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PRINCIPAL INVESTIGATOR: Beatrice S. Knudsen, M.D., Ph.D.

CONTRACTING ORGANIZATION: Fred Hutchinson Cancer Research Center
Seattle, WA 98109-1024

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Beatrice S. Knudsen, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Fred Hutchinson Cancer Research Center
Seattle, WA 98109-1024

E-Mail: bknudsen@fhcrc.org

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The hypothesis of this grant proposal is that androgen-ablative therapy paradoxically increases growth factor secretion from bone stromal cells and that this may stimulate the growth of prostate cancer metastases. We proposed to test this hypothesis using IL-6 and HGF/SF as paradigms of androgen regulated growth stimulators. However, two separate experimental approaches did not demonstrate an increase in IL-6 or HGF/SF gene expression in androgen-deprived mouse bone. As a result of this initial negative investigation in task 1 we decided to take a broad approach to identify genes up-regulated by androgen deprivation in mouse bone. This approach has revealed multiple genes overexpressed upon androgen-ablation that may regulate growth factor systems involved in the crosstalk between the bone environment and prostate cancer cells.

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SUBJECT: Annual Report for Award Number DAMD17-02-1-0159

"Hepatocyte Growth Factor and Interleukin-6 in Prostate Cancer Bone Metastasis"

INTRODUCTION: The hypothesis of this grant proposal is that androgen-ablative therapy paradoxically increases growth factor secretion from bone stromal cells and that this may stimulate the growth of prostate cancer metastases. We proposed to test this hypothesis using Interleukin-6 (IL-6) and hepatocyte growth factor/scatter factor (HGF/SF) as paradigms of androgen regulated growth stimulators. However, two separate experimental approaches did not demonstrate an increase in IL-6 or HGF/SF gene expression in androgen-deprived mouse bone. As a result of this initial negative investigation in task 1 we decided to take a broad approach to identify genes up-regulated by androgen deprivation in mouse bone. This approach has revealed multiple genes overexpressed upon androgen-ablation that may regulate growth factor systems involved in the crosstalk between the bone environment and prostate cancer cells.

BODY:

Summary of work completed in the first year of award: The first task (months 1-12) was "To determine whether hepatocyte growth factor/scatter factor synthesis by bone stromal cells is regulated by androgen". To test this hypothesis we conducted (1) measurements of HGF/SF m-RNA by RT-PCR in normal and orchiectomized mice and did not find an increase in HGF/SF upon androgen withdrawal. However, we detected increased Met (HGF/SF receptor) expression in orchiectomized mice by Western blotting.

For this task, we developed a. a method to extract intact RNA from mouse bone; b. a semi-quantitative RT-PCR to measure HGF, IL-6, th ebone specific transcription factor: Cbfa-1, and osteocalcin; Using this methods, we were not able to detect significant differences in gene expression between normal and orchiectomized bone.

In **task 2** (months 12-36), we planned "To determine whether IL-6 regulates HGF/SF synthesis in the murine bone stroma and in cultured human bone cells". Since we were not able to demonstrate up-regulation of IL-6 or HGF/SF RNA in orchiectomized mice, we did not move to studying the effects of IL-6 in mice. The focus of the project and the critical question in the treatment of patients with androgen-ablative therapies are the effects of androgen-ablation on bone cells. To address this question, we moved to task 4 of the proposal.

In **task 3** (months 24-36), we proposed "To determine the role of IL-6 as a mediator of the orchiectomy triggered HGF/SF synthesis". In the next year, we will determine the effects of elevated serum IL-6 levels on serum HGF/SF levels in mice.

Task 4: DNA array of mouse bone and bone marrow m-RNA, Months: 24-36

Goal of task 4: Identifying androgen regulated and IL-6-regulated growth factors in mouse bone.

Reasons for proceeding to task 4:

The hypothesis of this grant proposal is that androgen-ablative therapy increases the synthesis of growth factors in mouse bone, either directly, or indirectly through IL-6. Further, we reasoned based on published reports that one of the androgen-suppressed growth factors could be HGF/SF. However, experiments in task one did not support androgen regulation of HGF/SF in C57BL6 mice. Therefore we decided

1. to survey the entire transcriptome of mouse bone for its androgen regulation using a prostate and bone-specific c-DNA array platform

2. to translate and confirm array results directly to human tissue samples.

