed prior to YR 3: Subtask listed as it was modified from the original SOW.

Subtask 1b: Transfection of combinations of precursor (Pre-) miRNAs will be used to decrease the level of MKK4 *Cell Lines Used:* CWR22Rv1, C4-2B
Status: Completed prior to YR 3: Subtask listed as it was modified from the original SOW.

Subtask 1c: antisense (AS-) miRNAs (antagomirs) will be used to increase the level of MKK4 *Cell lines used:* PC 3, C4-2B
Status: Completed prior to YR 3: Subtask listed as it was modified from the original SOW.

Subtask 1e: Validation of MKK4 depletion by treatment with miRNAs using WI38 human fibroblasts a control. *Cell Lines Used:* WI38
Status: Completed prior to YR 3: Subtask listed as it was modified from the original SOW.

Subtask 1f: Lentiviral vectors will be constructed that express: 1) AS-miRNAs to stably deplete endogenous MKK4 protein; and 2) precursor (Pre-) miRNA(s) to stably upregulate endogenous MKK4 protein.
Status: To be done [during an EWOF]

Subtask 1g: Lentiviral vectors will be used to express: 1) shRNAs to stably deplete endogenous MKK4 protein; and 2) MKK4 cDNA to stably express ectopic MKK4 protein.

Status: To be done [during an EWOF]

☐**Major Task 2: Selection and characterization of CWR22Rv1 and C4-2B prostate cancer cell sublines with increased "bone-seeking" ability for use *in vivo* metastasis assays.**

☐ **Subtask 1a:** Construct and characterize CWR22Rv1and C4-2B parental lines with pGL4-luc2 luciferase evaluate photon flux cell lines *in vitro*. *Cell Lines Used:* 2CWR2Rv1, C4-2B

☐ *Status:* Completed in YR 3

Summary of Work completed in Support of Subtask 1a:

We began by examining the metastatic potential of CWR22Rv1 cells, which are a human prostate cancer cell line derived from the original CWR22 cancer cell line. This cell line has been reported to form bone metastases following intracardiac injection. They are representative of advanced, castrate resistant prostate cancer, express a mutant form of the androgen receptor, and do not express high levels of prostate specific antigen (PSA). We are also interested in using the C4-2B cell line, as it has been reported to also be bone-trophic. As a first step, the cells were engineered to express luciferase by lentiviral transduction with the pGL4-Hygro-luc2 vector (Promega), creating CWR22Rv1-luc2 and C4-2B-Luc2 cells. Luc2 is a synthetic luciferase which was codon optimized for optimal expression in mammalian cells. Expression of luciferase in the cancer cells allows for surrogate monitoring of cancer growth *in vivo* by analyzing light emission following administration of the luciferase substrate, luciferin. Representative data from the CWR22Rv1 cell line is shown in Figure 1.
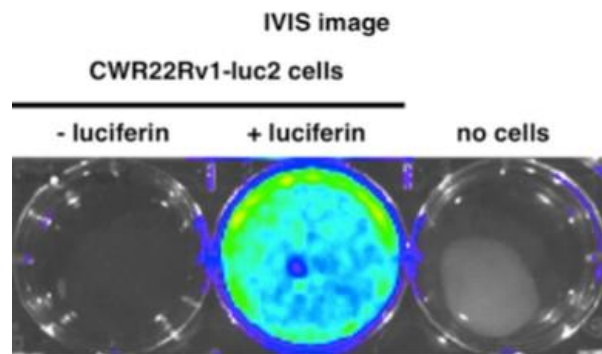

Figure 1. Ectopic expression of synthetic Luc2 luciferase in CWR22Rv1 cells. Luciferase activity was confirmed by plating CWR22Rv1-luc2 cells ± luciferin, or no cells, imaged on the IVIS system.

☐ **Subtask 1b**: Validate the distribution of luciferase-tagged cells after intracardiac injection (state-of-the-art approach for localization of these cell lines to bone). *Cell Lines Used:* CWR22Rv1-luc2, C4-2B-luc2.

