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

CONTRACTING ORGANIZATION: Fred Hutchinson Cancer Research Center
Seattle WA 98109-4417

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

E-Mail: bknudsen@fhcrc.org

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14. ABSTRACT

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. Based on the presence of androgen receptor regulatory elements in the promoter regions of the Interleukin-6 (IL-6) and hepatocyte growth factor/scatter factor (HGF/SF) genes and the presence of nuclear factor IL-6 binding sites in the promoter of the HGF gene, it is the premise of this project that IL-6 and HGF genes may serve as paradigms of genes that are increased in expression under androgen-suppressed conditions. However, initial experiments failed to reveal regulation of HGF and IL-6 by androgen suppression. An analysis of global gene regulation in castrated mice demonstrated that 159 genes were regulated and that Insulin growth Factor Binding Protein-5 demonstrated the most consistent increase in expression. The support provided by the grant fostered the PI's career development to an independent investigator in the field of prostate cancer metastasis to bone. There were several publications supported by the funds from this grant.

15. SUBJECT TERMS
Prostate cancer metastasis, bone, growth factors, castration, IL-6

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INTRODUCTION:

The hypothesis of the 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 propose to test this hypothesis using Interleukin-6 (IL-6) and hepatocyte growth factor/scatter factor (HGF/SF) as paradigms of androgen regulated growth stimulators for prostate cancer cells [1,2]. In particular, based on circumstantial reports in the literature, IL-6 and HGF are normally suppressed by steroid hormones [3-5]. Consequently, these cytokines should be overexpressed when androgen levels are reduced. When we tested this hypothesis in two separate experimental systems – human bone stromal cells and mouse bone and bone marrow – we were unable to demonstrate that androgen-deficient conditions cause an increase in IL-6 or HGF/SF gene expression. As a result of this initial negative result in the first task of the grant we decided to take a broader approach for identifying the target genes of androgen suppression. Using a specific mouse DNA array platform enriched in sequences that are expressed in mouse prostate and bone, we discovered multiple genes that are up-regulated upon castration. Insulin growth factor binding protein-5 (IGFBP-5) was most consistently increased after androgen suppression and selected to further investigate the crosstalk between the bone environment and prostate cancer cells.

We realize that the experimental design of the project is limited to effects that are mediated by the transcriptional activity of the androgen receptor (AR) and androgen regulated gene expression. We reasoned that measuring the changes in transcription by androgen represents a direct measure of androgen concentration and AR activity. To evaluate whether IL-6 and HGF are targets of androgen suppression, we only evaluated changes in IL-6 and HGF expression at the transcript level. Our experiments were not designed to detect translational regulation of IL-6 and HGF by androgen/AR. There is recent evidence that androgen/AR increases protein translation of FGF-2 by an internal ribosomal entry site dependent mechanism [6]. Therefore there is a recent novel paradigm by which androgen and AR can increase protein expression without effecting gene transcription. However, detecting protein expression expression changes by androgen and AR requires a proteomics approach. In summary, the research that was funded by this grant application focused (1) on the activity of androgen and the AR to regulate gene transcription, (2) on the effects of androgen signaling in the bone marrow and on (3) the targets of androgen-deprivation therapies.

BODY:

Below we list the tasks of the grant application and describe the work that we conducted for each task. A submitted publication of the work is appended.

Appreciate **Task 1** was "To determine whether hepatocyte growth factor/scatter factor synthesis by bone stromal cells is regulated by androgen". To test this hypothesis we conducted 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 (reported in Annual

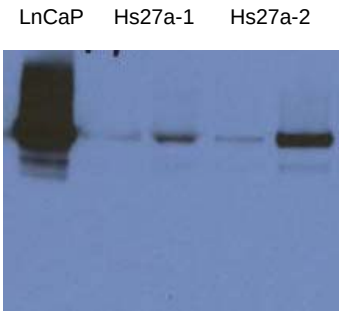
Report for year 2).

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, the bone specific transcription factor: Cbfa-1, and osteocalcin. Using these methods, we were not able to detect significant differences in gene expression between normal and orchiectomized bone in these four genes. Thus, we concluded that IL-6 and HGF/SF are not significantly regulated in the bone and bone marrow of C57/BL6 mice by androgen. While we considered this to be a strain-specific problem[7], we decided not to interrogate other strains. Since the ultimate goal of our studies is a translation to human, we were concerned that a strain-specific mechanism may not be sufficiently robust to be translatable to human bone marrow. Further, mouse strains other than C57/BL6 provide fewer opportunities for subsequent experiments with knockout mice. Therefore, we decided to identify additional androgen-responsive gene expression changes in the bone marrow by using DNA arrays[8,9] (**Task 4**).

Unpublished work and reagents under **Task 1**:

a) Increased expression of Met protein in castrated mouse bone marrow (data in annual report of year 2).
b) *In-vitro* differentiation of human bone marrow stromal cells and analysis of differentiation markers in cultured human bone marrow stromal cells.

In **Task 2** (months 12-36), we proposed "To determine whether IL-6 regulates HGF/SF synthesis in the murine bone stroma and in cultured human bone cells". We cloned the AR and inserted the c-DNA into pBabe and pQCXIN. Using 273T cells for packaging of the retrovirus, we generated a high titer retrovirus and infected Hs27a human bone marrow stromal cells. A Western blot measuring AR expression in the resulting Hs27a cell populations is shown below. Two separate transfections are compared to LnCaP cells. After stimulation of the Hs27a-AR cells with R1881-androgen, we were not able to demonstrate up-regulation of IL-6 or HGF/SF RNA. In addition, we did not observe increases of IL-6 and HGF RNA in orchiectomized mice. Thus, we did not move to studying the effects of IL-6 in cultured mouse bone stromal cells. As the androgen receptor (AR) field has matured, the gene expression changes caused by expression of AR protein in in-vitro cultured cells frequently do not recapitulate in-vivo gene activation by the AR. For example, genes regulated by androgen in human prostate epithelium and prostate cancer in-vivo do not show a significant overlap with the androgen-regulated transcriptome in LNCAP cells [10-12] (and personal communication). We wanted to be in a better position to evaluate the validity of an in-vitro system of bone marrow stromal cells and wanted to accomplish this by first understanding AR-target genes in-vivo. Therefore we undertook an in-vivo experiment in mice to identify AR-regulated genes in mouse bone marrow stroma and subsequently confirmed observations from the mouse system in the Hs27a human bone marrow stromal cell line. The gene that we found to be most consistently androgen regulated in mouse bone marrow was insulin growth factor binding protein-5 (IGFBP-5) (see attached manuscript).



Unpublished work and reagents under **Task 2**:

a) Construction of an AR retroviral construct
b) AR expression bone marrow stromal cells

In **Task 3** we proposed "To determine the role of IL-6 as a mediator of the orchiectomy triggered HGF/SF synthesis". We proposed to analyze the regulation of HGF via IL-6 in response to androgen suppression. For this experiment there are available IL-6 knockout mice. However, since we could not demonstrate regulation of the two cytokines by androgen suppression, this task is not feasible.

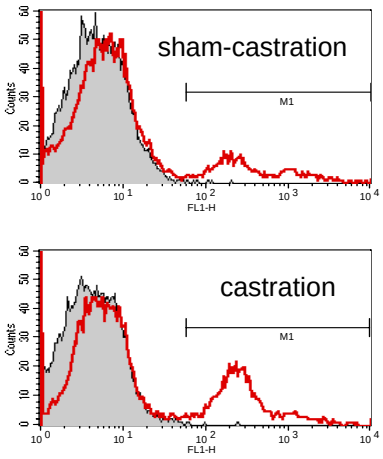
The goal of **Task 4** is to perform a DNA array study of mouse bone and bone marrow to identify androgen-regulated gene expression (months 24-36).