The move to the Fred Hutchinson Cancer Research Center has opened novel translational avenues that did not exist at Cornell Medical College, when this grant was written. We decided to take advantage of novel technological advances that are available within the environment at FHCRC to address an important biological question, the androgen-regulation of bone. Through the prostate SPORE program, human tissues from prostate cancer metastases are collected by a rapid autopsy service team. Directly translating the results from the mouse models to human tissues will greatly accelerate the identification of therapeutically relevant targets that may improve the treatment of metastatic disease in patients with prostate cancer.

Based on the hypothesis that orchiectomy triggers the synthesis of growth factors in the bone, we undertook a broad analysis of changes in the bone-associated transcription profile after androgen-deprivation using PEDB arrays[1]. 4 mouse pairs were used in this study.

Experimental methods used in Task 4:

Isolation of mouse bone and bone marrow: C57BL/6J mice were purchased from the Jackson Laboratory at 8 weeks of age. Mice were either castrated or sham-operated at week 7. Mice were kept for 10 weeks after shipment at FHCRC before euthanasia and tissue collection. Femurs, tibiae and humeri are collected and cleaned from attached muscles and tendons. Bone marrow is separated from the bone by flushing several times with phosphate buffered saline and pelleted by centrifugation at 3000 rpm for 5 minutes. Bone marrows for RNA extraction are stored in TRIZOL™ at –80°C. Bones are snap frozen in liquid nitrogen and stored at –80°C.

Mouse prostate cDNA arrays (mPEDB) as a platform for measuring gene expression in bone. Arrays of DNA molecules offer a high-throughput, quantitative method for measuring differences in RNA expression levels between treated and untreated mice. Thousands of genes can be assessed simultaneously and subjected to a rigorous statistical analysis. The transcriptome of the mouse prostate has been expanded through sequencing of prostate-derived expression sequence tags (EST). 5000 prostate genes are represented within the (mouse Prostate Expression Data Base) mPEDB array, in addition to another 5000 random genes[1]. We reasoned that using a mouse prostate array platform is a good starting point for analyzing androgen-regulated expression changes in bone, since (1) both tissues are regulated by androgen, (2) prostate cancer favors growth in bone, suggesting similarities between the bone and prostate microenvironments, and (3) up-to-date annotation of genes on the array is available through the bio-informatic team of the Nelson lab.

RNA isolation and labeling:

Long bones from normal and orchiectomized mice were separated into bone and bone marrow. RNA was extracted from bone marrow or pulverized bone using TRIZOL® (Invitrogen). The RNA is further cleaned and DNase-I treated using RNeasy® kit (Qiagen®). Quality of purified RNA was evaluated using $OD_{260/280}$ ratio and an Agilent 2100 bioanalyzer (Agilent Technologies). Sixty to 100μg of RNA was extracted from bone marrow and 5 to 10μg from the bone of each mouse. Since RNA from bone was not sufficient for array studies, 2μg RNA were amplified in one round using RiboAmp® RNA Amplification Kit (Acturus), which generated approximately 40 μg bone-derived amplified RNA (aRNA). RNA and aRNA were stored in aliquots at -80 °C. Total RNA was labeled with Cy3 or Cy5 and competitively hybridized to the mPEDB array.

Array hybridization:

Either 30 μg of total RNA or 2 μg of aRNA are reverse transcribed with oligo-dT primers with amino-allyl-dUTP added using SuperScript® II, RNase H, and Reverse Transcriptase. Amino-allyl-cDNA are labeled with Cy3 or Cy5 fluoro (Amersham) and hybridized to array at 65°C for 16 hours in SSC buffer with poly-A blockade. To exclude dye bias, a dye-swap is conducted for each paired sample. Fluorescent images are

collected for both Cy3 and Cy5 by using a GenePix 4000A fluorescent scanner (Axon Instruments). Hybridized signals are extracted and analyzed using GENEPIX 3.0 microarray analysis software.

