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Award Number: W81XWH-06-1-0171

TITLE: "Androgen, Estrogen and the Bone Marrow Microenvironment"

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CONTRACTING ORGANIZATION: Fred Hutchinson Cancer Research Center
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REPORT DATE: December 2009

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
              Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

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REPORT DOCUMENTATION PAGE

1. REPORT DATE *(DD-MM-YYYY)* 01-12-2009	2. REPORT TYPE Final	3. DATES COVERED *(From - To)* 29 NOV 2005 - 28 NOV 2009
4. TITLE AND SUBTITLE "Androgen, Estrogen and the Bone Marrow Microenvironment"		**5a. CONTRACT NUMBER** W81XWH-06-1-0171
		5b. GRANT NUMBER *
		5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S) Beatrice Knudsen, M.D., Ph.D. gO CKN<dmpwf ugpB hj ete0qti "		**5d. PROJECT NUMBER**
		5e. TASK NUMBER
		5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Fred Hutchinson Cancer Seattle, WA 98109-1024 *		**8. PERFORMING ORGANIZATION REPORT NUMBER**
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, MD 21702-5012		**10. SPONSOR/MONITOR'S ACRONYM(S)**
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
We have accomplished the following:
1) Characterized androgen responsive genes in mouse bone marrow (BM) via castration (androgen ablation) and estrogen stimulation.
2) Measurements of testosterone, dihydrotestosterone and of genes that regulate the local androgenconcentration in samples of human bone marrow.
3) Generating a human model system for studies of androgen regulation of the bone marrow stroma and its effects on cancer cells.
4) Established a 3D system of bone marrow stromal cells and DU145 cancer cells; we report the outcomes that are relevant to each of the tasks listed in the grant: We are organizing the animal work for the human bone marrow xenograft studies in mice by generating the appropriate stromal cell line/androgen response system and determining the most effective controls to address the complexity of the mouse:human heterologous system.

15. SUBJECT TERMS
Cancer, Bone Marrow, Prostate

16. SECURITY CLASSIFICATION OF: Unclassified			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	21	**19b. TELEPHONE NUMBER** *(include area code)*

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SUBJECT: Annual Report for Contract Number W81XWH-06-1-0171

"Androgen, Estrogen, and the Bone Marrow Microenvironment"

INTRODUCTION:

In this project we proposed to analyze androgen- and estrogen-responsive gene expression in the bone marrow. In males, the main source of estrogen is through conversion of androgen by aromatase. We postulate that gene and protein expression in the bone marrow microenvironment is subject to regulation by androgen and estrogen and could affect the growth and progression of micrometastatic prostate cancer cells. When prostate cancer cells leave the circulation through fenestrations in the bone vasculature, they lodge in the fertile soil of the bone marrow. Interactions between prostate cancer cells and the bone marrow regulate the early steps of metastasis formation. This environment differs from the environment of established prostate cancer metastasis, in which a fibrotic bone marrow stroma surrounds the cancer cells and cancer cells stimulate an osteoblastic response in adjacent bone. Almost nothing is known about the initial interactions of micrometastatic prostate cancer with the bone marrow microenvironment (BM-ME). During this period critical decisions in the fate of micrometastatic cancer cells occur that determine their latency, survival and proliferation. Most likely, factors in the BM-ME play a major role in regulating the progression of micrometastatic disease. While model systems exist for several steps in metastasis formation, including interactions of prostate cancer cells with endothelial cells and osteoblastic and osteoclastic bone cells, there is no *in-vivo* system to investigate the interactions between prostate cancer cells and the BM-ME. Therefore, there are many unanswered questions related to events that will ultimately determine who develops lethal prostate cancer metastases. In this grant application we will begin to investigate mechanisms that control the fate of prostate cancer cells when they first enter the BM.

Early androgen ablation has a significant survival benefit in patients at risk for prostate cancer recurrence or with increasing PSA levels after surgery or radiation therapy. At the initiation of androgen ablative therapy, the disease is often not apparent by conventional radiographic methods. However, the majority of patients will have micrometastatic disease outside the prostate. Therefore it is important to understand if and how androgen ablative therapy affects the bone marrow cells that surround the micrometastatic cancer cells. In this study, we plan to work to: determine if castration-induced gene expression changes in mouse bone marrow are caused by the deficiency of testosterone or estrogen; analyze androgen- and estrogen-sensitive cytokine and gene expression changes in human bone marrow transplanted into NOD/SCID mice, and; examine androgen- and estrogen-sensitive gene expression in the bone marrow of patients with low and high circulating testosterone levels.

BODY:

This research project was subject to second-level review by the U.S. Army Medical Research Material Command's Human Subjects Research Review Board (HSRRB). Because of this review, study implementation was delayed until we met specific requirements for compliance with human subjects protection and received approval from our local IRB and then the HSRRB. We received final approval from the HSRRB on November 21, 2006.

Task 1: To characterize androgen and estrogen regulated gene and protein expression in mouse bone marrow

- Determine if specific genes are regulated by androgen or estrogen in bone marrow

- Measure global gene regulation by androgen and estrogen in mouse bone marrow

- Correlate gene and protein expression for genes of interest

We completed the experiments for this task. We published the androgen regulation of the bone marrow Xu, et al. (2007) this grant [W81XWH-06-1-0171] was referenced in the publication, **see attached manuscript**), but did not publish the genes regulated by estrogen. To summarize the androgen regulated gene expression data, castration of mice increases expression of 159 genes (including 4 secreted cytokines) and suppresses expression of 84 genes. IGFBP5 is most consistently increased and the increase in

expression is reversed by testosterone administration. IGFBP5 protein is expression was analyzed by immunohistochemistry and is consistent with the results from the RNA expression analysis. IGBP5 protein is detected in vivo in osteoblasts, BM stromal cells, and endothelial cells. Primary human bone marrow stromal cell cultures secrete IGFBP5. In vitro, treatment of immortalized human marrow stromal cells with charcoal stripped serum increases IGFBP5 mRNA expression, which is reversed by androgen supplementation. IGFBP5 is incorporated into the extracellular matrix. Further, IGFBP5 immobilized on extracellular matrices of stromal cells enhances the growth of immortalized prostate epithelial cells.

Treatment of mice with DES for 3 weeks had a dramatic effect on the bone. 518 genes were upregulated by DES by at least 1.5 fold with a false discovery rate of < 5%. The genes that are 5-fold or greater overexpressed after DES treatment are:

procollagen, type XI, alpha 1	21.71	13.225554	33.67597	0.007597324
osteoblast specific factor 2 (fasciclin I-like)	15.19	12.364223	18.805717	0.001737353
procollagen lysine 2-oxoglutarate 5-dioxygenase 2	14.34	7.13059	20.761808	0.016561646
thrombospondin 4	13.45	8.957121	19.22137	0.006922916
procollagen type I alpha 1	10.72	7.1679716	14.533561	0.007230753
collagen binding protein 1	10.20	6.8017654	16.632366	0.011901953
secreted acidic cysteine rich glycoprotein	9.96	6.4937263	16.356506	0.012843502
osteoblast specific factor 2 (fasciclin I-like)	9.58	6.717266	11.977482	0.005546464
carbonic anhydrase 3	9.57	6.729155	13.689311	0.007458016
procollagen type I alpha 2	9.32	6.914127	14.515159	0.009389743
ESTs, Weakly similar to p190-B [M.musculus]	9.15	5.1857786	16.174885	0.020606289
lysyl oxidase	8.70	3.8938096	19.470554	0.042362962
Mus musculus matrix metalloproteinase 13 (Mmp13)	8.55	7.208835	9.475321	6.50E-04
tenascin C	8.41	5.874074	10.539991	0.006191831
Mus musculus matrix metalloproteinase 13 (Mmp13)	7.67	7.349513	8.165846	3.50E-08
immunoglobulin superfamily member 4	7.46	5.889755	8.951681	0.002399894
ESTs, Highly similar to protocadherin [R.norvegicus]	7.46	6.30951	9.614442	0.002694553
calcium channel beta 3 subunit	6.94	5.1022305	9.344099	0.006413208
bone morphogenetic protein 1	6.64	4.524218	10.723218	0.015817951
sushi-repeat-containing protein, X chromosome	6.62	6.3569093	7.023357	9.81E-06
sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A	6.15	4.349518	8.719176	0.009838916
slit homolog 2 (Drosophila)	6.01	3.8076353	10.836443	0.026326934
procollagen type VI alpha 3	5.96	3.3533673	8.322061	0.02297356
cadherin 2	5.90	4.419258	7.5463037	0.005129278
Mus musculus pigment epithelium-derived factor	5.64	4.568989	6.4149084	0.001219857
procollagen type VI alpha 1	5.63	4.631811	6.225177	7.85E-04
ESTs, Moderately similar to putative transcription regulation nuclear protein [H.sapiens]	5.63	4.8378706	6.5366893	3.29E-04
Wnt inhibitory factor 1	5.59	5.132187	5.846894	5.24E-07
Mus musculus pigment epithelium-derived factor	5.51	4.550973	6.160566	6.60E-04
lysyl oxidase-like	5.43	4.050051	6.8484035	0.005171978
hepatic lipase	5.41	4.886305	5.9013877	1.35E-06
tenascin C	5.33	2.6838875	7.612739	0.0367242
ESTs, Moderately similar to NEUROMEDIN B PRECURSOR [Homo sapiens]	5.21	4.7380557	6.0448394	7.12E-05
stromal cell derived factor 5	5.10	3.8982677	6.0784297	0.003327382

Estrogen opposes the effects of androgen and both castration and estrogen treatment kills cancer cells. Therefore we hypothesized that certain gene expression changes should be the same in the castrated and estrogen treated mice. Comparing the effects of DES treatment to castration we observed some overlap, but we also noted many genes that are downregulated by DES (green in the Figure below), but upregulated by castration (red in the Figure below).

The gene expression analysis demonstrates a subset of genes that are concordantly regulated by castration and estrogen and that could account for the effects of hormonal treatment on the environment of prostate cancer cells in the bone, which is responsible for the inhibition of tumor growth,
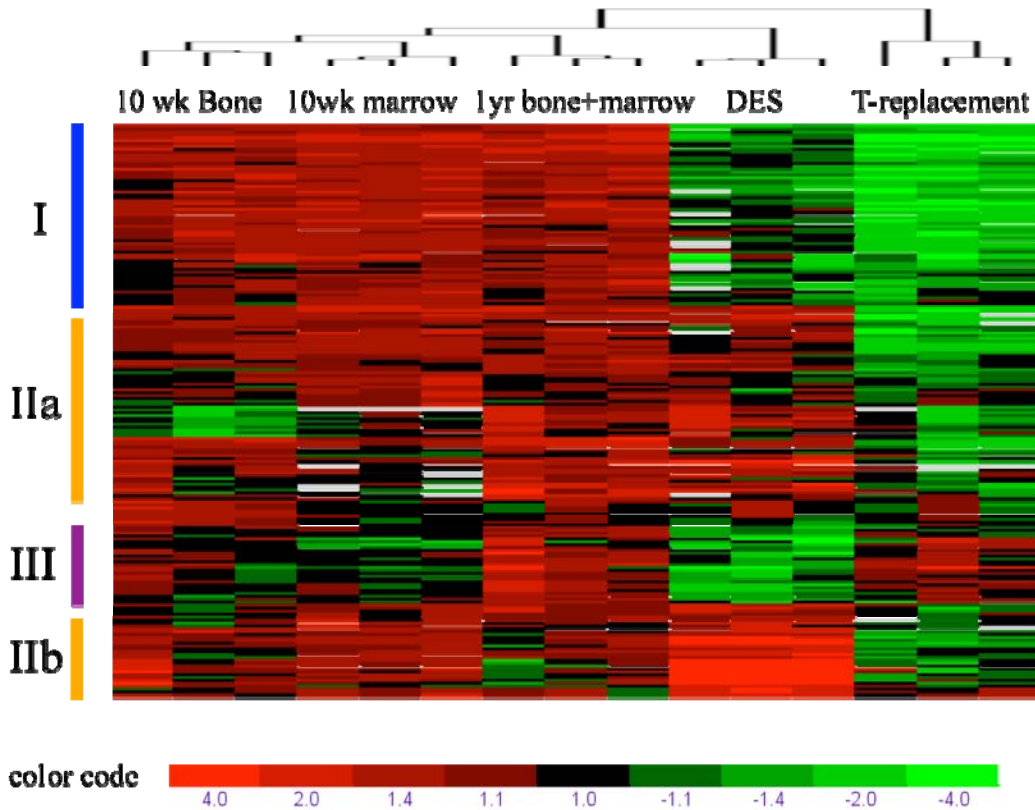
Gene categories responsive to castration and DES treatment. Three experiments were compared and each experiments represents the everage data from 3 – 4 mice. Category-I are genes that are upregulated after castration and downregulated by DES or testosterone replacement. Category-II genes are upregulated by castration as well as by DES and category-II genes are upregulated after castration and after testosterone replacement and downregulated by DES.

The third sub-task is to determine whether the effects of androgen on the BM stroma are mediated through the conversion of androgen to estrogen. In this case, the genes that we found regulated through androgen ablation would be a consequence of the low estrogen levels. This information is important to knw to know because patients could receive estrogen supplementation. To analyze the effects of estrogen on the mouse bone/bone marrow, we treated mice with DES. DES treatment caused massive production of bone and the array data demonstrated a large increase in the expression of genes driven by connective tissue derived growth factor (CTGF).

Based on these data, we realized that androgen and estrogen exert their primary activity on the mesenchymal cells in the bone marrow. This observation causes a problem for the mouse model in aim 2 (see below).