☐ *Status:* Completed in YR 3

☐ **Subtask 1c:** Conduct dose (cell number injected) response (metastatic yield) curves on luciferase-tagged cells to determine both optimal dose and time-to-endpoint for studies (using IVIS imaging over period of 7 weeks) *Cell Lines Used:* CWR22Rv1-luc2, C4-2B-luc-2.
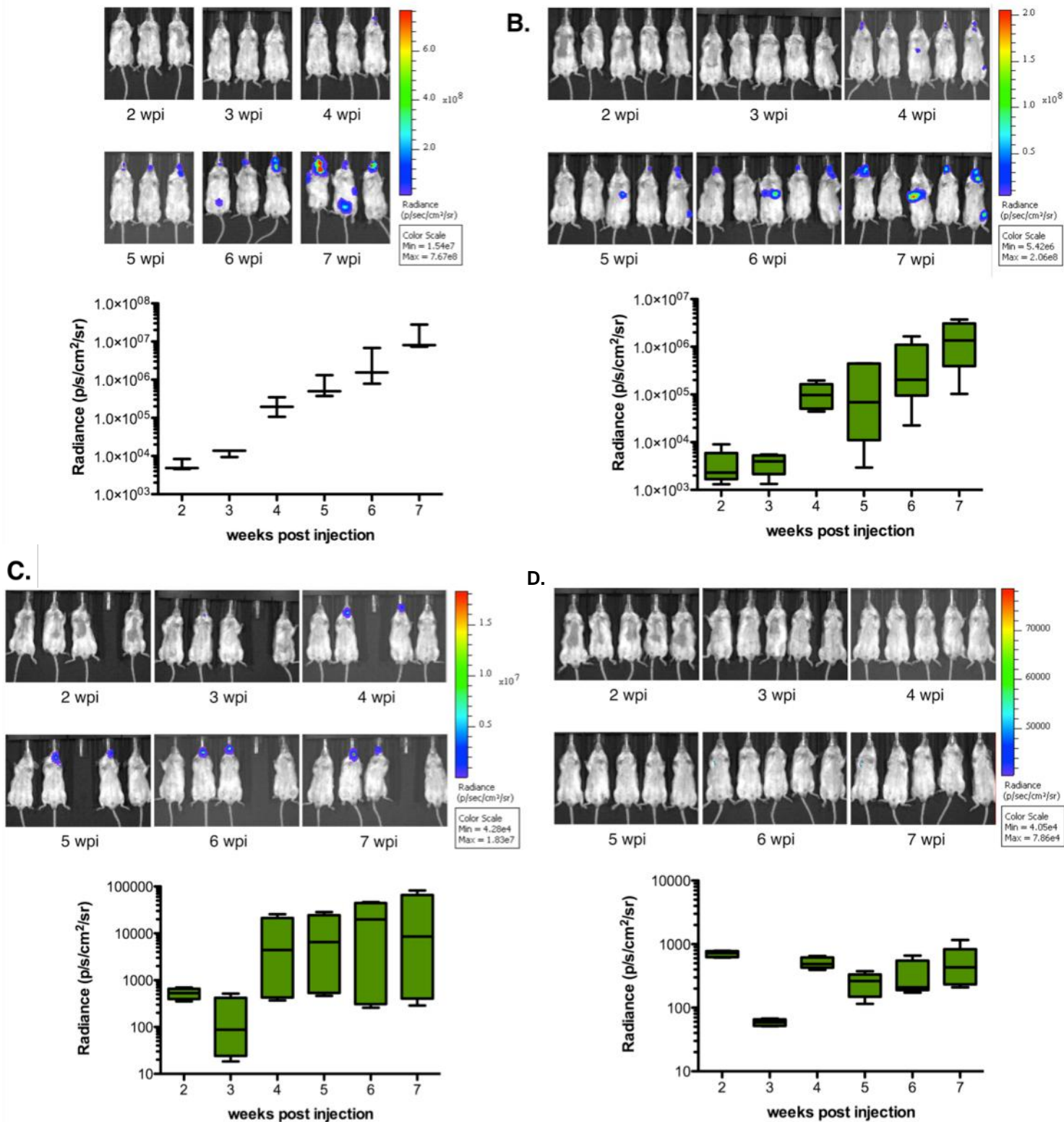
☐ *Status:* Completed in YR 3

