Identification and characterization of genes that are common in prostate cancer and adipocyte cell lines

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Contents

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Abbreviations

5α-dihydrotestosterone (DHT)
Acetyl CoA Carboxylase (ACC)
Adipocyte Determination and Differentiation factor 1 (ADD1)
Adipocyte-specific fatty acid binding Protein 2 aP2
Alzheimer Disease (AD)
Androgen Receptor (AR)
Androgen Response Elements (AREs)
Bax Inhibitor 1 (BI-1)
Bcl-2 Associated X protein (Bax)
Brown Adipose Tissue (BAT)
Calorie Restriction (CR)
CCAAT/Enhancer Binding Protein α (C/EBPα)
Chole Cysto Kinin (CCK)
c-Jun N-terminal Kinase (JNK)
DNA Binding Domain (DBD)
Endoplasmic Reticulum (ER)
Epidermal Growth Factor (EGF)
Estrogen Receptor-related Receptors α (ERRα)
Extracellular-signal Regulated Kinase (ERK)
Fatty Acid Synthase (FAS)
Fibroblast Growth Factor (FGF)
Formyl Peptide Receptor-Like-1 (FPRL1)
Glucagon-Like Peptide-1 (GLP-1)
Glucocorticoid Receptor (GR)
Glucose Transporter 4 (Glut 4),
Glycerophosphate Dehydrogenase (GAPDH)
Homeodomain-containing transcription factor 3.1 (NKX.3.1)
Hormone Response Element (HRE)
Humanin (HN)
ImmunoFluorescence (IF)
Insulin like Growth Factor -1 (IGF-1)
Insulin Receptor (IR),
interleukin 1 (IL-1)
ligand-binding domain (LBD)
lipoprotein-lipase (LPL)
Open Reading Frame (ORF)
Peroxisome Proliferator Activated Receptor (PPAR)
Platelet-Derived Growth Factor (PDGF),
Prostate Cancer (PCa)
Prostate Specific Antigen (PSA)
Prostate Specific Library (PSL)
Prostate-Derived Ets Factor (PDEF)
Phosphotase and Tensin Homolog Deleted on Chromosome Ten (PTEN)
Reactive Oxygen Species (ROS)
Retinoic acid receptor-related Orphan Receptor-γ (RORγ)
Retinoid X Receptor (RXR)
small interfering RNAs (siRNA)
SREBP-Cleavage-Activating Protein (SCAP)
Sterol Regulatory Element Binding Proteins (SREBPs)
Suppression Subtractive Hybridization (SSH)
Testicular Enhanced Gene Transcript (TEGT)
Thiazolidinediones (TZDs)
Tumor Necrosis Factor α (TNF-α)
Ubiquitin ligases (E3)
Ubiquitin-conjugating enzymes (E2)
Ubiquiting-activating enzymes (E1)
Uncoupling protein (UCP)
Vascular Endothelial Growth Factor (VEGF)
White Adipose Tissue (WAT)

General Introduction

Androgens and Androgen Receptor

Androgens are steroid hormones derived from cholesterol and synthesised primarily in the testes prior to transport to target tissues (1). The main function of androgens is to mediate male phenotypic differentiation during the fetal period. During the neonatal period it also exerts effects upon the central nervous system, and during puberty and adulthood androgens have stimulatory effects on growth and functionality of the testes and the spermatogenesis. Although testosterone is one of two main androgen hormones, there is evidence that it might not be the active male sex hormone in certain tissues (2). Testosterone appears to be responsible for promoting the formation of the male reproductive structures (i.e., the epididymis, seminal vesicles, and vas deferens). However, it is not directly involved in the development of the male urethra, prostate, penis, or scrotum. Development of such tissues rely instead on 5α-dihydrotestosterone (DHT) which is converted to testosterone by 5-α reductase produced by basal and secretory luminal cells of the prostate and other sites as well, including liver.

Androgen and the androgen receptor (AR) are essential for prostate development and proper function of the normal prostate (1). Binding of DHT to AR activates the receptor and, together with co-activators and co-repressors, regulates transcription of target genes. Approximately 80–90% of prostate cancers (PCa) are dependent on androgen at initial diagnosis, and endocrine therapy of PCa is directed toward the reduction of serum androgens and inhibition of AR function (3). Increased levels of testosterone or its metabolite, DHT, may increase risk for PCa (4), although such findings are inconsistent and controversial. For example, a nested cohort study of Norwegian subjects found no association between levels of DHT and PCa (5).

In both the rat and human genome the gene encoding AR is located on the long arm of the X chromosome (q11-12) and consists of eight exons spanning a region of 170-180 kb of genomic DNA (6) (See Figure 1). The promoter of the *AR* gene is a TATA-less type of promoter and contains GC-rich elements as bindings sites for the transcription factor Sp1 (7), a homopurine domain, a cAMP response element, and binding sites for other transcription factors (6). The original structural analysis of the AR protein revealed

that it contains four functional domains, similar to other members of the steroid receptor superfamily: a conserved DNA binding domain (DBD), a hinge region, a ligand-binding domain (LBD), and a less conserved amino-terminal domain (8,9). Further analysis of AR structure revealed two transcriptional activation function (AF) domains, including the N-terminal ligand-independent AF-1 domain and the C-terminal ligand-dependent AF-2 domain. Upon binding of androgen to AR in the cytosol, the LBD undergoes conformational changes which result in a more compact structure (10). Parallel with this conformational change is the release of several previously bound molecular chaperones (e.g. hsp90 and hsp70) and the translocation of the receptor to the nucleus. The regulation of gene expression by AR requires the specific tethering of the AR homodimer to the promoter and/or enhancer sequences of target genes. Recognition and binding of AR to target genes is mediated by the DBD (amino acids 550–624), which is comprised of two zinc-binding modules, where four cysteine residues coordinate each Zn ion (6). The main features of the DBD are two α-helices positioned perpendicular to each other; of these the N-terminal helix is the 'recognition helix' and is positioned within the major groove of the DNA. This helix contains the key amino acids glycine 568, serine 569 and valine 572, which are collectively termed the 'P-box residues', and are directly involved in DNA sequence recognition. The P-box residues found within the AR are identical to those at the corresponding positions within the glucocorticoid, mineralocorticoid and progesterone receptors (6).

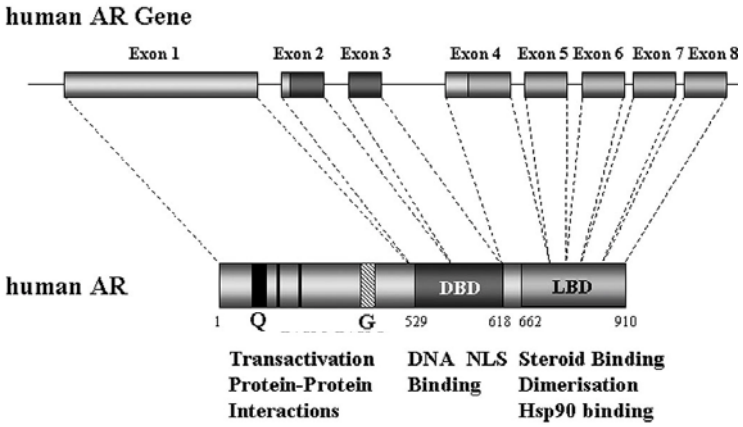

FIGURE 1. *AR gene organization and domain structure of the protein. The AR gene (top) consists of eight exons that give rise to the characteristic domain structure of the receptor protein (bottom). LBD, ligand-binding domain; DBD, DNA-binding domain. Q and G represent polyglutamine and polyglycine repeats respectively. Figure from reference 6.*

Androgen regulated genes

Since prostate epithelial cells are highly androgen sensitive, they have been model systems for the identification of AR target genes (11). The most important androgen-regulated gene related to clinical application is Prostate Specific Antigen (PSA or Kallikrein 3) (12). PSA is a protease that is secreted from the prostate and may function in the liquification of the seminal fluid (13). Today PSA is used worldwide as a marker for PCa (14). However, because serum PSA levels differ significantly among men with no cancer, there is still strong debate among clinicians regarding its use as a marker for early cancer detection (15). Identification of better markers for early cancer detection that is more stable and specific than PSA is ongoing. Homeodomain-containing transcription factor 3.1 (NKX.3.1) is another androgen-regulated gene. NKX.3.1 is shown to interact with prostate-derived Ets factor (PDEF) a prostate-specific transcription factor that positively regulates *PSA* gene expression (16) and is thought to be essential for normal prostate development (17). The NKX.3.1 has previously been reported to map to chromosome band 8p21, which is a region frequently lost in PCa and in up to 78% of metastatic PCa cases NKX.3.1 expression is lost (18). However, new insight have questioned this view by the findings that there is no significant change in *NKX3.1* mRNA levels during prostate cancer progression which suggests that *NKX3.1* may not be associated with the allelic loss (19). Recently, microarray technology has been used to identify a large number of novel androgen-regulated genes involved in protein folding, trafficking and secretion, metabolism, the cytoskeleton, cell-cycle regulation and signal transduction (6). As expected, a common feature of AR-regulated genes is the presence of one or more Androgen Response Elements (AREs) AR or hormone response elements (HRE), together with binding sites for housekeeping genes, and inducible and tissue-specific transcription factors. Thus, androgen-regulated gene expression is likely to involve the coordinated interactions of the receptor protein as well as many other transcription factors.

Androgen regulation of lipid metabolism

The group of Guido Verhoeven was the first to report that androgens regulate lipid metabolism genes in the LNCaP prostate cancer cell line (20,21). Later they found more direct evidence by the observation that exposure of LNCaP cells to androgens lead to massive accumulation of neutral lipids (triglycerides and cholesteryl esters), which are storage products of fatty acid and cholesterol (22). In support for involvement of androgen receptor the androgen antagonist Casodex (bicalutamide) abolish the stimulatory effects of androgens and do not change the lipid profile in AR-negative prostatic cell lines. Analysis of the origin of the accumulated lipids revealed that these lipid accumulations are the result of a major androgen-induced increase in the synthesis of fatty acids and of cholesterol, the majority of which is used for membrane synthesis (22). Induced synthesis of fatty acids and cholesterol is governed by androgens through stimulation of the expression of whole sets of lipogenic enzymes, covering the entire pathways of fatty acid and cholesterol synthesis (23). Key players in the regulation of these pathways are the sterol regulatory element binding proteins (SREBPs). SREBPs are a family of three basic helix–loop–helix leucine zipper lipogenic transcription factors (SREBP-1a, SREBP-1c, SREBP-2) that are synthesized as inactive precursor proteins anchored to the membranes of the endoplasmic reticulum (ER) (24). There they interact with an SREBP-cleavage-activating protein (SCAP), which is retained in the ER by Insig retention proteins (25,26). The SCAP/SREBP/Insig complex is stabilized by cholesterol. When sterol levels are low, the SREBP–SCAP complex is released from the Insig retention protein and travels to the Golgi apparatus where an amino-terminal SREBP fragment is released by a two-step mechanism of regulated intramembrane proteolysis. This transcriptionally active fragment is translocated to the nucleus and depending on the SREBP isoform activates the transcription of multiple genes involved in the synthesis, binding, metabolism and uptake of fatty acids and cholesterol.

Mounting evidence demonstrates that the primary sites of action of androgens on the SREBP pathway are SCAP and Insig retention proteins (see figure 2). Androgens markedly stimulate the expression of SCAP and cause a switch in the isoform expression of Insig (27). This results in a change in the balance of the SREBP–SCAP complex on one hand and the retention protein complex on the other hand. The fraction of SCAP that

is not retained by the retention protein would be free to escort the SREBP precursor to the Golgi apparatus leading to proteolytic maturation and activation of lipogenic gene expression. Androgens also stimulate the expression of SREBP-1c and SREBP-2 precursors, but these effects are thought to be secondary to the proteolytical activation of SREBPs. A similar mechanism of action as described for androgens has been proposed to explain the lipogenic effects of progestagens in adipocytes and in breast cancer cells (28).

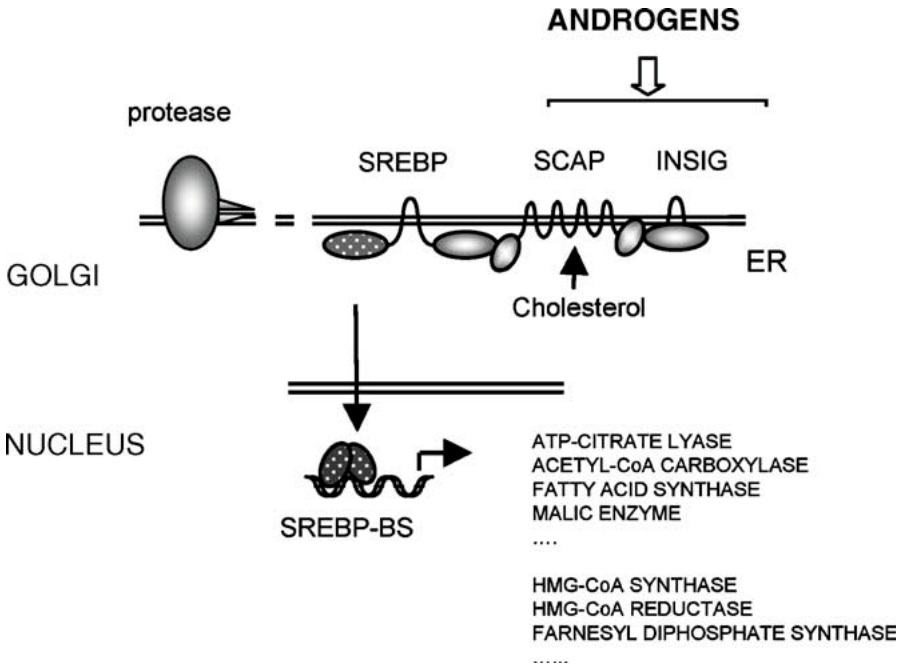

***FIGURE 2**. Androgens stimulate lipogenic gene expression. Lipid homeostasis in mammalian cells is largely regulated by the SCAP/SREBP pathway. Sterol regulatory element-binding proteins are lipogenic transcription factors, synthesized as inactive precursors. SREBPs interact with SCAP, a SREBP cleavage-activating protein, which functions as a cholesterol sensor and which is retained in the ER by Insig retention proteins. Androgens stimulate the expression of SCAP and modulate the balance of SCAP and the Insig protein. Part of the SCAP pool is not retained by Insig and escorts the SREBP precursor to the Golgi, where a set of proteases cleaves the SREBP precursor. The amino-terminal fragment is released and is translocated to the nucleus where it binds to SREBP-binding sites (SREBP-BS) in numerous genes involved in lipid metabolism and activates their transcription. Figure from reference 21.*

The prostate

The prostate is located in the pelvis, posterior to the bladder and superior to the rectum. The normal function of the prostate gland is not clearly understood but it produces seminal fluid and may facilitate sperm motility (29). The prostate is a network of branching glands, composed of ducts lined with secretory epithelial cells and basal

cells. Scattered neuroendocrine cells are also present and are thought to provide a paracrine function in the gland (30). Secretory epithelial cells represent the major cell type in the gland. They are androgen-dependent for growth, and secrete PSA. The basal cell layer is not dependent on androgen for growth and is believed to contain the stem cell population for the epithelial prostate cells. Surrounding the gland is a stroma that includes fibroblasts, smooth muscle, nerves, and lymphatic cells (31).

