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Identification, properties, and clinical significance of putative stem-like cell populations in prostate cancer

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“The whole of science is nothing more than a refinement of everyday thinking”

Albert Einstein

This work was carried out in collaboration with the department of Urology and the Institute for Pathology from the Basel University Hospital.

My thesis consists of an **introduction** covering several aspects related to my work followed by a result section which is composed by unpublished results (**Chapter I**), an accepted publication (**Chapter II**), and a submitted manuscript (**Chapter III**).
Major **findings and perspectives** are summarised and discussed in the last section.
Every section is concluded by its own list of references.

Abstract

The notion that tumour initiation and heterogeneity might be driven by small population of tumour-initiating cells (TIC) has gained high significance since the pioneering identification of TIC in leukaemia. This has led to a worldwide research effort to further identify TIC in solid tumours.

In prostate cancer (PCa), however, demonstration of the existence and identification of TIC have been hampered by a lack of consistent *in vitro* and *in vivo* models. **Chapter I** of this thesis presents several models of human PCa and their respective significance to study TIC in PCa. This chapter also describes my attempts to establish more relevant models by generating and characterising short-term primary cultures derived from clinical PCa specimens.

TIC are thought to share some properties of normal stem cells and to express genes typically expressed by embryonic stem cells. Based on this hypothesis, I investigated the expression of stemness-associated genes in PCa. Findings are presented in **Chapter II** of the thesis and described in the published manuscript entitled *"Klf4 transcription factor is expressed in the cytoplasm of prostate cancer cells"*.

Additionally, same as normal stem cells, TIC might display high activity of aldehyde dehydrogenase (ALDH) enzyme. **Chapter III** reports the characterisation of a cell subset exhibiting high ALDH activity in PCa. In particular, features, prevalence and clinical significance of these cells in PCa are presented in the manuscript entitled *"Characterization and clinical relevance of ALDH^{bright} populations in prostate cancer".*

Taken together, my thesis highlights the complexity of the TIC concept and the urgent need for more accurate models, paving the way for further studies aiming at identifying TIC in human PCa.

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Introduction

I. Prostate biopathology

1. The normal prostate gland

The prostate is a walnut-sized male gland localised at the base of the bladder and surrounding the urethra. Its most important function is the production of main components of the seminal fluid. The human prostate is composed of distinct tissue zones, defined as the central, transitional, periurethral, peripheral, and fibromuscular zones [1]. Importantly, these distinct zones can harbour different pathologic processes (Figure 1 and cfr part I.2). At the histological level, the prostate gland is composed by a pseudo-stratified epithelium surrounded by stromal tissue. More precisely, the prostatic epithelium comprises three types of cells referred to as basal, luminal and neuroendocrine cells ([2] and Figure 2). In particular, basal cells, directly contacting the basement membrane, are characterised by the

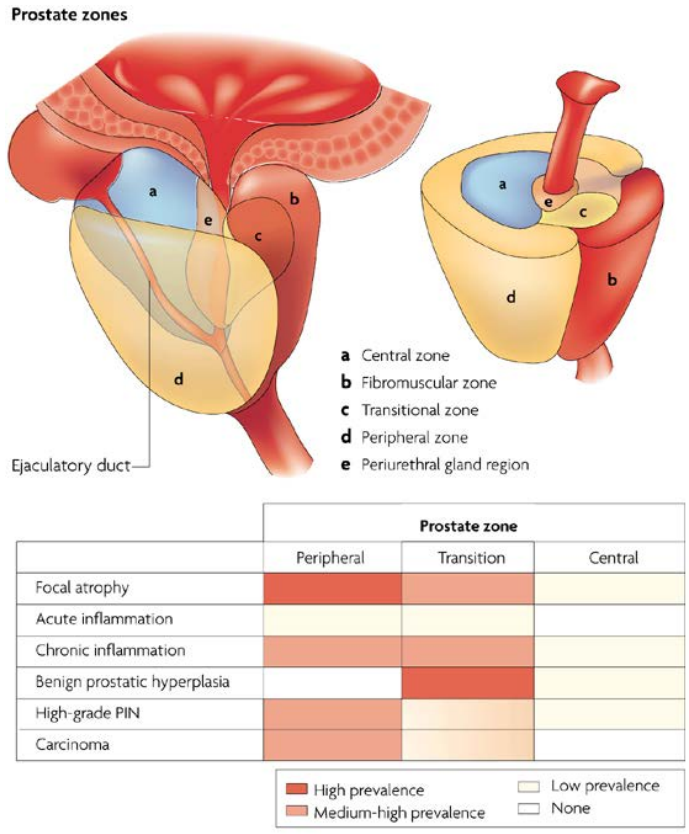

Figure 1: Zones of the prostate and predisposition to prostate disease

Most cancer lesions occur in the peripheral zone of the gland, fewer occur in the transitional zone and almost none arise in the central zone. Most benign prostate hyperplasia (BPH) lesions develop in the transitional zone. PIN: prostatic intraepithelial neoplasia. *Adapted from de De Marzo et al., Nature Reviews Cancer 2007* [1].

expression of epithelial-specific cytokeratins such as CK5 and CK14 as well as other markers such as p63 [3,4]. Luminal cells are terminally differentiated secretory cells positive for both cytokeratins CK8 and CK18 and highly expressing the androgen receptor (AR) [3]. Finally, neuroendocrine cells (NE) are characterised by the expression of neuroendocrine factors such as chromogranin A (CgA) and synaptophysin A. NE cells are thought to be AR negative and their origin as well as their role in normal prostate development and pathogenesis are unclear [5].

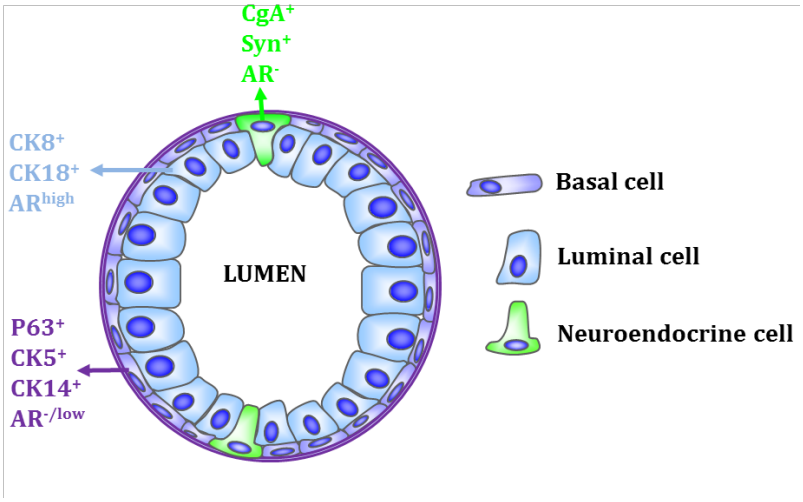

Figure 2: The prostate epithelium

The normal prostate epithelium is composed of basal cells, secretory luminal cells as well as rare neuroendocrine cells. Phenotype of each cell population is also indicated. AR: androgen receptor; CgA: chromogranin A; Syn: Synaptophysin; CK: Cytokeratin.

The stromal compartment of the normal prostate is mainly composed of smooth muscle cells and fibroblasts as well as endothelial cells, nerves, and immune cells. The prostate represents a good example of an organ that relies on its surrounding stroma for its development. Indeed, stromal and epithelial cell interactions are required for prostate growth and maintenance of its functions under the influence of androgens [6].

2. Pathologies of the prostate

The prostate is the site of several pathologies, mostly occurring in ageing men. Among the most common prostatic diseases, **benign prostatic hyperplasia**

(**BPH**) and **prostate carcinoma** (**PCa**) mainly arise in the transitional zone and the peripheral zone, respectively (Figure 1).

2.1 Benign prostatic hyperplasia

Benign prostatic hyperplasia (**BPH**) is characterised by a benign enlargement of the transitional prostatic zone. It is the most frequent benign neoplasm in ageing men and one of the most common chronic conditions in the male population. The histological prevalence rises from 50% in men aged 50–60 years to over 90% in men over 80 years. Histologically, BPH is characterised by an androgen-dependent tissue remodelling that involves both epithelium and fibromuscular stroma. Clinically, BPH is often associated with lower urinary tract symptoms. [7,8]. Even if BPH is not considered as a precursor of PCa, the relationship between BPH and PCa is highly debated [9].

2.2 Prostatic intraepithelial neoplasia

Prostatic intraepithelial neoplasia (**PIN**) is generally considered as a precursor of prostate cancer [10]. Histologically, PIN lesions are characterised by an expansion of luminal cells and a perturbation of the basal layer, as revealed by the alterations in expression of specific markers (importantly, basal cells decrease in number but do not disappear in contrast to prostate cancer, see Figure 3). Some of the key morphological features characterizing PIN also include changes in nuclear and nucleolar morphology, as well as in the chromatin structure [2,11].

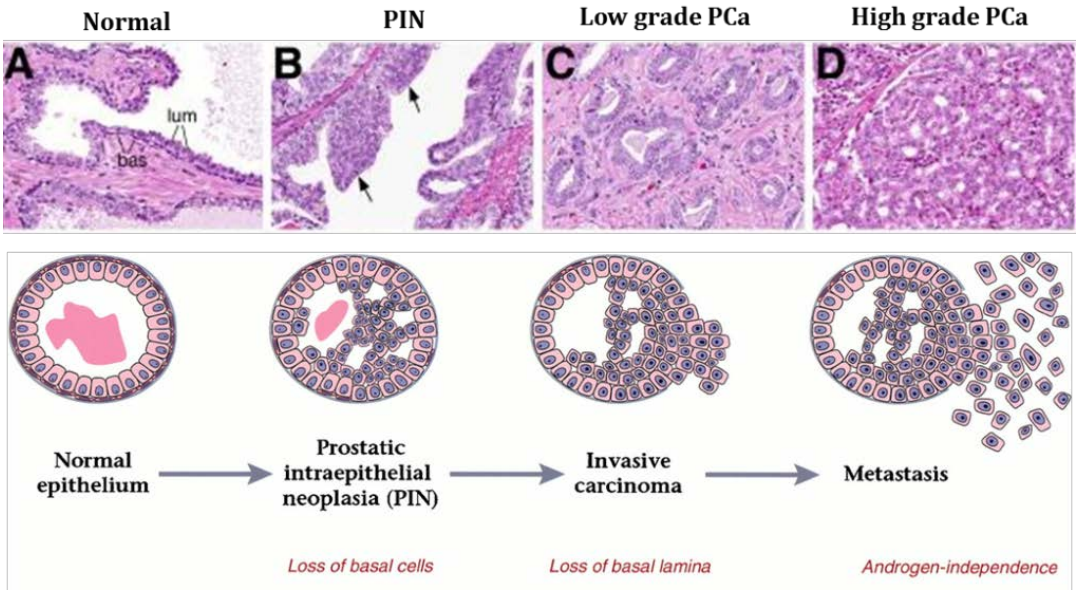

Figure 3: Progression stages from normal prostate to PCa.

Stages of progression are shown together with the typical histology of each stage (pictures above). Histological and molecular changes characterising each stage are indicated in red.
Adaptated from Abate Shen and Shen., Genes Dev. 2010 [2].

2.3 Prostate cancer

Prostate cancer: a clinical challenge

Prostate cancer (**PCa**) is one of the most prevalent malignancies affecting men worldwide and represents the most frequently diagnosed male solid neoplasm in Europe [12]. While all risks factors for PCa are still poorly understood, factors such as increasing age, ethnicity and heredity have been identified [13]. One of the main challenges in the diagnosis and treatment of PCa remains the discrimination between indolent PCa which does not require treatment, and aggressive potentially lethal PCa [14]. Elucidating molecular pathways involved in early events of carcinogenesis is a pre-requisite to identify such patients and provide new opportunities for early detection and treatment. Unfortunately, studies aiming at

investigating early events leading to cancer have so far been limited by the lack of appropriate models and the limited access to relevant clinical specimens.

Detection and diagnosis

Besides digital rectal examination, **prostate-specific antigen** (**PSA**) screening has been widely used as the main diagnosis tool over the past three decades. PSA, encoded by the KLK3 gene, is a kallikrein-related serine protease normally responsible for liquefying the seminal fluid [15]. PSA is synthesised by luminal secretory cells from the prostatic epithelium under the tight control of androgens [16]. Importantly, in healthy tissue, prostate architecture keeps PSA tightly confined and it is almost exclusively released into seminal fluid. In contrast, in men with PCa, PSA is released in the circulation, resulting in a boost of PSA level in the blood of the patients. This increased release of PSA is thought to arise from the disruption of the normal prostate architecture characterising prostate tumours (i.e loss of basal layer, cell polarity; see [16]). Importantly, an increase of PSA in the blood is also observed in patients with BPH and the threshold of PSA discriminating patients with BPH or PCa is difficult to define [13].

In order to confirm the diagnosis, men with elevated PSA levels generally undergo **biopsies**, defining a histopathological grade for the prostate tissue. To define PCa grade, the most accepted system is the **Gleason score**. Briefly, the Gleason grading system is based on the evaluation of the degree of differentiation of glands characterising the malignant tissue. The two most predominant architecture patterns are allocated within a grade between 1 (well differentiated) and 5 (poorly differentiated). The sum of the two grades defines the final Gleason score [17].

Clinically localised PCa vs Castration-resistant PCa

In case of **clinically localised PCa**, several treatment options are available. Active treatment includes **radical prostatectomy** (**RP**), external-beam radiation therapy, and brachytherapy [13]. In case of **metastasised PCa**, **androgen deprivation therapy** (**ADT**) is the treatment of choice because of the dependence of prostate cancer on androgen receptor signalling. While it initially results in a decrease of serum PSA level accompanied by a tumour regression, the tumour ultimately recurs and evolves towards a **castration-resistant PCa** state (**CR PCa**), which so far remains incurable [18,19]. The molecular mechanisms leading to CR PCa are still poorly understood [2]. We know that even if the disease progresses toward castration-resistance (previously termed androgen-independence), androgen-receptor (AR) signalling still is sustained. In fact, castration-resistant tumour cells still express AR as well as AR-related genes such as PSA, suggesting that AR signalling pathways are still maintained [20]. Understanding the role of androgen receptor signalling in prostate tumorigenesis has proven to be much more complex than initially anticipated and represents a pre-requisite to elucidate mechanisms leading to CR PCa.

Metastasis sites

In PCa, the most common sites of distant metastasis are bone, lung and liver [21]. In particular, prostate cancer metastases to the **bone** occur at high frequency in patients with advanced disease and are largely responsible for the significant PCa **morbidity and mortality** [22].

Histological and morphological characteristics of PCa

Many epithelial tumours, such as breast cancer, are characterised by the existence of distinguishable histopathological subtypes which are associated with different prognosis and treatment responses in patients. In contrast, PCa is characterised by a **lack of such histopathological subtypes** and most PCa (≥95%) are classified as **adenocarcinomas**, while variants such as small cell carcinoma or mucinous carcinoma are infrequent [23].

At the histopathological level, PCa is characterised by the disappearance of the basal lamina and basal epithelial cells as revealed by a loss of expression of p63 and CK5/14 basal-specific proteins (Figure 3). In addition, expansion of the luminal compartment can be revealed by high expression of luminal markers such as AMACR, typically overexpressed in PCa [24].

PCa: A model of clinical and genetic heterogeneity

Even in the absence of defined histopathological subtypes, prostate cancer is characterised by a remarkable clinical heterogeneity between patients, resulting in differences in term of therapeutic response and prognosis. This **intertumour heterogeneity** can be partially explained by the existence of **distinct molecular subtypes** of PCa. Indeed, genomic analyses have highlighted the identification of molecular signatures associated with distinct subtypes which are associated with specific patient outcomes and response to therapy [25,26]. In particular, chromosomal rearrangements such as the **TMPRSS2-ERG fusion gene** appear to be present in ~50 % of PCa [27] and have been suggested to be associated with a specific survival outcome [25]. Other molecular events occurring in a large

percentage of PCa include **loss of Nkx3.1 function** [28], **amplification of Myc** oncogene [29]and/or **PTEN deletion** [30]. **Stem cell-like signatures** have been also shown to characterise aggressive subtypes of PCa [25]. These distinct molecular subtypes have convincingly been shown to be associated with poor prognosis in PCa [25,31,32].

Additionally, PCa is often multifocal and characterised by **intratumour heterogeneity**, as suggested by the concomitant presence of different Gleason grades within an individual PCa specimen. Importantly, this heterogeneity can be observed at the histopathological level but also at the molecular level, with foci exhibiting distinct molecular features within the same sample (e.g TMPRSS2-ERG fusion (+) and (-) within the same patient)[33].

This heterogeneity highlights the complexity of PCa and the challenges inherent with the development of effective treatments.

3. Experimental models to study prostate cancer

Elucidating molecular events leading to human prostate cancer requires both *in vitro* and *in vivo* relevant experimental models. *Since this thesis deals with human prostate cancer, the use of mouse models of PCa will not be discussed in detail here.*

3.1 Established PCa cell lines

Establishing cell lines derived from PCa has proven to be much more difficult than for other cancers in spite of the wide research effort made in this direction over the past 30 years [3,34]. The most commonly used established PCa cell lines remain the androgen-insensitive **PC3** [35] and **DU145** [36] cell lines, as well as the **LNCaP** and **VCaP** androgen-sensitive cell lines [37,38]. Notably, all the aforementioned cell lines

have been derived from PCa metastases, which reflect the difficulties inherent with the establishment of PCa cell lines derived from primary tumours. Moreover, whether they might represent a good model for primary PCa is highly debated, since they appear not to represent the different phenotypes characterising PCa cells and extensive long-term culture might have affected their properties [3]. Other studies have reported the establishment of novel PCa cell lines but many of them have been later shown to result from the contamination with other cell lines [39,40].

3.2 PCa clinical specimens and PCa-derived primary cultures

In view of the difficulties associated with the use of long-term established cell lines, many studies have aimed at generating short-term primary PCa cultures. Most of them are derived from primary PCa samples obtained after radical prostatectomy and have been generated using a variety of techniques (explant, xenograft, 3D cultures…; [3]). Surprisingly, while PCa is characterised by the disappearance of basal cells and the expansion of luminal cells, most investigators report that primary cultures display a rather basal-like phenotype. This indicates that culture conditions may favour the expansion of specific cell subsets which do not exhibit a typical PCa phenotype. One hypothesis could be that culture conditions may provide a growth advantage to benign cells concomitantly present with cancer cells within the clinical specimens [41]. *Findings related to the generation of primary cultures derived from primary PCa specimens are presented in chapter one of this thesis* (Chapter I.part 3).

3.3 Xenograft models of PCa

Xenograft models of PCa have been widely used to perform *in vivo* investigations. Usually, these studies involve the use of established PCa cell lines or

primary tumours which are injected either subcutaneously or orthotopically in immunodeficient mice (i.e nude, NOD/SCID, or NOD/SCID gamma). In some cases, fragments of surgically excised tumour tissues can be implanted directly subcutaneously or orthotopically and can only be passaged *in vivo* [42]. The xenograft model, however, presents some limitations due to the lack of endogenous immune system and the absence of human microenvironment (murine stroma). Moreover, in case of PCa, xenografts derived from primary tumours are relatively difficult to obtain given the inefficiency of PCa cells grafting [43]. Yet xenografts still represent an interesting *in vivo* system since they involve cells of human origins which are likely to recapitulate human PCa events and reflect tumour heterogeneity more closely than genetically engineered mouse models.

