OSTEOBLAST DERIVED FACTORS INDUCE ANDROGEN INDEPENDENT Proliferation AND Expression of PROSTATE SPECIFIC Antigen IN HUMAN PROSTATE CANCER CELLS

by

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ABSTRACT

Prostate cancer metastasizes to the skeleton to form osteoblastic lesions. Androgen ablation is the current treatment for metastatic prostate cancer. This therapy is palliative and the disease will return in an androgen independent form that is preceded by a rising titer of prostate-specific antigen (PSA). Factors secreted by osteoblasts have been implicated in mediating progression to androgen independence. Here we investigated the possibility that human osteoblasts might secrete factors that contribute to the emergence of androgen independent prostate cancer.

Primary cultures of human osteoblasts were used as a source of conditioned medium (OCM). Proliferation, expression of androgen-regulated genes, and transactivation of the androgen receptor (AR) were monitored in LNCaP human prostate cancer cells in response to OCM using a proliferation assay, Northern blot analysis and reporter gene constructs. Levels of interleukin-6 (IL-6) present in OCM were measured and its contribution to proliferation and expression of PSA was investigated by neutralization studies with anti IL-6 antibodies.

OCM increased the proliferation and the expression of PSA at both the protein and RNA levels in LNCaP cells. Synergistic increases in the activities of PSA(6.1kb) and pARR$_3$-tk-luciferase reporters were measured in cells co-treated with both OCM and androgen. OCM targeted the N-terminal domain of the AR. The effect of OCM on transcriptional activity of the AR was inhibited by an antiandrogen. Neutralizing antibodies to IL-6 blocked proliferation and expression of PSA by OCM.
Osteoblasts secrete factors such as IL-6 that cause androgen-independent induction of PSA gene expression and proliferation of prostate cancer cells by a mechanism that partially relies on the AR. Therefore, other AR-regulated genes involved in proliferation and regulatory mechanisms may also be activated in the absence of androgens by OCM. This suggests that the AR may play a role in the progression of prostate cancer to androgen independence by a mechanism initiated by factors secreted from the bone, such as IL-6. Identifying such molecular mechanisms may lead to improved clinical management of metastatic prostate cancer, which has a poor prognosis and significant morbidity and mortality.
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<tr>
<td>ACE</td>
<td>antichymotrypsin inhibitor protein</td>
</tr>
<tr>
<td>AF</td>
<td>activating function</td>
</tr>
<tr>
<td>Akt</td>
<td>serine/threonine protein kinase</td>
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<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>ARACaP</td>
<td>prostate cancer cell line derived from ascites fluid</td>
</tr>
<tr>
<td>ARF</td>
<td>activation resorption formation</td>
</tr>
<tr>
<td>BAP</td>
<td>bone-specific alkaline phosphatase</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenic protein</td>
</tr>
<tr>
<td>BPH</td>
<td>benign prostatic hyperplasia</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C4-2B</td>
<td>prostate cancer cell line</td>
</tr>
<tr>
<td>CAT</td>
<td>computer assisted tomography</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate signaling molecule</td>
</tr>
<tr>
<td>CKII</td>
<td>casein kinase II</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic AMP response element binding protein</td>
</tr>
<tr>
<td>CSF</td>
<td>colony stimulating factor</td>
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<tr>
<td>CXCR12</td>
<td>CXC 12-receptor</td>
</tr>
<tr>
<td>CXCR4</td>
<td>CXC 4 (amino acid motif) receptor</td>
</tr>
<tr>
<td>D3</td>
<td>vitamin D3</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
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<td>DBD</td>
<td>DNA-binding domain</td>
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<tr>
<td>DHT</td>
<td>dihydrotestosterone</td>
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<tr>
<td>DPD</td>
<td>deoxypiridinoline</td>
</tr>
<tr>
<td>DU-145</td>
<td>brain metastatic prostate cancer cell line</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>MAP (mitogen activated protein) kinase</td>
</tr>
<tr>
<td>ET1</td>
<td>endothelin 1</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>HBME</td>
<td>human bone marrow endothelial cells</td>
</tr>
<tr>
<td>HGF</td>
<td>heparin growth factor</td>
</tr>
<tr>
<td>Hk2</td>
<td>human kallikrein 2</td>
</tr>
<tr>
<td>HOBIT</td>
<td>human osteoblast like cell line</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>ICAT</td>
<td>isotope coded affinity tags</td>
</tr>
<tr>
<td>IGF(BP)</td>
<td>insulin-like growth factor (binding protein)</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>IP3</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>KGF</td>
<td>keratinocyte growth factor</td>
</tr>
<tr>
<td>Ki 67</td>
<td>Ki-67 biomarker - a proliferation antigen</td>
</tr>
<tr>
<td>LBD</td>
<td>ligand-binding domain</td>
</tr>
<tr>
<td>LFA1</td>
<td>leukocyte function-associated antigen one</td>
</tr>
<tr>
<td>LHRH</td>
<td>luteinizing hormone releasing hormone</td>
</tr>
<tr>
<td>LNCaP</td>
<td>lymph-node carcinoma of the prostate cell line</td>
</tr>
<tr>
<td>LuCaP</td>
<td>prostate cancer cell line</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MDA</td>
<td>metastatic bone derived androgen independent prostate cancer cell line</td>
</tr>
<tr>
<td>Mdm2</td>
<td>mouse double minute 2 gene</td>
</tr>
<tr>
<td>MDP</td>
<td>methylene diphosphonate</td>
</tr>
<tr>
<td>Me2SO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>MEM</td>
<td>minimal essential media</td>
</tr>
<tr>
<td>Met family</td>
<td>met receptor tyrosine kinase - the prototypic member of a small subfamily of growth factor receptors that when activated induce mitogenic and morphogenic cellular responses</td>
</tr>
<tr>
<td>MMPs</td>
<td>matrix metalloproteases</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MTT</td>
<td>(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide)</td>
</tr>
<tr>
<td>NES</td>
<td>nuclear export signal</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>NTD</td>
<td>N-terminal domain</td>
</tr>
<tr>
<td>NTX</td>
<td>N-telopeptide</td>
</tr>
<tr>
<td>OCM</td>
<td>osteoblast conditioned media</td>
</tr>
<tr>
<td>OPG</td>
<td>osteoprotegrin</td>
</tr>
<tr>
<td>PBS-</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PC-3</td>
<td>bone metastatic prostate cancer cell line</td>
</tr>
<tr>
<td>PCIB</td>
<td>serum type I collagen C terminal propeptide</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PMO</td>
<td>primary mouse osteoblasts</td>
</tr>
<tr>
<td>PSA</td>
<td>prostate-specific antigen</td>
</tr>
<tr>
<td>PSMA</td>
<td>prostate-specific membrane antigen</td>
</tr>
<tr>
<td>PTHR</td>
<td>parathyroid receptor</td>
</tr>
<tr>
<td>PThrP</td>
<td>parathyroid related protein</td>
</tr>
<tr>
<td>R1881</td>
<td>synthetic androgen</td>
</tr>
<tr>
<td>RANK(R)</td>
<td>receptor activator of NF-Kappa B (receptor)</td>
</tr>
<tr>
<td>RAS</td>
<td>cytoplasmic GTPase</td>
</tr>
<tr>
<td>RGD</td>
<td>arginine-glycine-aspartate (RGD) sequence motif</td>
</tr>
<tr>
<td>Rho-C</td>
<td>proteins that control the organization of the actin cytoskeleton</td>
</tr>
<tr>
<td>Rho-GTPase</td>
<td>GTPase enzyme controlling Rho protein activity</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>RPMI</td>
<td>red phenol minimal essential media</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency disorder</td>
</tr>
<tr>
<td>SDF1</td>
<td>stromal derived factor 1</td>
</tr>
<tr>
<td>Src kinase</td>
<td>rouse sarcoma virus kinase</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TGF α/β</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TPA</td>
<td>transientsly proliferating/amplifying cells (between the epithelial differentiated and stem cells)</td>
</tr>
<tr>
<td>TR</td>
<td>transretinoic acid receptor</td>
</tr>
<tr>
<td>TRMP2</td>
<td>complement-associated protein SP-40</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid stimulating hormone</td>
</tr>
<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
</tr>
</tbody>
</table>
There are some without whom this thesis would have been impossible

BUT

There are many without whom it would have been a lot easier
ACKNOWLEDGEMENTS

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DEDICATION

I dedicate this thesis to

Marianne Sadar,
James Goldie and
Nicholas Bruchvsky

Exceptional mentors and friends
CHAPTER 1: INTRODUCTION

1.1 PROSTATE CANCER:

1.1.1 The Challenge

In Canada an estimated 145,500 new cases and 68,300 cancer related deaths are predicted in 2004. The most frequently diagnosed cancer in Canadian men is prostate cancer, contributing almost 27% (USA 33%) to overall cancer incidence and third in contributing almost 12% (USA 13%) to overall cancer related deaths. Among the 2 million men living in B.C. approximately 10,000 new cancer diagnoses and 4,500 deaths are noted yearly. Of those over 3,000 diagnoses and 540 deaths will be prostate cancer related. The average lifetime risk of developing prostate cancer is about 1 in 7.6 but only 1 in 26 will die of the disease. This translates to a lifetime incidence risk of 13.2% and a lifetime mortality risk of 3.8% (McLaughlin, 2004).

Prostate cancer is a disease predominantly affecting the older population with more than three quarters of the diagnoses and deaths occurring in males over 60. In particular, 46% of new cases and 85% of deaths occur past the age of 70. The total age standardized prostate cancer incidence and mortality rates per 100,000 Canadian males are 449 and 121 respectively.

A sharp increase in Canadian prostate cancer incidence was noted between the years 1989-1993, attributed to a novel screening regimen employing the serum tumor marker called prostate-specific antigen (PSA). However this detection pool has now been
largely exhausted and the rising trend for prostate cancer has once again decreased to pre-
PSA screening days. Although the rise in prostate cancer cases over the years has been
attributed to PSA screening and an ageing population, changes in unidentified risk and
protective factors, although largely hypothetical almost certainly contribute to today’s
overall prostate cancer statistics. Overall, PSA screening has reduced the number of
patients first presenting with metastatic prostate cancer from 17% in 1990 to 4% in 1998,
while early tumor detection has increased from 14% to 51% (Crawford, 2003). Androgen
ablation, the most successful treatment for prostate cancer no longer confined to the
prostate, slows the growth of the disease. The average time of progression to therapy
resistance, termed androgen independence, is on the order of 2.5 years. (Pollen et al.,
1981; Soloway et al., 1988). One of the most frequent metastatic sites in prostate cancer
is bone where the lesions are blastic in 65%, lytic in 16% and mixed in 23% of cases
(Berruti et al., 2000). Survival is closely correlated to the extent of bone metastases,
where 96% of patients with fewer than 6 lesions and less than 43% with more than 6
lesions survive over 30 months (Mettlin et al., 1996).

1.1.2 The Prostate Gland

1.1.2.1 Anatomy and function

The prostate gland is an exocrine gland between the urinary bladder and pelvic
floor that is approximately 3cm in length, 2cm in depth and 4cm in width, weighing
about 18g (Figure 1) (Campbell and Walsh, 2002; DeVita et al., 2001). Its external
anatomy divides it into anterior, posterior and lateral areas. The transitional zone is
Figure 1. Zonal anatomy of the prostate. The prostate is divided into several distinct regions, which differ slightly in terms of glandular architecture. The TZ-transitional zone gives rise to few carcinomas, the CZ-central zone gives rise to about a quarter of malignancies and the peripheral zone (PZ) contributes the bulk.

most often the origin of benign prostatic hyperplasia and gives rise to less than 20% of adenocarcinomas. The central zone comprises roughly about 25% of tissue volume in the prostate. The individual glands in this region are somewhat different from glands in the rest of the prostate which is the basis for speculation that this region of the prostate is of a different embryonic origin. This central zone gives rise to only about 1-5% of adenocarcinomas. The majority of the prostate is composed of the peripheral zone, stretching out along the posterior and lateral aspects of the gland. The peripheral zone is the most common site for prostatic adenocarcinoma (Campbell and Walsh, 2002; DeVita et al., 2001). The prostate and accessory glands produce most of the volume of the seminal secretions which extend the viability and decrease the impact of the environmental shock the sperm encounter in the vagina. Prostatic secretions are also thought to protect the lower male urinary tract from infections (Lepor and Lawson, 1993).

1.1.2.2. Cellular components of the prostate

The prostate is composed of 2/3 glandular tissue and 1/3 fibromuscular supporting stroma which forms part of the capsule. The glandular secretory epithelium is composed of mostly cuboidal or columnar tubuloalveolar secretory cells, under and among which are the basal cells surrounded by stroma and connective tissue (Figure 2). The luminal secretory cells express cytokeratin 8 and 18, are androgen dependent and are the most common epithelial cell type in the prostate. The basal cells attach to the stroma and contain high molecular weight cytokeratins such as cytokeratin 5, some are androgen
sensitive but not dependent, and make up less than 10% of the entire glandular population (Lepor and Lawson, 1993; Schalken and van Leenders, 2003; Xue et al., 1998). In between the basal and differentiated secretory luminal cells is a continuum of intermediately differentiated populations of secretory cells, transiently proliferating/amplifying (TPA) cells intermediate to the stem cells and fully differentiated cells, that are most commonly characterized by staining for cytokeratin 5 and 18 and c-met (Schalken and van Leenders, 2003). The third secretory cell type, neuroendocrine cells, contain chromogranins and secrete products such as serotonin and thyroid stimulating hormone (TSH) which indirectly may be involved in prostatic cell growth and function (Abrahamsson and Lilja, 1989; di Sant'Agnese, 1992; di Sant'Agnese and Cockett, 1996). Stromal cells aid in the control of epithelial development and differentiation by producing growth factors which have direct and indirect influence on prostate growth. These cells are also key players responsible for the cell interactions that promote epithelial cell survival. Stromal cells are derived from the mesenchyme and have the potential to change their cellular phenotype based on the environment. There are two types of stromal cells in the prostate: 1) smooth muscle cells that express smooth muscle α-actin that are negative for vimentin; and 2) fibroblasts that express vimentin and are negative for α-actin. The smooth muscle cells produce inhibitory substances for epithelial cells while fibroblasts produce stimulatory factors, which may explain regional apoptosis and mitosis of epithelial cells in various regions of the gland (Naz, 1997).
Figure 2. Cellular composition of the prostate. The prostate is composed of glandular tissue comprised of cuboidal epithelial cells. Under the epithelial cells is the basal and stem cell layer that separates the epithelium from the stromal cells. The arrows within the same cell type indicate autocrine stimulation and in between cell types indicate paracrine signaling. Testosterone and DHT affect both the epithelial and stromal cells.
1.1.2.3 Growth and maintenance

Factors contributing to the growth and maintenance of the prostate include: 1) endocrine secretions which act over long ranges and are generally transported in the blood such as steroids and polypeptides; 2) neuroendocrine factors such as acetylcholine and norepinephrine from neighbouring neuroendocrine cells; 3) paracrine factors such as basic fibroblast growth factor (bFGF) which are short range mediators generally produced by neighbouring cells; 4) autocrine factors which act on the same cell as they are produced; and 5) intracrine factors which are never secreted but act inside the cell in which they are produced (Figure 3) (Lepor and Lawson, 1993). Among the different substances that mediate regulation of cellular activities such as differentiation, proliferation and apoptosis are various polypeptides which are usually produced in a paracrine fashion (Aaronson, 1991). These polypeptide growth factors include epidermal growth factor (EGF), insulin like growth factor (IGF), transforming growth factor alpha (TGFα), transforming growth factor beta (TGFβ), bFGF, and keratinocyte growth factor (KGF) (Sherwood et al., 1992). KGF is mostly produced by fibroblasts and is generally stimulatory (Sugimura et al., 1994). TGFβ stimulates stromal growth, angiogenesis and epithelial apoptosis (Lepor and Lawson, 1993). It is generally produced by the smooth muscle cells (Ilio et al., 1995). bFGF is produced at high levels in the normal adult prostate. It promotes angiogenesis and acts as a broad spectrum mitogen and inducer of various genes. EGF is produced at lower levels and its significance in growth has not been clearly determined (Sherwood et al., 1992). TGFα is an autocrine growth factor which acts through the EGF receptor (Ilio et al., 1995). IGF I and II promote prostate cell
growth and stimulate osteoblasts to lay down new bone in a paracrine fashion but also act as an endocrine factor carried by blood, with overall growth stimulatory properties (Peehl et al., 1995). Platelet-derived growth factor (PDGF) is also mitogenic and promotes responses to other growth factors (Rijnders et al., 1985). Many of the various factors are sequestered by the extracellular matrix and do not exert their effects until released (Lepor and Lawson, 1993). Androgen, and more specifically its active metabolite dihydrotestosterone (DHT), is the most potent mitogen in the prostate and appears to influence cells differently in different regions of the prostate. In the distal acinar compartment of the prostate gland, DHT promotes proliferation while in the proximal ductal compartment DHT stimulates apoptosis. The outcome of androgen action seems to rely on the composition of the adjacent stroma (Naz, 1997). Androgen deprivation promotes epithelial atrophy but some cells always remain and can regenerate the prostate (Bockrath et al., 1981).
Figure 3. Types of growth control. Mechanisms of cellular and environmental communication include various and diverse ways of conducting signals. Intracrine refers to the fact that the signaling molecules do not leave the target cell, autocrine means the molecules leave the cell of origin but act on it externally, while the same signal acting on the cells neighbours is termed paracrine signaling. Endocrine is signaling through a plethora of factors secreted into blood. Cytokine signaling is mediated mostly by immune cells and neuroendocrine signaling through neural and neural like cells.