Prostate Cancer

Prostate cancer is the second most commonly diagnosed cancer in men in the US and Western Europe that leads to significant numbers of death or impaired quality of life. The estimated annual incidence in 2000 was 543,000 cases worldwide (32). The number of incidents in 2005 is predicted to be 232 080 in the United States alone (33). In Norway the age-adjusted incident rate was 76 cases per 100,000 inhabitants in 2001 (34). In the US the lifetime risk for PCa is 1 in 6, and the risk for development to metastatic PCa is 1 in 30 (35). The incidence of PCa has increased in the past 50 years, with recent dramatic increases most likely due to early detection methods, such as the measurement of serum PSA, rather than true differences in underlying risk (36).

The transformation of the prostate from a normal to an aggressive hormone insensitive cancer state is a complicated process that is still not fully understood. In recent years, a more comprehensive picture of the physiological and molecular state of the disease has been revealed. Briefly, three different cellular origins for prostate cancer have been postulated (37). In the first scenario, androgen-independent basal stem cells acquire somatic genetic changes which results in continuous growth stimulation. Alternatively, the tumour can arise from androgen-independent cells from this basal layer. In both scenarios, such cell types are potential cancer origin cells. Acquisition of AR expression in these cells confers androgen dependent growth properties. Androgen ablation therapy is effective at eliminating cells, but leaves androgen- independent basal cells intact. Growth of androgen-independent cells is thought to give rise to malignant growth. The reduction of serum and prostatic DHT levels by castration also results in a loss of 70% of the prostate secretory epithelial cells due to apoptosis, but the basal

epithelia and stromal cell populations are relatively unaffected (38). As a consequence, low circulating amounts of androgen will allow continued proliferation of the precursors to reach a hormone refractory state. Finally, in a third scenario, prostate cancer might originally develop from an AR positive luminal cell. Such a cancer could be completely abolished by ablation therapy but due to the genetic instability of cancer cells, some of the androgen-dependent cells somehow lose their dependency on androgen to become androgen-independent (37). By the time of PCa diagnosis, prostate cells may have undergone many somatic mutations, gene deletions, gene amplifications, chromosomal rearrangements, and changes in DNA methylation patterns. These alterations probably accumulate over a period of several decades (39).

Risk factors for prostate cancer

Despite its importance, the cause of PCa development is still not well understood. Nutrition and diet is believed to play an important part, but other factors also influence (36). In general, risk factors for PCa can be divided into two categories: the endogenous factors that consist of family history, hormones, aging, oxidative stress and race, and exogenous factors are related to diet, environmental factors and exercise. A more detailed discussion of some the most relevant risk factors follow below.

Family history. PCa appears to have a stronger familial aggregation than colon or breast cancer, two malignancies with well recognized familial components (36). There is a reported 2- to 4-fold increased risk among men whose father or brother were also PCa patients after adjustment for age and dietary factors (40). Polymorphic variation in the trinucleotide repeat lengths of the AR NH_2-terminal is associated with altered AR transcriptional activity *in vitro* (41), and may also contribute to PCa risk or progression.

Age and oxidative stress. The frequency of PCa increases dramatically with age, beginning with low frequencies in middle-aged men and progressing to >90% by the age 90 (42). During aging there is a progressive accumulation of DNA adducts and an increase in DNA frequency strand-breaks in most tissues (43). It is believed that these

age-related changes are caused by accumulation of oxidative stress (44). Energy metabolism has for many years been coupled to oxidative stress by generation of reactive oxygen species (ROS). During cellular respiration in the mitochondria ROS can occasionally be generated as by-products (45,46). ROS are highly reactive and potentially damaging to cells because they directly impair macromolecule structure and organelle function. Improper disposal of ROS can lead to greater genomic instability. Whether the prostate accumulates more ROS than other tissues remains an unanswered question.

Race – There is reportedly large differences in PCa incidence among various races. Between 1988 and 1992, race-specific incidence rates in the U.S. ranged from 24.2 per 100, 000 for Koreans, 89.0 per 100, 000 for Hispanics, 134.7 per 100, 000 for whites, and 180.6 per 100, 000 for African Americans. Black men in the U.S. are more likely to present with advanced-stage cancers than white men, and their stage-specific mortality is significantly greater, especially among younger men (36). In the last few years, a large increase in incidence in Asia has been observed, and has been explained by the adaptation of lifestyle and diet habits more similar to western countries. Japanese immigrants to the U.S. for example, have a higher incidence rate than native Japanese.

Diet. Dietary fat is a strong risk factor for PCa (47). A large body of descriptive epidemiological studies of migrants, geographic variations, and temporal studies supports the hypothesis that dietary factors may contribute to PCa development. However, not all studies have found an association between dietary factors and PCa risk (48). A cohort study measuring fat intake did not note any connection between increased fat intake and the risk of advanced PCa (49). It is unclear how dietary fat may increase the risk of PCa, but a number of mechanisms have been proposed. These include dietary fat-induced alterations in hormonal profiles, the effect of fat metabolites as protein or DNA reactive intermediates, and dietary fat-induced elevation of oxidative stress. It is likely that the relationship between dietary fat and PCa risk is complex, involving interplay of fat with other dietary factors, such as antioxidants, vitamins and minerals, as well as genetic factors that influence disease susceptibility (36). There is a growing body of evidence that obesity is related to others malignancies as well, especially colon cancer and post

menopausal breast cancer (50). But also PCa has been associated with obesity (Ref). Since a major goal of this thesis is to understand how obesity is a risk factor leading to cancer, a basic understanding of the nature of obesity is necessary, described below.

Lipogenesis in prostate cancer

In recent years, numerous reports have demonstrated overexpression of lipogenic enzymes such as fatty acid synthase (FAS) and acetyl-CoA carboxylase, in a wide variety of cancer types including cancer of the breast, endometrium, ovaries, lungs, colon, oral cavity, several soft tissues, and the prostate, (reviewed in (51)). In the prostate, overexpression of FAS has been studied most intensively and is found in the earliest stages of neoplastic transformation (PIN lesions) and in nearly all invasive carcinomas (52-54).

How and why lipogenic proteins are overexpressed in prostate cancer cells remains poorly understood. With respect to the mechanism underlying high level expression of FAS, it is evident from the earlier mentioned observations that androgens and dysregulated androgen receptor function play an important role. In LNCaP prostate cancer cells it has been shown that a mutation in the tumor suppressor gene encoding Phosphatase and Tensin Homolog Deleted on Chromosome Ten (PTEN) leads to constitutive Akt signaling and substantially contributes to the high level expression of FAS. Exposure to growth factors such as epidermal growth factor (EGF) further enhances lipogenic enzyme expression (55). Similar to the lipogenic effects of androgens, growth factor-induced lipogenesis is in part governed at the transcriptional level and involves activation of SREBPs. In several instances the effects at the protein level are more pronounced than those at the transcriptional level, suggesting that translational and/or post-translational effects further enhance lipogenic enzyme expression and activity (53,56)

In most tumor cells examined the majority of newly synthesized lipids are phospholipids (54,57). As phospholipids are the major building blocks of membranes it has been speculated that increased lipogenesis in cancer cells reflects the high rate of membrane synthesis in rapidly dividing cells. In most clinical prostate cancers, however,

only a fraction of the cancer cells are at one moment engaged in an active cell cycle, while nearly all cancer cells express high levels of FAS (52). Moreover, in contrast to the lipids derived from the diet which are relatively rich in polyunsaturated fatty acids, the newly synthesized phospholipids are enriched in saturated and in monounsaturated fatty acyl chains (57). Together with cholesterol these phospholipids tend to partition into detergent-resistant membrane microdomains (57,58). These are raft-aggregates implicated in key cellular processes including intracellular trafficking, signal transduction and cell migration (59). Hence, it is expected that increased lipogenesis in cancer cells affects multiple key aspects of tumor cell biology and actively contributes to the development and the progression of cancer.

So far we have discussed androgens and AR as important factors for prostate cancer progression and growth. Androgens induce synthesis, metabolism and transport of fatty acids and cholesterol activating a whole set of lipogenic enzymes which is highly upregulated in PCa. Epidemiological and molecular evidence connects PCa and high fat diet but a connection between up-regulation of lipogenic protein and diet has not been confirmed in PCa. A growing body of evidence does however link obesity to malignancies, especially colon cancer and post menopausal breast cancer, and PCa also has been associated with obesity (50). Since a major goal of this thesis is to understand how obesity is a factor leading to cancer, a basic understanding of the nature of obesity is necessary as reviewed below.

Obesity

Obesity is defined medically as a state of increased body weight, more specifically adipose tissue, of sufficient magnitude to produce adverse health consequences (60). The most widely used index of obesity is the body mass index (BMI), calculated by dividing the body weight in kilograms by the square of the height in meters (kg/m^2). According to this system, people with a BMI of 18.5 to <25 is considered to be of normal weight, BMI of 25 to <30 are considered "overweight" and BMI ≥30 are considered "obese" (50). Obesity has reached epidemic proportions globally and continues to increase. In the US, the prevalence of obesity rose from 12 to 20% between

1978 and 1990 (61). In Norway, a population-based study among men during a two year period showed an increase in obesity from 9.6 to 14% (62).

The increase in obesity during the last 30 years is associated with a dramatic increase in prevalence of serious health conditions, such as type 2 diabetes. Globally, the number of people with diabetes is predicted to rise by almost 50% in 10 years from 151 million in the year 2000 to 221 million in 2010 (63). The most potent predictor for the risk of diabetes, apart from age, is BMI. A high BMI is also associated with higher blood pressure and risk of hypertension, higher total cholesterol, LDL cholesterol, triglyceride levels, and lower HDL cholesterol levels. The overall risk of coronary heart disease and stroke, therefore, increases substantially with weight gain and obesity (63).

Obesity results from an imbalance between energy expenditure and energy intake. The epidemic of obesity is clearly related to nutrition and energy intake (64), and the underlying cause is thought to be a combination of excess caloric intake and insufficient physical activity (65). Nevertheless, many individuals manage to resist obesity. Accordingly, a key goal of ongoing research in this field is to identify mechanisms by which environmental factors interact with specific genes, either to promote, or facilitate resistance to obesity.

Energy expenditure can be divided into three major components. The largest of these is the necessary energy spent on basic cellular and physiologic functions that require ATP, followed by physical activity and the diet induced thermogenesis (50). The first of these, basal metabolism, is related to the types of foods eaten, adaptive capacity of the body and rate of energy expenditure. The maintenance of energy balance requires that the body oxidizes the food eaten. The capacity for storage of carbohydrate is very limited, and the capacity to store protein is also restricted (50). Only fat stores can readily expand to accommodate increasing levels of energy intake above those required for daily energy needs. Secondly, energy expended in physical activity is directly related to body weight. Physical activity gradually declines with age, and maintaining a regular exercise program is difficult for many people, particularly as they get older. Adaptation to a change from a low- to a higher-fat diet takes time and can be accelerated by exercise (66). The thermic effect of food is the third component of energy expenditure (50). After food is ingested, there is a rise in energy expenditure, which accounts for approximately 10% of the day's

energy expenditure. The sympathetic nervous system controls part of this process. The control of sympathetic activity and its noradrenergic output offers a possible strategy for treating obesity by raising energy expenditure. Brown adipose tissue (BAT), which is rich in the uncoupling protein 1 (UCP1), has a well established role in temperature and body weight regulation in rats and mice (67). Increased expression or activation of this protein uncouples oxidative phosphorylation, resulting in the conversion of energy to heat. The importance of this molecule in humans has always been questioned because of the very low levels of brown fat in adult humans. Recently the identification of two additional uncoupling proteins (UCP2 and UCP3) that are highly expressed in adult human muscle tissue has attracted considerable interest. Nonetheless, many think that these proteins are not critical for involvement in whole body energy expenditure because energy homeostasis is normal in mice lacking both proteins (68,69). However, they are under heavy focus especially related to calorie restriction (CR) which will be covered in detail below.