II. A hierarchical model of cancer

1. Models of tumour heterogeneity

Prostate cancer, same as various other cancer types, is characterised by a remarkable heterogeneity including cells with different phenotypic characteristics, as well as various degrees of proliferation and differentiation [2]. To explain the cellular origin and the heterogeneity of cancers, two models are currently proposed. The classical **stochastic model** postulates that all cancer cells can proliferate extensively and that any cell within a tumour possesses the potential to form a new tumour ([44], Figure 4).

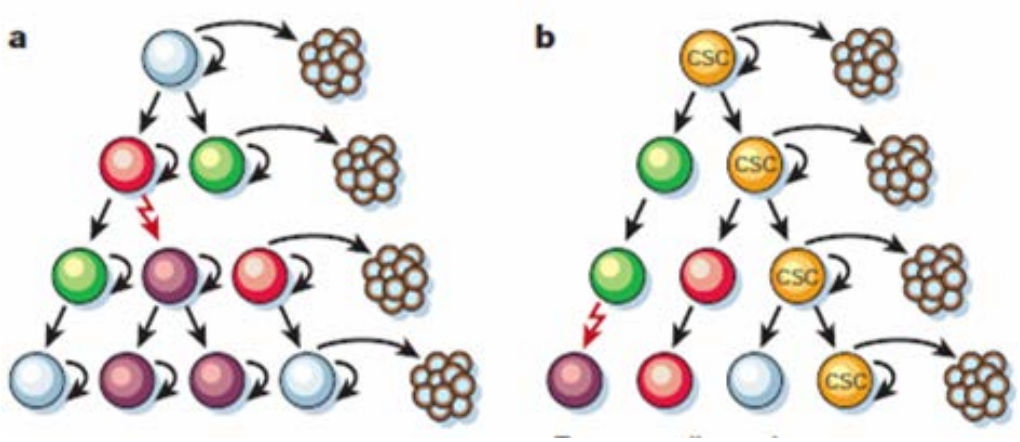

Figure 4. Two models of cancer heterogeneity.

(**a**) **The stochastic model**.
(**b**) **The hierarchical model** involves **cancer stem cells** (**CSC**) at the top of the hierarchical pyramid constituting the tumour.
Adapted from Reya et al., Nature 2001[44].

In contrast, the **cancer stem cell** (**CSC**) model assumes that a tumour is organised as a **hierarchy** that originates from a small population of cells. The CSC model postulates that most cancer cells possess a limited proliferative potential, and that only a subset of cells, potentially endowed with stem-like properties, intrinsically possesses the potential to extensively proliferate and to reproduce heterogeneous tumours ([44]; Figure 4). Based on their specific capacity to initiate a tumour in transplanted mice, CSC are also referred to **as tumour-initiating cells** (**TIC**); *controversies regarding terminology and definitions are further discussed in part II.2 of the introduction*). Notably, the two above-mentioned models are not necessarily mutually exclusive.

Supporting the CSC model, John Dick and colleagues first demonstrated in acute myeloid leukaemia (AML) the existence of rare CD34+CD38- leukaemia initiating-cells exclusively able to transfer and reconstitute a tumour in

immunodeficient mice [45]. Following this seminal paper, the same group demonstrated that AML is organized as a hierarchy that originates from a primitive hematopoietic cell, endowed with differentiation and self-renewal capacities [46]. These two studies provided a paradigm to support the hypothesis of a similar hierarchy in solid tumors. In 2003, the first demonstration of the existence of CSC in solid tumours was accomplished by Michael Clarke and colleagues, in breast cancer [47]. A year later, the team of Peter Dirks reported the identification of human brain tumour-initiating cells specifically expressing CD133 [48]. Following these pioneering studies, a flurry of publications described the identification and isolation of putative CSC in human solid tumours including colon [49,50], pancreatic [51], and liver [52] cancers as well as melanoma [53] (Table1).

Table 1: Phenotypes of TIC populations isolated from primary solid tumours
Seminal studies

Tumour type	**Phenotype**	**Reference**
Breast	$Lin^{-}CD44^{+}CD24^{low/-}$	[47]
	$ALDH^{high}$	[54]
Brain	$CD133^{+}$	[48]
Pancreas	$CD44^{+}CD24^{+}ESA^{+}$	[51]
	$CD133^{+}$	[55]
Colon	$EPCAM^{high}CD44^{+}$	[56]
	$CD133^{+}$	[49,50]
Liver	$CD90^{+}$	[52]
Lung	$CD133^{+}$	[57]
Melanoma	$ABCG5^{+}$	[53]

2. CSC versus TIC: blurry definitions and confusing nomenclature

The cancer stem cell theory suggests that only a subset of cells is enriched for the ability to form new tumours, while the cells composing the bulk of the tumour are devoid of this ability [44]. This CSC subset is generally thought to exhibit stem-like properties such as **self-renewal and differentiation capacities**, based on the observation that the spectrum of phenotypes present within the initial tumour is recapitulated in the transplanted tumour. Yet, in solid tumours, many aspects of this model remain speculative since the demonstration of self-renewal and differentiation capacities would require the tracking of cell fate at clonal level. Thus, stem-like properties putatively characterizing CSC remain hypothetical in many human tumours.

Additionally, the term "cancer stem cell" generates much confusion and misunderstandings in the tumour biology field since it suggests that CSC might be derived from normal stem cells. Yet scientific evidences supporting this hypothesis are rare and CSC might be derived from stem, progenitors, or differentiated cells [58]. Thus, CSC can be reliably defined as cells that have **the ability to initiate and re-grow the tumour from which they were isolated** [58]. This definition implies that these cells can only be defined experimentally *in vivo*, which suggests that the term **tumour-initiating cell** (**TIC**) is more accurate.

3. TIC: Properties and isolation

Based on the hypothesis that TIC might share some properties with normal stem cells (NSC), methods commonly used to isolate NSC have been similarly applied to identify and isolate putative TIC (Figure 5). As an example, TIC subsets have been

isolated in various tumours, using surface markers previously shown to mark normal stem cells. Probably the best example is represented by the CD133 marker, which has been used to identify normal stem cells and tumour-initiating cells in the brain [48,59], colon [49] or prostate [60,61]. Nevertheless, the accuracy and specificity of this marker to select for TIC appears to be limited and still is highly debated [62–64]. Using cell sorting (FACS) strategies, various combinations of markers have been used to isolate TIC from primary solid tumours (Table 1).

Other properties such as the expression of stemness-associated genes [65,66] or the capacity to grow as 3D structures (so-called spheroids) [67] have been proposed to characterise TIC and are exploited to isolate them using functional assays (see Figure 5). Nevertheless, given that TIC are operationally defined by their capacity to re-grow a tumour, the gold standard assay remains the xenotransplantation of the cells in immunodeficient mice ([68]; Figure 5).

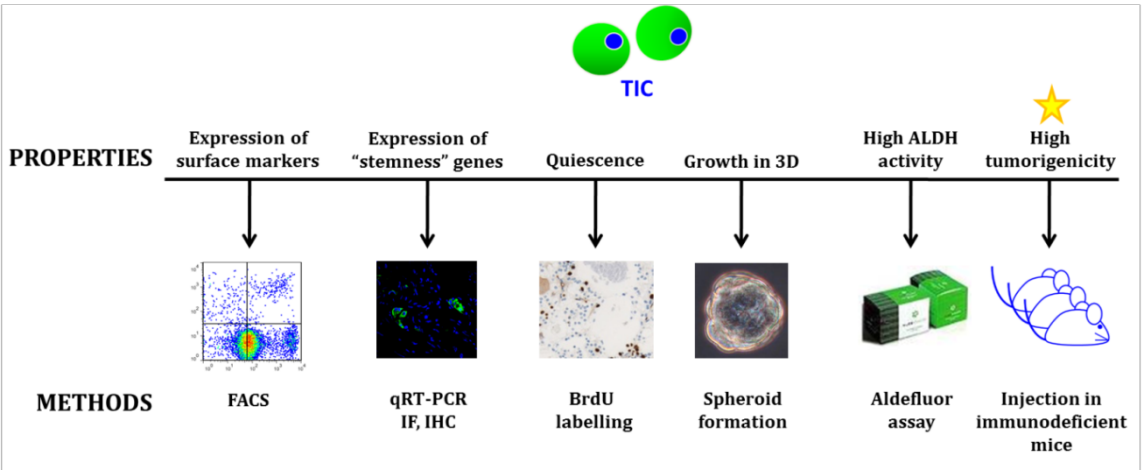

Figure 5: Examples of methods to identify TIC/CSC based on their putative properties.

Phenotypic and functional assays have been established by exploiting putative properties of TIC. Xenotransplantation of cells populations remains the gold standard to assess the tumour-initiating potential which is defining a TIC.

Some of these properties will be described in detail and discussed in the experimental part of the present thesis.

Notably, until a few years ago, tumour-initiating cells were considered to represent a minor population of cells. Recent studies, however, have suggested that TIC frequency can dramatically increase when using more permissive xenotransplantation conditions [69,70]. These studies led to the conclusions that TIC are not necessarily rare and that their frequency might depend on the tumour type [68,71]. This also raised the issues of the universality of the CSC model (implying that it might only be applicable for some tumour types), and of the necessity to improve the assays currently in use.

4. Clinical relevance of TIC

In various tumours including PCa, patients favourably respond to therapies but do relapse at short or long-term after the initial remission. This might indicate that current therapies do not efficiently target TIC, which represent the only cells able to re-initiate and sustain the tumour growth. The CSC model and the notion of hierarchy hold great implications for clinical practice. In the case of PCa for example, the tumour grade (Gleason) is determined as a function of the level of differentiation of the cells, with the most undifferentiated (immature) tumours being the most advanced and aggressive ones [17]. In other cancers, the number of TIC has been shown to correlate with aggressiveness of the tumour, but also with the molecular and phenotypic sub-group of the tumour, and the probability to relapse, thus raising the necessity to elaborate specific targeted therapies [72–74]. In this context, a flurry of studies describing the clinical and prognostic relevance of TIC/CSC populations

have been published in the last years, holding promises of a new cellular target for therapies [54,75–77].

Yet elaborating therapies efficiently targeting TIC might be an ambitious step to reach since these cells appear to exhibit properties allowing them to escape conventional treatments. Indeed, TIC are thought to be resistant to chemo- and radio-therapies thanks to their slowly proliferating nature [78], high expression of ABC transporters allowing the efflux of anticancer drugs [79], and high resistance to oxidative and DNA damages [80]. Nevertheless, a worldwide research effort is now addressing ways to overcome this resistance and establish cellular-specific therapies. As a proof of concept, different therapeutic strategies have already been proven successful at killing or inhibiting the activity of TIC populations in several tumours types [81–83].

III. State of the art in the prostate

1. Adult stem cells in the normal prostate

During late embryogenesis, the prostate epithelium arises from the differentiation of a stem cell population localised in the embryonic urogenital sinus (UGS) epithelium from which prostatic epithelial buds develop. This development requires the influence of UGS mesenchyme interactions and the control of testicular androgens [84]. Once adult, in contrast to organs with a constant turnover such as the intestine, the prostate does not necessitate rapidly cycling stem cells to replenish the organ every few days. Nevertheless, prostate stem cells are needed to replenish the adult organ after routine cell death and maintain homeostasis of the gland [84].

Importantly, there is still no clear consensus about prostatic cell lineage. Some hypotheses and evidences are presented in the following paragraph.

2.4 Evidences for basal stem cells

The existence of adult stem cells in the prostate has long-time been supported by the aptitude of the prostate to undergo repeated cycles of extensive regression in response to androgen deprivation, followed by complete regeneration after androgen restoration. This observation has led to the hypothesis that prostate stem cells should be **castration-resistant** i.e. responsive but not dependent on androgens for their survival [85]. Notably, after androgen deprivation, most of the AR(+) luminal cells undergo apoptosis, while basal and NE cells, being AR(-)^{low} are able to survive. Based on these observations, prostate stem cells have been assumed to reside within the basal compartment of the epithelium. In two pioneering studies, Collins and colleagues have used human normal prostate tissue to purify a highly proliferative basal stem cell population, exhibiting a **CD44^{+}α2β1^{+}CD133^{+}** phenotype and able to generate prostate-like acini *in vivo* [60,86]. Besides, Goldstein and colleagues have later shown that basal human **Trop2^{+}CD49^{+}** cells are endowed with tissue-regenerative and spheres-forming capacities and are therefore enriched in stem cells [87]. Notably, studies performed with human cells have often been inspired by studies using mouse tissue, where several phenotypes of basal stem cells have been proposed [88,89].

2.5 Evidences for luminal stem cells

In contrast, recent evidences have supported the presence of putative stem cells in the luminal compartment of the prostatic epithelium. In particular, in the

mouse, Wang and colleagues have identified a rare luminal population of castration-resistant cells (called **CARNs** and positive for **Nkx3.1**), which are able to regenerate prostatic tissue following single cell transplantation [90].

2.6 Proposed hierarchy

Importantly the two above-mentioned hypotheses regarding prostate stem cells localisation are not mutually exclusive. Based on combined evidences, one possibility could be that a stem cell within the basal layer can give rise to a basal multipotent progenitor. This progenitor is likely to give rise to NE cells, mature basal cells and luminal progenitors, which might give rise to mature luminal cells [91].

2. Cell of origin in PCa

The concept of "cell of origin" in cancer has recently taken importance. While the term "cell of origin" is often confused with "cancer stem cell" or "tumour-initiating cell", their definitions are nevertheless different. Indeed, the cell of origin of a cancer is defined as the **normal cell type** from which a tumour arises following oncogenic transformation [92]**,** while the terms TIC/CSC identify **cancer cells subsets** involved in initiation and growth of the tumour**.**

Given their ability to self-renew and their long-term persistence, normal stem cells have been proposed to represent good targets for oncogenic transformation and have therefore been proposed to be cells of origins of cancer [44]. In particular, in the prostate, populations enriched in stem cells contained within both luminal and basal compartments have been shown to be able to give rise to PCa in two elegant studies [90,93].

3. TIC/CSC in prostate cancer

In a seminal study from 2005, Collins and colleagues investigated the presence and phenotype of human prostate CSC [61]. Interestingly, they successfully identified a minor population of primary prostate CSC exhibiting, such as normal stem cells, a **CD44+α2β1+CD133+** phenotype. These cells were highly clonogenic in culture and possessed the capacity to self-renew and differentiate *in vitro* [61]. Following this pioneering paper, a series of publications aiming at characterizing CSC in the prostate have emerged [94–98]. Nevertheless, most of these studies have been performed using established PCa cell lines as a model. Given the long-term culture and the metastatic origin of these cell lines, it is likely that these studies might not reflect what is happening in the physiological conditions. Moreover, when using primary prostate cancer cells, such as in the study by Collins et al., tumorigenicity *in vivo* was not demonstrated [61]. The probable reason is represented by the difficulties inherent with the grafting of human prostate cancer cells in immunodeficient mice ([43] and see part **I.3.3)**). Thus, the most important property characterising CSC/TIC has never been demonstrated in studies using primary prostate cancer cells. Therefore, while their existence has been assumed, evidence supporting the presence of TIC in the prostate is still lacking.

IV. Presentation of the experimental work

In this project, we aimed at identifying and assessing functional properties and clinical relevance of cell populations potentially associated with stem-like properties in prostate cancer.

In **Chapter I**, we investigated the expression of surface markers previously shown to be associated with TIC properties in prostate cancer. In particular, we examined the expression of these markers in different models of PCa, including established PCa cell lines, freshly excised PCa samples, and PCa derived cultures. In this part, we also analysed and tested methodologies used to generate PCa-derived primary cultures. Limitations and pitfalls of these techniques are presented and discussed.

In **Chapter II**, we investigated the expression of putative stemness-associated signatures in PCa. Based on the CSC model and on the fact that the level of differentiation of tumour cells inversely correlates with tumour aggressiveness, we hypothesised that increased expression of stemness-associated factors might be associated with PCa and may have prognostic significance (Figure 6: *Rationale*). In particular, we investigated the expression of 5 transcriptions factors previously shown to be associated with cellular self-renewal and pluripotency capacity. Results are presented and discussed in the paper entitled "Klf4 transcription factor is expressed in the cytoplasm of prostate cancer cells", currently in press in *European Journal of Cancer*.

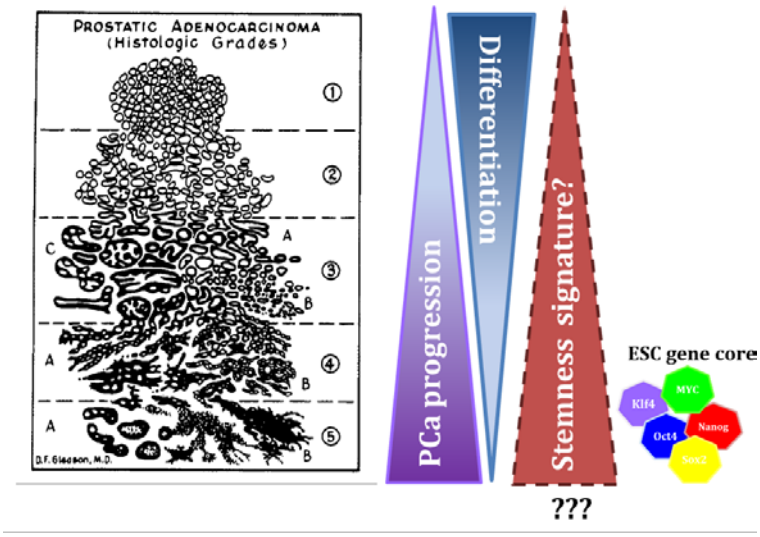

Figure 6: Parallels between cellular differentiation and PCa progression (Chapter II)

According to the Gleason scale, PCa aggressiveness inversely correlates with differentiation level of the cells. Here, we hypothesised that increased aggressiveness might be associated to a "stemness state" characterised by increased stemness-specific signatures. This stemness signature might include genes associated with self-renewal and pluripotency capacities, constituting the so-called embryonic stem cell core (ESC core).

Our results and results published by others indicate that the use of surface markers might not represent the most suitable method to enrich in TIC. In **Chapter III**, we therefore focused on a functional property previously suggested to be associated with TIC properties. Selecting for cells with high aldehyde dehydrogenase activity (so-called ALDH^{bright} cells) has been shown to identify normal stem cells and TIC in various tissues. In PCa, ALDH bright cells were most recently shown to select for TIC in established PCa cell lines. In this chapter, we assessed the existence, phenotype, and clinical significance of ALDH^{bright} cells in PCa primary samples (Figure 7: *experimental design*). Results are presented and discussed in the paper entitled "Characterization and clinical relevance of ALDH ^{bright} populations in prostate cancer" recently submitted for publication and currently in revision (*Clinical cancer Research*).