1.2 Androgens and the Androgen Receptor

1.2.1 Androgens

Androgens are steroid hormones that are required for the differentiation, maturation and maintenance of male reproductive organs. In the prostate, DHT regulates cell proliferation and cell death. Production of androgens in males occurs primarily in the testis by Leydig cells with minor amounts contributed by the adrenal glands. Testosterone is converted to 5α-DHT in some peripheral tissues by two isoforms of the low abundance enzyme 5α-reductase (Figure 4). Expression of 5α-reductase has a highly variable tissue distribution which contributes to the variable distribution of DHT (Kemppainen et al., 1992; Wilbert et al., 1983; Zhou et al., 1995). 5α-reductase type II is predominant in the prostate (Andersson et al., 2002; Steers, 2001). Levels of 5α-reductase are induced by DHT (Torres and Ortega, 2003). Androgens are lipophilic and are transported in the blood bound to plasma proteins such as albumin (50%), sex hormone binding globulin (44%), and corticosteroid-binding globulin (4%) (Baker, 2002; Beato and Klug, 2000). It is generally assumed that androgens enter the cell by diffusion across the plasma membranes (Hogeveen et al., 2002).

The action of androgens is mediated by the intracellular ligand-activated transcription factor called the androgen receptor (AR). The AR is a member of a large family of nuclear receptors that are structurally and functionally similar (Evans, 1988; Truss and Beato, 1993). After translation the AR becomes phosphorylated and associated with heat shock proteins (HSPs). Testosterone and DHT are ligands of the AR.
Figure 4. Hormone structure: Structures of androgen precursors, androgens (testosterone) and the active form of androgen-DHT. For comparison structures of the synthetic androgen R1881 (used in these studies and not degraded like DHT in the cells) and bicalutamide, an inhibitor that prevents androgens from binding their receptor, are shown.
However, DHT is more potent in inducing transactivation of the AR because it has a higher affinity, lower dissociation constant and thus has a more stable receptor occupancy as compared to testosterone. Different ligands of the AR such as testosterone and DHT activate partially distinct genes in the prostate, which is thought to involve different conformations of the AR resulting in preferential interactions with different DNA sequences or co-activators (Avila et al., 1998; George et al., 1991). The binding of ligand to the AR induces a more compact structure due to a realignment of helix 12 (Matias et al., 2000). This conformational change is associated with the release of molecular chaperones (hsp90 and hsp70) and the translocation of the AR to the nucleus. The receptor then dimerizes and binds DNA of androgen regulated genes at androgen response elements (AREs) (Kuil et al., 1995) (Figure 5).

Furthermore, nongenomic actions mediated by the AR are now being recognized as important in addition to genomic actions. For example, androgens can signal inside cells through surface receptors or other cytoplasmic (possibly including the AR) receptors to modulate second messengers such as cyclic AMP (cAMP), inositol triphosphate (IP3), phospholipase C, diacylglycerol (DAG) and calcium ions (Ca$^{2+}$). Each of these second messengers can activate various kinases including Ca$^{2+}$ which for example increases activity of Rous Sarcoma Virus kinase (Src kinase). Involvement in various signaling cascades independent of transcription in the target cell allows androgens to affect many more regulatory networks than would be permitted by genomic mechanisms alone (Walker, 2003).
Figure 5: Activation of the AR via ligand binding. Upon binding DHT the inactive AR dissociates from the HSP chaperones and translocates to the nucleus where it serves as a transcription factor to enhance expression of androgen regulated genes such as PSA.

Source: M. Sadar
1.2.2 Structure and functional domains of the AR

The AR gene is located on the q11-12 X chromosome arm. There are two different initiation start sites which produce two AR transcripts of 10 and 7kb (Faber et al., 1989; Tilley et al., 1990). The promoter lacks TATA and CAAT activation sequences but contains pur/pyr and GC box SP-1 binding sites (Chen et al., 1997; Tilley et al., 1990). Regulation of the promoter occurs through hypermethylation, growth factor pathways and ligand concentration. For example, high androgen levels decrease androgen gene mRNA production but at the same time increase protein stability (Chung et al., 1999; Henttu and Vihko, 1993; Izbicka et al., 1999; Robel et al., 1983; Tan et al., 1988).

The AR gene has 8 coding exons (Figure 6). Exon 1 encodes the transactivation domain in the N-terminal domain (NTD) and there are several polymorphic regions in this domain (Faber et al., 1989). Exons 2 and 3 encode the DNA binding domain (DBD) while exons 4-8 encode the ligand-binding domain (LBD). The whole DNA spanning region is 180kb and the protein is 919 amino acids long depending upon the length of polymorphic tracts (Figure 6) (Brinkmann et al., 1989).

Nuclear receptors are unique ligand-dependent transcription factors that have an NTD, a central DBD, a hinge region and a C terminus which contains the LBD as illustrated in the upper panel of Figure 6. The N-terminal domain (NTD) contains an activation function 1 (AF-1) transactivation region of the AR that is constitutively active in the truncated receptors which lack the LBD (Miesfeld et al., 1987; Simental et al., 1991). The NTD contains polymorphic repeats of polyglutamine, polyproline and polylysine.
Figure 6. The AR. The upper panel shows the AR is composed of several distinct domains, with their associated functions listed below each domain. The lower panel shows 8 genomic exons of the AR, also grouped into functional domains. Both panels are identically color coded for ease of comparison.

One well characterized region on the AR is the glutamine repeat where an increase in its length results in decreased AR transactivation while a decrease in its length is associated with increased transcriptional activity. It has been shown that the NTD contributes to receptor DNA complex stability as well by interacting with co-regulators (De Vos et al., 1994; Kallio et al., 1994). Ligand-dependent intramolecular N terminal-C terminal interaction which map to 23-FXXLF-27 and 433-WXXLF-437 motifs respectively occur during receptor dimerization that result in an antiparallel arrangement of androgen receptor monomers (He et al., 2000; Langley et al., 1995).

The DBD is highly conserved, rich in cysteine, lysine and arginine. It includes 2 zinc fingers, encoded by two different exons which each bind a zinc atom between 4 cysteine residues (Hard et al., 1990; Katahira et al., 1992; Lee et al., 1993; Luisi et al., 1991; Schwabe et al., 1990). The first zinc finger contacts the major groove of the DNA and is important for specific recognition of DNA binding, while the second zinc finger stabilizes binding by contact with the sugar backbone of adjacent sequence (Schoenmakers et al., 1999). The AR recognizes the same DNA consensus sequence as the glucocorticoid receptor 5'-AGAACAnnnTGTTCT-3'. However, it is important to note that AREs identified in natural promoters differ from the consensus sequence and the sequence confers receptor specificity as discussed by Verrijdt et. al., 2003.

The LBD is also highly conserved and contains the ligand-dependent transactivation function AF-2. The LBD is capable of binding numerous ligands with various affinities. For example if DHT binding is set as 100%, testosterone binds 84%,
progesterone 24%, DHT metabolites 17%, corticosteroids 7% and cortisol 1% to mention a few. The synthetic androgen R1881 also binds the AR with high affinity and is more stable than testosterone and DHT molecules and therefore preferentially used for *in vitro* experiments (Murthy et al., 1986). The LBD binds antagonists or antiandrogens such as bicalutamide which are used for treatment of advanced prostate cancer, albeit with much lower affinities range from 0.1-0.4 % (Steinsapir et al., 1991). Antagonists such as the non-steroidal bicalutamide, demonstrate their antiandrogen properties by binding the LBD and preventing N and C terminal interactions which are important in dimerization (Hakimi et al., 1997; Jenster et al., 1995; Kemppainen et al., 1999; Murthy et al., 1986; O'Malley, 1990; Waterman et al., 1988).

The amino acids in the hinge region have homology to nuclear localization signals (NLS) of the SV40 large T antigen and nuclear plasmin (Mitchell and Tjian, 1989; Simental et al., 1991). Under normal conditions, ligand influences subcellular localization of the AR from the cytoplasm to the nucleus. *In vivo*, the AR is only transiently cytoplasmic, immediately post synthesis, and then translocates to the nucleus (Jenster et al., 1992). Deletion of the hinge NLS still allows some ligand-dependent nuclear translocation which implicates the existence of a second NLS in the LBD (Jenster et al., 1992; Poukka et al., 2000). Recently a ligand-regulated nuclear export signal (NES) has been identified in the LBD of the AR (Saporita et al., 2003).
1.2.3 Post Translational Modification of AR

After translation most proteins undergo covalent modification. There are 100 or more forms of such modification known (Han and Martinage, 1992; Krishna and Wold, 1993). In nuclear receptors, disulfide bond formation, glycosylation, transamination, phosphorylation, acetylation, sumoylation and ubiquitination can occur. One of the best studied modification of steroid receptors is phosphorylation through protein kinases which regulate cell function. R1881, probably like testosterone in vivo, increases the phosphorylation of the AR in the cytosol, but also in the nucleus such that the ratio of phosphorylated to non-phosphorylated protein is the same in both compartments (Kuiper et al., 1993). AR is phosphorylated within 15 min of translation and shows a shift from 110 to a 112 kDa (Kuiper et al., 1991). Both non- and phosphorylated forms can bind hormone (van Laar et al., 1991).

Approximately 90% of phosphorylation occurs in the N-terminal domain and majority of these are serine residues (Kuiper and Brinkmann, 1994; Kuiper and Brinkmann, 1995). Ten phosphorylation sites have been identified in the AR (Gioeli et al., 2002; Lin et al., 2001; Wen et al., 2000; Yeh et al., 1999). Changes in phosphorylation of the AR have been shown in response to androgens, forskolin (stimulates the PKA pathway), EGF, and phorbol 12-myristate 13-acetate (Gioeli et al., 2002; van Laar et al., 1991). Serines 213 and 791 are phosphorylated by MAPK and serine/threonine protein kinase-Akt (Lin et al., 2001; Wen et al., 2000; Yeh et al., 1999). Phosphorylation of the AR by Akt reduces its transcriptional activity (Lin et al., 2001),
while phosphorylation by MAPK is suggested to enhance transcriptional activity (Yeh et al., 1999). In support of this, inhibition of MAPK activity blocks both ligand-dependent and ligand-independent transcriptional activity of the AR (Ueda et al., 2002a; Ueda et al., 2002b). Phosphorylation of the AR may also alter receptor turnover as suggested by studies examining Akt and Mouse double minute 2 protein (Mdm2) (Lin et al., 2002). Other posttranslational modifications of the AR include acetylation (Fu et al., 2002), ubiquitination (Sheflin et al., 2000) and sumoylation (Poukka et al., 2000).

1.2.4 Ligand Independent Activation of the AR

The AR can be activated in an androgen independent manner by a number of factors including interleukin-6 (IL-6), interleukin-2 (IL-2), bombesin, vasoactive intestinal peptide, heregulin, leutenizing hormone releasing hormone (LHRH), bone morphogenic proteins (BMPs), triiodothreonine, vitamin D, phenylacetate, butyrate, IGF I, IGF II, KGF, EGF, forskolin and cAMP (Bova et al., 2001; Chackal-Roy et al., 1989; Gleave et al., 1991; Hobisch et al., 1998; Kimura et al., 1996; Lang et al., 1995; Orr et al., 2000; Ritchie et al., 1997; Sadar et al., 1999; Ueda et al., 2002a; Ueda et al., 2002b).

The mechanism involved in ligand-independent activation of the AR by IL-6 and stimulation of the protein kinase A (PKA) pathway in the absence of androgens has been shown to target the NTD of the AR (Sadar, 1999; Sadar et al., 1999; Ueda et al., 2002a; Ueda et al., 2002b). In the case of IL-6, this mechanism appears to involve protein-protein interactions between the NTD of the AR and signal transducers and activators of transcription STAT 3 and is dependent upon the MAPK pathway (Ueda et al., 2002a;
Ueda et al., 2002b). LHRH increases AR activation in a ligand-independent manner via the second messenger cAMP. Meanwhile, sex hormone-binding protein may also activate unliganded protein complexed AR to increase androgen independent gene expression (Culig, 1997).

1.2.5 Androgen-Regulated Genes

Many genes from various tissues can be induced or suppressed by androgens via the AR at both the protein and mRNA level (Chang et al., 1989; Saltzman et al., 1987). Analyses of the proteome and transcriptome in prostate cancer cells in response to androgens have led to the identification of androgen-regulated genes involved in protein-folding, metabolism, protein trafficking and secretion, cytoskeleton, and regulation of cell-cycle and signal transduction (Eder et al., 2003; Meehan and Sadar, 2004; Nelson et al., 2002). In the prostate, the prostate-binding protein, the prostate spermine-binding protein (a secretory glycoprotein that is a substrate for PKA), cyclin dependent kinase 2 and 4 (CDK2 and 4) and casein kinase I (CK1p16), important in cell cycling, NKx3.1 (a development and differentiation product), and prostate specific antigen (PSA) are regulated via androgens and the AR (Brooks et al., 1986; Celis et al., 1993; Chang et al., 1989; Lu et al., 1997; Prescott et al., 1998). In addition, the trans-retinoic acid (TR2 and 3) orphan receptors are under the control of the AR (Lin et al., 1993). Examples of genes negatively regulated by androgens include AR mRNA, early growth response gene alpha, glutathione S-transferase, maspin and serpin (Blok et al., 1995; Henttu and Vihko, 1992; Quarmby et al., 1990). Hallmarks of genes regulated by androgens is the presence of one
or more AREs, together with a binding site for Sp1, CCAAT and NF-1 and/or NF-kB, Ets and AP-1.

In terms of other more widely expressed genes, levels of IL-4, -5 and interferon gamma (T cell cytokines) are influenced by DHT (Araneo et al., 1991). TGF-β, an AR regulated growth factor predominating in the thymus, has been shown to limit growth in the prostate and decrease apoptosis in mutant mice. TGF has also been shown to be important in growth and maintenance of the prostate gland (Olsen et al., 1993). Androgens are also important in regulating IGF I in some epithelial cells, KGF in stromal cells, as well as casein kinase II (CKII) and complement-associated protein SP-40 (TRPM2) (Sahlin et al., 1994; Yan et al., 1992). Integrin-mediated extracellular adhesion protein in prostate has shown AR dependence and has been suggested to play a role in metastasis (Bonaccorsi et al., 2000). Interestingly, bone development and maintenance also involves androgens (Melton et al., 2000). Many genes “normally” regulated by androgens become re-expressed in androgen-independent tumours (Gregory et al., 2001). AR protein is generally expressed in most clinical samples of prostate cancer and is retained at similar or increased levels in hormone refractory disease (Culig et al., 1998; Pertschuk et al., 1994; Takeda et al., 1996). Thus the AR has been suggested to play a role in hormone refractory disease.

1.2.6 PSA

PSA is a representative, well studied, model of genes “normally” induced by androgen through an activated AR, and is one of the best clinical tumor markers in
monitoring disease progression during androgen ablation therapy (Luke and Coffey, 1994). In the normal prostate, AR expression levels follow those for PSA mRNA. However, growth factors and the extracellular matrix also contribute to PSA regulation (Goldfarb et al., 1986; Guo et al., 1994; Sica et al., 1999; Young et al., 1991).

The PSA gene belongs to the human kallikrein family, located on chromosome 19q13.4, found among 15 genes, all of which are serine proteases, highly expressed in prostate and possibly prostate cancer (Diamandis et al., 2000; Takayama et al., 2001). The PSA gene is composed of 5 coding exons, the first contains the 5' untranslated region, the second contains the histidine, the third the aspartic acid and the fifth the serine- key residues of the PSA enzyme catalytic triad. The fourth exon has no remarkable features. The promoter of the gene contains classic activation sequences including the TATA box and polyadenylation signals, as well as steroid receptor binding consensus sequences (AREs) which determine AR specificity (Figure 7) (Diamandis et al., 2000).

Normally PSA is secreted into the glandular lumen of epithelial cells at 10-50μmol/L, and the extracellular levels are much lower at <0.1nmol/L. High extracellular levels, which in cancer may be 1000 times normal physiological levels, are indicative of damage to the prostatic glandular ductal system (which usually protects the outside environment from proteases) and possibly other contributing factors such as failure of clearance mechanism including renal malfunction (Lilja, 2003). Furthermore, in adenocarcinoma of the prostate, PSA tends to be preferentially complexed with the
antichymotrypsin protease (ACT) inhibitor which aids to distinguish prostate cancer from BPH (free PSA) where there is also an increase in serum PSA.

Initially, as the adenocarcinoma regresses following pharmacological or surgical castration, serum PSA levels fall. However, eventually there is a relapse of the disease in an androgen independent form indicated by a rising titer of serum PSA. At this point the cancer has adapted to grow without androgens and increasing PSA serum levels imply that alternative factors are activating the AR and therefore AR regulated genes (Culig et al., 2002b). Finally, it worthy to note that PSA may in fact be much more than just a tumor marker, it may actually actively contribute to cancer invasion and progression by promoting angiogenesis, growth factor release and extracellular matrix modification (Lilja, 2003).
**Legend**

- Biologically Active Site
- Putative Site
- ARE Androgen Response Element
- ARR Androgen Response Region
- CRE Cyclic AMP Response Element

**Figure 7. PSA Promoter.** Regulatory regions of prostate specific antigen untranslated/regulatory sequence. The DHT bound AR binds AREs and ARRs.

1.3 Bone-Structure Function and Cell Types

1.3.1 Physical Anatomy of Bone

Bone is a dynamic connective tissue that responds to metabolic and mechanical forces and as a result changes throughout life. There are three main functions for this highly specialized tissue. First, it serves as a mechanical and structural support for the body and attachment for muscle. Second, it serves a protective function where it surrounds vital organs such as the heart and lungs and houses the bone marrow within its cavities. Third, it serves a metabolic/homeostatic function by storing reserves of calcium and phosphate and maintaining their balance in blood (Baron, 1999). There are two major components to bone, the cells and the extracellular matrix. The matrix is composed of collagen fibers, non-collagenous proteins and has the unique ability to calcify.

