Immunobiological aspects in prostate cancer progression

by

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Yishan Liu

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Selected abbreviations

AA	Anti-androgens
APC	Antigen presenting cell
AS	Active surveillance
ADT	Androgen deprivation therapy
BCF	Biochemical failure
BRCA	Breast cancer susceptibility gene
CAFs	Carcinoma associated fibroblasts
CCL	Chemokine (c-C) motif ligand
CSCs	Cancer Stem Cells
DCs	Dendritic cells
DRE	Digital rectal examination
EAU	European Association of Urology
EGF	Epidermal Growth Factor
EMT	Epidermal-mesenchymal transition
EtBr	Ethidium bromide
ePLND	Extended pelvic lymph node dissection
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
Gy	Gray
H&E	Hematoxylin & Eosin
IFN	Interferon
IL	Interleukin
ISUP	International Society of Urological Pathology
LHRH	Luteinizing Hormone Releasing Hormone
LPS	Lipopolysaccharide
MHC I/II	Major histocompatibility complex class I/II
MIP	Macrophage inflammatory protein
MRI	Magnetic Resonance Imaging
mtDNA	Mitochondrial DNA
NoPCR	Norwegian Prostate Cancer registry
PCa	Prostate cancer
PD	Programmed death
PD-L	Programmed death ligand
PDGF-β	Platelet derived growth factorβ
PSA	Prostate specific antigen
SCID	Severe combined immunodeficiency
RP	Radical prostatectomy
RT-PCR	Reverse transcription-poly chain reaction
TCR	T cell receptor
TGF-β	Transforming growth factor-β
TLR	Toll-like receptors
TMA	Tissue micro array
TNM	Tumour Node Metastasis
Treg	T regulatory cell
TREM	Triggering receptor expressed on myeloid cells
TRUS	Trans rectal ultrasound

List of original papers

This thesis is based on the following papers, which are referred to in the text by the Roman numerals I-IV:

Paper I

Is the clinical malignant phenotype of prostate cancer a result of a highly proliferative immune-evasive B7-H3-expressing cell population?
Liu Y, Vlatkovic L, Sæter T, Servoll E, Waaler G, Nesland JM, Giercksky K-E, Axcrona K. International Journal of Urology. (2012), doi: 10.1111/j.1442-2042.2012.03017.x

Paper II

Dendritic and Lymphocytic Cell Infiltration in Prostate Carcinoma
Liu Y, Sæter T, Vlatkovic L, Servoll E, Waaler G, Axcrona U, Giercksky K-E, Nesland JM, Suo Z, Axcrona K. (*accepted* Histology & Histopathology Feb 2013)

Paper III

Blocking mtDNA replication upregulates the expression of stemness-related genes in prostate cancer cell lines
Liu Y, Wu X, Li X, Kvalheim G, Axcrona U, Axcrona K, Suo Z. (*accepted* Ultrastructural Pathology Jan 2013)

PART I: BACKGROUND

Chapter 1 – Prostate cancer

1.1 The Prostate Gland

The prostate is both an exocrine and endocrine gland surrounding the urethra as the most proximal part entering out from the urinary bladder. Adjacent to the prostate, the seminal vesicles are located whose ducts enter the prostate. The seminal fluid consists of fluid arising in the seminal vesicles, the exocrine products of the prostate and the semen. Thus the seminal fluid is a transport medium for the sperms to reach the egg in the course of conception thought to consist of a source of energy for the sperms. There is evidence suggesting that the seminal fluid is also giving the sperms protection during the course of migration. The prostate's exocrine function consists in contribution of e.g. zinc, thought to stabilize the sperm chromatin, and acid phosphatases. Secretions from the seminal vesicles contribute with fructose as a source of energy for the sperm, peptides and proteins as well as prostaglandins (1, 2). It has been suggested that the seminal fluid elicits a transient state of peripheral immune tolerance in the female reproductive organs (3, 4). The seminal fluid has also been demonstrated to contain the different forms of TGF-β, a potent immuno-modulating protein, for review see (5).

The endocrine function of the prostate consists in the synthesis of androgens from dehydroepiandrosterone (DHEA), that is a precursor of sex steroids produced by the adrenal glands (6). It has been estimated that amongst others the prostate contributes with approximately 40% of the total androgen pool with dihydrotestosterone being the predominant androgen produced from DHEA, for review see (7).

1.2 Prostate Cancer Epidemiology

Prostate carcinoma (PCa) is the most commonly diagnosed male malignancy in the western world. In Norway, close to one-third of all cancers diagnosed for men in 2008 were primarily occurring in the prostate (8). With extended PSA testing of asymptomatic men, the incidence of PCa is rapidly increasing in Norway (9). Although more patients die with PCa than die from the disease, Norway's PCa-specific mortality is among the highest worldwide (10, 11). Among all the deaths from cancer among men in Norway in 2009 (5636 deaths), PCa was the second reason in terms of cancer mortality numbers; just followed after lung cancer (1230 deaths), see also Figure 2. 3500 primary prostate carcinomas are diagnosed every year and 1090 deaths of the disease were registered in 2007 (Cancer in Norway 2009). Approximately one out three patients diagnosed with PCa die of the disease. The clinical course is variable. Men with metastases at diagnosis and men with locally advanced cancer have an unfavorable prognosis. For men with locally disease radical prostatectomy, radiation therapy or observation and close follow up are the treatment options.

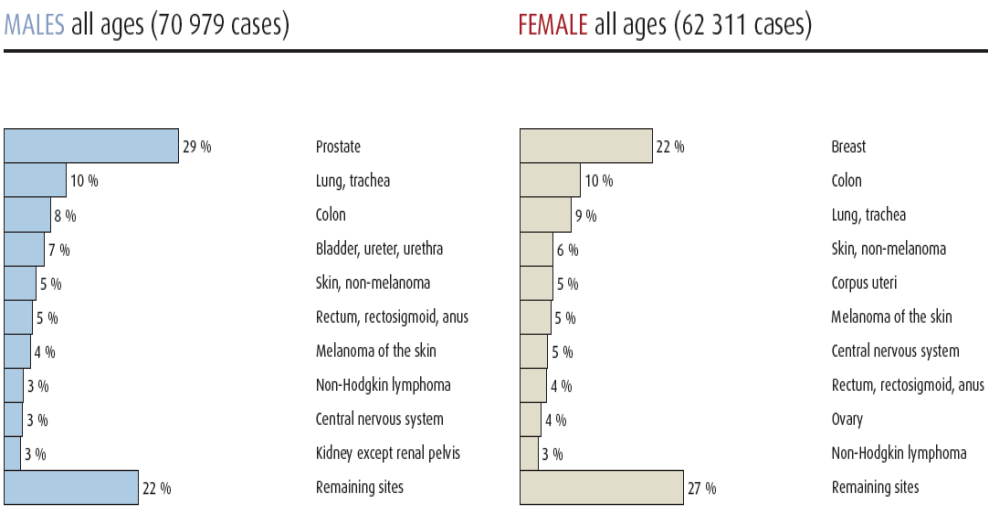


Figure 1: The most frequent incident cancers by sex in all ages in Norway during 2005-2009 (source: Statistics Norway), (from www.kreftregisteret.no).

1.3 Prostate cancer etiology

Our understanding of molecular mechanisms involved in PCa development is rapidly improving. PCa does not occur in eunuchs and in men that have been castrated as adults. Thus, androgen stimulation is important for PCa development.

1.3.1 Age

Age is an established risk factor. PCa is the most frequent cancer in men aged over 50, and one in eight men will develop this cancer before the age of 75 (8). In Norway, just 4% of all new cases in year 2004 were men of age 55 years or younger, but long-term survival among patients diagnosed aged under the age of 50 is actually lower than for patients diagnosed aged 50-59. The median (range) age of all patients with PCa diagnosed during 2004 in Norway was 72 (43–96) years (10).

1.3.2 Ethnicity

PCa occurs more often in African-American men than in men of other races (12). African-American men are also more likely to be diagnosed at an advanced stage, and are more than twice as likely to die of PCa as white men. PCa occurs less often in Asian-American and Hispanic/Latino men than in non-Hispanic whites. PCa is diagnosed more often in North America, northwestern Europe, Australia, and on Caribbean islands than in Asia, Africa, Central America, and South America. More intensive screening in some developed countries very likely accounts for at least part of this difference, but other factors like lifestyle (diet, etc.) are likely to be important as well. For example, men of Asian descent living in the United States have a lower risk of PCa than white Americans, but their risk is higher than that of men of similar backgrounds living in Asia. Constitutional differences in populations may also

likely contribute (12). Dorff and co-workers (13) reported ethnic variation in neuroendocrine cell expression in prostate carcinomas.

1.3.3 Hereditary factors

A family history of PCa is an important risk factor. A man who has a father or brother with clinically diagnosed PCa is one and a half to three times more likely to develop the disease than a man with no family history.

Family history and PCa risk		
Family history	**Estimated relative risk**	**Estimated lifetime risk**
No PCa	—	8%
Father diagnosed after age 60	1.5%	12%
One brother diagnosed after age 60	2.0%	15%
Father diagnosed before age 60	2.5%	20%
One brother diagnosed before age 60	3.0%	25%
Two relatives with PCa	4.0%	30 %
Three or more relatives with PCa	5.0%	35%–45%
Relative risk = Increase in risk in comparison to men with no family history of PCa Lifetime risk = Overall chance of developing PCa during a man's lifetime Source: Bratt, O. *Journal of Urology* 2002, vol. 168, p. 907.		

Some inherited gene changes raise the risk for more than one type of cancer. Inherited mutations of the BRCA1 or BRCA2 genes are well established for breast and ovarian cancer patients. Mutations in these genes may also increase the PCa risk, but only in a limited number of cases.

1.3.4 Lifestyle factors

The exact role of diet in PCa is not clear. No relation between red meat consumption and PCa incidence and mortality has been described so far (14). Fish intake has been associated with decreased PCa mortality (14).

Asian food, with high intake of soya plant fibers etc., might have a protective role, and explain the lower incidence of prostate carcinoma among Asian populations. The food content of oestrogens, isoflavonoids, etc. are also having an important impact of development of PCa. Increased physical activity is associated with a small increase in PCa incidence and modest to strong decrease in PCa mortality (15, 16). Alcohol and sexual activity, have all been analysed but without clearcut answers (14).

Some studies have suggested that men who consume a lot of calcium (through food or supplements) may have a higher risk of developing advanced PCa (14). Most studies have not found such a link with the levels of calcium found in the average diet, and it's important to note that calcium is known to have other important health benefits. Also, most studies have not found a link between smoking and the risk of developing PCa (14). Some recent research has linked smoking to a 14% increase in PCa mortality (17).

1.3.5 Medication & Prevention

The molecular link between chronic inflammation and cancer development is becoming more and more evident (18). Macrophages and other inflammatory cells provide access of growth factors and cytokines for stem cell and stem cell niches and stimulate growth. Clinically aspirin has been in use for more than 100 years and in the review of epidemiological studies Mahmud and coworkers (19) showed some indications that both aspirin and other non steroidal and anti inflammatory drugs (NSAIDs) have a protective role, but the results are not conclusive.

Studies have demonstrated that inhibition of the conversion of testosterone to di-hydrotestosterone by medication with 5 alpha-reductase inhibitors (5ARIs) significantly reduces the incidence of low-grade PCa but increases to some extent development of high-grade PCa and at the cost of sexually related adverse events (20, 21).