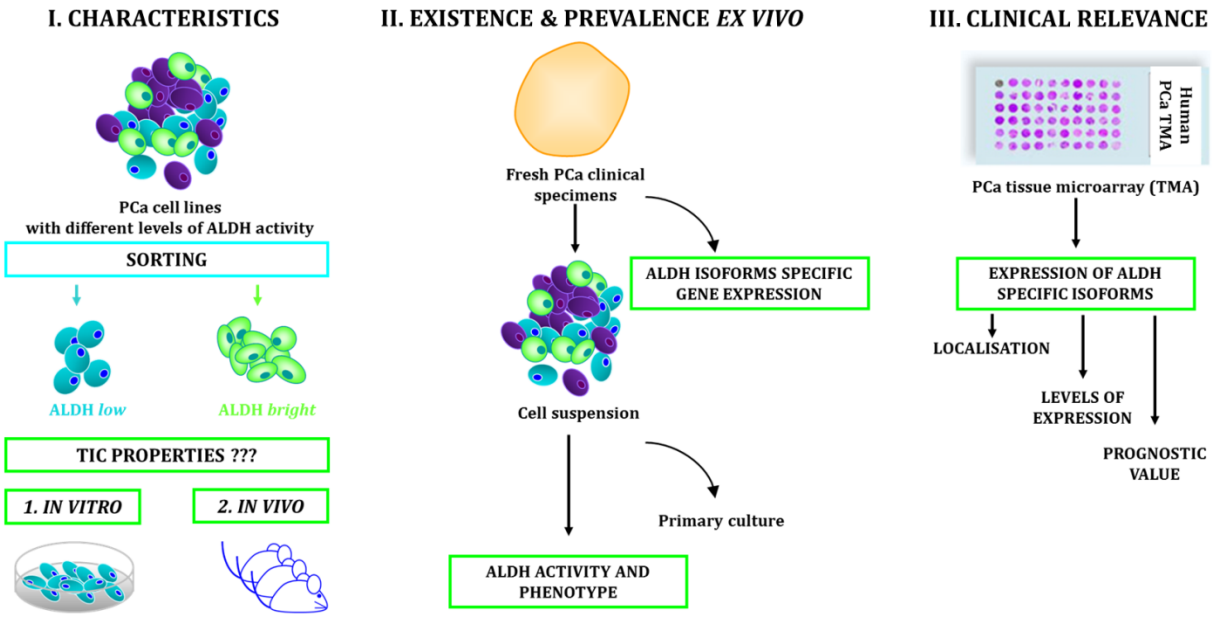

Figure 7: Characterisation and clinical relevance of ALDH^{bright} populations in PCa (Chapter III)

Experimental design of the project. (I) We assessed putative TIC characteristics displayed by ALDH^{bright} cells using established PCa cell lines. (II) We tested existence, prevalence, and phenotype of ALDH^{bright} populations in freshly excised clinical PCa specimens. (III) Finally, expression of ALDH1A1 specific isoform was tested on a panel of TMAs to assess clinical relevance of ALDH1A1 positive cells.

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Chapter I

Prostate TIC: inadequacy of model systems

Introduction

Since decades, established PCa cell lines have been widely used to investigate many aspects of PCa pathogenesis [1]. More recently, they have also been used as a model to study tumour-initiating cells (TIC) in PCa [2,3]. Yet, due to long-term culture and phenotype selection, established cell lines might not reflect the *in vivo* situation. In particular, they might fail to reproduce the vast heterogeneity of cell phenotypes characterising PCa patients.

Using cells isolated from freshly excised PCa specimens might therefore represent a better alternative to investigate characteristics of distinct PCa cell populations. This type of studies is nevertheless hampered by a limited access to primary PCa specimens and a frequent "contamination" of normal cells in the sample. Given the lack of PCa specific surface markers, the purity of the sample is therefore mainly dependent on an accurate pre-screening performed by expert pathologists. Even when all conditions are optimised, phenotypes of patients' tumours are characterised by a remarkable heterogeneity. For all these reasons, evidence demonstrating the presence of sub-populations of cells expressing putative TIC markers is rare [4].

Additionally, elucidating molecular mechanisms leading to human cancer requires both *in vitro* and *in vivo* robust assays. In this context, culturing PCa primary cells *ex vivo* represent a unique opportunity to study properties characterising different subsets of cells. Besides, it would allow recapitulating the heterogeneity observed in patients. Generation of such cultures has been the aim of an important research effort in the PCa field. Yet only rare studies report the generation of

primary cultures derived from PCa primary samples and, in these studies, a full characterisation of the cells in culture is often lacking (reviewed in [5]). In particular, when cells are characterised, most of the investigators report phenotypes resembling to those of basal cells. Yet, PCa being a luminal cell disease, these results indicate that cells in culture do not accurately reflect *in vivo* tumour biology. Thus, modelling and adapting the culture conditions appears to represent a pre-requisite to address these issues and succeed at culturing prostate cancer cells.

In this chapter, we first investigated the expression of putative markers of TIC in established PCa cell lines. In parallel, we investigated the expression of the same markers in cells derived from freshly excised PCa clinical specimens. Finally, we focused on the generation of primary cultures derived from freshly excised PCa specimens. To characterise the cells in culture, we examined the presence of the different populations which compose the prostatic epithelium including previously described TIC populations. Limitations of the different models are discussed at the end of the chapter.

Material and Methods

Clinical specimens

We used a series of samples obtained from 26 patients with pT2a-pT3b stage PCa patients who underwent radical prostatectomy (RP) at the Department of Urology of the University Hospital of Basel (Switzerland). Written informed consent was obtained from all patients in accordance with the requirements of the local Ethical Committee (EKBB, Ref.Nr.EK: 176/07).

Established cell cultures

Established prostate cancer cell lines Du145, PC3, and VCaP (LGC Standards, Molsheim, France) were routinely cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, Penicillin 10 U/ml and Streptomycin 10 µg/ml (Pen/Strep, Invitrogen Carlsbad, CA).

Isolation of primary cells and generation of primary cultures derived from freshly excised surgical PCa specimens

Prostate tissues were screened for the presence of tumour tissues by experienced pathologists. PCa samples were chopped, washed and then digested in a mixture containing DMEM, 5 % Knockout Serum Replacement (KO serum, Gibco, Paisley, UK), 1% Pen/Strep and 200IU/ml of type I collagenase (Worthington, Lakewood, NJ). After a 12-16 hour incubation at 37°C, digested tissues were washed and underwent a series of differential centrifugations, as previously described to separate epithelial and stromal fractions [6]. Cell pellets enriched in epithelial fraction were re-suspended in PBS. On one hand, cells were passed through a 100 µm cells strainer in order to obtain single cell suspensions. Resulting cells were immediately used for

FACS analysis to assess surface markers expression (see "Phenotypic characterisation"). On the other hand, organoids retained in the cell strainer after filtration, were washed out and re-suspended in CnT-52 medium (CELLnTEC, Bern, Switzerland). Organoids were then plated in small Petri dishes and further expanded after passaging and re-plating in bigger dishes.

Phenotypic characterization

Cells were incubated with the following antibodies recognizing isotype-matched immunoglobulins or surface markers:

- Phycoerythrin (PE)-labelled: anti-CD44 (BD Biosciences, San Josè, CA), anti-CD133/1 (Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD24 (BD Biosciences), anti-CD49b (BD Biosciences), anti-CD166 (BD Bioscience), and anti-CD31 (BD Biosciences).
- Allophycocyanin (APC)-labelled: anti-EpCAM (BD Biosciences), anti-Trop2 (R&D Systems, Cambridge, UK), and anti-CD133/1 (Miltenyi Biotec).
- Fluorescein isothiocyanate (FITC)-labelled anti-CD49f (Serotec, Dusseldorf, Germany), anti-CD44 (BD Biosciences), and anti-CD45 (Miltenyi Biotec).

Briefly, cells were re-suspended in a small amount of buffer and then stained with antibody concentrations recommended by the manufacturer. Following a 30 minute incubation at 4°C, cells were then washed, re-suspended in buffer, and analysed using a dual laser BD FACS Calibur (BD Biosciences, San Josè, CA). Dead cells were excluded based on propidium iodide (PI) incorporation.

Quantification of gene expression by quantitative Real-Time PCR (qRT-PCR)

Cultured cells were collected and washed in PBS. Prostatic tissues were collected by an experienced pathologist, immediately submerged in RNAlater (Ambion, Foster City, CA) and stored at -70°C until further processing. Total cellular RNA was extracted and DNase treated from tissues, by using NucleoSpin® RNA II (Macherey-Nagel, Oensingen, Switzerland). RNAs were then reverse transcribed by using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen). Quantitative real-time PCR assays were performed in the ABI prism™ 7700 sequence detection system, using Taqman® Universal PCR Master Mix (Applied Biosystems, Rotkreuz, Switzerland). Specific gene expression was normalized and evaluated using the $2^{-\Delta\Delta C}$T method and GAPDH housekeeping gene as reference. GAPDH and c-Myc primers and probes sequences were derived from existing literature [7,8].

TMPRSS2-ERG primers and probe sequences, derived from existing literature [9] are the following:

Forward: CTGGAGCGCGGCAGGAA

Reverse: CCGTAGGCACACTCAAACAACGA

Probe: TTATCAGTTGTGAGTGAGGAC

Primers and probes for prostatic markers such as PSA, AR, and CgA and surface markers such as CD44, CD133, and EpCAM were provided by Assays-on-Demand, Gene Expression Products (Applied Biosystems).

Tumorigenic capacity in vivo

In vivo experiments were approved by the Basel Cantonal Veterinary Office. NOD/SCID mice, initially obtained by Charles River Laboratories (Germany), were bred and maintained under specific pathogen free conditions in the animal facility of the Basel University Hospital. 200 000 cells were re-suspended in Matrigel Matrix (BD Biosciences) and injected subcutaneously into the flank of recipient mice (8-10 weeks old mice; n>3 per culture). Tumour growth kinetic was monitored weekly by palpation.

Results

1. Expression of putative TIC markers in PCa established cell lines

Considering the lack of experimental models, established PCa cell lines have been widely used to study molecular mechanisms underlying cancer initiation and progression. In particular, they have been shown to contain sub-populations exhibiting stem-like characteristics [2,3]. Yet it appears that the properties displayed by PCa cells depend on cell culture conditions and on the cell line under investigation.

We therefore investigated the expression of surface markers previously shown to identify TIC populations in various solid tumours including PCa. Interestingly, we observed that expression of putative TIC markers is heterogeneous among PCa cell lines. In particular, the TIC/CSC population previously described by Collins and colleagues and exhibiting a **$CD44^{+}\alpha2\beta1^{+}CD133^{+}$** phenotype [4] was not reliably detectable in the PCa cell lines tested (Figure 1). While this project was on-going, other putative TIC or NSC populations have been described [10,11]. We therefore also investigated the expression of additional markers such as Trop2, CD49f, EpCAM, CD44, or CD24 in the previously tested cell lines. Results for Du145 and PC3 (most commonly used cell lines) are presented in Figure 1. Strikingly, PC3 cell line was characterised by 2 subpopulations exhibiting different levels of EpCAM and Trop2 markers. In particular Trop2 level of expression was overall not high. Given the specificity of these 2 markers for epithelial cells and the supposed

epithelial-nature of this cell line, these observations are puzzling. In addition, except for EpCAM and Trop2, established PCa cell lines were characterised by a lack of distinct cell sub-populations.

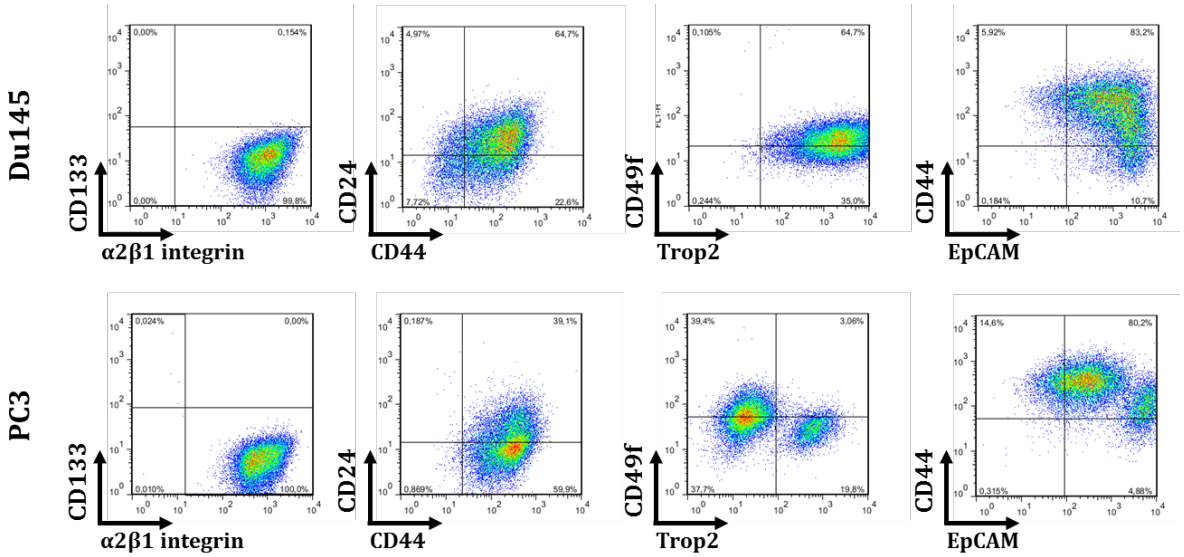

Figure 1: Phenotypic characterisation of Du145 and PC3 cell lines

Representative staining profiles of the expression of CD133, α2β1 integrin, CD44, CD24, CD49f, Trop2, and EpCAM in Du145 and PC3 cell lines.

2. Expression of putative markers of TIC in PCa clinical specimens

We assessed the expression of putative markers of TIC in cell suspensions derived from freshly excised PCa specimens (n=20). To do so, we first generated single cell suspensions following mechanical and enzymatic tissue dissociation (cfr material and methods). After an enrichment in epithelial cells, we investigated the phenotype of live cells (PI (-) cells). Strikingly, except for one patient, we never could detect a sub-population significantly positive for CD133 (Figure 2). We also

investigated the expression of epithelial-specific markers, lineage-specific markers and other putative markers of TIC. Representative results are presented in Figure 2.

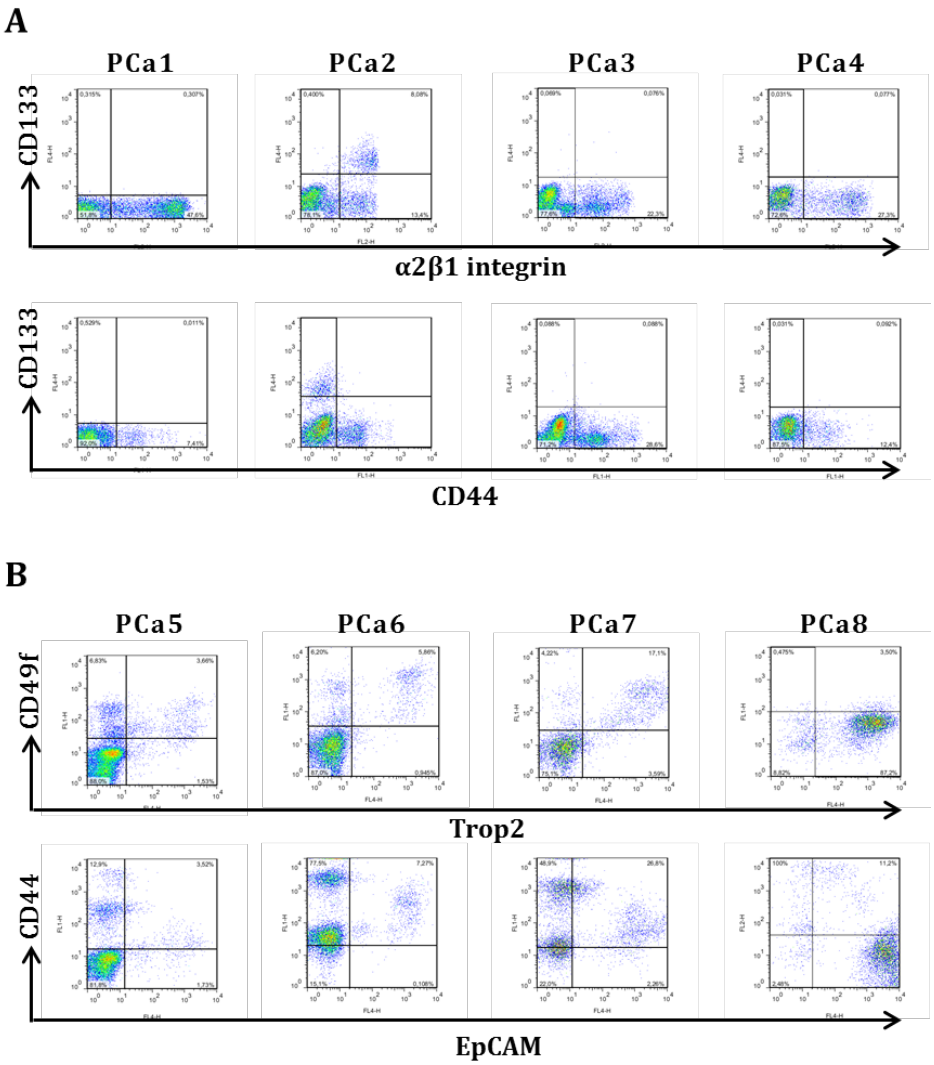

Figure 2: Phenotypic characterisation of cells derived from PCa samples

A: Expression of CD44, CD133, and α2β1 integrin in cell suspensions derived from 4 representative patients (PCa 1-4).
B: Expression of CD44, EpCAM, Trop2, and CD49f in cell suspensions derived from 4 other representative patients (PCa 5-8).

Overall, expression of the markers was highly variable across patients, consistent with the heterogeneity characterising PCa.

3. PCa-derived primary cultures: just an illusion?

3.1. Generation of short-term primary cultures

In an effort to generate PCa experimental models that would better reflect cancer pathophysiology than established cell lines, we attempted to establish short-term primary cultures derived from freshly excised PCa primary specimens.

Until recently, most of the investigators reporting the generation of primary cultures have been using stem cell medium (a keratinocyte specific medium supplemented by multiple stem cell factors), in combination with feeder cells (mouse embryonic cells) [6]. These feeder cells are known to favour the maintenance/growth of prostate epithelial cells. Recently, a new medium commercialised in Switzerland (CELLnTEC) has held new promises to generate PCa-derived primary cultures. In particular, the use of this medium does not require the addition of a feeder layer and has recently been proven to be successful to culture PCa cells [12,13].

Here, we combined and adapted experimental procedures briefly described in previous studies [5,6,13], to generate primary cultures derived from clinical PCa specimens. The full procedure is described in detail in "Material and Methods" and schematised in Figure 3.

Outcome

We generated 21 short-term primary cultures isolated from 9 clinical PCa specimens (for some patients, several cultures were generated). Due to technical and timing issues, characterisation experiments could not be performed for all primary cultures.