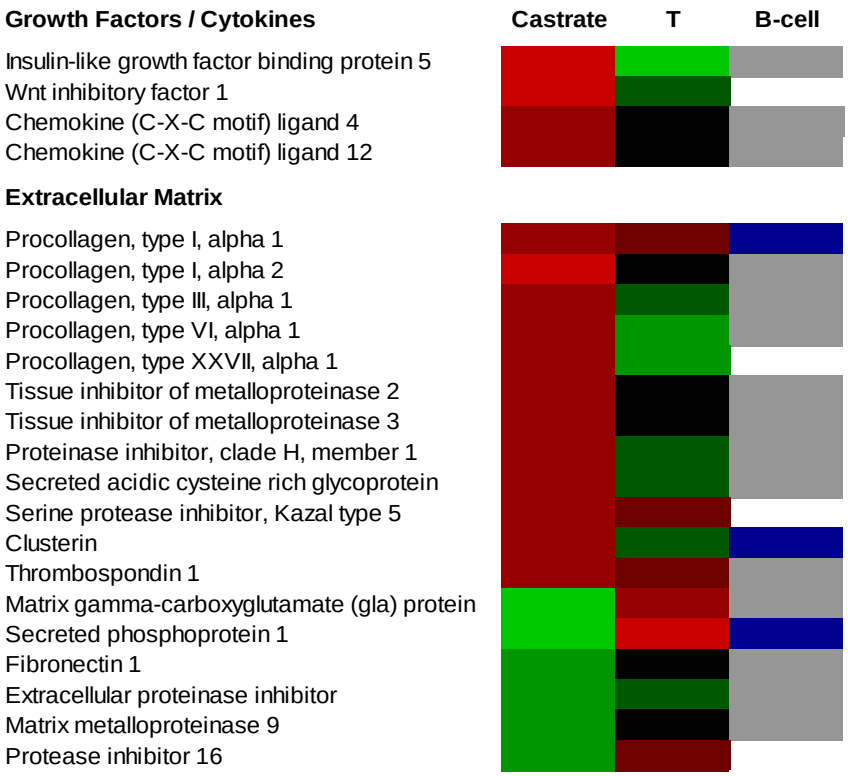
The results from the comparison of normal and androgen-suppressed bone marrow are in the appended manuscript. Briefly, we observed consistent gene expression changes after orchiectomy and there were twice as many genes upregulated compared to downregulated. We reasoned that the primary response to androgen suppression should occur in the mesenchymal cell compartment, which consists of bone marrow stromal cells and osteoblasts. The expression of the AR in osteoblasts has clearly been demonstrated [13-19]. In contrast AR expression in bone marrow stromal cells is not as well established, but implied through indirect effects of androgen on hematopoeisis [20-23].

The interactions between the bone marrow and the cancer cells are mediated through secreted growth factors, cytokines, or extracellular matrix. Thus the analysis of array data focused on categories of secreted genes and identified IGFBP-5 as the most consistent androgen-regulated gene in bone marrow stromal cells. The majority of results are included in the paper. Results that are not included in the manuscript are presented below.

Unpublished work under **Task 4** and future work to better address the research topic:

a) Unpublished observations in bone marrow from orchiectomized mice:





1. *Androgen ablation causes a B-lymphocyte hyperplasia.*

Analysis of bone marrow from castrated mice showed a large increase in the B-lymphocytic lineage. In human, androgen ablation after autologous hematopoeitic stem cell transplantation enhanced lymphoid recovery[22]. Recently we observed the B-cell hyperplasia in the array data, by FACS analysis and immunohistochemical staining. The quantitative analysis of the BM was facilitated by B220, which is an antibody that specifically binds to the CD45R protein on the B-cell surface. The results from the FACS analysis are shown in the above figure in the 2 left panels. The M1 fraction, which represents B220 high expressing cells, is markedly increased after castration. The percentage of B-cells in the BM increased from 13% to about 26%. Thus after orchiectomy the B-cell compartment doubled in size. The increase in the B-cell compartment was also noted in the array data. Approximately half the genes that were overexpressed after castration were expressed in B-cells. To exclude the B-cell specific genes, we performed a virtual subtraction. We identified array data sets of bone marrow B-cell precursors within the gene expression omnibus (GEO) database (record # GDS44 and GDS52)[24] and excluded the genes represented in these data from our analysis. The B-cell development data span five consecutive stages of B-cell development and thus we are confident that we excluded most B-cell specific genes. This approach significantly enriched our array data sets for gene regulation that is unrelated to the expansion of the B-cell compartment. The results from the genes that we identified are shown in the above figure. Genes marked by a blue color in the right column are expressed in B-cells and stromal cells and were therefore included.

b) Future work to better address the research topic:

To obtain a better understanding of how various androgen-ablative drugs affect the bone marrow, we treated mice with drugs that are used for the treatment of patients with bone marrow metastases. In all cases, we used a measurement of the size of the prostate and seminal vesicles as an indicator of the anti-androgenic activity of a given drug and the PEDB array platform for evaluation of drug effects on the bone marrow.

2. *Androgen deprivation causes estrogen deprivation and thus estrogen and not androgen could be a major regulator of the bone marrow environment.*

- *Effect of DES on the bone marrow*

DES was the first treatment for metastatic prostate cancer and many patients experienced a dramatic clinical response. Because of vascular side effects, DES was replaced by androgen inhibitors. However, recently estrogenic therapy became popular again. There are novel estrogenic compounds in clinical trials for metastatic prostate cancer that do not possess the side effects of DES. Therefore understanding the effects of estrogen on the bone marrow is important.

The effects of DES on mouse bone marrow have not been reported. We treated mice with DES to determine whether this treatment causes gene expression changes in bone marrow. The treatment consisted of daily doses of 83 g DES/day administered by subcutaneous injection. The dose corresponds to a 200 mg / day dose, which is in the range of human treatment regimens. In mice after the one month treatment, the given DES dose reduced the size of seminal vesicles by 90%, demonstrating a complete ablation of androgen signaling. At this dose in the bone marrow, 1959 genes were differentially expressed, 830 were upregulated and 1129 were down-regulated. Histologic analysis of DES treated sections of bone revealed massive new bone formation. A detailed analysis of array data demonstrated that gene expression changes in 12 genes in common between the DES, castration, and androgen supplementation groups are shown in **Table 1**. There were 72 more genes in common between the DES and the castration groups and 3 more genes in common between the DES and androgen supplementation group. These data demonstrate gene expression changes in common between castration and DES treatments. Interestingly, the genes that are regulated in the same direction by testosterone and DES are expressed in B-cells. This suggests that DES does not cause the b-cell hyperplasia that is noted with androgen ablation.

Table 1. Differential expression of annotated and androgen-sensitive genes.

(genes with expression differences of > 1.5)		Castration			Testosterone	DES
Systematic	Description	Young Bone Marrow	Young Bone	Old Bone & Marrow		
Igfbp5	insulin-like growth factor binding protein 5	2.4	1.8	2.0	1.0	1.7
Vpreb3	pre-B lymphocyte gene 3	2.3	2.1	2.3	-6.7	-1.6
Pou2af1	POU domain class 2 associating factor 1	2.1	1.6	2.2	-4.4	-1.5

Pafah1b3	platelet-activating factor acetylhydrolase isoform 1b alpha1 subunit	2.1	1.7	1.9	-3.8	-1.4
Sox4	SRY-box containing gene 4	2.0	1.5	1.6	-3.6	2.3
Cd79b	CD79B antigen	2.0	1.7	2.1	-4.0	-2.2
Bach2	BTB and CNC homology 2	2.0	2.6	2.2	-4.7	-1.9
Lef1	lymphoid enhancer binding factor 1	1.9	1.7	1.7	-2.6	NA
Sbk	SH3-binding kinase	1.9	1.7	1.7	-3.5	-1.2
Il7r	interleukin 7 receptor	1.9	2.1	1.8	-3.5	-1.4
Foxo1	forkhead box O1	1.8	1.5	1.7	-2.5	-1.2
Glcci1	glucocorticoid induced transcript 1	1.6	1.6	1.5	-2.6	-1.2
Blk	B lymphoid kinase	1.6	1.7	1.7	-2.9	NA
Scd1	stearoyl-Coenzyme A desaturase 1	1.6	1.9	2.7	-1.7	1.6
Siglec10	sialic acid binding Ig-like lectin 10	1.6	1.6	1.9	-2.7	-2.3

Table 2. Functional annotation of selected genes from Table 1.

	Genes (abbreviation)	Functional category	Effects on metastatic tumor
1	IGFBP5[25]	increases IGF activity, unique receptor	increases tumor growth
2	PAF acetylhydrolase (Pafah1b3)[26]	Decreases pro-apoptotic PAF	stimulates survival and invasion of tumor cells
3	SRYbox gene 4 (Sox4)[27]	transcription factor, patterning	Mesenchymal growth factors
4	interleukin 7 receptor[28]	IL7R triggers IL-6 synth. by BM stroma	Incr. tumor growth through IL-6
5	serine (or cysteine) protease Inhibitor ,cladeH (Serpinh1)[29]	= hsp47, stablizes and increases collagen secretion from stromal cells	stimulates tumor growth through increasing cell adhesion
6	Thrombomodulin[30]	receptor for activation of protein C	Incr. growth through active prot. C
7	Clusterin (Clu)[31]	secreted protein stimulating cell survival	stimulates tumor cell survival
8	Amyotrophic lateral sclerosis 2 (ALS2)[32]	GEFfor Rab and Rho, regulating vesicular transport and cytoskeleton	promotes secretion of GFs from bone
9	Platelet factor 4 (PF4)[33]	binds FGF and stabilizes activity	Incr. tumor growth through FGF
10	Cxcl12, chemokine lig.[34]	binds to CXCR4 on tumor cells	tumor cell activation and invasion
11	cAMP-phosphodiesterase 4B (PDE4B)[35]	Decr. cAMP, promotes cytokine secretion, PDE4B inhibitor effective in MM	promotes tumor growth
12	Osteonectin/SPARC[36]	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.