The Endocrine System and Obesity

The endocrine and autonomic nervous systems are major efferent control systems involved in regulating appetite and energy storage (see figure 3). The hypothalamus is the primary locus for integration of the various signals secreted from the adipose tissue, gonads, intestine or pancreas. Several critical hypothalamic circuits and signalling pathways are reported so far (64). Three major endocrine components involved in obesity are the intestine, the pancreas and the sex hormone secreting gonads; these will be covered briefly below, while the white adipose tissue (WAT) will be extensively discussed.

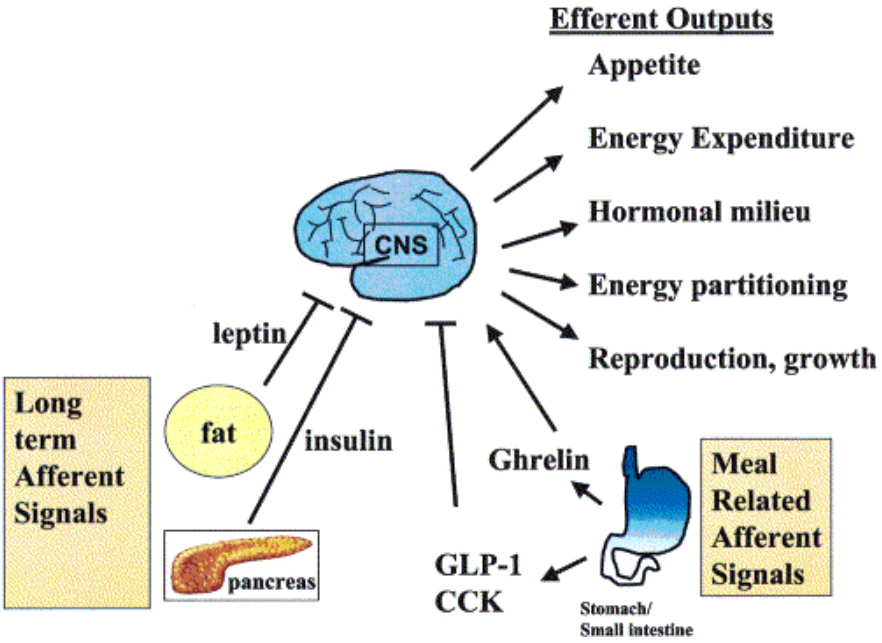

FIGURE 3. *The energy balance system involves long-term afferent signals from fat (leptin) and pancreatic β cells and short-term, meal-related afferent signals from the gut, including inhibitors of feeding (GLP-1, and CCK), and the stimulator of feeding (ghrelin). These inputs are integrated within the brain. Efferent outputs regulate appetite, energy expenditure, hormonal milieu, energy partitioning, and the status of reproduction and growth. Figure from reference 64.*

WAT is an important component of the body's system of energy balance and therefore plays a central role in obesity and type 2 diabetes (60). Until the 1980s adipose tissue was viewed to function as a depot for fat in the body where hormone stimulation would change lipid balance in the tissue. Discoveries made since then have revolutionized this concept and adipose tissue is now thought to also function as an important part of the endocrine system. Adipose tissue is dispersed throughout the body but accumulates in loose connective tissues positioned subcutaneously between muscles and dermis. Fat also surrounds every internal organ (70). Nearly all initial work on adipocytes was conducted in mouse preadipocyte fibroblast cell lines such as 3T3-L1 and 3T3-F442A, both of which can be induced to differentiate into adipocytes (71). In general this *in vitro* model system is thought to faithfully recapitulate *in vivo* adipocyte differentiation. However, one striking difference is that molecules secreted by the adipocyte, leptin and tumor necrosis factor α (TNF-α), are poorly expressed in these cell lines compared to adipocytes *in vivo*. More recently, studies have been performed using pluripotent mesenchymal stem cells that can be induced to yield adipocytes in addition to cells of several other lineages. Other studies have also been carried out in mouse models

in which important adipose genes were overexpressed or knocked out. Adipsin, adiponectin, TNF-α, leptin, and plasminogen activator inhibitor-1 are all produced by adipocytes (72-79). Interestingly, these factors can be regulated by feeding, fasting and obesity (80-82). Among the adipocytokines, leptin is probably the most well characterized. Leptin is a 16-kDa protein and was initially viewed as an adipocyte-derived signal that functioned primarily to prevent obesity by controlling satiety. It is now understood that leptin also serves as an important signal generated by adipose tissue to inform the brain, by falling below threshold amounts, that the body is starving (83); this function is likely as important or perhaps even more so, than its antiobesity role (64). In obese patients with functional *ob* genes, leptin levels are increased in proportion to body fat (84), and this hyperleptinemia reflects a state of leptin resistance. The mechanism for resistance to the weight reducing effects of leptin in obesity has received considerable attention, but is still unclear ten years after initial identification of the leptin receptor (85). Resistance to leptin action is typical in obesity, but is a less severe condition than that seen in rare cases where the receptor is totally lacking. There is therefore increased interest in the precise signalling pathways downstream of the leptin receptor that mediates its actions on energy balance and endocrine function. Initial attention focused on the JAK/STAT intracellular signalling pathway and its regulation of gene expression. However, additional pathways downstream of JAK, including MAP kinase and PI3 kinase, that are also capable of being activated by leptin *in vitro* and *in vivo* are being considered (86).

Adiponectin is an adipocyte-secreted protein that circulates at a high concentration in plasma (79). Levels of adiponectin are reduced during obesity, and the suppression correlates with insulin resistance in obesity and related disorders (87). Replacement of deficient adiponectin has a variety of beneficial effects, including reducing glucose and lipid levels, increasing lipid oxidation rates, and maintaining vascular tone (88). Adiponectin appears to act in part by activating the enzyme AMP kinase (89). Levels are also induced by treatment with anti-diabetic thiazoladinediones (TZDs) (90). The cognate receptor for adiponectin has recently been identified (91), and its physiologic role is receiving great attention.

The first stage of differentiation from fibroblasts into fat cells involves growth arrest achieved by cell-cell contact inhibition. Addition of prodifferentiative hormones stimulate a few cycles of cell division and activate the transcription factors Peroxisome Proliferator Activating Factor γ (PPARγ) and CCAAT/Enhancer Binding Protein α (C/EBPα). These transcription factors bring the cells to permanent growth arrest and start the transcriptional regulation of a number of important genes essential for development to a fully differentiated adipocyte phenotype (71). Some of these genes are glycerophosphate dehydrogenase (GAPDH), fatty acid synthase (FAS), acetyl CoA carboxylase (ACC), malic enzyme, glucose transporter 4 (Glut 4), the insulin receptor (IR), and adipocyte-specific fatty acid binding protein 2 (aP2) (92).

PPARγ is a member of the nuclear receptor protein subfamily that form heterodimres with the retinoid X receptor (RXR). It regulates genes by binding to response elements as a heterodimer complex with RXRs (93). PPARγ exists in two isoforms that are created by alternative promoter usage and alternative splicing at the 5' end of the gene. PPARγ_2 contains 30 additional amino acids at the N terminus compared with PPARγ1 (94). While many tissues express low levels of PPARγ_1, PPARγ_2 is highly fat-selective and is expressed at very high levels in this tissue (95). Use of the high-affinity, selective PPARγ agonist TZD or mice null for PPARγ have been important for establishing PPARγ as the most important factor for adipogenesis (71).

C/EBP proteins are basic-leucine zipper transcription factors. They act as homo- or heterodimers, and their tissue distribution is not limited to adipose tissue (96). However, regulation of the expression of several C/EBP family members is seen during adipogenesis, and recent gain- and loss-of-function studies indicate that these proteins have a profound impact on fat cell development. In cultured preadipocytic cell lines that have been induced to differentiate, C/EBPβ and δ mRNA and protein levels rise early and transiently. C/EBPα, on the other hand, is induced later in the differentiation process, slightly preceding the induction of most of the end-product genes of fat cells (97). The emerging picture is therefore that C/EBPβ and δ induce the expression of PPARγ by direct binding to the promoter of PPARγ which then induces expression of C/EBPα (71). The whole picture has become more complicated recently as new factors are discovered to play important roles during adipogenesis. These include transcription factors such as

adipocyte determination and differentiation factor 1 / sterol regulatory element-binding protein 1 (ADD1/SREBP1) (98), PPARδ (99) , retinoic acid receptor-related orphan receptor-γ (RORγ) (100) , estrogen receptor-related receptors α (ERRα) (101), GATA-2 and GATA-3 (102).

The literature concerning extracellular and intracellular signals that influence adipogenesis is enormous. Only some major pathways that likely are of great importance will be discussed below. Insulin is a product of the pancreatic β cells, but induces major effects upon adipose tissue. Insulin is required for differentiation into adipocytes *in vitro* and also increases the accumulation of lipids inside the cells (103). Since adipocytes express few insulin receptors, cross- activation of the Insulin like Growth Factor -1 (IGF-1) receptor is the most likely target for the hormone (71). IGF-1 and insulin induce expression of Ras and protein kinase B (PKB or Akt) which both strongly induce further differentiation via downstream signalling (71). Glucocorticoids also stimulate adipogenesis by activating the glucocorticoid receptor (GR) which has been shown to induce expression of C/EBPδ (104). Many cytokines and growth factors have been shown to inhibit fat cell development. The most studied candidates are TNF-α, interleukin 1 (IL-1), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF). Their downstream effector are most likely the mitogen activated protein (MAP) kinases, such as extracellular-signal regulated kinase (ERK1 and 2) and c-Jun N-terminal Kinase (JNK); it is interesting to note that JNK phosphorylates and inactivates PPARγ2 and RXR (105,106).

The gonads that secrete the sex steroids (testosterone and estrogens), are known to influence body composition, although the molecular mechanisms and their involvement in energy expenditure is not well understood (50). At puberty, the production of testosterone in males is associated with a reduction in the percentage of body fat (107). Testosterone increases lean mass relative to fat, and estrogen has the opposite effect. Testosterone levels fall when men grow older, and there is a corresponding increase in visceral and total body fat and a decrease in lean body mass. This may be compounded by the decline in growth hormone that is also associated with an increase in fat relative to lean mass. Both androgens and estrogens are involved in obesity and fat distribution. In females, the increase in estrogens is involved in the remodelling that produces the female

shape. When the ovaries are removed surgically in animals, obesity frequently develops and can be reversed by giving injections of estradiol. The importance of this mechanism has been highlighted by the study of animals with transgenic defects in their hormone receptors. Animals without estrogen receptor α are obese (50). Studies have also shown that testosterone inhibits lipid uptake and lipoprotein-lipase (LPL) activity in adipocytes. Moreover, testosterone stimulates catecholamine-induced lipolysis by increasing the number of beta-adrenergic receptors (108,109). An indirect sign of these effects is the decrease of adipocyte leptin production. Leptin, as described above, stimulates the brain to trigger a "stop eating" reaction, but can also act to prevent fat accumulation in non-adipose tissue by increasing mitochondrial oxidation of fatty acids (110). The fact that high leptin levels prevalent in hypogonadal men are reduced by testerosterone replacement therapy, offers further indication of lipolytic effects of testosterone (111). Testosterone also inhibits the differentiation of adipocyte precursor cells. In a study of the effect of sex hormones effect on rat adipogenesis, androgens (testosterone, DHT, androstanediol, and androstenediol) were shown to have no effect on the preadipocyte growth in male rats (112). Moreover, testosterone and DHT did not change PPARγ_2 expression in male rats, suggesting that these androgens are negative effectors of preadipoctye maturation.

The intestine secretes factors that stimulate the energy homeostasis in various ways. One of the gastrointestinal peptides that have been studied as potential regulators of satiety is cholecystokinin (CCK). This was one of the first peptides shown to reduce food intake in both rodents and humans (113). CCK is released from the small intestine into the circulation in response to luminal nutrients such as fatty acids, and influences satiety by acting on CCK receptors located on peripheral vagal afferent terminals, which transduce neural signals to the brainstem. Ghrelin is another peptide that was recently discovered to be produced in the stomach. It is believed to be the natural ligand for the growth hormone secretogogue receptor (114). This peptide stimulates food intake and with repeated administration will produce obesity. Its concentrations are lower in the serum of obese versus lean subjects, but in both cases there is a decrease with food intake.

Several pancreatic peptides modulate feeding. Both glucagon and its 6–29 amino acid analogue, glucagon-like peptide-1 (GLP-1), reduce food intake in animals and

humans (115). GLP-1 also enhances the release of insulin by the pancreatic beta cell in the presence of glucose. Analogs or small molecules that might influence GLP-1 receptor release or duration of action would be interesting for treating both obesity and diabetes. In addition to the direct impact on adipose differentiation, insulin also affects food intake and is the master metabolic switch between the fed and fasted states with regard to metabolic fuel deposition and use. The levels of insulin are well known to fall with fasting, and rise with obesity, similar to leptin (64). This led to the proposal many years before the cloning of the *ob* gene, that insulin might be the dominant signal of fuel status to the brain (116). When brain insulin levels increase, food intake is reduced. When the insulin receptor in the hypothalamic part of the brain is disabled by antisense oligonucleotides, animals eat ravenously (117).

Calorie restriction

The opposite of obesity is food scarcity. Although not intuitively thought of as healthy, pioneering intensive work during the 1930s in the model organism *Saccharomyces cerevisiae*, and later *Caenorhabitis elegans* and *Drosophila melanogaster*, have led to the theory that calorie restriction (CR) in fact extends life span (118). Early in the birth of this field a link between CR and decreased generation of ROS was suggested. Oxygen radicals are highly reactive chemical species that carry an unpaired electron, which can damage surrounding macromolecules, lipids, proteins and DNA. In eukaryotes, mitochondria are the prime site of ROS generation, because complex I and complex III of the electron transport chain occasionally generate these toxic by-products during normal respiration. ROS is strongly implicated in several diseases, such as prostate cancer (as discussed above). A molecular model for how CR is related to aging is still in early phases but there is strong evidence that it requires proteins coded by the genes in the *sirtuin* family (119). These are NAD dependent histone-deacetylases with sequence similarity to the yeast *Sir2* gene. Early studies suggested that adiposity was not an important factor in determining the lifespan of rodents (120), but more recent findings show that mice that have been genetically engineered to be lean live longer (121). In a breathtaking study by Picard et al., the mammalian homolog of Sir2,

SIRT1, was shown to inactivate PPARγ in WAT by docking to the negative cofactors of the nuclear receptor and thereby downregulating genes for adipogenesis, such as *aP2* (122). When taken together with the finding that calorie restricted fat cells secrete less TNF-α and more adiponectin, concomitant with increased insulin sensitivity and decreased glucose uptake, these data suggest a proposed pathway for CR where SIRT1 is activated in WAT by food scarcity with the consequence that the fat stores are reduced. This changes the hormone levels to a more physiologically favourable state (119).