1.3.6 Hormones

Androgens have been considered to be the major sex hormones regulating the normal and malignant growth of the prostate. However, recent epidemiologic findings and experimental data suggest that estrogens and their mimics can be responsible for the pathogenesis of PCa, for review see (22). The carcinogenicity of estrogens in the prostate during adulthood is believed to be mediated by the combined effects of the hormone-induced unscheduled cell proliferation and epigenetic silencing of antitumor genes, along with the bioactivation of estrogens to genotoxic carcinogens (22). Thus, individuals or ethnic groups with polymorphisms in genes encoding ERs and/or estrogen-metabolizing enzymes can modify the risk for PCa caused by altered responsiveness to the hormone and exposure to its carcinogenic metabolites during a lifetime (22). The age-dependent hormonal shift from androgen to

estrogen could also be an important contributing factor to increased estrogen bioavailability (22). Although PCa has a long latency and starts to develop in men around middle age, recent data strongly suggest that PCa risk could be determined even as early as during prenatal and perinatal life stages by a process known as estrogen imprinting (23). Thus, primary PCa prevention should probably begin in early life. Among the various cellular mediators, ER-β seems to be a key determinant in the pathogenesis, progression, and metastasis of PCa (24, 25). Therapeutic approaches targeting its activation/inactivation may have important ramifications in the prevention and treatment of PCa (26). Epigenetic mechanisms such as DNA methylation play important roles in regulating the expression of the 2 ER subtypes (27, 28). A change in the methylation status of proximal promoters of these genes constitutes an on/off switch for reversible gene regulation. Moreover, the differential expression of different ER-spliced variants (isoforms) could explain some conflicting observations related to estrogen action in the initiation and progression of PCa (29). Apart from the canonical genomic action of ER-α and ER-β, the therapeutic potential of ER and its variants that function in multiple non-genomic pathways, such as membrane ERα, mitochondrial ER, ERRs, and GPR30, may further contribute to the pathogenesis of PCa (30, 31). Various estrogenic/antiestrogenic/SERM-like compounds have demonstrable efficacies in causing PCa regression through various pathways and treatment of advanced PCa with transdermal estrogen has gained in popularity. Several SERMs, including toremifene, have shown promise as chemopreventive or therapeutic agents in clinical trials (32). Although data from clinical trials are not conclusive, phytoestrogen supplements, including dietary soy, continue to be used by patients as complementary alternative medicine for PCa. With a greater understanding of the molecular mechanism underlying estrogen carcinogenicity in the prostate, the applicability of estrogen/antiestrogen-based prevention and treatment therapies, as first-line or adjuvant therapies, will be used more in the clinic (22). Thus, the devising of a

new generation of estrogenic/antiestrogenic therapies with higher specificity against PCa and fewer off-target effects is timely.

1.3.7 Molecular mechanisms

The dominant opinion has been that carcinomas are caused by mutations in epithelial cells. On average, it has been claimed that four to five mutations occur in "driver genes" are needed for carcinoma development. However, whole genome sequencing has shown that sometimes no mutations can be found, indicating a role for epigenetic mechanisms.

Recently the key role of cancer stem cells and the stem cell niches are being high lightened. Changes in the stem cell populations and related stroma, are the key drivers for carcinoma development. The immune system including stromal macrophages provides growth factors and cytokines. Identification of reliable stem cell markers is an ongoing process. At present we assume that at least two stem cell populations are present, together with various progenitor cells. A stem cell population must give rise to all differentiated cell types, and at the same time be able to cell renewal. Progenitor cells can also give the same pattern, but cell renewal stops after some cell divisions. No marker is specific, but Goldstein and coworkers identified in the basal layer a stem cell population-giving rise to various epithelial phenotypes including neuroendocrine cells (33). Recently, Clevers presented an overview over markers used for characterization of stem cell populations (34). However, at present no marker is specific for stem cell populations, but CD24 negativity (35), CD44 positivity (35) and ALDH1 expression all characterise stem cells, but in different stages. CD 133 is also used to characterize proliferative cells with stem cell features in many organs (34). A stem cell population with both CD44 and ALDH1 expression is characterising an active, EMT linked stem cell population. Beta catenin signaling in the Wnt pathway is important in stem cell regulation.

Bmi1 is important in various stem cell populations including the ones in the PCa Bmi1 belongs to the mRNA 200-family, being down-regulated in EMT. However, Bmi1 is up-regulated. Bmi1 promotes histone ubiquination and histone remodeling. Targeted therapy has so far been focusing on differentiated cell populations and the clinical observation of tumour volume reduction. Since the cell populations responsible for tumour progression are found in the stem cell populations, focus is now on markers characterizing these cell populations (36). We know that ALDH1 is linked to steroid hormone regulation, as well as HER2 amplification in the breast. Her2 affects stem cell population. IL8 receptor is increased in stem cells. Thus, chronic inflammation, cell injury release IL8, and the use of anti-inflammatory drugs in stem cell targeted therapy is in ongoing clinical trials. Oxygenation regulates carcinoma stem cells, but anti-angiogenic therapy has turned out to give only a moderate effect. Carcinoma stem cells reside in hypoxic niches, like bone marrow and invade hypoxic areas. We do know that Wnt/NOTCH pathways are stimulated by hypoxia, so a potential therapeutic target could be to combine NOTCH inhibitors with anti-angiogenic therapy.

Carcinoma associated fibroblasts (CAFs) have a central role in epithelial-mesenchymal transition (EMT). The majority of CAFs arise from local fibroblasts and can be activated through various mechanisms, including TGF-β and MMP8, activated fibroblasts synthesize collagen and remodel stroma, promote invasion, tumor growth. YAP expression in CAFs is involved stiffening of stroma through remodeling. YAP works together with TAZ as a transcription co-activation. YAP turns out to be required for CAFs tumor promoting abilities, involved in angiogenesis, regulate myosin light chain.

Hypoxia is a driver for both stem cell population and for activation of carcinoma- associated fibroblasts in stem cell niches. NOTCH 1 can block the hypoxic influence. PDGF-β is produced in CAFs and has a key role in collagen production. Liao and coworkers have

recently documented the significant role of CAFs in prostate carcinoma development from the stem cell populations (37). In mixed cultures of CAFs and prostate carcinoma stem cells, neoplastic glandular structures appeared with high proliferation activity (37). Gregg and coworkers explored gene expression in prostate carcinoma cells and stromal elements separately and reported a series of genes highly expressed in stromal elements, including among others genes linked to extracellular matrix and to the immune system and to inflammation (38).

1.4 Prostate Cancer Screening

Screening activities for cancer aim at cancer detection at an early stage and treatment with the goal to reduce mortality in a cancer group. As PCa is the second leading cause of death in cancer in men, the question arose whether it would be of benefit to screen a population (of healthy men at risk) for PCa in order to reduce cancer death. Screening for PCa is usually organized as part of a clinical trial and is initiated by the screener. As, however, organized PCa is not performed as a general offer to the male population, and awareness of this cancer disease has risen amongst the male population, so called opportunistic also wild-screening has emerged, i.e. a screening for PCa initiated by the male patient or his physician. Measurement of the Prostate-specific antigen (PSA) level is the basis for PCa screening. In asymptomatic men an age of 50 years has been used as a first time point to start PSA screening in the Goteborg randomized population-based prostate-cancer screening trial (39). The PSA level at which further diagnosis started was set at 2.5 ng/ml.

One early study demonstrating the possible benefit of PCa screening was performed in Tyrol (Austria) where patients were included non-randomized (40). The PCa death rate decreased with 33% in Tyrol compared to Austria in general.

Lately, results from three large prospective randomized control trials were published: The Prostate, Lung, Colorectal and Ovary (PLCO) trial conducted in the US (41), the European Randomized Study of Screening for PCa (ERSPC) (42) and the Goteborg randomized population-based prostate-cancer screening trial, which is a section of the ERSPC trial (39). In the PLCO trial more than 70.000 men were included for PCa screening. The PLCO study did not show any significant difference in PCa mortality at 10 years follow-up in the screening arm compared to the standard care arm. It is, however, been pointed out that 42% of men included in the PLCO study had been PSA tested on beforehand (43). The ERSPC study included more than 160.000 men. The death rate from PCa at 10 years of follow-up was reduced with 20%, however, at a high risk of over-diagnosis. To save the death from PCa for one man 1.410 men needed to be screened and 48 men needed to be treated (42). At the follow-up at 11 years in the ERSPC study it was noted that there was a 41% reduction in development of metastases in the screened arm (44). The Goteborg PCa screening trial included 20.000 men, and in the latest update of that trial with a follow-up of median 14 years, PCa mortality decreased with 50%. 293 men needed to be screened and 12 men needed to be treated to save one life (39).

1.5 Prostate Cancer Classification

PCa is classified according to the well-known TNM (Tumour Node Metastasis) classification (45), see also Table 1.

Evaluation of the primary tumor – T stage

TX: Primary tumor cannot be evaluated
T0: No evidence of primary tumor
T1: Tumor present, but not detected clinically or with imaging[1]
- **T1a**: Tumor was incidentally found in less than 5% of prostate tissue resected
- **T1b**: Tumor was incidentally found in greater than 5% of prostate tissue resected
- **T1c**: Tumor was found in a needle biopsy, e.g. because of an elevated serum PSA

T2: Tumor can be palpated on examination, but has not spread outside the prostate[2]
- **T2a**: Tumor is in half or less than half of one of the prostate gland's two lobes
- **T2b**: Tumor is in more than half of one lobe, but not both
- **T2c**: Tumor is in both lobes but within the prostatic capsule

T3: Tumor has spread through the prostatic capsule[3]
- **T3a**: Tumor has spread through the capsule on one or both sides
- **T3b**: Tumor has invaded one or both seminal vesicles

T4: Tumor has invaded other nearby structures

Evaluation of the regional lymph nodes – N stage[4]

NX: cannot evaluate the regional lymph nodes

N0: there has been no spread to the regional lymph nodes

N1: there has been spread to the regional lymph nodes

Evaluation of distant metastasis – M stage[5]

MX: Distant metastasis cannot be evaluate
M0: No distant metastasis
M1: Distant metastasis
- **M1a**: Cancer has spread to lymph nodes beyond the regional ones
- **M1b**: Cancer has spread to bone
- **M1c**: Cancer has spread to other sites (regardless of bone involvement)

[1]Tumor found in one or both lobes by needle biopsy, but not palpable or visible by imaging, is classified as T1c.
[2]Tumor has to be palpated in both in lobes to be classified as T2c. Tumors found on biopsy in both lobes but not palpable bilaterally, should not be classified as T2c.
[3]Tumors invading into the prostatic apex or into the capsule (but not beyond) should be classified as T2.
[4]Metastasis no larger than 0.2cm can be designated pN1mi.
[5]The most advanced site of metastasis should be used.

Table 1 The 2009 Tumour Node Metastasis classification of PCa

1.6 The Diagnosis of Prostate Cancer

PCa is a common disease of elderly men and often does not cause symptoms initially. The presence of clinical symptoms usually means that the disease has already spread beyond the prostate. The characteristic symptoms are urinary problems, haematuria, haemospermia, and

reduced ejaculation. The more locally advanced tumour is also often associated with impotence and diffuse pain. Metastases to the bone marrow can give pain and anemia, loss of weight. It is not uncommon to diagnose a metastatic prostate carcinoma as the cause of low back pain in elderly men. However, benign prostatic hyperplasia (BPH) can cause many of the same symptoms as PCa.

The diagnosis of PCa is mainly obtained with a prostate biopsy demonstrating prostatic carcinoma (see also 1.7- Histopathological Analysis and Grading of Prostatic Carcinoma). However, for the diagnosis of PCa a triad of examinations is usually used.

- The Digital rectal examination (DRE)
- The Prostate-specific antigen (PSA) level
- The result of the Histopathological examination of the Prostate biopsy - The highest Gleason sum from a positive biopsy.