Summary of Work in Support of Subtask 1b and 1c:

To identify the optimal number of cells required to yield quantitative metastasis data at the experimental endpoint, a dose vs. time course experiment was performed. Specifically, 5.0×10^5, 2.5×10^5, 2.5×10^4 or 2.5×10^3 CWR22Rv1-luc2 cells were injected into the left ventricle of 6-8 week old male SCID mice with 5 animals in each group. Injection of cells into the left ventricle delivers the cells into full body circulation, allowing them to localize and grow in the environment they find most suitable to growth. The process by which the cells lodge within, survive, and grow into metastases is known as *metastatic colonization*. This is the process that the MKK4 metastasis suppressor specifically disrupts. From a clinical standpoint, it is important to test the ability of MKK4 to disrupt or suppress prostate cancer metastatic colonization of the bone microenvironment. Immediately following injection, the animals were intraperitoneally (ip) injected with luciferin and imaged on the IVIS *in vivo* imaging system (Caliper), which confirmed the accuracy of the injection (data not shown)[1].

Mice were then imaged weekly to monitor the location and growth of putative CWR22Rv1-luc2 metastases. The highest cell dose, 5.0×10^5 CWR22Rv1-luc2 cells, lead to rapid formation of metastases in all animals (Figure 2A). The second dose, 2.5×10^5 cells, lead to formation of metastases in all animals with a slight delay before luciferase signal was detectable (Figure 2B). The two lowest doses were slow to form metastases, when they formed at all (Figure 2C, D). Based on these results we chose 2.5×10^5 CWR22Rv1-luc2 cells for future experiments. At this number of injected cells all animals formed metastases, a desirable result, and there was a delay in metastasis formation. Since we desire to find genes that interfere with metastatic outgrowth in future experiments, this delay is necessary for future work.

In addition to our work on CWR22Rv1, have completed a first round of studies on C4-2B. To note, C4-2B cells were obtained from the lab of Dr. John Issacs at Johns Hopkins. The cells were engineered to express luciferase 2 as with the CWR22Rv1 cells. 5×10^5 C4-2B-luc2 cells were injected into the left ventricle of 6-8 week old male SCID mice. Unfortunately, these cells did not form detectable bone metastases, even after extended times post injection (monitored for bioluminescence for 9 months). To address this, we have proposed preconditioning the cells via direct injection and growth in the tiba (selection via passaging within the bone microenvironment). This additional step should increase the probability of establishing a cell line that will be sufficiently robust to undergo selection via intracardiac injection (strategy described for CWR22Rv1 cells). These additional steps are now included in Major Task 2, Subtasks 1g, 1h, and 1i.

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[1] It should be noted that an accurate injection delivers cells into the circulation and is seen as whole-body distribution of light production by luciferase/luciferin interaction. In contrast, in the case of misinjection, a concentration of cells will be seen in a single site, most often the lung. This is an important quality control step that unfortunately is overlooked in many studies. Although infrequent, in our studies misinjection does on occasion occur. These mice are excluded from the study.

Figure 2. CWR22Rv1-luc2 optimal dose determination. A. 5.0×10^5 CWR22Rv1-luc2 cells were injected into the left ventricle of 6-8 week old male SCID mice. Animals were injected ip with luciferin and imaged immediately to ensure injection of the cells into the circulation. Animals were imaged weekly post injection (wpi) and total luciferase radiance was calculated using Living Image software. **B.** 2.5×10^5 CWR22Rv1-luc2 cells were injected and animals imaged as in A. **C.** 2.5×10^4 CWR22Rv1-luc2 cells were injected and animals imaged as in A. **D.** 2.5×10^3 CWR22Rv1-luc2 cells were injected and animals imaged as in A. *Line,* mean, *box*, 25th and 75th percentile, *bars*, range.