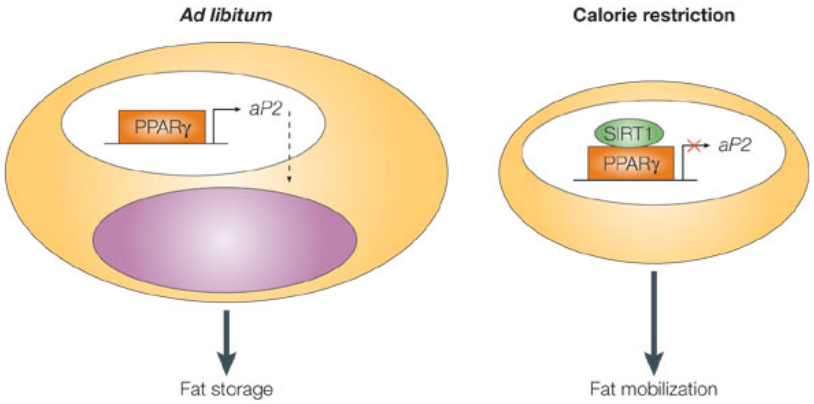

FIGURE 4. *Calorie restriction, SIRT1 and WAT. Under ad libitum (or freely fed) conditions, the fat-specific nuclear hormone receptor PPARγ transcribes the aP2 gene, which encodes a protein that binds to triglycerides and thereby assists fat storage (pink droplet). Under calorie-restriction conditions, SIRT1 binds to PPARγ at the aP2 promoter in white adipose tissue, thereby repressing PPARγ activity, lowering the expression of PPARγ target genes such as aP2, and reducing fat storage. Figure obtained from reference 119.*

Although SIRT1 seems to have an important function during life span regulation, it does not explain the connection between ROS and CR. The uncoupling proteins on the other hand are good candidates as they were shown to be positively correlated to lifespan (123). UCPs are believed to function in the mitochondria where their function is to partially depolarize the mitochondria membrane by lowering the proton gradient, and potentially reduce the rate of generation of oxidative damage (124). In small animals like rodents, CR may up-regulate UCPs assuming it is advantageous for the animal to use a greater portion of the energy to produce heat.

Obesity and PCa

Epidemiological evidence of a relationship between obesity and PCa is lacking. A study of 135, 000 Swedish construction workers found a positive correlation but a stronger relationship to mortality than incidence (125). A recent study from 47, 781 American men showed that a body mass index of more than 30 kg/m^2 (ie, the obese state) in men younger than 60 yrs and with a familiy history of PCa was associated with a lower risk of PCa compared to that of BMI 23-24.9 kg/m^2. For more sporadic cancers, BMI had a non-statistically significant positive correlation to PCa (126). However, a growing body of evidence from population-based studies supports the hypothesis that obesity may influence the development and progression of PCa. An attempt to estimate the rate obesity increases the risk for PCa suggests a 6% increase in risk of PCa for an overweight man compared with a normal weight and a 12% increase in risk for an obese man. This indicate that 4% of European men with PCa is attributable to this cause, corresponding to 5000 new cases a year (127). A different estimate for the US showed that men with a BMI of greater than 30 kg/m^2 have a 20% to 34% greater risk of PCa death than men with a BMI of 18 to 25 kg/m^2 (128). It is thought that obesity may not directly cause the onset of PCa, but instead worsen the prognosis and lethality of the disease (129).

A molecular link connecting PCa development and adipogenesis has not yet been extensively studied. One such candidate is leptin. Several reports have shown elevated leptin levels in men with PCa (130-132). Saglam et al. measured leptin and PSA levels of patients diagnosed with PCa compared to a control group and found significantly higher leptin and PSA levels in the PCa group. (130). However, others have not found any association between leptin and PCa (133). *In vitro,* leptin has been demonstrated by several groups to cause proliferation of androgen-independent PCa cells (129,134-138). The presence of the leptin receptor mRNA splice variants, human OB-Ra and human OB-Rb, in PCa cell lines and their expression levels in response to various leptin treatments have been documented (138). A possible mechanism of how leptin can directly stimulate PCa growth is by inducing high expression of vascular endothelial growth factor (VEGF), which is a potent mitogen and can stimulate migration, angiogenesis, and microvascular permeability (129). VEGF has been shown to correlate with tumor stage, grade, and clinical outcome in PCa and is also expressed in androgen independent PCa

cells (139,140). Further work is needed to conclusively assess the possible role of leptin in PCa.

Finally, expression of lipogenic enzymes like FAS is as previously described highly upregulated in PCa (21). Fatty acids have obviously a important function for cancer growth and maybe the high level of lipogenesis in cancer cells reflects the high rate of membrane synthesis in rapidly dividing cells as proposed by Johannes Swinnen et al. (21). Is lipogenic overexpression in prostate associated with higher accumulation of fat in adipose tissue triggered by obesity? So far, it is too early to conclude and no association between FAS expression in adipocytes and prostate tissue has so far been reported.

Aim of the study

As documented in the introduction presented above, prostate cancer and obesity are two major health problems facing the Western world. It was recently proposed that there is a molecular connection between increasing amounts of adipose tissue and progressive growth of the prostate. A description of some candidates have been introduced but most likely there are several other factors restricted to prostate or adipose tissue that influences the biochemical and genetic milieu in these tissues that may give rise to these pathologies. Towards this end, the goal of this master project was to identify genes that are regulated by androgens in the prostate cancer cells and during adipocyte differentiation.

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Manuscript

Identification and characterization of genes that are common in prostate cancer and adipocyte cell lines

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Summary

Prostate cancer (PCa) and obesity are currently two of the most prevalent health problems in the western world. Some epidemiological studies suggest a connection between PCa and fat intake and obesity. In addition, androgens, which are required for prostate growth and maintenance, as well as being necessary for the beginning stages of PCa, induce lipogenic phenotype and gene expression profile in PCa cells. Furthermore, androgens play a regulatory role in fat cells. We therefore hypothesized that there are common gene expression patterns between prostate and adipocytes. To assess this possibility, we used cDNA microarrays made from a prostate enriched library to identify genes that are commonly regulated by androgens in the PCa cell line LNCaP and during differentiation of 3T3-L1 cells into adipocytes. This screen identified Humanin (HN) and Bax Inhibitor 1 (BI-1) as two such genes, and their expression profiles were verified by quantitative RT-PCR. HN was found to be upregulated during adipogenesis and dose-dependently regulated by androgen in LNCaP cells. BI-1 was only slightly regulated by androgen in LNCaP cells and not regulated during adipogenesis in 3T3-L1 cells. A HN specific antibody was used to detect the predicted 3kD peptide in prostate and adipocyte cell extracts. In both tissues, we identified a peptide of 10 kD which is likely due to post-translational modification of the HN peptide in these tissues. Further studies are needed to assess the functional role of HN in these tissues.

Introduction

PCa is the most common type of cancer among men in Norway with 2841 new reported incidence in 2001 (1). In 2005, PCa is predicted to be the cause of death for 30 350 men in the United States and therefore the second leading cause of cancer death (2). The incidence rate of PCa varies significantly among various race groups. These could be explained by differences in environmental exposure, detection rate and methods, genetics and in particular dietary factors (3). High fat consumption has been reported to be one of the most important dietary factors which influences prostate cancer onset (4). Studies on human PCa mouse models have demonstrated that high fat diet gives rise to a substantially more aggressive PCa type (5). Epidemiological studies have been more inconsistent, but several studies report a positive correlation between consumption of dietary fat and increased risk of PCa (3). Accordingly, it has been hypothesised that obese men have a higher risk for developing PCa. Recently, three large studies found this to be the case but another report has found an inverse association for the age group <60 yrs or those with family history of PCa (6). Taken together, a growing body of evidence suggests that obesity may impact upon risk, detection and outcome with regard to prostate cancer. Obesity is a global epidemic affecting an staggering number of people each year and is an ever-spreading disease predicted to cause a decline in life expectancy during the 21st century (7,8). It is therefore important to enhance our molecular understanding of the link between PCa and obesity. Toward this end, we sought to identify genes that are expressed and regulated in prostate cancer progression and adipocyte differentiation. Using a prostate-specific microarray, we identified two candidate genes, Humanin (HN) and Bax Inhibitor 1 (BI-1) which fit this description and may therefore elucidate a link between prostate cancer and obesity.

HN is a 3 kD short anti-apoptotic peptide initially discovered from a cDNA library prepared from the occipital lobe of an autopsy-confirmed Alzheimer Disease (AD) brain (10,11). Death trap screening, which is a functional screening strategy to identify molecules that enable dying cells to survive (12), was used to identify HN as a gene that suppressed neuronal cell death. In this physiological context, HN is secreted from neuronal cells and stimulates extracellular cell receptors to induce intracellular signalling pathways (11). The receptor for HN is reported to be the G-protein-coupled

formylpeptide receptor-like-1 (FPRL1) (13). In the context of Alzheimer's disease, HN competes for binding to the 42-aa beta amyloid peptide, Aβ(42), receptor which induces plaque and neuronal death. HN can also act intracellularly by directly binding and inactivation of the pro-apoptotic Bcl-2 family members Bcl-2 associated X protein (Bax), Bid, or the extra long isoform of Bim (BimEL), thereby rescuing cells from programmed cell death (14-16).

The coding origin for transcription and translation of HN is not clear because DNA sequences homologous to HN have been found both in the nuclear genome and in the small genome of mitochondria (17). Two different theories for HN expression have although been postulated. Based on the homology between HN cDNA and mitochondria DNA (i.e., 99% similarity to the coding region of 16S rRNA in the mitochondrial genome) it is possible that the HN peptide is an artificial protein encoded by a non-functional ORF in 16S rRNA. If this is the case, the HN-open reading frame (ORF)-corresponds to the 16S rRNA containing a poly-A tail. An early study support this possibility by demonstrating that all mitochondrial rRNA transiently attach a polyA tail during transcription (18). The short HN-ORF of 75 bases, which encodes the 24 amino acid peptide, is located 950 bases downstream of the 5′ end of the cDNA. Since there are at least seven putative in-frame ORFs in this 5' region each with a termination codon (17), it is also possible that alternatively transcribed forms of HN exist, although this has not been thoroughly investigated. The HN sequence is 92–95% homologous to regions in the human chromosomes 5, 11, and X (11). Therefore, the source of HN cDNA may be nuclear and the HN peptide natively produced from HN mRNA (17). Indeed, some of the HN-ORFs are 99% identical to certain registered human mRNAs. In this case, since most cells contain poly-A 16S rRNA, the HN-ORF contains a mixture of HN mRNA and poly-A 16S rRNA. So far it is evident that transcriptional activation of the long HN cDNA leads to the production of HN peptide in mammalian cells, despite its unusual structure for peptide production. Immunoblots of various mouse tissues show that the HN peptide *in vivo* can be detected in testis and colon of three week old mice but only in the testis of 12 week old mice (17). Protein expression is also detected in the occipital lobe of AD patients. Despite this, it is still unclear if HN is expressed in physiologically functional levels *in vivo* in humans (19).

BI-1 was initially cloned from an adult rat testis and also referred to as testicular enhanced gene transcript (TEGT) (20,21). It was renamed when a functional screening for human proteins that suppresses cell death in yeast induced by ectopic expression of mammalian Bax, identified BI-1 as a anti-apotototic protein (22). BI-1 is well conserved in animals and plant species, and BI-1 like protein sequences have been found in unicellular eukaryotes supporting the hypothesis that BI-1 was as an ancient suppressor of programmed cell death (23). BI-1 is overexpressed in breast and prostate malignant tumours and down regulation of BI-1 by small interfering RNAs (siRNA) promotes apoptosis in LNCaP, DU-145 and PC-3 cells (24). Gene ablation studies of BI-1 show that BI-1 suppresses Bax activation through an apoptotic pathway linked to endoplasmic reticulum (ER) stress (25). It has also been hypothesized that BI-1 block the transmission of death signals or signals from damaged ER/Golgi to the mitochondria.

In this study we show the expression profile of HN and BI-1 in the androgen dependent PCa cell line LNCaP and the murine adipocyte cell line 3T3-L1. We also provide evidence for *in vivo* expression of the HN peptide in LNCaP and 3T3-L1 cell lines. The functional role for HN *in vivo* in these tissues is discussed.

