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14. ABSTRACT

Our proposal tests the idea that Core Binding Factor (CBF) should be targeted to achieve curative effects on AIPC. CBF is composed of a non-DNA binding component termed CBF β and one of the DNA binding RUNX (RUNX1-3) proteins. CBF is a master transcriptional regulator of stem cell self-renewal, differentiation and homeostasis; defects in these regulatory pathways are the hallmark of all cancers, including prostate cancer. The purpose of this research is to test whether CBF contributes to the malignant phenotype of AIPC and to define gene expression changes associated with alterations to CBF. We have completed gene expression analysis of CBFbeta knockdown prostate cancer cells and find that malignant reversion is associated with altered expression of several microRNAs (miRNAs) including miRNA-663, a tumor suppressor in gastric cancer.

15. SUBJECT TERMS

Core Binding Factor beta, microRNAs, malignant reversion, prostate cancer

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Prostate cancer is the most common malignancy in men and is the second leading cause of male cancer-related deaths in the United States. The events that lead to the initiation and progression of the disease are poorly understood, although androgen and the androgen receptor (AR) play a critical role. At diagnosis, most prostate cancers are dependent upon androgen for growth; however, androgen ablation treatment can result in the selection of androgen-independent prostate cancers (AIPC)(Brinkmann and Trapman 2000). AIPC remains the primary cause of mortality in these patients. Treatment strategies for AIPC include radiation and chemotherapy, but often these modalities fail to control tumor growth(Brinkmann and Trapman 2000). Our proposal tests the idea that Core Binding Factor (CBF) should be targeted to achieve curative effects on AIPC. CBF is composed of a non-DNA binding component termed CBF β and one of the DNA binding RUNX (RUNX1-3) proteins. CBF is a master transcriptional regulator of stem cell self-renewal, differentiation and homeostasis; defects in these regulatory pathways are the hallmark of all cancers, including prostate cancer(Coffman 2003). The regulatory roles played by CBF place it at the pinnacle of transcriptional cascades central to tumor formation. The purpose of this research is to test whether CBF contributes to the malignant phenotype of AIPC and to define gene expression changes associated with alterations to CBF. This proposal will also develop a high-throughput DNA binding assay to screen chemical libraries with the intent of defining small molecule inhibitors of CBF for use in preclinical studies. We proposed the following specific aims.

Specific Aim 1. Determine the effects of CBF β knockdown on the tumorigenicity of androgen insensitive prostate cancer cells using human prostate tumor xenograft models and identify gene expression changes associated with CBF β knockdown.

- 1.1. Determine the effect of CBF β knockdown on growth and metastasis of PPC1, DU145 and LNCaP C4-2 B4 xenograft tumors
- 1.2. Identify gene expression changes associated with CBF β knockdown.

Specific Aim 2. Identify small molecule inhibitors of CBF

- 2.1. Develop a microtiter plate based DNA binding assay for CBF
- 2.2. Identify small molecule inhibitors of CBF

Body of Text

The following narrative describes our research accomplishments in relation to the statement of work (SOW). Excerpts from the SOW appear in **bold**. Figure legends are *italicized*. References to the 09/10 update are underlined.

Aim 1.1. Determine the effect of CBF β knockdown on growth and metastasis of xenograft tumors.

Task 1. Determine the effect of CBF β knockdown on growth and metastasis of human prostate xenograft tumors (specific aim 1).

1a. Establish stable luciferase expressing androgen insensitive prostate cancer cell lines that also express control non-target shRNA and CBF β shRNA from lentiviral vectors. (months 1-12)

Task 1a was finished on time and reported in last years update.

1b. Establish xenograft assays using prostate cancer cell lines and monitor tumor growth and metastasis using a variety of methods, including *In-Vivo* Luminescence And Fluorescence Imaging (IVIS). (months 2-24)

Task 1b is mostly finished and was reported in last years update. We do have yet to perform cardiac injections.