Summary of Work in Support of Subtask 1d: Bone metastases are the major source of metastatic growth in men with prostate cancer thus, the possibility of developing a CWR22Rv1 subline that models this pattern of growth would be invaluable to both our studies and the greater prostate cancer research community. To explore this possibility we needed to characterize the lesions corresponding to the luciferase signal in these mice. Thus, at the experimental endpoint, long bones showing luciferase positivity were harvested to confirm the presence of cancerous lesions using histology.

To this end, we dissected out the leg bones of animals that exhibited luciferase expression in the legs, processed the bone for histology, sectioned the processed tissue, and performed H&E staining. As shown below, on the left is the IVIS image of an animal that exhibits strong luciferase signal in the leg region (Figure 3A). We were excited to find that the CWR22Rv1-luc2 cells were growing in the marrow space of the femur in this animal (Figure 3B). On the top left is an H&E stained section of a normal bone. Observe the linear structure and uniform thickness of the bone itself (light pink). On the top right is a section of bone with CWR22Rv1-luc2 cells growing in the marrow space. Importantly, it appears that the metastases formed by these cells are osteoblastic, which is consistent with the metastatic phenotype observed in clinical disease.
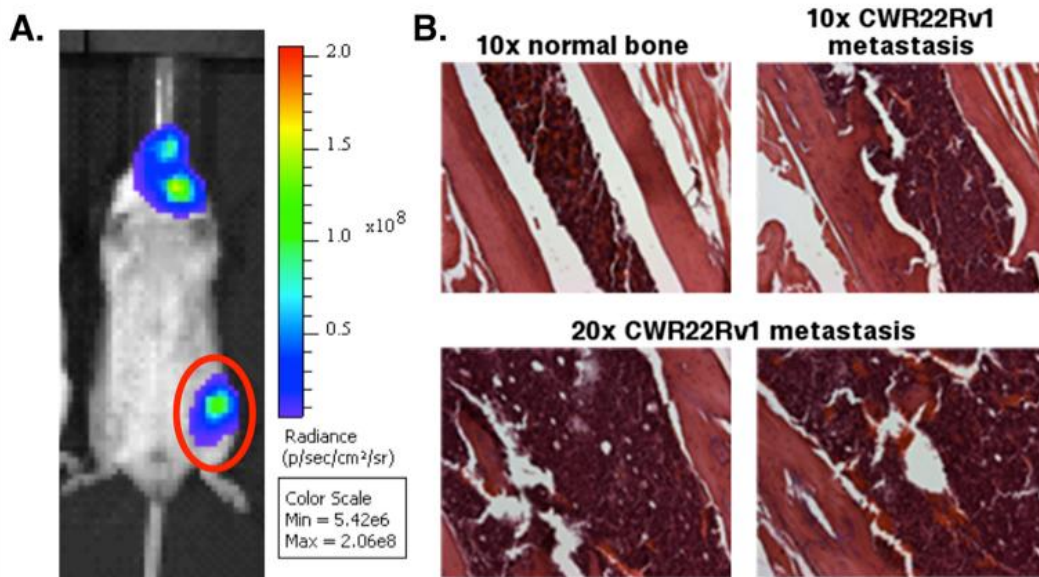

Fig. 3. Validation of CWR22Rv1-luc2 metastatic growth in bone marrow of long bones. A. Close-up of animal seen in Figure 1B, IVIS imaging at 7 weeks post injection. **B.** Femurs of animal dissected out, fixed and decalcified. *Upper left*, H&E of normal bone without CWR22Rv1 growth. *Upper right*, H&E of left femur of animal in A. Malformed bone growth along with metastatic growth in marrow space can be seen. *Lower*, close-up of CWR22Rv1 metastatic growth in bone marrow space.