1.3.2 Bone Forming Cells

1.3.2.1 Osteoprogenitors

The marrow and stroma generally give rise to stem cells for the haematopoietic (including osteoclast) and non haematopoietic (including osteoblasts) lineages. Thus the bone marrow along with the periosteum is the source of the mesenchymal progenitor cells which eventually give rise to preosteoblasts, osteoblasts and osteocytes. The pluripotent mesenchymal cells which give rise to osteoprogenitors have limited renewal capacity but an extensive proliferative capacity (Aubin, 1996). The growth and differentiation of stem cells to osteoblasts is largely under the control of various cytokines, hormones and growth factors (Figure 8). The influence of many of these
Figure 8. Osteoblast maturation. The growth and differentiation of stem cells to osteoblasts is largely under the control of various cytokines, hormones and growth factors of which some are indicated above.

factors is not purely inhibitory or stimulatory but is often complex and chronologically and geographically dependent. For example FGF and TGFβ are potent mitogens under certain circumstances but that depends on their concentration and on the progenitor age and type (Moseley, 1996). BMP-2, -4, -7 and parathyroid hormone (PTH) on the other hand are more defined in their actions and induce osteoclastogenesis (Stein, 1996). Glucocorticoids promote growth and differentiation. Such signalling factors affect cell specific regulatory factors such as steroid receptors, fos, jun, helix-loop helix proteins, leucine zipper proteins, zinc finger proteins and homeodomain proteins thereby influencing the cell’s state (Banerjee et al., 1997; Komori et al., 1997; Machwate et al., 1995; McCabe et al., 1996; Ogata et al., 1991; Otto et al., 1997; Royai et al., 1996; Ryoo et al., 1997; Sumoy et al., 1995).

1.3.2.2 Osteoblasts

Osteoprogenitor mesenchymal cells first differentiate into preosteoblast cells, which are characterized by some markers of mature osteoblasts such as alkaline phosphatase and by their proximity to surface osteoblasts which line the layer of bone they produce. These cells appear as spindle shaped osteoprogenitors and then become large cuboidal osteoblasts on the surface of bone. These cells are characterized by a large round nucleus at the base, basophilic cytoplasm, enlarged Golgi and extensive, and prominent endoplasmic reticulum (ER) that is typical of secretory connective tissue cells. Upon final differentiation, osteoblasts are responsible for the biosynthesis and organization of the extracellular matrix (ECM) (Baron, 1999). The temporal expression of proteins in osteoblasts reflects their stage of differentiation. The differentiated cells
secrete type I collagen, specialized bone matrix proteins and ground substance towards the mineralizing front of the tissue. Cell matrix and cell-cell interactions which take place via integrins and cadherins are important for differentiation and mineralization by the cells. Responsiveness to systemic factors and mechanical forces is ultimately manifested by signalling from the ECM to the nucleus and cell action. Increased ECM production leads to a decrease in cell growth and no growth leads to matrix maturation which involves alkaline phosphatase and specialized bone proteins which make the ECM ready for mineralization. Levels of alkaline phosphatase and osteocalcin in serum are good indicators of bone function and disease. In mature osteoblasts the plasma membrane is rich in steroid receptors, alkaline phosphatase, vitamin D₃ receptors (receptors for the active form of vitamin D-1,25 dihydroxy vitamin D₃), integrins and cytokine receptors (Baron, 1999; Lian, 1999).

1.3.2.3 Osteocytes

At the end of their secretory phase many osteoblasts become trapped in the bone matrix that is later calcified. This is the terminal differentiation phase of osteoblasts at which point they are termed osteocytes and are now largely responsible for supporting bone structure. The osteocytes are located in osteocytic lacunae which are special cavities in the calcified matrix. The osteocytes have many thin cytoplasmic processes throughout the calcified matrix in canals termed canaliculi. The canaliculi and the lacunae protect and nourish the cells in a specialized extracellular fluid. These processes are in contact with other such processes and other osteocyte bodies and thus there is direct communication between surface and other osteoblasts deep within the matrix, or a
continuum of cells and networks throughout the matrix permeating the entire bone (van der Plas et al., 1994). At points of contact the cells and processes are connected via gap junctions and specifically connexins which allow cell communication by facilitating calcium ion flux across the junctions (Civitelli et al., 1993; Rubin and Lanyon, 1987). Osteoblast morphology is therefore largely dependent on their differentiation state which is influenced by their age and the extracellular environment.

1.3.3 Bone Resorbing Cells

Remodelling of normal bone requires resorption which is accomplished by osteoclasts derived from the mononuclear/phagocytic lineage. Mature osteoclasts are giant and multinucleated and in contrast to acting in clusters like osteoblasts they are usually found as one or two per remodelling site. Mature osteoclasts express proteins such as esterases, lysozyme, colony stimulating factor (CSF1), calcitonin and vitronectin receptors (Lian, 1999). Osteoclasts contact the calcified bone directly and at the zone of contact between the calcified matrix surface the osteoclasts have a prominent ruffled border with deep folds of the plasma membrane surrounded by a ring of contractile proteins – termed the sealing zone. These cells are physically located in lacunae or pits of their own resorbtive activity. Cell attachment to bone is maintained via matrix proteins binding integrin receptors on the osteoclast. The cytoplasm is rich in foamy vacuoles, abundant Golgi and mitochondria. The vacuoles are vesicles that contain lysosomal enzymes and metalloproteinases such as collagenase and gelatinase which are secreted through the ruffled border into the extracellular bone resorbing compartment. This compartment is equivalent to a secondary lysosome where secretion of the contents under
low pH can dissolve calcified crystals thereby exposing the matrix substrate for degradation by lysosomal enzymes. Specifically the links between collagen and hydroxyapatite crystals (the major mineral complex of bone) are dissolved followed by digestion of collagen fiber (Lian, 1999). This is not only a remodelling mechanism but also contributes to maintaining calcium and phosphate concentration in blood. Urine measurements of hydroxyproline and N-terminal collagen peptide concentrations are useful indicators of bone resorption (Gehron, 1996). At the end of resorption, osteoclasts undergo apoptosis and are subsequently completely removed from the remodelling site (Gehron, 1996).

1.3.4 Growth Factors in Regulating Bone Cell maturation and activity

Systemic hormones and local factors affect osteoblast and osteoclast replication, differentiation and recruitment to appropriate sites, as well as their activity. Growth factors abundant in bone matrix regulate bone formation and resorption. Most factors are produced by bone forming cells but some are produced by stromal and immune cells (Canalis, 1993; Manolagas and Jilka, 1995). They can act as systemic regulators in skeletal metabolism when in general circulation.

One family of such factors include IGF-I and -II which are produced by the liver under control of growth hormone as well as in multiple tissues under diverse hormonal triggers (Delany et al., 1994; Jones and Clemmons, 1995). IGF-I is more potent than IGF-II to enhance bone collagen type I synthesis by stimulating osteoblast replication and therefore increasing cell number as well as enhancing individual cell function. IGF-II
inhibits collagen degradation by downregulating expression of matrix metalloproteinase-13 (Canalis et al., 1995). Production of IGF-I is regulated by hormones and growth factors while IGF-II is regulated only by growth factors (Canalis et al., 1995; Delany et al., 1994). In the circulation, these growth factors are bound by insulin like growth factor binding proteins (IGFBPs) which prolong their half-life as well as enhance biological activity and transport to target sites. Of the 6 different IGFBs, IGFBP-3 is the most abundant (Jones and Clemmons, 1995).

Another family of growth factors include TGFβ1, TGF and TGFβ3. TGF stimulates precursor replication of the osteoblastic lineage and has direct effects on bone collagen synthesis by increasing the number of cells and their activity (Canalis, 1993; Centrella et al., 1991). TGF also induces osteoclast apoptosis and decreases resorption. Similarly, TGFβ suppresses bone resorption and increase bone formation. BMPs share sequence homology with TGFβ and induce differentiation of osteoblasts.

Among other factors, FGF is important in angiogenesis, wound healing and bone repair and also activation of bone cell replication which results in increased collagen synthesis (Nakamura et al., 1995). There are two types of FGF, basic (bFGF) and acidic. bFGF increases expression of matrixmetalloproteinase-13 which may increase degradation and remodelling of bone tissue. PDGF expressed by platelets in blood is involved in early phase wound repair (Heldin and Westermark, 1987). PDGF is a systemic and local regulator of tissue growth. Other cells including osteoblasts produce PDGF which increases their replication and synthesis of collagen (Hock and Canalis,
1994; Rydziel et al., 1994). However, PDGF also increases bone resorption by stimulating osteoclasts and production of matrix metalloprotease (MMP)-13 by osteoblasts. Synthesis of PDGF is regulated by growth factors and the protein is controlled via binding osteonectin, thus decreasing its binding to specific receptors (Rydziel et al., 1994).

In addition there are numerous cytokines that regulate bone cell maturation and remodelling including IL-1, -4, -6 and -11, macrophage and granulocyte stimulating factor, CSF, and tumor necrosis factor (TNF). These cytokines stimulate bone resorption and enhance recruitment of osteoclasts (Manolagas and Jilka, 1995). IL-1 is especially important in malignancy-related hypercalcemia. IL-6 and CSF stimulate bone resorption and are produced by stromal cells of osteoblastic origin. CSF also stimulates maturation of osteoclast cells. TNFα is cytostatic, cytotoxic, antiviral and stimulates bone resorption and replication.

1.3.5 Matrix and Mineralization

Bone comprises the largest portion connective tissue in the body. Approximately 90% of bone matrix are collagen type I fibers, while III, V, X may be present in tiny amounts. The matrix also contains non-collagenous proteins. The crystal responsible for calcification of collagen fiber is hydroxyapatite \((3\text{CH}_3(\text{PO}_4))(\text{OH})_2\), and is deposited between collagen fibers and the ground substance. The ground substance is comprised of glycoproteins and proteoglycans which are highly ionic and fix crystals to the collagen, thus assisting in calcification. In order to achieve the highest collagen per volume density
bone is lamellar in structure. This means that the fiber orientation alternates from layer to layer. Lamellar bone structure can be seen in trabecular bone where the lamella are parallel and in harversian systems in which the lamella are concentric around harversian canals which house blood vessels. There is another type of bone, termed woven bone whose collagen is thin, fibery, loosely packed and almost random in orientation. This is the first type of bone usually laid down especially in rapidly forming bone of fractures and malignancies. Not much is know about the mechanical properties of woven bone, except that it is certainly mechanically inferior to lamellar bone (Baron, 1999). Woven bone may serve as a scaffold that becomes highly mineralized for quick growth. Woven bone is usually replaced by lamellar bone during bone development and healing (Currey, 2003).

1.3.6 Bone Remodeling

There is constant growth and turnover in trabecular bone and in harversian systems. Most of the turnover occurs on the endosteal surface of the bone. Normally growth of new bone can occur only where resorption has previously occurred. The sequence if events in bone remodeling is termed ARF for short (Haynesworth et al., 1992). ARF stands for Activation of osteoclast precursors, Resorption by osteoclasts and Formation of new bone by osteoblasts (Figure 9).

The two types of bone, cortical and cancellous, are remodeled slightly differently. Bone remodeling on cancellous bone is in contact with the marrow cavity and thus potent
Figure 9. Bone remodeling. Bone is constantly remodeled. Most of the turnover occurs on the surface of the bones. Normally growth of new bone can only occur where resorption has taken place. The sequence in remodeling is termed ARF and stands for Activation of osteoclast precursors, Resorption by osteoclasts and Formation of new bone by osteoblasts. After resorption a phase termed reversal occurs where osteoclasts undergo apoptosis and osteoblasts mature. During the resting phase bone is not remodeled. In the above diagram the large cells with one ruffled edge are the osteocytes that resorb bone-and they can be seen eroding the bone (dark pink). In the formation phase the cells on the surface of their newly laid down bone (light pink) are the bone producing cells-the osteoblasts. As they lay down new bone the osteoblasts become trapped in it at which point they are known as osteocytes-(green cells in the light pink matrix in the resting phase).

local osteotrophic factors play a role. Cortical bone is distant from such cytokines and resorption is probably controlled by systemic hormones such as PTH and vitamin D3.

Cortical remodelling occurs in denser bone. In fact 85% of bone in the body is cortical, found in the long bone shafts such as the appendicular skeleton. Cancellous bone comprises only 15% of bone and is most prone to loss during lytic malignant disease such as myeloma and breast cancer and to a small degree in prostate cancer. Malignant cells produce factors that stimulate osteoclasts (Mundy and Poser, 1983; Mundy et al., 1974). Cancellous bone is also lost with age due to perforation and fragmentation. A number of steps can be described in cancellous bone remodelling. First there is osteoclast activation. The rate of remodelling depends on the activation frequency of osteoclasts. Briefly, the osteoclasts must fuse, polarize and form a ruffled border (Hughes et al., 1995). The interesting question is how do osteoclasts recognize changes in mechanical properties of aging bone that require replacement. It is speculated that osteoclasts may recognize certain changes in bone matrix proteins via integral membrane proteins (Miyauchi et al., 1991). Resorption lasts approximately 10 days, followed by apoptosis of the osteoclasts (Hughes et al., 1995). Secondly a team of osteoblasts repairs the resorbed site, which takes about 3 months. Initially, osteoblast precursors increase their proliferation rate and are attracted to the resorption site through chemotaxis via factors release from the bone matrix due to resorption. The osteoblasts then mature, form new mineralized bone and finally cease their secretory activity. Normally osteoblasts lay new bone down only once old bone has been resorbed but there are exceptions such as in osteoblastic metastases, in
which case new bone is formed on an unresorbed surface. Normally though, resorption is always coupled to reformation. The coupling may be humorally mediated via factors released from bone matrix during resorption which stimulate osteoblast and new bone formation (Howard et al., 1981). Also it is possible that direct factors that stimulate resorption stimulate reformation as well (Manolagas and Jilka, 1995). Important regulators of remodelling are the mesenchymal stem cells in bone. It is also speculated that osteoblasts are normally present on the bone surface after osteoclast apoptosis and they recognize resorption sites via cell surface molecules upon which they differentiate, proliferate and repopulate the resorption site.

1.3.7 Factors involved in remodeling

There are a number of factors involved in bone remodeling. Among them are systemic hormones such as PTH, dihydroxy-vitamin D₃ and calcitonin. PTH contributes to the differentiation of committed progenitors by causing them to fuse into giant multinucleated osteoclast, while stimulating already formed osteoclasts to begin resorption (most likely with the aid of osteoblast-derived factors (McSheehy and Chambers, 1986). Dihydroxy-vitamin D₃ also stimulates osteoclast differentiation and fusion into giant cells, while activating mature osteoclasts (Roodman et al., 1985). Calcitonin is a polypeptide hormone which is an inhibitor of osteoclastic resorption but its activity is transient because the receptor is rapidly down-regulated (Takahashi et al., 1995; Wener et al., 1972).
Other factors involved in bone remodeling are grouped as local hormones and many may be more important than systemic factors because remodeling occurs in a discrete focal temporal pattern. For example, IL-1 α and β from activated monocytes, osteoblasts and tumour cells stimulate osteoclastogenesis and in vivo increase plasma calcium concentrations (Pfeilschifter et al., 1988; Sabatini et al., 1988). Lymphotoxin and TNF are produced by activated T lymphocytes and macrophages (TNF is an important mediator of cachexia) and are responsible for osteoclast progenitor proliferation, fusion into multinucleated cells, activation and hence resorption (Thomson et al., 1987). Other factors include CSF which is required for normal osteoclast formation and IL-18 which is an inhibitor of osteoclasts (Felix et al., 1990; Horwood et al., 1998). Free osteoprotegrin is an inhibitor of bone resorption - an antagonist for osteoclastic bone resorption. Yet upon the binding of osteoprotegrin the receptor activator of NF-KappaB (RANK) stimulates osteoclastic bone resorption is stimulated (Wong et al., 1997; Yasuda et al., 1998). IL-6 is also an important local factor produced in greatest amount by osteoclasts but it is a weak stimulator of osteoclast formation. It is a cytokine, secreted by normal bone in response to osteotropic hormones including PTH, vitamin D3 and IL-1 (Feyen et al., 1989). Furthermore, there is interferon γ, a lymphokine from activated T lymphocytes which inhibits osteoclastic bone resorption and differentiation of committed precursors (Gowen and Mundy, 1986; Gowen et al., 1986; Takahashi et al., 1987). One factor that has already been mentioned, namely TGFβ which bone cells can express, inhibits mature osteoclast activity and stimulates osteoblast differentiation and thus may be key in remodelling control (Noda and Camilliere, 1989). In fact, much of osteoblast maturation
may be due to TGF-β produced during osteoclast mediated resorption and apoptosis (some of the factors are compiled in Figure 10).