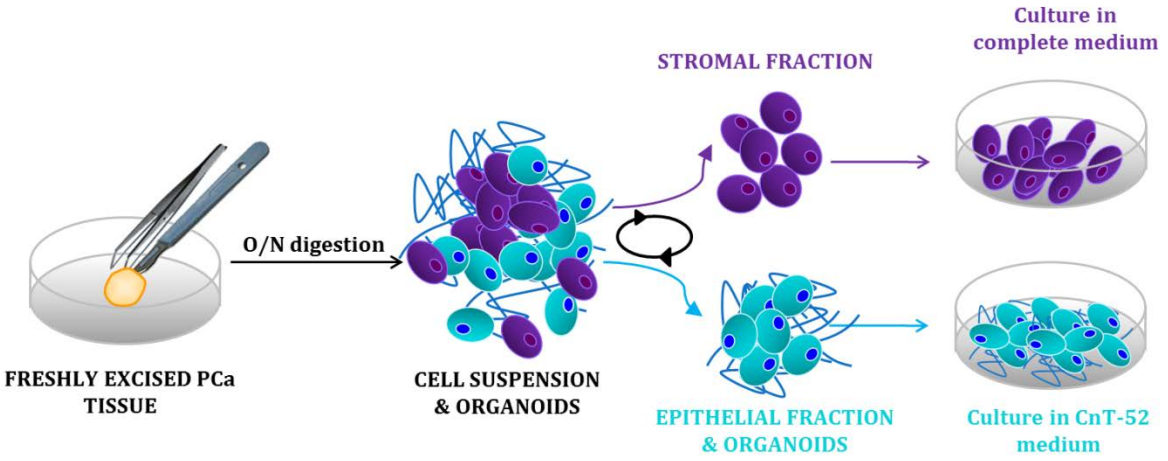

Figure 3: Experimental procedure used to generate PCa-derived primary cultures.

PCa tissues obtained from radical prostatectomy specimens are mechanically and enzymatically (overnight: O/N) dissociated to obtain a single cell suspension mixed with organoids. Cells undergo differential centrifugations to separate and enrich epithelial and stromal populations. The epithelial-enriched fraction is kept with organoids and is cultured in a selective serum-free medium (CnT-52), favouring the growth of epithelial cells. If needed, the stromal fraction can also be cultured and expanded in a medium supplemented with 10% FBS.

3.2. Morphological characteristics

After 24 hours of culture, initiation of epithelial-like colonies could already be observed. These colonies were emerging from organoids which had mainly adhered to the plastic of the plate (Figure 4A-B). After 72 hours, cell colonies expanded in size (Figure 4C-D), until covering the totality of the plate surface within 1-3 weeks (the time of expansion is donor-dependent).

Notably, some tissue-like structures growing in suspension could also be observed in several cases (Figure 4E-F). Once confluent, cells were detached and re-plated in new dishes, where they clearly exhibited an epithelial-morphology (Figure 5F).

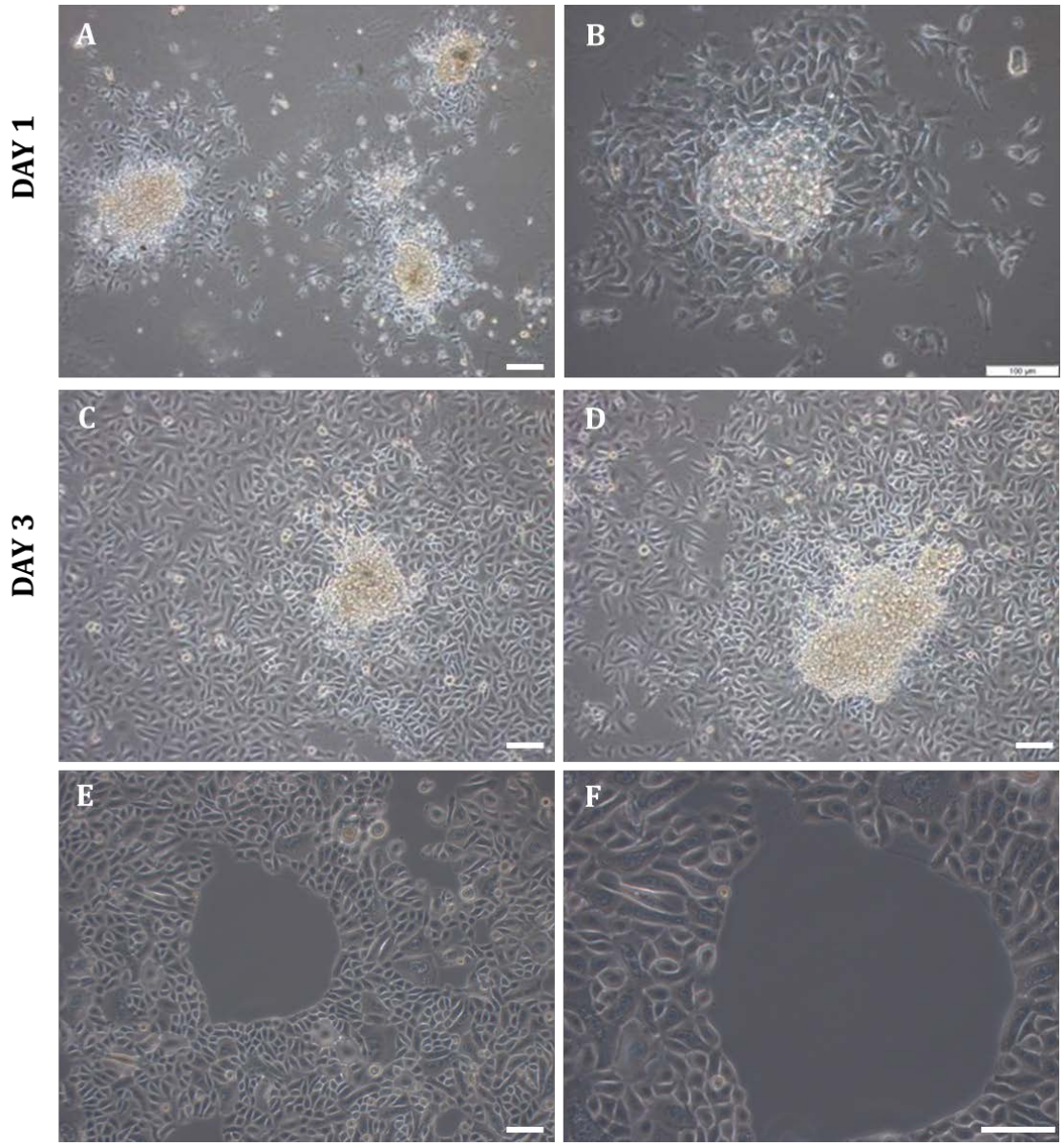

Figure 4: Primary culture generation.

After one day of culture, colonies are already emerging from organoids, similarly to an explant culture (**A-B**), and slowly expanding until confluence (**C-D**). Some "organised" structures (**E-F**) can also be observed in suspension after few days (depending on the donor). Scale bar: 100 µm.

As a proof of concept, we cultured the stromal fraction isolated from one clinical PCa specimen (Figure 5A-C). As expected, after a few days of culture, colonies with a fibroblast-like morphology started to expand. As shown in Figure 5, the

morphology of the stromal culture was totally distinct with that of the epithelial culture.

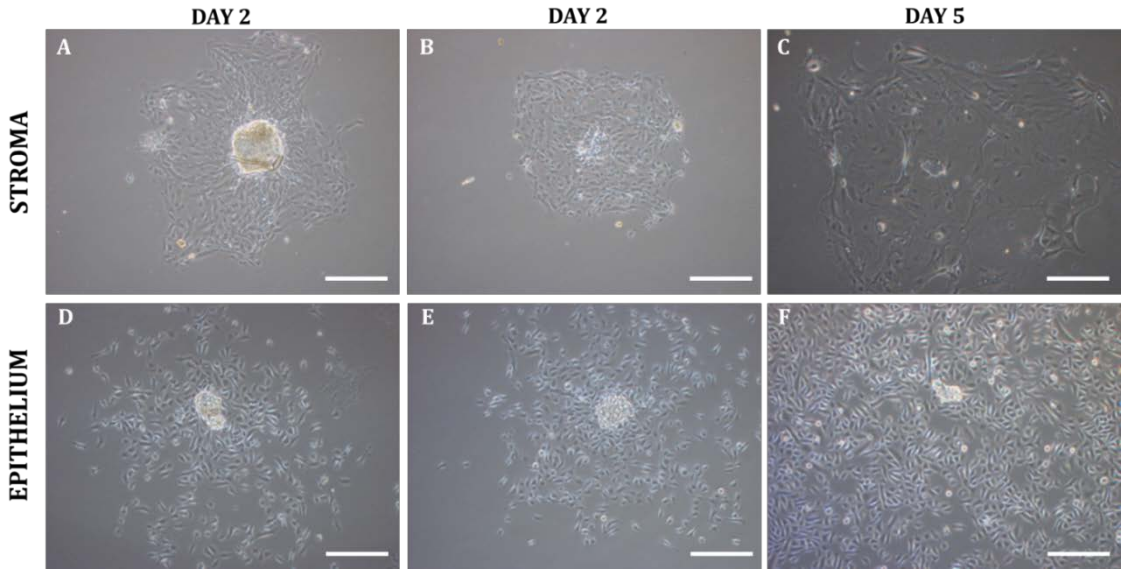

Figure 5: Morphology of the colonies derived from the epithelial and stromal fraction.

Stromal-like colonies (**A-C**) and epithelial-like colonies (**D-F**) are observed at 2 days and 5 days of culture. Scale bar: 200 µm.

3.3. Gene expression analysis

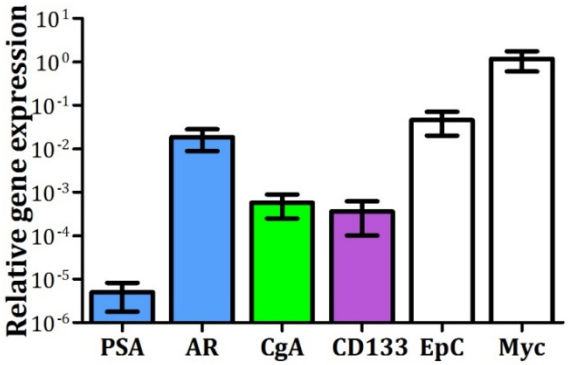

Figure 6: Expression of differentiation specific markers in PCa derived primary cultures.

Results are reported as relative values as compared to the housekeeping gene GAPDH (n= 6).
PSA: prostate specific antigen; AR: androgen receptor; CgA: Chromogranin A; EpC: EpCAM.

Prostatic epithelium is composed of three types of cells, i.e. basal, luminal and neuroendocrine cells, each of them being characterised by the expression of specific markers (see Introduction).

In case of PCa, the architecture of the epithelium is disrupted and the cell composition/ratio is modified. These changes are mainly reflected by the disappearance of basal cells and the expansion of luminal cells. Yet it appears that luminal cells are challenging to expand *in vitro* and to graft *in vivo*. Most investigators indeed report that normal prostate and PCa-derived primary cultures do not express AR and PSA, which are both specific markers of luminal cells. Notably, this is also observed for most of established PCa cell lines [5].

Based on this background, we investigated the expression of basal, luminal, and neuroendocrine cells specific markers of prostate in our primary cultures (passage 0; n total= 6). In all cultures, we found a relatively high expression of the gene encoding the neuroendocrine-specific marker Chromogranin A, indicating the putative presence of neuroendocrine cells (Figure 6). The gene encoding the basal-specific marker CD133, whose expression has been proposed to be restricted to CSC in the prostate, was also expressed. Likewise, CD44, mainly expressed by basal cells, was highly expressed in PCa cultures (data not shown). Notably, the gene coding for AR was expressed in all primary cultures tested, suggesting the putative presence of luminal cells within the primary cultures. PSA gene, specifically expressed by luminal cells, was only weakly detectable in 2 out of 6 primary cultures.

To understand whether cell passaging could alter the cell composition or select for defined phenotypes, we investigated gene expression in one primary culture (C192) before the first passage (passage 0: p0), and after re-plating and re-growth in a new plate (passage 1: p1). Interestingly, the expression of luminal markers such as PSA and AR was decreased after passaging, suggesting that passaging may have impaired the maintenance of the luminal compartment.

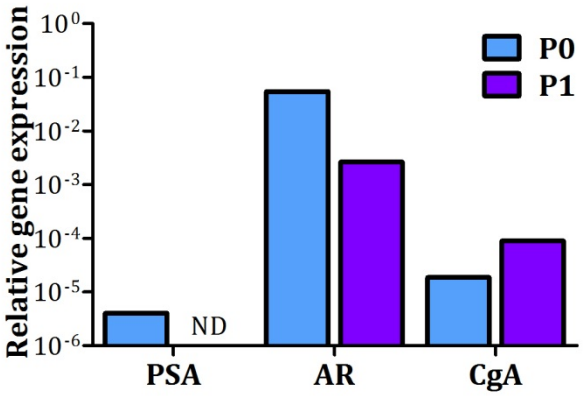

Figure 7: Expression of differentiation specific markers in the C192 primary culture at passage 0 and passage 1.

Results are reported as relative values as compared to the housekeeping gene GAPDH. PSA: prostate specific antigen; AR: androgen receptor; CgA: Chromogranin A; N.D: not detected.

3.4. Phenotypic characteristics

Cell suspensions derived from fresh PCa specimens contain cell components belonging to the stromal and epithelial compartments. Despite the epithelial enrichment procedure and the use of a selective medium, primary cultures may include a variable mixture of diverse cell populations. We therefore investigated phenotypes of cultured cells using a panel of surface markers allowing the identification of defined cell populations.

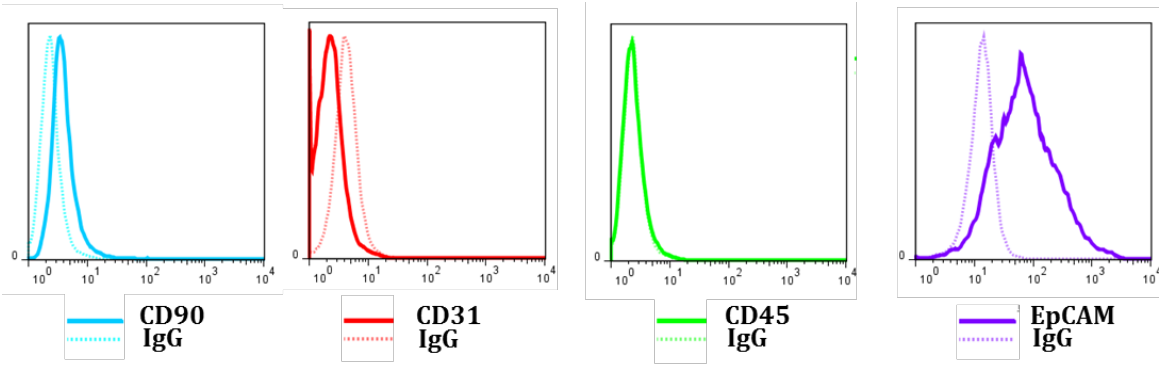

Figure 8: Phenotypic characterisation.

Flow cytometry analysis of one representative cell culture (C182 P0). Cells were tested for the expression of stroma-associated markers (CD90, CD31, CD45) or the epithelial specific marker EpCAM. Cells stained with isotype-matched immunoglobulins (IgG) antibodies were used as negative controls and are also shown.

We assessed the expression of CD45, CD90, and CD31 markers in order to identify putative haematopoietic, fibroblastic, and endothelial populations, respectively. In all tested cultures, the expression of these markers was not significantly detectable (n=7; representative staining in Figure 8). In rare cases, the presence of a minor population (≤1%) of cells positive for either CD31 or CD90 could be observed. Confirming the epithelial nature of the cells, all cultures were largely positive for the epithelial specific markers EpCAM (Figure 8) and Trop2 (data not shown).

Next, we assessed whether cells in culture do reflect the range of prostate cancer cell phenotypes. We therefore tested the expression of a combination of markers previously shown to be associated with a basal, luminal or "stem" phenotype. Considering the small amount of cells typically present at passage 0, the full phenotypic characterisation of the cells was mainly addressed after cell expansion (from passage 1). Notably, as shown in Figure 9, the identification of well-defined sub-populations, as seen in non-expanded cells freshly derived from patients, was unclear. Yet the phenotype of the cells was relatively heterogeneous when comparing cultures derived from different PCa patients.

The combined expression of Trop2 and CD49f has been shown to discriminate distinct basal and luminal populations of the prostate epithelium [11]. Using this combination of markers, we could observe $Trop2^{+}CD49f^{+}$ cells, indicating a basal phenotype. Yet in some cases, we could observe $Trop2^{+}CD49^{low/-}$ cell populations, suggesting a luminal lineage (Representative example in Figure 9). We also investigated the presence of the $CD44^{+}\alpha2\beta1^{+}CD133^{+}$ putative stem population [4]. Notably, as seen in established PCa cell lines and despite detectable expression at

the gene level, we did not observe any CD133⁺ cells within the primary cultures (Figure 9).

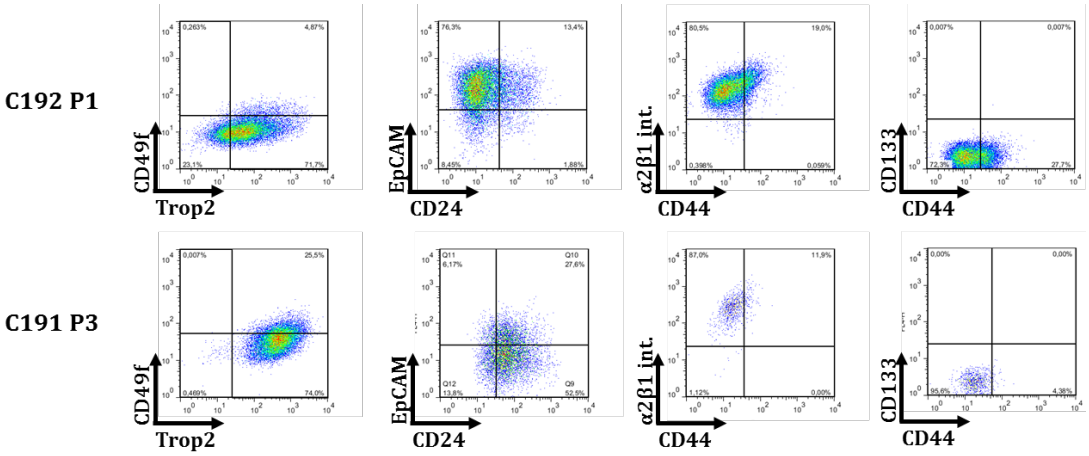

Figure 9: Expression of differentiation and stem-associated markers in primary cultures.
Flow cytometry analysis of 2 independent cultures (C192P1, C191P3) showing heterogeneous expression of several markers. Thresholds of positivity were defined according to negative controls using cells stained with isotype-matched immunoglobulins (not shown).