The light pink bone is thicker than in the normal control, and is clearly malformed. Note the growth of the bone into the marrow space, with an almost stalagmite appearance. The close-up views of the metastases presented on the bottom show the abnormal growth of the cells, indicated there is metastatic growth. Further, the cells Further, the cells appear to be forming glandular structures, an observation that warrants further investigation. Taken together, these findings are very promising; however, the percentage of mice that develop bone metastases is low. This is not surprising given the difficulties inherent in developing bone-trophic prostate cancer cell lines. In order to address this we will we will use an approach of serially passaging the cells through mice to select for metastatic variants (subtask 1e). Thus, in addition to making a reagent that we need to assess the affect of modulating MKK4 levels, this approach will also develop a CWR22Rv1 subline that has increased metastatic efficiency will be a valuable resource for other researchers.

Subtask 1e: Serial passaging of CWR22Rv1-luc2 cells through SCID mice via intracardiac injection to select for a subline with increased propensity for bone metastases.

Status: Started in YR 3, [continue during an EWOF]

Subtask 1f: Characterize CWR22Rv1-luc2-FDIC cells prior to use in Aim 2 (i.e. growth rate, kayotyping, etc.).

Status: To be done [during an EWOF]

Subtask 1g: Serial passage of C4-2B-luc2 cells *via* direct intratibial injection a subline with increased propensity for growth in bone.

Status: To be done [during an EWOF]

Subtask 1h: Serial passaging of C4-2B-luc2-FD cells *via* intracardiac (IC) injection to select for a sublinewith increased propensity for formation of "spontaneous" bone metastases

Status: To be done [during an EWOF]

Subtask 1i: Characterize C4-2B-luc2-FDIC cells prior to use in Aim 2 (i.e. growth rate, kayotyping, etc.).

*Status:* To be done [during an EWOF]

☐ **Subtask 1j**: Development of a sensitive and specific quantitative reverse transcription PCR (qRTPCR) assay to quantitate the number of prostate cancer cells within the bone. *Cell Lines Used:* (genomic DNA: CWR22Rv1, ID-8).

☐ *Status:* Completed in Year 3

Summary of Work in Support of Subtask 1j: As we continue to develop bone trophic cell line derivatives (summarized in Fig. 6), started to develop a method to sensitively and specifically quantitate human prostate cancer cells in the bone microenvironment. This method was key to

our previous studies of MKK4's effect on ovarian cancer metastatic colonization of the omentum.[2]   To this end, a qRT-PCR based approach was developed to distinguish human cells from murine cells. Taqman primer/probe mixes were purchased from Applied Biosystems and their specificity and sensitivity was evaluated. First, we determined that each set of primer/probe detected only the species it was directed toward (Fig. 8). The human GAPDH primers were unable to amplify when provided with only murine template, and the murine UBE2i primers were unable to amplify when provided with only human template. This indicates that these primers can be used to distinguish human from mouse because they are species specific.