Via experimentation we have observed that, using our proposed panel, mice generally respond differentially to hormonal manipulation compared to people. Although mouse models have been previously established using aromatase inhibitors (reviewed in Brodie, et al. 2005), our experience has been such that we are not able to appropriately adjust doses of androgen inhibitory drugs (i.e. we did not see a difference between treated and untreated animals on DNA arrays). In contrast, mice appeared to be extremely sensitive to DES and in our pilot study to determine the dose and using concentrations that are proportional to human

doses, we could not find a dose that avoids the massive response in the bone. We concluded that the hormal system in mice is significantly different from human. We decided to pursue human systems that we build with our primary cell models.
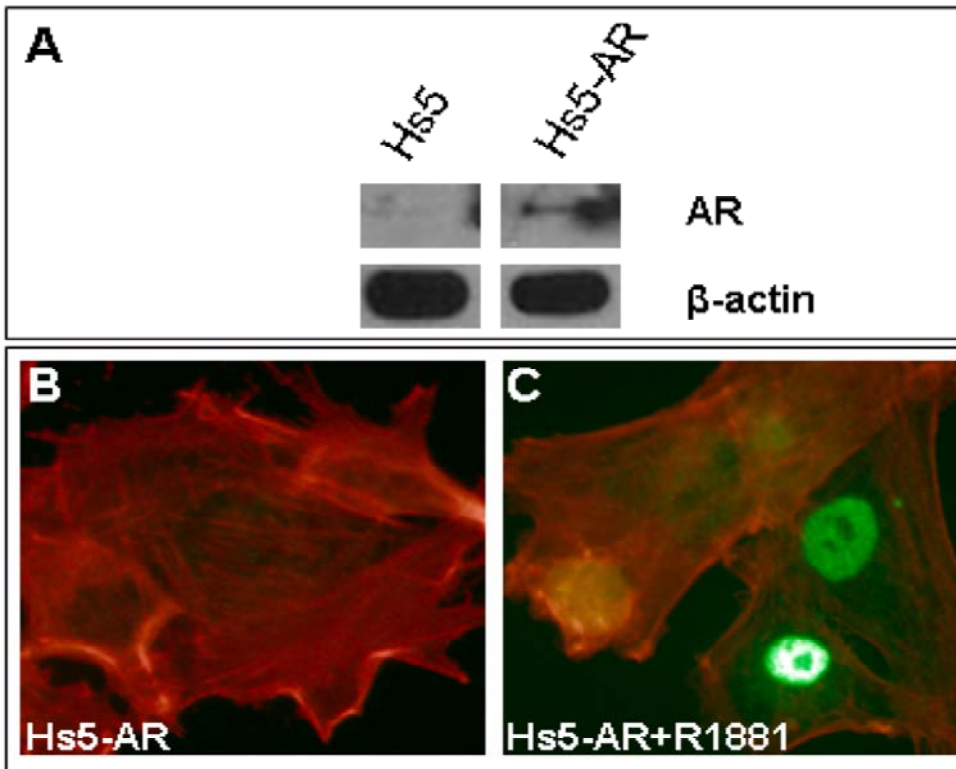
Task 2: To characterize androgen or estrogen regulated cytokines and gene expression in human bone marrow

Proposed predicted outcomes, products and deliverables:
⇒ Establishment of mouse model for studies of prostate cancer metastases using human cells
⇒ Androgen and estrogen sensitive gene expression changes in human bone marrow
⇒ Androgen and estrogen sensitive cytokine expression changes in human bone marrow

In the grant application, we proposed to generate a mouse model of prostate cancer metastasis that consists of human bone marrow (BM) in immunodeficicnt mice. SCID/hu model by transplanting human CD34+ cells into severely immunodeficient mice. The methods for this task involve reconstituting human bone marrow in the NOD-SCID mouse model. Based our data we realized that the model system in Aim 2 will unlikely generate conclusive results for the following reasons:

- The transplantation of CD34+ cells reconstitutes the BM, but does not the BM stroma, in which we observed the greatest effect of androgen regulation. CD34+ replenish the hematopoietic BM, but cannot differentiate into mesenchymal cells. Therefore, the transplantation model would determine effects of androgen ablation on the mesenchymal mouse BM stroma, which we already know. If the mouse factors interact with human cells, there might be a secondary effect on human hematopoietic cells.

- The analysis of human cytokines would reflect a secondary effect from the mouse stroma and the indirect regulation by androgen. We would not be able to distinguish between expansion of cell lineages secreting normal amounts of a cytokine per cell and the hypersecretion of cyctokines by a lesser cell number.

- The mouse cells that are the androgen responsive cell type in the reconstituted mice are heavily pretreated with radiation. We don't know whether the radiation effect interferes with the response to androgen.

Based on data that we generated after submitting the grant, we realized that the CD34+ transplantation model is not to be the best way to address the effects of androgen on human BM, since we expect the major effect on human BM stromal cells, which are not transplanted with the CD34+ cells. We therefore decided to continue with the immortalized human BM stromal cells (Hs5 and Hs27a) to build an ex vivo system of the human BM environment. As a first step, we increased the expression of AR in the Hs5 BM stromal cells. We generated an androgen responsive human bone marrow stromal cell line by expressing the

human Androgen Receptor (AR) in the Hs5 human bone marrow stromal cell line (**Figure 1).**

Figure 1. Exogenously expressed AR in Hs5 BM stromal cells becomes concentrated in the nucleus when stimulated by exposure to the synthetic testosterone (R1881). A. Western blot demonstrating increased AR expression in AR transfected cells. B. Immunofluorescence showing the dramatic increase in nuclear translocation of AR in transfected cells treated with the androgen precursor R1881.[anti-AR (green) and F-actin (red)]

Although we have generated a stable Hs5 cell line expression human AR, the expression of AR is not stable and decrease over time. We will therefore are attempting to express the AR in mesenchymal precursors, because these cells may be able to tolerate higher and more stable AR expression. In addition we plan to alter culture methods to retain AR expression. This is accomplished by charcoal-stripped fetal calf serum. In the last year, more effective methods of using NOD/SCID mouse reconstitution have been developed. We are currently considering establishing one of these models in collaboration with the bone marrow transplantation group at the Fred Hutchinson Cancer Center (D. Beverly Torok-Storb is a collaborator on the grant application). Thus, we will continue the project that was started with funding from this DOD award and are currently seeking to obtain support form other funding sources.

Task 3: To test the association between serum and bone marrow testosterone levels and expression of androgen or estrogen sensitive genes in bone marrow aspirates from patients

- Androgen-sensitive gene expression changes in human bone marrow

- Correlation between serum and bone marrow testosterone

To pursue this task, we obtained human samples of bone marrow from twenty patients with prostate cancer from Dr. Vessella, a collaborator on the grant. To evaluate the association between testosterone (T) and dihydrotestosterone (DHT) levels in serum and bone marrow, we obtained supernatants of BM aspirates. However, we quickly realized that there is an unknown dilution factor of BM aspirates when during their collection from the patients. This prohibited the accurate determination of T and DHT concentration. We have not been able to find a suitable normalization strategy to correct for the dilution factor. Normalizing to total protein did not work, because of variable amount of red cell lysis in the samples.

The androgen-responsive cells in the BM are believed to be the stromal cells (BMSC). Therefore, we used samples that were concentrated for BMSC. The Vessela lab isolates disseminated cancer cells from BM aspirates through serial depletion of CD45+ and BerH4+ cells. The remaining cells in BM aspirates should be enriched in BMSC since BMSC are negative for CD45 and BerH4 expression. Thus the remaining samples were used for isolation of mRNA.

The regulation of T and DHT concentration in the BM is complicated. Levels are regulated by (1) diffusion from blood (2) local synthesis and (3) catabolism. To determine whether T levels could be locally decreased by an increase in T catabolism, we analyzed gene expression levels of enzymes that regulate the derivatization and inactivation of DHT. The genes are: AKR1C1, AKR1C2, AKR1C3, RODH-4, RODH-5, and RL-HSD).