During the last decade MRI has progressively been introduced both in the diagnosis and clinical staging of PCa.

DRE: Approximately 80% of the PCas are located in the peripheral zone of the prostate (46, 47) and thus some of those cancers can be detected with digital rectal examination. As Richie et al demonstrated 18% of all newly diagnosed PCa could be detected with DRE only (48).

PSA: PSA was first described by Ban et al. to be secreted by the prostate, and it was also shown to be proteolytic enzyme (49). PSA was later demonstrated to be a kallikrein-like serine protease (50). Abrahamsson et al. demonstrated with immunohistochemical methods that PSA was present in the epithelial cells of acini and ducts of the prostate (51). They also demonstrated that the incidence of PSA producing cells was lower in moderately and lower

differentiated cells than in highly differentiated prostatic carcinomas. In 1987 Stamey et al. demonstrated the potential use of serum PSA levels as a marker for adenocarcinoma of the prostate (52). They demonstrated not only that a significant proportion of patients with elevated PSA levels had significant cancers, but they also demonstrated that a proportion of patients without any signs of PCa had an early cancer. Levels of PSA were correlated to tumor volume, but a proportion of patients with benign prostate hyperplasia also had elevated PSA levels. Because PSA is also expressed in benign prostate tissue, it is also elevated in men with enlarged prostates; and PSA levels are often increased in patients with prostatitis, i.e. in patients with an inflammation of the prostate. The PSA level as an independent measurement per se was described to be a better predictor for PCa than suspicious findings on DRE (53).

Serum PSA levels correlate with tumour burden and clinical stage in patients diagnosed with prostate carcinoma. A serum PSA higher than 4.0 ng/ml means presence of a carcinoma in 70-80 % of the men; but a PSA level up to 10.0 ng/ml can be caused by benign lesions.

Serum PSA is used to monitor patients under treatment and in the follow-up situation. A rapid increase in serum PSA indicates PCa progression.

The Prostate biopsy: The definitive diagnosis of PCa is set by prostate biopsies demonstrating prostatic carcinoma as the result of histo-pathological evaluation. If prostate biopsies should be taken, is determined by a suspicious DRE and/or an elevated PSA level. It should, however, always be kept in mind that any invasive investigation on a patient should have a further diagnostic and/or therapeutical consequence. That means that the patient's biological age and comorbidity should be considered when decision about taking prostate biopsies should be taken (54). Prostate biopsies are usually taken with ultrasound guidance. Biopsies can be taken with the transrectal or the transperineal approach (55, 56). It is recommended to take 12 biopsies.

If indication for taking prostate biopsies in a patient is found, and initial transrectal biopsies are negative, investigation of the patient with an MRI following directed TRUS biopsies (57), with transrectal saturation biopsies (58) or with transperineal saturation biopsies (59) should be considered.

Magnetic Resonance Imaging: As approximately 20% of the prostate surface is palpable when performing a DRE of the patient it is obvious that the diagnostic accuracy is rather scarce. At the same time clinical staging of the patient is extremely important in stratification of the patient to radical treatment, either radical prostatectomy or radiation treatment. During the last five years application of MRI in PCa staging has 'exploded'. Just very recently the European Society for Urogenital Radiology has published MRI guidelines (60). Thus MRI is used in detection of suspicious PCa foci in patients at risk for PCa (57) prior to further investigation with prostate biopsies. MRI is used increasingly as a tool in patients who are candidates for active surveillance (AS)(60). MRI is finally used in PCa staging - in both treatment decision making, operative procedure planning and radiation treatment planning (60).

MRI of the prostate comprises investigation with a set of modalities:

- The T2-weighted imaging, also called T2WI
- Dynamic contrast enhanced MRI, also referred to as DCE-MRI
- Diffusion weighted MRI, also referred to as DWI
- MR spectroscopic imaging, referred to as MRSI

The radiologist usually investigates the above-mentioned components when MRI of the prostate is performed. The combined evaluation of these modalities is therefore referred to as multiparametric MRI.

1.7 Histopathological Analysis and Grading of Prostatic Carcinoma

Histopathology

The majority of PCas are adenocarcinomas arising in the posterolateral parts of the gland (for details, see WHO classification 2004). Adenocarcinomas in the central zone are uncommon, constituting 5% of all carcinomas. Multifocality is common. More than 50% of all adenocarcinomas are multifocal and with heterogeneity in morphological features.

The adenocarcinomas can be a challenge to diagnose, especially in needle biopsies. The search for invasion pattern, mitotic figures, accumulation of small glands with hyper chromatic cells and mitotic figures favors a malignant diagnosis. Faint bluish mucinous material in the glandular lumens and crystalline figures are also in favor of a carcinoma. Corpora amylacea are present in benign lesions. An infiltrative growth is a key pattern in highly differentiated tumors. Prominent nucleoli are usually seen in prostate adenocarcinomas. It is not uncommon to observe atypical glands in a biopsy, but not enough to diagnose a cancer. Then a repeat biopsy is recommended. Atrophy, crush artifacts or atypical neoplasia in the glandular epithelium (high-grade prostatic intraepithelial neoplasia/HGPIN) must all to be ruled out. Immunostaining can be of diagnostic help. Alpha-methylacyl-CoA racemase (AMACR) is highly upregulated in prostate adenocarcinomas and its product p504s can be demonstrated by immunostaining, showing a strong cytoplasmic expression. In normal and benign glands only a weak or negative staining is observed (61). Loss of the basal cell layer is most often seen in adenocarcinomas, but in some Gleason grade 3 tumors, the basal layer can be partially present (62). The basal epithelial cell markers p63 and cytokeratin 34βE12 are not present in adenocarcinomas, indicating absence of basal cells.

Other markers in clinical use are prostate specific acid phosphatase (PAP) and prostate specific antigen (PSA).

A molecular classification of prostate carcinomas has been established (63), being statistically independent of Gleason score. Various approaches are ongoing, but main focus at present is to characterize stem cell populations and related stromal elements (64). Surplus material from needle biopsies has been analyzed with gene microarray and RT-PCR and could be applied in routine diagnostics (65). The expression profile in normal elements adjacent to carcinoma areas can detect increased activity in interleukins, chemokines, and various growth factors of importance for stem cell growth (66).

Stromal alterations both in tumor near areas and in more remote parts of the prostate gland are of clinical relevance (67). The extent of collagen deposition, activation of carcinoma associated fibroblasts, presence of immune cells, including macrophages, mast cells are more pronounced in the more aggressive carcinomas (high Gleason grade – see below). These reactive changes demonstrate the close link between chronic inflammation and cancer development. Increased hyaluronan has been observed (68), together with cancer-associated fibroblasts, platelet-derived growth factor receptor beta increase (69), and increased angiogenesis (70). Vascular invasion and perineural infiltration are both not infrequent in prostate adenocarcinomas.

Gleason grade and score

Donald Gleason introduced the first histopathological classification of prostate adenocarcinomas based on architectural features in 1966 (71). The score is at present the most important prognosticator for clinical use. A low Gleason score (up to 6) is associated with a favorable clinical outcome (72). The grading system is mainly based upon evaluation of the

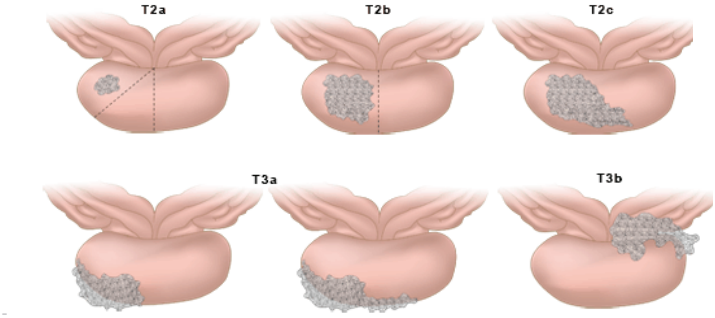
morphological architecture. The sum of the two most frequently occurring growth patterns, referred to as Gleason grade, is reported as the definite Gleason score. Gleason pattern 1 is including circumscribed nodule of closely packed medium sized acini. Gleason pattern 2 embraces features like in 1 but with additional minimal infiltration at the edge of the nodule. Gleason pattern 3 reveals discrete glandular units and with smaller glands showing variation in size and shape. A clear infiltration is observed. Gleason pattern 4 shows fused small glands with ill-defined lumina, cribriform pattern. In Gleason pattern 5 no glands are seen, only solid sheets, cords and single cells. Comedonecrosis can be present. The Gleason score ranges from 2 to 10. However, based on updated criteria for Gleason grade evaluation, Gleason score below 6 is rarely reported in prostatectomy specimens and never in prostate biopsies (73, 74). The updated criteria for Gleason score 6 and 7 have caused an upgrading of the tumors (73). It was suggested to explore incorporation of the tertiary growth pattern in radical prostatectomy specimens and later clinicopathological studies have shown a clinical impact (75, 76).

1.8 Clinical Staging of Prostate Cancer and Risk Stratification

The clinical staging of a patient's PCa is the prerequisite for later treatment decision-making. The clinical stage is determined through assessment of the clinical

- T-stage, i.e. assessment of tumour extent, see also Figure 2.
- N-stage, i.e. lymph node involvement,
- M-stage, i.e. establishment of whether metastases are present.

Figure 2 – Clinical T categorization of PCa



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The clinical **T-stage** is determined with DRE. Accuracy is, however, low- and below 50% of the cases were staged correctly when related to the pathological stage (77). Most often TRUS is used in combination with DRE in assessment of the clinical stage (78). Three dimensional mapping biopsies with the transperineal technique has shown to give a more accurate assessment of tumor location and tumor extent demonstrating an up-staging in 45% up-grading of the Gleason score in 27% (79). Another study, however, showed that up-grading of the Gleason scored occurred in 46% of the cases, even if patients were investigated with transperineal biopsies (59). Lately, also MRI has been introduced in clinical T-staging, and endorectal MRI (e-MRI) has been demonstrated to add value in clinical T-staging (80). e-MRI was also shown to substantially help in pre-operative assessment of locally advanced PCa disease, both adding information in extraprostatic extension of the tumor (81) and extension of PCa to the seminal vesicles (82).

The **N-stage**, or lymph node stage is determined in patients who are eligible for curative treatment. Often nomograms are used, i.e. tables or computer or internet-based tables who are based on true patient populations, to predict lymph node metastases. The risk of lymph node metastases is determined by several parameters, e.g. the PSA value, clinical stage, the biopsy

Gleason grade or score. An example of such a nomogram is the Partin table (83). Recently, in accordance to development MRI staging, also a nomogram incorporating results of MRI in PCa has been presented (84). The most accurate N-staging is performed with an operative lymphadenectomy, with either an open, laparoscopic or robot-assisted technique. The extended pelvic lymph node dissection (ePLND) should be preferred since it yields the most accurate lymph node staging (85).

The **M-stage** is determined with radiologic methods aiming at analysis of the skeleton since the skeleton is the most frequent target of metastasation in PCa. An elevated serum-alkaline phosphatase might be an indicator of skeletal metastases (86). According to the EAU-guidelines on PCa a bone scan should be performed in patients with Gleason score >4+3=7b or patients with newly diagnosed PCa and PSA levels above 20 ng/ml (87).

Stratification of PCa patients to risk groups is to reflect prognostic categories. Risk groups are based on the assumption that they do not have any metastases at the time of diagnosis. Risk groups are stratified to predict PCa specific mortality in patients undergoing radical PCa treatment, either radical prostatectomy or radiation treatment. To decide on curative treatment patients are stratified into risk groups. The risk stratification is based on evidence from many retrospective and prospective studies, and the depicted stratification shown below is a summary from the European Association of Urology (EAU) guidelines on PCa, see also Table 2 (87). EAU guidelines recommend that decision on treatment stratification on high-risk and very high-risk patients should be made in multidisciplinary teams involving urologists, urologic oncologists and radiologists in which benefits and side effects of radical treatment should be considered on an individual base and according to the patients circumstances (87).