3.5. Malignancy of the cells

Notably, although specific markers have been shown to be up-regulated in PCa as compared to normal prostate [14], there is currently no cancer-specific marker allowing the discrimination between cancer and normal cells in culture. Expression of the TMPRSS2-ERG fusion gene could represent a good alternative since it has been demonstrated to be exclusively expressed in a major subtype of PCa but not in benign prostate [15]. Notably, in the patient cohort analysed in our laboratory, we found expression of TMPRSS2-ERG fusion gene in around 61 % of fresh PCa specimens (n=112 PCa tested) but never in BPH specimens (n=59 BPH tested), confirming the cancer specificity of this “marker”. Strikingly, we never

observed TMPRSS2-ERG expression in our primary cultures (n= 7). In particular, even for tumours with positive fresh tissue, the expression of TMPRSS2-ERG was not detectable in primary culture, suggesting that culture conditions might be unfavourable for the growth of these specific cancer cells.

Additionally, for 2 cultures, *in vivo* tumorigenic capacity was assessed following subcutaneous injection of the cells in immunodeficient mice. Notably, none of the tested cultures gave rise to tumours in recipient mice.

3.6. Perspectives

As we observed that adherent cell cultures do not appear to sustain tumourigenic cells growth, we decided to culture the cells in suspension. Using the same methodology as for adherent primary cultures, we cultured the cells (n=3 patients) on Ultra-Low Attachment Surface plates (Corning Tewksbury MA, USA). After a few days, spheres-like structures started to expand (Figure 10).

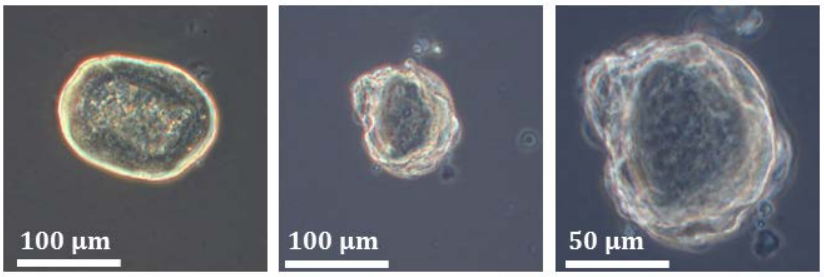

Figure 10: Sphere culture derived from a PCa sample (C203).

Cells were plated on a ultra-low adherent plate in a serum free medium (CnT52). Representative pictures are shown after 15 days of culture.

Unfortunately, the sphere-forming efficiency being low, we did not get enough material to perform gene expression analysis. Additional experiments aimed at allowing us to obtain enough spheres material to perform phenotypic and gene expression analysis are currently ongoing.

Conclusions and discussion

Since the pioneering identification of TIC in leukaemia, numerous investigators have reported the identification of TIC in diverse solid tissues, using a combination of surface markers (reviewed in "Introduction" Table 1).

Here, we have first investigated the expression of TIC markers in established PCa cell lines. Our results highlight the absence of distinct cell populations in these cell lines, consistent with the selection pressure potentially inherent with long-term culture. Importantly, we did not reliably detect the putative $CD133^+$ TIC population, in accordance with previous published studies [3,16]. In cells freshly derived from PCa clinical specimens, however, we observed a heterogeneous expression of several putative markers of TIC, consistent with the phenotypic heterogeneity typically characterising PCa. In our hands, the CD133+ population was not detectable in the large majority of tested samples (a $CD133^+$ population was only found in one sample (Figure 2)). Yet this population has been described to mark TIC in the prostate and would therefore be expected to be virtually present in all PCa samples tested [4].

The use of surface markers as a tool to identify cells with tumour-initiating capacity has been recently questioned in the cancer stem cell field. Indeed, it appears that TIC, as many other cells, are characterised by a remarkable phenotypic plasticity [17–19], and that the use of surface markers might therefore not be the most suitable method to identify and isolate them. CD133 expression, in particular, has been highly debated and has been shown to not be restricted to TIC populations in various tissues, including prostate [20–22].

Thus, the prostate CSC field is facing two challenges: the lack of reliable markers to identify TIC and the lack of experimental models which truly recapitulate patients' heterogeneity. In this context, the generation of short-term primary cultures derived from PCa specimens appears to represent an appealing model. Using and adapting published methods, we have generated primary cultures derived from 9 PCa clinical specimens. Notably, using our system, we observed an efficient expansion of the cells in culture. These cells exhibited an epithelial-like morphology and did express epithelial-specific markers at both RNA and protein levels, confirming their epithelial nature. Additionally, expression at RNA level of prostate differentiation and stem associated-markers such as AR, CgA, and CD133 was also demonstrated.

Full characterisation of cultured cells is required to claim the successful generation of primary cultures derived from PCa tissues. In particular, the lineage nature of the cells has to be investigated. In our culture models, we clearly detected the expression of AR gene, which represents a hallmark of luminal cells. Yet the expression of the gene encoding PSA, another marker of luminal cells, was more questionable. Additionally, our preliminary evidences indicate that un-passaged cultured are more likely to resemble a luminal phenotype than passaged cells. Nevertheless, expression of luminal genes at protein level should be performed in order to confirm the nature of these cells.

Surface markers such as Trop2 and CD49f have been successfully used to discriminate the different cell populations constituting the prostate [11]. Testing these markers, we found a high heterogeneity between cultures. Notably, we could observe the presence of $Trop2^{+}CD49f^{-/low}$ populations in several cultures, suggesting

a luminal nature for these cells. When comparing uncultured PCa-derived cell suspensions and primary cultures derived from the same patient, we observed that cultures selected for some phenotypes initially representing a sub-population from the uncultured cells. This is likely to be due to the culture conditions that might favour the growth of specific cells.

Unfortunately, no surface marker that can reliably discriminate cancer from normal cell has been identified so far [5]. Thus, the demonstration of the cancerous nature of the cells could only be performed using other methods such as chromosomal aberration analysis. Here, we have tested the *in vivo* tumorigenic capacity of 2 generated primary cultures. Unfortunately, none of the tested cultures did induce a tumour in immunodeficient mice. Nevertheless, since primary prostate cancer cells are well known for being inefficiently transplantable in immune-compromised mice, these results do not fully exclude the cancerous nature of these cells.

The use of such cultures for further experiments would require the full characterisation of their functional properties as well as their genotypic and phenotypic traits. For this reason, we did not use our short-term primary cultures in the following chapters of the thesis. Nevertheless, we are confident that the generation of well-characterised primary PCa cultures could be successfully achieved by careful modulation of defined culture parameters.

Taken together, our results highlight the scarcity and the inadequacy of models systems to study tumour-initiating cells in prostate cancer.

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Chapter II

Looking for a stemness signature in PCa

Research article:

Klf4 transcription factor is expressed in the cytoplasm of prostate cancer cells

Le Magnen et al., European Journal of cancer 2012

Klf4 transcription factor is expressed in the cytoplasm of prostate cancer cells

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Short title: Klf4 expression in prostate cancer cells

Abstract

BACKGROUND: Cancer initiation and progression might be driven by small populations of cells endowed with stem cell like properties. Here we comparatively addressed expression of genes encoding putative stemness regulators including c-Myc, Klf4, Nanog, Oct4A and Sox2 genes in benign prostatic hyperplasia (BPH) and prostate cancer (PCa).

METHODS: Fifty-eight prostate cancer (PCa) and thirty-nine benign prostatic hyperplasia (BPH) tissues samples were used for gene expression analysis, as evaluated by quantitative RT-PCR. The expression of specific Klf4 isoforms was tested by conventional PCR. Klf4 specific antibodies were used for protein detection in a tissue microarray including 404 prostate samples

RESULTS: Nanog, Oct4A and Sox2 genes were comparably expressed in BPH and PCa samples, whereas c-Myc and Klf4 genes were expressed to significantly higher extents in PCa than in BPH specimens. Immunohistochemical studies revealed that Klf4 protein is detectable in a large majority of epithelial prostatic cells, irrespective of malignant transformation. However, in PCa, a predominantly cytoplasmic location was observed, consistent with the expression of a differentially spliced Klf4α isoform.

CONCLUSION: Klf4 is highly expressed at gene and protein level in BPH and PCa tissues but a cytoplasmic location of the specific gene product is predominantly detectable in malignant cells. Klf4 location might be of critical relevance to steer its functions during oncogenesis.

Introduction

Prostate cancer (PCa) is a leading cause of cancer related death in men (1). Initially, most patients favourably respond to commonly used anti-androgen treatments, but tumors frequently recur and evolve towards castration-resistant stage, for which therapeutic options are scarce.

Tumor initiation and progression has been suggested to be driven by small populations of cells endowed with stem-like properties and therefore defined as cancer stem cells (CSC) (2). Interestingly, CSC may share properties of normal stem cells, such as self-renewal and differentiation potential, and utilize molecular pathways typically used by pluripotent embryonic stem cells (ESCs) (2, 3). Notably, the prognostic significance of ESC gene expression signatures in solid tumors, including PCa has been successfully demonstrated (3-5).

Stem cell-like pluripotency has been successfully induced in differentiated fibroblasts upon reprogramming by transfecting a limited number of genes, including Sox2, Oct4A, c-Myc, and Klf4 transcription factors, and further selection by using Nanog cell marker (6). The expression of these genes might therefore represent a stemness specific gene signature.

In this study, we comparatively evaluated the expression of Sox2, Oct4A, c-Myc, Nanog and Klf4 stemness-associated genes in PCa cell lines and in surgically excised PCa and benign prostatic hyperplasia (BPH) samples.

Patients, Material, and Methods

Clinical samples

We investigated specimens from a consecutive series of 39 patients with BPH and 58 with T1-pT3b stage PCa referred for treatment to the Department of Urology of the University Hospital of Basel, Switzerland, from 2008 to 2011. Patients bearing BPH underwent conventional transurethral resection (TUR-P), while patients bearing PCa underwent either palliative TUR-P or radical prostatectomy (RP). Pathological characteristics of PCa patients included in the gene expression study are recapitulated in supplemental Table 1 (S1).Written informed consent was obtained from all patients in accordance with the requirements of the local Ethical Committee (EKBB, Ref.Nr.EK: 176/07).

Cell cultures

Certified, established prostate cancer cell lines Du145 (DSMZ, Braunschweig, Germany), PC3, VCaP, and LNCaP (LGC Standards, Molsheim, France) were routinely cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, Penicillin 10 U/ml and Streptomycin 10 µg/ml (Invitrogen Carlsbad, California, USA).

Quantification of gene expression by quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from cell lines and tissues, by using the RNeasy® Mini kit protocol (Qiagen, Basel, Switzerland). RNA from CH-ES3 human embryonic stem cell line (passage 14) was kindly provided by Dr. Sterthaus (University Hospital, Basel, Switzerland). Pooled RNA from normal human prostate samples was purchased from

Clontech (Mountain View, CA, USA). All RNAs were treated by Deoxyribonuclease I (DNAse I; Invitrogen, Carlsbad, CA, USA), and reverse transcribed by using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen, Carlsbad, CA, USA).

Quantitative real-time PCR assays were performed by using an ABI prism™ 7700 sequence detection system, utilizing Taqman® Universal PCR Master Mix (Applied Biosystems, Forster City, CA, USA). Specific gene expression was evaluated using the $2^{-\Delta\Delta C}$T method (7). Gene expression was normalized using GAPDH house-keeping gene as reference.

The following primer sequences were derived from existing literature, as indicated below:

GAPDH (8)

Fwd: ATGGGGAAGGTGAAGGTCG

Rev: TAAAAGCAGCCCTGGTGACC

Probe: FAM-CGCCCAATACGACCAAATCCGTTGAC-TAMRA

c-Myc (9)

Fwd: GCCACGTCTCCACACATCAG

Rev: TCTTGGCAGCAGGATAGTCCTT

Probe: FAM-ACGCAGCGCCTCCCTCCACTC-TAMRA

Sox2, Nanog, Oct4A, Klf4, and CD133 gene specific primers and probes were provided by Assays-on-Demand, Gene Expression Products (Applied Biosystems, Foster City, CA).

Detection of Klf4 specific isoforms by PCR

Detection of differentially spliced Klf4 isoforms was performed by conventional RT-PCR using the following primers, designed to detect the entire coding region of Klf4 mRNA, and derived from existing literature (10):

Fwd: 5'-ATGGCTGTCAGCGACGCGCTGC-3'

Rev: 5'TTAAAAATGCCTCTTCATGTGTAAGGCG-3'

Expected sizes for Klf4 wild-type and Klf4α are 1410 and 440 bp, respectively. GAPDH gene was used as a loading control and amplified by using the following primers (11):

Fwd: 5'-CAACAGCCTCAAGATCATCAGC-3'

Rev: 3'-TTCTAGACGGCAGGTCAGGTC-3'

Tissue Micro-array (TMA) and immunohistochemistry

The prostate tissue micro-array (TMA) used in this study was previously described (12). Interpretable specimens (n=404) included a) transurethral resections from 46 patients with BPH as controls, b) samples from 46 high grade prostatic intra-epithelial neoplasias (PIN) and c) 111 clinically localised PCa from transurethral resections (T1a/b; n= 41) and radical prostatectomy specimens (pT2a-pT3b; n= 70), d) transurethral resections from 83 castration-resistant local recurrences, e) 78 metastatic lesions from liver, lung, and lymph nodes, and 40 samples for which pathological staging data were not available.

Immunohistochemistry was performed by using standard indirect immuno-peroxidase procedures. A specific polyclonal rabbit antibody (sc20691, Santa Cruz Biotechnology, Heidelberg, Germany) was used to detect Klf4 protein on prostate and skin sections. Klf4 specific staining was scored as previously described (13).

Immunofluorescence

Human skin sections and cells from established PCa cell lines attached to multiwell chamber slides (BD Biosciences, San Josè, CA) were stained by anti Klf4 polyclonal rabbit antibody preparation (see above) following incubation with a blocking solution (0.3% Triton, 2% goat serum in PBS). Specific binding was then revealed by incubation in the presence of a rabbit immunoglobulin specific, fluorochrome (Alexa Fluor® 488) labelled goat reagent (Invitrogen, Basel, Switzerland). Nuclei were counterstained by DAPI (Invitrogen). Samples were then mounted in an aqueous medium (Dako, S3025) and analysed on an Olympus BX61 fluorescence microscope (Olympus, Homburg, Germany).

Statistical analysis

Statistical analyses were performed by using SPSS software (version 17.0, SPSS Inc., Chicago, IL, USA). Each panel of samples was first tested for the normality of the populations. Parametric T-test or non-parametric Mann-Whitney test were used, for normal or non-normal population, respectively. The correlation of specific expression between two groups was assessed using Pearson correlation test. P-values <0.05 were considered statistically significant.

Results

Expression of stemness related genes in PCa established cell lines

Cancer cell subsets have been suggested to share defined molecular features with normal embryonic stem cells (3). In order to investigate specific gene signatures, we evaluated the expression of Sox2, Nanog, Oct4A, Klf4, and c-Myc genes, encoding pluripotency-associated transcription factors (6, 14), in four established human PCa cell lines. As positive control, we used cDNA from a human embryonic stem cell line, CH-ES3 (see "Materials and Methods").

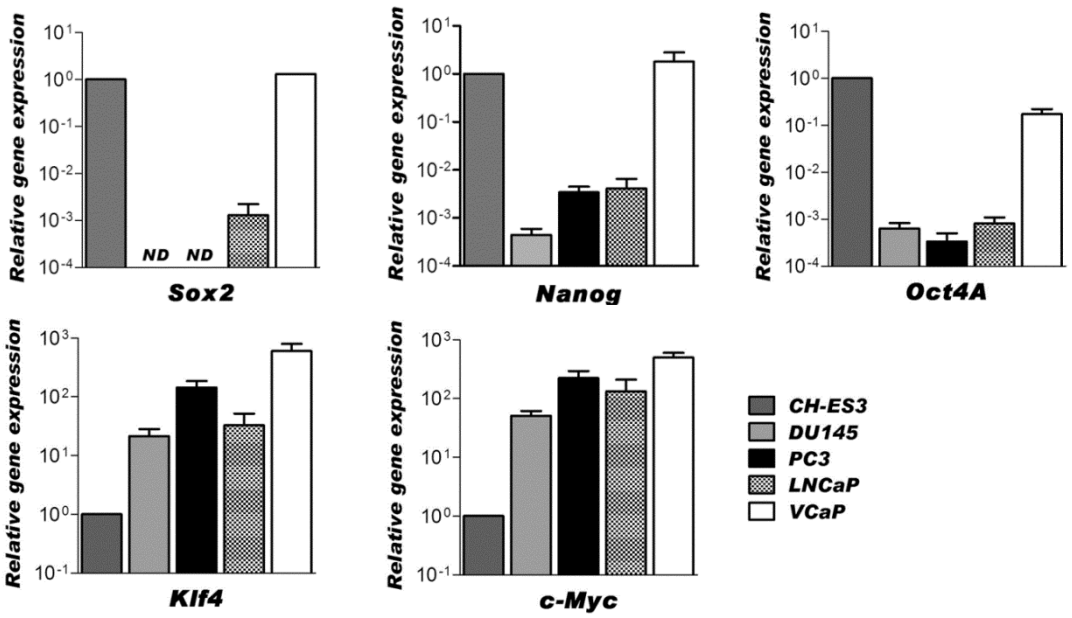

Figure 1: Expression of stemness-related genes in established prostate cancer cell lines.

Total cellular RNA was extracted from Du145 ( ), PC3 ( ), LNCaP ( ), and VCaP ( ) PCa established cell lines, reverse transcribed and tested in quantitative RT-PCR assays for the expression of Sox2, Nanog, Oct4A, Klf4, and c-Myc genes. Gene expression levels are reported as relative values as compared to the human embryonic stem cell line CH-ES3 ( ). ND: non detected. Mean values refer to at least 2 independent experiments, each performed in duplicates. Standard deviations are indicated for each cell line.

Positive control CH-ES3 cells expressed to high extents the five transcription factors under investigation. Expression of Nanog, Oct4A, Klf4, and c-Myc genes could be observed to different extents in all PCa cell lines. In contrast, expression of Sox2 gene was undetectable in Du145 and PC3, weak in LNCaP, but high in VCaP cell line (Figure 1A). Remarkably, all PCa cell lines showed similar or lower expression of Sox2, Nanog and Oct4A genes, but a markedly higher expression of Klf4 and c-Myc genes, as compared to CH-ES3 cells.

CD133 has been proposed as marker of normal and cancer stem cells in prostate (15, 16). In agreement with previously published data (17), CD133 gene expression was only weakly detectable in DU145 and VCaP cell lines (data not shown).

Expression of stemness-associated genes in prostatic tissues from patients bearing either BPH or PCa

Capitalizing on data from established cell lines, we analysed the expression of pluripotency-associated genes in clinical samples freshly obtained from patients diagnosed with either BPH or PCa.

Nanog and c-Myc genes were expressed in all samples tested, irrespective of malignant transformation (figure 2). Oct4A transcripts were detected in 24/24 (100%) BPH tissues investigated and in 41/43 (95.3%) PCa tissues, while Sox2 gene was expressed in 29/36 (80.5%) BPH and in 51/54 (94.4%) PCa samples. Klf4 gene was found to be expressed in 100% of PCa (n=54) and in 94.3% of BPH tissues (33/35).