1c. Inject lentivirus engineered to express control non-target shRNA and CBF β shRNA into pre-established xenograft tumors in SCID/Beige mice and monitor tumor growth and metastasis using IVIS. (Months 2-24)

At the last annual update we were making large batches of virus for purification to perform this task. We had difficulty deriving consistently high titers and it was technically difficult to get a titer that we were confident in due to the virus being sticky (thus serial dilutions of the virus for titrating purposes was difficult). This was an important issue for this experiment since it was imperative that the non-target and CBF β -2 shRNA viruses be of the same titer when injecting preformed tumors. To get around this issue we decided to turn to an alternative method that we had discussed in the grant proposal. We have moved the non-target and CBF β -2 shRNAs into the lentiviral vector pTETpuro, which we purchased from Addgene. pTETpuro drives expression of the shRNAs under tight control of a doxycycline-inducible promoter. We have made virus, infected the prostate cancer cell lines and are now deriving cell lines that we can inject into mice to complete this task. In this experimental design, the cell lines will be injected into the mice as usual, tumors will be allowed to grow for a week, and then we will induce expression of the shRNAs by feeding doxycycline to the mice. The remainder of the experiment will be performed exactly as written in the proposal.

Task 2. Identify gene expression changes associated with CBF β knockdown (specific aim 1).

2a. Extract RNA from cell lines expressing control non-target shRNA or CBF β -2 shRNA. (Months 13-24)

2b. Perform microarray analysis using Agilent human 4 X 44 K microarrays, representing 41,000+ unique human genes and transcripts. (Months 20-30)

In our preliminary data section of the proposal we discussed a preliminary gene expression analysis using total RNA isolated from three independent cultures of PPC1 cells expressing control non-target and CBF β shRNAs. The gene expression profiles for the PPC1 cells were analyzed using the Agilent Whole Genome Array and completed on the first year of the grant (and reported in the first years update).

To complete this analysis, we obtained gene expression profiles for a second androgen insensitive prostate cancer cell line (PC-3) expressing the non-target shRNA or the CBF β -2 shRNA using the Agilent Whole Genome Array. The results for 545 genes with altered expression are shown in heat map format (Fig 1).

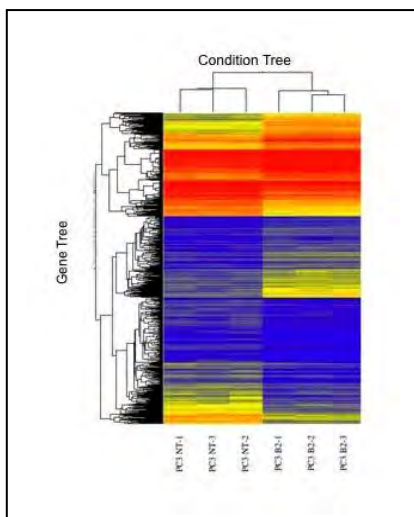


Fig 1. Heatmap for differentially expressed genes. Genes found to be differentially expressed (> two-fold, $p < 0.05$) in three independent PC-3 cell pools expressing the CBF β shRNA (shRNA CBF β #1, #2,

and #3) versus three independent cell pools expressing the non-target shRNA (shRNA NT #1, #2, and #3) are shown. The results were normalized to the median gene expression across six samples (>2 fold, $p<0.05$). Red indicates high expression, yellow indicates median expression, and blue/gray indicates low expression. The gene array was performed using the Agilent whole genome 4X44K arrays, and the results were analyzed using the Agilent Feature Extraction and GeneSpring GX v7.3.1 software packages.

An examination of the gene array data derived from the PPC1 and PC-3 cells uncovers nine genes with increased expression in both cell lines when CBF β is knocked-down and six genes whose expression is decreased. Examples of genes whose expression is altered in both the PPC1 and PC-3 cell lines are shown in figure 2.

Fig. 2. Examples of genes whose expression is altered by CBF β knockdown in both PC-3

Increased Expression			
IFI27 interferon, alpha inducible protein 27		3.85 (6.45E-03)	17.16 (2.63E-03)
IFITM1 interferon induced transmembrane protein 1		2.16 (2.79E-02)	3.73 (2.19E-03)
IFI44L interferon induced protein 44-like		1.72 (2.46E-02)	2.72 (5.76E-02)
Decreased Expression			
ALDH3A1	aldehyde dehydrogenase 3	0.41 (5.65E-03)	0.47 (2.37E-02)
CFL2	cofilin 2	0.32 (1.91E-02)	0.31 (1.29E-02)
UGT1A6	UDP glucuronosyltransferase 1 family	0.51 (9.20E-03)	0.42 (2.24E-03)

and PPC1 cell lines. Shown are three genes whose expression increases upon CBF β knockdown and three genes whose expression decreases upon CBF β knockdown.

The gene names are followed by the relative change of expression and significance in PC-3 (first set of numbers) and PPC1 cell lines.