A number of other factors involved in bone remodelling include retinoids such as vitamin A which are stimulatory for osteoclasts and increase bone resorption leading to hypercalcemia (Fell and Mellanby, 1952). Neutral phosphate and calcium inhibit osteoclast activity (Raisz and Niemann, 1969). Prostaglandins are linked to hypercalcemia and increase bone resorption in malignancy. The E series of prostaglandins stimulate osteoclast bone resorption in culture but inhibit formation of human osteoclasts and cause cytoplasm contraction of osteoclasts in vivo. Other factors include leukotriens, thyroxin and triiodothyronine which stimulate osteoclastic bone resorption (Mundy et al., 1976). In addition, glucocorticosteroids inhibit osteoclast formation and resorption in vitro. However, in vivo, they indirectly increase resorption via inhibition of calcium absorbance from the gut. A lack of steroid hormones such as androgen or estrogen leads to an increase in bone resorption via direct and indirect effects (Girasole et al., 1992; Jilka et al., 1992; Oursler and Osdoby, 1988; Pacifici et al., 1989). Finally, a number of pharmacological agents inhibit resorption including plicamycin, gallium nitrate and various bisphosphonates by being cytotoxic to and inducing apoptosis in osteoclasts (Mundy, 1983; Sato et al., 1991).
<table>
<thead>
<tr>
<th><strong>OSTEOBLAST FACTORS</strong></th>
<th><strong>PROSTATE/CaP FACTORS</strong></th>
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<tr>
<td>VITAMIN D</td>
<td>•HRG</td>
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<tr>
<td>•INTERFERONS</td>
<td>•Mitogenic neuropeptide hormones</td>
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<tr>
<td>•IL 3, 4, 18</td>
<td>•Serotonin, bombesin, calcitonin, gastrin</td>
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<td>•OSM, LIF, CNTF</td>
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- •IGF I, IGF II
- •b,a FGF
- •TGF B
- •BMP
- •IL-6

- •M-CSF, GM-CSF
- •TNF
- •VEGF

- •PDGF AA, BB AB
- •IL 1, 8, 10
- •EGF
- •TRANCE
- •ESTROGEN, ANDROGEN
- •PTH, PTHrP
- •Osteonectin
- •Osteocalcin
- •Osteopontin
- •MMP, TIMP
- •Ca2+

**Figure 10. Factors produced by bone and prostate.** A plethora of factors have been shown to be involved in the growth of prostate and bone. The factors produced by both cell types are in the middle column indicated in blue. Both the factors in blue and black are candidates for promoting metastases.
Factors released during resorption, including fragments of matrix and released protein such as collagen and osteocalcin are also important in the formation/chemotaxis phase of ARF (Deuel et al., 1982; Senior et al., 1983). After chemotaxing to the remodelling site, proliferation of osteoblasts is enhanced by growth factors released from the matrix such as previously mentioned TGFβ, PDGF, IGF-I and BMP-2. Next osteoblasts differentiate as indicated by the appearance of markers of differentiation such as alkaline phosphatase, type I collagen and osteocalcin. After the cessation of osteoblast activity the resorption lacunae are almost completely repaired. A important note is that many of the above factors including TGFβ, BMPs IGFs, FGFs and other locally released factors due to resorption of the matrix, are important in coupling resorption to formation and probably act upstream in regulating events in further bone formation (Canalis et al., 1989).

1.4 Prostate Cancer Migration and Establishment as Bone Metastases

1.4.1 General mechanism of metastasis

Metastasis is the process whereby the neoplasm spreads to organs other than the organ of origin. In prostate cancer metastases occurs predominantly to bone. The mechanism of metastases involves a number of steps in a cascade. Generally it is thought that local malignant invasion occurs as the tumour alters the normal architecture of the tissue and the malignant cells become less differentiated. Malignant cells must intravasate or cross the endothelial vascular boundary and survive in lymphatic tissue and the blood stream. To do this the cells have to be anchorage independent, or resistant to anoikis, able to evade immune recognition and survive a variety of physical and biochemical stresses.
Once the cells are in the distant target organ they must adhere in the vasculature, proliferate and rupture the vessel or extravasate via the endothelium. Finally the cells must colonize the secondary site. This requires attachment, survival, proliferation and angiogenesis (Gohji and Kitazawa, 2003; Poste and Fidler, 1980) (Figure 11).

1.4.2 Prostate Cancer Metastasis

Detection of micrometastases of prostate cancer in early stage disease is challenging long held assumptions that only poorly differentiated cells metastasize (Funke and Schraut, 1998). Studies employing polymerase chain reaction (PCR) indicate that micro-metastases may occur in as many as 20-70% of prostate cancers initially diagnosed as localized (Cher et al., 1999). Other evidence for early malignant dissemination has been shown in patients that die due to metastatic lesions with no identification of primary disease. Only upon autopsy have the primary foci been identified as small, well differentiated prostatic tumors. Furthermore, evidence for early metastases was shown in a heart transplant case. The heart was obtained from a donor who had received treatment for localized prostate cancer. The recipient of the heart developed bone metastatic prostate cancer which suggests that prostate cancer cells had been arrested in the donor heart despite the presumably successful initial treatment for his early disease (Riethmuller and Klein, 2001). Thus the metastatic cascade and arrival at the secondary site may be much more efficient than previously assumed. The limiting step in metastasis may be the control of growth and adaptation of the cells to the new environment at the secondary site (Cher et al., 1999).
Figure 11: Steps in development and spread of prostate cancer:
1. Development: oncogenes (erbB, erbB-2, c-met) and tumor suppressor genes (Rb, p53, APC, WT-1, KAI1, PTEN, nm-23).
2. Neovascularization at site of origin: angiogenic factors (bFGF, VEGF, angiopoetins, Tie2, HGF) and inhibitors of angiogenesis (α/β–IFN, endostatin, angiostatin).
3. Cell detachment and Invasion: Adherence molecules (E-cadherin, catenin, CD44, integrins, ICAMs, VCAM-1) and proteases (MMPs, MT-MMPs, TIMPs, uPA, PAIs, heparanase) and chemotaxis and motility factors (Rho, HGF, AMF).
4. Intravasation: adhesion molecules (CD44, integrins, ICAM-1, VCAM-1) and proteases (MMPs, MT-MMP, TIMPs, uPA, PAI, heparanases), chemotaxis/motility factors (HGF, Rho, AMF).
5. Arrest/Adherence: adhesion molecules (selectin, SLα/e, CD44, integrin, VCAM-1, ICAM-1), platelet, coagulation factors.
6. Extravasation: proteases (MMPs, MT-MMP, TIMPs, uPA, PAIs, heparanase) chemotaxis/motility factors (Rho, HGF, AMF).
8. Further metastases: neovascularization, angiogenic factors (bFGF, VFGF, AGF, angiopoetin) and inhibition of neovascularization (α/β–INF, endostatin, angiostatin)
Animal studies have provided useful information about growth and progression of metastatic disease. Techniques such as *in vivo* videomicroscopy and fluorescent tagging of various cell epitopes allows careful monitoring of simulated metastases. These studies indicate that upon intravenous injection of tumour cells, 70-90% of these cells survive in the circulation, arrest and extravasate in the microvasculature of various organs. Prostate cancer cells can first be detected in the capillaries of cancellous bone. After 24 hours most malignant cells are detected in target organs. However, subsequent growth is inefficient and less then 0.1% of micrometastases survive (Chambers et al., 2002; Luzzi et al., 1998). The target metastatic tissue for prostate cancer is bone marrow. *In vitro*, bone marrow specifically stimulates proliferation of prostate cancer cells while other tissues such as lung, liver and skin have little effect thus indicating the specificity of target organ soil (Aslakson et al., 1991; Chackal-Roy et al., 1989). Further support for the relevance of Paget's seed and soil hypothesis, which states that various cancers have a predisposition to seed in specific tissue, as relevant to prostatic bone lesions can be drawn from studies where injected human prostate cancer cells preferentially homed to human bone that was subcutaneously implanted in immunocompromised mice rather than endogenous mouse bone (Cher et al., 1999).

*In vivo*, the tumour cells invade the marrow and develop their own stroma and blood supply enhancing formation of woven bone (Clarke et al., 1991). The new woven bone in the marrow does not form mature bone and is not laid down by mature
osteoblasts. Instead the mineral producing cells are spindle shaped in appearance and are presumed to be preosteoblasts.

Cancer cells can promote two responses from the bone: 1) proliferation with the establishment of osteoblastic lesions causing new bone formation and increased bone density; or 2) osteolytic lesions causing bone destruction (Athanasou et al., 1992; Quinn and Athanasou, 1992). Often a mixed response is observed and in the case of prostate cancer a mixed response is postulated to occur in early metastatic disease with progression to a predominantly osteoblastic lesion (Mundy, 2002). Key factors influencing prostate cancer bone metastases likely include the plethora of growth factors and other various secreted or membrane bound factors produced by bone cells and found in the ECM. It is possible that prostate cancer cells react to bone- and ECM-derived factors and respond reciprocally by secreting or releasing factors to which osteoblasts are reactive. Such interactions are classified as paracrine, and promote a vicious cycle between bone and prostate cancer cells where each is dependent on the other (Sung and Chung, 2002; Yeung and Chung, 2002). In addition, some of the factors produced by each cell type likely influence the cell itself to produce other factors in an autocrine loop (Wood and Banerjee, 1997). A number of these factors have already been discussed and include TGFβ, BMPs, FGF, PDGF, and IGF (Gleave et al., 1991; Lang et al., 1995; Lee et al., 2003; Mundy, 1997; Orr et al., 1995). These and other factors including cytokines and chemokines are important in adhesion, key to the development of metastatic disease by directing migration of cells expressing the cytokine/chemokine receptors up the chemokine gradient. Cytokine and chemokine gradients influence not only cancer
migration but also angiogenesis, immune responses and survival of cancer cells. (Balkwill, 2003).

1.4.3 Chemokines and migration

Recently CXCR4/ SDF1 and CXCR12 chemokines have been shown to be involved in the migration of prostate cancer to bone. These chemokines appear to be upregulated in cell lines derived from bone metastatic disease and in clinical samples (Sun et al., 2003; Taichman et al., 2002). Among the steps that determine homing of cancer cells to secondary sites, the chemokine/cytokine axis may be more important than the mechanical trapping of the cells in vasculature or, the blood volume passing through the site (Asosingh et al., 2000; Cooper and Pienta, 2000). Also of importance, PTHrP, has been described as the most active bone cytokine in skeletal lesions (Kakonen et al., 2002). PTHrP is expressed in the prostate and may be elevated in prostate cancer. Although PTHrP is predominantly thought to be involved in lytic lesions, it has been demonstrated in mixed and blastic lesions with elevated levels in early metastatic prostate cancer (McCauley and Schneider, 2004). PTHrP acts through the PTH receptor to stimulate PKA/PKC signalling, c-AMP response element binding protein (CREB) phosphorylation and c-fos expression. PTHrP also increases RANKL (receptor activator of NF-KappaB ligand) expression, thus promoting bone resorption and osteopenia. On the other hand though, decreased PTHrP leads to increased OPG expression (a negative regulator of RANKR) thus promoting classical bone formation or osteosclerosis in prostate cancer(Pearman et al., 1996). It has also been shown that the cytokine IL-6 is induced in bone cells due to increasing OPG (Brown et al., 2001).
1.4.4 Factors involved in metastasis

Growth factors alter the tumour phenotype by stimulating \textit{in vitro} and \textit{in vivo} growth, osteoblast differentiation, matrix degradation and deposition (Figure 12) (Brown et al., 2001). Other important factors involved in promoting metastasis are MMPs thought to be required for invasion of the primary and secondary organs. MMPs degrade extracellular matrix and basement membranes including collagenous proteins such as collagen I in the ECM and collagen IV in the basement membrane (BM) (Sternlicht and Werb, 2001). MMP levels are high in invasive tumours, both primary and metastatic (Bonfil et al., 2004). Interfering with MMPs (which are also secreted by osteoclasts) via broad spectrum inhibitors can decrease bone matrix turnover as well as cancer colonization. (Nemeth et al., 2002). ET-1 is a factor produced by prostate cancer cells, acts in a paracrine manner on bone and autocrine manner on the tumour and has been documented to increase MMP levels and cancer invasiveness. The paracrine effects on bone provide a favored growth environment for the tumour in the bone. ET-1 is a vasoconstrictor, and mediates its action through its 7 transmembrane G protein (Cyr et al., 1991). It causes mitogenesis in osteoblasts and acts with other factors to increase bone formation through increasing osteopontin and osteocalcin expression, increasing matrix calcification and regulating bone cell migration in a dose dependent manner (Nelson et al., 1995; Stern et al., 1995). ET-1 also functions in cancer progression by
Figure 12. An in depth view of prostate cancer migration and establishment in the bone environment. The cancer cells are first attracted to the local bone vasculature where chemokine gradients and specific adhesion molecules facilitate intravasation, migration and establishment at the bone. Prostate cancer cells produce a plethora of factors which not only degrade the extracellular matrix but also stimulate osteoblasts and osteoclasts to lay down new and destroy old bone in a haphazard fashion. This makes the bone mechanically weak which is often painful. The arrows indicate how each cell type can influence itself as well as the other cell types (both stimulation and inhibition are encompassed). The inset in the top right corner illustrates what new sclerotic woven bone formation looks like. The dark areas are new bone (indicated with b); the o indicates active osteoclastic bone destruction and the white speckled areas are tumor stroma with relatively low malignant cell density.


increasing mitogenic effects of various other growth factors (Nelson et al., 1996). Increased levels of ET-1 are also correlated with androgen independent metastatic disease. While androgens may decrease ET-1 expression, bone factors may stimulate its expression when prostate cancer cells come in contact with bone at metastatic sites (Chiao et al., 2000).

Other factors involved in metastasis are cell adhesion molecules (CAMs), cadherins and selectins. Induction of some of the adhesion molecules in the cancer cells and local endothelium is mediated by osteoblasts through various factors such as IL-1 (Cooper and Pienta, 2000). E-cadherin suppresses invasion and its decrease in prostate cancer corresponds to poor patient outcome (Paul et al., 1997). Integrin adhesion molecules, normally important in development, appear significant in cancer (Mizejewski, 1999). Integrins bind noncollagenous proteins including bone sialoprotein, osteopontin, vitronectin, osteocalcin and osteonection. It would appear that the key integrin mediating the invasive prostate cancer phenotype may be αvβ3, as it exhibits highest staining. Promotion and inhibition of angiogenesis also appears to be partly mediated via integrins (Fornaro et al., 2001). Regulation of integrins has been partly shown to occur via BMP1-7, which induces cartilage and bone formation. The effects of the BMPs on integrins is likely similar to a related family member TGFβ, and specifically BMP4 may mediate increased cell binding to bone endothelium (Cooper and Pienta, 2000). TGFβ, a previously mentioned growth factor, increases adhesion to type I collagen in various cancers including prostate cancer by increasing homing, adherence and proliferation of cancer cells partly through up-regulation of α3β1 and α2β1 integrin receptors (Kiefer et
al., 2004). Type I collagen may also play a role in prostate cancer. Besides being important for integrin mediated interactions, microarray profiles have shown that progression to androgen independence and changes that occur upon interactions of CaP with bone ECM may also be specially influenced by type I collagen. Type I collagen has been shown to alter cellular signaling, increase cell metabolism, transcription, translation, proliferation, ECM/cytoskeletal/adhesion and structural interactions (Kostenuik et al., 1996; Kostenuik et al., 1997). Type I collagen has also been demonstrated to facilitate metastatic behavior via promoting enigma (protein scaffold) interactions with cell actin filaments, CD44 (a transmembrane adhesion receptor), binding to ECM components such as hyaluronate and osteopontin and up regulation of ezrin (a membrane cytoskeletal linker). In general, observations that CaP cells prefer to attach to human bone marrow endothelial cells (HBME) cells via various factors, discussed above, lends strong support to the hypothesis that organ specific vasculature favors arrest and invasion by specific cancers (Scott et al., 2001). In fact, collective data from adhesion molecule studies suggests an existence of a docking and locking mechanism in cancer targeted local vasculature (Honn and Tang, 1992). Adhesion factors identified in docking include gelactin-3 T-antigen, vascular cell adhesion molecule (VCAM) and Leukocyte Function-Associated Antigen (LFA1), locking factors include arginine-glycine-aspartate (RGD) sequence motifs, β1 integrin, CD44, increased C cellular lecthin and hyaluronan (Scott et al., 2001). After locking, other factors assist invasion (transendothelial migration) such as protease activated receptor 1 (activated by thrombin) which increases cancer motility and MMP expression (Chay et al., 2002).
A large number of other mechanisms that enhance metastasis include involvement of Rho-GTPases (GTPase enzyme controlling Rho protein activity) metastatic suppressor genes, scatter factors such as heparin growth factor (HGF), and semaphorins which interact with tyrosine kinase receptors of the Met (Met receptor tyrosine kinase) family. Signalling through Met receptors increases invasiveness and alters the transcription, cellular localization and activation of MMPs, cadherins and integrins (Kauffman et al., 2003). Rho C (protein family that controls the organization of the actin cytoskeleton) increases metastatic potential in poorly differentiated cell lines, is unregulated in cancer and promotes shape, migration and adhesion changes through constant reorganization of the cytoskeleton (Clark et al., 2000).

1.4.5 Bone Metastatic Prostate Cancer

Skeletal metastases are one of the most significant contributor to cancer-related morbidity and mortality. An estimated two thirds of patients that die of cancer have involvement of the skeleton (Greenlee et al., 2001). A number of tumours exhibit skeletal metastases including breast, prostate, melanoma, kidney, lung, myeloma and lymphoma. The major complications resulting from bone involvement are bone, brain, spinal cord compression (Figure 13), pathological fractures, paralysis, hypercalcemia, cachexia and death (Mundy, 1997; Mundy, 2002). Paget’s seed and soil theory appears most convincing among other explanations as to why cancers displaying a predilection for certain organs as targets for metastases. The theory also proposes cancer cells influence bone remodeling and bone influences cancer colonization, with emphasis on
Figure 13. Spinal Cord Compression. Pressure on the spinal nerves can cause paralysis.

bone as a unique connective tissue that when remodeled releases a number of growth factors from the insoluble extracellular matrix (Fidler and Johnson, 2001).

1.4.6 Clinical features and assessments of bone metastatic prostate cancer

Metastatic disease is usually the terminal stage of cancer and most patients die of metastases and not of primary organ confined malignancy. Treatment for bone metastases is limited in scope and duration (Jemal et al., 2002). To avoid metastatic progression of disease emphasis has been placed on early detection of disease. However, metastases may occur earlier and more frequently than once believed (Kauffman et al., 2003). Not all secondary sites of cancer seeding develop overt metastatic disease and this is a very important regulatory step in the early metastatic cascade during which disseminated cancer cells are subject to stringent growth controls at the secondary site. If these controls can be identified then diagnosis and certainly treatment could be improved (Cher et al., 1999).