Future studies are needed to determine the effects of estrogenic drugs on the bone marrow. We have defined several endpoints (gene expression to detect B-cell hyperplasia, osteogenesis, and the reactive stromal cell compartment) that can be used to evaluate the activity of these drugs in pre-clinical trials and to guide the development of anti-cancer agents that also have desirable activities on the bone marrow. (Annual Reports 2004 and 2005).

- *The effects of flutamide, an AR antagonist, on the bone marrow.*

Since orchiectomy decreases both androgen and estrogen levels, we thought to investigate gene regulation that is specifically caused by androgen suppression. In these experiments, we treated mice with flutamide. Flutamide is a commonly used drug in the clinic. It binds the AR and in part inhibits its activity. Recent studies demonstrated that the inhibitory activity is gene specific [37-40]. In this capacity it functions as a partial AR antagonist. In contrast to orchiectomized animals, flutamide treated animals have normal levels of estrogen. At the time of the experiment, only flutamide and not the more potent biclutamide (Casodex) was available for preclinical studies. At a dose of 50mg/day, we observed approximately 50% reduction in the size of the seminal vesicles and of the anterior lobe of the prostate, suggesting that flutamide did not completely inhibit the androgen axis. This explains why we did not detect consistent gene expression changes in the BM, when we compared BM from mice treated with flutamide and control BM. We were unable to increase the dose of flutamide because of toxicity to the animals. Therefore, determining the effects of estrogen depletion on the bone marrow will be resolved in future experiments.

- *The effects of an LHRH agonist on the bone marrow environment.*

Another strategy of reducing androgen levels is by the downregulation of GnRH receptors in the hypothalamus. This is accomplished by the LHRH receptor agonist, Lupron®. Therefore we treated mice with Luprolide, which has the same activity as Lupron. However, we did not detect an inhibition of androgen production, even at a dose that corresponds to 100-times of the human dose. In the dose titration experiment, we observed an increase in prostate size, even at the highest drug concentration. The strategy that we will take in the future is to analyze the effects of a GnRH-receptor inhibitor.

KEY RESEARCH ACCOMPLISHMENTS:

Reagents:

- AR construct in a retroviral vector
- AR expressing bone marrow stromal cells
- Extracellular matrix form bone marrow stromal cells

Experimental results (please see attached manuscript):

- Effects of several androgen-ablative agents on mouse bone.
- Array data sets of androgen-regulated bone marrow deposited in the GEO database
- Regulation of the IGF axis and IGFBP-5 by androgen signaling in the bone marrow

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. This grant was not funded.
- 2004 AACR abstract # 172
- Prostate Cancer Foundation Abstract # 45
- AACR Molecular Diagnostics, Abstract
- 2006 SBUR meeting, abstract # 36
- DOD New Idea Award 2005: Androgen, estrogen and the bone marrow environment
- Paper under review by ONCOGENE: Androgen Suppression Increases Insulin-like Growth Factor Binding Protein 5 in the Bone Marrow Microenvironment which Supports Growth of Prostate Cancer Cells

CONCLUSIONS:

The project that we conducted with the funding from this award analyzed the effects of androgen-ablative therapies on the bone marrow environment. We systematically tested the hypothesis, that androgen ablative therapy paradoxically increases the secretion of growth factors in the bone marrow microenvironment by treating mice with various androgen-suppressive and anti-androgenic agents. While we did not observe effects of androgen ablation on the anticipated gene targets, HGF and IL-6, we noted a consistent increase in expression of IGFBP-5. Orchiectomy as an androgen ablative strategy as well as DES caused increased the expression of IGFBP-5, suggesting that IGFBP-5 is upregulated by two androgen-suppressive treatments that encompass two entirely different mechanisms of androgen suppression. The results of this study are appended in a manuscript.

The importance of the study lies in its clinical application. The manuscript contains preclinical data to support the development of anti-sense IGFBP-5 therapeutics. This drug is under development by Dr. Martin Gleave's group for treatment of refractory and advanced prostate cancer. The drug is thought to be efficacious when combined with androgen-suppressive treatments. The results from our study suggest that the anti-IGFBP-5 agent will not only inhibit the growth of local prostate cancer, but also reduce tumor growth in the bone marrow. In particular, it will inhibit the stimulation of tumor cells by factors from the environment.

LIST OF PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT:

- Dr. Glenn Gmyrek, Urology resident, post-degree training
- Dr. David Chen, Urology resident, post-degree training
- Dr. Douglas Scherr, Urology fellow, clinical fellowship training
- Bosede Adenekan, Undergraduate student, BS from University of Pennsylvania
- Dr. Chang Xu, post-doctoral fellow

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APPENDICES:

- Manuscript: “Androgen Suppression Increases Insulin-Like Growth Factor Binding Protein 5 in the Bone Marrow Microenvironment which Supports Growth of Prostate Cancer Cells”
- Abstract: “Androgen-regulation of the Bone Marrow Microenvironment targets IGFBP-5”
- Abstract: “Androgen Ablation Increases the Synthesis of Growth Factors in the Bone Microenvironment”
- Abstract: “Surgical Castration Increases the Synthesis of Growth Factors in the Bone Microenvironment”

Title: Androgen Suppression Increases Insulin-like Growth Factor Binding Protein 5 in the Bone Marrow Microenvironment which Supports Growth of Prostate Cancer Cells

Running Head: Bone Marrow Environment, Androgen Ablation, IGFBP5

Authors: Chang Xu [1], Lynn F. Graf [1], Ladan Fazli[2], Ilsa M. Coleman[1], Denise E. Mauldin[1], Peter S. Nelson[1], Martin Gleave[2], Stephen R. Plymate[3], Michael E. Cox [2], Beverly J. Torok-Storb[1], Beatrice S. Knudsen[1]

Institutions: 1. Fred Hutchinson Cancer Research Center, Seattle, WA. 2. Vancouver General Hospital, Vancouver, BC. 3. University of Washington and GRECC VAPSHCS, Seattle, WA.

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Corresponding Author: Beatrice S. Knudsen, M.D., Ph.D., Public Health Sciences, M5-A864, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. N., Seattle, WA 98109. Tel: (206) 667-1823. Fax: (206) 667-2537. E-mail: bknudsen@fhcrc.org.

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Abstract

Metastatic prostate cancer is commonly localized in bone and treated with androgen ablation, which is initially highly effective, but eventually results in resistant cancer cells. The decreased dependence on androgen is associated with activation of the androgen receptor (AR) via other growth factor receptor pathways. The current study analyzed global gene expression in bone from castrated and sham operated C57BL/6 mice to identify factors that may be responsible for androgen signaling. Androgen suppression by castration increased the expression of 159 genes with insulin-like growth factor binding protein 5 (IGFBP5) being the most consistently increased. IGFBP5 protein was detected by immune histochemistry in osteoblasts, bone marrow stromal cells and endothelial cells. In vitro treatment of marrow stromal cells with charcoal-stripped serum increased IGFBP5 mRNA expression approximately 25-fold and was reversed by androgen supplementation. Endogenous IGFBP5 was incorporated into extracellular matrix. In addition, treatment of extracellular matrices with exogenous IGFBP5 and IGF-1 or IGF-2 enhanced the growth of immortalized human prostate cells. These results suggest that androgen ablative therapy may increase IGFBP5 in the marrow microenvironment, thereby providing support for the development of therapy resistance prostate cancer cells.

Introduction

Prostate cancer commonly metastasizes to the skeleton.[1] Disseminated cancer cells reach the bone through the circulation and enter the bone marrow through bone marrow sinusoids.[2] Prostate specific antigen (PSA)-expressing cells are detectable in bone marrow specimens of 54% of patients at the time of prostatectomy, indicating that cancer cells disseminate early on [3]. However, disease progression is often delayed by years, suggesting that disseminated cancer cells can be in a state of dormancy. It is likely that quiescence and proliferation of micrometastatic prostate cancer cells are determined by genetic changes within cancer cells as well as by the marrow microenvironment.

Androgen suppressive therapy has proven a significant benefit when administered in an adjuvant setting together with radiation therapy for the treatment of prostate cancer.[4] The systemic androgen deprivation kills disseminated prostate cancer cells; however, some cells survive the treatment. Survival under androgen deficient conditions maybe an inherent property of certain cancer cells but may also be stimulated by factors in the microenvironment. Hypothetically the reaction to androgen suppression of cells in the bone marrow microenvironment could create a permissive milieu for "androgen independent" growth. In the bone marrow microenvironment the androgen receptor is expressed by bone marrow stromal cells, osteoblasts, endothelial cells, osteocytes and chondrocytes.[5,6] Androgens increase the thickness of bone, augment the hematocrit and regulate the expansion of B-cells. It is likely that the effects of androgen on hematopoeisis are, in part, mediated indirectly through the androgen receptor activity in bone marrow stromal cells [7]. However, the androgen regulated factors that

might mediate the interactions between the bone marrow stroma and neighboring cell types have not been reported.