Representative array results of bone marrow and bone, comparing androgen-deprived and sham-operated mice, are shown as in scatter plots in Figure 1 and Figure 2. The individual spots of the scatter plot lie on a curve with minimal concavity, as is found in spotted c-DNA array of high quality. The outliers are highly reproducible amongst the four mouse pairs and consist of 108 differentially expressed genes. The scatter plots indicate that the differences in the transcriptomes of the bone and bone marrow in the castrated and sham-operated mice are quite small as most outlier genes are less than a two-fold difference in the castrated compared to sham-operated mice. The difference in expression level was confirmed for Insulin growth factor binding protein (IGFBP)-5 and SRY Box transcription factor, Sox-4, Figure 1. Scatter plot of a representative mouse bone marrow array. Each spot represents the relative hybridization of Cy3 and Cy5 labeled probes to a single gene on the array slide. X-axis is the background corrected fluorescent intensity of the Cy5 labeled cDNA derived from RNA in sham-operated mouse bone marrow and Y-axis is the background corrected fluorescent intensity of the Cy3 labeled cDNA derived from RNA in castrated mouse bone marrow.

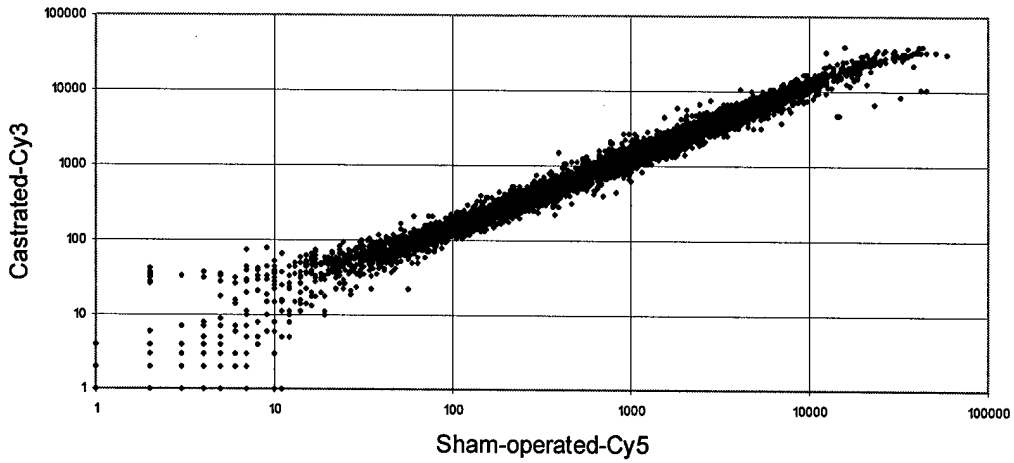

Figure 1. Scatter plot of a representative mouse bone marrow array. Each spot represents the relative hybridization of Cy3 and Cy5 labeled probes to a single gene on the array slide. X-axis is the background corrected fluorescent intensity of the Cy5 labeled cDNA derived from RNA in sham-operated mouse bone marrow and Y-axis is the background corrected fluorescent intensity of the Cy3 labeled cDNA derived from RNA in castrated mouse bone marrow.