Risk	Clinical T stage		Gleason score		PSA ng/ml
Low-risk	cT1-T2a	and	6	and	<10
Intermediate-risk	cT2b-T2c	or	7	or	10-20
High-risk	cT3a	or	8-10	or	>20
Very high-risk	cT3b-T4				

Table 2 - Risk stratification according to the EAU guidelines.

1.9 Treatment Stratification of Prostate Cancer

Decision on treatment modality of PCa is dependent on several factors: The patients' age, life expectancy, the type of PCa, i.e. the grade of the cancer and the stage. According to these the EAU has elaborated guidelines for PCa treatment (see also Table 3).

Treatment	Watchful Waiting	Active surveillance	RP	Low dose BT	RT
Low-risk	X	X	X	X	X
Intermediate-risk			X		X
High-risk			X[1]		X
Very high-risk			X[2]		X

Table 3 – Treatment options for PCa patients in relation to risk according to the EAU guidelines. [1]For selected patients with low volume, high-risk, localized PCa (cT3a or Gleason score 8-10, or PSA >20 ng/ml. [2]For highly selected patients with very high-risk, localized PCa (cT3b or T4 N0 or any T1) in the context of multimodality treatment.

The patient with a newly diagnosed PCa should have a life expectancy for at least 10 years to be eligible for a radical and curative treatment (88).

For many years a so-called "**watchful waiting**" (WW) strategy regarding PCa treatment has been practised. During WW patients are observed and treatment is directed towards symptoms. This option is practised in patients who have a localised and slowly growing cancer or patients who are older and comorbid, and have a limited life expectance (89). For patients randomized to either WW or radical prostatectomy (RP) there was not seen any difference in overall survival or metastases free survival for men older than 65 years (90). Thus, a substantial proportion of patients with PCa who are treated are overtreated, since many of the patients never would develop any life threatening PCa disease (91).

In several studies the lead-time, i.e. the time from discovering PCa disease, has been estimated to be about 10 years (91, 92). Due to detection of early PCa, i.e. low volume and low grade PCa, through PSA screening, **active surveillance** or active monitoring has emerged as a treatment option during the last decade (93, 94). In an active surveillance program PCa are followed with PSA measurements were patients are monitored with repeat clinical staging, i.e. DRE, PSA measurements for establishment of PSA doubling time, and eventually repeat prostate biopsies are performed to monitor the histopathological PCa grade (93).

For patients with PCa there are in general terms two main "gold-standard" radical or curative treatments: Surgical and radiation therapy. Until these days there have never been performed any randomized control trials to compare radical prostatectomy (RP) and radiation treatment with regard to long-term oncologic results.

Low-risk PCa: Patients with low-risk and intermediate-risk PCa should be informed of the results of active treatment vs. side effects of treatment and the randomized control trial comparing radical prostatectomy and WW (95). It has to be taken into account that the survival benefit is equal in these risk groups at nine years of follow-up, and that the numbers to treat is 1 in 15 overall and 1 in 7 in men younger than 65 years. Patients can be offered treatment with either radical prostatectomy, i.e. surgical complete removal of the prostate.
Patients can also be offered low-dose brachytherapy with perineal permanent implantation of iodine-125 or palladium-103 (87). The implanted dose seems to impact on the recurrence rate, and one study has demonstrated a significant benefit in treating patients with doses of >140 Gy in a four year follow-up (96). Use of neo-adjuvant or adjuvant ADT did affect results (97). This treatment option is not present in Norway, but in other European countries and also the United States.

Patients might be offered EBRT, and a minimum dose of 74 Gy is recommended (87). It seems that higher doses up to 79.2. Gy even improve long-term EBRT results in low-risk patients (98). A dose escalation to 78-80 Gy seems to achieve the same effects as lower radiation doses in combination with ADT (99-101). The last decade improved EBRT with the use of intensity modulated external beam radiotherapy (IMRT) and also three-dimensional conformal radiotherapy (3D-CRT) has been introduced. These days IMRT is regarded the gold standard radiotherapy for PCa (87).

Intermediate-risk PCa: Radical/curative treatment should be offered to these patients since disease specific mortality is decreased significantly if patients are treated with radical prostatectomy (95). If the estimated risk for positive lymph nodes exceeds 5% ePLND should

be performed (102). 15-year cancer-specific survival ranges from 85% to 91% in low- and intermediate risk patients (95, 103).

Several studies have analysed the effect of neo-adjuvant and adjuvant ADT in combination with EBRT in intermediate-risk PCa, and concluded that long-term oncological results are improved with this combination treatment. Therefore ADT is now recommended as neo-adjuvant and adjuvant therapy in conjunction with EBRT (104). The length of ADT as neo-adjuvant and adjuvant treatment is practised differently at various oncologic departments.

High-risk PCa: According to the EAU guidelines RP is the reasonable first step in selected patients with low tumor volume disease (87), i.e. localised high-risk disease. For patients with locally advanced disease EBRT combined with ADT is often the treatment of choice. Due to higher numbers of positive resection margins, lymph node metastases and distant relapse urologist have been reluctant to treat this group of patients (105, 106). However, no trial with EBRT has ever been shown to be superior to RP (107). The 10-years cancer-specific survival for patients with high-risk PCa operated with RP has been reported to be in the range from 57% (108) to 92% (109).

Patients with short-life expectancy, and metastatic PCa disease:

The dependence of prostate cells on testosterone to stimulate growth and function is well known. The effect of testosterone depletion from the body was demonstrated already in 1941 by Huggins and Hodges (110). One option is surgical castration, or as has evolved through the recent decades, chemical castration with LHRH-agonists. Hormonal treatment palliates symptoms of advanced PCa disease there is no evidence that it prolongs life.

Patients with a limited life expectancy, either due to age or comorbidities, are often recommended hormonal treatment when diagnosed with locally advanced PCa. Patients with both symptomatic and asymptomatic metastatic PCa disease are recommended ADT (87).

2.0 Preparation of Radical Prostatectomy specimens

The histo-pathological report of the radical prostatectomy specimen is meant to give definite information of the type of carcinoma, i.e. grade of the cancer, the stage, i.e. extension of the carcinoma disease with respect to the boundaries, and the surgical resection margins. The prostate is fixed in 10% buffered formalin for two to three days. The prostate is sliced according to a standardized protocol, and one of the protocols widely used has been described by Bennett et al (111). The prostate is paraffin embedded, and 5 μm thick slices are processed for hematoxylin-eosin staining.

Chapter 2 – Cancer Stem Cells (CSCs)

The concept of CSCs

CSC is defined as a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor (112). CSCs are distinctly rare populations found within tumors. According to this concept, CSCs have the normal SCs properties such as self-renewal and differentiation, in addition to the capability to initiate new tumors. Actually, the concept of CSC is firstly characterized in hematological malignancies where cell lineages are clearly defined and classified, and it was firstly identified in acute myeloid leukemia showing that the isolated subpopulations of $CD34^{+}/CD38^{-}$ in acute myeloid leukemia were able to initiate tumors in NOD/SCID mice with the similar histology to donor (113). Various solid tumor CSCs have also been reported during the past few years (36, 114-118).

Properties of CSCs

According to the CSC concept, CSCs should have the following features: 1): Normal stem cell properties like capabilities of self-renewal and differentiation, and keeping in a relatively dormant status when the situation requires. 2): Tumorigenic ability that is the main difference than the normal stem cells. 3): Therapeutic resistance, a definition developed during the past years CSC research and discussions (119-121).

The self-renewal is a hallmark of CSCs through which identical daughter cells with the same biologic properties as the parent cells during cell division are generated (122). The attribution of self-renewal is especially crucial for tumorigenesis and tumor development (123, 124). Accordingly, the major difference of cancer growth from normal tissue is that cancers with

disorder function mostly fail to expand normally and undergo maturation arrest (125). Normal SCs can modulate and balance between self-renewal and differentiation during different development process, whereas cancer could be considered to be a disease with dysregulated self-renewal. However, it is still largely unknown about the dysregulated self-renewal in CSCs. Functionally, self-renewal *per se* in the CSCs and normal SCs should be largely similar.

Like normal stem cells, CSCs are also able to differentiate (112, 126, 127). Under special conditions, epithelial originated carcinomas may manifest sarcoma features, and in special condition sarcoma may originate from epithelial cells as well. The hallmark of the tumor cells differentiation capability may reflected by their histological grade, a status revealing whether the tumor cells, in general, resemble the rather progenitors or the rather mature cells in the corresponding cell and tissue type. Heterogeneity is another common feature for tumors, in consideration of differentiation capability. It has long been known that tumor cells display disordered differentiation. For example, squamous cell carcinoma may have tumor cells with adenocarcinoma features, and vice versa.

Theoretically, CSCs preferentially exist in a quiescent status similar to their normal SCs and divide infrequently unless activation. Clinically, this theory is supported by the fact that most chemotherapeutic reagents targeting dividing tumor cells fail in killing CSCs (124, 128, 129). It should be noted that CSCs could be in a dormant status or proliferative stage, depending on the niche situation. It is well known that all the aggressive tumors may manifest a “disease free” period followed the removal of primary tumor and combined adjuvant therapy. During this period, the micro-metastatic tumor cells hidden in bone marrow or other places in the body are in dormant status, remaining a great clinical challenge for curing of tumors. And when the situation permits, these dormant tumor cells are activated and begin to proliferate,

although the mechanism about exactly when and how these tumor cells are provoked is not understood.

Origin of CSCs

The origin of CSCs has been a hot topic in the CSC research community. Theoretically, CSCs should be able to originate from normal stem cells, progenitor, or differentiated cells. In order to avoid confusion about the origin of CSCs, these cells are termed as "tumor-initiating cells" or "cancer-initiating cells", but not "cancer stem cells". It has long been illustrated in literature that tumors may originate from the transformation of normal cells through the accumulation of genetic modifications. However, it is worthy of notice that not all normal cells can be transformed. From the pathological point of view it is understandable that all the cells in the glandular epithelium may be transformed, but the superficial layer cells, or called keratinized cells in the squamous cell epithelium should not be possible to have such transformation, since these cells are already in the late apoptotic status (Figure 3).