Insulin-like growth factors (IGF) are involved in normal and malignant growth of prostate epithelial cells.[8] Metastatic prostate cancer cells maintain expression of IGF receptors (IGFR), albeit at diminished numbers compared to localized prostate cancer.[9] IGFs are abundant growth factors in bone and activation of the IGF pathway may lead to ligand-independent activation of the androgen receptor (AR).[10-12] The bioavailability of IGF is regulated by a group of IGF binding proteins (IGFBP1 to 6). Androgen regulates the expression of IGFBP2, 3, 4, 5 in the prostate.[13-15] In the bone, IGFBP4 and IGFBP5 are the two major binding proteins that modulate the IGF activity [16] and IGFBP5 is sequestered by the bone matrix. IGFBP5 may also act independently of IGF as a growth stimulator for osteoblasts, through binding to a separate receptor on the cell surface.[17,18] In human bone and bone marrow, IGFBP5 is expressed in chondrocytes, osteoblasts and osteocytes, which also express androgen receptors.[19-22]

In this study we sought to measure the effects of androgen suppressive therapy on the bone marrow environment. To determine global androgen regulated gene expression, we compared transcriptional profiles of bone and bone marrow cells in castrated and sham operated mice. We observed that androgen suppression causes an increased expression of genes that may stimulate the proliferation of prostate cancer cells. Specifically, IGFBP5 expression was up-regulated. Subsequent in vitro experiments with human cells confirmed IGFBP5 regulation by androgen in bone marrow stromal cells and demonstrated the functional relevance of elevated IGFBP5 for stimulation of prostate cancer cell proliferation.

Materials and Methods

Mice: Castrated or sham-castrated C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) after surgery at 7-weeks of age. Femural, tibial and humeral bones were collected from 17-week old (young mice, 10 weeks after castration) and 59-week old (old mice, 52 weeks after castration) mice. Testosterone or placebo pellets (12.5 mg 60 day slow release, Innovative Research of America, Sarasota, FL) were implanted for 4 weeks. The experiment was performed in accordance with an approved Animal Care and Use Committee (IACUC) protocol.

Cells: Human primary bone stromal cells HB5, HB6, and HB15 were derived from 3 different individuals under an approved IRB protocol. The cells were maintained in MEM-alpha medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS, Hyclone, Logan, UT). Human immortalized bone stromal cells HS27a, prostate epithelial cells P69, and LNCaP prostate cancer cells were propagated in RPMI 1640 10% FBS. The human sarcoma cell line MG63 and MG63 expressing IGFBP5 were maintained in DMEM (Invitrogen, Carlsbad, CA) with 10% FBS. Human primary prostate stromal and epithelial cells were cultured as described.[23]

RNA isolation: Total RNA was isolated from pulverized bone or cultured cells using TRIZOL® (Invitrogen, Carlsbad, CA) and cleaned with RNeasy® kit (Qiagen, Valencia, CA). Quality of RNA was evaluated using Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA).

Microarrays: Microarray hybridization and processing of raw data is described in [24] Genes with differential expression > 1.5 fold and false discovery rate (FDR) < 5% were selected and analyzed by hierarchical clustering using Cluster 3.0.[25]

Reverse transcription and quantitative real-time PCR (qPCR): First strand cDNA was synthesized using SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Primers for qPCR spanned across intron-exon junctions and the sequences are listed in Supplementary Table 1. qPCR conditions for detection with Platinum® SYBR® Green in an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) were: 10 minutes at 95ºC, followed by 40 cycles of amplification at 95ºC for 15 seconds, 60ºC for 30 seconds, and 72ºC for 30 seconds.

Immunohistochemical staining: Mouse femurs were fixed in 10% buffered formalin at 4ºC overnight and decalcified in EDTA bone decalcifier (Decal Corp., Tallman, NY). For antigen retrieval, slides were incubated with EDTA, pH=8 at 95 ºC for 8 minutes. The goat polyclonal anti-IGFBP5 antibody (sc-6006, Santa Cruz Biotechnology Inc., Santa Cruz, CA) was diluted 1:100, immunohistochemical staining was conducted by Vantana autostainer model Discover XT ™ (Vantana Medical System, Tuscan, Arizona) with enzyme labeled biotin streptavidin system and solvent resistant DAB Map kit (company).

Serum testosterone measurement: Mouse blood samples were collected by cardiac puncture and sent for measurement of serum testosterone to the Center for Reproductive Biology,

Washington State University, Pullman, WA (Website). The sensitivity of the measurement was 0.10 ng/ml.

Preparation of cell lysate, conditioned medium (CM), and extra cellular matrix (ECM): Cell lysate samples were prepared in RIPA buffer [26] containing protease and phosphatase inhibitors (Roche Applied Science, Mannheim, Germany). Serum-free medium was conditioned for 48 hours, mixed with RIPA buffer, and concentrated with an Amicon Ultra-15, 5kDa Centrifugal Filter (Millipore, Billerica, MA). ECM was prepared as described by Knudsen et al.[27] ECM on plates was used immediately for growth assays or solubilized in RIPA buffer.

Immunoprecipitation and Immunoblotting: 500 ug RIPA lysates were precipitated with 10 µl anti-IGFBP5 serum (Catalog # 06-110, Chemicon International Inc., Temecula, CA) overnight at 4ºC and protein G agarose beads (Sigma, Saint Louis, MO) for 2 hours. Beads were washed 3-times with RIPA buffer and eluted with Laemmli sample buffer. Samples without the addition of anti-IGFBP5 were used as a negative control. 40 µg total protein or immunoprecipitated proteins were analyzed on 12% NuPAGE or 4-12% Bis-Tris Gels (Invitrogen, Carlsbad, CA) and transfer to Immobilon-P PVDF membrane (Millipore, Billerica, MA). Membranes were blocked with 5% powdered nonfat milk and probed with 1:1000 anti-IGFBP5 antiserum. Blots were developed with a horseradish peroxidase conjugated secondary antibody and the Supersignal West Pico Chemiluminescent (Pierce Biotechnology, Inc., Rockford, IL.)

Regulation of IGFBP5 expression in HS27a: HS27a cells were cultured in Phenol red-free medium with 10% charcoal-stripped FBS. Charcoal stripped serum was prepared by stirring 100

ml of fetal bovine serum with 15 g of dextran-treated charcoal (Sigma, Saint Louis, MO) at 4ºC overnight. The serum was clarified by centrifugation and filtration with a 0.22 μm MILLEX®GV (Millipore, Billerica, MA). Methyltrienolone (R1881, PerkinElmer Life And Analytical Sciences, Inc, Wellesley, MA) was added as a synthetic androgen.

P69 proliferation in response to IGFBP5: HS27a ECM in 24-well plates was incubated with human recombinant IGFBP5 without or with 10 ng of human recombinant IGF1 (Sigma, Saint Louis, MO) or 10 ng of mono-biotinyl IGF2 (GroPep Limited, Adelaide, SA, Australia) for 4 hours at room temperature. After the plates were washed, 40,000 P69 cells were seeded per well in serum free medium containing 10ng/ml IGF1 or IGF2. MTS assays were performed after 48 hours using the CellTiter 96® AQ_{ueous} cell proliferation assay kit (Promega, Madison WI). The experiment was repeated 4 times. Statistical analysis was conducted using ANOVA.

Results

Gene expression changes in mouse bone and bone marrow after androgen deprivation

The regulation of gene expression by androgen suppression in the bone marrow has not been reported. Therefore we undertook a global approach to analyze gene expression changes in mouse bone marrow and bone that occur upon castration. We initially collected bone and bone marrow from 17 weeks old castrated and sham-operated C57BL/6 mice for comparison in array experiments. The bone marrow was removed by flushing long bones and analyzed separately from the bone. Purified RNA from bone or bone marrow cells was labeled and hybridized to mPEDB arrays. Results obtained with bone samples were similar to those obtained with bone marrow samples, therefore we did not separate bone and bone marrow in subsequent experiments. In later experiments, we measured castration related gene expression changes in pairs of castrated and sham-operated old mice (59 weeks old) and in young castrated mice supplemented with testosterone using whole bones.

Castration significantly lowered serum androgen levels. In addition, the size of the seminal vesicles was greatly reduced, indicating that the castration procedure was effective **(Table 1)**. Analysis of array data revealed that 243 genes exhibited significant and consistent differential expression. Of these, 159 were up-regulated and 84 were down-regulated in castrated compared to sham-operated mice. Gene expression changes in the various conditions (bone marrow, bone, young mice, and old mice) were compared (**Figure 1A**). Of the up-regulated genes, 25/159 were differentially expressed across all arrays. These experiments

clearly demonstrate that androgen suppression affects gene expression in the bone marrow, that most of the responsive genes are increased in expression upon androgen suppression and that the expression changes are similar in young versus old mice. Quantitative real time PCR (qPCR) was used to confirm the expression changes from array data for 22 genes. The fold changes were highly correlated between the two methods (r = 0.97; **Figure 1B**).