Materials and Methods

Cell cultures and treatment

The human prostate cell line LNCaP was obtained from the American Type Culture Collection (Rockville, MD). All cells were routinely maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 5 mg/ml penicillin/streptomycin, and 200 mM L-glutamine. The cells were incubated at 37°C in a humidified 5% CO_2, 95% air incubator. The passage number of LNCaP cells was 20–25. For androgen induction cells were serum starved for 48 h in RPMI containing 2% charcoal-treated (CT)-FCS to remove steroids, followed by 24 h in RPMI containing 0.5% CT-FCS. The synthetic androgen R1881 (10^{-8} M) (Dupont-NEN) was then added and cells were collected at indicated time points. Control cells were stimulated with vehicle (ethanol) only. Tunicamycin treatment was conducted in non-starved LNCaP cells. Three different concentrations of chemical (purchased from Sigma) or vehicle

(ethanol) was added to 80% confluent cells and incubated for 24h or 48h. 3T3-L1 cells, also obtained from ATCC, were grown in monolayer culture in DMEM supplemented with 10% FCS. Confluent 3T3-L1 fibroblasts were differentiated to adipocytes in DMEM containing 10% FCS, 0.5mM isobutylmethylxanthine (IBMX), 1 μM dexamethasone, and 5μg/ml insulin for two days (all purchased from Sigma Aldrich). After 48h, cells were reefed with DMEM supplemented with 10% FCS and 100ng/ml insulin for an additional 12 days when typically 60-80% of the cells exhibited adipocyte morphology. For all cell types, RNA pellets were isolated with TRIzol (Invitrogen) at indicated time points.

Oil Red O staining

Adipogenesis was measured at indicated time points using the triglyceride-specific dye, Oil red O (Sigma-Aldrich). Cells were fixed in 0.5% gluteraldehyde/PBS and stained with 0.15% Oil red O until aggregates start to precipitate (approximately 15 minutes), and then rinsed with distilled water prior to photography using a phase contrast microscope (Olympus).

cDNA Microarray analysis

Microarray slides were made in-house (The Norwegian Radium Hospital – University of Oslo microarray consortium) from a prostate specific library (26). Total RNA was isolated from cells with TRIzol (Invitrogen). RNA concentrations were determined spectrophotometrically and RNA integrity was confirmed by 1% agarose gel electrophoresis. RNA was collected from untreated LNCaP (control, 0h), cells stimulated with R1881 for 6h and 24h. RNA was collected from 3T3-L1 cells at 2 days pre induction (control) or 4 and 2 days post induction. For each experiment, cDNA was synthesized from 15μg total RNA from the reference sample and labelled with Cy3 (GE health) using SuperScriptII (Invtrogen). cDNA was synthesized from 15μg total RNA from the test samples and labelled with Cy5 (GE health) using SuperScriptII (Invitrogen). Excess dye was removed with BIO Spin 6 columns (Bio-Rad, Hercules, CA). These two labelled populations of cDNA were then mixed and hybridized manually to the pre-hybridized microarray slides overnight at 65°C. After washing the slides two times with

0.2 μm-filtered 0.5x SSC, 0.01x SDS, 10^{-3}M DTT solutions and two times with 2μm-filtered 0.06 X SSC, 10^{-3}M DTT solutions, the slides were scanned and visualized with a GenePix 3000 scanner (Axon Instruments). Finally, the results were computationally analyzed with Bio Array Software Environment (BASE), and all genes commonly expressed in both 3T3-L1 and LNCaP were selected and clustered.

cDNA synthesis and quantitative real-time RT-PCR

1 to 5 μg of total RNA was DNase-treated with RNase-free DNase I (Promega) before total RNA was precipitated with ammonium acetate and ethanol to inactivate the DNase, and processed for cDNA synthesis by SuperScript II (Invtirogen). Primers used in real-time PCR was designed by the primer design program, Primer3 (27). Information of primers is enclosed in table 1. Real time PCRs were analyzed by Light Cycler instrumentation (Roche) in capillaries with 10μl reaction volumes containing 0.25x LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics), 2-4 mM $MgCl_2$, 0.5 μM of each primer, and 1 μl of cDNA template. $MgCl_2$ concentrations and temperature conditions were optimized for each primer pair. Cycling conditions included an initial denaturation step at 95 °C for 10 min, followed by 45 cycles of 95°C denaturation for 10 sec, 62-66°C annealing for 5 sec (see table 1), and 72°C extension for 20 sec. A negative control reaction in the absence of template was also performed for each primer pair. Melting curve analysis was performed for each reaction to confirm exclusion of non-specific PCR by-products. Dilution standard curves were used as external standards. For each sample, the ratio between the relative amounts of target gene and housekeeping gene was calculated to compensate for variations in the quantity or quality of the starting mRNA as well as for differences in reverse transcriptase efficiency. The change in fluorescence of SYBR Green dye in every cycle was monitored, and the crossing point (CP) values, defined as the points at which the fluorescence increased appreciably above background was used to determine the relative expression of the gene of interest. All gene quantities were normalized to a housekeeping gene. In 3T3-L1 cells 36B4 was used as housekeeping gene and ATP-6 was used in LNCaP cells. All data analysis was performed with Excel (Microsoft).

Western blotting

Protein tissue samples were prepared by homogenization of mouse testes or subcutaneous adipose tissue (kindly provided by Arne Klungland, The National Hospital, University of Oslo, Norway) with lysis buffer [0.5% Triton X, 50mM Tris/HCl (pH7.5), 1mM EDTA]. After centrifugation, the supernatant was mixed with reducing SDS sample buffer [187.5mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 0.03% phenol red, 1.25M DTT]. Whole cell extracts from LNCaP and 3T3-L1 cells were prepared by resuspending the cells in 50-100 µl of lysis buffer [10mM Tris-HCl(pH 7.5), 1mM EDTA, 1% Triton X-100 and 0.5 mM PMSF]. The suspension underwent two freeze-thaw cycles, followed by centrifugation at 13000rpm for 10 min at 4° C. The supernatant was collected and stored at –80°C. All protein concentrations were determined by the Bio-Rad protein assay. Reducing SDS sample buffer [187.5mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 0.03% phenol red, 1.25M DTT] was mixed with protein extracts at room temperature over-night before the samples were electrophoresed on a 16% separating, 10% spacer and 4% stacking SDS-PAGE gel. A Precision Plus Protein dual protein standard (Bio-Rad) was used to decide the molecular weight of the bands. The proteins were transferred to a PVDF membrane (Bio-Rad) soaked in 10mM cyclohexylaminopropanesulfonic acid (CAPS) (Sigma) and 10% methanol transfer buffer. The blotted membrane was blocked with 10% milk for 1 h, followed by incubation with the primary antibody at 4°C overnight. The antibodies used are anti-Humanin rabbit polyclonal antibody (1:2500 dilution) (a generous gift from Ikuo Nishimoto, Department of Pharmacology and Neurosciences, Keio University School of Medicine, Tokyo, Japan), anti-PSA goat polyclonal antibody (1:200) (Santa Cruz Biotech), anti-Tubulin (1:1000) monoclonal mouse antibody (Sigma Aldrich), Horseradish peroxidase (HRP) conjugated to anti-mouse IgG (Sigma Aldrich), HRP conjugated to anti-rabbit IgG (Sigma Aldrich), and HRP conjugated to anti-goat IgG (Santa Cruz Biotech.) PSA goat monoclonal antibody (1:200 dilution), Tubulin mouse polyclonal antibody (1:2000), aP2 polyclonal antibody (1:2000) (a generous gift from Gökhan Hotamisligil, Department of Genetics and Complex Diseases, Harvard School of Public Health, Boston, Massachusetts, USA). The enhanced chemiluminescence kit

(Amersham Pharmacia) was used for detection according to the manufacturer's recommendations.

Competition assay

A synthetic HN peptide (MAPRGFSCLLLLTSEIBLPVKRRA) (Sigma Aldrich) and KLK4 NH_2-terminal peptide (QIINGEDCSPHSQPW) (Medprobe) was dissolved in 1 x TBS/0.1% Tween. 150µg peptide was pre-incubated with 1:2500 HN-Ab for 1 hour rotating at 4°C before incubated with western blot membranes.

Name	Annealing temp (°C)	[$MgCl_2$] (mM)	Sequence
HN (human)	66	4	Forw: 5'- aaacctaccgagcctggtga – 3' Rev: 5'- ggcttatgcggaggagaatg – 3'
HN (mouse)	64	3	Forw: 5'- taccctaaccgtgcaaaggt – 3' Rev: 5'- aagctccatagggtcttctc -3'
BI-1 (human)	64	3	Forw: 5'- gcatccttcccactgctttc – 3' Rev: 5'- ccatgttcggccttttcaat -3'
BI-1 (mouse)	64	3	Forw: 5'- cccacataactccctcgaca -3' Rev: 5'- aaggttccccagagaggaca -3'
36B4	64	3	Forw: 5'- aagcgcgtcctggcattgtct -3' Rev: 5'- ccgcaggggcagcagtggt -3'
ATP-6	62	3	Forw: 5'- cagtgattataggctttcgctctaa -3' Rev: 5'- cagggctattggttgaatgagta -3'
aP2	64	3	Forw: 5'- ggtcaccatccggtcagagag -3' Rev: 5'- tcgactttccatcccacttc -3'
PSA	66	4	Forw: 5'- ccctgagcacccctatcaac -3' Rev: 5'- tgagtgtctggtgcgttgtg -3'

TABLE 1. Information of primer used in experiments.

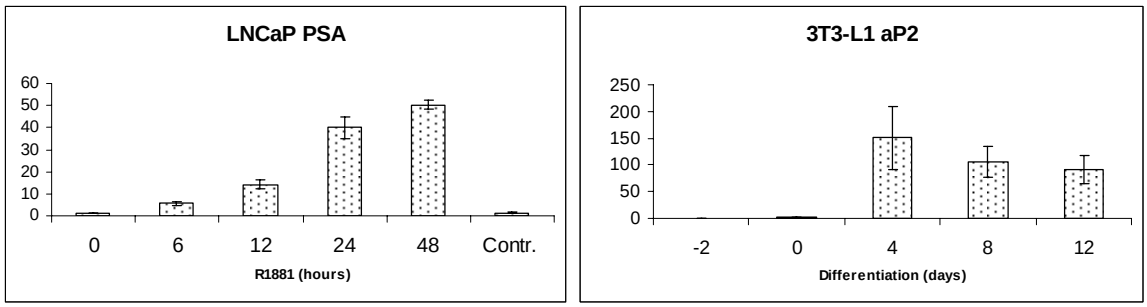
Results

Identification of Humanin and Bax Inhibitor 1-expression in LNCaP and 3T3-L1 cells by microarray analysis

To identify new candidate genes that are expressed and regulated during adipocyte differentiation and PCa development we took advantage of a prostate specific library (PSL) that previous laboratory members had constructed (26). Since adipose tissue was not among the tissues that were subtracted from the prostate DNA, we expected to identify adipose tissue genes among those identified through this screen. All the cDNA sequences from the PSL were spotted onto microarrays plates (The DNR-UiO Microarray Core Facility). To identify genes relevant to prostate cell progression and adipocyte differentiation, RNA was harvested from untreated or androgen-treated LNCaP cells, and undifferentiated and differentiated 3T3-L1 cells. Upregulation of PSA gene expression in response to androgen was used to verify androgen activation of LNCaP cells, as shown in Figure 1A. Gene expression analysis and lipid staining was used to confirm differentiation of pre-adipocytes into mature adipocytes. Upregulation of adipocyte-specific fatty acid binding protein (aP2) mRNA were determined by quantitative real–time RT-PCR. aP2 is induced early in adipogenesis by the transcription factor Peroxisome Proliferator Activated Receptor γ (PPAR γ) (30). As shown in Figure 1B, the levels of aP2 mRNA increased during adipocyte differentiation beginning at day 4 post induction. Oil red O staining of fixed 3T3-L1 cells upon differentiation show accumulation of lipid by red staining of triglycerides and cholesteryl oleate (31). Pictures of the cells during differentiation show that pre-adipocytes initially harbour very small amount of lipid droplets, but accumulates significant amounts only 4 days after induction (Figure 1C).

RNA extracts from androgen stimulated LNCaP cells and differentiated 3T3-L1 cells were then used in microarray experiments to determine differences in gene expression induced by R1881 or during adipogenesis, respectively. We measured expression ratios of 0h vs. 6h and 0h vs. 24h of R1881 treatment in LNCaP cells, and -2 days vs. +4 days and -2days vs. +12 days in differentiated 3T3-L1 cells. Analysis of the scanned hybridized microarray slides by the BASE software (32) showed that 713 cDNA

sequences were expressed and/or regulated in both tissues (Figure 2). Clones in the PSL library corresponding to these genes were PCR amplified using T7 and SP6 primers. After PCR purification, the samples were sequenced and the sequencing data were analysed with the BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST/). Some of the clones that we sequenced are listed in Table 2. The criterion in our microarray clustering analysis was that only genes expressed in LNCaP and 3T3-L1 cells were subjects of interest. Literature searches were conducted on certain genes identified by this screen to confirm that these genes have previously been shown to be highly enriched in the prostate and androgen regulated. The microarray analysis showed that PSA cDNA was highly upregulated in LNCaP cells, but not expressed in 3T3-L1, demonstrating the integrity of the samples used in this analysis and the ability of the bioinformatics analysis to selectively choose androgen- regulated genes. Curiously, we also identified many other genes known to be expressed ubiquitously and previously not known to be androgen regulated (e.g. MDM2 and RGS10). Based on the obtained data, we were particularly interested in further investigating Humanin (HN) as a candidate link between prostate cancer and obesity because it is positively regulated by androgen in LNCaP cells and during differentiation of 3T3-L1 cells. Bax Inhibitor 1 (BI-1) was also selected as a negative control because of it plays a similar role as HN in apoptosis, but was neither regulated by androgen in LNCaP cells or during adipogenesis.