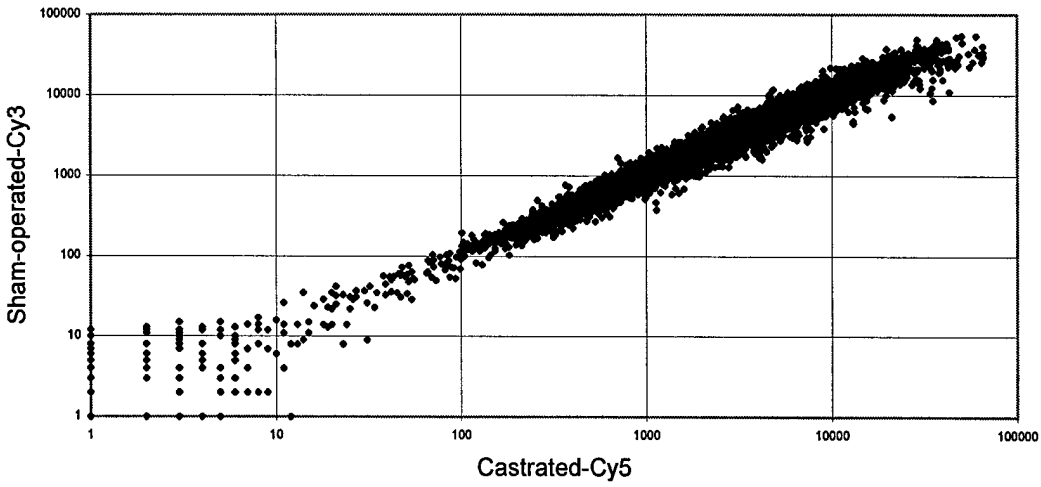

Figure 2. Scatter plot of a representative mouse bone array result. Each spot represents a gene probe on the array slide. X-axis is the background corrected fluorescent intensity of the Cy5 labeled cDNA derived from RNA in castrated mouse bone and Y-axis is the background corrected fluorescent intensity of the Cy3 labeled cDNA derived from RNA in sham-operated mouse bone. The fluorescence is much enhanced using amplified RNA (aRNA) compare with un-amplified RNA (figure 1).

Analysis of array data:

For each spot on the array, the Cy3 or Cy5 expression signal is defined as subtracting the median foreground with the median background. Flagged and poorly hybridized spots with intensity levels less than 2 standard deviations above the local background are excluded from analysis. Array data are normalized and analyzed using GeneSpring® 5.0 software (Silicon Genetics). Intensity dependent locally weighted regression scatter plot smoothing (Lowess) is applied to eliminate dye-related artifacts in two-color system. Measurements on each chip are also normalized to 50 percentile of the value on the chip to allow inter-array comparison. To evaluate significantly differentially expressed genes, a p-value of 0.05 in t-test is used as a cut-off.

Results of c-DNA array analysis contrasting gene expression in bones of orchiectomized and sham-operated mice.

Data analysis revealed 108 genes that are differentially expressed in androgen-deprived and normal bone. 98 were up-regulated in orchiectomized bone and 10 were down-regulated. Annotation was possible for 60/108 genes. 32 genes were more than 1.5-fold increased. The up-regulated genes were functionally classified into three groups: group-I is linked to tumor growth in bone (12 genes, **Table 1**), group-II is connected to the B-cell hyperplasia that occurs with androgen-deprivation (10 genes) and group-III is unrelated (10 genes). The overexpressed genes facilitate activation of specific growth-regulatory mechanisms. Several genes were elevated in bone and bone marrow, suggesting that they may be regulated in bone stromal cells (**Table 2**). Some of the genes (IGFBP-5, Sox-4) are normally expressed in mesenchymal cells.

Table 1. Genes up-regulated by androgen-deprivation in mouse bone that potentially relate to the stimulation of growth and invasion of metastatic prostate cancer.

	Genes (abbreviation)	Functional category	Effects on metastatic tumor
1	IGFBP5[2]	increases IGF activity, unique receptor	increases tumor growth
2	PAF acetylhydrolase (Pafah1b3)[3]	Decreases pro-apoptotic PAF	stimulates survival and invasion of tumor cells
3	SRYbox gene 4 (Sox4)[4]	transcription factor, patterning	Mesenchymal growth factors
4	interleukin 7 receptor[5]	IL7R triggers IL-6 synth. by BM stroma	Incr. tumor growth through IL-6
5	serine (or cysteine) protease Inhibitor ,cladeH (Serpinh1)[6]	= hsp47, stablizes and increases collagen secretion from stromal cells	stimulates tumor growth through increasing cell adhesion
6	Thrombomodulin[7]	receptor for activation of protein C	Incr. growth through active prot. C
7	Clusterin (Clu)[8]	secreted protein stimulating cell survival	stimulates tumor cell survival
8	Amyotrophic lateral sclerosis 2 (ALS2)[9]	GEFfor Rab and Rho, regulating vesicular transport and cytoskeleton	promotes secretion of GFs from bone
9	Platelet factor 4 (PF4)[10]	binds FGF and stabilizes activity	Incr. tumor growth through FGF
10	Cxcl12, chemokine lig.[11]	binds to CXCR4 on tumor cells	tumor cell activation and invasion
11	cAMP-phosphodiesterase 4B (PDE4B)[12]	Decr. cAMP, promotes cytokine secretion, PDE4B inhibitor effective in MM	promotes tumor growth
12	Osteonectin/SPARC[13]	ECM protein stim. migration and osteoblast differentiation	tumor invasion and tumor-bone interactions