To further confirm the regulation of gene expression by androgen, we treated castrated mice with testosterone. The treatment increased serum testosterone levels above those in control mice (13 ng/ml versus 0.7 ng/ml, see **Table 1**) and the seminal vesicles grew to the size found in sham-operated controls (**Table 1**). Elevated serum testosterone levels reversed the castration effects for a subgroup of genes. Of the 159 genes that were up regulated in castrated mice, 69 were reversed by testosterone treatment (**Figure 1A**).

The array data were examined to identify genes that encode for secreted proteins, capable of interacting with metastatic prostate cancer cells. We identified 4 genes for growth factors/cytokines, and 18 genes that encoded proteins that are associated with the extracellular matrix (**Figure 1C**). In the group of growth factors/cytokines, IGFBP5 was consistently overexpressed in castrated mice across all arrays and suppressed after treatment with testosterone. Thus we further explored the regulation of expression, localization and growth stimulatory activity for prostate cancer cells of IGFBP5.

Expression and regulation of IGFBP5 expression in bone and bone marrow

IGFBP5 is highly expressed in bone.[29] IGFBP4 is also expressed by cells in bone and bone marrow stroma and antagonizes the activity of IGFBP5. However in contrast to IGFBP5, IGFBP4 mRNA expression did not change after castration. The fold expression change for IGBP5 mRNA as determined by qPCR in three pairs of mice was 2.7 $\pm$ 0.88 in young mice and 2.0 $\pm$ 0.17 in old mice. In the same samples the fold expression change for IGFBP4 was 0.86 $\pm$ 0.50 in young mice and 1.14 $\pm$ 0.41 in old mice. To identify cell types expressing IGFBP5 we used immunohistochemistry. IGFBP5 expression in sections of mouse bone and bone marrow was observed in osteoblastic cells lining the bone and in endothelial cells (**Figure 2**). While we observe weak diffuse staining in the bone marrow stroma, individual cells are difficult to define by morphologic criteria alone. The expression of IGFBP5 in bone marrow stroma is supported by the subsequent experiments in cultures of primary bone marrow stromal cells.

To determine whether IGFBP5 is expressed in human bone and bone marrow stroma, we measured IGFBP5 mRNA expression in cultures of primary human bone marrow stromal cells (**Figure 3A**). IGFBP5 RNA expression was detected in cells from three separate individuals and in immortalized HS27a bone marrow stromal cells. While IGFBP5 was expressed in primary cultures of prostate stromal cells, it was not expressed in prostate epithelial cultures under standard growth conditions. IGFBP5 protein was secreted from primary marrow stromal cells (**Figure 3B**) and HS27a cells (**Figure 3C**) and accumulated in the conditioned medium. In addition, IGFBP5 became incorporated into the HS27a extracellular matrix (**Figure 3C**). To exclude the possibility that the IGFBP5 antibody cross-reacts with other IGFBPs or that IGFBP5 is derived from fetal calf serum, we used MG63 osteosarcoma cells transfected with an IGFBP5 containing plasmid (MG63-BP5) to validate the specificity of the antibody. A band of the size

expected for IGFBP5 was only detected in the MG63-BP5 cells, but not in the parent control cells (**Figure 3B**).

To determine whether IGFBP5 expression was regulated by androgen in human bone marrow stromal cells, we first confirmed expression of the AR in HS27a cells. Both AR mRNA and protein (**Figure 4A**) were detectable in cultures of HS27a cells, although to a lesser amount than in fresh prostate tissue. When HS27 cells were cultured in serum that was depleted of steroid hormones by incubation with surface-activated charcoal, IGFBP5 mRNA increased 25-fold after 3 days and IGFBP5 protein increased in parallel (**Figure 4B**). The increase of IGFBP5 mRNA was reversed by addition of physiological concentrations of androgen (**Figure 4C**). These results demonstrate that the level of AR expression in Hs27a cells is sufficient to regulate IGFBP5 expression.

IGFBP5 stimulates the growth of prostate cancer cells

In contrast to IGFBP5 in conditioned medium which is growth inhibitory, IGFBP5 in extracellular matrix promotes cell growth.[30] Therefore we tested whether IGFBP5 bound to extracellular matrix stimulated the growth of P69 prostate cancer cells, extracellular matrix from HS27a cells was incubated with recombinant IGFBP5 and IGF1 or IGF2. The unbound proteins were removed by washing and P69 cells were plated on the extracellular matrices in serum-free medium (**Figure 5A**). Two days later, P69 cell numbers were determined using an MTS assay. Compared to untreated matrix, larger numbers of P69 cells were observed on matrices incubated with IGFBP5 alone. Addition of IGF1 or IGF2 to IGFBP5 further increased cell growth. The effects of IGFBP5, IGF1 and IGF2 on growth induction of P69 cells reached a plateau,

suggesting a saturable mechanism (**Figure 5B**). While growth increased in a linear fashion up to incubation with 25ng/ml IGFBP5, there was no further increase with 50ng/ml or 100ng/ml IGFBP5. IGF1 or IGF2 in the absence of exogenous IGFBP5 also stimulated cell growth, possibly binding to endogenous IGFBP5.

Discussion

We identified gene expression changes in bone and bone marrow after castration of C57BL/6 mice. The expression of 159 genes was increased. The most significant and consistent increase in expression occurred for IGFBP5. Immunohistochemical staining indicated that bone marrow stromal cells, osteoblasts, endothelial cells in bone and bone marrow express IGFBP5. In the human immortalized bone stromal cell line HS27a, IGFBP5 gene and protein expression were increased by treatment with charcoal-stripped serum and were inhibited by addition of androgen. In-vitro, IGFBP5 was secreted into the medium and deposited into extracellular matrix by primary human bone marrow stromal cells and HS27a. When bound to extracellular matrix, IGFBP5 increased the proliferation of P69 immortalized prostate epithelial cells. We conclude from these results that IGFBP5 can promote the growth of prostate cancer cells in the bone marrow environment and that its expression increases after androgen ablation.

Androgens are known to augment the thickness of bone and accordingly, the AR is expressed in osteoblasts, osteocytes and at sites of endochondrial ossification in proliferating, mature and hypertrophic chondrocytes.[5] In several reports, AR copy numbers range between 150 and 5000 per cell in cultured human bone marrow stromal cells, which include osteoblastic cells.[31,32] Consistent with these results, we detected expression of AR mRNA and protein in the immortalized HS27a bone marrow stromal cell line. In vivo androgens regulate cells types that lack detectable AR expression presumably through the bone marrow stroma. Anecdotally, androgens were used to treat anemia and the higher hematocrit in men compared to women is attributed to differences in circulating androgen levels.[33] Androgen but not estrogen regulates

the maturation and expansion of the B-cell compartment.[34,35] In castrated animals, stromal cells expressing the AR were able to promote the expansion of B-cells from mice afflicted by testicular feminization (Tfm), which possess non-functional AR. In the reverse situation, stromal cells from Tfm mice did not cause changes in B-cell numbers after castration [7]. Interestingly, the activity from the stroma is specific for B-cells and does not affect the T-cell compartment in the bone marrow.[36]

The androgen receptor (AR) is a transcription factor that is activated by androgen and that promotes or suppresses gene transcription dependent on the presence of AR co-activators and co-repressors.[37] Array results from androgen-stimulated mouse prostate or human prostate cancer cells clearly demonstrate increases in message abundance for many genes.[38] These analyses have focused on the epithelial cells and identified several androgen-responsive genes that are epithelial cell-specific and encode secretory proteins for prostatic function.[37] While approximately 50% of human prostate stromal cells express AR, the regulation of gene expression by androgen has not been characterized in-vivo. Similarly, the regulation of gene expression in AR positive mesenchymal cells in the bone and bone marrow has not been analyzed. When evaluating the stimulatory effects of androgen in our array studies, we found that 254 genes were up-regulated in the bone and bone marrow (FDR < 5%, fold change > 1.5) after treatment with testosterone (data not shown). Only 8 of these were reversed by castration. These results suggest that androgen-stimulation and castration are not completely reciprocal and the regulation of gene expression by the AR is complex and factors besides androgens can have a major impact on gene expression. In addition, there might be a transcription-independent non-genomic component of AR stimulation. Another complication is that our experiments are

confounded by the metabolism of androgen into estrogen, which is a major stimulus of gene expression in the bone and bone marrow of C57BL/6 mice (data not shown). The proportion of genes in our array results that are regulated by estrogen suppression and not androgen suppression is uncertain.