Testosterone levels for twenty samples from bone marrow of patients with prostate cancer were analyzed and subdivided into the top and bottom quartiles (see table).

Table 1. Testosterone levels in patients with prostate cancer

	sample i.d.	BM T - ng/ml
1	23316K	0.2
2	23343R	0.56

Top and bottom quartiles from Table 1

Bone Marrow	BM

					T - ng/ml
3	23334D		0.68	Testosterone levels	
4	23290M		0.69	1	23316K	0.2
5	23289C		0.72	2	23343R	0.56
6	23333L		0.82	3	23334D	0.68
7	23349C		0.82	4	23290M	0.69
8	23283K		1.03	5	23289C	0.72
9	23329L		1.03	6	23253G	1.31
10	23331K		1.03	7	23293M	1.4
11	23319B		1.08	8	23291C	1.41
12	23300B		1.14	9	23320H	1.66
13	23340K		1.25	10	23328J	1.94
14	23301H		1.26		
15	23285C		1.28	low Testosterone Levels	
16	23253G		1.31	High Testosterone Levels	
17	23293M		1.4		
18	23291C		1.41		
19	23320H		1.66		
20	23328J		1.94		

B) Two bone marrow samples were selected from the top and bottom quartiles for evaluation of five androgen metabolising genes (Table 2).

Table 2. Bone Marrow gene expression levels for selected androgen metabolising genes

Bones Marrow Sample	Testosterone (ng/ml)	AKR1C1	AKR1C2	AKR1C3	RL-HSD	RODH-4
23334D	0.680	0.160	0.062	3.204	0.002	0.006
23343R	0.560	10.056	0.010	0.505	0.026	0.002
23328J	1.940	0.003	0.004	0.039	0.002	0.018
23253G	1.310	7.499	0.317	0.201	0.005	0.034
PEC control	n/a	0.027	0.054	0.867	0.000	0.000

All gene expression values are de-logged Ct's normalized to ACTB	low testosterone	high testosterone

We did not observe an association between expression of DHT catabolizing genes and T levels, suggesting that T levels are not regulated through catabolism of DHT.

KEY RESEARCH ACCOMPLISHMENTS:

1) Characterized androgen responsive genes in mouse bone marrow (BM) via castration (androgen ablation) and estrogen stimulation[1].

2) Gene list of diethylstilbesterol (DES) responsive genes in mouse bone/bone marrow

3) Comparison of genomic effects of castration and DES treatment in the bone/bone marrow

4) Measurements of testosterone, dihydrotestosterone and of genes that regulate the local androgenconcentration in samples of human bone marrow.

5) Generating a human model system for studies of androgen regulation of the bone marrow stroma and its effects on cancer cells.

6) established a 3D system of bone marrow stromal cells and DU145 cancer cells

Below we report the outcomes that are relevant to each of the tasks listed in the grant: We are organizing the animal work for the human bone marrow xenograft studies in mice by generating the appropriate stromal cell line/androgen response system and determining the most effective controls to address the complexity of the mouse:human heterologous system.

REPORTABLE OUTCOMES:

Publications:

Regulation of global gene expression in the bone marrow microenvironment by androgen: androgen ablation increases insulin-like growth factor binding protein-5 expression. Xu C, Graf LF, Fazli L, Coleman IM, Mauldin DE, Li D, Nelson PS, Gleave M, Plymate SR, Cox ME, Torok-Storb BJ, Knudsen BS. Prostate. 2007 Nov 1;67(15):1621-9.

Mesenchymal-to-epithelial transition in prostate bone metastases is regulated by Zeb1. Putzke AP, Ventura AP, Opoku-Ansah J, Bailey AM, Akture C, Celiktas M, Hwang M and Knudsen BS (in preparation)

Cell adhesion and androgen receptor expression in prostate cancer metastasis: an immunohistochemical study. Akture C., Chen C., Ventura AP, Morrissey C., Roudier M., True L. and Knudsen BS (in preparation)

Meeting presentations:

Xu C, Graf LF, Fazli L, Nelson PS, Plymate SR, Cox ME, Torok-Storb BJ,
Knudsen BS (2007) Androgen-regulation of the Bone Marrow
Microenvironment targets IGFBP-5. Innovative Minds in Prostate Cancer
Today (IMPaCT). Atlanta, Georgia. P8-7.

Xu C, Graf LF, Fazli L, Nelson PS, Plymate SR, Cox ME, Torok-Storb BJ,
Knudsen BS (2006) IGFBP-5 in the Bone and Bone Marrow is a Therapeutic
Target for Metastatic Prostate Cancer. Molecular Diagnostics in Cancer
Therapeutic Development. Chicago, Illinois. B38.

Chang Xu, Peter S. Nelson, Beatrice S. Knudsen (2004) Androgen ablation
increases the synthesis of growth factors in the bone microenvironment.
95th American Association for Cancer Research Annual Meeting. Orlando,
Florida #172.

Chang Xu, Peter S. Nelson, Beatrice S. Knudsen (2004) Surgical
castration and the bone microenvironment. Prostate Cancer Foundation.
#45.

Project continuation: preliminary data for RO1 grant application for the Tumor Microenvironment (T-MEN) Program at NCI

Invited review by Journal of Cellular Biochemistry based on the published paper

Employment or research opportunities

Golovaty, I: undergraduate student, currently in Medical School
Karademos, J: undergraduate student
Schulte, B: undergraduate student, applying to Medical School

Allen, A: technician, moved to Arizona and works at TGEN
Ventura, A: technician, Knudsen lab

C. Xu, Ph.D.: post-doc, currently staff scientist at Fred Hutchinson Cancer Research Center
A. Putzke, Ph.D.: post-doc, Professor at Hope College, MI
S. Rajaram, Ph.D.: post-doc, currently works for FDA

B. Torok-Storb, Ph.D.: co-investigator
B. Knudsen, M.D., Ph.D.: PI, promoted to Associate Professor with the help of this ward

CONCLUSIONS:

1. We have generated a panel of androgen responsive genes, including cytokines, which are increased/decreased in the absence of androgen in a mouse bone marrow (Xu, et al., 2007). We focused on IGFBP5, which is the most consistently increased gene in the panel and show that its response is reversed by administration of androgen. During the course of the work, we observed a significant of androgen on B-cell development and expansion of the B-cell compartment, which we corrected for in our data analysis. This observation has since been confirmed 19164450. We observed expression of IGBP5 in stromal cells and endothelial cells. The androgen regulation of endothelial cells and its role in prostate cancer progression has since been studied in more detail [2-5]

2. Androgen metabolizing genes were examined in the bone marrow samples from patients with prostate cancer. There is no positive correlation between testosterone concentration and levels of androgen metabolizing genes. A limitation of the data is that the testosterone concentrations may be inaccurate due to dilution of the sample during aspiration from the patient.

3. The complexities involved in the mouse NOD-SCID/human bone marrow reconstitution model have forced us to find interim solutions for studies of prostate cancer/bone interactions in a human system. Hs5 cells transfected with AR are more suitable to investigate the effects of androgen on cancer cell/bone marrow stroma interactions. We are continuing to work with the bone marrow transplantation group to improve the hu/mouse models for studies of bone metastatic prostate cancer.