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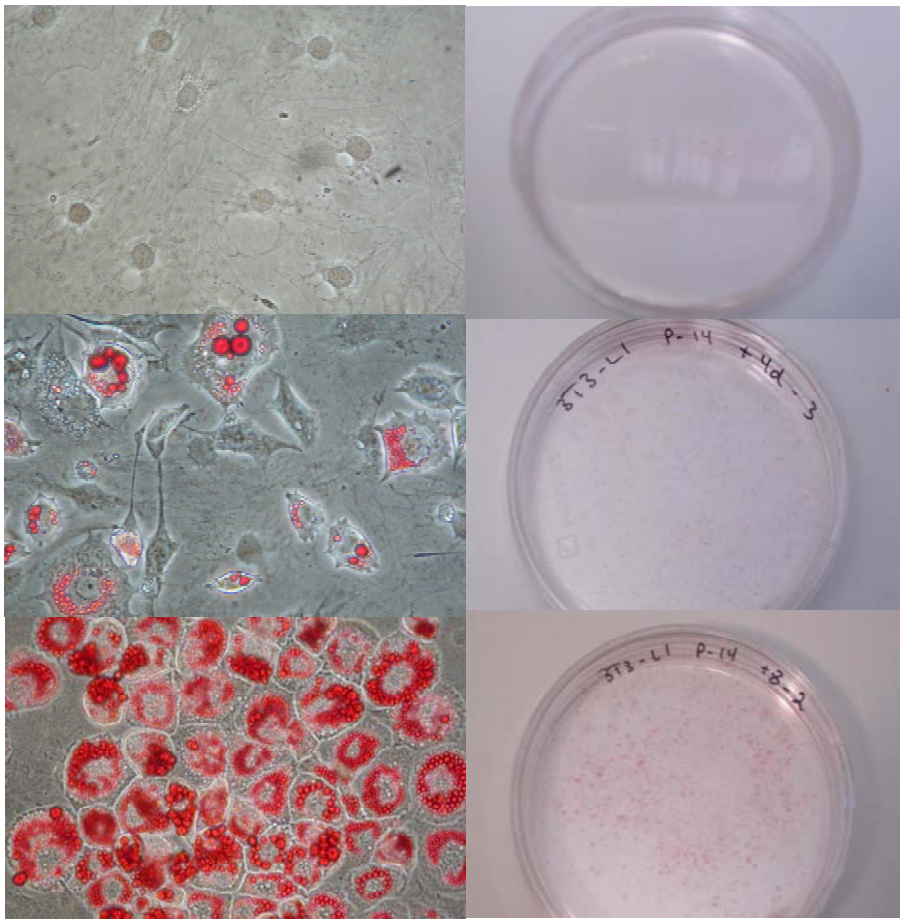

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FIGURE 1 *– Verification of activated AR in LNCaP and complete differentiation of 3T3-L1 cells.* ***A)*** *PSA is induced by androgens in LNCaP cells. Cells were treated with 10^{-8}M R1881 synthetic androgen for indicated time points. Isolated total RNA was converted to cDNA and PSA expression (relative to ATP-6 expression) was measured by quantitative real-time RT-PCR. The average of three independent experiments each in triplicate +/-SE is shown.* ***B)*** *aP2 is induced in 3T3-L1 four days after induction of adipogenesis. Isolated total RNA was converted to cDNA and aP2 expression (relative to 36B4 expression) was measured by quantitative real-time RT-PCR. The result presented is the average of three independent experiments +/- SE.* ***C)*** *Fatty acids accumulate during adipogenesis in 3T3-L1 cells. Cells differentiated for 0, 4 and 8 days were fixed and stained with OilRed O. The left pictures are photographs at 20x magnification. Red dots show staining of accumulating fat.*

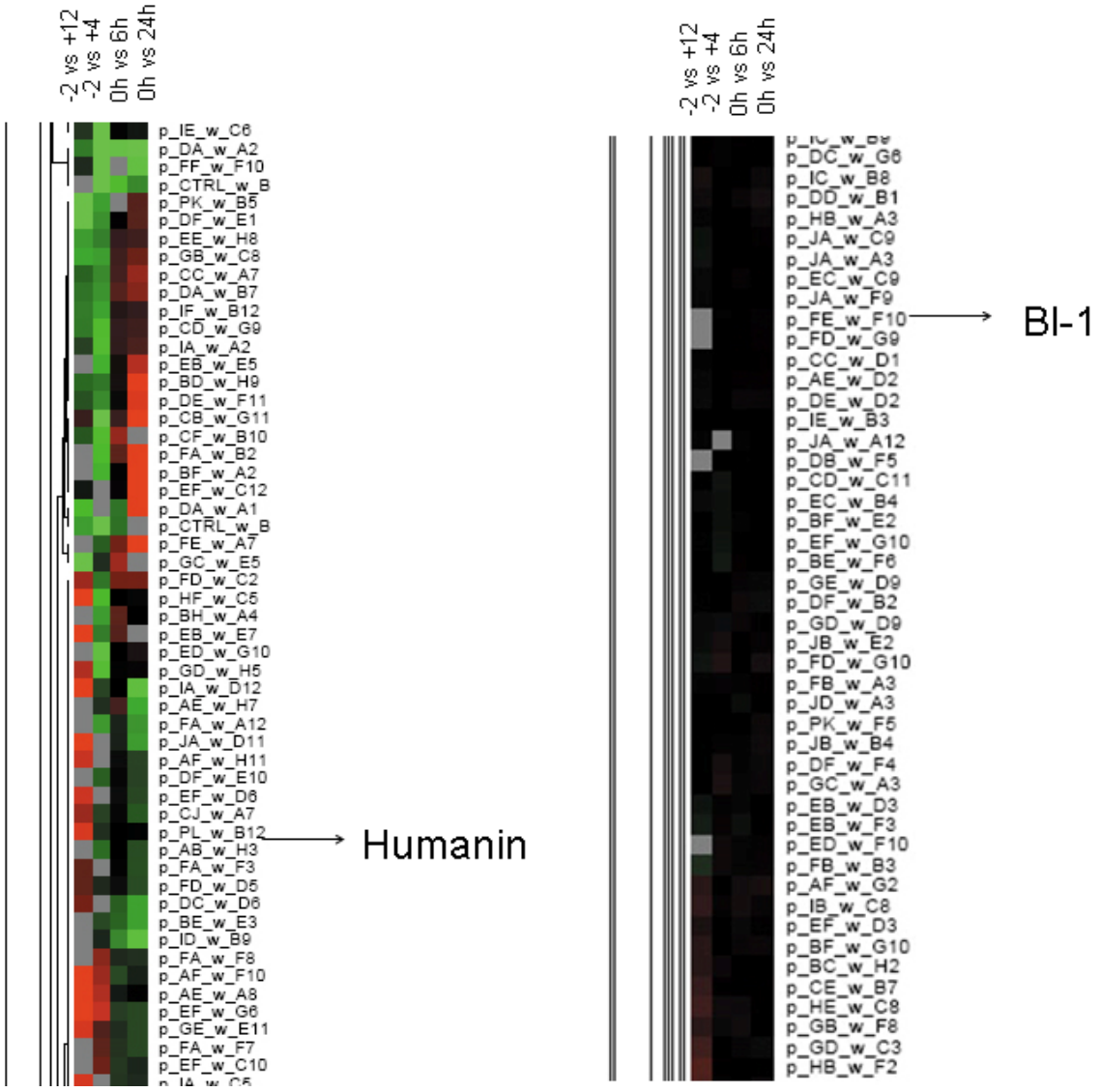


FIGURE 2 *– HN and BI-1 are expressed in LNCaP and 3T3-L1 cells.*
RNA was collected from untreated LNCaP cells (reference, 0h), or cells stimulated with R1881 for 6h and 24h. RNA was collected from 3T3-L1 cells at 2 days pre induction (reference) or 4 and 12 days post induction. cDNA was synthesized from 15µg total RNA from the reference and test sample and labelled with Cy3 and Cy5, respectively by SuperScriptII enzyme. The samples were manually hybridized on the slides at 65C overnight, washed, scanned by a GenePix3000 scanner and analysis was performed using the BASE software. The figure shows two pieces of the obtained clustering map identifying HN and BI-1. HN is highly upregulated in 12 days differentiated 3T3-L1 cells but not in LNCaP cells. BI-1 is not regulated.

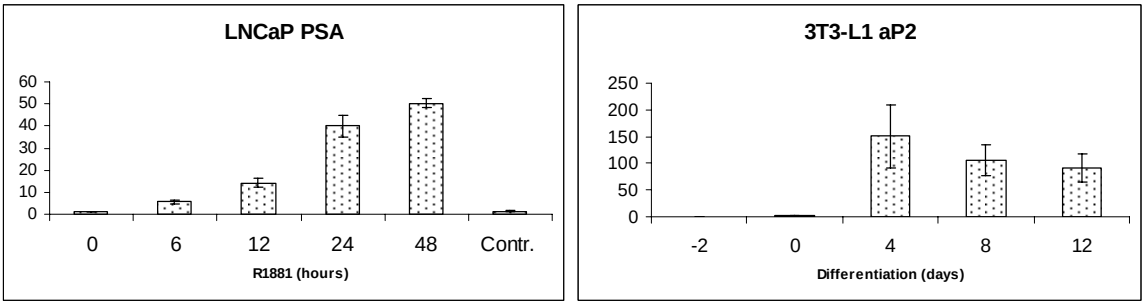
Name	Clone number	Accession number	3T3-L1	LNCaP
Humanin	PL B12	AY029066	Up regulated	Weakly up
BI-1	FE F10	NM_003217	Not regulated	Not regulated
MDM2	BC C10, CC E1, HC D7, JB B2	NM_006879	Weakly up	Weakly down
RGS 10	EB A11, EC A8, EE D3, IB D9, JB E2	NM_001005339	Weakly up	Down regulated
PSA	AE E10, BF A2, CE E11, CK A6	NM_001002231	Not detected	Upregulated

TABLE 2. *Some of the clones identified by the microarray experiments.*
MDM; murine double minute-2, RGS 10; regulators of G-protein signalling 10.

HN and BI-1 are expressed in LNCaP and differentiated 3T3-L1 cells

To verify the microarray data of HN and BI-1 and to more accurately quantify their regulation, we analyzed the relative expression of these by quantitative real-time PCR. Gene quantities were normalized to relevant housekeeping genes which are expressed at stable levels in each cell type. The mitochondrial gene ATP synthase 6 (ATP-6) has previously been used as a housekeeping gene in LNCaP cells (33) and our results confirm those studies (see Figure 3A, left panel). In 3T3-L1 cells the ribosomal protein 36B4 has been shown to be not regulated during adipogenesis (34) and our results confirmed this (Figure 3A, right panel). HN is expressed and upregulated 5-fold four days after induction of differentiation in 3T3-L1 cells (Figure 3B, right panel). In LNCaP cells no significant time-dependent regulation of androgen was seen (Figure 3B, left panel). However, HN expression was down regulated in a dose-dependent fashion in LNCaP cells treated with various concentrations of R1881 (10^{-12}M to 10^{-8}M) for 24h (Figure 3C, left panel). As a positive control, expression of PSA was assessed which

increased in a dose dependent fashion (Figure 3C, right panel). Expression levels of BI-1 mRNA were also determined in LNCaP cells treated with 10^{-8}M R1881 at various timepoints, or during differentiation of 3T3-L1 cells. In agreement with the microarray analysis we found that BI-1 is not regulated during adipogenesis (Figure 3D, left panel) and only slightly induced, approximately two-fold, by androgen in LNCaP cells (Figure 3D, right panel).



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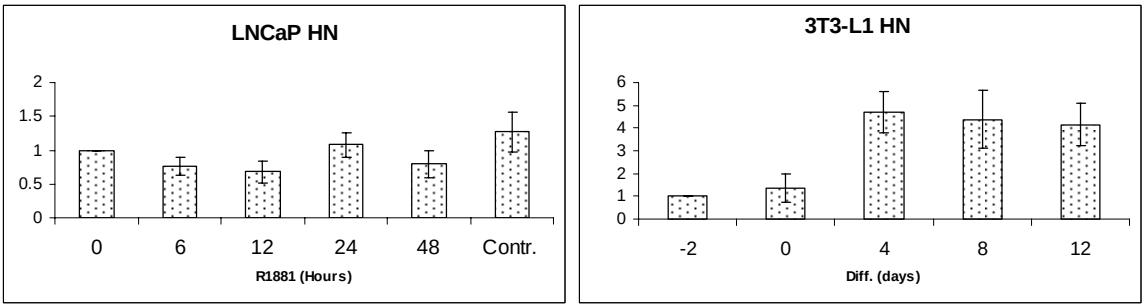

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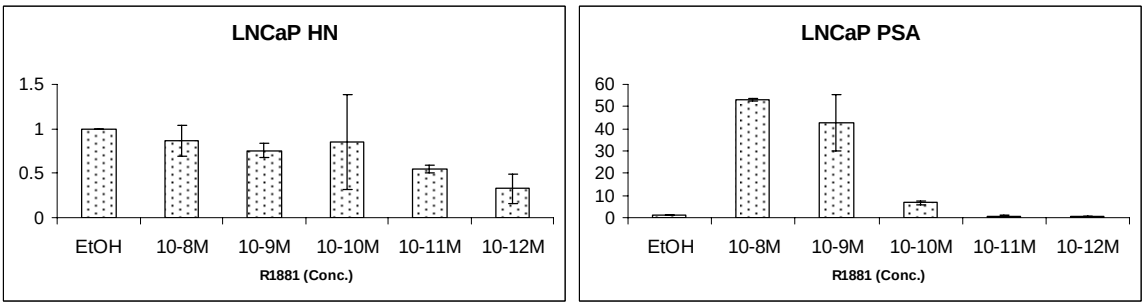