The first observation that IGFBP5 is an androgen responsive gene occurred in the Shinogii and LNCaP cell culture models.[39] IGFBP5 secretion was increased after androgen deprivation and this caused an increase in the growth of cell line xenografts. An immunohistochemical study using sections of prostate from patients who were treated with androgen-ablative therapy prior to radical prostatectomy demonstrated increased IGFBP5 staining in normal prostate epithelial cells and in prostate cancer cells.[40] Together these studies suggest that androgen ablation can augment IGFBP5 expression in epithelial cells. In this context, castration may influence an autocrine mechanism in which epithelial derived IGFBP5 facilitates tumor growth. Since IGFBP5 expression increases in both the cancer cells and the microenvironment of the cancer, it represents a promising therapeutic target for treatment of metastatic prostate cancer and IGFBP5 targeted therapies are under development.[39] Because IGFBP5 might stimulate the progression to androgen independence in cancer cells, early administration of IGFBP5 antagonizing agents may augment clinical responses resulting from androgen ablation. In addition, baseline IGFBP5 levels in the bone and bone marrow might differ in patients with high or low endogenous serum testosterone levels. It is therefore conceivable that natural fluctuations in IGFBP5 levels in the bone marrow microenvironment could be responsible for the progression of early metastatic disease or for the release of dormant prostate cancer cells. Thus, IGFBP5 secreted by cells in the bone and bone marrow could be an

important regulator of the progression of prostate cancer metastasis under conditions of low androgen.

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Table 1. Serum testosterone and seminal vesicle size.

	Testosterone (ng/ml)	Seminal Vesicle (g)
Sham-operation	0.69 ± 0.52	0.1337 ± 0.0234
Castration	< 0.1	0.0021 ± 0.0005
T-replacement	13.17 ± 1.94	0.1527 ± 0.0213

Serum samples and seminal vesicles from mice 10 weeks after castration or sham-operation and from castrated mice with testosterone replacement are collected. Serum testosterone is determined using a RIA kit with a detection limit of 0.1ng/ml. Measurements are the average ± standard deviation of tissue samples from 3 mice.

Figure 1. Differentially expressed genes in bone and bone marrow after androgen deprivation.

A. Array results of gene expression changes. Gene expression differences in paired castrated versus sham-operated mouse tissues: young bone (YB1-3, 10 weeks after castration), young bone marrow 10 weeks after castration (YM1-3,), old bone and bone marrow 52 weeks after castration (OBM1-3), and bone and bone marrow after castration and testosterone replacement (T1-3). The displayed genes possess an expression difference of at least 1.5 fold and a false discovery rate < 0.05 as determined by SAM (Significance Analysis of Microarrays). A hierarchical cluster analysis is applied using Cluster 3.0 software. *B. Confirmation of Expression changes by qPCR.* Fold changes in selected gene expression are determined using three pairs of castrated and sham-operated mice after normalization to mouse GAPDH. Average fold changes in gene expression from qPCR or microarray measurements are plotted on the x-axis and y-axis, respectively. The Pearson's correlation coefficient (r) is calculated. *C. Genes for secreted and extracellular matrix proteins.* Genes encoding secreted or extracellular matrix proteins in young bone (YB), young bone marrow (YM), old bone and marrow (OBM), and in bone and marrow from castrated mice with testosterone replacement (T). The color code for average expression changes is the same as in Figure 1A.

Figure 2. Expression of IGFBP5 in mouse bone and bone marrow-IHC staining.

Decalcified sections of femoral bone marrow are stained with anti-IGFBP5 antibodies and the expression of IGFBP5 is visualized by a brown color. OB: osteoblasts and End: endothelial cells. The scale bar measures 30 microns. Control: secondary antibody only.

Figure 3. RNA and protein expression of IGFBP5 in human bone stromal cells.

A. RNA expression of IGFBP-5 in human primary and immortalized bone stromal cells. Steady state IGFBP-5 mRNA is measured by qPCR in human primary bone stromal cells HB5, HB6, and HB15, human immortalized bone stromal cells HS27a, human primary prostate epithelial cells (PEC) and primary prostate stromal cells (PSC). Gene expression is normalized to ß-actin. IGFBP-5 expression levels relative to expression in PSC are shown. Error bar indicates the standard deviation of the mean of 3 measurements. *B. Secretion of IGFBP5 protein from human primary bone stromal cells.* Conditioned medium was concentrated approximately 10 fold and loaded directly onto 4-12% SDS-PAGE. Lane 1: Recombinant IGFBP5 (rBP5); Lanes 2 -4: conditioned medium from human primary bone stromal cell cultures; Lane 5: conditioned medium from the human sarcoma cell line MG63 expressing IGFBP5 (MG63/BP5); and Lane 6: conditioned medium from MG63 cells. *C. IGFBP-5 protein expression by HS27a cells.* IGFBP5 protein is immunoprecipitated from conditioned medium (left panel) or extracellular matrix (right panel) of HS27a cells. Lane 1: Recombinant IGFBP-5 (rBP5); Lane 2: Protein G bead control; and Lane 3: IGFBP5 immunoprecipitation. The upper arrow points to IGFBP-5, while the lower arrow indicates the position of the immunoglobulin light chain (Ig LC).

Figure 4. Regulation of IGFBP5 expression in HS27a cells by androgen.

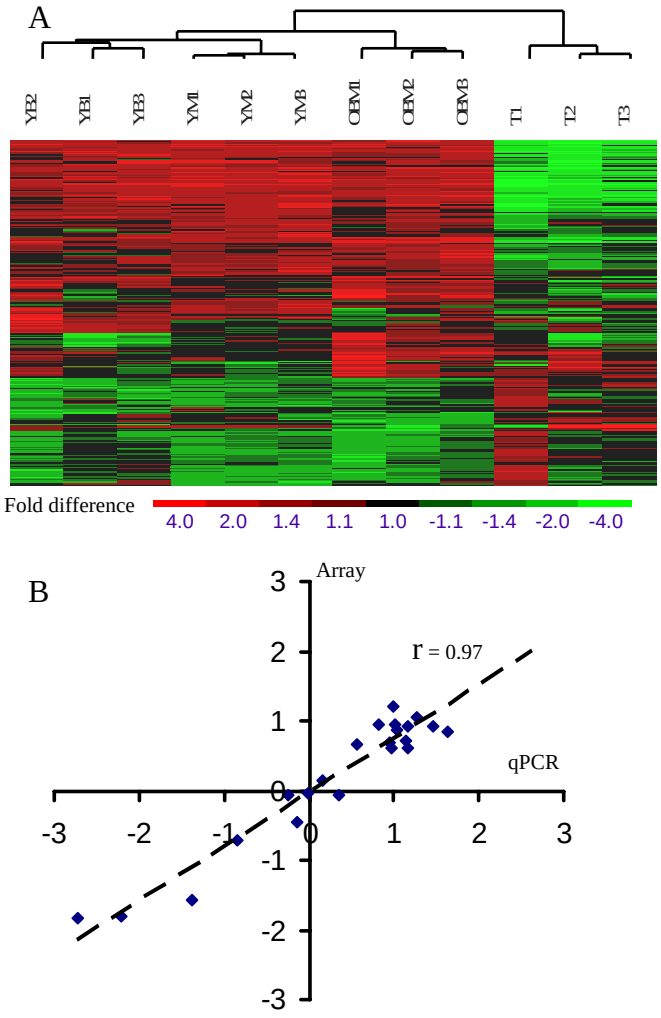
A. Androgen receptor (AR) RNA expression (left panel) and protein expression (right panel) in HS27a grown in 10% FCS. A 292 base fragment with in the AR mRNA (panel A) is amplified by PCR and detected by agarose gel electrophosis. Lane 1: HS27a; lane 2: prostate tissue, lane 3: negative control. AR protein is detected by Western blot in whole cell lysates of HS27a cells or prostate tissue. *B. Induction of IGFBP-5 expression by charcoal stripped serum.* In upper

panel, HS27a cells are cultured in charcoal-stripped serum for indicated time periods. The mRNA expression of IGFBP5 is measured by qPCR and compared to cells grown in regular serum. The expression is normalized to ß-actin and the fold difference between regular and charcoal-stripped medium is depicted on the y-axis. In lower panel, HS27a cells are cultured in charcoal stripped serum (CS) or regular serum (R) for 2 and 4 days. IGFBP5 expression in equal amounts of whole cell lysate is measured by Western blotting. *C. Suppression of IGFBP5 expression by androgen.* HS27a cells are cultured in regular serum (R), charcoal-stripped serum (CS) or CS serum supplemented with R1881 testosterone for 24 hours. The fold difference of IGFBP5 RNA expression is calculated as described in B.

Figure 5. Growth stimulation of P69 prostate cells by IGFBP5

A. Cartoon of the experimental design. Extracellular matrix (ECM) is prepared from HS27a cells and treated with increasing amounts of recombinant IGFBP5 (BP-5) and 10 ng/ml IGF1 or IGF2. After washing, P69 cells are added together with 10ng/ml IGF1 or IGF2 and cell numbers are measured 2 days later. *B. Growth of P69 cells.* The increase in P69 cell numbers on treated matrices is calculated relative to growth on untreated matrix. Values represent the average % increase in cell numbers from 4 experiments ± standard deviation. The ANOVA test indicates that addition of IGFBP5 significantly increases P69 cell growth ($p < 0.001$) and that IGF1 or IGF2 further increase cell growth ($p < 0.001$).