The Agilent whole genome array examines the expression of genes that code for protein. To complete our examination of transcriptional changes associated with CBF β knockdown we examined microRNA (miRNA) gene expression. miRNAs are active small RNAs that are not translated but do alter target gene expression via effects on mRNA stability or translation(Kwak, Iwasaki et al.). To do this, we extracted total RNA from non-target and CBF β -2-expressing PPC1 and PC-3 cells using the mirvana RNA isolation kit (which captures small RNAs better than conventional isolation kits) and sent the RNA to LC Sciences (Houston, Texas) for subsequent analysis. LC Sciences provides genome-wide miRNA expression analysis using labeled probes. In PC-3 cells 26 miRNAs exhibited altered expression while in the PPC1 cell line 29 miRNAs showed altered expression (Fig. 3). For example, in the PC-3 cell line, miR-1246, a p53-regulated microRNA, expression increased nearly 16 times in cells knocked-down for CBF β -knockdown while miR-1915 increased 16 times in PPC1 cells knocked-down for CBF β (Fig.3)(Zhang, Liao et al.).

Fig. 3. Changes in miRNA expression in prostate cancer cell lines knocked-down for

A.

No.	Reporter Name	p-value	Group 1 PC3-NT Mean	Group 2 PC3-R2 Mean	Log2 (G2/G1)
80	hsa-miR-1245	7.70E-05	1,453	19,827	3.77
778	hsa-miR-4333	9.93E-05	294	1,983	2.53
367	hsa-miR-23a	6.50E-05	11,456	2,041	-2.49
311	hsa-miR-25a	7.86E-05	394	267	-1.90
274	hsa-miR-1915	1.09E-04	607	2,276	1.91
372	hsa-miR-24	7.79E-04	4,034	949	-2.09
416	hsa-miR-3656	2.14E-04	1,096	4,077	1.90
187	hsa-miR-1469	5.70E-04	167	679	-2.03
519	hsa-miR-1196	1.09E-04	891	3,960	2.00
18	hsa-miR-7c	1.26E-03	1,212	1,205	0.14
313	hsa-miR-21	1.43E-03	5,536	12,711	1.20
36	hsa-miR-1275	1.76E-03	4,340	1,741	-1.63
185	hsa-miR-222	1.89E-03	6,106	2,837	-1.11
353	hsa-miR-221	1.89E-03	1,931	889	-1.12
20	hsa-miR-100	1.01E-03	5,406	1,897	-1.52
1151	hsa-miR-762	2.81E-03	99	867	-3.12
369	hsa-miR-23b	2.99E-03	4,394	1,944	-2.07
249	hsa-miR-16	3.17E-03	1,614	696	-1.21
4	hsa-miR-7c	3.07E-03	3,145	6,299	-0.44
1097	hsa-miR-438	3.05E-03	1,844	6,395	-1.73
122	hsa-miR-1275	4.43E-03	330	1,879	-2.46
596	hsa-miR-3176	4.49E-03	269	648	-1.30
396	hsa-miR-29a	4.91E-03	8,498	2,073	-2.04
580	hsa-miR-29b1	5.17E-03	729	812	-1.79
456	hsa-miR-3963	7.43E-03	12,364	16,327	-0.42
6	hsa-miR-7c	8.99E-03	1,726	3,295	-0.93
742	hsa-miR-923-3p	9.69E-03	513	492	-0.30

B.

No.	Reporter Name	p-value	Group 1 PPC1-NT Mean	Group 2 PPC1-R2 Mean	Log2 (G2/G1)
274	hsa-miR-1915	7.05E-05	49	3,495	4.06
1157	hsa-miR-762	7.14E-05	49	521	3.68
779	hsa-miR-4281	7.50E-04	639	1,653	-1.39
288	hsa-miR-2881	8.17E-04	82	612	-2.93
1114	hsa-miR-663	8.95E-04	16,036	5,397	-1.57
519	hsa-miR-3156	9.73E-04	457	4,047	-3.15
629	hsa-miR-5963	1.07E-04	1,147	36,179	-2.39
325	hsa-miR-21	7.78E-04	4,403	1,513	-1.54
321	hsa-miR-29a	8.79E-04	1,854	561	-1.49
1007	hsa-miR-438	9.09E-04	461	4,905	-1.17
80	hsa-miR-124b	2.83E-04	211	45,145	-2.74
232	hsa-miR-17	3.19E-04	1,813	535	-1.72
533	hsa-miR-3206	7.72E-04	665	372	-0.84
132	hsa-miR-1273	7.47E-04	399	1,386	-2.18
616	hsa-miR-3859	7.33E-04	553	4,141	-2.93
96	hsa-miR-125b	9.03E-04	2,958	1,516	-0.96
1141	hsa-miR-726	1.93E-04	3,085	6,315	-1.94
10	hsa-miR-7c	2.05E-04	2,393	949	-1.65
79	hsa-miR-1584	2.09E-04	1,641	358	-2.26
6	hsa-miR-7c	2.09E-04	9,832	2,830	-1.80
227	hsa-miR-130b	2.36E-04	1,111	184	-1.77
4	hsa-miR-7c	2.40E-04	6,949	3,682	-0.84
8	hsa-miR-7c	2.46E-04	7,818	2,468	-1.72
430	hsa-miR-31	6.47E-04	622	1,103	-0.83
532	hsa-miR-3308	7.47E-04	999	607	-0.61
20	hsa-miR-100	7.51E-04	4,332	1,324	-1.71
298	hsa-miR-29a	7.73E-04	949	398	-1.88