Abbreviations: IGFBP: insulin growth factor binding protein; PAF: platelet activating factor; Sox 4: SRY Box gene 4, GF: growth factor.

Other genes were significantly increased (>1.5 fold) and are related to the B-cell expansion, triggered by androgen-ablative therapy. These are: The pre-B lymphocyte gene 3 (VpreB3), a B-cell specific transcription factor [14]; Fc receptor related protein X (FcRX), a cell surface receptor for B-cell activation [15]; immunoglobulin-associated beta (Igb); BTB and CNC homology 2 (Bach2), a B-cell specific transcription factor [16]; heavy chain of IgM (Igh6); SH3 binding kinase (Sbk)[17], forkhead box 1 (Foxo1) and B-lympoid kinase (Blk) [18,19]; lymphoid-restricted membrane protein (Lrmp) [20]; and the transcription factor E2F2a [21]. Several significantly overexpressed genes were of uncertain relationship to tumor growth. These include myosin light chain alkali cardiac atria[22], stearoyl-Coenzyme A desaturase 1 (Scd1)[23], erythrocyte protein band 4.1-like 4b (Epb4.1l4b)[24], C-type lectin-like receptor 2[25] and f-box and leucine-rich repeat protein 12 (Fbxl12)[26].

Table 2. Comparison of up-regulated genes in androgen-deprived bone marrow and bone.

	Gene name (abbreviation)	fold increase in bone marrow	P-value in bone marrow	fold increase in bone	P-value in bone
1	insulin-like growth factor binding protein 5 (IGFBP5)	2.4*	0.001	1.8*	0.010
2	platelet-activating factor acetylhydrolase (Pafah1b3)	2.1*	0.004	1.7*	0.006
3	SRY-box containing gene 4 (Sox4)	2.0*	0.003	1.5*	0.043
4	interleukin 7 receptor	1.9*	0.004	2.1*	0.005
5	serine (or cysteine) proteinase inhibitor,cladeH (Serpinh1)	1.8*	0.011	1.6	0.110
6	Thrombomodulin (Thbd)	1.8*	0.003	1.2	0.149
7	Clusterin (Clu)	1.7*	0.013	1.1	0.681
8	Amyotrophic lateral sclerosis 2 (ALS2)	1.6*	0.032	1.4	0.102
9	Platelet factor 4 (PF4)	1.6*	0.036	1.1	0.596
10	chemokine ligand 12 (Cxcl12)	1.5*	0.029	1.0	0.944
11	cAMP-phosphodiesterase 4B (PDE4B)	1.5*	0.030	1.1	0.686
12	Osteonectin/SPARC	1.5*	0.021	1.5	0.188

Interpretation of data:
Our data show that androgen deprivation causes the up-regulation of genes in the mouse bone.
Several of the genes can be linked to the promotion of tumor growth in the bone environment.
Most notably, IGFBP-5, a positive regulator of insulin-like growth factor (IGF), and Sox-4, a mesenchymal transcription factor that may regulate the fibroblast growth factor axes, are up-regulated after androgen ablation.

These data support our hypothesis that androgen-ablative therapy causes the expression of genes that can potentially promote the growth of prostate cancer metastases in the bone environment.