4. The funding has generated a publication and established a project in the laboratory in which we will continue with studies on the interactions between prostate cancer cells and the environment in the bone marrow. In particular, we are studying the effects of androgen in this system.

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2. Foresta, C*., et al.* Androgens stimulate endothelial progenitor cells through an androgen receptor-mediated pathway. *Clin Endocrinol (Oxf)* **68**, 284-289 (2008).
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"

Appendices – On next page.

Regulation of Global Gene Expression in the Bone Marrow Microenvironment by Androgen: Androgen Ablation Increases Insulin-Like Growth Factor Binding Protein-5 Expression

Chang Xu,[1] Lynn F. Graf,[2] Ladan Fazli,[3] Ilsa M. Coleman,[4] Denise E. Mauldin,[4] Danbin Li,[3] Peter S. Nelson,[4] Martin Gleave,[3] Stephen R. Plymate,[5] Michael E. Cox,[3] Beverly J. Torok-Storb,[2] and Beatrice S. Knudsen[1]*

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[2]*Division of Clinical Research, Fred Hutchinson Cancer Research Center, Seattle, Washington*
[3]*Clinical Research, The Prostate Centre, Vancouver General Hospital, Vancouver, British Columbia, Canada*
[4]*Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington*
[5]*Department of Medicine, Division of Gerontology and Geriatric Medicine, University of Washington and GRECC VAPSHCS, Seattle, Washington*

BACKGROUND. Prostate cancer frequently metastasizes to bone. Androgen suppression treatment is initially highly effective, but eventually results in resistant cancer cells. This study evaluates the effects of androgen suppression on the bone and bone marrow (BM). In particular we questioned whether the androgen therapy could adversely facilitate prostate cancer progression through an increase growth factor secretion by the bone microenvironment.

METHODS. Global gene expression is analyzed on mPEDB DNA microarrays. Insulin-like growth factor binding protein-5 (IGFBP5) is detected by immunohistochemistry in mouse tissues and its regulation measured by qPCR and Western blotting in human BM stromal cells. Effects of extracellular matrix-associated IGFBP5 on human prostate epithelial cells are tested in an MTS cell-growth assay.

RESULTS. Castration increases expression of 159 genes (including 4 secreted cytokines) and suppresses expression of 84 genes. IGFBP5 is most consistently increased and the increase in expression is reversed by testosterone administration. IGFBP5 protein is detected in vivo in osteoblasts, BM stromal cells, and endothelial cells. Primary human stromal cell cultures secrete IGFBP5. In vitro, treatment of immortalized human marrow stromal cells with charcoal-stripped serum increases IGFBP5 mRNA expression, which is reversed by androgen supplementation. IGFBP5 is incorporated into the extracellular matrix. Further, IGFBP5 immobilized on extracellular matrices of stromal cells enhances the growth of immortalized prostate epithelial cells.

CONCLUSIONS. Androgen suppressive therapy increases IGFBP5 in the BM microenvironment and thereby may facilitate the progression of prostate cancer. *Prostate 67: 1621–1629, 2007.* © 2007 Wiley-Liss, Inc.

KEY WORDS: bone marrow microenvironment; androgen suppression; IGFBP5; prostate cancer

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DISCOVER SOMETHING GREAT

INTRODUCTION

Androgen suppressive therapy has proven a significant benefit when administered in an adjuvant setting together with radiation therapy for the treatment of prostate cancer [1]. Systemic androgen deprivation kills disseminated prostate cancer cells; however, some cells survive the treatment. Survival under androgen deficient conditions may be an inherent property of certain cancer cells but may also be stimulated by factors in the microenvironment. Since prostate cancer commonly metastasizes to the skeleton [2], the environment consists primarily of bone marrow (BM) stroma and hematopoietic BM. Disseminated prostate cancer cells extraversate from the circulation through BM sinusoids [3]. Prostate specific antigen (PSA)-expressing cells are detectable in BM specimens of 54% of patients at the time of prostatectomy, indicating that cancer cells disseminate early [4]. However, disease progression is often delayed by years, suggesting that disseminated cancer cells can remain in a state of dormancy before renewing their growth. Here we investigate whether a decrease in androgen level affects the BM and whether it generates a permissive microenvironment for the growth of prostate cancer cells.

In the BM microenvironment, the androgen receptor is expressed by BM 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 [7,8]. The effects of androgen on hematopoiesis are, to a large extent, mediated indirectly through the androgen receptor activity in BM stromal cells [9]. However, androgen-sensitive factors that regulate hematopoiesis are unknown.

Insulin-like growth factors (IGF) and their binding proteins (IGFBP 1–6) are involved in normal and malignant growth of prostate epithelial cells [10]. While there were conflicting results about the expression of the IGF1 receptor (IGFR) in localized and metastatic prostate cancer in formalin-fixed tissues [11–14], a recent study using frozen tissues clearly demonstrates high IGFR expression in localized and metastatic cancer as well as in stromal cells surrounding the tumor [15]. In addition, pre-clinical studies with an inhibitory IGFR antibody (A12) reduces the growth of prostate cancer xenografts [16]. IGFs are abundant growth factors in bone and activation of the IGF pathway may lead to ligand-independent activation of the AR [17–19]. The bioavailability of IGF is regulated by a group of IGF binding proteins (IGFBP 1–6). Androgen regulates the expression of IGFBP 2, 3, 4, and 5 in the prostate [20–22]. In the bone, IGFBP 4 and 5 are the two major binding proteins that modulate the

IGF activity [23] 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 [24,25]. In human bone and BM, IGFBP5 is expressed in chondrocytes, osteoblasts and osteocytes. These cell types express androgen receptors [26–29], however only androgen regulation of IGFBP 2, 3, 4 and not of IGFBP5 has been examined in-vitro [30].

In this study we sought to measure the effects of androgen suppressive therapy on the BM environment by transcriptional profiling of castrated and sham operated mice. We observed a predominant increase in gene expression after androgen suppression and in particular of IGFBP5. Subsequent in vitro experimentation confirmed IGFBP5 regulation by androgen in human BM stromal cells and demonstrated the functional relevance of elevated IGFBP5 in the BM microenvironment.

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. Testosterone or placebo pellets (12.5 mg 60-day slow release, Innovative Research of America, Sarasota, FL) were implanted for 4 weeks. Femoral, 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. The experiment was performed in accordance with an approved Animal Care and Use Committee (IACUC) protocol.

Cells

Primary bone stromal cells HB5, HB6, and HB15 were derived from three individuals with IRB approval and maintained in MEM-alpha medium with 10% FBS (Hyclone, Logan, UT). Human immortalized bone stromal cells, HS27a [42] and prostate epithelial cells, P69 [43] were propagated in RPMI1640 with 10% FBS. Human sarcoma MG63 cells were maintained in DMEM with 10% FBS. Human primary prostate stromal and epithelial cells were cultured as previously described [44].

RNA Isolation and Microarrays

Total RNA was isolated from pulverized bone or cultured cells using TRIZOL® (Invitrogen, Carlsbad, CA) and the RNeasy® kit (Qiagen, Valencia, CA). Microarray hybridization and processing of raw data is described in Ref. [45]. Differentially expressed genes

were analyzed by hierarchical clustering using Cluster 3.0 [46]. The microarray data have been submitted to the Gene Expression Omnibus (GEO) public database at NCBI. The accession numbers are GSE5775 for castration versus sham-operation and GSE5776 for testosterone replacement versus placebo.