Bone is the second most common metastatic site in cancer overall and is a major site of metastasis in prostate cancer. Up to 85% of advanced prostate cancer cases exhibit skeletal involvement (Galasko, 1986). In the past years a decrease has been observed in men first presenting with bone metastatic disease with a concomitant increase in early stage cancer. In newly diagnosed and untreated patients, bone metastases are detected in approximately 14% of prostate cancer patients (Chybowski et al., 1991). The likelihood of finding overt disease increases with increasing PSA, higher clinical stage and tumour grade (Mettlin et al., 1996). Approximately 13% of patients progress to bone disease
when monitored solely through watchful waiting (Johansson et al., 1997). Patients with more advanced disease who are treated conservatively progress to bone involvement in roughly 27% of the cases versus 5-13% in patients who have received radical prostatectomy (Holmberg et al., 2002). The first indicator of progressing disease is a rise in PSA, which generally occurs in 88% of cases. Only 9% of patients progress to bone metastatic disease without an increase in serum PSA levels (Newling, 1993). Bone metastases are an indication of poor prognosis. The median survival for patients with androgen dependent disease in the bone is 30 months (Soloway et al., 1988). Treatment for these patients is hormone ablation therapy. After hormone ablation therapy, bone lesions can be detected within approximately 2.5 years of an initial PSA rise. Patients with androgen independent disease and bone involvement have the shortest survival time of approximately 4 months (Pollen et al., 1981).

Histological features of prostatic bone lesions include 65% osteoblastic (sclerotic), 23% mixed and 12% lytic, although more recent statistics indicate the mixed phenotype may be much more common accounting for up to 40% of disease (Roudier et al., 2004). Bone sites affected by prostate cancer metastasis are the skull, axial skeleton and the bones of the pelvic and shoulder girdle. Figure 14 lists frequencies of metastases to various skeletal sites (Byar, 1977). The dissemination of prostate cancer appears to follow the route of the haematopoietic marrow of the axial skeleton, with predominant invasion of the marrow (Arguello et al., 1990). At first the invading cancer cells present
Figure 14. The most to the least common skeletal sites of prostate cancer metastases. Please note these numbers do not add up to 100% because there are usually several sites affected at once.

a number of primary tumour characteristics, but with androgen ablation the cytoplasm becomes clearer, there is nuclear atypia, decrease in the nuclear to cytoplasmic ratio, cytoplasmic swelling, vacuolization, scattered apoptosis and shrinkage of the actual prostate gland (if still present) (Harada et al., 1992; Reuter, 1997). However, even tumours in bone that have progressed to androgen independence on histological examination still present with a number of hormone sensitive primary cancer features (Roudier et al., 2004). Immunologically there is a wide range of heterogeneity of various markers even within the same patient and even the same site of metastases with for example PSA, prostate specific membrane antigen (PSMA) and the AR, which may indicate 2 clonal outgrowths from the original metastatic colony or two separate seeded clones from the primary tumour in close proximity (Roudier et al., 2004). PSA decreases with poorly differentiated disease and human kallikrein 2 (hk2) increases (Darson et al., 1997; Furuya et al., 2001). Roudier et al. have shown that although most cancers express PSA, there can be wide variability within the marker in one patient, making the marker somewhat difficult to use as an average representing all the cancer in the body. Other features that can be assessed immunologically include rising PSMA with androgen independence, AR expression and possibly neuroendocrine markers. In contrast, staining for the Ki-67 biomarker, a proliferation marker, is clearly indicative of aggressive metastatic disease, marked by tenfold stronger staining in metastases over primary tumour (Roudier et al., 2004).
1.4.6.1 Bone pain and symptoms

The clinical features of bone metastases are increased and imbalanced bone remodelling, especially at the bone surface (cancellous bone). The newly formed “woven bone” is physically weak because it is composed of loosely patched, randomly oriented collagen fibers (Rosol, 2000). The symptoms of bone metastases include bone pain, fracture, spinal cord compression, paralysis, wasting and death. Fractures happen generally if more than 50% of the cortical bone is destroyed a condition that occurs in about 4% of prostate cancer patients. Fractures also increases with androgen ablation therapy and are not due to the cancer itself but rather to osteoporosis caused by hormone withdrawal (Townsend et al., 1997). Prostate cancer is third among all cancers as the cause of metastasis-related spinal cord compression (Figure 13) (Gilbert et al., 1978). Life expectancy with spinal and cranial nerve compression is short but pain and many of the symptoms can be alleviated (Ransom et al., 1990).

1.4.6.2 Diagnosis of bone metastasis

There are various means of detecting bone metastases. Plain radiography requires a 50% increase in bone mineral density for detection. The amount and pattern of osteosclerosis indicates the growth rate of the tumour because the reaction is largely osteoblastic with denser patterns indicating slower growth and dense/lytic patterns indicating fast growth (Figure 15). Plain radiography has low sensitivity and is not commonly used currently, except to evaluate focal abnormalities detected on screening with bone scintilligraphy (Tu and Lin, 2004). More convenient, efficient, reproducible
Figure 15. Bone Scan. The dense black areas indicate increased bone cell activity which can be a result of new bone formation or repair after bone destruction.

and easy to use for follow up studies today is bone scintilligraphy. This technique involves the use of technetium 99- labeled methylene diphosphonate (MDP). This is a bisphosphonate with affinity for the hydroxyapatite crystals that are preferentially found in areas of new bone formation with scintilligraphy. Lesions can be detected 2-18 months earlier than with plain radiography (Galasko, 1986). The difficulty with this screening is that it is sensitive for hyperactive bone, such as is found in inflammation, fractures and osteoarthritis but is not specific. Thus patients with cancer and other foci of bone remodelling have only about 50% of the detected lesions actually being caused by cancer (Rosenthal, 1997). Specificity of bone scintilligraphy has recently improved with the use of radioimmunoscentilligraphy for PSMA which is prostate specific (Yao et al., 2002). In general PSA is still the determining factor for further evaluation of disease progression in patients, because less than 1% of patients with a PSA score of less than 20ng/ml in serum will have metastatic disease; therefore bone scans are generally performed when PSA is over 20ng/ml (Chybowski et al., 1991). One drawback to the use of MDPs is that its uptake is dependent on osteoblast activity thus osteolytic lesions may not be detected (although biochemical changes due to lytic lesions can cause bone scans to light up). Furthermore, an extensive bone scan may appear normal because lesions can affect the entire skeleton thereby producing a uniform image. To improve detection of bone metastases computer assisted tomography (CAT), scans are being used to detect hot spots of metastasis while magnetic resonance imaging (MRI) is used to detect the medullary component of the disease in early metastatic disease. Positron emission tomography (PET) screening with fluorodeoxyglucose detects CaP with 65% sensitivity and 98% specificity (Tu and Lin, 2004). In general, evaluation of treatment with radiography is
also not straightforward because osteoblastic lesions often remain osteosclerotic even on remission and osteolytic disease may be repaired by an osteosclerotic response which may be mistaken for progression rather than healing. Therefore, in addition to imaging studies laboratory markers of bone turnover are also being implemented. These markers include serum calcium, phosphorus and alkaline phosphatase. None of these markers are specific for metastatic disease but rather for bone formation and resorption and can be measured in blood to determine relative bone formation and degradation. Early markers of osteoblast proliferation include serum type I collagen C terminal propeptide (PCIB), matrix maturation involved bone-specific alkaline phosphatase (BAP) and late bone formation osteocalcin, of which BAP is most reliable (Maeda et al., 1997). Markers of degradation and breakdown products from collagen cross links include N-telopeptide (NTX), deoxypiridinoline (DPD), and are good predictors of bone turnover (Ikeda et al., 1996; Maeda et al., 1997; Takeuchi et al., 1996). Sometimes it is especially difficult to evaluate patients on androgen ablation therapy because testosterone depletion promotes osteoclastic and osteosclerotic activity. Lytic components, although believed to be important in prostate cancer, are contributed to by both osteoclasts and cancer cells and are difficult to correctly identify with imaging techniques (Taube et al., 1994).

1.4.7 Treatment for bone metastases

Bone metastases are difficult to manage because they are commonly refractory or respond only for a limited time to the majority of current treatments. Therefore novel therapies are actively being pursued such as for example, targeting angiogenesis and MMP’s (Folkman, 2002; Mundy, 1997). Most of the therapy used for bone metastases is
aimed at prolonging life and controlling pain. Radiation is primarily used for pain relief and there is usually a response in about 80% of patients within 10-14 days, along with some control of tumour growth and decreased use of analgesia narcotics. $^{89}\text{Sr}$ can be used in radiotherapy. It is incorporated into bone instead of calcium, it is a beta emitter, with short penetrance and can cause response rates of 75% for up to 6 months (Porter et al., 1993; Quilty et al., 1994). $^{153}\text{Sm}$ can also be used with response rates of 72% within 1-2 weeks after treatment and maintained response in 43% of patients 16 weeks after treatment (Serafini et al., 1998). Moreover, surgery can be used to relieve painful and life threatening fractures and lesions especially in long bones and the vertebral column. Prophylactic surgery can also be used to fix impending fractures before they happen. Many of the bone metastases are still hormone dependent therefore an effective treatment is androgen ablation, and if primary androgen ablation has been applied earlier, then secondary ablation can be administered followed by cytotoxic chemotherapy (Oh, 2000). Unfortunately, hormone ablation is best for primary tumours and soft tissue metastases with complete response rates of over 80% versus 30% for bone metastases. The relapse after androgen ablation occurs primarily in bone (85% vs. 23% in other organs) (Goldenberg et al., 1988). Androgen ablation therapy employs LHRH agonists and bilateral orchiectomy. However, application of estrogen in place of LHRH agonists and orchiectomy may decrease the risk of osteoporosis associated with these latter therapies.

Once prostate cancer has progressed to androgen independence, chemotherapy can be implemented, although alone it may not be very efficient but combined with hormone ablation shows much better efficacy. The use of mitoxantrone (a topoisomerase
II inhibitor) together with corticosteroids does not increase life span of the patient but does increase the time to progression, enhance the quality of life and reduces pain. Estramusine is another chemotherapeutic agent which interferes with mitosis, and increases progression free survival but not overall survival (Parvez et al., 2003). It is best used with taxanes which also affect microtubule formation (Oh, 2000). Vinblastine, etoposide, paclitaxel, docetaxel, carboplatin and estradiol with nitrogen mustard have also shown some efficacy in malignant disease. In addition suramin has been shown to inhibit stimulation by binding some growth factors, thalidomide decreases angiogenesis and increase apoptosis, yet alkylating agents such as cyclophosphamide are not efficient (Parvez et al., 2003). The most important risk factors that may justify early preventative treatment using chemotherapy with hormonal therapy for metastatic cancer include high stage cancer, elevated PSA, and a high Gleason grade (Oh, 2000).

1.4.7.1 Bisphosphonates

Bisphosphonates suppress osteoclastogenesis as they have strong affinity for bone and promote apoptosis in existing osteoclast cells. Data from animal models suggest that administration of zelendronic acid before detection of metastatic disease blocks osteoclastogenesis and osteosclerosis suggesting that osteolysis is necessary even in osteosclerotic disease (Yoneda et al., 2004). In line with this, bisphosphonates have gained increasing use as a therapy for prostate cancer. Pamidronate and zelendronic acid may delay the time to first skeletal events and reduce pain even though they have not shown to benefit survival time (Oh, 2000). The mechanism by which bisphosphonates cause a response in prostate cancer is unknown.
1.5 Models of Prostate Cancer

The development and characterization of good models that mimic a neoplasm is a key prerequisite in gaining an understanding of the malignant events involved in its initiation, progression and dissemination. Unfortunately, at this time an ideal model for prostate cancer does not exist. An ideal model would have to mimic the clinical aspects of the disease. For prostate cancer this would mean that the model would have to have the following characteristics: be of human origin; have a slow-doubling time; be androgen-dependent initially or respond to androgens; express an AR; secrete PSA; metastasize to lymph nodes and bone; and grow in the absence of androgens after castration (Royai et al., 1996). Here we limit our review to models using human cells, although a multitude of animal models exist that encompass the use of tissue recombination, transgenic mice, canines which are one of the few mammals that spontaneously develop prostate cancer, and various other models.

In total, there are approximately 200 human cell lines and sublines used in prostate cancer research including ones derived from primary tumors, metastases and normal prostate tissue (Sobel and Sadar, 2005a; Sobel and Sadar, 2005b). One of the most common cell lines used for prostate cancer research is the human LNCaP cell line. This cell line was derived from a lymph node metastasis from a 50 year old male with prostate cancer (Carroll et al., 1993; Isaacs and Carter, 1991; Webber et al., 1997). LNCaP cells have a doubling time of 60-72h, low anchorage potential, have a modal chromosome number of 76, express PAP and PSA and a high affinity, mutated, AR that binds to estradiol and progesterone (Berns et al., 1986; Langeler et al., 1993; Schuurmans
et al., 1991; Webber et al., 1995). LNCaP cells secrete EGF and TGFα and have increased expression of epidermal growth factor receptor (EGFR) which appears to be hormone modulated via growth factors. The cells do not produce detectable TGFβ, little bFGF, are not stimulated by IGF, are cytokeratin 8 and 18 positive and thus of epithelial origin, tumorogenic in mice and have a mutated p53 gene (Connolly and Rose, 1990; Iwamura et al., 1993; Schuurmans et al., 1991). These cells can be grown as a monolayer as well as xenografts in immunocompromised mice. When injected orthotopically, LNCaP cells will metastasise in SCID mice, but not in Nude mice (Sato et al., 1997). Sites of metastasis include lymph nodes, lungs and possibly the axial skeleton (Sato et al 1997). LNCaP cells progress to androgen-independence in castrated hosts, thereby resembling the pathogenesis of human prostate cancer (Gleave et al., 1992).

Two additional cell lines derived from an androgen independent bone metastasis are the MDA-PCa2a and 2b models. The lines form tumors in nude mice upon subcutaneous and intraprostatic implantation and exhibit androgen responsiveness, express AR and PSA, and show an abrogated growth response upon castration. The two cell lines are slightly different as they were derived from different areas of the same lesion, thus reflecting tumor heterogeneity. These cells also appear to be a good model for bone lesions and importantly when grown in mice retain original tumor morphology, i.e. normal p53, Bcl2, Rb, p16, and p21. These characteristics are relevant clinically as p53 is normal in 40-50% of bone metastases and 50-70% do not over express Bcl2, while p21 mutations are rare (Navone et al., 1997; Navone et al., 2000). The MDA cells are osteoblastic in SCID mice upon bone injection and eventually replace trabecular bone
with new woven bone in vivo, while exhibiting an increase in PSA. The 2b cells can also be used as an vitro osteoblastic model, when the cancer cells are co-cultured with primary mouse osteoblasts. Co-culturing osteoblasts and MDA cells leads to primary mouse osteoblasts (PMO) proliferation and differentiation (Navone et al., 1997; Navone et al., 2000; Yang et al., 2001). The PMOs conversely stimulate PCa-2b proliferation, as well as ET-1 production through a PMO differentiation/ mitogenic factor whose action can be inhibited with inhibitors to the ET-1. OPG was decreased and RANK was increased upon co-culture with PMOs suggesting osteoclast activation. In vitro, the cancer cells increased alkaline phosphatase levels and calcified matrix production by PMOs (Fizazi et al., 2003). As such, the in vitro model is a convenient first step in identifying genes and cellular pathways that lead to osteoblastic responses and metastases.

To recreate prostatic bone lesions in vitro, a co-culture model using osteoblasts with prostate cancer cells has been employed. Generally osteoblast interactions with prostate cancer cells have shown increased growth rates (Pinski et al., 2001). Co-cultures of primary prostate cancer or BPH and bone cells have shown that bone growth is preferentially stimulated by cancer over BPH (Lalani et al., 1997). Initial studies were conducted by Gleave at al in 1991 where bone or prostate fibroblast conditioned media was used on LNCaP cells and mitogenic assays revealed 200-300% reciprocal stimulation of proliferation. Several paracrine mediator growth factors were elucidated in this research as important in prostate cancer growth and progression including bFGF. Another pioneer of the in-vitro metastases model of prostate cancer was Lang et al 1995. He used conditioned media from osteoblast like cells and demonstrated enhanced growth
of androgen sensitive LNCaP cells as well as androgen insensitive PC-3 and DU-145, but not with bone marrow or human skin fibroblasts. At the same time conditioned media from osteoblasts had no proliferative effect on breast, bladder and liver cancer cell lines. This supports the idea that skeletal factors promote metastatic growth of prostate cancer in bone. Attachment, migration and proliferation were studied by stimulating LNCaP and DU145 cells with crude protein extracts (Hullinger et al., 1998). In addition, Zheng Fu in 2002 showed that HOBIT (human osteoblast like cell line) conditioned media induces differential expression of 30 genes in LNCaP cells and some 35 in C4-2B prostate cancer cell line. Of these genes, 19 were differentially expressed in C4-2B as compared to untreated LNCaP but paralleled expression in HOBIT treated LNCaP cells.