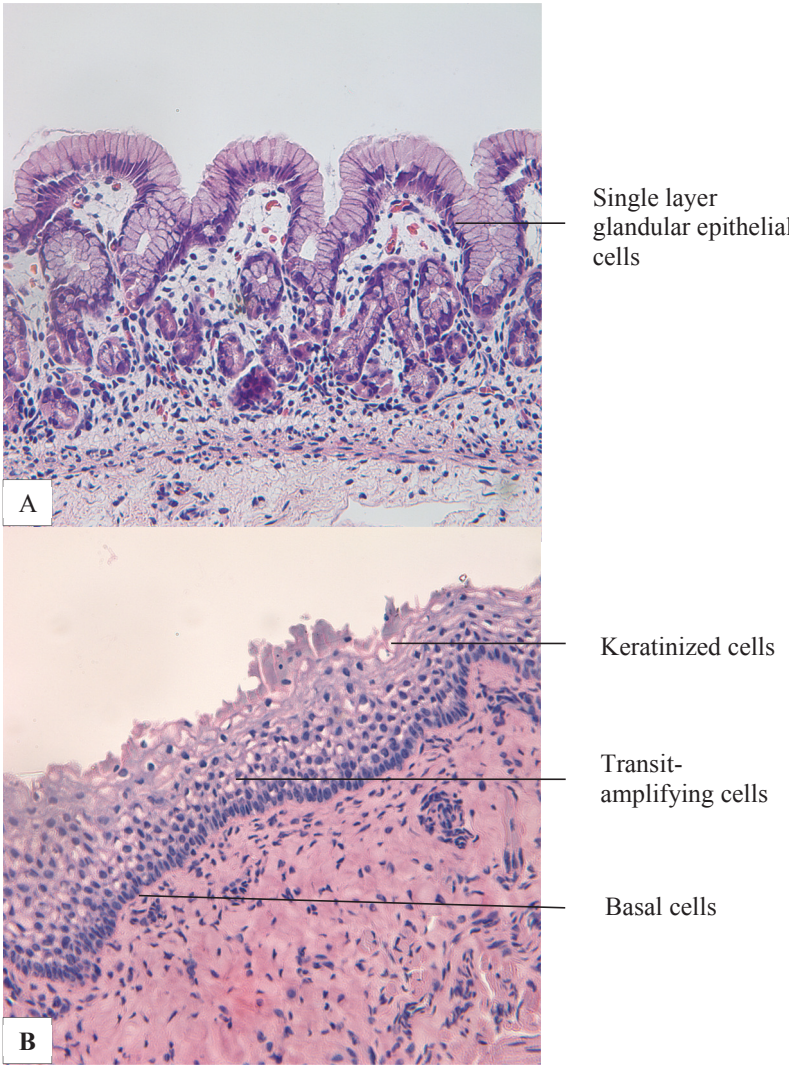

Figure 3: The single layer glandular epithelial cells (A) share similar morphology, and all these cells may be transformed when facing carcinogenic attacks. The basal layer cells in the squamous cell epithelium (B) morphologically mimic stem cells with less cell organelles and smaller in size. It has been documented in literature that it is this cell layer containing normal stem cells. The transit-amplifying cells may also be transformed since these cells keep active

proliferating activity. However, the keratinized cells in the super layers of the squamous cell epithelium should not have the possibility of transformation, because the cells in these layers have non-recoverable apoptosis. All photos are taken in 40x magnification.

Therefore, it is reasonable to believe that CSCs are derived from normal stem/progenitor cells at least for the squamous epithelial cells. As illustrated in the Figure 3B, the progenitor cells can be found in the transit-amplifying cells or in the basal layer cells, but the stem cells should be only identified in the basal layer cells. However, for other non-epithelial tissues it may be difficult to judge where the stem cells or progenitors may exist. Dysregulation of self-renewal/proliferation and differentiation in stem/progenitor cells may trigger carcinogenic process that may result in tumor formation. It is well documented that oncogene activation or suppressor gene inactivation may dysregulate cells´ self-renewal capacity and carcinogenic (130). In supporting this assumption, Cozzio et al. have shown that transient repopulated progenitor cells can initiate myeloid leukemia in response to a mixed lineage leukemia (131).

Microenvironment of CSCs

CSCs, similar to the SCs, preferentially reside in a distinctive and specific niche for maintaining their unique properties. Actually, niche is a phrase loosely used in the stem cell research field referring the microenvironment in which SCs locate. It is documented that microenvironment factors in the niche interact with SCs to regulate stem cell fate. The well studied niche factors include oxygen, growth factors, cytokines, chemokines etc. It has been revealed during the past years that cellular pH, ionic strength (e.g. Ca^{2+} concentration) and metabolites like ATP, ADP etc are also important niche factors for stem cell molecular and biological regulations. All these factors are either already confirmed or in extensive studies now for the CSCs. Low levels of oxygen are confirmed to be an important factor in cancer

cell stemness maintaining and up-regulation (132, 133). Within the human body, SC niches maintain adult stem cells in a quiescent state, but after tissue injury, the surrounding micro-environment factors may actively signal to stem cells to either promote self renewal or differentiation to form new tissues. Some cytokines are important for upregulating of PCa cells *in vitro* (134). The niche factors, especially those factors produced by stromal cells, are in focus for CSC research now (135, 136).

Immuno-editing of CSCs

The concept of immuno-surveillance of tumors dominated in the immunology of cancer study during the past decades (137). Increasing evidence reveals drawbacks of this conception and this conception is evolved to a new term cancer immuno-editing (138-142). The cancer immuno-editing concept is supported by strong experimental data derived from murine tumor models and from human cancer studies. The main point of this new concept is that the immune system not only protects the host against development of primary carcinogenesis but also sculpts tumor immunogenicity. According to this new concept, cancer immuno-editing is referred to a process of three phases: elimination (i.e., cancer immuno-surveillance), equilibrium, and escape (139-142). It is clear form the new concept that the cancer immuno-surveillance is only one step of the three cancer immuno-editing phases. Since CSC may be the core issue of tumors, immuno-editing of tumors may also fit CSCs.

Immuno-editing of CSCs is still in its early stage of study, since many issues around CSC and tumor immuno-editing are either unknown or debating. As mentioned earlier, although there is great progress in CSC study, many key issues around CSC are not solved. For example, searching for universal CSC makers has been a focus for many years. However, many potential CSC markers are either only conditionally verified, or still debating. CD133 protein

is often positively detected in different types of potential CSCs, but CD133 positive cells are also reported in some studies to be non-CSCs as well. Therefore, a speculation is presented that CSC may be a transient status of cancer cells (143).

However, one of the important issues in CSC immunological studies is still how CSCs can effectively evade host immune surveillance. Increasing evidence strongly suggests that CSCs are significantly associated with tumor progression and therapy resistance. Although the question how CSCs evade immuno-surveillance remains, interesting progress is already made during the past studies. It is clear that the mechanisms of CSC immune recognition and their consequent immunological destruction are actively disturbed by a number of processes, such as altered immunogenicity of CSCs, production of CSC-derived regulatory molecules, and interaction of CSCs with tumor-infiltrating immune cells.

Stem cell and CSCs in PCa

It has been demonstrated in animal experiments that prostate tissue regresses and regenerates following depletion of testosterone or restitution of testosterone levels, respectively (144). It has therefore been speculated that prostate stem cells constitute the basic pool of cells responsible for generation of more mature prostate cells. Prostate stem cells are thought to differentiate into three distinct types of prostate cells, i.e. basal, luminal and neuroendocrine cells (145). It is thought that these three cell types constitute the different histological/morphological epithelial compartments of the prostate. The basal cells and neuroendocrine cells are thought to constitute the basal layer of the prostate gland, whereas the luminal cells constitute the luminal layer/compartment of the prostate. It is further thought that these cells differentiate to more mature cells. In other words these three types of cells might be/give rise to differentiated stem cells with different kinds of cell characteristics and

biological behavior, see also Figure 4. Further research during the last decade has identified several cell surface markers as potential candidates for selection of stem cells. Prostate cells have been sorted with fluorescence-activated cell-sorting based on expression of several cell surface markers. The sorted cells were shown to grow *in vitro* in prostaspheres or spheroids. Markers used for selection of such populations include α2β1 (146), CD133 (147), CD44 (148), Sca-1 (149) and ABCG2 (150).

In 2005 Collins et al. demonstrated the existence of tumorigenic prostate cancer stem cells (151). Based on the prostate stem cell markers CD133, CD44 and α2β1 PCa cells were isolated and shown to have a high capacity of self-renewal. In addition, these cells could differentiate to androgen receptor positive cells. Leong et al. also demonstrated that Sca-1^{+}/$CD133^{+}$/$CD44^{+}$/$CD117^{+}$ cells were able to produce wild-type prostatic acini in the mouse (152).

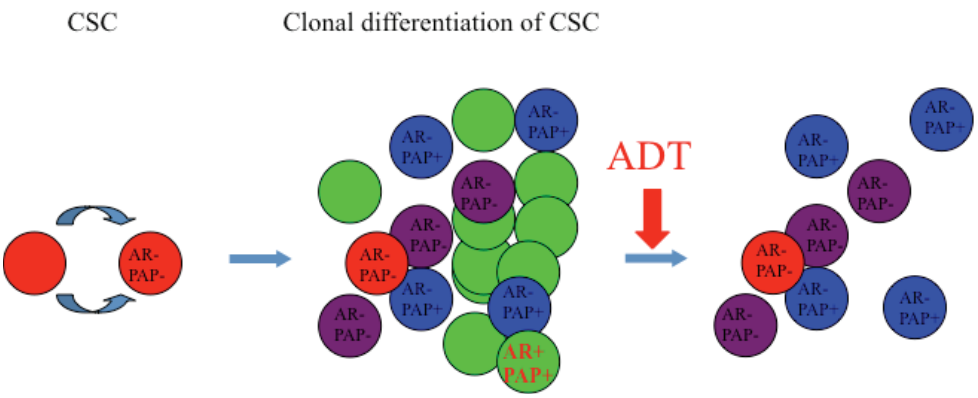

Figure 4.

Schematic overview of a PCa stem cell concept with clonal and hierarchical expansion of cells with different molecular signatures. A majority of PCa cells are initially controlled with ADT, however in the long-term run cancer cells expand due to increased resistance.

Thus, treatment of patients with PCa illustrates a good model for the current treatment options of cancer patients in general. The cancer disease is initially well controlled with ADT. The more differentiated cells expressing AR are targeted with this therapy, however, with time the patients become ADT resistant. Thus, it is becoming increasingly evident that the definitive cancer treatment will be dependent on targeting the CSCs. As a future perspective of the CSC concept increasing research on characterization of patients CSCs signatures and relation of these to clinical outcome will be seen. This will also lead to evolvement of different kinds of treatment depending on these signatures, i.e. personalized medicine.

Chapter 3 – The Immune System and Tumor Immunology

The Immune System

The immune system in vertebrates is composed of the innate and the adaptive, or specific, immune system.

The innate immune system is directed against common foreign structures/molecules not present in vertebrates and is mediated through 1. Natural killer (NK) cells; 2. Phagocytic cells in all tissues, including macrophages, monocytes, granulocytes, dendritic cells (DCs), mast cells; 3. The complement system; 4. Cytokines; and 5. Protection through the skin and mucosal membranes.

Specific immunity in vertebrates is the further development of natural immunity and directed against specific peptides, also called antigens. Specific immunity is mediated through specialized cells, also called lymphocytes or effector cells: B-cells – or antibody producing cells; and – T-cells – $CD4^+$ helper (immune-regulating) cells and $CD8^+$ cytotoxic cells. The action of these cells is tightly regulated in interplay between antigen presenting cells (APCs) and the above-mentioned effector cells. Major interaction between antigen presenting cells and effector cells, and fine-tuning of an immunological response is taking place in secondary

lymphoid organs, i.e. the spleen, lymph nodes and the mucosa-associated lymphoid tissue. Exertion of specific immunity includes rendering of immunologic memory.

The innate and adaptive immune system is among others communicating through innate DCs which can activate T cells.

The immune system also has a self-regulating potential, which secures that immune reactions are aborted once the goal of eradicating a microbe or virus is achieved. On the other hand suppressor functions also guarantee protection against auto-immunity.

Antigens are presented to the immune system on specialized proteins, called the Major Histocompatibility Complex (MHC) from the cell surface. The most potent APCs are the DCs (153), however, also activated macrophages and B-cells can activate the immune system through activation of T cells (154).

Endogenous antigens are presented to the immune cells on MHC class I molecules by well-characterized mechanisms (155, 156). Peptides presented on MHC class I are activating CD8+ T cells through the T cell receptor (TCR).