Reverse Transcription and Quantitative Real-Time PCR (qPCR)

cDNA was synthesized using SuperScript™ II Reverse Transcriptase (Invitrogen). Primers for qPCR spanned across intron–exon junctions and the sequences are listed in Supplementary Table I. qPCR conditions with Platinum® SYBR® Green in an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) were: 10 min at 95°C, 40 cycles at 95°C for 15 sec, 30 sec at 60°C, 30 sec at 72°C.

Immunohistochemical Staining

Mouse femurs were fixed in 10% buffered formalin at 4°C overnight and decalcified in EDTA (Decal Corp., Tallman, NY). Antigens were retrieved with EDTA, pH8 at 95°C for 8 min. The anti-IGFBP5 antibody (sc-6006, Santa Cruz Biotechnology Inc., Santa Cruz, CA) was diluted 1:100 for incubation in the Vantana autostainer model Discover XT™ (Vantana Medical System, Tuscan, AZ).

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. The sensitivity of the measurement was 0.10 ng/ml.

Preparation of Cell Lysate, Conditioned Medium (CM), and Extracellular Matrix (ECM)

Cells were lyzed in RIPA buffer [47] containing protease and phosphatase inhibitors (Roche Applied Science, Mannheim, Germany). Serum-free medium was conditioned for 48 hr and concentrated with an Amicon Ultra-15, 5 kDa Centrifugal Filter (Millipore, Billerica, MA). ECM was prepared as described by Knudsen et al. [48]. ECM on plates was used immediately for growth assays or solubilized in RIPA buffer.

Immunoprecipitation and Immunoblotting

Proteins (500 µg) were precipitated with 10 µl anti-IGFBP5 (Catalog # 06-110, Chemicon International, Inc., Temecula, CA) overnight and proteinG agarose beads (Sigma, Saint Louis, MO) for 2 hr. Total proteins (40 µg) were analyzed on 12% NuPAGE or 4–12% Bis-Tris Gels

(Invitrogen) and transferred to Immobilon-P (Millipore). Membranes were blocked with 5% milk and probed with 1:1,000 anti-IGFBP5. Blots were developed with the 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 (100 ml of FBS stirred with 15 g of dextran-treated charcoal at 4°C overnight and sterilized). 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 rIGFBP5 without or with human rIGF1 (Sigma) or mono-biotinyl IGF2 (GroPep Limited, Adelaide, SA, Australia) for 4 hr. After the plates were washed, 40,000 P69 cells were seeded per well in serum free medium containing 10 ng/ml IGF1 or IGF2. MTS assays were performed after 48 hr using the CellTiter 96® AQ$_{ueous}$ cell proliferation assay kit (Promega, Madison WI). The experiment was repeated four 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 BM has not been reported. Therefore we undertook a global approach to analyze gene expression changes in mouse BM and bone that occur upon castration. We separated bone and BM from young (17 weeks) castrated and sham-operated C57BL/6 mice for comparison in array experiments. Purified RNA from bone or BM cells was labeled and hybridized to mPEDB arrays. Results obtained with bone samples were similar to those obtained with BM samples; therefore we tested combined bone and BM preparations in subsequent experiments. In these experiments, we compared the effects of castration in old (59 weeks) and young (17 weeks) mice. In addition, we analyzed castrated mice with and without testosterone supplementation.

Analysis of array data revealed that 243 genes exhibited significant and consistent differential expression in bone and BM of young castrated compared to sham-operated mice. Of these, 159 were up-regulated and 84 were down-regulated in. The effectiveness of the castration procedure was documented by a reduction in serum androgen levels and seminal vesicle size

TABLE I. 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

*Measurements are the average ± standard deviation of tissue samples from three mice.

(Table I). Next, gene expression changes in BM and bone from young and old mice were compared (Fig. 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 BM, 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 ($r = 0.97$; Fig. 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 vs. 0.7 ng/ml, see Table I) and the seminal vesicles grew to the size found in sham-operated controls (Table I). 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 (Fig. 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 encode proteins that are associated with the extracellular matrix (ECM; Fig. 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 [31]. IGFBP4 is also expressed by cells in bone and BM 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 ± 0.88 in young mice and 2.0 ± 0.17 in old mice. In the same samples the fold expression change for IGFBP4 was 0.86 ± 0.50 in young mice and 1.14 ± 0.41 in old mice. To identify cell types expressing IGFBP5 we used immunohistochemistry. IGFBP5 expression in sections of mouse bone and BM was observed primarily in osteoblastic cells lining the bone and in endothelial cells (Fig. 2). While we observed weak diffuse staining in the BM stroma, individual BM cells were difficult to discern by morphologic criteria. Thus, the expression of IGFBP5 in BM stromal cells was demonstrated subsequently in cultures of primary BM stromal cells.

To determine whether IGFBP5 is expressed in human bone and BM stroma, we measured IGFBP5 mRNA expression in cultures of primary human BM stromal cells (Fig. 3A). IGFBP5 RNA expression was detected in cells from three separate individuals and in immortalized HS27a BM 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 (Fig. 3B) and HS27a cells (Fig. 3C) and accumulated in the conditioned medium. In addition, IGFBP5 became incorporated into the HS27a ECM (Fig. 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). A band of the size expected for IGFBP5 was only detected in the MG63-BP5 cells, but not in the parent control cells (Fig. 3B).

To determine whether IGFBP5 expression is regulated by androgen in human BM stromal cells, we first confirmed expression of the AR in HS27a cells. Both AR mRNA and protein (Fig. 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 (Fig. 4B,C). The increase of IGFBP5 mRNA was reversed by addition of physiological concentrations of androgen (Fig. 4D). These results demonstrate that the level of AR expression in Hs27a cells is sufficient to regulate IGFBP5 expression.

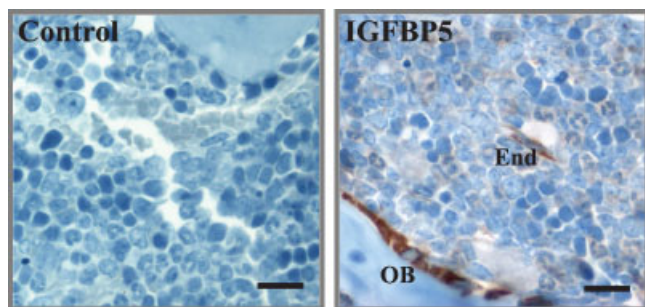## IGFBP5 Stimulates the Growth of Immortalized Prostate Epithelial Cells