Fig. 8. qRT-PCR primers are species specific. CWR22Rv1 (human prostate cancer) and ID-8 (murine ovarian cancer) cells were cultured and genomic DNA (gDNA) was extracted. Following quantitation, gDNA from each cell line was left unmixed or mixed at the indicated percentages. qRT-PCR was performed using the mixed gDNA as template, and TaqMan primer/probe mixes from Applied Biosystems. Unmixed samples exhibited no amplification – human GAPDH was undetectable in the 100% ID-8 sample, and murine Ube2i was undetectable in the 100% CWR22Rv1 sample. CT values are plotted for samples that exhibited amplification. Human GAPDH is represented by red boxes, murine Ube2i is represented by gray circles. *Points*, mean of five technical
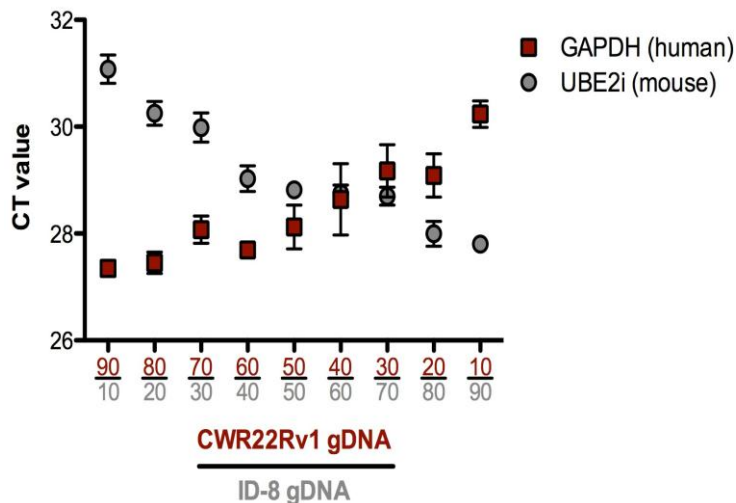
Subtask 1k: Confirmation that qRT-PCR assay/primers is efficient and linear over a range of template concentrations. *Cell Lines Used:* (genomic DNA: CWR22Rv1, ID-8).

 Status: Completed in Year 3

Summary of Work in Support of Subtask 1k: We next sought to determine if each set of primer/probe is efficient. Analysis of qRT-PCR data assumes that the target sequence is doubled during each cycle, and it is important to verify this assumption. By performing qRT-PCR over a range of template concentrations, the efficiency of the primers can be calculated. Primers that perfectly double the target sequence concentration each cycle will have an efficiency of 100%; however, anything within 90-110% efficient is considered to validate the assumption of doubling. Both primer sets were found to have high efficiency (Fig. 9)

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2 Lotan Hickson *et al.* Cancer Res. 2008 Apr 1;68(7):2166-75.

UBE2i Efficiency

y = -3.6241x + 27.297
R^2 = 0.9914
Efficiency = 94.4%

GAPDH Efficiency

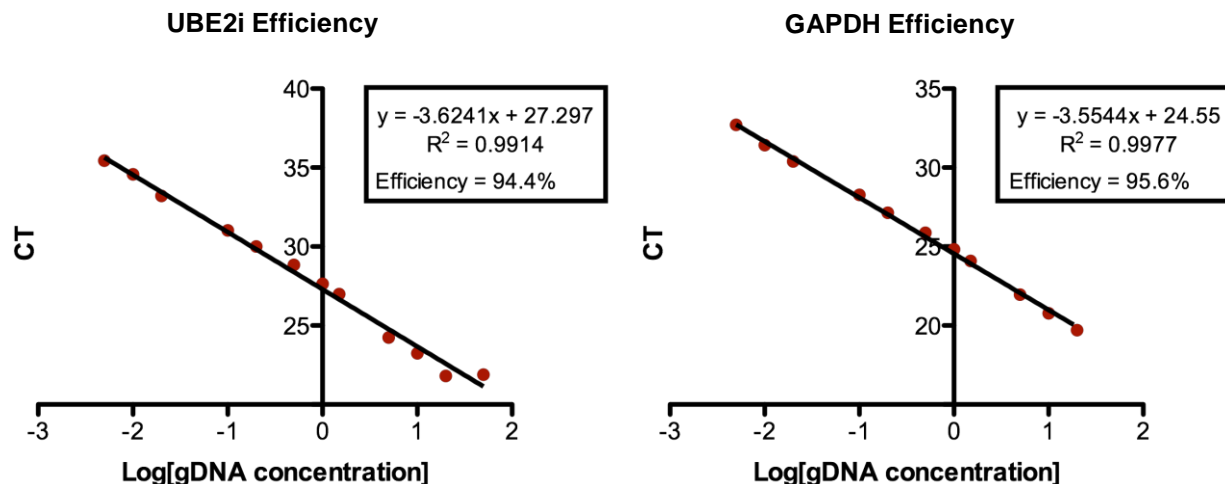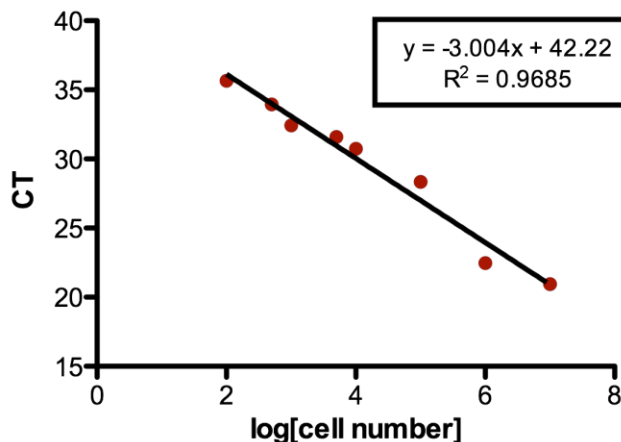y = -3.5544x + 24.55
R^2 = 0.9977
Efficiency = 95.6%