KEY RESEARCH ACCOMPLISHMENTS:

- We identified the changes in the transcriptome of mouse bone resulting from androgen-ablation using a mouse prostate specific expression array.
- We identified 98 genes that are up-regulated upon castration and 10 that are downregulated.
- We found that the upregulated genes belonged to three broad categories: category 1 is associated with growth factor receptor systems and could contribute to androgen-independent growth of prostate cancer cells in bone (12 genes); category 2 contains genes associated with the B-cell hyperplasia that is induced by castration (32 genes) and category 3 are all other genes and all genes that are not annotated.
- We identified IGFBP5 and Sox-4 as two genes that are up-regulated by androgen and that are associated with the IGF and FGF axis, respectively, making them excellent candidates for further studies of androgen-dependence in the bone/bone marrow stroma and potential stimulators of prostate cancer growth.

REPORTABLE OUTCOMES:

- The data generated in the mouse system were used as preliminary results in a post-doctoral grant application to the prostate cancer DOD program in 2004.
- 2004 AACR abstract # 172

CONCLUSIONS:

Several conclusions related to the statement of work can be drawn from the data obtained in the last year:

1. androgen ablation induced gene expression changes that may regulate several different growth factor systems that may enhance metastatic growth.
2. IL-6 and HGF/SF were not amongst the genes that are significantly up-regulated by androgen ablation.
3. the reason for the lack of up-regulation of IL-6 and HGF/SF could be the mouse strain and we had proposed in the original grant to analyze two separate mouse strains.
4. since resources of human bone metastases have become available, it appears that analyzing expression human tissues would provide more information than analysis of another mouse strain.
5. the results obtained so far provided data for grant applications and will hopefully lead to a publication in the final year of funding.

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172 Androgen ablation increases the synthesis of growth factors in the bone microenvironment.

Chang Xu, Peter S. Nelson, Beatrice S. Knudsen. *Fred Hutchinson Cancer Research Center, Seattle, WA.*

Background. Androgen ablative therapy is the principal treatment for advanced prostate cancer, which in most cases has metastasized to bone. Upon androgen deprivation, metastatic prostate cancers frequently progress to androgen independence, which renders the disease incurable. Resistance to anti-androgen therapy may in part be caused by factors from the bone stroma that promote tumor growth. These include IL-6, hepatocyte growth factor, and TGF-_, since their synthesis is suppressed by steroid hormones and since the bone cells expressing these factors also express androgen receptors. Their production could potentially be increased on androgen-ablative therapy. To identify growth factors that are negatively regulated by androgen, we applied cDNA array technology to two complementary model systems, in-vivo mouse bone and cultures of human primary bone-derived cells, stably expressing the AR. Here we present our findings in the mouse model.

Methods. Three pairs of C57BL/6J mice were castrated or sham-operated at week 7 and sacrificed at week 20. Long bones were collected and the bone marrow was separated from bone by flushing with 1xPBS. Either total (marrow) or amplified RNA (bone) from each pair was used to generate cy3 or cy5 labeled samples for hybridization to our custom-made cDNA array that contained 10,000 genes with enrichment of genes expressed in mouse prostate. Sample labeling was altered with both dyes to exclude dye bias. Array data were analyzed with GeneSpring (Silicon Genetics).

Results. Gene expression profiles from the three pairs of samples were highly consistent. Of the 10,000 genes examined, 52 genes in bone and 76 genes in bone marrow were significantly altered in androgen deprived versus control mice ($P < 0.05$ as in student t-test). These included 47 up-regulated and 5 down-regulated genes in bone, and 71 up-regulated and 5 down-regulated genes in bone marrow. There were 15 genes in both samples in common, which was unrelated to bone marrow contamination, as indicated with marrow-specific markers Runx1 and Cbfb. Since this cDNA approach was designed to identify growth factor systems that were up-regulated upon androgen deprivation, we specifically searched for growth factor related mRNAs in the gene list. Among candidates emerged were members of the insulin-like growth factor (IGF) axis and the Sox family transcription factors that regulate the activity of fibroblast growth factor-4.

Conclusions. Androgen-deprivation affects the expression of a variety of genes in bone and bone marrow. The stimulation of the IGF axis upon androgen withdrawal may paradoxically lead to increased growth and survival of prostate cancer cells in the bone environment and may be the underlying cause for treatment failure and progression of metastatic prostate cancer to androgen-independence.

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