In contrast to IGFBP5 in conditioned medium, which is growth inhibitory, IGFBP5 in ECM was shown to
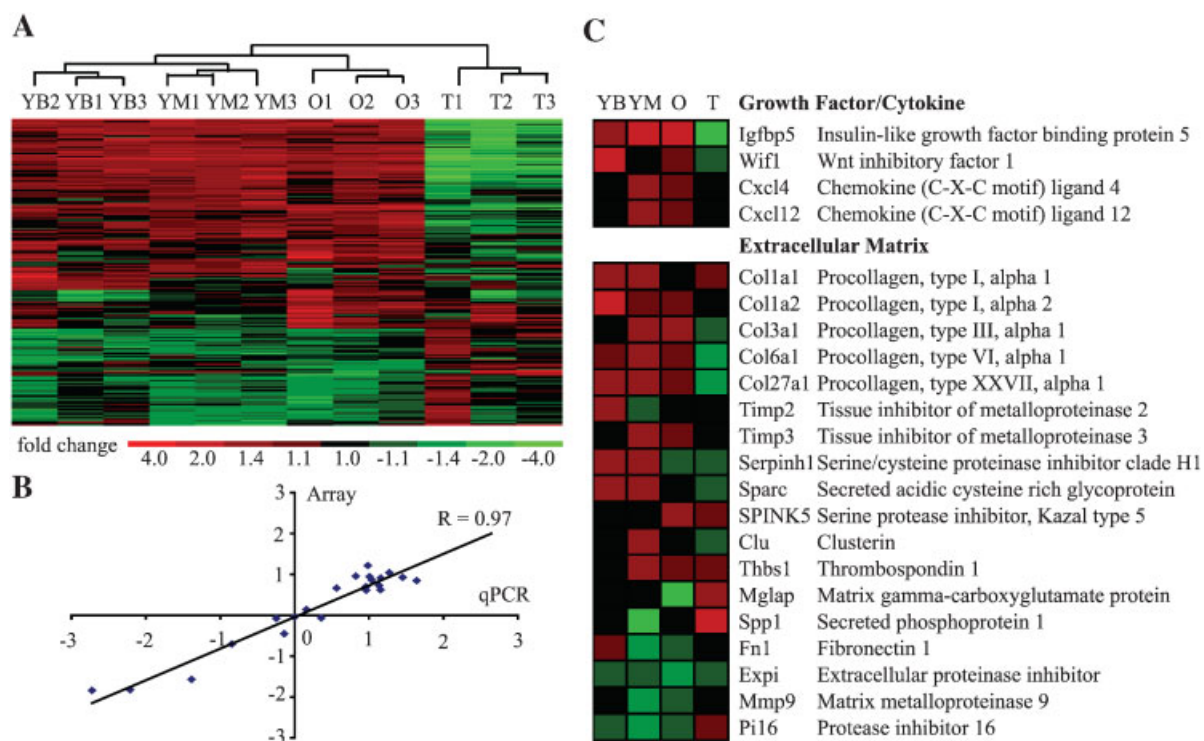
Fig. 1. Differentially expressed genes in bone and bone marrow after androgen deprivation. **A**: Hierarchical clustering of genes that are differentially expressed in paired castrated versus sham-operated mouse tissues: young bone (YB1-3, 10 weeks after castration), young BM 10 weeks after castration (YM1-3), old bone and BM 52 weeks after castration (O1-3), and bone and BM 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. **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 ECM proteins.

promote the growth of fibroblasts [32]. We therefore tested whether IGFBP5 bound to ECM could also stimulate the growth of epithelial cells and used P69 immortalized prostate epithelial cells to evaluate the growth-promoting activity of matrix-bound IGFBP5. P69 cells are immortalized human prostate epithelial cells, deficient in Rb and p53 protein function and are

highly responsive to IGF1. ECM from HS27a cells was incubated with recombinant IGFBP5 and IGF1 or IGF2. The unbound proteins were removed and P69 cells were plated on the ECM in serum-free medium (Fig. 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 (Fig. 5B). While growth increased in a linear fashion up to incubation with 25 ng/ml IGFBP5, there was no further increase with 50 or 100 ng/ml IGFBP5. IGF1 or IGF2 in the absence of exogenous IGFBP5 also stimulated cell growth.


Fig. 2. Expression of IGFBP5 in mouse bone and bone marrow. Decalcified sections of femoral BM are stained with anti-IGFBP5 antibodies and the expression of IGFBP5 is visualized by DAB (brown color). OB: osteoblasts; End: endothelial cells. The scale bar measures 30 μM. Control: no primary antibody.

DISCUSSION

We identified gene expression changes in bone and BM after castration of C57BL/6 mice. The expression of 159 genes increased and the greatest and most
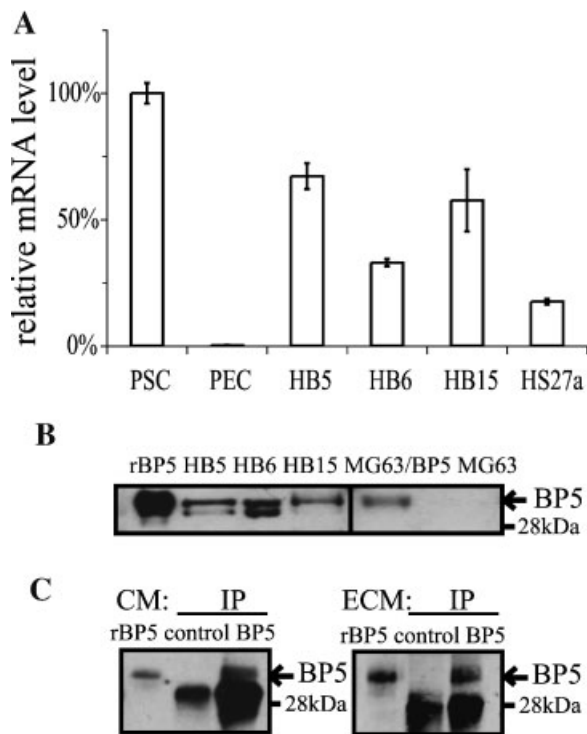
Fig. 3. RNA and protein expression of IGFBP5 in human bone stromal cells. **A**: Steady state IGFBP5 mRNA is measured by qPCR in human primary bone stromal cells HB5, HB6, and HBI5, human immortalized HS27a bone stromal cells, human primary prostate epithelial cells (PEC) and stromal cells (PSC). Expression is normalized to β-actin and is plotted relative to expression in PSC. Results are the average of three measurement replicates. Error bar indicates the standard deviation. **B**: Detection of IGFBP5 protein from conditioned medium of human primary bone stromal cells. A representative Western blot is shown. **lane I**: Recombinant IGFBP5 (rBP5, 25 ng); **lanes 2 – 4**: primary bone stromal cell culture; **lane 5**: human sarcoma cell line MG63 expressing IGFBP5 (MG63/BP5); and **lane 6**: MG63 control cells. **C**: IGFBP5 protein is immunoprecipitated from conditioned medium (CM, **left panel**) or extracellular matrix (ECM, **right panel**) of HS27a cells. Lane I: Recombinant IGFBP5 (rBP5, 25 ng); lane 2: Protein G bead control; and lane 3: IGFBP5 immunoprecipitation. Arrow: IGFBP5.

consistent increase was observed for IGFBP5. Immunohistochemical staining indicated that BM stromal cells, osteoblasts and endothelial cells 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 ECM by primary human BM stromal cells and HS27a cells. When bound to ECM, IGFBP5 increased the growth of P69 immortalized prostate epithelial cells. We conclude from these results that IGFBP5 expression increases after androgen ablation, which

may promote the growth of prostate cancer cells in the BM environment.