Exogenous peptide antigens are presented to immune cells on MHC class II molecules. MHC class II is expressed on APCs including DCs, macrophages, monocytes and B cells, although also some epithelial cells and some tumor cells do express that molecule (157, 158). Peptides presented on MHC class II molecules activate CD4+ T cells through their TCR. Exogenous lipid antigens can be presented on CD1 presented by DCs and Langerhans cells, a professional APC mainly characterized in the skin (159). Five types of CD1 molecules are described, CD1a-e (for review, see (160)). It has been demonstrated that antigens presented on CD1 can activate both CD4-CD8-cytolytic T cells (159) as well as invariant NKT cells (161).

T cell activation by DCs occurs in a concerted activation pathway, see Figure 4. Only mature DCs are able to activate T cells (for review, see (162)). Generally immature DCs are located in peripheral tissues adjacent to the bodies' outer borders, e.g. the skin, the mucosa of the

gastro-intestinal tract, and the airways. Inflammation and inflammatory cytokines released trigger chemokine receptors as CCL5 and MIP to trigger recruitment of DCs to the place of inflammation (162). Tissue damage or microbial signals, e.g. specific antigens, or pro-inflammatory cytokines and cell surface expressed activating receptors induce maturation of DCs. Examples of antigens and tissue damage signals inducing DC maturation by activating TLRs or NOD like receptors are LPS, double-stranded RNA and methylated CpG DNA (162-164). Examples of pro-inflammatory cytokines inducing maturation of DCs are TNF-α (165), IL-1β (166) and IFN-γ (167). Example of a cell surface expressed activating receptor on DCs is CD40, which can be provided with its ligand the CD40L present on various immune cells (168). Also NK and NKT cells have the ability to activate DCs (for review, see (169)). Upon maturation DCs up-regulate the chemokine receptor CCR7, and the chemokines CCL19 and CCL21 induce responsiveness, thus inducing migration of DCs to secondary lymphoid organs (170-172).

As DCs mature a number of different cell surface proteins are upregulated, including MHC molecules, and adhesion and co-stimulatory cell surface proteins. This DC cellular phenotype enables interaction with innate T cells and activation of T effector cells, i.e. CD4+ T helper cells- and/or CD8+ cytotoxic cells, memory T cells and suppressor regulatory T cells. General principles of DC and T cell interaction and T cell activation is depicted in Figure 4. The crucial step in this interaction is the recognition of the antigen presented by the DC by its proper T cell bearing the correct T cell receptor (TCR) that recognizes its antigen. This correct antigen recognition secures the immune systems' specificity both as effector cells and memory cells. In this process the CD4 molecule anchors to the MHC class II, whereas the CD8 molecule anchors to its counterpart, the MHC class I. Adhesion molecules interacting are ICAM-1 and LFA-1, and LFA-3 and CD2 (173). Interactions of the co-stimulatory

molecules CD40 and CD40L, and CD80/86 and CD28 or CTLA-4 are securing a cross-talk between these cells rendering an activation and concerted interplay inducing T cell memory cells, T cell effector cells and/or T cell suppressor cells. CD80 and CD86 were initially described as B7-1 and B7-2 and these are present on various APCs including activated B cells (174), activated macrophages (150) and splenic DCs (175). While co-stimulation of T cells by CD80 and CD86 through CD28 activates T cell and is a necessary co-stimulus in addition to signaling through the TCR, interaction of CD80/CD86 with CTLA-4 acts as an inhibitor for T cell proliferation (for review, see (176)).

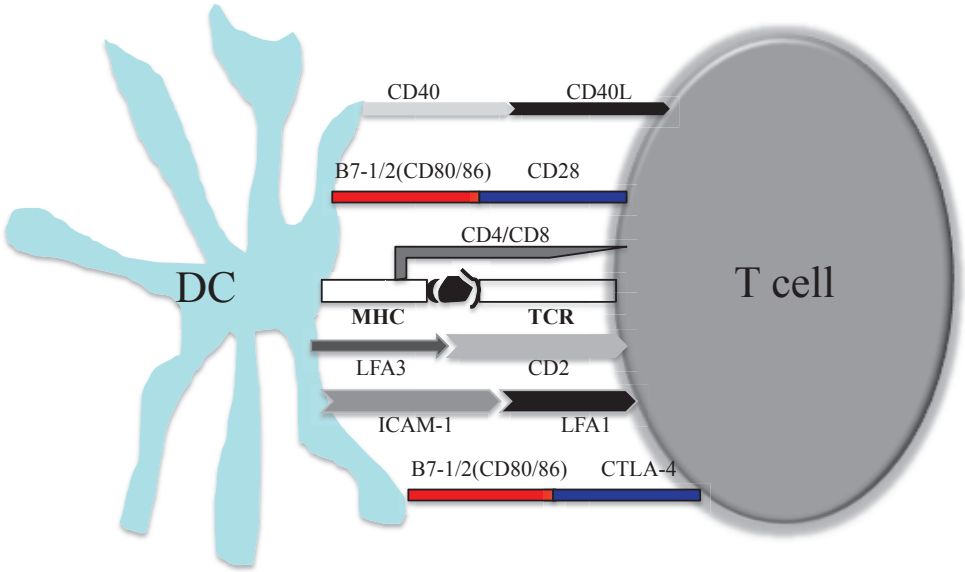

Figure 4. T cell activation by DC. T cell activation requires presentation of an antigen on MHC class I/II and recognition of antigen by the TCR. CD4 or CD8 binds to MHC class I or II. Costimulatory molecules on the DC and the T cell lead to activation and later inactivation as well as immunologic memory in a concerted action.

The above-described mechanisms of T cell activation lead to initiation of memory T cells. Upon eradication of the target, e.g. viral infection or tumor, memory T cells remain in the body for future challenges. Upon new stimulation by the antigen, which the T cell is responsive to, these cells have the property to expand rapidly giving rise to effector T cells and thus act rapidly (177). Both CD4+ and CD8+ T cells are generated as memory T cells. The memory T cells have been described with various cell surface markers including CD45RA, CD45R0, CCR7, CD27, CD28 and CD62L (178). Two different kinds of memory T cells have been described: Effector memory T cells (T_{EM}) and central memory T cells (T_{CM}) (for review, see (179)). While T_{CM} express CD62L and CCR7, the T_{EM} lack these molecules. The presence of these molecules enables the T cells to migrate to lymph nodes, whereas T_{EM} are mostly present in tissues and are able to adopt an immediate T cell effector function as well as the ability to migrate to peripheral tissues.

In order to maintain immunologic homeostasis regulatory T (Treg) cells are also generated during an immune response. Both CD4+ and CD8+ Treg have been described (for review, see (180)). The role of these cells is to mediate peripheral tolerance and protect the body from autoimmune disease. Treg cells have been demonstrated to affect the immune response and to lead to a compromised immune response in both an anti-tumor response as well as in infections (181, 182). Two types of Treg exist: natural Treg (nTreg) and induced Treg (iTreg). The main feature of the nTreg cells is that they express the forkhead box p3 (FoxP3) transcription factor together with CD25 and either CD4 or CD8. The iTreg cells, which are induced in the periphery during the course of an inflammation, do not express FoxP3.

The B7 family of immuno-regulatory cell surface proteins

As mentioned above the B7 cell surface proteins B7-1 (CD80) and B7-2 (CD86) are involved in T cell co-stimulation and cross talk. Following these proteins, also five other members of the family were discovered, and named B7-H1, B7-DC, B7-H2, B7-H3 and B7-H4 (for review, see (183, 184)). Expression of the members of the B7 family of proteins has also been shown to affect immune responses to tumors (for review, see (185, 186)). The molecule B7-H3 had in addition to immune-related features also been shown to have non-immune-related functional properties favoring the metastatic process (187). It has been proposed to divide the B7 family members to three different groups according to their functions (188).

- Group I comprises B7-1, B7-2 and B7-H2. These proteins are involved in T cell activation and inactivation. Whereas B7-1 and B7-2 act through binding to CD28 and CTLA-4, B7-H2 binds to ICOS, a family member of CD28, which is expressed on T and B cells (189-191).

- The group II B7 molecules comprise B7-H1 (PD-L1) and B7-DC (PD-L2), which have been described as ligands of the programmed cell death-1 (PD-1) receptor (192-194). B7-H1 is expressed on various cells and tissues (for review, see (188)); B7-DC is expressed on immune cells (188). The receptor of B7-H1 and B7-Dc is expressed on immune cells, including activated T cells, B cells, DCs, NKT cells and activated monocytes (188). A function of these proteins has been proposed to mediate immune tolerance (195).

- The group III B7 family members comprise the B7 family members B7-H3 and B7-H4. Both proteins are expressed on various peripheral organs and lymphoid tissues (188). Immunologic function of these proteins has mostly been described in inhibition on T cell function. B7-H3 has also been described to inhibit proliferation, cell migration and adhesion in PCa (196) and to affect the process of metastasation in melanoma cells (187). The receptors for B7-H3 and B7-H4 have not yet been characterized very well. Just recently Hashiguchi et al. reported that

B7-H3 bound to the Triggering receptor expressed on myeloid cells (TREM)-like transcript 2 (TLT-2, TREML2) (197).

Tumor Immunology

The interaction between tumor cells and the immune system is quite intricate. On one hand the immune system is trained to respect "self"; when self is not recognized an autoimmune process is started leading to an autoimmune disease. However, the immune system is trained to recognize foreign antigens, e.g. virus and bacteria. The cells of the immune system are also trained to respect "self" and cells are specifically eliminated when they recognize self. It is also recognized that certain immune cells, a sub-fraction of $CD4^+$ T cells have the function to regulate the immune system in order not to let the immune exert a too exaggerated immune response. Thus, tumor cells are often recognized by the immune system if they are induced by virus- but cancer cells that are not, not necessarily display a phenotype, enabling the immune cells to recognize them as foreign. Nevertheless, immune responses are exerted by tumors, and certain types of tumor antigens and T cells reacting to tumors have been identified. Some data also indicate that tumors exhibit a phenotype "repelling" the immune system, by either expressing proteins on their cell surface that "down-regulate" the immune system or produce soluble factors that act in a paracrine fashion and that "down-regulate" the immune system. To describe the exact mechanisms would go beyond the scope of this thesis.

The B7 family of proteins and cancer

Not surprising, since members of the B7 family of proteins are involved in immune regulation, members of the B7 family of proteins are expressed in various hematopoietic malignancies. However, it has been demonstrated, that these proteins are also expressed on various solid cancers whereas they are not expressed in the corresponding normal tissue, so

called aberrant expression (for review, see (188)). Mostly, aberrantly expressed members of the B7 family of proteins on cancer cells are linked to aggressive cancer behavior, poorer survival and more advanced cancer stages.

Of the B7 family of proteins, expression only of B7-H3 and B7-H4 proteins by PCa cells, has been reported (198, 199). Expression of both proteins was analyzed on radical prostatectomy samples and clinical follow-up from American patients. Zang et al. demonstrated that expression of B7-H3 and B7-H4 in PCa was associated significantly with poor outcome, metastatic spread, and increased risk of recurrence and death. Roth et al demonstrated that expression of B7-H3 was associated with poor clinical outcome, disease progression and that expression of B7-H3 in PCa was associated significantly with Gleason score. PCas with higher Gleason scores expressed B7-H3 at a higher level. Later it has been demonstrated that B7-H3 expression is also expressed in metastases from PCas and that B7-H3 expression is not affected by neo-adjuvant ADT in patients operated with radical prostatectomy (200). As in other cancers (187) it has also been demonstrated in a PCa cell line that down-regulation of B7-H3 expression was associated with decreased cell adhesion, decreased cell migration and decreased cell invasion (196).