In accordance with previous studies (18), the expression level of c-Myc gene was significantly higher in PCa than in BPH (*P*=0.03). Interestingly, Klf4 gene expression was also significantly increased in PCa as compared to BPH (*P*=0.04).

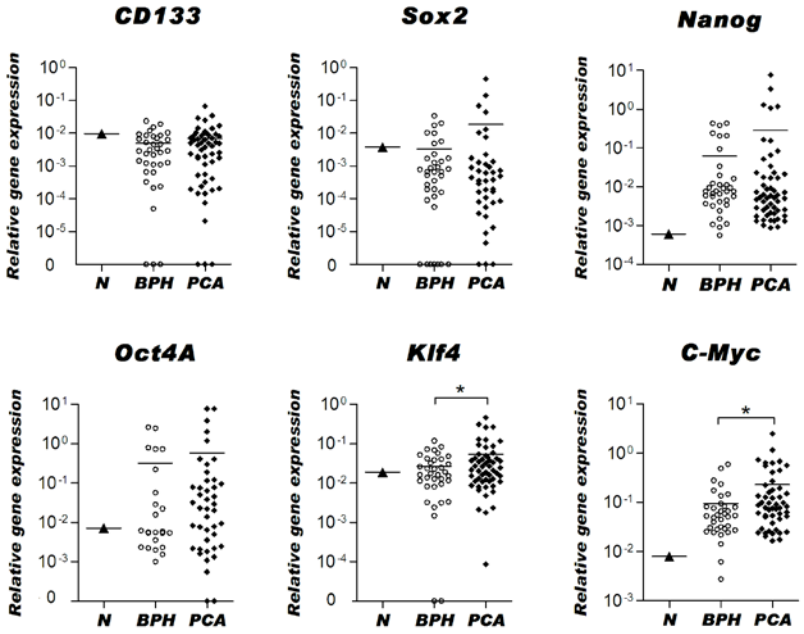

Figure 2: Detection of CD133, Sox2, Nanog, Oct4A, Klf4, and c-Myc gene expression in human BPH and PCa tissues

Total cellular RNA was extracted from BPH (○, n=35), or T1-pT3b PCa (♦, n=54) surgical specimens, reverse transcribed and tested in quantitative RT-PCR assays for the expression of the indicated genes. Expression levels are reported as relative values, using GAPDH housekeeping gene as reference. Significant differences between BPH and PCa groups are indicated (*: p< 0.05). As control, a cDNA pool from normal prostates was also tested (▲). Mean values (—) are indicated for each group.

Minor increases in expression of Nanog and Sox2 in samples from patients bearing PCa, as compared to BPH tissues (*P*=0.16 and *P*=0.15, respectively) did not reach the threshold of statistical significance.

CD133 gene was expressed in 33/36 (91.7%) BPH and in 51/54 (94.4%) PCa tissues and no significant difference in expression levels was observed between BPH and PCa (*P*=0.29).

We performed a similar analysis on RNA from a pool of normal prostate tissues. Interestingly, Oct4A, Nanog, and c-Myc genes were markedly less expressed in normal prostate tissues as compared to a large majority of BPH and PCa tissues. Instead, for Klf4 and Sox2 genes, higher expression appeared to be limited to PCa specimens.

Interestingly, Klf4 gene expression correlated with the expression of Sox2, Oct4A, c-Myc and Nanog in PCa, suggesting a close relationship between these genes (Supplemental figure S1).

Klf4 expression is not restricted to a minor subset of cells within prostate tissues

Prompted by these data, we investigated Klf4 expression at the protein level by staining a PCa "progression" TMA including 404 specimens.

As positive control, we used sections from skin, known to strongly express Klf4 (19). As expectable for a transcription factor, a predominantly nuclear signal was detected upon Klf4 specific staining in dermal and epidermal cells (Figure 3I).

In prostate tissues, we observed that Klf4 protein expression in both BPH and PCa was not limited to small cell subsets, but rather detectable in a large majority of epithelial cells, irrespective of malignant transformation (Figure 3). Most interestingly, Klf4 location was predominantly nuclear in BPH samples (panels A-B), although cytoplasmic staining was also detectable. On the other hand, in cancerous tissues, irrespective of clinical stages, a prevailingly cytoplasmic location of Klf4 protein could be observed (figure 3, panels C-H). Accordingly, a predominantly cytoplasmic Klf4 location was also detectable in established PCa cell lines (Supplementary figure 2).

Klf4 expression was then comparatively investigated, at the protein level, in BPH and tumors at different clinical stages. Importantly, in the 364/404 specimens for which pathological staging was available, a significant ($P \leq 0.001$) increase in Klf4 total staining score was detectable in PIN, PCa, CR PCa and metastatic lesions as compared to BPH samples (Figure 3, panel J).

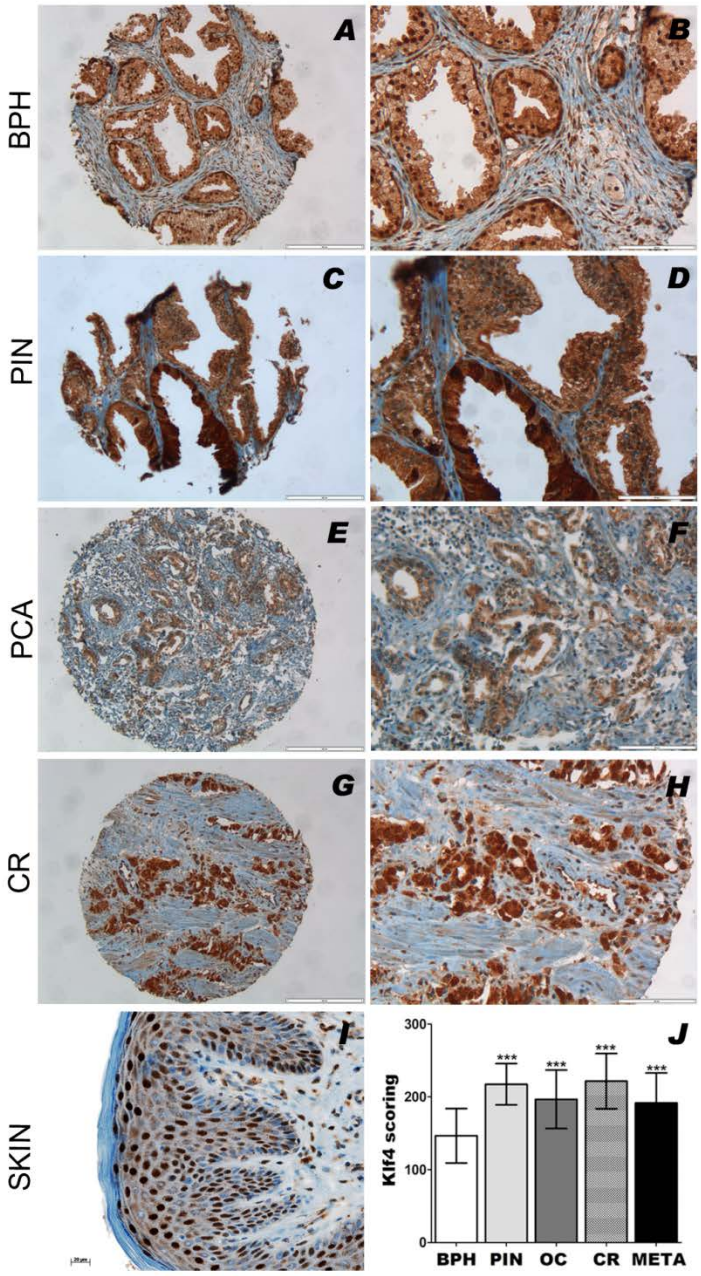

Figure 3: Klf4 expression is not restricted to a minor cell subset in prostate surgical specimens

A TMA containing benign prostatic hyperplasia (BPH: **A-B**), prostatic intraepithelial neoplasia (PIN: **C-D**), organ-confined prostate cancer (PCa: **E-F**), and castration-resistant prostate cancer (CR: **G-H**), was stained with a Klf4 specific reagent (see "Materials and methods). Representative pictures at 100x (left panels) and 200x (right panels) magnification are shown. As positive control, skin tissue was also stained using the same procedure (I). The TMA was then analysed and scored by an experienced pathologist as described in "materials and methods". Means of Klf4 scores are reported for patients with BPH ( ), PIN ( ), clinically localised PCa ( ), castration-resistant PCa (CR:), and PCa metastases (meta:). Standard deviations and significance (***: P≤0.001) of the observed differences are also reported.

Expression of Klf4 isoforms in PCa

Recently, different Klf4 isoforms have been described in pancreatic cancer (10). At least one of them, Klf4α, is devoid of nuclear localization signal and is therefore primarily located in the cytoplasm of tumor cells. Full length sequence of Klf4α isoform and splicing sites are shown in figure 4A. To gain insights into the molecular background underlying intracellular localization of Klf4 in prostate samples, as detected by antibodies recognizing multiple protein isoforms (10), we performed PCR analysis using a primer set amplifying full-length Klf4 mRNA.

As positive control, we used HT29 colorectal cancer cell line, previously shown to express several Klf4 specific isoforms (10). In 8/12 PCa surgical specimens, we could detect several bands (Figure 4). In particular, the size of a high molecular weight (MW) band corresponded to that of wild-type Klf4 (1410 bp), while that of a lower MW band corresponded to the size of the putative Klf4α isoform (440 bp), missing exon 3, as previously reported (10). Notably, the low MW Klf4α band was also detectable in cDNA from 3/11 BPH samples, and prominently in Du145 PCa cell line. Representative data are reported in figure 4B. To confirm the specificity of Klf4α amplification, we harvested, cloned and sequenced the low MW band obtained from two PCa clinical specimens. Sequencing analyses (supplementary figure 3) showed that this amplicon fully shares Klf4α sequence.

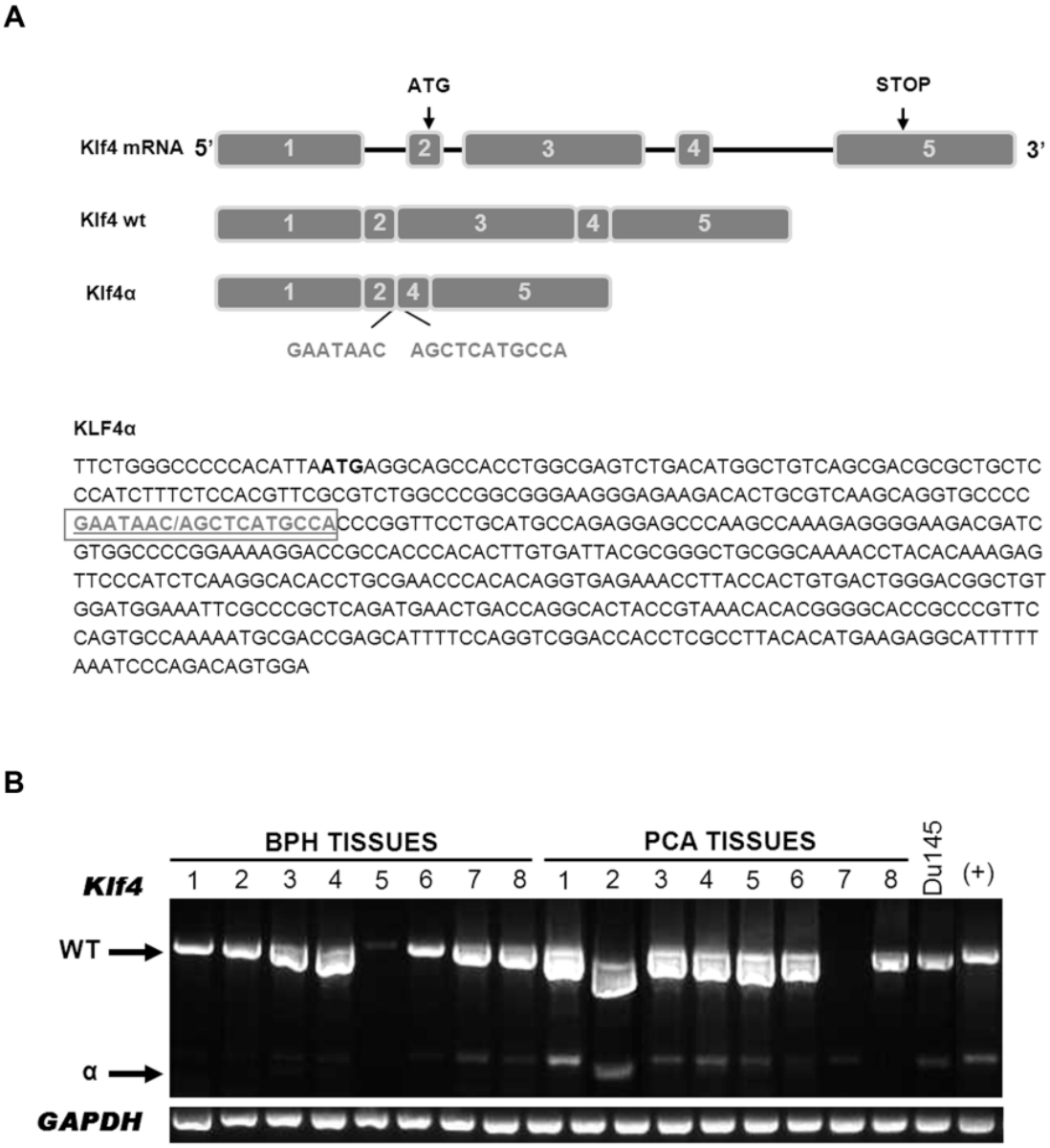

Figure 4: Expression of Klf4 specific isoforms in prostate tissues and cell lines

A: Klf4 splicing sites and Klf4α sequence. **B**: cDNA obtained from human prostate samples including BPH and PCa and Du145 PCa cell line were used for PCR analysis using Klf4 primers amplifying the entire cDNA sequence. As positive control, cDNA from HT29 colorectal cell line (+) was tested in the same conditions. Two predominant bands were detected, corresponding to Klf4 « wild type » (« wt »: 1410 bp; upper band), and Klf4α (« α »: 440 bp; lower band).

Discussion

Expression of genes encoding pluripotency-associated transcription factors, including Oct4A, Sox2, c-Myc, and Klf4, typically detectable in embryonic cells, might be considered as representing a "bona fide" stemness signature.

Interestingly, specific embryonic signatures have been reported in different solid tumors, including breast and bladder cancers (3, 20). Regarding PCa, microarray data set analysis has recently shown that the expression of stemness signatures correlates with poor outcome, when associated with p53 and PTEN inactivation (4). The expression of Nanog, an ESC marker involved in the self-renewing process, has also been shown to be increased in PCa (21), while data on Klf4 gene expression are controversial (5, 22-24).

In this study, in keeping with recently published data (25), we could confirm that a specific ESC signature can be observed in established PCa cell lines. Remarkably, we have observed a relatively high expression of stemness-associated genes in BPH and PCa, as compared to normal prostate tissues from non-age matched healthy donors. Furthermore, here we show that c-Myc and Klf4 genes are expressed to significantly higher extents in PCa than in BPH tissues from age matched patients. The detection of increased c-Myc gene expression is in full agreement with its putative role in prostate carcinogenesis and with studies by others (26). In contrast, the detection of increased Klf4 gene expression in PCa is more puzzling.

Klf4 is a transcription factor involved in many cellular processes including regulation of proliferation and differentiation. Klf4 has been suggested to prevent

ESC differentiation (27, 28). In addition, recent studies are consistent with a potential role of Klf4 in cancer, depending on cell context and tissue specificity (29). While Klf4 has been demonstrated to be a potential tumor suppressor gene in colon and lung carcinogenesis (30, 31), it acts as an oncogene in breast and skin cancers (22, 32).

Limited and controversial literature is presently available on Klf4 gene expression in normal, hyperplastic and cancerous prostate. To the best of our knowledge, the only study addressing the expression of Klf4 in BPH reported a non-significant difference of expression between BPH (n=9) and normal tissues (23). Furthermore, a study based on a minute database (n=4) (22), reported a lower expression of Klf4 gene in PCa samples as compared to adjacent "uninvolved" tissue. In contrast, Schoenhals and colleagues found an overexpression of Oct4, Sox2, c-Myc, and Klf4 genes in PCa, as compared to normal tissues (5). In particular, Klf4 gene expression was associated with tumor grade. Most recently, based on the finding of a decreased expression in PCa with evidence of lymph-node metastases, as compared to tumors without lymph-node metastases, Klf4 has been proposed to be an inhibitor of tumor cell growth and migration (24).

Our findings led us to investigate the expression of Klf4 at protein level in a TMA including substantial numbers of BPH and PCa at different clinical stages.

Surprisingly, we observed that Klf4 protein is detectable in a wide majority of epithelial cells in both BPH and PCa tissues. These findings argue against the hypothesis that this gene product might serve as marker of minor cells subsets possibly involved in cancer initiation.

Moreover, we observed that PIN, organ confined and castration-resistant PCa are characterised by a significant increase in Klf4 total protein detection, as compared to BPH. Most importantly, immunohistochemistry data indicate for the first time the presence of a modified compartmentalization of the specific gene product since a preferential cytoplasmic location is clearly detectable in PCa, but not in BPH.

Cytoplasmic location is relatively unexpected for a transcription factor, and, indeed, Klf4 staining is mainly detectable in nuclei of healthy skin cells. However, differentially spliced isoforms of Klf4 have recently been described in pancreatic cancer (10). At least one of them, Klf4α, is devoid of nuclear localization signal encoded in exon three, and is therefore primarily located in the cytoplasm of tumor cells. Remarkably, while Klf4 wild-type has been associated with tumor-suppression in pancreas, cytoplasmic Klf4α was shown to act in an opposite manner and to promote tumorigenesis (10).

Here, we show for the first time that the expression of Klf4α isoform is frequently detectable in PCa, and, more rarely in BPH tissues. Importantly, the anti-Klf4 specific antibody preparation used throughout our study has been previously shown to recognize both Klf4 and Klf4α isoforms (10). Therefore, cytoplasmic Klf4 found in prostatic tissues might result from the expression of Klf4α, as suggested by PCR assays. Alternatively, we might hypothesize that, in the presence of Klf4α gene product, Klf4 wild type could also be "trapped" in the cytoplasm, as recently proposed based on co-transfection studies (10).

Notably, specific isoforms have also been identified for Klf6 transcription factor. At least one of them, Klf6-SV1, has also been found to be localised in the

cytoplasm of tumor cells and to be associated with prostate cancer progression and metastatic behaviour (33). Furthermore, Oct4A gene product has also been "atypically" detected in cellular cytoplasm in both prostatic and lung malignant tissues (34, 35).

Taken together, our data pave the way for additional studies aimed at obtaining mechanistic insights into the regulation of Klf4 gene expression in normal prostate, BPH and PCa. On the other hand, they raise the issue of the functional significance of differentially spliced cytoplasmic Klf4 in PCa.

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Conflict of interest statement

None declared.