Figure 1



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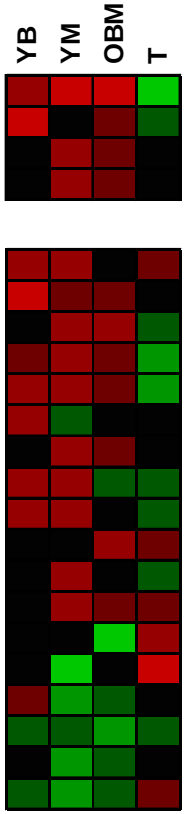


Growth Factor/Cytokine

Igfbp5	Insulin-like growth factor binding protein 5
Wif1	Wnt inhibitory factor 1
Cxcl4	Chemokine (C-X-C motif) ligand 4
Cxcl12	Chemokine (C-X-C motif) ligand 12

Extracellular Matrix

Col1a1	Procollagen, type I, alpha 1
Col1a2	Procollagen, type I, alpha 2
Col3a1	Procollagen, type III, alpha 1
Col6a1	Procollagen, type VI, alpha 1
Col27a1	Procollagen, type XXVII, alpha 1
Timp2	Tissue inhibitor of metalloproteinase 2
Timp3	Tissue inhibitor of metalloproteinase 3
Serpinh1	Serine/cysteine proteinase inhibitor clade H member 1
Sparc	Secreted acidic cysteine rich glycoprotein
SPINK5	Serine protease inhibitor, Kazal type 5
Clu	Clusterin
Thbs1	Thrombospondin 1
Mglap	Matrix gamma-carboxyglutamate (gla) protein
Spp1	Secreted phosphoprotein 1
Fn1	Fibronectin 1
Expi	Extracellular proteinase inhibitor
Mmp9	Matrix metalloproteinase 9
Pi16	Protease inhibitor 16

Figure 2

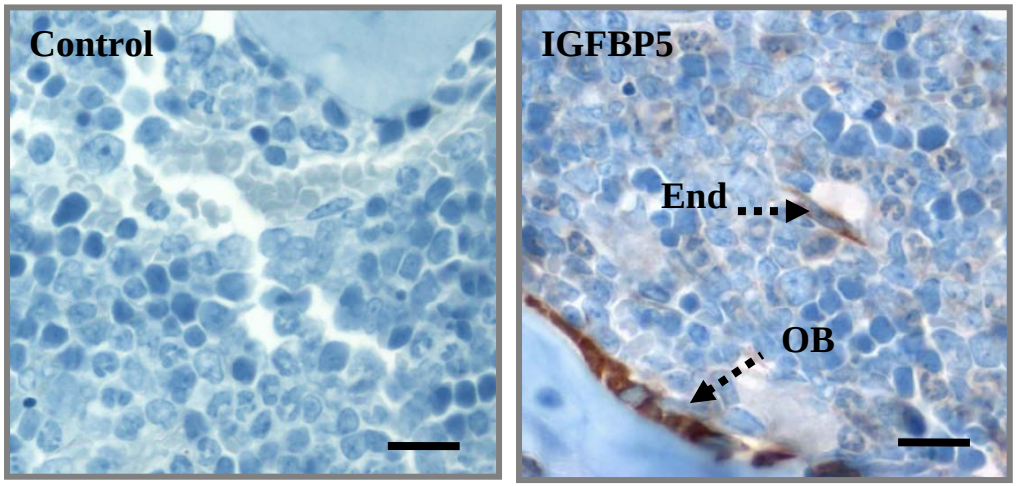
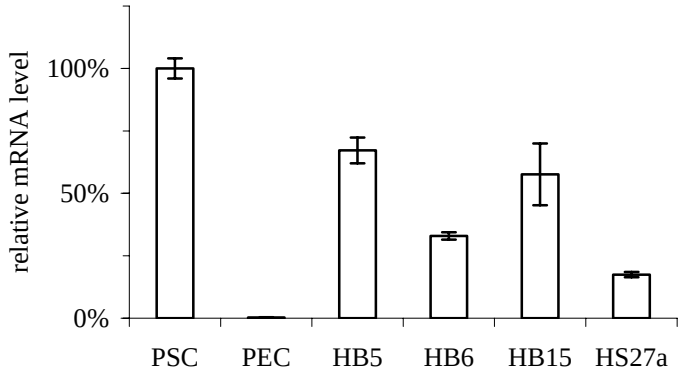
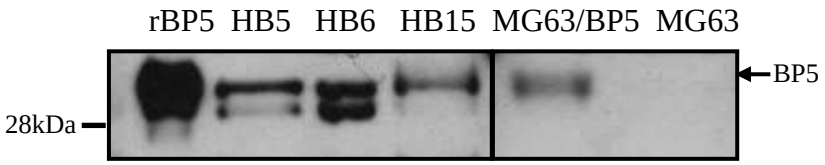


Figure 3

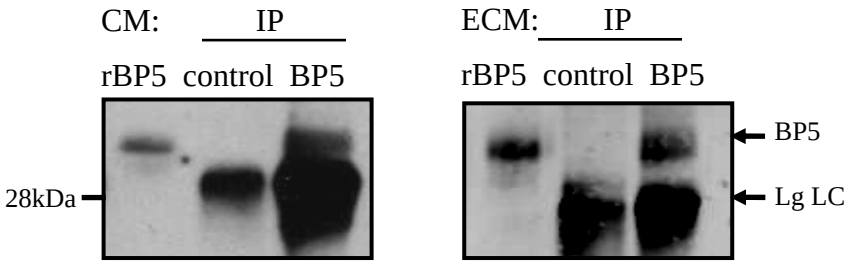
A


relative mRNA level
100%
50%
0%
PSC
PEC
HB5
HB6
HB15
HS27a

B


rBP5
HB5
HB6
HB15
MG63/BP5
MG63
BP5
28kDa

C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rBP5
control
BP5
ECM:
IP
rBP5
control
BP5
BP5
Lg LC
28kDa

Figure 4

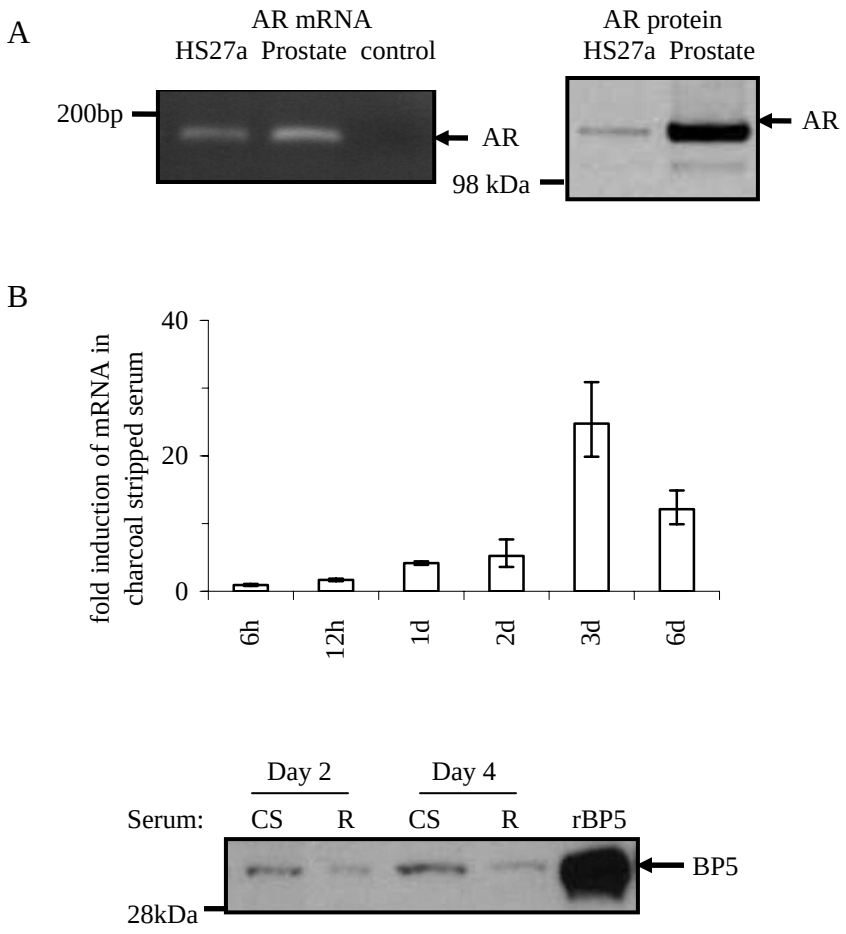
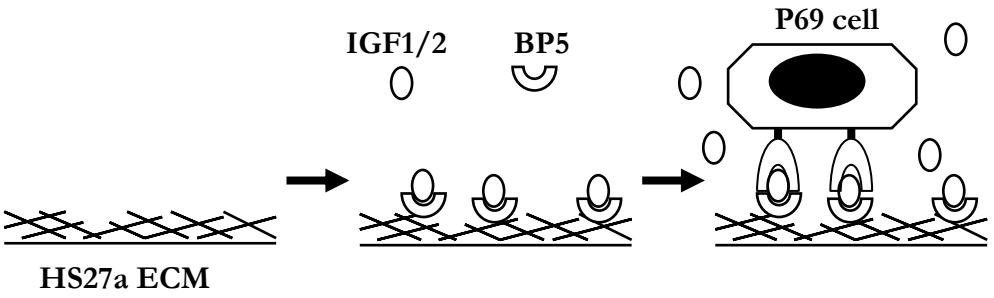

A
AR mRNA
HS27a Prostate control
AR protein
HS27a Prostate
200bp
AR
98 kDa
AR
B
fold induction of mRNA in charcoal stripped serum
40
20
0
6h
12h
1d
2d
3d
6d
Day 2
Day 4
Serum:
CS
R
CS
R
rBP5
BP5
28kDa
C
fold induction of mRNA ir charcoal stripped serum
3
2
1
Serum:
R
CS
R1881:
--
--
10nM
100nM

Figure 5.