PART II - THE CURRENT THESIS

Background for the Thesis and General Aims

Prostate carcinoma is a disease with a high variety of clinical outcome. Far from all men diagnosed with this disease will die from it. A proportion of men diagnosed with PCa are bearing on genetic defects, estimated to be approximately 15% (201). From other malignancies it is well known that the immune system can play a role for the outcome (202). Just recently, the United States Food and Drug Administration (FDA) approved the first cancer vaccine treatment for a malignancy, Provenge™ (203). The interaction between PCa cells and the immune system have been explored both in animal studies and in humans, but still many questions are not answered. Do PCa cells exhibit tumor escape mechanisms? Do PCa cells exhibit a cell surface phenotype that directly interacts with the immune system? Do PCa cells secrete substances that influence the immune system negatively in order to escape the host's immune system? Do PCa cells influence regulatory T cells? Can PCa cells be influenced to exhibit a more immunogenic phenotype?

We investigated primarily a potential immune system regulating molecule known from the family of B7 molecules, the B7-H3. Two research groups had independently reported the aberrantly induced expression of B7-H3 in PCa in 2007 (198, 199). They observed that both expression of B7-H3 was correlated to non-favorable clinicopathological parameters and to unfavorable clinical outcome.

The content of the various immune cells in PCa has been investigated to some extent (204). In smaller series it was demonstrated that $CD1a^+$ cells were present in PCa specimen. It was also noted that there were fewer $CD1a^+$ cells in high-grade PCa samples (205).

As the above-mentioned tasks are of scientific descriptive nature, a further aim of this thesis was to investigate the possibility to culture PCa cells *in vitro*. The further aim would be to being able to set up *in vitro* systems to investigate the functional interactions between prostate carcinoma cells and the cells from the immune system.

Specific aims

1. To investigate earlier findings of aberrant expression of B7-H3 in PCa cells in radical prostatectomy specimens from Norwegian patients treated with radical prostatectomy. To investigate the nature of B7-H3 expressing PCa cells with regard to proliferation- i.e. correlation to the proliferation marker Ki-67.
2. To examine primary immune cells involved in tumor immunology in radical prostatectomy specimens. The content of APCs ($CD1a^+$), $CD4^+$ and $CD8^+$ T lymphocytes, were analyzed in radical prostatectomy specimens.
3. To investigate whether the expression of B7-H3 on PCa cells can be regulated.

Material and Methods

Paper I and Paper II

Arendal Hospital located in the South of Norway is a community hospital serving approximately 100.000 inhabitants. It is one of the first Norwegian hospitals implementing radical retropubic prostatectomy (RRP) as curative treatment for localized PCa from 1985. In the period from 1985 until 2003 patients had done frozen sections of the pelvic lymph node staging and if metastatic disease was found a RRP was not performed. From 1985 until 2006 151 patients were operated with RRP and these patients were followed-up with clinical controls and registered prospectively. The corresponding radical prostatectomy specimens were sent to the Norwegian Radium Hospital for histo-pathological evaluation.

Pathology

All the prostates were re-examined by a uro-genital pathologist with regard to Gleason score according to the International Society of Urological Pathology (ISUP) Consensus on Gleason grading (74), surgical margin status, seminal vesicle invasion and extraprostatic extension. The diagnosis of adenocarcinoma was set on H&E stainings.

Tissue micro arrays (TMAs) were prepared with 0.6 mm punches in triplets from matched slides with corresponding tumors in the paraffin blocks. Five μm thick sections from the TMA blocks were cut and the Dako FLEX system (Dako Denmark, Glostrup, Denmark) was applied with an automatic Dako immunostainer. In paper 1 the primary antibodies were the polyclonal goat anti-B7-H3 antibody (R&D systems Europe, Abingdon, UK) and the mouse monoclonal antibody MIB1 (Dako). Both had been tested prior to the experiments and diluted 1:200. The secondary antibodies were a mouse anti-goat IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-mouse IgG monoclonal antibody diluted at 1:100. In paper 2 whole mount sections from radical prostatectomy specimens were immunostained. The primary antibodies CD1a (Novocastra NCL-CD1a-235, Leica Microsystems A/S, Ballerup, Denmark), CD4 (Novocastra NCL-CD4-368) and CD8 (Novocastra NCL-CD8-4B11) were diluted at 1:25, 1:50 and 1:150. Positive and negative controls were tested with satisfactory results. All stainings and cell counts were performed without the knowledge of the patients' clinical outcome. Staining with B7-H3 was grouped into 3 groups regarding staining intensity: group I: No staining/weak staining; group II: Moderate staining; group III: Strong staining. Immunoreactive cells were counted in 10 randomly selected 40x magnification fields. Regarding $CD4^+$ and $CD8^+$ lymphocytes both intratumoral and peritumoral cells were counted.

Statistical analysis

To evaluate the relationship between B7-H3 expression, Ki-67 expression and clinicopathological features the Spearman's rank correlation was used. To evaluate the correlation between CD1a, CD4 and CD8 cells to clinicopathological parameters the number of CD1a cells was dichotomized to a median value (17 cells/10 fields). Continuous data were compared between the groups. Where appropriate the Spearman's rho correlation test, Pearson χ^2 or Fisher's exact test was applied. For survival analyses the Kaplan Meier method and Cox proportional hazards regression was used. A two-sided p-value less than 0.05 was considered statistically significant. All analyses were carried out using SPSS 18.0 (SPSS, Chicago, IL, USA).

Paper III

mtDNA replication blocking and cancer stem cell factors

Human prostate cancer cell lines PC-3 and DU145 (American Type Culture Collection, USA) cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin at 37°C in a humidified incubator of 5% CO_2. To block mtDNA replication, 50ng/ml and 500ng/ml of EtBr (Sigma, St. Louis, MO, USA) were added to the medium and the cells were cultured for about two weeks before harvested for further quantitative RT-PCR or flow cytometry analysis.

Quantitative real-time PCR

Total RNA of cells was extracted using the RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction before RNA concentrations were quantified using a spectrophotometer (Nanodrop ND-1000, USA) at OD260/280 before complementary DNA (cDNA) was subsequently synthesized from 5µg total RNA using the Multiscribe reverse

transcriptase (Applied Biosystems, Foster City, CA, USA). The conditions for reverse transcription were: 25°C for 10 minutes, 37°C for 12 minutes, 85°C for 5 minutes, followed by holding at 4°C. The mRNA expressions of *Nanog1*, *Nanogp8* and GAPDH were measured by quantitative real-time PCR using a Taqman ABI 7900 Sequence Detector System (Applied Biosystems) according to the published literature [20]. The primers and probes for detection of *Nanog1*, *Nanogp8* are: *Nanog1*: forward primer-5′-CGCCCTGCCTAGAAAAGACATTT -3′, *Nanog1*: reverse primer-5′-AGAAGCCGTCTCTGGCTATAGATAA -3′, *Nanog1:* probe-CTGCTAAGGACAACATTGAT; *Nanogp8*: forward primer-5′-CGCCCTGCCTAGAAAAGACATTT-3′, *Nanogp8*: reverse primer-5′-ACGAGTTTGGATATCTTTAGGGTTTAGAATC-3′, *Nanogp8*: probe-CCTTGGCTGCCGTCTCTG. All the primers and probes labeled with FAM-MGB were purchased from Applied Biosystems. The GAPDH quantitative RT-PCR kit (4352934E, Applied Biosystems) was used as an internal control and the Ct values of the cells without EtBr treatment were used as calibrators for evaluating *Nanog1* and *Nanogp8* expression levels in response to mtDNA blocking.

A reverse transcription – polymerase chain reaction (RT-PCR) was performed to verify the presence of stem cell factors in the cell cultures. Stem cell factors to be identified were the transcription factors Oct 3/4 and Sox-2 (206). Primers for the reaction were designed to give PCR products of 297 base pairs (bp) (Oct 3/4) and 75 bp (Sox-2). As a positive control for the reaction the housekeeping gene GAPDH was chosen. The amplified PCR products were separated by 7,5% polyacrylamide gel electrophoresis, stained with Gel Red, and visualized with a Syngene image system (Syngene, Cambridge, UK).

Main results

Summary of the paper and main results

Paper I –

We assessed the expression of the cell surface protein B7-H3 in 130 prostate carcinomas, and its association to clinicopathological parameters after radical prostatectomy. The median clinical follow up was 8 years. We observed a high expression of B7-H3 in pathological stage T3a and T3b, high Gleason score, extraprostatic extension, seminal vesicle invasion and high proliferative activity. In univariate analysis we found that a high expression level of B7-H3 correlated with biochemical failure and clinical relapse, and with the expression of Ki-67. A high expression level of Ki-67 was associated with clinical progression and a tendency towards higher rates of prostate-specific antigen relapse in multivariate analyses.

Paper II –

The amount of cells involved in the anti-tumor immune response was counted in RP specimens. Analyses revealed a correlation of low numbers of $CD1a^+$ cells with high Gleason score and pathological stage T3. The amount of $CD1a^+$ cells was significantly correlated with intratumoral and stromal $CD8^+$ and stromal $CD4^+$ T lymphocytes. The expression of $CD1a^+$ cells and tumoral $CD4^+$ T lymphocytes correlated inversely with B7-H3 expression in PCa cells. Patients with low numbers of $CD1a^+$ cells showed a tendency toward impaired biochemical progression-free survival in Kaplan-Meyer analyses.

Paper III –

Based on indications in the literature and own preliminary tests, we treated both PC-3 and DU145 cell lines with 50ng/ml and 500ng/ml of ethidium bromide (EtBr) for two weeks.

During the first week of culture, cells treated with EtBr grew to 80% confluence in three days for both cell lines, which was similar to the control cells. A slightly slower and dose-independent growth, with 80% confluence in four days, was observed for both PC-3 and DU145 cells during the second week of culture with EtBr. Immunocytochemically, cytoplasmic ABCG2 staining could be identified in the ABCG2 seminoma positive control. Comparatively weak ABCG2 immunoreactivity was seen in the DU145 and PC-3 cell lines cultured in normal medium, while more intensive ABCG2 immunostaining could be observed in the EtBr (500ng/ml) treated cells. For Oct3/4 immunostaining, the normal medium cultured DU145 and PC-3 cells revealed a punctual colorization in the nucleus, while the EtBr treated DU145 and PC-3 cells demonstrated more condensed nuclear staining. For B7-H3, only weak immunostaining could be seen on the cell membrane of the normal medium cultured DU145 and PC-3 cells, while the EtBr treated DU145 and PC-3 cells displayed stronger immunostaining. To verify the immunocytochemical results of B7-H3 expression, flowcytometry was further performed. It was shown that both cell lines revealed a significant EtBr dose-dependent increase of B7-H3 expression ($p < 0.05$). Furthermore, an EtBr dose-dependent increase of CD44 expression intensity was repeatedly observed in these two cell lines ($p<0.05$ for both cell lines). Quantitative RT-PCR revealed an EtBr dose-dependant induction of Naog1 and Nanogp8 was repeatedly demonstrated in these two cell lines as well.

Discussion

Methodological considerations

As the Gleason grading system has changed continuously over time, the RP specimens have been re-evaluated by a dedicated and experienced urological pathologist according to the ISUP Consensus conference in 2005. The resection margins, the pathological stage regarding extraprostatic extension, and seminal vesicle infiltration were also re-examined. Of the 151

patients initially included in the study the diagnosis of PCa could not be given since the carcinoma never could be found. Eighteen of the patients' TMAs could not be analyzed due too limited or missing tumor. Thus 130 patients were included in the studies involving TMA.