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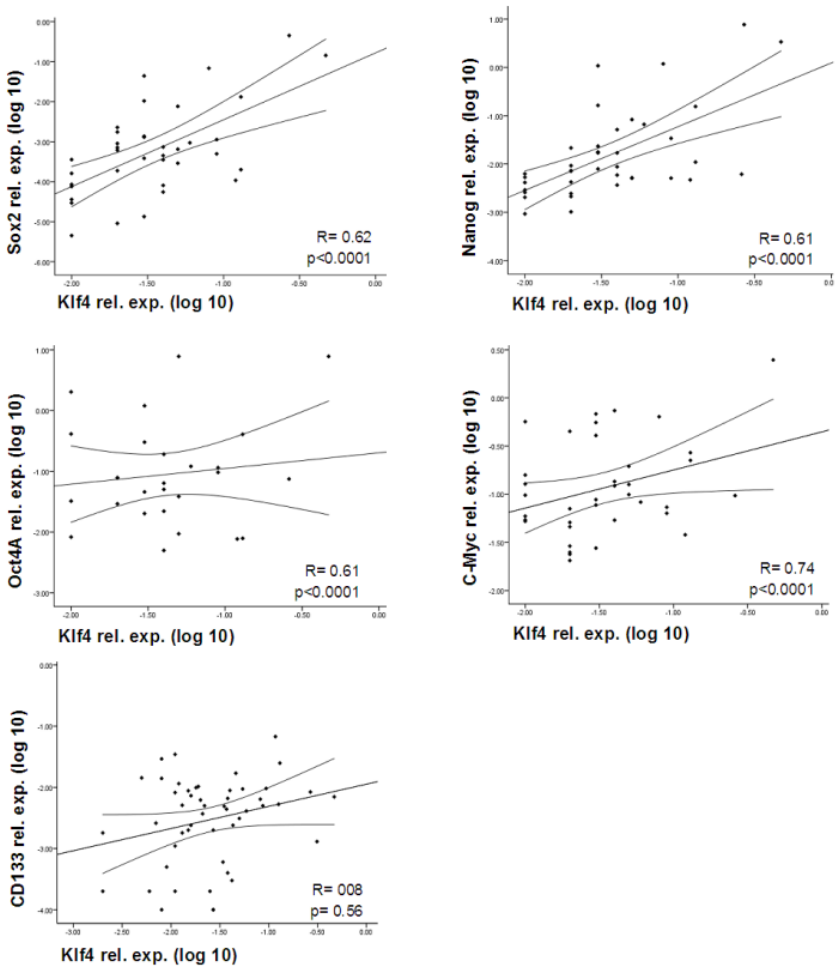
Supplementary Table 1

TABLE S1. Characteristics of PCa patients (n=58)		
		N patients (%)
Stage		
	T1	1 (1.7%)
	pT2a	8 (13.8%)
	pT2b	3 (5.2%)
	pT2c	35 (60.3%)
	pT3a	4 (6.9%)
	pT3b	4 (6.9%)
	NA	3 (5.2%)
Gleason score		
	5-6	19 (32.8%)
	7	31 (53.5%)
	8	2 (3.4%)
	9	5 (8.6%)
	NA	1 (1.7%)
Nodal status		
	N0	43 (74.1%)
	Nx	3 (5.2%)
	N1	1 (1.7%)
	NA	11 (19.0%)

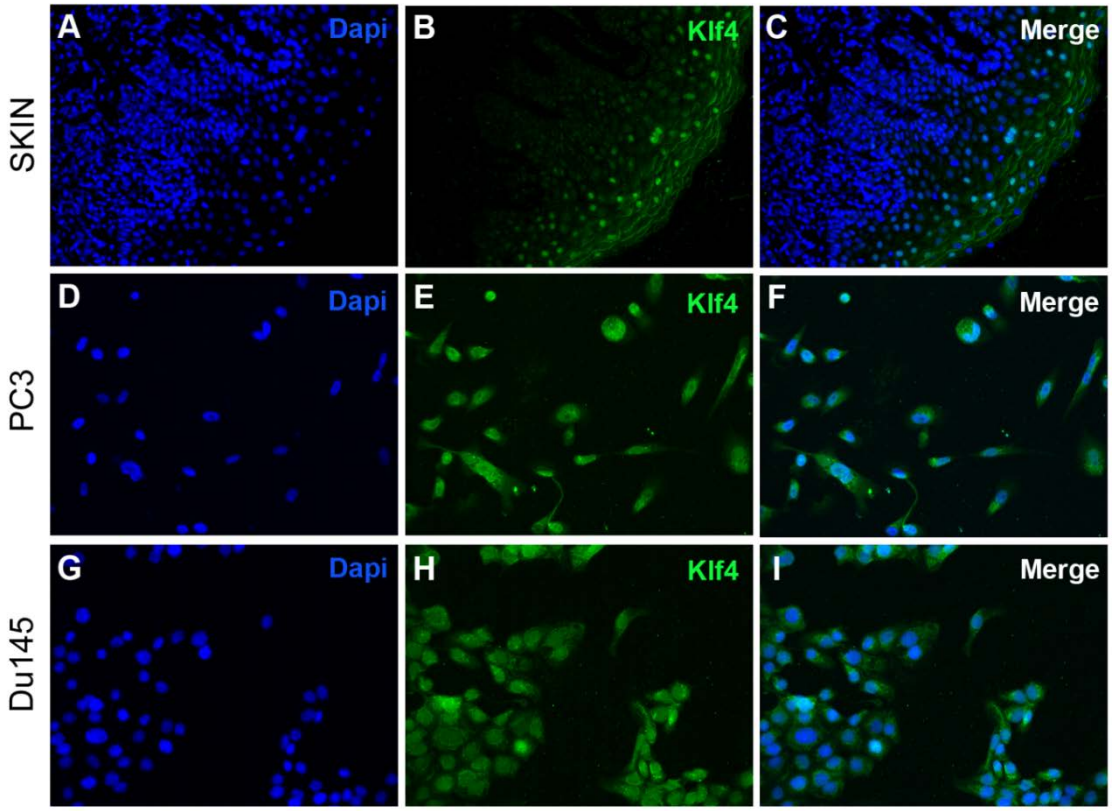
NA: not available

Supplementary Figures



Supplementary Figure 1: Klf4 gene expression correlates with that of other stemness-associated genes

The association of Klf4 relative gene expression (rel. exp.) with that of Sox2 (**A**), Nanog (**B**), Oct4A (**C**), c-Myc (**D**), and CD133 (**E**) genes, as detected in Figure 2, was investigated in PCa tissues (n=54) by Pearson correlation analysis. R correlation coefficients and p values are reported in each panel.



Supplementary figure 2: Klf4 detection in established PCa cell lines

Cells from PC3 (panels **D-F**) and Du145 (panels **G-I**) established PCa cell lines, were stained with a Klf4 specific polyclonal antibody preparation (see "materials and methods"). Specific binding was revealed by a fluorescent anti-rabbit immunoglobulin reagent. Nuclei were counterstained by DAPI. Skin sections (panels **A-C**) were also stained to provide positive control specific nuclear staining.

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Wei-α  46   ATGGCTGTCAGCGACGCGCTGCTCCCATCTTTCTCCACGTTCGCGTCTGGCCCGGCGGGA  105
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C149c  1    ATGGCTGTCAGCGACGCGCTGCTCCCATCTTTCTCCACGTTCGCGTCTGGCCCGGCGGGA  60

Wei-α  106  AGGGAGAAGACACTGCGTCAAGCAGGTGCCCCGAATAACAGCTCATGCCACCCGGTTCCT  165
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C149c  61   AGGGAGAAGACACTGCGTCAAGCAGGTGCCCCGAATAACAGCTCATGCCACCCGGTTCCT  120

Wei-α  166  GCATGCCAGAGGAGCCCAAGCCAAAGAGGGGAAGACGATCGTGGCCCCGGAAAAGGACCG  225
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C149c  121  GCATGCCAGAGGAGCCCAAGCCAAAGAGGGGAAGACGATCGTGGCCCCGGAAAAGGACCG  180

Wei-α  226  CCACCCACACTTGTGATTACGCGGGCTGCGGCAAAACCTACACAAAGAGTTCCCATCTCA  285
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C149c  181  CCACCCACACTTGTGATTACGCGGGCTGCGGCAAAACCTACACAAAGAGTTCCCATCTCA  240

Wei-α  286  AGGCACACCTGCGAACCCACACAGGTGAGAAACCTTACCACTGTGACTGGGACGGCTGTG  345
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C149c  241  AGGCACACCTGCGAACCCACACAGGTGAGAAACCTTACCACTGTGACTGGGACGGCTGTG  300

Wei-α  346  GATGGAAATTCGCCCGCTCAGATGAACTGACCAGGCACTACCGTAAACACACGGGGCACC  405
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C149c  301  GATGGAAATTCGCCCGCTCAGATGAACTGACCAGGCACTACCGTAAACACACGGGGCACC  360

Wei-α  406  GCCCGTTCCAGTGCCAAAAATGCGACCGAGCATTTTCCAGGTCGGACCACCTCGCCTTAC  465
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C149c  361  GCCCGTTCCAGTGCCAAAAATGCGACCGAGCATTTTCCAGGTCGGACCACCTCGCCTTAC  420

Wei-α  466  ACATGAAGAGGCATTTTTAAA  486
            ||||||||||||||||||||
C149c  421  ACATGAAGAGGCATTTTTAAA  441
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Supplementary Figure 3: Alignment of predicted and PCa sample derived Klf4α sequences.

Total cellular RNA from a PCa sample (C149c) was extracted, reverse transcribed and amplified by 35 cycles RT-PCR in the presence of primers spanning through the entire Klf4 cDNA sequence and run on agarose gels (see "materials and methods"). A low, 441 bp band was then excised and DNA was purified and sequenced by standard methods. The sequence obtained fully matched the predicted Klf4α isoform sequence (Wei-α) (10).

Chapter III

Characterization and clinical relevance of ALDH^{bright} populations in prostate cancer

Research article

Le Magnen et al., in revision for Clinical Cancer Research

Characterization and clinical relevance of $ALDH^{bright}$ populations in prostate cancer

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6 Figures

Supplementary data: 2 Figures, 4 Tables

Conflict of interest: none

Statement of translational relevance

Tumor-initiating cells (TIC) are highly tumorigenic cells, putatively responsible for resistance to conventional therapies and tumor relapses. High ALDH activity has been proposed to represent a functional marker of TIC in various types of tumors, including prostate cancer (PCa). Yet clinical relevance of $ALDH^{bright}$ populations has not been investigated in detail so far in PCa. Here, using a large cohort of multi-stage PCa, we show that $ALDH^{bright}$ populations are present and heterogeneously distributed across PCa tissues. Furthermore, we demonstrate that expression of ALDH1A1 specific isoform, at the gene and protein level is associated with advanced clinical stage and unfavorable prognosis in "hormone therapy naïve" PCa. Our findings highlight the importance of ALDH in PCa pathogenesis and suggest that its clinical significance might be specific for sub-groups of patients. These results may help to improve stratification and identification of high-risk patients with PCa.

Abstract

PURPOSE: High aldehyde dehydrogenase (ALDH) activity has been suggested to select for cells endowed with tumor initiating capacity in prostate cancer (PCa). Yet existence of cells with high ALDH activity ($ALDH^{bright}$) in fresh PCa primary specimens has not been demonstrated so far. We investigated presence, phenotype, and clinical significance of $ALDH^{bright}$ populations in clinical PCa specimens.

EXPERIMENTAL DESIGN: We used the Aldefluor™ technology and FACS staining to identify and characterize $ALDH^{bright}$ populations in cells freshly derived from clinical PCa specimens. Expression of genes encoding ALDH specific isoforms was evaluated by quantitative real-time PCR in normal, benign prostatic hyperplasia (BPH) and PCa tissues. ALDH1A1 specific expression and prognostic significance were assessed by staining two tissue microarrays (TMA) including over 500 samples of BPH, prostatic intraepithelial neoplasia (PIN), and multi-stage PCa.

RESULTS: Here, we show for the first time that $ALDH^{bright}$ cells are detectable in freshly excised PCa specimens (n= 39), and that they are mainly included within the $EpCAM^{(+)}$ and $Trop2^{(+)}$ cell populations. Although several ALDH isoforms are expressed to high extents in PCa, only ALDH1A1 gene expression significantly correlates with ALDH activity ($p<0.01$), and is increased in cancers with high Gleason scores ($p=0.03$). Most importantly, ALDH1A1 protein is expressed significantly more frequently and to higher extents in advanced as compared to low stage PCa and BPH. Remarkably, ALDH1A1 positivity is associated with poor survival ($p=0.02$) in "hormone therapy naïve" patients.

CONCLUSIONS: Our data indicate that ALDH contributes to the identification of subsets of PCa cells of potentially high clinical relevance.

Introduction

Despite the availability of several therapeutic options, prostate cancer (PCa) remains a leading cause of cancer-related death in men [1]. PCa is characterized by a remarkable cellular heterogeneity, and includes cells with different phenotypes, proliferative capacities and differentiation states [2]. Tumor cell phenotypic and functional heterogeneity might be supported by small populations of cells endowed with tumor-initiating capacities and therefore referred to as tumor-initiating cells (TIC). TIC can be defined functionally as cells exclusively capable of initiating tumors in immunodeficient mice [3]. Interestingly, although their molecular characterization is debated, TIC are thought to share functional properties , such as self-renewal and differentiation capacities, with normal stem cells [4].

Several groups have previously identified putative TIC in hematopoietic cell malignancies [5], and solid tumors such as breast [6], or colorectal cancers [7]. Regarding prostate cancer, several putative TIC populations have been identified and isolated from different compartments of the prostatic epithelium. In a pioneering study, Collins and co-workers first demonstrated, that “stem-like” prostate cancer cells can be identified in the basal epithelial layer, using a combination of surface markers including CD44, α2β1, and CD133 [8]. In recent years, several groups have successfully identified prostate cancer cells with tumor-initiating properties, using different combinations of markers [9-11]. In particular, Wang and colleagues elegantly demonstrated that murine prostate cancer can be re-initiated after castration by castration-resistant Nkx3.1 expressing cells (CARNs), exhibiting a luminal phenotype [10]. More recently, a functional luminal CARN-like population

was identified in the BM18 xenograft model of human PCa [12]. Besides, Goldstein and colleagues have shown that human PCa might be initiated by basal cells, positive for CD49f and Trop2 [13]. Altogether, these studies suggest the co-existence of several potential TIC subsets in prostate cancer. Importantly, TICs might possess a plastic phenotype. Therefore, the use of surface markers might represent a limited approach to identify them.

Aldehyde dehydrogenase (ALDH) enzyme is responsible for the oxidization of cellular aldehydes resulting in the production of retinoic acid [14]. Notably, ALDH has been shown to be involved in stem cell protection and differentiation and high levels of ALDH activity have been found in several stem cell populations [15]. Thus, high ALDH activity has been used to select and identify normal stem cells and TIC in a variety of solid tissues including breast [16] and colon [7].

In the prostate, high ALDH activity has first been shown to represent a functional marker for murine normal progenitor/stem cells [17]. More recently, cells exhibiting high ALDH activity (referred to as "$ALDH^{bright}$") have been identified in human PCa cell lines and expanded primary PCa cultures [18]. Interestingly, when isolated from established PCa cell lines, $ALDH^{bright}$ cells were associated with increased clonogenicity, invasiveness, and tumorigenic and metastatic capacities [18, 19]. Nevertheless, culture conditions might modulate cell characteristics, potentially favouring selection of specific phenotypes, and PCa cell lines may therefore not adequately reflect the biology of human prostate cancer[20].

Importantly, evidence of the existence and functional characterization of $ALDH^{bright}$ populations in uncultured clinical PCa specimens is still missing.

Moreover, it is still unclear whether these cells may have clinical and prognostic relevance in PCa.

In this study, we have identified, quantified, and characterized ALDH^{bright} populations in epithelial cells freshly isolated from established PCa cell lines and a series of PCa specimens directly retrieved from surgery. We show that these populations are heterogeneous across patients and are associated with high tumorigenicity *in vivo*. Finally, we have assessed localization and clinical relevance of cells expressing ALDH1A1 isoform using two distinct tissue microarrays (TMAs). Our results support an association between ALDH1A1 positivity and poor prognosis in PCa**.**

Material and Methods

Clinical specimens

We investigated a series of 38 patients with BPH and 71 patients with pT2a-pT3b stage PCa referred for treatment to the Department of Urology of the University Hospital of Basel (Switzerland) from 2008 to 2012. Patients with BPH underwent conventional transurethral resection (TUR-P), while patients with PCa underwent radical prostatectomy (RP). Written informed consent was obtained from all patients in accordance with the requirements of the local Ethical Committee (EKBB, Ref.Nr.EK: 176/07).

Supplementary table 1 (Table S1) summarizes clinical and pathological data characterizing PCa patients used for the present study.

Isolation of primary cells freshly derived from PCa surgical specimens

Prostate tissues were screened for the presence of tumor tissues by experienced pathologists. PCa samples, were chopped, washed and then digested in a mixture containing DMEM, 5 % Knockout Serum Replacement (KO serum, Gibco, Paisley, UK), 1% Pen/Strep and 200IU/ml of type I collagenase (Worthington, Lakewood, NJ). After a 12-16 hour incubation at 37°C, digested tissues were washed and underwent a series of differential centrifugations, as previously described to separate epithelial and stromal fractions [21]. Cell pellets enriched in epithelial fraction were re-suspended in PBS and passed through a 100 μm cells strainer in order to obtain single cell suspensions. Resulting cells were immediately used for FACS analysis to assess ALDH activity and surface markers expression (see below).

Cell cultures

Established prostate cancer cell lines Du145, PC3, and VCaP (LGC Standards, Molsheim, France) were routinely cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, Penicillin 10 U/ml and Streptomycin 10 µg/ml (Pen/Strep, Invitrogen Carlsbad, CA).

Quantification of gene expression by quantitative Real-Time PCR (qRT-PCR)

Cultured cells were collected and washed in PBS. Prostatic tissues were collected by an experienced pathologist, immediately submerged in RNAlater (Ambion, Foster City, CA) and stored at -70°C until further processing. A normal human RNA prostate pool was purchased from Clontech (Clontech Laboratories Inc., Mountain View, CA). Total cellular RNA was extracted and DNase treated from cell lines and tissues, by using NucleoSpin® RNA II (Macherey-Nagel, Oensingen, Switzerland). RNAs were then reverse transcribed by using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen). Quantitative real-time PCR assays were performed in the ABI prism™ 7700 sequence detection system, using Taqman® Universal PCR Master Mix (Applied Biosystems, Rotkreuz, Switzerland). Specific gene expression was normalized and evaluated using the $2^{-\Delta\Delta C}$T method [22] and GAPDH housekeeping gene as reference. GAPDH and c-Myc primers and probe sequences were derived from existing literature [23, 24].

TMPRSS2-ERG primers and probe sequences, derived from existing literature [25] are the following:

Forward: CTGGAGCGCGGCAGGAA

Reverse: CCGTAGGCACACTCAAACAACGA

Probe: TTATCAGTTGTGAGTGAGGAC

Primers and probes for stemness-associated genes such as Sox2, Nanog, Oct4A, and ABCG2 (Supplementary data), and ALDH specific isoforms such as ALDH1A1, ALDH1A3, ALDH3A1, ALDH4A1, ALDH7A1, ALDH9A1, ALDH18A1 were provided by Assays-on-Demand, Gene Expression Products (Applied Biosystems).