Prostate cancer metastasizes to bone at a rate of 65-75% in advanced disease (Coleman, 1997). Early on, the disease is often mixed osteolytic and osteoblastic but as it progresses it becomes predominantly osteoblastic with new bone formation. The new bone is mostly woven bone that forms on surfaces of pre-existing medullary trabecular bone and this comprises a purely osteoblastic response. There are several model categories available in prostate cancer research including spontaneous, syngeneic transplant of spontaneous, chemically induced, transgenic and xenograft tumor models (Rosol et al., 2004).
1.6 INTERLEUKIN 6 (IL-6)

Clinical and experimental evidence suggests that one of the more important factors in progression of prostate cancer to androgen independence and development of osseous metastases is IL-6. Elevated levels of IL-6 have been found in sera from patients who have metastatic and or hormone refractory disease (Adler et al., 1999; Drachenberg et al., 1999; Kovacs, 2001; Shariat et al., 2001; Twillie et al., 1995). IL-6 was first characterized as a modulator of an immune response that is produced by T cells and involved in haematopoiesis and pro- and anti-inflammatory processes (Kishimoto et al., 1992). However, IL-6 has been subsequently shown to be produced by many other cell types, including normal and malignant prostate epithelial cells, and bone cells (Bellido et al., 1995; Klein et al., 1989; Lee et al., 2003; Lu and Kerbel, 1993; Miki et al., 1989; Okamoto et al., 1997; Yee et al., 1989). IL-6 is, in fact, important in many aspects of cell activity including survival, apoptosis, differentiation and proliferation. IL-6 has the potential to act in both a paracrine and autocrine manner. Importantly, IL-6 has been shown to enhance proliferation of prostate cancer cells including LNCaP (Okamoto et al., 1997; Ueda et al., 2002a) and induce transcription of androgen-regulated genes such as PSA by a mechanism of ligand-independent activation of the AR via a pathway dependent on MAPK (Hobisch et al., 1998; Ueda et al., 2002a).

Specifically, IL-6 has several paths of signal trasduction which ultimately all lead to stimulation and inhibition at the gene transcription level. The cytokine first binds to the
alpha subunit of the IL-6 transmembrane receptor. The receptor/cytokine complex recruits the signalling subunit gp130 and allows activation and dimerization of gp130. Gp 130 is ubiquitously expressed in tissues but the alpha receptors have a more limited and specific tissue distribution. The cytokine itself is a helix bundle cytokine with four long alpha helices. Each subunit of gp 130 binds to JAK1 through JAK’s box1/box 2 domains, which is a very tight and long lasting interaction, not transient. The receptor is therefore a docking and activating station for JAKs. With activated JAKs, there is a transient association of STATS to gp130 receptors, which induces STAT phosphorylation on tyrosine residue and dimerization. STAT can also be phosphorylated on serine residues and methylated to increase DNA binding capacity. Dimerization of activated STATs potentiates their active transportation to the nucleus via their NLS. Here the STATs act as transcription factors and regulate the expression of various genes (Heinrich et al., 2003). Dimerization of gp130 can also activate the MAPK cascade, activation by JAK’s links the receptor to Grb2-SOS and or Gab1 which induce ERK signalling via RAS-GTPase activation (Heinrich et al., 2003). An additional mechanism involves the PI3K pathway which upon activation via the gp130 receptor modifies some membrane phosphatidylinositides and promotes membrane Akt recruitment and phosphorylation. Akt acts on its downstream signals such as the family of forkhead transcription factors and the anti-apoptotic Bad, whose upregulation leads to increased growth and survival (Heinrich et al., 2003). Both the STAT and MAPK cascades are stimulatory to ligand independent activation of the AR, but PI3K is inhibitory to the former two pathways and to activation of the AR itself. Therefore the large body of evidence for both pro- and anti-proliferative and pro- and anti-stimulatory properties of IL-6 depends on the balance of
these signalling cascades and the end result may be activation or inhibition depending on the cell type, genetic alterations and environment. Thus, IL-6 derived from either bone or cancer cells may initiate and/or sustain progression of CaP to androgen independence (Figure 16 demonstrates the IL-6 interactions with the AR ).
Figure 16. IL-6 and the AR. IL-6 signalling activates the AR to upregulate expression of androgen regulated genes in the absence of androgen or in androgen independent disease. Activation of gene expression occurs via MAPK or STAT3 AR activation, while PI3K-Akt activation inhibits STAT and MAPK activation.

1.7 RESEARCH HYPOTHESIS AND AIMS

Hypothesis: The working hypothesis tested in this thesis is that the bone cells secrete factors that allow prostate cancer cells to grow in the absence of androgens.

1.7.1 Specific Aims

1) To develop an *in vitro* model system to study the effects of soluble bone factors from bone on prostate cancer cells. Work towards this objective will validate the biological relevance of the model to prostate cancer.

2) To determine whether conditioned media from osteoblasts cells affects proliferation and expression of PSA in prostate cancer cells in the absence of androgens. Studies in this direction will establish if our model mimicks these two important clinical characteristics of prostatic bone metastases not responding to androgen ablation therapy.

3) To distinguish whether the AR plays a role in the response of prostate cancer cells to bone derived factors. Work towards this goal will highlight the importance of the AR in the androgen independent growth of prostate cancer.

4) To evaluate the role of IL-6 as a potential factor derived from osteoblasts in the proliferation and induction of PSA observed in prostate cancer cells. Work rewards this aim will provide insight into an alternative pathway of growth regulation that can supplant androgens in prostate cancer.
CHAPTER 2: OSTEOBLAST-DERIVED FACTORS INDUCE ANDROGEN-INDEPENDENT PROLIFERATION AND EXPRESSION OF PROSTATE-SPECIFIC ANTIGEN

Adapted from:
2.1 INTRODUCTION

Localized carcinoma of the prostate (CaP) can potentially be cured by surgery or radiation therapy. However, the only treatment available for advanced metastatic disease is the withdrawal of androgens which are essential for the survival of prostate epithelial cells (for review see Culig et al., 2003b). Clinical response to androgen ablation therapy is temporary with the ultimate development of androgen independent disease that is preceded by an increasing titer of PSA. Unlike most malignancies, up to 85% of CaP metastases occur in the bone as osteoblastic (bone forming) lesions (which prior to androgen ablation are androgen responsive). Osteoblastic lesions are the major cause of CaP-related morbidity, and mortality and even small, bone-limited tumour burden in patients is strongly correlated to cachexia and death (for review see Rana et al., 1993).

Bone is mostly composed of an acellular collagen matrix and is abundant in immobilized growth factors. Dispersed throughout this matrix are osteoblasts and osteoclasts which are the cells responsible for bone maintenance. In bone remodelling osteoclasts degrade the matrix to release growth factors which stimulate osteoblasts to lay down new bone (for review see (Bova et al., 2001; Marks and Odgren, 1996). Many of these growth factors increase proliferation of prostate cancer cells. In vitro studies have shown increased proliferation of prostate cancer cells stimulated by conditioned media from or co-cultured with osteoblasts, growth factor extracts, and individual growth factors derived from bone such as IL-6, IGF-I and IGF-II, EGF, KGF, BMPs, FGFs, TGFβ, PDGF, vascular endothelial growth factor (VEGF), and endothelin-1 (Bentley et al., 1992; Chackal-Roy et al., 1989; Gleave et al., 1991; Kimura et al., 1996; Lang et al.,
Many of these growth factors may be involved in circumventing the need for androgen in advanced disease by a mechanism involving ligand independent activation of the AR (Culig et al., 1994; Hobisch et al., 1998; Sadar, 1999; Ueda et al., 2002a; Ueda et al., 2002b).

The AR is a transcription factor that binds androgens and regulates gene expression required for normal male sexual development and maintenance of secondary sex characteristics (for review see (Culig et al., 2002a). It is expressed in the majority of prostate cancer tissue specimens, including androgen independent, or hormone refractory disease and is therefore a strong candidate for mediating androgen resistance (Hobisch et al., 1995; Sadi et al., 1991; Wilding, 1995). The AR is composed of three domains. Centrally located is the DBD that binds to AREs in upstream regulatory regions of androgen regulated genes, such as PSA. The most C-terminal region comprises the LBD that binds androgens and antiandrogens such as bicalutamide. The LBD contains a weak AF-2 region and is separated from the DBD by a hinge region which mediates nuclear localization. The NTD is the most variable in sequence homology between species and contains the AF-1 region required for transactivation (for review see (Culig et al., 2002a; Culig et al., 2002b). The AR can be activated in an androgen independent manner by a number of factors including IL-6, IGF I, IGF II, KGF, EGF, forskolin and cAMP, and some factors such as IL-6 and cAMP have been shown to mediate activation via the AR NTD (Hobisch et al., 1998; Sadar, 1999; Ueda et al., 2002a; Ueda et al., 2002b). Since the bone environment is rich in many of these growth factors, it has been suggested that
bone-derived factors may facilitate survival and progression of CaP to androgen independence by cross-talk with the AR and alternative signal transduction pathways (Gleave et al., 1991; Lang et al., 1995; Sadar et al., 1999).

Clinical and experimental evidence suggests that one of the more important factors in prostate cancer progression to androgen independence and development of osseous metastases is IL-6. Elevated levels of IL-6 have been found in sera from patients who have metastatic and/or hormone refractory disease (Adler et al., 1999; Drachenberg et al., 1999; Kovacs, 2001; Shariat et al., 2001; Twillie et al., 1995). IL-6 was first characterized as a modulator of an immune response that is produced by T cells and involved in haematopoiesis and inflammation (for review see (Kishimoto et al., 1992). However, IL-6 has been subsequently shown to be produced by many other cell types, including normal and malignant prostate epithelial cells, and bone cells (Bellido et al., 1995; Klein et al., 1989; Lee et al., 2003; Lu and Kerbel, 1993; Miki et al., 1989; Okamoto et al., 1997; Yee et al., 1989). It is clear that IL-6 has the potential to act in both a paracrine and autocrine manner. Importantly, IL-6 has been shown to enhance proliferation of prostate cancer cells including LNCaP (Okamoto et al., 1997; Ueda et al., 2002a) and induce transcription of androgen-regulated genes such as PSA by a mechanism of ligand-independent activation of the AR (Hobisch et al., 1998; Ueda et al., 2002a). Thus, IL-6 derived from either bone or cancer cells may initiate and/or sustain progression of CaP to androgen independence. Here we examined the effects of osteoblast-derived factors on the proliferation, expression of PSA, and ligand-
independent activation of the AR in prostate cancer cells and confirm that IL-6 secreted from osteoblasts may be an important factor underlying androgen independent disease.

2.2 MATERIALS AND METHODS

2.2.1 Cell Culture and Materials

LNCaP human prostate cancer cells between passage 37 to 55 were maintained in RPMI 1640 supplemented with 5% v/v Fetal Bovine Serum (FBS, Invitrogen), 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen) and incubated at 37°C in an atmosphere of 5% CO₂. All other chemicals were purchased from Sigma unless otherwise stated. Human recombinant IL-6 and antibodies to human IL-6, the IL-6 receptor, and IL-1β used in neutralization studies were purchased from R&D systems. All experiments were performed using serum-free and phenol red-free conditions.

2.2.2 Culturing of Osteoblast-like Cells and Preparing OCM-MEM

Human osteoblast-like cells were cultured from trabecular femoral bone head trabecular explants obtained from osteoarthritis patients undergoing hip or knee replacement surgery. To minimize possible patient variation, donors were restricted to males, under 65 years of age. Trabecular bone was scraped into bone chips and further processed with a mortar and pestle. Bone chips were cultured in minimal essential media (MEM) containing 20% FBS at 37°C in the presence of 5% CO₂ (Rickard et al., 1996).
Figure 17. Human Osteoblast like Cells. H&E staining of osteoblast-like cells maintained for 3 months in culture and then grown for 3 days on glass coverslips.
The outgrowth of the osteoblast-like cells was monitored visually under the microscope with the aid of Gram Safaran staining and H & E (Figure 17). H& E staining was performed by dipping cells grown on glass cover slips in 95% alcohol for 2 minutes and 70% alcohol for 2 minutes. The cells were then washed briefly in distilled water and stained in Mayer hematoxylin solution for 8 minutes and rinsed again in distilled water once and dipped 10 times in 95% alcohol. To counterstain the cells were dipped in eosin-phloxine solution for 45s and dehydrated in two 5 minute absolute alcohol baths. The cells were then mounted in oil and viewed under the microscope.

Cells were characterized using the reverse transcriptase polymerase chain reaction (RT-PCR) for expression of osteoblast markers: type I procollagen, alkaline phosphatase and osteocalcin (Figure 18). Briefly, for the RT-PCR and for RNA extraction in general, Trizol from Invitrogen was used. Cells were directly treated with Trizol, 2ml per 10cm dish, scaled to different sized dishes. The RNA was then extracted using 0.2ml chloroform per 1ml Trizol, centrifuged at high speed, washed in 70% DEPC ethanol and dissolved in an appropriate volume of water depending on pellet size. cDNA was synthesized with 4μg RNA, containing 1X reverse transcriptase buffer and 2mM dNTPs, and 10 U of AMV reverse transcriptase with 200pmol random hexamers for 3h at 42°C (all materials from Invitrogen). The cDNA was amplified with 10 pmol primers, in PCR buffer containing dNTPs and Taq polymerase. For all samples, an initial denaturation step was performed for 2min at 94°C followed by 30 cycles of 94°C, 55°C and 72°C for 30s, 2min and 2min respectively. The products were analyzed using agarose gel electrophoresis and ethidium bromide staining. The primer sequences are as follows:
Alkaline phosphatase-5’ACGTGGCTAAGAATGTCATC and
3’CCAAGTAAGTCCAACGAAAG, osteopontin-5’CCAAGTAAGTCCAACGAAAG
and 3’GGTGATGTCCTCGTCTGTA, osteocalcin-5’CATGAGAGCCCTCACA and
3’AGGCCAGCCAGCATAATGGAA, type I procollagen-
5’TGACGAGACCAAGAACTG and 3’CCATCCAAAACCACCTGAAAACC.

When the osteoblast-like cells were confluent, they were washed with phosphate buffer
saline (PBS) (3 x 20 ml) and cultured in 20 ml of serum-free MEM. After 48 h, the OCM
was collected and centrifuged or filtered through 0.22 μm Nalgene units to remove the
cellular debris prior to storing at -80°C until use. OCMs from primary cultures of
osteoblasts prepared from different patients were never pooled, but rather used
individually. Primary cultures of osteoblasts were grown and maintained in Falcon
Primaria T75 flasks, with a surface of 75 cm². The average protein concentration in OCM
was 0.28 +/- 0.05 mg/ml (S.E.M). To determine protein concentration 1X Biorad Red dye
was used with 20μl protein samples, determined using a standard curve generated for
Bovine Serum Albumin (Biorad). Fibroblast conditioned media was collected and used
similarly to OCM (Lang et al., 1995). Skin fibroblasts were cultured in DMEM/F12
nutrient medium with 10% FBS at 37°C in 5%CO₂, until confluent, followed by
conditioning media according to the osteoblast protocol.

2.2.3 Proliferation Assay

LNCaP cells (1x10⁴) were plated in 96-well Falcon tissue culture plates in RPMI
containing 0.5% FBS in a final volume of 0.1 ml. When the cells reached 60%
confluence, usually within 24h, they were treated with R1881 or OCM collected from preparations of osteoblasts from three individual patients. After 5 days of culture, cell proliferation was assessed by adding 50μl of MTT dye (1mg/ml) in serum-free media to the cells. The MTT assay is based on the enzymatic reduction of the tetrazolum salt MTT in living but not dead cells. After 4h of incubation the cells were solubilized in dimethyl sulphoxide (Me₂SO) (150μl/well) on a shaker at room temperature prior to reading the absorbance at 570nm using a Dynex Technologies Microplate Reader (Ueda et al., 2002a; Ueda et al., 2002b).

2.2.4 PSA Protein and mRNA Expression

LNCaP cells were seeded at 1.5x10⁶ cells per 10cm² or 4x10⁶ cells per 15cm² dish in RPMI-1640 containing 5% FBS and cultured for 48h prior to incubation in 10 or 20ml of serum-free and phenol red-free RPMI-1640 for an additional 48h. This medium was removed and the cells were pre-treated with 5 or 10ml of fresh RPMI for 2h prior to adding an equal volume of treatment medium containing either OCM in MEM, 0.2nM R1881 or both for a final concentration of 50% OCM and 0.1nM R1881. After incubation for an additional 48 h, total RNA was extracted from LNCaP cells with Trizol (Invitrogen) and 15μg of RNA was electrophoresed on a 1% denaturing agarose gel before transfer to Hybond-N+filters (Amersham Pharmacia Biotech) by capillary diffusion. Northern blots were probed with a α³²P-labeled (Amersham-radioactive probe and Invitrogen-random labelling kit) 1.4 kb Eco RI PSA probe, quantitated using a
Figure 18. Osteoblast Markers: After several weeks of culture the osteoblast like cells were characterized using the reverse transcriptase polymerase chain reaction (RT-PCR) for expression of osteoblast markers: type I procollagen, alkaline phosphatase and osteocalcin. As can be seen from the size profiles of the PCR in lane A, C and E and from their respective sequence digests, in vitro cultures of bone cells appear osteoblast like.
STORM 860 Phosphoimager (Molecular Dynamics) and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was probed with $\alpha^{32}$P-labeled 1.0 kb Bam HI GADPH fragment (Sato et al., 1996).

Quantitation of secreted PSA protein by LNCaP cells was performed using $1.0 \times 10^5$ cells/well seeded in 12 well plates and treated as described above for the Northern blot experiment. Cells were cultured in 1.0ml of medium for 48h, serum-starved in 0.6ml of RPMI for an additional 48h before an equal volume of treatment media was added.

Supernatants from treated cultures were collected after 72h of incubation. Total cellular DNA was extracted using the DNeasy kit (Qiagen) and DNA was quantitated by absorbance at 260nm by a spectrophotometer and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was probed with $\alpha^{32}$P-labeled 1.0 kb Bam HI GADPH fragment (Sato et al., 1996). PSA protein in the culture medium (supernatant) was quantitated using the IMX total PSA kit (Abbot Laboratories) and normalized to total cellular DNA.