CBF β . A. PC-3 cells expressing NT and CBF β shRNAs were examined for altered expression of 1000 miRNA genes. B. miRNA gene expression in PPC1 cells expressing NT and CBF β shRNAs. In both A and B panels, the name of the miRNA is given

(reporter name; the pre-fix “hsa” indicates that we tested expression of human miRNA genes) while p-values are recorded in red. The mean expression of each miRNA whose expression is significantly altered when CBF β expression is knocked-down is shown for both control and knockdown cells. Gene expression changes are presented as Log2 (group2/group1).

Nine miRNAs exhibited changes in expression in both prostate cancer cells lines (Fig. 4). For example, miR-663 expression is increased approximately 8 fold in PPC1 and PC-3 cells knocked-down for CBF β . miR-663 is a tumor suppressor associated with retinoic acid induced cellular differentiation(Pan, Hu et al.). Thus, Core Binding Factor may directly regulate the expression of miR-663. These studies **complete the major aspects of task 2** by identifying gene expression changes associated with CBF β knockdown and place CBF upstream of miR-663, an important tumor suppressor.

Fig. 4. miRNAs whose expression changes as a result of CBF β knockdown in both PC-3 and PPC1 cell lines.

No.	Reporter Name	p-value	Group 1 NT Mean	Group 2 B2 Mean	Log2 (G2/G1)
375	hsa-miR-25	7.30E-03	801	495	-0.70
197	hsa-miR-1469	1.06E-01	110	445	1.99
20	hsa-miR-100	3.03E-02	4,879	1,611	-1.61
122	hsa-miR-1275	4.11E-03	320	1,602	2.31
1151	hsa-miR-762	5.16E-02	74	744	3.40
29	hsa-miR-106a	5.17E-02	1,003	229	-2.03
232	hsa-miR-17	5.47E-02	1,133	358	-1.59
1124	hsa-miR-663	6.50E-02	53	527	3.32
321	hsa-miR-20a	8.55E-02	1,424	974	-1.67

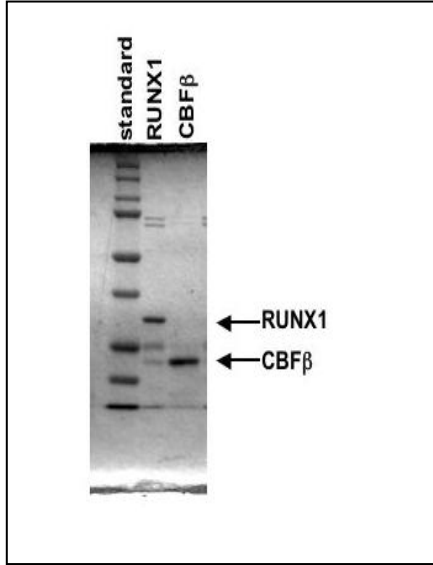
Task 3. Identify Small molecule inhibitors of CBF β using high throughput screening (specific aim 2).

3a. Develop a 96 well microtiter-based fluorescent DNA binding assay for CBF to be used in high-through put screening of chemical libraries. (months 20-30)

3b. Screen for small molecule inhibitors of CBF β or CBF. (months 25- 36)

3c. Confirm the activity of small molecules using electrophoretic mobility shift analysis (EMSA) and transient transfection and transcription assays to demonstrate that putative small molecules inhibitors display physical and functional inhibition of CBF β and CBF activity *in vivo*. (Months 25-36)

We have begun this task. We cloned CBF β and a shortened form of RUNX1 into



bacterial expression vectors in frame with a 6Xhis tag. These proteins were produced in E.coli, purified and resolved on SDS-page (Fig. 5). These proteins were then tested by EMSA for their ability to bind to DNA (not shown). These proteins are being used to develop a high throughput DNA binding assay to screen for small molecule inhibitors.