A



B

BP5 concentration (ng/ml)	Matrix Treatment		
	BP5	BP5+IGF1	BP5+IGF2
0	100 ± 0	147 ± 10	154 ± 20
6.25	170 ± 28	190 ± 53	170 ± 16
12.5	236 ± 50	256 ± 53	231 ± 29
25	233 ± 56	303 ± 88	276 ± 81
50	223 ± 47	330 ± 69	319 ± 76
100	228 ± 54	328 ± 73	312 ± 85

Supplement Table 1. Primers used in qPCR.

Gene	forward primer	reverse primer
mouse:		
Igfbp5	ACTGTGACCGCAAAGGATTC	GAATCCTTTGCGGTCACAGT
Igfbp4	CACGAAGACCTCTTCATCATCC	GATCCACACACCAGCACTTG
Sox4	CTTTATGGTGTGGTCGCAGA	GAACGGAATCTTGTCGCTGT
Il7r	GCAACTGGACGCATGTATCTT	GACTCCACTCGCTCCAGAA
Pafah1b3	TACTCTGGCGGCTTGAGAAC	TGATGCCACCTGTCACTTGT
Cxcl12	CGGCTGAAGAACAACAACAG	GGCGTCTGACTCACACCTCT
Clu	CAGCTGGCTAACCTCACACA	AACAGCTTCACCACCACCTC
Cxcl4	AGTCCTGAGCTGCTGCTTCT	CCCAGAGGAGATGGTCTTCA
Col1A1	GAGAGCATGACCGATGGATT	GCTACGCTGTTCTTGCAGTG
Mmp9	TCGCGTGGATAAGGAGTTCT	GTTCACCTCATGGTCCACCT
Rps16	AGGAGCGATTTGCTGGTGTGGA	GCTACCAGGCCTTTGAGATGGA
Actb	ACTGCTCTGGCTCCTAGCAC	ACATCTGCTGGAAGGTGGAC
Gapdh	GTGGACCTCATGGCCTACAT	GCCTCTCTTGCTCAGTGTCC
human:		
AR	CTCACCAAGCTCCTGGACTC	AATGCTTCACTGGGTGTGGAAATAGAT
IGFBP5	ATTGTGACCGCAAAGGATTC	AGGTGTGGCACTGAAAGTCC
ACTB	ACAGGATGCAGAAGGAGATCA	AGTACTTGCGCTCAGGAGGA

Androgen-regulation of the Bone Marrow Microenvironment targets IGFBP-5

Xu C[1], Graf LF[1], Fazli L[2] , Nelson PS[1], Plymate SR[3], Cox ME[2], Gleave M, Torok-Storb BJ[1], Knudsen BS[1]. 1. Fred Hutchinson Cancer Research Center, Seattle, WA. 2. Vancouver General Hospital, Vancouver, BC. 3. University of Washington, Seattle, WA.

Introduction: The androgen receptor (AR) is expressed in mesenchymal cells in the bone marrow and bone. Androgen-suppressive therapy is the most common treatment for metastatic prostate cancer. Androgens and other steroid hormones suppress the expression of cytokines and growth factors that stimulate the growth of prostate cancer cells. Therefore, androgen-suppressive treatment might adversely facilitate the progression of prostate cancer. Here we examine the effects of androgen-suppression on the bone marrow microenvironment.

Methods: Gene expression in bone and bone marrow samples from castrated and sham-operated C57BL/6J mice was analyzed using a microarray with 16,000 cDNA probes. Differentially expressed genes were determined as with a 1.5 fold difference and a false discovery rate < 5% using SAM. Cells expressing IGFBP-5 in mouse bone and bone marrow were determined by IHC. Expression of IGFBP-5 in human primary and HS27a immortalized bone marrow stromal cells was measured by qPCR and Western blot. Proliferation of P69 immortalized prostate epithelial cells on extracellular matrices that were treated with recombinant IGFBP-5 was determined by MTS assay.

Results: Expression of 249 genes changed in bone and bone marrow after castration, including 22 genes encoding extracellular proteins. Most changes were reversed by androgen supplementation. The most significant and consistent change was an increase in IGFBP-5 expression. IHC indicated that bone marrow stromal cells, osteoblasts and endothelial cells express IGFBP-5 in mouse bone marrow. IGFBP-5 was secreted and deposited into extracellular matrix by human primary bone marrow stromal and HS27a cells and expression of IGFBP-5 was regulated by androgen. When bound to extracellular matrix, IGFBP-5 sequestered IGF-1 or IGF-2 and increased the proliferation of P69 prostate epithelial cells.

Conclusions: We conclude from these results that androgen ablative therapy increases the expression of IGFBP-5 in the bone and bone marrow microenvironment and that this might promote the growth of prostate cancer cells. Therefore, therapeutic approaches targeting IGFBP-5 are indicated as an adjunct to androgen withdrawal. Inhibition of IGFBP-5 would prevent the development of androgen-independent prostate cancer cells and inhibit the progression of metastatic prostate cancer.

ANDROGEN ABLATION INCREASES THE SYNTHESIS OF GROWTH FACTORS IN THE BONE MICROENVIRONMENT

Chang Xu, Peter S. Nelson, Beatrice S. Knudsen*
Division of Public Health Sciences, Program in Cancer Biology, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave N. , Seattle, Washington, 98109
*Corresponding author: bknudsen@fhcrc.org

ABSTRACT

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-β, since their synthesis is suppressed by steroid hormones and since the bone cells elaborating these factors express androgen receptors. Their production could potentially up 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. Four 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 flushed. 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. As candidates emerged 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.

SURGICAL CASTRATION INCREASES THE SYNTHESIS OF GROWTH FACTORS IN THE BONE MICROENVIRONMENT

Chang Xu, Peter S. Nelson and Beatrice S. Knudsen
Division of Public Health Sciences, Program in Cancer Biology, M5-A864, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave N. , Seattle, Washington, 98109

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. Because certain growth factors and cytokines are normally suppressed by steroid hormones, we postulated that androgen-ablative therapies increase the local growth promoting activities in the bone marrow and that this may stimulate tumor growth and progression to androgen-independence.

Methods. C57BL/6J mice were castrated or sham-operated at week 7 and sacrificed at 17 weeks (young) or one year later (old). Long bones were collected and in one set of mice, the bone marrow and bone were analyzed separately. If amounts of RNA were insufficient, one round amplification was conducted before Cy3 or Cy5 labeling and hybridization onto customized PEDB arrays, enriched in prostate-expressed sequences. We corrected the local background by Lowess normalization using Silicon Genetics GeneSpring 6.2 software and identified and selected genes with a 1.5 fold expression difference and a false discovery rate (FDR) around 1% using SAM (Tusher et al., 2001) (http://www-stat.stanford.edu/~tibs/SAM/).

Results. Differentially expressed genes in paired castrated and sham-operated mice were highly consistent. A total of 116 of 10,000 genes were differentially expressed after castration for 10 weeks. In the bone marrow, 74 genes were up- and 30 were down-regulated, while in bone, 32 genes were up- and 3 were down-regulated. After one year castration, 53 genes were up- and 6 down regulated in the bone and bone marrow. Twenty of the up-regulated and none of the down-regulated genes were in common between, bone and bone marrow of young and and old mice in all 9 separate expression profiles. Four of these, insulin-like growth factor binding protein 5, platelet-activating factor acetylhydrolase isoform 1b, SRY-box containing gene 4 (Sox4), and interleukin 7 receptor are interesting because they may be directly involved in the regulation of tumor growth in the bone marrow microenvironment.

Conclusions. We demonstrate that surgical castration changes gene expression in the bone marrow. Several of the genes encode secreted or cell surface proteins and may regulate interactions between tumor cells and the bone marrow microenvironment. Thus, it is conceivable that androgen-ablative therapies increase growth factor concentrations in the bone marrow microenvironment and that this may promote progression of tumor cells that are resistent to the cytotoxic effects of androgen-ablative treatments.