### 2.2.5 Plasmids

The pARR$_3$-tk-luciferase reporter contains three tandem repeats of the rat probasin AREs (-244 to -96) upstream of a minimal thymine kinase promoter in the pT81 vector (American Tissue Cell Collection) (Snoek et al., 1998). The PSA(6.1kb)-luciferase reporter contains the 6.1kb promoter/enhancer region of the PSA gene and was kindly provided by Dr. J.-H. Hsieh (Department of Urology, Southwestern Medical School,
Dallas, Texas). The AR\textsubscript{1-588}-Gal4DBD, the Gal4DBD and p5xGal4UAS-TATA-luciferase have been described previously (Sadar, 1999; Ueda et al., 2002a; Ueda et al., 2002b).

### 2.2.6 Transfection and Luciferase Assays

Transient transfections were carried out using Lipofectin\textsuperscript{®} (Invitrogen) as previously described (Sadar, 1999; Ueda et al., 2002a). Briefly, LNCaP cells were seeded at 1.0\times10^5 cells/well in 12well NUNC tissue culture plates and cultured in RPMI 1640 supplemented with 5% FBS for 48h. Cells were starved in RPMI for 24h prior to transfection with 0.5 μg PSA(6.1kb)-luciferase or 0.5μg of pARR\textsubscript{3}-tk-luciferase and 1μg pGL2 basic vector (Promega) per well (to normalize the amount of DNA/well to 1.5μg) using 2.5 μl/well of Lipofectin in 0.6ml of serum-free RPMI 1640. Transactivation studies with the AR NTD were performed using 3.0\times10^5 cells seeded 24h prior to cotransfecting with 5xGal4UAS-TATA-luciferase (1μg/well) and AR\textsubscript{1-588}Gal4DBD (50ng/well) or Gal4DBD for an additional 24h. The total amount of transfected plasmid DNA was normalized to 3μg/well by addition of empty vector with 5μl of Lipofectin in 1.0 ml of serum-free RPMI 1640 using 6 well Falcon tissue culture plates. After 24h, the cells were supplemented with an equal volume of medium containing the appropriate treatment. For experiments using the PSA(6.1kb) and pARR\textsubscript{3}-tk reporters, OCM in MEM or MEM was added to 50% concentration with or without R1881 (0.1 or 10 nM), bicalutamide (20μM), or 5μg/ml (final concentration) of anti IL-6, anti IL-6R or anti IL-1β or both IL-6 antibody with bicalutamide and cultured for an additional 48h. For AR\textsubscript{1-588}-Gal4DBD transactivation and titration studies, forskolin, Me\textsubscript{2}SO (vehicle control for
forskolin), human recombinant IL-6, R1881 or OCM was added and cultured for an additional 24h. Harvested cells were resuspended in 1.5ml of PBS containing 1mM Ethylenediaminetetraacetic Acid (EDTA), pelleted by centrifugation, solubilized in passive lysis buffer and assayed for luciferase activity using the Dual Luciferase Assay System (Promega) consisting of The Luciferase Assay Reagent II and Stop and Glo substarted dispensed and quantitated with the EG&G Berthold multi-plate luminometer according to the operation manual. Luciferase activity was normalized to protein concentration, which was determined by the Bradford assay (Biorad) using γ-globulin as the standard (Bradford, 1976). Each assay was done in triplicate and experiments were repeated at least three times. Fold-induction represents the luciferase activity in the cells cultured in the treatment medium relative to that cultured in growth medium alone or vehicle control.

2.2.7 IL-6 ELISA

Anti-IL-6 monoclonal antibody (285ng from R&D Systems) was immobilized on a 96 well plate for 2h and the wells blocked with 1% BSA in phosphate buffer saline (PBS) containing 0.01M phosphate buffer, pH 7.4, 136mM NaCl and 2.7mM KCl. Human recombinant (R&D Systems), or osteoblast-derived IL-6 was captured by the monoclonal antibody and then detected with a 1:2500 diluted polyclonal antibody (Sigma). Goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (1:5000) was used to label the bound polyclonal antibody and o-phenylenediamine was used as the substrate in 0.1 M citrate-phosphate buffer, pH 5.0. Recombinant human IL-6 from R&D Systems was used to generate a standard curve for IL-6. The colorimetric development was monitored
spectrophotometrically at 450 nm using a MRX microplate reader (Dynex Technologies).

Antibodies, IL-6 standards or OCM were diluted in PBS containing 1% BSA and 0.05% Tween. Washes were carried out between incubations with PBS containing 0.05% Tween.

2.2.8 IL-6 Neutralization Assays

LNCaP cells were seeded at $2.5 \times 10^4$ cells/well (4 wells/treatment) in 24 well tissue (Primaria Falcon) culture plates and cultured in RPMI 1640 supplemented with 5% FBS for 48h, then starved in 0.5ml/well RPMI for an additional 48h. The cells were then supplemented with 0.5ml of medium containing OCM (50% final concentration) in MEM, MEM, and R1881 (10 nM final concentration). Neutralizing experiments employed 10 μg/ml of antibodies to IL-6 and IL-1β which were added to filtered OCM and then incubated for 30min with occasional shaking at 37°C in a 5% CO₂ incubator prior to addition to LNCaP cells for a final antibody concentration of 5μg/ml. This concentration is in the range previously reported for neutralization studies in prostate cancer cells (Okamoto et al., 1997). According to manufactures instructions, 0.2-0.5 μg/ml of antibody should neutralize up to 7.5 ng/ml IL-6 in media. Cells were harvested after 24h of treatment. Total RNA was isolated in 0.25ml Trizol (Invitrogen) reagent per well and processed according to the manufacturers instructions. Semi-quantitative RT-PCR for PSA was performed using total RNA (0.5μg) as previously described (Ueda et al., 2002a). PSA and GAPDH primers were as follows: PSA 5'-GGCAGGTGCTTTGAGCCTCTC-3'; PSA 5'-CACCCGAGGAGTGTCTTTGC-3';
GAPDH 5'-CCGAGCCACATCGCTCAGA-3' and GAPDH 5'-CCCAGCCTTCTCCTGGTG-3'. For quantitation 5μM PSA primers (1μl) were mixed with 2.5μM GAPDH primers (1μl), 0.5μg total RNA, and earlier described Invitrogen RT-PCR enzyme/buffer reaction mix. The fragments were then resolved on 1.3% agarose gel, and the bands were analyzed with ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA). The PSA fragments were normalized to GAPDH.

For proliferation studies, cells were harvested after 5 days of incubation and analyzed using the MTT assay as described above.

2.2.9 Statistical Analysis

The Student's t test was used for statistical analysis. The significance level was set at p<0.05, indicated by a * above the data point.
2.3 RESULTS

2.3.1 Osteoblast-derived factors induce proliferation of LNCaP prostate cancer cells

To investigate the mechanism underlying androgen independent growth of prostate cancer cells in response to osteoblast-derived factors, we first established whether LNCaP cells proliferated more rapidly in response to conditioned medium collected from primary cultures of human osteoblast-like cells as previously described (Chackal-Roy et al., 1989; Gleave et al., 1991; Ritchie et al., 1997). OCM was collected from osteoblast-like cells prepared from three different bone donors. As shown in Figure 19, LNCaP cells proliferated more rapidly in response to incubation with OCM (50% final concentration). A concentration of 50% OCM was previously determined to be optimal for proliferation assays in prostate cancer cells (Lang et al., 1995). Untreated LNCaP cells (control) did not proliferate over the five day experiment. Androgen-treated cells (R1881, positive control) increased in proliferation as expected and previously reported (Sadar, 1999; Ueda et al., 2002a; Ueda et al., 2002b). This suggests that there are factors present in OCM that promote proliferation of prostate cancer cells.

2.3.2 Osteoblast-derived factors induce expression of PSA

Increasing levels of serum PSA indicate increased tumor burden and elevation in serum PSA over nadir levels precede clinical indication of recurrent, androgen
Figure 19. Osteoblast-conditioned medium (OCM) increases the proliferation of LNCaP cells. LNCaP cells were treated with R1881 (10 nM) or OCM (50% volume for volume) from three different preparations of osteoblasts labeled 26, 28, and 29. After 5 days in culture, cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Absorbance at 570 nm was measured, and error bars signify the mean ± SD of six independent experiments. *, significantly increased over the control values; $P < 0.05$. 
independent prostatic bone lesions (Bruchovsky et al., 2001; Bruchovsky et al., 2000; Huber et al., 1987; Lange and Vessella, 1998; Mundy, 1997). Thus we addressed the question whether osteoblast-derived factors have an effect on expression of PSA in LNCaP cells maintained in vitro. To do this, LNCaP cells were serum-starved prior to incubation with OCM (50% v/v final concentration). Protein levels of PSA that were secreted into the culture media in response to OCM are shown in Figure 20 A. The basal level of secreted PSA protein was below 20ng protein per μg of cellular DNA. OCM induced a 2.9-fold increase in the secreted level of PSA protein over control when normalized to cellular DNA content. R1881 induced a 7.4-fold increase in the secreted level of PSA as compared to control. These values were consistent with changes in levels of PSA mRNA in response to OCM. PSA mRNA was elevated 2.4-fold ± 0.2 S.E. in LNCaP cells incubated with OCM based on 5 independent experiments (Fig 20 B).

2.3.3 Induction of androgen responsive reporter gene constructs by OCM

To determine whether the induction of PSA mRNA and secreted protein by OCM is regulated at the transcriptional level, we employed several reporter gene constructs that contain functional AREs. LNCaP cells were transfected with a PSA(6.1kb)-luciferase reporter which contains several well characterized AREs (Schuur et al., 1996; Yeung et al., 2000). Optimal concentrations of R1881 (10nM) activated this promoter approximately 100-fold (Figure 21 A) over control which was consistent with previous reports (Sadar, 1999; Ueda et al., 2002a; Ueda et al., 2002b). OCM caused a 3-fold induction of the PSA (6.1 kb)-luciferase reporter. A mixture of R1881
Figure 20. Osteoblast-conditioned medium (OCM) increases levels of prostate-specific antigen (PSA) mRNA and secreted protein in LNCaP cells. In A, levels of secreted PSA protein normalized to total cellular DNA from LNCaP cells treated for 72 h with R1881 (0.1 nM), OCM (50% volume for volume), or a combination of R1881 and OCM. Error bars signify the mean ± SD of three independent experiments. *, significantly increased in OCM and R1881 over the control values; P < 0.05. In B, Northern blot analysis of PSA mRNA levels normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in LNCaP cells exposed to OCM (50% volume for volume) for 48 h. At the end of the incubation period, cells were harvested, RNA was isolated, and Northern blots were performed using radiolabeled probes for PSA and GAPDH. RNA bands corresponded to PSA at 1.5-kb pairs. Each lane contains 15 μg of total RNA.
Figure 21. Osteoblast-conditioned medium (OCM) induces androgen-regulated reporters. LNCaP cells were transiently transfected with PSA (6.1 kb)-luciferase (0.5 µg/well; A and C) or ARR3-tk-luciferase (0.5 µg/well; B and D) for 24 h before treatment with R1881 (A, 10 nM; B, 0.1 nM) or OCM (50% volume for volume) for an additional 48 h under serum-free conditions. The cells in C and D were treated with OCM from osteoblasts prepared from several different bone donors for 48 h. The error bars represent the mean ± SE of three independent experiments. *, significantly different; P < 0.05 between OCM and R1881 compared with control and OCM + R1881 compared with R1881.
(10nM) and OCM induced a synergistic increase in reporter activity that was approximately 250-fold over the control value. The ARR3-tk-luciferase reporter was next tested to determine if another androgen-responsive reporter gene construct could be induced by OCM. This reporter consists of three repeats of the ARE1 and ARE2 region (six AREs in total) of the probasin gene ligated in tandem with the minimal thymidine kinase promoter in a luciferase reporter thus making it highly sensitive to R1881 (Snoek et al., 1996) (Figure 21). R1881 induced activity of the ARR3 reporter over 400-fold (Figure 21B) in transfected LNCaP cells. OCM induced this reporter approximately 10-fold over control. Co-treatment of cells with R1881 and OCM resulted in a synergistic increase of pARR3-tk-luciferase activity greater than 800-fold over control (Fig 21B).

Variability in the induction of reporter gene constructs was observed between OCM obtained from osteoblasts prepared from different bone donors. Induction of PSA(6.1kb)-luciferase activity by OCM from different bone donors ranged from 3-fold to approximately 24-fold (Figure 21C). Similarly, induction of activity of ARR3-tk-luciferase by OCM ranged from 8-fold to 23-fold (Figure 21D). Despite the variability, induction of reporters was clearly observed using OCM from all preparations of osteoblasts.

2.3.4 Bicalutamide blocks OCM induction of androgen responsive reporter gene constructs by OCM

Induction of both PSA(6.1kb)- and pARR3-tk-luciferase reporters by OCM suggests the involvement of the AR since both of these reporters contain AREs. To further investigate the involvement of the AR, bicalutamide, a non-steroidal antagonist of the
AR, was used in combination with either OCM or R1881. As expected, bicalutamide blocked the induction of PSA(6.1kb)-luciferase and pARR\textsubscript{3}-tk-luciferase reporter activity by R1881 treatment from approximately 60- to -6, and 38- to 4-fold respectively (Figure 22 A,B). The inhibition of OCM induction of these reporters was less, but remained statistically significant, i.e. from approximately 3- to 1.5-, and 30- to 5-fold respectively (Figure A, 22B). Together our results indicate that bicalutamide is not as effective in blocking the induction of androgen responsive reporters by osteoblast-derived factors when compared to blocking the effects of androgen.

2.3.5 OCM activates the human AR NTD

It has been shown previously that other compounds such as forskolin that activate the protein kinase A (PKA) signalling pathway and IL-6 increase expression of PSA by targeting the AR NTD (Sadar, 1999; Ueda et al., 2002a; Ueda et al., 2002b). Therefore transactivation studies were employed to examine if OCM also activated the AR NTD. To do this, LNCaP cells were transfected with plasmids coding for either the AR\textsubscript{i}.\textsubscript{558}Gal4DBD fusion protein, or Gal4DBD control, and the luciferase reporter construct that contains the 5XGal4UAS binding element as previously described (Ueda et al., 2002a). Forskolin (50 μM) was included as a positive control and it stimulated luciferase activity over 5-fold (Figure 23) which was consistent with previous reports using this optimal concentration (Sadar, 1999; Ueda et al., 2002a). A 4-fold stimulation of the reporter was observed with OCM demonstrating that OCM transactivates the AR.
Figure 22. Bicalutamide partially inhibits osteoblast-conditioned medium (OCM) activation of androgen-regulated reporter gene constructs. LNCaP cells were transiently transfected with prostate-specific antigen (PSA; 6.1 kb)-luciferase reporter (0.5 μg/well; A) or ARR3-tk-luciferase (0.5 μg/well; B) for 24 h before pretreatment for 2 h with bicalutamide (BIC, 20 μM) and then incubated for an additional 48 h with MEM (control), OCM (50% volume for volume), or R1881 (A, 10 nM; B, 0.1 nM). Cells were harvested, and relative luciferase activity was determined. The error bars represent the mean ± SD of three independent experiments. *, significantly different; \( P < 0.05 \) between bicalutamide, OCM and R1881 compared with the control, OCM + BIC compared with OCM alone, and R1881 + BIC compared with R1881 alone.
Figure 23. Effect of osteoblast-conditioned medium (OCM) on transactivation of the androgen receptor (AR) NH2-terminal domain. Transactivation assays were performed in LNCaP cells cotransfected with the 5XGal4UAS-TATA-luciferase reporter (1 μg/well) and AR 1-558Gal4DBD (50 ng/well) or Gal4DBD control(50 ng/well) for 24 h before incubation with forskolin (FSK, 50 μM), OCM (50% volume for volume), Me2SO (DMSO control vehicle for FSK), or control (MEM) for an additional 24 h. Cells were harvested, and relative luciferase activity was determined and reported as fold-induction. The error bars represent the mean ± SD of three independent experiments. *, significantly different between OCM compared with the control; $P < 0.05$. 
2.3.6 Levels of IL-6 in OCM and activation of the AR NTD by recombinant human IL-6

The AR NTD can be activated by IL-6 and osteoblasts secrete IL-6 (Bellido et al., 1995; Birch et al., 1993; Ueda et al., 2002a; Ueda et al., 2002b). It is therefore possible that the observed effects of OCM on PSA gene expression and activation of the AR NTD might be attributed to IL-6. Levels of IL-6 in OCM obtained from preparations of primary osteoblasts from four different patients were quantified by ELISA and were shown to range from 1.69 to 3.40 ng/ml (Table I) which correlates to activating levels reported in literature (Bellido et al., 1995; Kovacs, 2001; Okamoto et al., 1997; Shariat et al., 2001; Ueda et al., 2002a). This range of concentrations of IL-6 have been shown to be sufficient to activate the AR NTD (Ueda et al., 2002a; Ueda et al., 2002b). Results in Figure 24, show a 6-fold increase in luciferase activity was obtained using 1ng/ml of recombinant IL-6, while a 13-fold induction was achieved with 10ng/ml IL-6. R1881 was included as a negative control since androgen binds to the LBD which was not present in the AR\textsubscript{1-558}Gal4DBD chimera. Together these results strongly suggest that IL-6 at levels present in OCM is sufficient to transactivate the AR NTD.