The main method used in the studies was immunohistochemical staining of TMAs and whole sections. The TMAs were prepared according to standard protocols in triplets from the index tumor. The antibodies used in the studies were all commercially available, and used under standardized conditions. All antibodies were checked with positive and negative control stainings. For the counts of immune cells it was necessary to stain whole mount sections, which per se gives a better impression of the general topographical location of the immunostained cells. Two investigators without knowledge of clinical data performed evaluation of all immunostaining and cell counting independently. The subjective evaluation of the immunostained sections is always a methodological limitation. But, on the other hand, evaluation of immunostained whole sections provides a possibility to explore the cellular sociology. Hence, we used a graded staining intensity (B7-H3) into "no staining/weak staining"; "moderate staining" and "strong staining" (198, 199). The cytoplasmic/membraneous B7-H3 staining were evaluated. A Ki-67 staining score into three groups depending on percentages of Ki-67 positive nuclei was applied (207). In order to achieving a more complete picture and precise cell count whole mount sections from the corresponding patients from whom TMAs had been analyzed for B7-H3. In this case whole mount sections from 118 patients had been analyzed, since there was limited or missing tumor in thirty cases. Cell counting of $CD1a^+$ Langerhans/dendritic cells, $CD4^+$ and $CD8^+$ T lymphocytes was performed by counting of 10 randomly chosen fields at 40x magnification. The cut-off into low/high numbers of $CD1a^+$ cells was set statistically using the Mann-Whitney *U* test.

In order to explore whether cancer cell stemness is influenced by mitochondrial function, we treated the prostate cancer cell lines, PC-3 and DU145, with EtBr for two weeks. Analysis of stemness related genes demonstrated significantly higher levels of ABCG2, Oct3/4, Nanog and CD44 expression after EtBr treatment. Since all of these factors are closely related with cell stemness, or cancer stem cells, the increased expression of these factors may indicate an upregulation of stemness of these cancer cells in vitro after mitochondrial function blocking.

ABCG2 expression has been associated with cancer stem cells' multidrug resistance. In line with our present finding, it has been reported that a human hepatoma cell line SK-Hep1 became resistant to both doxorubicin and cisplatin treatments when the mtDNA was depleted by 100ng/ml EtBr treatment for at least 20 generations (208). It has also been documented that mtDNA depletion could induce radioresistance in human pancreatic cancer cells (209). Oct3/4 and Nanog play an important role in the transcriptional network for maintenance of embryonic stem cell and primordial germ cells self-renewal (206, 210). In our current study, blocking mitochondrial function by blocking mtDNA replication induced the expression of Oct3/4 and Nanog in the prostate cancer cell lines PC-3 and DU145, which is in line with our previously reported results on hypoxia influence in these cell lines (133). Oct3/4 and Nanog are considered as markers for undifferentiated cells and play an essential role in sustaining capacity of self-renewal in adult stem cells (211, 212). It has been shown that invasive tumor cells acquire a stem-like genomic signature expressing a number of stem cell genes, including Oct3/4 and Nanog. These cells are more tumorigenic compared to their non-invasive counterpart (213). Therefore, our results may indicate that blocking mtDNA replication in prostate cancer cells upregulates the cell stemness and endorses the cells with higher cell stemness. In support of this, it has been shown in a study by Cheon et al (214) that loss of

mtDNA enhances the angiogenic and invasive potential of hepatoma cells. In these cells, blocking mtDNA replication induced the expression of HIF-2α and consecutively the expression of vascular endothelial growth factor (VEGF). It should be explored in the future whether similar signaling pathways are triggered in the prostate cancer cell lines upon mtDNA replication blocking.

Main results

During the last decades the field of tumor immunology has evolved rapidly. Immunology is one of the research fields that have had major impact on development and implementation of molecular biological techniques. The reason is probably the ease to study cells, as these are easily accessible. It has been increasingly evident that the immune system plays an important role in tumor development. The concept of immunoediting has been launched (for review, see (202)). The pillars in this concept are i. elimination or cancer immuno surveillance; ii. equilibrium or expansion of carcinoma cells held under control of the immune system and iii. escape of carcinoma cells and expansion of these.

In Paper I we studied B7-H3 - a family member of the B7 molecules – and involved primarily in immunological cross-talk. In urologic carcinomas B7-H3 was discovered to be aberrantly expressed in renal and prostatic carcinomas. These findings were described in an American based population (198, 199). It was therefore important to validate the findings in a homogeneous Norwegian population. We have demonstrated that the more aggressive prostatic carcinomas express higher levels of B7-H3 making it tempting to suggest that this molecule contributes to breaking the equilibrium of a carcinoma and/or to the escape of the carcinoma. The significant association between B7-H3 and Ki-67 expression suggests that

B7-H3 could contribute to the malignant phenotype of the prostate carcinoma rather than displaying a separate carcinoma entity. Thus, the expression of the B7-H3 molecule could be a strong feature/indicator of a slow cycling or growing tumor. Therefore, changes in the expression levels of the B7-H3 molecule could contribute to the equilibrium and escape of carcinoma cells in the context of immunoediting. One limitation of the study is the relatively low number of 130 patients included. However, the hypothesis of a subgroup of slow cycling carcinoma displaying a B7-H3 phenotype could still be valid.

The median follow-up time of 7 years of the patient cohort analyzed in this study might constitute another limitation of the study. PCa is generally a slowly growing cancer type, also demonstrated in the PSA screening trials (39).

Immune cell infiltration has been studied in a variety of tumors previously (for reviews, see (204)). However, in PCa only a few studies have been performed with regard to immune cell infiltration, clinicopathological parameters, biochemical recurrence free period and survival (204). In an early study by Bigotti et al from 1991 a possible relation of DC infiltration in PCa, and putatively lower number of DCs in high-grade PCa has been described (215). The antibody used in that study was directed against protein S-100, a marker that is not specific for DCs. In 1998 Troy et al. identified DCs in fifteen PCa cases (205). The group reported a lower number of DCs in PCa compared to normal prostate tissue, in contrast to our findings in Paper II.

An interesting finding in our study is the observation of a continuous decrease in numbers of $CD1a^+$ cells to a progressively aggressive carcinoma phenotype. As the Gleason grading

system mirrors the aggressiveness of PCa, this tumor might be a good model to study biodynamic remodeling of the concept of immunoediting.

The concept of immunoediting is further strengthened by our finding of a significant correlation of $CD1a^+$ DCs to the numbers of stromal $CD4^+$ and intratumoral and peritumoral $CD8^+$ T lymphocytes. Furthermore, numbers of $CD1a^+$ cells were found to be significant inversely correlated to the expression of B7-H3 by PCa cells, i.e. in relation to Gleason score. It has been proposed earlier that B7-H3 is a negative regulator of the immune response in cancer (199, 216). The observation of an inverse relationship between $CD1a^+$ cells, $CD4^+$ and $CD8^+$ T lymphocytes on one hand and B7-H3 expressed by PCa cells on the other hand is a strong indicator of interference of the immune response to cancer. Thus, our findings support the hypothesis that a local anti-tumor response is generated at the tumor site and immune cells are directly affected by a progressive carcinoma phenotype. Thus, one could foresee affection of the equilibrium in terms of immunoediting in the low-grade PCa from high immune cell numbers to escape in high-grade PCa with low immune cell numbers.

We did not observe any significant correlation between immune cell infiltration and biochemical-free survival or PCa specific survival. However, a trend towards improved biochemical-free survival for patients with high numbers of $CD1a^+$ cells was noted. In this context the median follow-up time of 8 years might be a factor of limitation of this study. We would like to speculate that a longer follow-up time would give a clearer result with respect to giving a significant improved biochemical-free survival in patients with high numbers of $CD1a^+$ cells.

In view of the potential malignant contribution of B7-H3 expression is was important to explore if B7-H3 expression in PCa is static or if it could be altered. In Paper III, we show that blocking mtDNA replication could induce the expression of B7-H3, which was verified by both immunocytochemistry and flow cytometry. It has been shown that B7-H3 is expressed in melanoma (187), breast cancer (217, 218), osteosarcoma cells (219) and prostate cancer (198, 199), and its expression may be associated with tumorigenesis. A functional study has shown that siRNA-downregulation of hB7-H3 reduces cell adhesion to fibronectin of melanoma and breast cancer cells by up to 50 %, and migration and matrigel-invasion by and breast cancer cells by up to 50 %, and migration and matrigel-invasion by more than 70%. Most importantly, it is recently shown that B7-H3 expression in tumor and endothelial cells correlates with the grade of malignancy and with poor survival in gliomas (220). Both soluble 4IgB7H3 in the supernatant of glioma cells and cell-bound 4IgB7H3 are functional and suppress natural killer cell-mediated tumor cell lysis. Gene silencing showed that membrane and soluble 4IgB7H3 convey a proinvasive phenotype in glioma cells and glioma-initiating cells in vitro, strongly indicating its immunosuppressive and proinvasive function (220). Tumor cell immunosuppression has been shown to be a key issue of cancer stem cell. Our finding of increased expression of B7-H3, together with the increased expression of stemness factors in prostate cancer cells after mitochondrial function blocking may indicate the involvement of B7-H3 in cancer stem cell immunosuppression.

Conclusions

Paper I –

- Previous findings of aberrantly expressed B7-H3 in American PCa patients with high-grade and locally advanced PCa were validated in Norwegian PCa patients.
- Expression of B7-H3 was significantly associated expression of the proliferation marker Ki-67.
- In a univariate analysis expression of B7-H3 was correlated with biochemical–free survival and clinical progression.

Paper II –

- The number of CD1a expressing cells, a cell surface molecule that present lipid antigens to the immune system and expressed on LCs or DCs, is associated with Gleason grade/score. Low numbers of $CD1a^+$ cells are associated with high-grade and locally advanced (pT3) PCas, whereas a high number of $CD1a^+$ cells are associated with low-grade PCas. Benign prostate tissue contains a low number of $CD1a^+$ cells.
- Numbers of $CD1a^+$ cells is correlated with stromal $CD4^+$ and stromal and intratumoral $CD8^+$ T lymphocytes.
- The number of CD1a cells is significantly and inversely correlated to the expression of B7-H3, a member of the B7 family of immunologically active cell surface proteins and aberrantly expressed on PCa cells.
- A tendency towards improved biochemical-frees survival was noted in patients with a high number of $CD1a^+$ cells.

Paper III –

- Inhibition of mtDNA replication with ethidium bromide up-regulates stemness-related genes ABCG2, Oct3/4, Nanog1 and Nanogp8 in the PCa cell lines PC-3 and DU145.
- mtDNA inhibition also lead to an up-regulated B7-H3 expression in the PCa cell lines PC-3 and DU145.

Future perspectives

The major finding presented is the dependence of numbers of immune cells, i.e. LCs, and $CD4^+$ and $CD8^+$ T lymphocytes, on the grade of the prostatic carcinoma. High-grade and locally advanced PCas contain a lower number of $CD1a^+$ cells, whereas normal prostate tissue contains low numbers of these cells. In other words, it seems as aggressive PCas influence the immune system in a negative way. One could hypothesize that PCas secrete paracrine substances "repelling" immune cells. It is known that carcinomas may predispose an environment favoring the induction of regulatory T cells as has been demonstrated in aggressive breast carcinomas (221). Aggressive PCas could also impair the function of LCs either through autocrine actions or through direct cell-cell interactions, such as we hypothesize that B7-H3 does.

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Errata

1. There is a typing error in Paper I, in Table 1: Gleason score >7 should be replaced by ≥8.
2. There is a typing error in Paper I, in Figure 3, the figure text: "(a) Little, (b) moderate and (c) strong immunostaining intensity." Should be replaced with Ki-67 staining score: (a) no immunoreactive cells/<10% positive cells; (b) 10–50% positive cells; and (c) >50% positive cells.

I

II

III