Fig. 9. qRT-PCR primers efficiently amplify over a range of template concentrations. CWR22Rv1 (human prostate cancer) and ID-8 (murine ovarian cancer) cells were cultured and genomic DNA (gDNA) was extracted. qRT-PCR was performed with TaqMan primer/probe mixes from Applied Biosystems. Murine UBE2i primer/probe was used to on the ID-8 template, while human GAPDH was used on the CWR22Rv1 template. Template concentration varied from 0.005 ng/μL to 300 ng/μL. *Points*, mean of five replicates, *bars*, standard error of the mean. Efficiency was calcuated as $0.5*10^{(-1/slope)}*100$

Subtask 1l: Determination of the limit of detection for human prostate cancer cells in the mouse femur. *Cell Line Used:* CWR22Rv1

Status: Completed in Year 3

Summary of Work in Support of Subtask 1l: Finally, we sought to identify the limit of detection of human cells in the long bones. To this end, we added known numbers of CWR22Rv1-luc2 cells to murine femurs and extracted the gDNA. We performed qRT-PCR using both the UBE2i and GAPDH primers. As shown in Fig. 10, this assay can detect as few as 100 CWR22Rv1-luc2 in a whole femur. While is this quite good, we are working on increasing the sensitivity of the assay by using higher input template concentrations.

Fig. 10. As few as 100 CWR22Rv1 cells are detectable in whole femur. CWR22Rv1 cell numbers ranging from 10,000,000 to 0 were pelleted and frozen. Whole femurs were frozen, ground up, and added to the cell pellets. gDNA from cell pellets and bones was extracted together and qRT-PCR performed as before. Human GAPDH was undetectable in samples containing 0 CWR22Rv1 cells. *Points*, mean of five technical replicates, *bars*, standard error of the mean.

y = -3.004x + 42.22
R^2 = 0.9685

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Summary of Work in Support of Subtask 1l: Finally, we sought to identify the limit of detection of human cells in the long bones. To this end, we added known numbers of CWR22Rv1-luc2 cells to murine femurs and extracted the gDNA. We performed qRT-PCR using both the UBE2i and GAPDH primers. As shown in Fig. 10, this assay can detect as few as 100 CWR22Rv1-luc2 in a whole femur. While is this quite good, we are working on increasing the sensitivity of the assay by using higher input template concentrations.

Subtask 1m: Quantitating skeletal distribution of CWR22Rv1-luc2 cells at discrete time points using qRT-PCR.

Status: Status: To be done [during an EWOF]

Aim 2: To test the hypothesis that modulation of MKK4 levels by miRNAs affects *in vivo* measures of malignant potential.

☐**Major Task 1: Modulation of MKK4 protein levels in prostate cancer cell lines with increased propensity for bone metastasis (Derived in Aim 1)**

Subtask 1a: Use vectors described in Aim1 Major Task 1, subtasks 1f and 1g to modulate MKK4 protein levels in CWR22Rv1-luc2-FDIC cell lines

Status: To be done [during an EWOF]

Subtask 1b: Characterize cell lines generated in Aim 2, Major Task 1, Subtask 1a: 1) confirm MKK4 depletion, and 2) determine *in vitro* growth rates.