A limitation in the interpretation of castration induced gene expression changes is that castration alters the levels of several hormones, and not only decreases circulating T levels. Thus, changes in other hormones could be responsible for the regulation of gene expression in the BM. Bone is specifically responsive to estrogens, which are decreased in castrated mice and increased with testosterone supplementation. Castration also increases FSH/LH. The distinction between the effects of androgen and estrogen in-vivo would require blockade with an aromatase inhibitor. While the effects of decreased androgen and estrogen cannot be distinguished in-vivo, in-vitro, IGFBP5 induction by charcoal-stripped medium is suppressed by R1881, which cannot be aromatized. These data suggest that the regulation of IGFBP5 expression is mediated by androgen; however it does not exclude a contribution of estrogen in-vivo.

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 5,000 per cell in cultured human BM stromal cells, which include osteoblastic cells [33,34]. Consistent with these results, we detected expression of AR mRNA and protein in HS27a cells. In vivo androgens regulate cell types that lack detectable AR expression presumably through the BM 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 [35]. Androgen, but not estrogen or IGF1, regulates the maturation and expansion of the B-cell compartment [8,36,37]. 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 [9]. Interestingly, the activity from the stroma is specific for B-cells and does not affect the T-cell compartment in the BM [38].

Several other studies analyzed the response of IGFBP5 expression to androgen stimulation or androgen suppression and the results are inconsistent. The reason for the discrepancies lies in the difference in cell type and source (cell cultures, xenografts, patient tissue samples), and in the reagents and methods that were used for the analysis. In the prostate and bone, the expression of IGFBP5 RNA is observed in the mesenchymal cells, for example, prostate stromal cells, BM stromal cells and osteoblasts (Ref. [14] and Fig. 3).
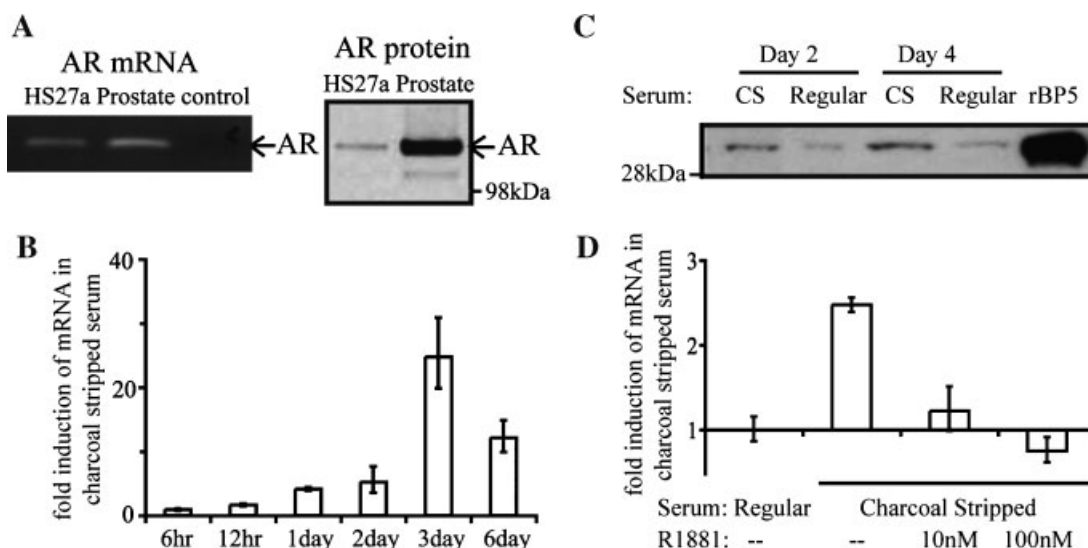
Fig. 4. Regulation of IGFBP5 expression in HS27a cells by androgen. **A**: AR RNA expression (**left panel**) and protein expression (**right panel**) in HS27a. **Lane 1**: HS27a; **lane 2**: prostate tissue, **lane 3**: negative control. AR protein is detected by Western blot in whole cell lysates of HS27a cells or prostate tissue. **B**: Induction of IGFBP5 expression by charcoal stripped serum. 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. Expression is normalized to β-actin. **C**: HS27a cells are cultured in charcoal stripped serum (CS) or regular serum for 2 and 4 days. IGFBP5 expression in equal amounts of whole cell lysate is measured by Western blotting. **D**: Suppression of IGFBP5 expression by androgen. HS27a cells are cultured in regular serum or charcoal-stripped serum with R1881 testosterone for 24 hr. The experiment was repeated twice with similar results. The fold difference of IGFBP5 RNA expression is calculated as described in B.

Our primary prostate epithelial cultures, which are of the basal/intermediate cell types, did not express IGFBP5 RNA. An immunohistochemical study found about 4% of prostate epithelial cells were stained positive for IGFBP5 protein after 18–43 days of androgen suppressive therapy, an increase compared to the 0.2% as in the placebo group [39]. The regulation of IGFBP5 expression by androgen was also analyzed in xenografts. While IGFBP5 expression increased after

castration in the Shinogii xenograft [40], it increased with androgen stimulation in the CWR22 xenograft [41]. This study is the first to examine the regulation of IGFBP5 expression by androgen in bone and BM stromal cells. The results of this study are consistent between in-vivo and in-vitro systems and between mouse and human. Androgen withdrawal clearly increased IGFBP5 expression and the increase in bone and BM in-vivo is sustained for at least 1 year (Fig. 1).

Both, the IGF1 receptor and IGFBP5 are targeted by novel drugs that are undergoing clinical trials for treatment of metastatic prostate cancer [16,40]. This study suggests that the drug target, IGFBP5, is expressed in the microenvironment of metastatic cancer cells. This may increase the opportunity of
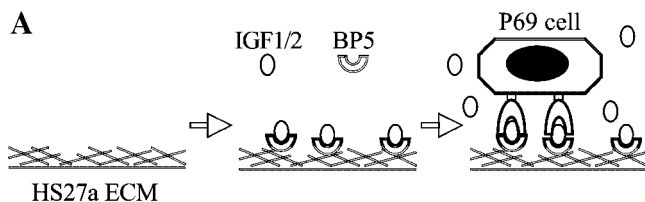
BP5 concentration (ng/ml)	Matrix Treatment		
	BP5	BP5+IGF1	BP5+IGF2
0	100 ± 0	147 ± 10	154 ± 20
6.25	170 ± 28	190 ± 53	170 ± 16
12.5	236 ± 50	256 ± 53	231 ± 29
25	233 ± 56	303 ± 88	276 ± 81
50	223 ± 47	330 ± 69	319 ± 76
100	228 ± 54	328 ± 73	312 ± 85

Fig. 5. Growth stimulation of P69 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 IGF1 or IGF2 and cell numbers are measured 2 days later. **B**: Growth P69 cells on HS27a matrix. 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 four 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). The experiment was repeated twice with different passage numbers of HS27a cells.

IGFBP5 targeted therapies of treating bone metastatic disease. Because IGF1 and IGFBP5 stimulate the progression of cancer cells to androgen independence, early administration of drugs that inhibit their activities may augment the clinical response to androgen ablative treatment. In addition to its therapeutic interest, IGF1 and IGFBP5 levels in the bone and BM could affect the progression of micrometastatic disease. Studies are under way to determine whether progression of micrometastatic disease at the time of radical prostatectomy is increased in men with low serum testosterone levels due to an elevated IGFBP5 concentration. In summary, IGFBP5 functions as a key androgen-sensitive modulator of the BM microenvironment.

ACKNOWLEDGMENTS

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