2.3.7 Antibodies to IL-6 block the induction of PSA gene expression by OCM

OCM contains levels of IL-6 that are sufficient to cause transactivation of the AR NTD and induction of PSA promoter activity (Figure 24 and (Ueda et al., 2002b)
Table 1. Levels of IL-6 in OCM measured by ELISA, n = 3

<table>
<thead>
<tr>
<th>Bone donor #</th>
<th>IL-6 (ng/ml) ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>3.40 +/- 0.04</td>
</tr>
<tr>
<td>19</td>
<td>2.99 +/- 0.08</td>
</tr>
<tr>
<td>20</td>
<td>1.69 +/- 0.04</td>
</tr>
<tr>
<td>21</td>
<td>2.34 +/- 0.03</td>
</tr>
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</table>
Figure 24. Titration of recombinant interleukin (IL)-6 on transactivation of the androgen receptor NH₂-terminal domain. Transactivation assays were performed in LNCaP cells and cotransfected with the 5xGal4UAS-TATA-luciferase reporter (1 μg/well) and AR₁₅₈₈Gal4DBD (50 ng/well) or Gal4DBD control for 24 h before incubation with R1881 (10 nM), IL-6 (1, 10, 50 ng/ml), or vehicle for an additional 24 h. The total amount of plasmid DNA transfected was normalized to 3 μg/well by the addition of empty vector. The error bars represent the mean ± SD of three independent experiments. *, significantly increased over the control values; *P < 0.05.
Therefore, neutralizing antibodies to IL-6 and IL-6 receptor were applied to confirm the role of IL-6 in the mechanism of induction of PSA gene expression by OCM. As shown in Figure 25A, induction of PSA(6.1kb)-luciferase activity with OCM was not affected by an antibody to IL-1β. However, addition of antibodies to IL-6 or its receptor resulted in attenuated induction of PSA(6.1kb)-luciferase activity by OCM from approximately 3-fold to slightly above 1-fold. A combination of bicalutamide and neutralizing antibodies to IL-6 did not result in significantly greater attenuation of induction of PSA(6.1kb)-luciferase activity by OCM from that achieved with IL-6 antibody alone.

To ensure that neutralization of IL-6 in OCM had an effect on endogenous expression of PSA, semi-quantitative RT-PCR was employed using total RNA isolated from cells. As expected, 10 nM R1881 (positive control) induced levels of PSA mRNA, as did OCM (Figure 25B, lanes 2 and 3). Neutralizing antibodies to IL-6 completely blocked induction of PSA mRNA by OCM (lane 4), while neutralizing antibodies to IL-1β had no inhibitory effect (lane 5). These results are consistent with the reporter gene assays and suggest that IL-6 present in OCM contributes to the induction of PSA gene expression.

2.3.8 Antibodies to IL-6 reduce proliferation of LNCaP cells in response to OCM

As shown in Figure 19, the proliferation of LNCaP cells was stimulated by OCM (50% final concentration). To determine the role of IL-6 in the enhanced proliferation of
LNCaP cells in response to OCM, neutralizing antibodies to IL-6 and IL-1β were employed. Neutralization of IL-1β in OCM had no effect the proliferation of LNCaP cells in response to OCM (Figure 26, compare lanes 3 and 5). However, neutralizing IL-6 in OCM significantly reduced proliferation as compared to OCM treatment alone (compare lanes 3 and 4). These results suggest that IL-6 is involved in the proliferative response of LNCaP cells in response to OCM.
Figure 25. Anti-interleukin (IL)-6 antibodies block the induction of prostate-specific antigen (PSA; 6.1 kb)-luciferase activity by osteoblast-conditioned medium (OCM). In A, LNCaP cells were transiently transfected with PSA (6.1 kb)-luciferase (0.5 µg/well) for 24 h and pretreated for 2 h with bicalutamide (20 µM) before the addition of OCM (50% volume for volume) for an additional 48 h. Neutralization studies were performed using OCM that was pretreated with antibodies for IL-1β, IL-6, and IL-6R (5 µg/ml final concentration) before the addition to LNCaP cells. Cells were harvested, and relative luciferase activity was determined and reported as fold-induction. The error bars represent the mean ± SD of three independent experiments. *, significantly different; \( P < 0.05 \) between OCM compared with the control and OCM + IL6R antibody (Ab), OCM + IL6 Ab, and OCM + IL6 Ab + bicalutamide (BIC) compared with OCM. In B, LNCaP cells were serum starved for 48 h, then treated with OCM, R1881, or OCM treated with neutralizing antibodies to IL-6 and IL1-β antibodies (5 µg/ml final concentration). Cells were harvested after 24 h, total RNA was isolated, and semiquantitative reverse transcription-PCT for PSA was performed with 0.5 µg of total RNA. Fold-induction of PSA is with respect to the control levels and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This is a representative experiment of \( n = 7 \).
Figure 26. Anti-interleukin (IL)-6 antibodies block proliferation of LNCaP cells in response to osteoblast-conditioned medium (OCM). LNCaP cells were treated with R1881 (10 nM), OCM (50% volume for volume), or OCM (50% volume for volume) pretreated with neutralizing antibodies to IL-6 or IL1-β. Cell proliferation after 5-day incubation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Absorbance at 570 nm was measured, and error bars signify the mean ± SD; n = 6. *, significantly different; P < 0.05 between the control and R1881 and OCM and OCM compared with OCM + IL6 antibody (Ab).
DISCUSSION

Prostate cancer has the propensity to metastasize to the bone and form osteoblastic lesions. Treatment for advanced metastatic disease is androgen ablation therapy which causes a temporary reduction in tumor burden concomitant with a decrease in serum PSA. Unfortunately prostatic bone lesions will begin to grow again in the absence of androgens to form androgen independent disease (Bruchovsky et al., 2001; Bruchovsky et al., 2000; Huber et al., 1987; Lange and Vessella, 1998; Mundy, 1997). Androgen independent disease is biochemically characterized before the onset of symptoms by a rising titer of serum PSA (Miller et al., 1992). It has been suggested that rising levels of IL-6 in the serum of prostate cancer patients have also been suggested to be a surrogate marker for androgen independent disease (Twillie et al., 1995). Here we examined the molecular mechanisms that may underlie the proliferation and expression PSA in androgen-deprived prostate cancer cells in response to factors secreted by osteoblasts and have made the following observations: 1) OCM increased the proliferation of LNCaP prostate cancer cells; 2) OCM induced PSA gene expression; 3) OCM transactivated the AR; 4) IL-6 was present in OCM at levels sufficient to transactivate the AR; and 5) neutralizing antibodies to IL-6 and its receptor attenuated the induction of PSA gene expression and blocked proliferation of LNCaP cells in response to OCM.

Localized CaP is generally slow growing; however, once the disease becomes androgen independent, particularly in the bone, it becomes more aggressive (Jacobs, 1983; Rana et al., 1993). The effect of OCM on the growth of LNCaP cells (Figure. 24) is
consistent with clinical observations and provides experimental evidence that factors secreted by bone stimulate the proliferation of prostate cancer in the absence of androgens. Thus, osteoblast-derived factors appear to be in place to substitute for androgens by promoting the growth of metastatic prostate cancer cells in bone.

Serum level of PSA is directly correlated to tumor burden (Huber et al., 1987; Stamey et al., 1987). Here we show that in addition to the proliferative response observed in the LNCaP cells exposed to OCM, PSA gene expression was also elevated. Induction of the PSA reporter gene construct was consistent with the elevated endogenous levels of PSA mRNA and secreted protein in LNCaP cells exposed to OCM (Figures 25-27). These data correlate to the clinical observations of recurrent, androgen independent disease. Interestingly, PSA gene expression appeared to be additive or synergistic when the synthetic androgen R1881 and OCM were used together and mRNA or reporter activity measured. Previous studies have observed synergistic increases in PSA gene expression in LNCaP cells in response to forskolin and IL-6, R1881 and IL-6, R1881 and butyrate (Sadar and Gleave, 2000; Ueda et al., 2002a; Ueda et al., 2002b). Typically additive or synergistic changes are interpreted to imply that multiple signalling pathways are being utilized.

The AR is expressed in the majority of metastatic and hormone refractory prostate cancer tissues and genes "normally" regulated by androgens become re-expressed in androgen independent prostate cancer cells (Gregory et al., 2001; Hobisch et al., 1995; Sadi et al., 1991; Wilding, 1995). Here we suggest a role for the AR in the up-regulation
of PSA gene expression by OCM due to the following observations. 1) OCM induced the transcription of both PSA and ARR3 reporter gene constructs and both of these reporters contain several well-characterized AREs (Riegman et al., 1991; Schuur et al., 1996; Yeung et al., 2000). 2) OCM increased transactivation of the AR. 3) The antiandrogen, bicalutamide, partially blocked the induction of PSA and ARR3 reporters by OCM. Together these data imply that factors present in OCM may activate the AR to induce genes containing AREs. This is consistent with the fact that several factors secreted by bone such as IGFs, KGF, EGF, and IL-6 have previously been shown to activate the AR (Culig et al., 1994; Hobisch et al., 1998; Ueda et al., 2002a; Ueda et al., 2002b). The precise mechanism for how the AR, and especially its N-terminal domain, is activated in the absence of its cognate ligand by cross-talk with these alternative pathways has not been elucidated, but may involve changes in the phosphorylation state of the AR or an interacting protein such as SRC-1 (Nazareth and Weigel, 1996; Sadar, 1999; Ueda et al., 2002b). Indeed it has recently been shown that mitogen activated protein kinase (MAPK) is required for both ligand-dependent and ligand-independent activation of the AR and that phosphorylation of SRC-1 by MAPK at serine 1185 and threonine 1179 are required for optimal ligand-independent activation of the AR in LNCaP cells (Ueda et al., 2002a; Ueda et al., 2002b). Our data showing that the antiandrogen bicalutamide only partially blocks the induction of these ARE-driven reporters by OCM may imply the involvement of additional mechanisms to the AR. Alternatively, antiandrogens that bind the LBD may not be effective antagonists for AR that is activated via its NTD by non-androgenic pathways.
IL-6 is important in normal bone turnover, is elevated in the serum of prostate cancer patients with androgen-independent disease, causes ligand-independent activation of the AR, induces PSA gene expression, increases proliferation of prostate cancer cells, and is secreted by the bone (Bellido et al., 1995; Drachenberg et al., 1999; Hobisch et al., 1998; Ueda et al., 2002a; Ueda et al., 2002b). Here IL-6 was shown to be present in OCM at concentrations that were sufficient to activate the AR (Table I, Figure 29, and (Ueda et al., 2002b)). Consistent with the idea of IL-6 being one of the main bone-derived mediators in promoting osseous lesions we have shown the following: 1) OCM induced PSA mRNA to similar levels reported using recombinant IL-6 (Hobisch et al., 1998; Ueda et al., 2002b). 2) Both OCM and IL-6 transactivate the AR NTD (Figures 28 and 29; (Ueda et al., 2002b)). 3) Both OCM and IL-6 increase the proliferation of LNCaP cells (Figures 24 and refs 14 and 28). 4) Neutralization studies with antibodies to IL-6 and its receptor blocked the induction of PSA reporter activity, endogenous PSA mRNA, and proliferation while an antibody to IL-1β did not show inhibition providing an indication of specificity. Together these data support a role for IL-6 as an important component secreted by bone in mediating androgen-independent expression of PSA in prostate cancer cells.

In conclusion, these studies show that factors secreted by primary cultures of human osteoblast-like cells stimulate proliferation and induction of PSA gene expression and androgen-regulated reporter gene constructs in prostate cancer cells devoid of androgens. OCM transactivated the AR suggesting that the AR may play a role in the progression of prostate cancer to androgen independence by a mechanism initiated by factors secreted
from osteoblasts, one of which appears to be IL-6. Further studies of factors such as IL-6 will provide insight into new alternative pathways of growth regulation that can supplant the requirement for androgens in prostate cancer and result in better methods to prevent or control androgen-independent disease.
3.1 SUMMARY

Prostate cancer has the propensity to metastasize to the bone and form osteoblastic lesions. Treatment for advanced metastatic disease is androgen ablation therapy which causes a temporary reduction in tumor burden concomitant with a decrease in serum PSA. Unfortunately prostatic bone lesions will begin to grow again in the absence of androgens to form androgen independent disease. Androgen independent disease is biochemically characterized before the onset of symptoms by a rising titer of serum PSA. In androgen independent disease, involvement of the AR appears to remain significant. Evidence for this is provided by the re-expression of genes normally regulated by androgens such as PSA and the presence of a functional AR in clinical specimens of advanced disease. Experimental and clinical data suggest that androgen independence may be favored by disease present in the skeleton. This is because bone is a rich source of growth factors that have been shown to stimulate the proliferation of prostate cancer. One such growth factor is IL-6. Rising levels of IL-6 in the serum of prostate cancer patients have been suggested to be a surrogate marker for androgen independent disease.

Currently there are no effective therapies for metastatic disease once it has become androgen independent. The long term goal is to improve the clinical management of bone metastatic prostate cancer such that it is treated as a chronic disease rather than a terminal malignancy. The short term objectives addressed in this thesis were to: 1) develop an in vitro model that mimics specific aspects of osteoblastic prostate cancer; 2) characterize the effects of osteoblast-derived factors on androgen-independent proliferation of prostate
cancer cells and expression of PSA; 3) distinguish the role of AR; and 4) evaluate the
contribution of IL-6 derived from osteoblasts in androgen-independent growth.

3.2 SUMMARY OF RESULTS

Work presented in this thesis shows the development and characterization of an *in vitro* model for osteoblastic prostate cancer. Primary cultures of osteoblasts were successfully grown and maintained as a monolayer. These cultures of osteoblasts provided a source of secreted factors shown to have biologically activity on prostate cancer cells. Clinical relevance of this model was provided by evidence of androgen independent proliferation of prostate cancer cells and induction of PSA gene expression in response conditioned media obtained from osteoblasts. Induction of PSA gene expression was at the level of transcription initiation as indicated through employment of a PSA-reporter gene construct. Androgen independent induction of other androgen-regulated reporter gene constructs indicated the possible role of the AR. Delineation of the role of AR in the androgen independent response to osteoblast-derived factors was provided by application of bicalutamide, an antagonist to the AR and direct measurement of transactivation of the AR. One potential candidate secreted by osteoblasts that is known to have an effect on transactivation of the AR is IL-6. The contribution of IL-6 as an important factor present in the milieu of growth factors secreted by osteoblasts was explored. IL-6 was shown to be present in conditioned media from osteoblasts at levels sufficient to transactivate the AR. Neutralizing antibodies to IL-6 and its receptor attenuated the induction of PSA gene expression and blocked proliferation of LNCaP.
cells in response to conditioned media from osteoblasts. Together these data support the use of this model system for osteoblastic prostate cancer and suggest the AR and IL-6 may be important players in the progression of prostate cancer to androgen independence disease in the bone environment.

3.3 FUTURE DIRECTIONS

This thesis describes a clinically relevant system for investigating certain aspects of bone metastatic prostate cancer. The newly developed system also offers a number of possibilities for continued research. Further delineation of factors secreted by osteoblasts and the pathways affected in prostate cancer cells in response to these factors will provide insight into the complex interactions between bone and prostate cancer and may reveal new therapeutic targets to prevent or control bone metastases and androgen-independent disease.

Identification of therapeutic targets and pathways affected in prostate cancer cells in response to osteoblast-derived factors can be examined by measuring global changes in gene expression at the RNA and protein levels. Global technologies may be useful in generating a signature profile using this model system that can be extrapolated to the clinic. However, candidate genes and proteins will have to be investigated individually to decipher their precise mechanisms of action.

In terms of global profiling, both the cancer cells and the bone cells should be investigated. RNA and protein from prostate cancer cells harvested after treatment with
OCM should be collected as well as RNA and protein from osteoblasts after treatment with conditioned media from prostate cancer cells. The RNA and protein can be used to generate a signature profile of changes in gene expression with respect to cells treated with regular media. Profiles using RNA can be generated using gene arrays, subtractive hybridization and multiple tissue northerns. Profiles using protein can be generated by application of Isotope-Coded Affinity Tag (ICAT) coupled to liquid chromatography tandem mass spectrometry (LC-MS/MS) technology, two dimensional gel electrophoresis followed by protein identification as well as tissue protein arrays. Validation of changes in expression levels of interesting candidates should first be performed in experimental samples then later in clinical samples by methods such as immunohistochemistry and in situ hybridisation/Fluorescent -Insitu Hybridization. In addition, the proteins secreted into the conditioned media by both the osteoblasts and prostate cancer cells can be identified and quantitated using MS and ICAT technology. On a smaller scale, specific screens for kinases and phosphatases can be performed on the protein extracts of cells to delineate signalling pathways involving changes in phosphorylation, an important post-translational modification of proteins.

In terms of small scale functional experiments, the first thing that can be investigated is the biological role of PSA as a protease in mediating the effects of osteoblast-derived factors on proliferation and AR/androgen controlled gene activation. For example, antibodies or gene silencing techniques can be applied to determine if PSA in particular may serve to release some growth factors in OCM that are otherwise not bio-active/bio-available to the treated cells.
Secondly, signalling pathways activated by OCM can be investigated with various inhibitors, especially pathways that have been shown to be important in ligand independent activation of the AR via forskolin and IL-6 such as PKA and MAPK. Other growth factors known to be secreted by osteoblasts can be quantitated and neutralization studies can be performed to examine their effects on growth, PSA and other androgen regulated gene expression, as well as on the AR and its various sub-domains. Specific regions of the AR can be investigated in detail as to their importance with various factors, and then once the general region is identified, the domain can be further dissected to identify the exact amino acids important in the interaction.

Other transcription factors besides the AR can also be investigated, as to being activated by OCM, based on literature and gene expression data generated. IL-6 can also be further investigated. For example, its production in osteoblasts can be blocked by gene silencing and the activity of the OCM on prostate cancer cells can be reassessed.

Moreover, AR involvement in activating AREs in prostate cancer cells in response to OCM can be validated by using gene silencing of AR. The activated AR can also be immunoprecipitated from prostate cancer cells and examined for changes in phosphorylation and interactions with co-regulators and co-repressors. In addition, the morphological changes taking place in the cancer cells as a result of OCM treatment can be further investigated. Finally, IL-6 neutralization studies can be undertaken in animal models of prostate cancer bone lesions expressing IL-6 as preliminary pre-clinical evaluations for treatment for osteoblastic prostate cancer.
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