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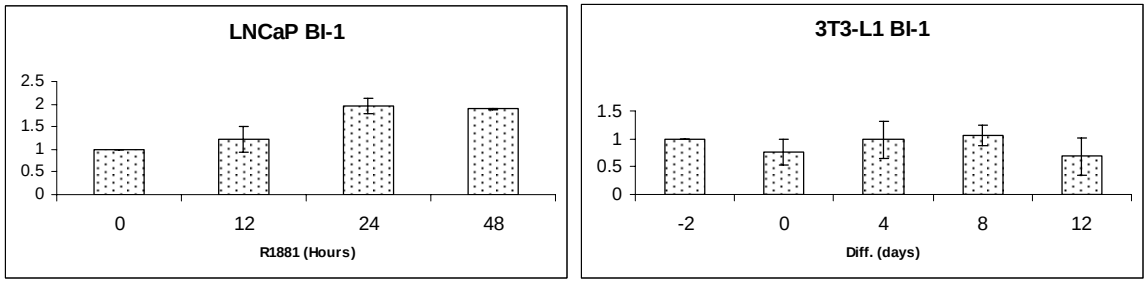

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FIGURE 3*.HN is regulated during adipogenesis in 3T3-L1 cells and dose-dependently in LNCaP cells. BI-1 is only slightly regulated during R1881 stimulated LNCaP cells and not during adipogenesis in 3T3-L1 cells.*
A) *ATP-6 (left) and 36B4 (right) are expressed in equal levels in androgen treated LNCaP and 3T3-L1 differentiated cells, respectively.* ***B)*** *HN is not time-dependently induced by androgens in LNCaP cells (left panel) but upregulated during adipogenesis in 3T3-L1 cells (right panel).* ***C)*** *Dose-dependent androgen treatment of LNCaP cells show a dose-dependent increase of PSA expression (right panel) and increase of HN expression from 10^{-12} to 10^{-8}M (left panel). Cells were treated with indicated concentrations of R1881 for24h. Isolated total RNA was converted to cDNA and HN expression (relative to ATP-6 expression) was measured by quantitative RT-PCR. The average of two independent experiments +/-SE is shown.* ***D)*** *BI-1 expression is induced in LNCaP R1881 treated cells (left panel). BI-1 is not regulated during 3T3-L1 adipogenesis (right panel) but regulated two fold in LNCaP cells after 24h (left panel). For* ***A****,* ***B*** *and* ***D*** *LNCaP cells were treated with 10^{-8}M R1881 androgen for indicated time points. Isolated total RNA was converted to cDNA and HN expression (relative to ATP-6 expression) was measured by quantitative real-time RT-PCR. The average of three independent experiments each in triplicate +/-SE is shown. 3T3-L1 cells were differentiated and isolated total RNA from indicated time-points during differentiation were converted to cDNA. Gene expression (relative to 36B4 expression) was measured by quantitative real-time RT-PCR. The result presented is the average of three independent experiments +/- SE.*

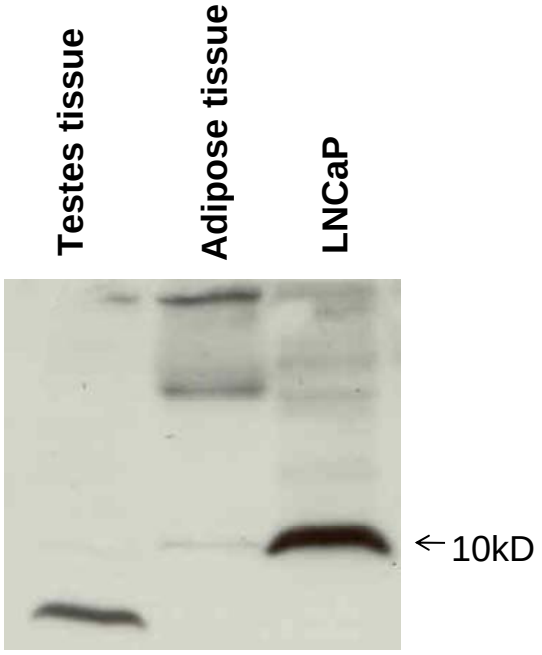
HN is detected as a 10kD peptide in LNCaP and 3T3-L1 cells

To investigate whether the HN mRNA was functionally translated to protein in PCa cells and adipocytes we examined whole cell extracts of LNCaP cells or subcutaneous adipose tissue by Western blot analysis using a HN antibody previously shown to detect this peptide in testes and colon (17). Protein extracts from testes of five-month old mice was prepared and used as positive control samples. As expected, HN was detected in the testes with a size of approximately 3kD (Figure 4A). A strong 10 kD band was detected in LNCaP extracts and a weaker band at the same size was observed in adipose tissue (Figure 4A). In testes we also see a band of 10kD (Figure 5 and 6).

To examine protein expression of HN in response to R1881, LNCaP cells were treated with R1881 for different time points. As expected, we see the same regulation of PSA at the protein level as we saw at the mRNA level (Figure 4B, upper panel). When

the same membrane was stripped and probed with HN antibody, we detected a strong band at approximately 10kD that was not regulated by androgen over time (Figure 4B, middle panel). Equal protein loading was confirmed by examination of tubulin protein expression (Figure 4B, lower panel).

A 10kD band was also detected in 3T3-L1 adipocytes differentiated for 4 and 12 days (Figure 4C). Proper differentiation of adipocytes was confirmed by aP2 protein expression.



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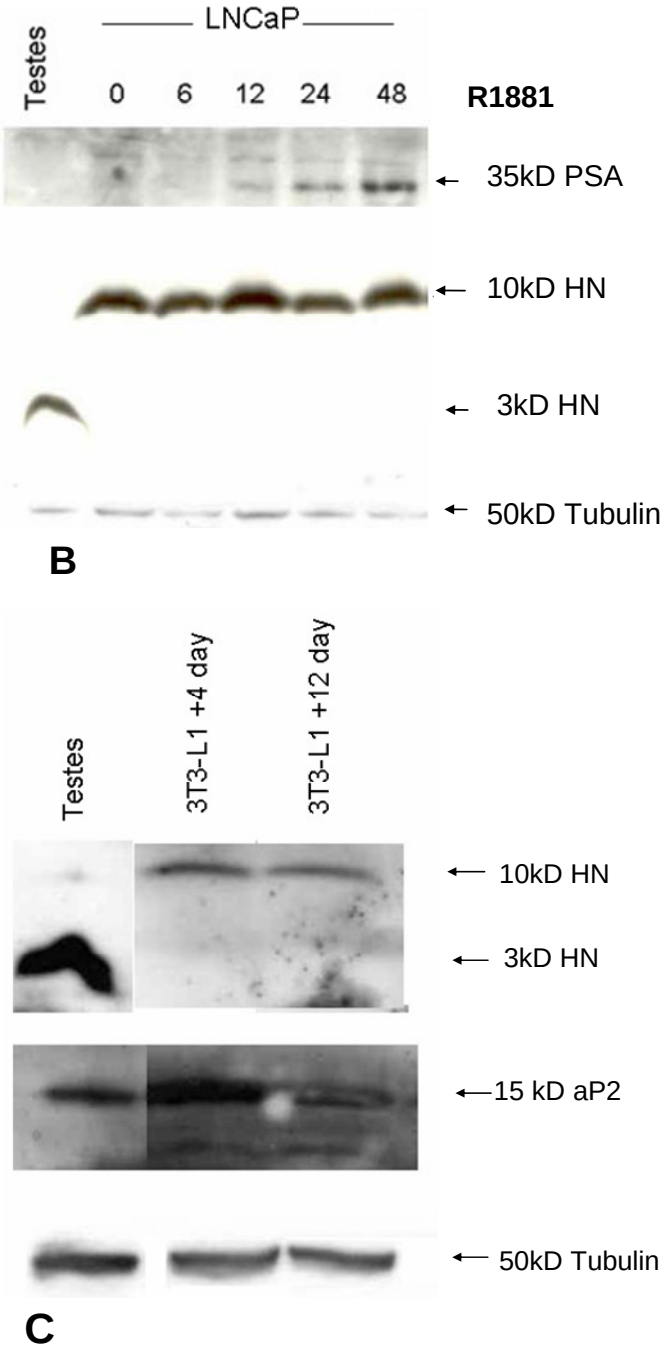

FIGURE 4*. A signal of approximately 10kD is detected in LNCaP and 3T3-L1 cells with a HN antibody.* ***A)*** *Protein extracts from untreated LNCaP cells (200µg), murine testes tissue(50µg) or subcutaneous adipose tissue(200µg) was collected and analyzed by Western blotting. A highly expressed band of approximately 3kD was detected with a HN-Ab in testes but not in adipose tissue and LNCaP cells. A 10kD band is strongly expressed in LNCaP cells and weakly in adipose tissue.* ***B)*** *PSA is regulated at the protein level in LNCaP cells but the 10kD band is not. LNCaP cells were charcoal treated and incubated with 10^{-8}M R1881 at indicated time points. Cell extracts were collected and probed with PSA, HN or Tubulin antibodies using the same membrane.* ***C)*** *A 10kD band is weakly detected in 3T3-L1 cells. +4 and +12 day 3T3-L1 adipocytes cell extracts were collected and probed with HN, aP2 or tubulin antibodies.*

HN antibody specifically recognizes the observed bands at 3kD and 10kD

Although HN has not been previously shown to be translated into a 10kD product, it is possible that HN undergoes unique post-translational modifications in LNCaP and 3T3-L1 cells. To rule out the possibility of non-specific binding by the antibody used, we titered the HN antibody pre-incubating it with the same peptide used for immunization of the rabbits in preparation of the antibody. Co-incubating with 150 μg of synthetic HN peptide resulted in complete loss of the 10kD band from LNCaP and testes extracts and almost complete loss of the 3kD band in testes (Figure 5, right panel), but no differences occurred when 150 μg of a control KLK4 peptide was co-incubated with HN antibody (Figure 5, middle panel). From this, we conclude that HN is functionally translated in LNCaP cells, but may be modified with post-translational moieties.

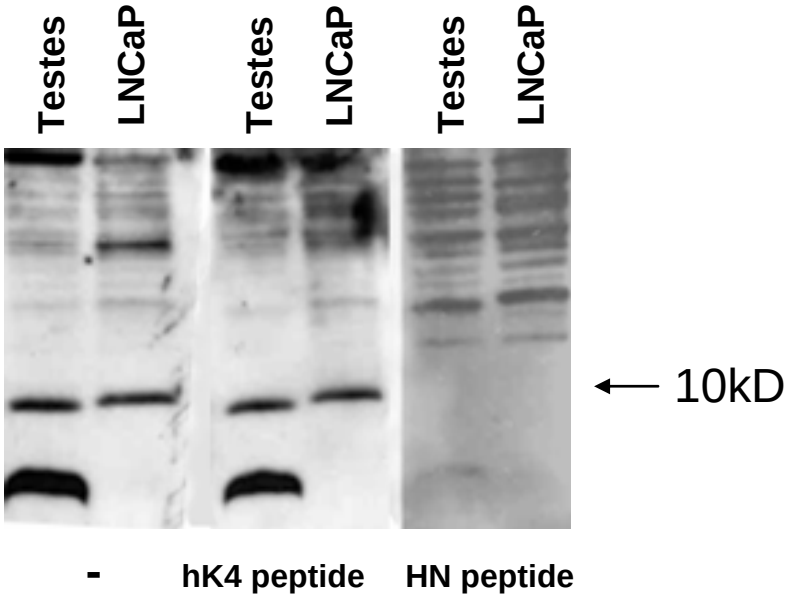

FIGURE 5*. HN antibody is specific for the 10kD modified form of Humanin.*
Triplicates of protein extracts from testes (50μg) and untreated LNCaP cells (200μg) were loaded to the same gel and blotted .The membrane was cut in three separate pieces and incubated with HN-Ab only, HN-Ab pre-incubated with 150 μg hK4 peptide or HN-Ab pre-incubated with 150 μg synthetic HN peptide. The membrane to the right activated the ECL solution stronger than the other two membranes but despite strong background the 10kD band disappears and the 3kD band is significantly reduced in strength compared to controls.

The 10kDa HN peptide is most likely not a dimer/trimer or glycosylated

We further investigated the reason for the significant size difference between HN in testes versus LNCaP and 3T3-L1 cells. Since the protein extracts used in these immunoblots were not denatured by heat, we tested the impact of using a stronger detergent and of boiling the extracts. Surprisingly, the 10kD band disappears upon heating or usage of the strong detergent 2-mercapthoethanol (Figure 6A), suggesting alteration of its secondary structure. We then hypothesized that HN could be glycosylated since it has previously been found to be secreted from cells transfected with a HN expression plasmid, as well as in F11 cells which are hybrid cells of E13 rat primary neuron with mouse neuroblastoma NTG18 cell (11). Addition of sugar chains during transport in the Golgi network prior to exocytosis could explain this increase in size. Tunicamycin is a well established as an inhibitor of glycosylation. Untreated LNCaP cells were therefore incubated with different amounts of tunicamycin or vehicle. PSA has previously been reported to be glycosylated and tunicamycin treatment reduces the detected size of PSA compared to untreated cells in LNCaP extracts (Figure 6 B, middle panel). However, the 10kD HN band did not exhibit a mobility shift upon tunicamycin treatment, indicating that glyosylation is not the reason for the increased protein size. The strength of the band was, however, weaker in tunicamycin-treated cells indicating that glycosylation or other functions of the compound do affect the expression of HN. Investigations to unveil reasons for the increased protein size are ongoing.