Fig. 5. His-tagged and purified proteins. Proteins were isolated from bacteria, purified using nickel resin (Bio-Rad) and resolved on 10% SDS-PAGE and then stained with Coomassie blue. Arrows indicate full length protein.

Key Research Accomplishments

- ✓ Completed analysis of gene expression changes in androgen insensitive prostate cancer cell lines in response to CBF β knockdown.
- ✓ Identified an important microRNA (miR-663) tumor suppressor down stream of CBF in prostate cancer cell lines.
- ✓ Prepared recombinant protein to use for drug screens
- ✓ Developed shRNA lenti constructs for doxycycline inducible expression in animal studies.

Reportable Outcomes

1. Manuscripts

This article was in press at the time of the last update; it is now published.

1. J. Nathan Davis, Donna Rogers, Lisa Adams, Thomas Yong, Jette S. Jung, Bing Cheng Katie Fennell, Erkut Borazanci, Yara W. Moustafa, Amanda Sun, Runhua Shi, Jonathan Glass , J. Michael Mathis, B. Jill Williams and Shari Meyers*. Association of Core Binding Factor β with the Malignant Phenotype of Prostate and Ovarian Cancer Cells. (2010). J Cell Physiol. 2010 Nov;225(3):875-87. *

*The journal's editor highlighted this article.

2. Yong T, Sun A, Henry MD, **Meyers S** and Davis, JN. Down regulation of CSL activity inhibits cell proliferation in prostate and breast cancer cells. J. Cell Biochem. Epub ahead of time.

2. Presentations

1. **Meyers S.**, Davis JN, and Williams BJ. Core Binding Factor Is Required For The Malignant Phenotype Of Prostate Cancer Cells, Innovative Minds in Prostate Cancer Today (IMPACT), Orlando, Fla. March 2011.

2. **Meyers S.** Core Binding Factor; Oncogenic Roles. Translational Urology Research Group (TURG), LSUHSC-S April 18, 2011

3. Informatics

The gene array data discussed in this annual report will be deposited in NCBI's Gene Expression Omnibus, (<http://www.ncbi.nlm.nih.gov/geo/>).

4. Products

We have produced the following reagents;

- Bacterial strains expressing his-tagged RUNX1 and CBF β for use in high throughput screening.
- Lentivirus (pTETpuro) expressing the non-target and CBF β shRNAs from a doxycycline inducible promoter.

Conclusions

To investigate the contribution of CBF to the malignant phenotypes of prostate cancer cells, we reduced CBF β expression using CBF β -specific shRNA, and tested the effect of CBF β knockdown on gene expression patterns in an effort to understand the mechanism behind the malignant reversion. Extensive gene expression studies reveal that CBF is upstream of miR-663, a microRNA that is known to function as a tumor suppressor. These data suggest that CBF promotes malignancy via repression of a small number of tumor suppressing microRNAs, including miR-663. Thus, loss of CBF (via knockdown of CBF β , may revert malignancy by allowing expressing of tumor suppressing miRNAs. Such RNAs could themselves be used as therapeutic intervention.

SO WHAT?

We are accumulating mounting evidence that CBF β and the RUNX proteins can act as oncogenes in solid tumors and alterations to CBF are continuing to be described in primary human tumor of epithelial origin. Our data demonstrates (and is the first to do so) that CBF β is required for AIPC tumor growth *in vivo* in animal models. These data strongly suggests that CBF β should be targeted by novel therapeutics in AIPC and other cancers. As we continue to understand the mechanism by which CBF controls malignancy, we will continue to uncover “drugable” proteins, as well as, small RNAs, such as miR-663 which can be developed as therapies.

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- Kwak, P. B., S. Iwasaki, et al. "The microRNA pathway and cancer." Cancer Sci **101**(11): 2309-15.
- Pan, J., H. Hu, et al. "Tumor-suppressive mir-663 gene induces mitotic catastrophe growth arrest in human gastric cancer cells." Oncol Rep **24**(1): 105-12.
- Zhang, Y., J. M. Liao, et al. "p53 downregulates Down syndrome-associated DYRK1A through miR-1246." EMBO Rep **12**(8): 811-7.