Status: To be done [during an EWOF]

Subtask 1c: Validate the distribution of cell lines characterized in Aim 2, Major Task 1, Subtask 1b using IVIS imaging.

Status: To be done [during an EWOF]

Subtask 1d: Determine the growth kinetics and metastatic site preference of cell lines characterized in Aim 2, Major Task1, Subtask 1b using IVIS imaging.

Status: To be done [during an EWOF]

Subtask 1e: Evaluate tissues at endpoint of study in Aim 2, Major Task1, Subtask 1d. Use appropriate methods to quantify metastasis formation (i.e. q-RTPCR, histology, histomorphometry, etc.).

Status: To be done [during an EWOF]

Subtask 1f: Use vectors described in Aim1 Major Task 1, subtasks 1f and 1g to modulate MKK4 protein levels in C4-2B-luc2-FDIC cell lines.

Status: To be done [during an EWOF]

Subtask 1g: Characterize cell lines generated in Aim 2, Major Task 1, Subtask 1f: 1) confirm MKK4 depletion, and 2) determine *in vitro* growth rates.

Status: To be done [during an EWOF]

Subtask 1h: Validate the distribution of cell lines characterized in Aim 2, Major Task 1, Subtask 1g using IVIS imaging.

Status: To be done [during an EWOF]

Subtask 1i: Determine the growth kinetics and metastatic site preference of cell lines characterized in Aim 2, Major Task1, Subtask 1g using IVIS imaging.

Status: To be done. Year 5, Month 9-12

Subtask 1j: Evaluate tissues at endpoint of study in Aim 2, Major Task1, Subtask 1i. Use appropriate methods to quantify metastasis formation (i.e. q-RTPCR, histology, histomorphometry, etc.)

Status: To be done. Year 5, Month 9-12

KEY RESEARCH ACCOMPLISHMENTS:

- Constructed and characterized luciferase-tagged CWR22Rv1 and C4-2B cell lines that allow for quantitative assessment of cancer cell localization and dissemination.

- Implemented and validated quantitative *in vivo* imaging technique for quality control of injection, lodging/dissemination, and growth of luciferase-tagged prostate cancer cells.

- Validated CWR22Rv1 bone metastasis formation using histologic assessment of luciferase-positive lesions detected by *in vivo* imaging.

- Developed a sensitive and specific PCR-based approach to quantitate the number of prostate cancer cells within the bone, or other tissue microenvironments.

- Determined the limit of detection of the PCR-based method to quantitate the number of prostate cancer cells within the bone.

REPORTABLE OUTCOMES:

None

CONCLUSIONS AND FUTURE DIRECTIONS: CONTINUING EFFORTS: A continuing challenge to researchers in the prostate cancer metastasis community is having models that closely parallel clinical disease. The CWR22Rv1 and C4-2B cell lines are improvements over many of the "work horse" lines, such as PC3, DuPro, and LNCaP. For our work, we were especially interested literature references and meeting discussions that purport bone-metastatic ability of the CWR22Rv1 and C4-2B cell lines.   Our preliminary studies show that the CWR22Rv1 cells make bone metastases after intracardiac injection. Thus it is anticipated that we can select for sublines with enhanced metastatic ability to which will enable quantitative

assessment of the effect of modulating MKK4 levels on metastasis formation. We will also work toward optimizing the C4-2B cell line, also it may be more difficult as it has slower *in vivo* growth kinetics and a lower take rate. Having a sensitive and specific method for quantitating human prostate cancer cells within the mouse bone is also critical for our studies of metastatic colonization. Thus, taken together our efforts this year have yielded the tools and established approaches that we need to move forward on our proposed studies. We are very excited about the progress of this research and look forward to sharing our results in our next report.

REFERENCES:

None

APPENDICIES:

None