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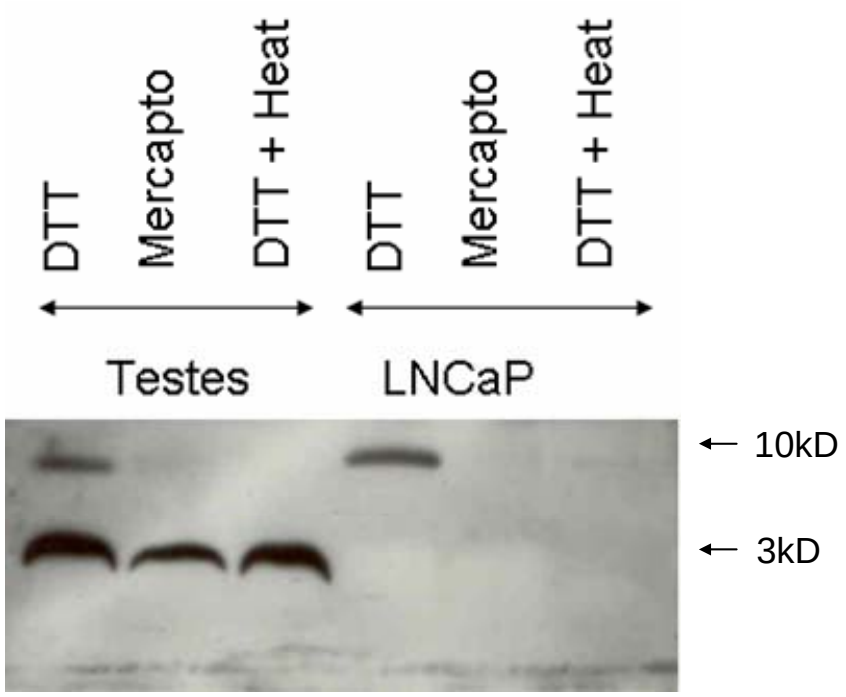
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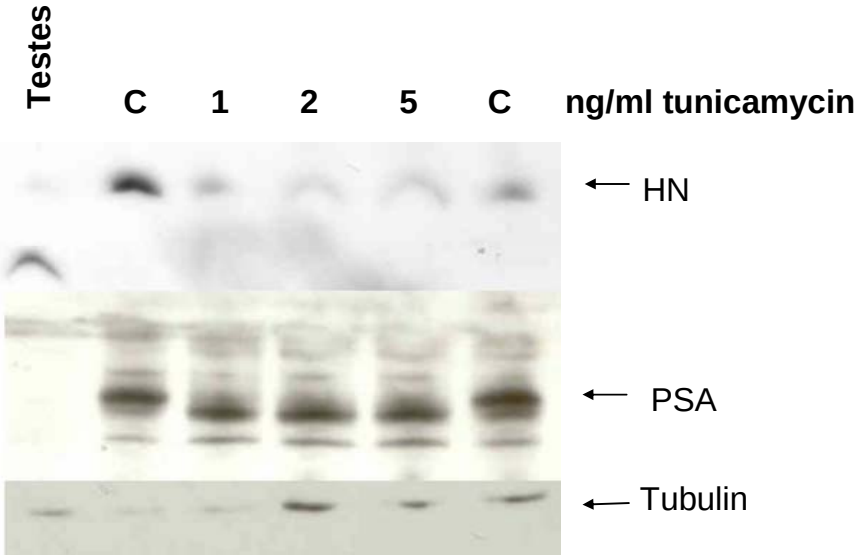
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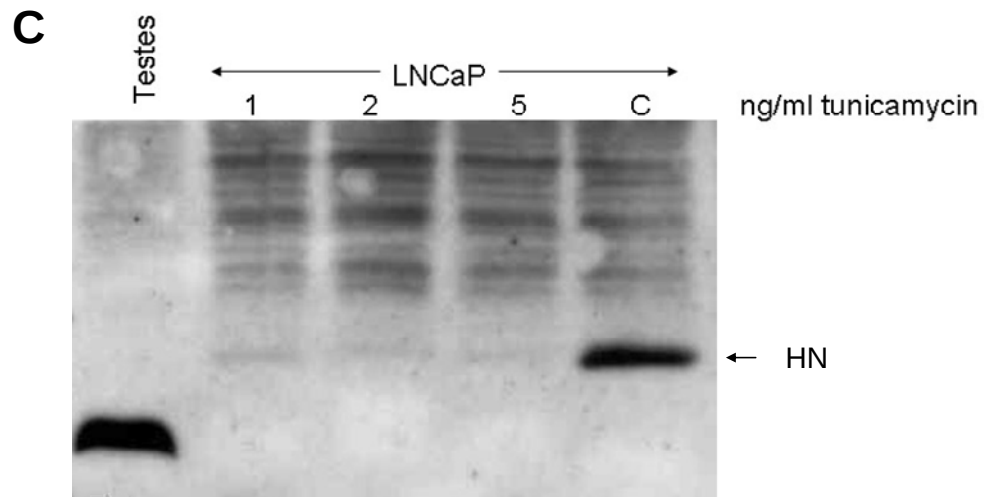

FIGURE 6*. 10kD HN is lost when heated and during tunicamycin treatment in LNCaP cells.* ***A)*** *Heat and treatment with a strong detergent result in loss of the 10kd HN form in LNCaP but not in 3kd HN from testes. Testes (50µg) or LNCaP (200µg) extracts were incubated with DTT (normal procedure) or 2-Mercaptoethanol at room temperature overnight or boiled at 95ºC for 3 minutes before analyzed by Western blotting.* ***B)*** *PSA is deglycosylated in LNCaP cells but HN is not. Indicated amounts of tunicamycin or vehicle control (MQ) were added to LNCaP for 24h hours. Extracts were analyzed by Western blotting with testes tissue as control. The same membrane was incubated with PSA, HN and tubulin Ab. Band shift was detected for PSA in tunicamycin positive cells but not in control indicating that glycosylation is inhibited. With HN-Ab no size differences are detected indicating that HN not is glycosylated.* ***C)*** *Humanin expression is inhibited by tunicamycin. The same cell extracts from B) analyzed by Western blotting probed with HN-Ab shows that tunicamycin inhibits translation of 10kD HN in LNCaP cells.*

Discussion

We have used a prostate-enriched cDNA library in microarray analysis to identify genes that are commonly expressed in adipocyte and PCa cell lines. Our high-throughput screening approach identified 713 cDNA clones which are expressed in both adipose and PCa cell lines. Of the genes that were identified, the work reported here has focused especially on HN and BI-1. Neither of these two genes exhibit tissue-specific expression in prostate and adipose tissue. However, Korkmaz et al. found in their initial report using the PSL library that of the 117 clones they investigated 76% were largely specific to the prostate for expression, and of these, approximately 40% were previously shown to be under androgen regulation (26). It is therefore no surprise that we detect genes that are not prostate specific among the thousands of clones that we investigated. In fact, since many clones housing common housekeeping genes were subtracted when the library was made the possibility for finding new candidates in our experiments may have increased.

On the other hand, elimination of these housekeeping genes may hastily have ruled out genes that might be relevant to both prostate cancer and adipogenesis, such as adhesion molecules, structural genes, or genes related to transformation or cell cycle.

We found BI-1 and HN particularly interesting because they have both been reported to inactivate the highly studied pro-apoptotic Bax and therefore behave like oncogenes (14,25). Evasion of programmed cell death or apoptosis is one of the well established hallmarks of cancer (35). Importantly, oncogenes are gene variants that stimulate cancer growth often through involvement in apoptotic evasion (36). Apoptosis during adipogenesis has not been established to the same degree as in PCa cells, although there is some evidence indicating that apoptosis indeed plays an important role in regulation of involution of adipose depots under certain conditions. Previously it was believed that adipocyte acquisition was permanent and that weight loss caused a decrease in cell size only (37). It is now hypothesized that adipocyte acquisition is balanced by a process also including cell deletion (38-40).

BI-1 was previously shown to be expressed at higher levels in PCa patients than in healthy subjects (24), but its functional role in PCa development is not known. In microarray analysis (Figure 2) BI-1 was not regulated in LNCaP cells and we observed only a slightly upregulation in LNCaP cells in response to androgen (Figure 3D left panel) by quantitative RT-PCR verifications. Further experiments at the protein level must be performed before we can conclude regarding the role of androgens in regulation of BI-1. In adipocytes we did not observe any significant regulation during adipogenesis (Figure 3D right panel). Still, BI-1 might have vital functions in both tissues. It would be interesting to explore if BI-1 is involved in the same ER stress induced pro-apoptotic pathway in PCa tissue as Chae et al. reported for liver and brain (25).

Our quantitative measurement of HN mRNA expression levels in 3T3-L1 and LNCaP do not disagree with the microarray data (Figure 2 and 3B). Indeed, levels of HN gene transcripts increase during adipogenesis. While HN mRNA does not seem to be regulated by androgen in LNCaP over time, a dose-dependent response to androgen is observed (Figure 3A and 3B). Since HN has sequence identity to the 16S rRNA in mitochondria, it is possible that the signals that have been measured are artifactual and actually correspond to 16S rRNA levels instead of HN levels. There is also one report in

the literature stressing that rRNAs do have a poly-A tail, debunking the general view that it only mRNA possess poly-A tails (18). Since we used oligodT primers to extract mRNA from the isolated total RNA pool, we did not expect that rRNA would be converted to cDNA by the reverse transcriptase. It is important to verify whether rRNAs do have a poly-A tail before any conclusion about the mRNA HN expression levels can be made. However, our data on HN protein expression do support that our data of HN mRNA expression is not artifactual evidence.

We detected a signal at approximately 10kD in prostate and adipocyte whole cell extracts by use of a HN specific antibody (Figure 4A and 4C). We surmise that the observed band is a modified form of the 24 amino acid HN peptide natively expressed in prostate and adipose tissue. In fact, a 10kD HN band is also detected in testes albeit at lower levels than the 3kD form. The competition assay clearly showed that the antibody is specific for both the 3kD and the 10kD bands in testes and LNCaP cells (Figure 5). We propose that the observed size difference is most likely due to post-translational modifications, which have indeed been reported for HN (42). The HN amino acid sequence has two serine residues (Ser 7 and Ser 14) that are potential sites for glycosylated, phosphorylated or ubiquitinylated. In addition Terashita et al. claims that synthetic HN injected into cells self- dimerize. Alteration of Ser14 to Gly14 eliminated dimerization and resulted in loss of neuronal cell death protection, indicating that this residue is necessary for dimerization, and that this residue is important for proper HN function. Normally, non-covalent bonds formed upon dimerization are disrupted by the harsh treatment of detergents and heat, which destroy the native structure of proteins. We have shown that boiling or incubation with stronger detergents results in degradation of the 10kD band in LNCaP cells, suggesting that this band represents a post-translationally modified form of HN that is stable at room temperature but unstable under denaturing conditions (Figure 6A). Although, we have so far been unable to separate the proposed dimer into monomers, we have not ruled out the possibility of a dimerization.

HN has been shown to be secreted from cells (11). Normally, this process involves transport through the Golgi apparatus where glycosylation occurs. If HN in LNCaP cells was glycosylated and therefore larger, we expected antibody recognition of a smaller HN peptide when tunicamycin inhibits addition of sugar chains. We observed

that tunicamycin treatment inhibits the expression of the 10kD protein, however did not observe size differences due to glycosylation (Figure 6B and 6C). Clearly, further experiments are necessary to elucidate why HN is expressed in a larger modified form. Interestingly, it has recently been reported that the intracellular level of HN is regulated through ubiquitin-mediated degradation by the RING ubiquitin ligase TRIM11, which binds and facilitates ubiquitination of HN (43). Ubiquitin (Ub) is activated by ubiquitin-activating enzymes (E1), passed onto ubiquitin-conjugating enzymes (E2), and then transferred to ubiquitin ligases (E3) which contain HECT or RING finger domains (44). In the light of the observation that the E3 ligase TRIM11 can bind HN, it is tempting to speculate that HN is ubiquitinated, although this has not been confirmed in our hands. Ub has a molecular weight of 8.5kD and addition of the 3kD expected size of HN results in a final product of 11.5 kD which is close to the size of the signal observed in our experiments with LNCaP and 3T3-L1 cell extracts. To further investigate whether HN is monoubiquitinated immunoprecipitation studies on HN could be performed on LNCaP or 3T3-L1 cells in conjunction with immunodetection of ubiquitin. Additionally, genetic knockdown of TRIM11 by RNA interference (RNAi) may result in a shift in molecular weight of HN detectable by Western blotting.

It is important to elucidate the exact function of HN in prostate. In addition to the already mentioned TRIM11 and the pro-apoptotic Bcl-2 family members Bax, Bak and BimEL protein, yeast two hybrid screening has also demonstrated the interaction of HN with insulin-like growth binding protein 3 (IGFBP-3). IGFBP induces and regulates apoptosis in various cancer cells, and is implicated in neurological disorders (45). Additional studies are required to verify these potential interactions but HN is clearly involved in several independent pathways associated with cell death suppression.

After Guo et al. reported that HN inactivated Bax (14), HN attracted much attention. Bax is an essential protein which participates in cell death during normal development and in various diseases (46). It is known that Bax resides in an inactive state in the cytosol of many cells. In response to death stimuli, Bax protein undergoes conformational changes that expose membrane-targeting domains, resulting in its translocation to mitochondrial membranes, where Bax inserts and causes release of cytochrome C and other apoptogenic proteins (47). Although this is an area of intense

research, it is still unknown what controls conversion of Bax from the inactive to active conformation (14); HN can be important for this conversion. Immunohistochemical studies of HN may provide more clues to its possible regulation of Bax. Preliminary immunofluorescence (IF) experiments with the HN specific antibody in LNCaP cells indicate that HN is located in all areas of the cells (data not shown). It is necessary to transfect cells with a transient expression vector containing a HN-GFP (Green Fluorescence Protein) construct to validate how specific the antibody is related to IF.

In conclusion, we have detected expression of the two anti-apoptotic genes HN and BI-1 in LNCaP and 3T3-L1 cells. HN mRNA expression is induced during adipogenesis, and dose-dependently in LNCaP whereas BI-1 is slightly regulated by R1881 in LNCaP cells. We have also observed endogenous expression of HN protein in LNCaP and 3T3-L1 cells, and propose that HN is post-translationally modified in these cells although further evidence is necessary to elucidate the exact modification involved. Future studies are required to elucidate the specific functions of these genes *in vivo* in prostate and adipose cells.

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