Identification and isolation of cells with high ALDH activity (ALDH^{bright} cells)

ALDH activity was assessed using the ALDEFLUOR™ assay system according to the manufacturers' recommendations (StemCell Technologies, Grenoble, France). Briefly, single cells obtained from PCa specimens or PCa cell lines were re-suspended in aldefluor buffer and incubated with ALDH substrate (Biodipy-aminoacetaldehyde: BAAA). As a "negative control", an aliquot of the treated cells was also incubated with the ALDH inhibitor, DEAB (diethylaminobenzaldehyde). Following a 35-40 minute incubation at 37°C (optimal time point tested for PCa cell lines), cells were then washed, re-suspended in assay buffer, and analyzed using a dual laser BD FACS Calibur (BD Biosciences, San Josè, CA). Dead cells were excluded based on propidium iodide (PI) incorporation. Results are presented as percentages of ALDH^{bright} cells or ratios of mean fluorescence intensity (MFI) as compared to cells incubated in the presence of DEAB control. To assess *in vitro* and in *vivo* cell characteristics, , ALDH^{bright} and low populations were sorted using a BD Influx cell sorter (BD Biosciences, San Josè, CA). For each subset, including the unsorted population, phenotypes were re-analyzed to confirm the purity of the populations under investigation.

Phenotypic characterization of ALDH^{bright} cells

Following treatment with ALDEFLUOR™ assay system, cells were incubated with antibodies recognizing surface markers or isotype-matched immunoglobulins such as phycoerythrin (PE)-labeled anti-CD44 (BD Biosciences, San Josè, CA), allophycocyanin (APC)-labeled anti-EpCAM (BD Biosciences), and APC-labeled anti-Trop2 (R&D Systems, Cambridge, UK). Briefly, ALDEFLUOR™ treated cells were re-suspended in a small amount of buffer and then stained with antibody concentrations recommended by the manufacturer.

Assessment of tumorigenic capacity in vivo

In vivo experiments were approved by the Basel Cantonal Veterinary Office. NOD/SCID mice, initially obtained by Charles River Laboratories (Germany), were bred and maintained under specific pathogen free conditions in the animal facility of the Basel University Hospital. After sorting, cell subsets were re-suspended in Matrigel Matrix (BD Biosciences) and injected subcutaneously into the flank of recipient mice (8-10 weeks old mice; n>3 per group). Tumor growth kinetic was monitored weekly by palpation and measured with a dial calliper. When tumors reached a maximum diameter of 10 mm, mice were sacrificed and tumors were collected for further analyses.

Tissue microarrays

The two prostate tissue micro-arrays (TMA) used in this study were constructed as previously described [26]. The “progression TMA” contained single tissue cores from prostate cancers from all stages [26]. The newly constructed “castration resistance TMA”, which addresses the progression from hormone-naïve to castration-refractory

prostate cancer, contained mostly palliative transurethral resection specimens represented by three tissue cores (diameter 0.6mm) whenever possible. The presence of tumor tissue on the arrayed samples was verified on hematoxylin-eosin-stained sections. Formalin-fixed and paraffin-embedded tumor and control specimens were obtained from the archives of the Institute for Pathology (University of Basel). Characteristics and number of samples included in the two TMAs are described in Supplementary Table 4.

Immunohistochemistry

After rehydration, sections underwent heat-mediated antigen retrieval. Primary antibodies for ALDH1A1 or ALDH7A1 were incubated at a 1/100 dilution (ab51028 and ab51029 respectively, both rabbit polyclonal from Abcam, Cambridge, UK). Immune-binding was detected by a biotinylated secondary antibody and using the appropriate Vectastain® ABC system. The red signal was developed with the Fast Red kit (Dako, Baar Switzerland) and sections were counterstained by hematoxylin. For immunohistochemical analysis of ALDH1A1 expression on the tissue microarrays, we used standard indirect immunoperoxidase procedures on the Ventana BenchMark XT autostainer (Roche Diagnostics, Pleasanton, CA). The same rabbit polyclonal anti-ALDH1A1 antibody (see above) was applied at a dilution of 1:200.

TMA analysis

The staining intensity was visually scored and stratified into four groups: negative (absence of staining), weak (weak but distinct immunoreactivity), moderate (between weak and strong) and strong (apparent even at small magnification; x2.5 objective). As previously described, a histoscore (H-score) was calculated by

multiplying the staining intensity (0, 1, 2, or 3) by the percentage of positive cells, leading to an H-score ranging from 0 to 300 [27]. A score >0 was considered as positive.

Statistical analysis

Statistical analyses were performed using GraphPad Prism5.0 (GraphPad Software Inc., La Jolla, CA) and SPSS softwares (IBM, New York, NY). To assess the equality of means, parametric T-test or non-parametric Mann-Whitney test were used, for normal or non-normal population, respectively. The correlation of specific expression between two groups was assessed using Pearson or Spearman correlation tests. Fisher and χ^2 tests were used to compare ALDH1A1 positivity frequency in different groups of patients. Survival curves were constructed according to Kaplan-Meier and compared using log-rank (Mantel Cox) tests. P-values lower than 0.05 were considered statistically significant.

Results

Detection of ALDH^{bright} populations in PCa cell lines

Recently, high ALDH activity has been shown to identify tumor-initiating and metastasis-initiating cells in human PCa cell lines [18]. We first investigated whether frequently used PCa cell lines do contain cells populations exhibiting high ALDH activity (Supplementary Figure 1A and B). Using the classical Aldefluor™ technology, we confirmed that PC3 and Du145 androgen-independent cell lines do contain minor ALDH^{bright} populations (mean±SD: 6.01±0.22% and 6.03±2.56% bright, respectively) [18]. Moreover, VCaP androgen-dependent cells were found to contain a major population of cells with high ALDH activity (mean±SD: 62.56±21.48%).

High ALDH activity selects for highly tumorigenic cells in vivo

TIC populations are defined as cells exclusively capable of forming tumors following xenotransplantation in immunodeficient mice [3]. ALDH^{bright} populations have been associated with tumor-initiating properties in several malignancies including PCa [18, 19]. In PCa, this association has been investigated in several PCa cell lines including PC3, PC-3M-Pro4-luc, C4-2B, and very recently, 22Rv1 but results are controversial [18, 28]. We thus decided to investigate tumor-initiating properties displayed by PCa cells from the Du145 cell line, whose properties regarding ALDH^{bright} populations have not been evaluated yet. To address functional features associated with ALDH activity, we sorted out cells displaying high levels (ALDH^{bright}) or low levels (ALDH^{low}) of ALDH activity.

Tumorigenic capacity was assessed by sub-cutaneous injection in immunodeficient mice. At high cell concentration, tumor incidence was reaching 100% for ALDH bright, low or unsorted Du145 cells, consistent with a high tumorigenicity likely related to the high number of cells injected (Supplementary Table 2). Strikingly however, when 100 cells only were injected, tumor incidence reached 100% upon $ALDH^{bright}$ cells injection (7/7; n=2 experiments) while $ALDH^{low}$ cells did not induce tumors in any mice (0/5; n=2 experiments). Moreover, tumors developed faster following injection with $ALDH^{bright}$ as compared to $ALDH^{low}$ or unsorted cells (data not shown). Thus, $ALDH^{bright}$ cells appear to be more tumorigenic than $ALDH^{low}$ cells.

We also assessed additional putative stemness related properties displayed by cell populations exhibiting high or low ALDH activity but no convincing difference was found in term of colony and spheroid formation capacities, and expression of stemness-associated genes between the two populations (Supplementary Figure 2). In summary, our results suggest that cells with high ALDH activity are enriched in putative tumor-initiating cells.

Epithelial cells freshly isolated from PCa clinical specimens do contain a heterogeneous $ALDH^{bright}$ population.

Presence and characteristics of $ALDH^{bright}$ cells have been investigated in several PCa established cell lines [18, 19]. Yet evidence of the presence of cells with high ALDH activity in human clinical PCa specimens is still missing. We therefore investigated the presence of $ALDH^{bright}$ populations in cells freshly isolated from 39 PCa specimens directly retrieved following surgery. Tissues were digested and processed in order to enrich the epithelial fraction (see “materials and methods” and

[29]). Cells were then tested for ALDH activity and stained for phenotypic markers. Representative stainings are shown in Figure 1A. Notably, as reported in Figure 1B, we could detect $ALDH^{bright}$ populations in all cells fractions obtained from clinical PCa samples. Importantly, percentages of $ALDH^{bright}$ cells were highly heterogeneous and varied from patient to patient (average±SE: 1.56±0.24% bright cells; MFI ratio: 257.2±40.87; n=39; Figure 1A-B). Upon gating on $EpCAM^{(+)}$ cells to identify the bulk of the epithelial population, percentages of $ALDH^{bright}$ cells turned out to be significantly higher and exceeded 15% of $EpCAM^{(+)}$ cells in several specimens (average±SE: 9.06±0.97% bright cells; MFI ratio: 292.3±32.99; n=31; Figure 1A-B). Notably, as shown in Figure 1C, we observed a trend toward a higher percentage of $ALDH^{bright}$ cells in high as compared to medium Gleason score (8-9 versus 7; p=0.09).

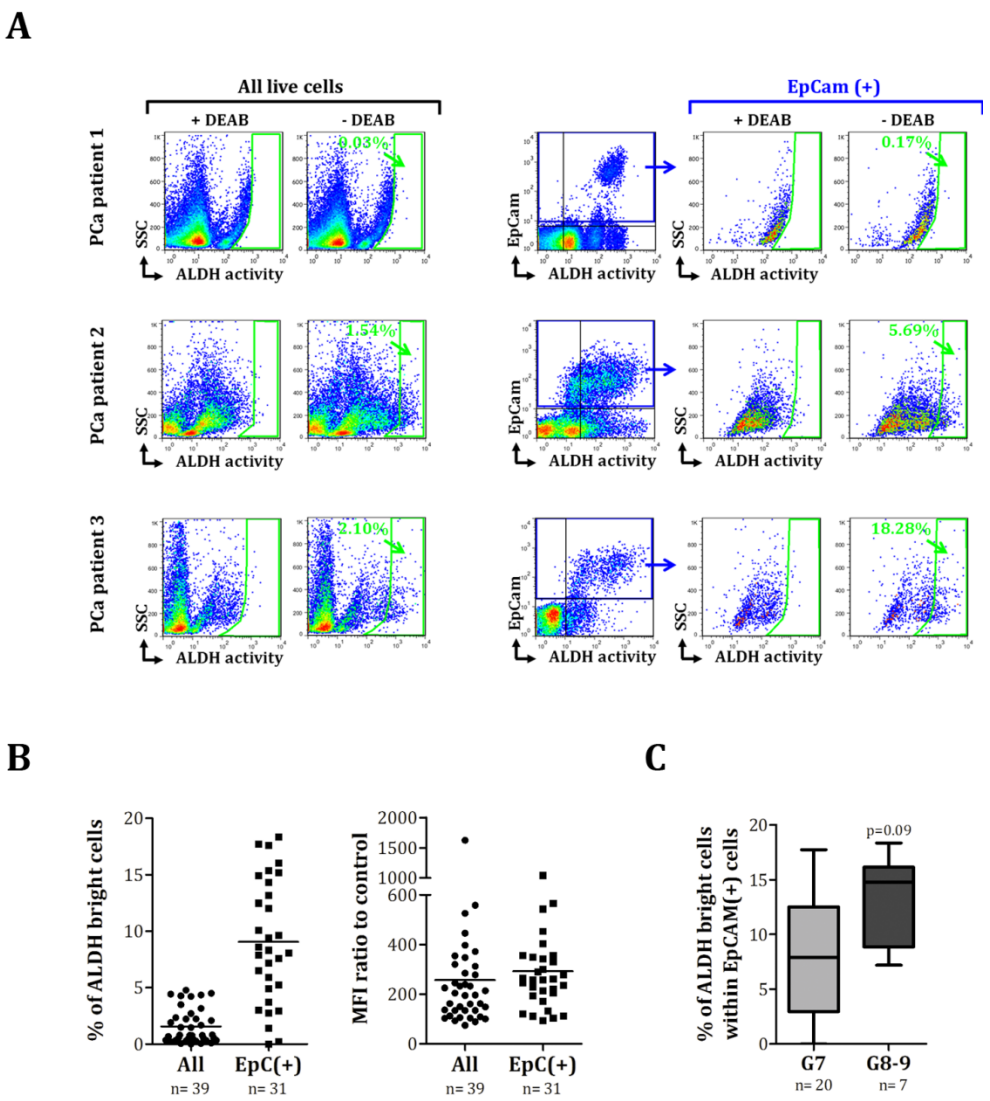

Figure 1: Identification of ALDHbright populations in cells freshly derived from PCa surgical specimens.

A. Flow cytometry analysis of 3 representative PCa patients (PCa patient 1-3). Single cell suspensions were obtained after digestion of PCa tissues. ALDH activity was tested on live cells (left panel) using the aldefluor™ technology. Cells were also tested for EpCam specific expression in order to identify ALDHbright populations within EpCam(+) cells (right panel).

B. Cells suspensions freshly derived from 39 PCa specimens were assessed for ALDH activity ("All"). For 31 specimens, ALDH activity was also tested within EpCam(+) populations ("EpC(+)"). Left panel: % of ALDHbright cells. Right panel: MFI ratio to control DEAB.

C. Percentage of ALDHbright cells within EpCam(+) populations in patients with Gleason 7 (G7) as compared to patients with high Gleason (8 to 9: G8-9). Patients with Gleason 5-6 were too few to be included in the analysis (n=4).

Phenotypic characterization of ALDH^{bright} cells present within PCa clinical specimens

The epithelial cell-specific marker EpCAM has been shown to be highly expressed in the vast majority of carcinomas including PCa [30] and has also been proposed to represent a TIC marker in various solid tumors [31]. Interestingly, we noted a correlation between percentages of EpCAM$^{(+)}$ cells and percentages of cells with high ALDH activity in PCa clinical specimens. Indeed, the majority of the ALDH^{bright} population (average±SE: 75.47±4.06% of EpCAM$^{(+)}$ cells; Figure 2 A-B) was also positive for EpCAM. We also tested expression of Trop2, another epithelial cell-specific marker previously shown to be expressed by cells with tumor-initiating potential in the prostate [11]. Likewise, a high proportion of ALDH^{bright} cells (average±SE: 81.41±4.94% Trop2$^{(+)}$cells, Figure 2B) was also positive for Trop2.

To gain additional insights into the phenotype of ALDH^{bright} cells in PCa, we co-stained aldefluor-treated PCa cells with antibodies recognizing EpCAM and CD44 in four different PCa. In all cases, ALDH^{bright} phenotype was heterogeneous, as illustrated by two representative staining profiles in Figure 2C. Interestingly, two populations of EpCAM^{high} cells were present within the ALDH^{bright} population, showing either positivity or negativity for CD44. Notably, in PCa 1 case, we found a EpCAM$^{(-)}$CD44^{high} population within the ALDH^{bright} subset. This EpCAM$^{(-)}$ population is likely to be constituted by stromal cells. However, since EpCAM has never been demonstrated to mark all epithelial cells in the prostate, we cannot formally exclude an epithelial origin for these cells.

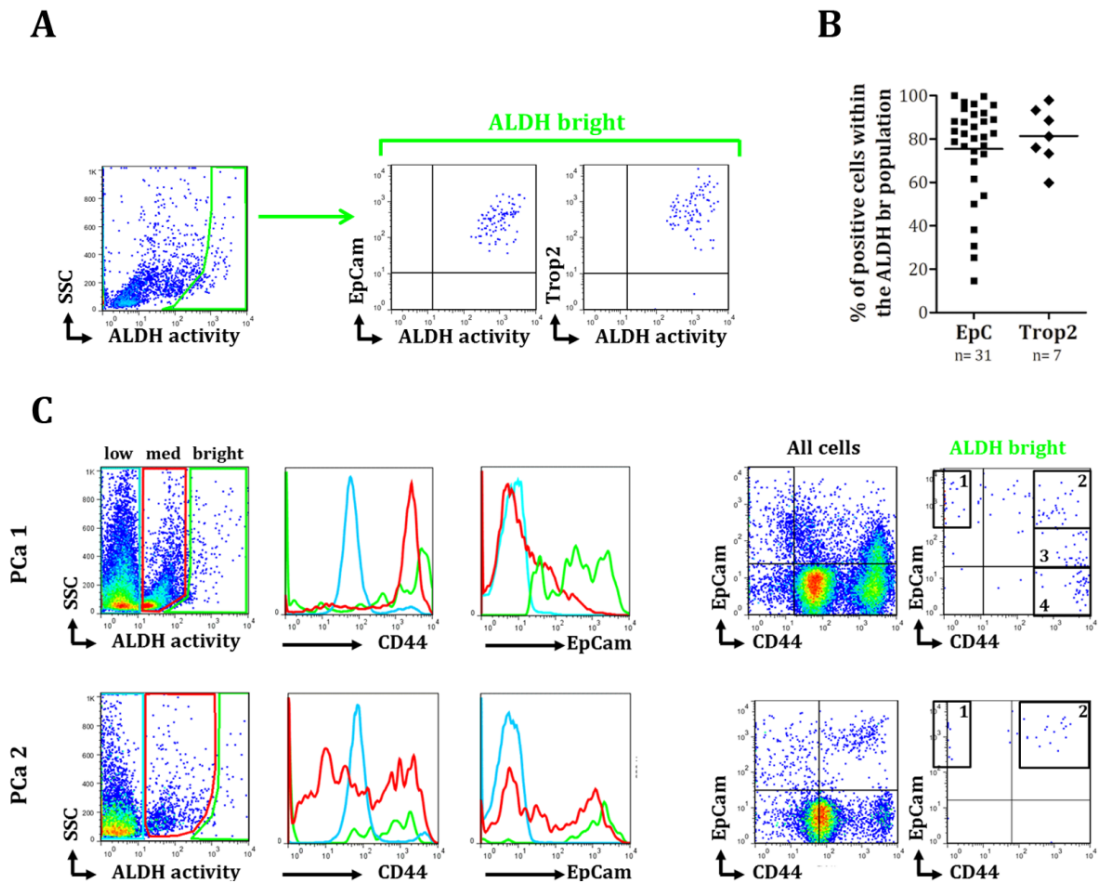

Figure 2: Characterization of ALDHbright populations derived from PCa surgical specimens.

A and B. In PCa tissues, ALDHbright subsets are mainly comprised within the EpCam(+) and the Trop2(+) populations. **A.** High expression of EpCam and Trop2 markers in the ALDHbright population derived from one representative patient.

B. Expression of EpCam and Trop2 in ALDHbright populations freshly derived from a panel of PCa samples (n=31 and n=7, respectively).

C. Left panel: Expression of CD44 or EpCam in cell populations with different levels of ALDH activity (blue: low, red: medium, green: bright). Right panel: Expression of CD44 and EpCam in all cells and gated on ALDHbright cells. Gate 1: EpCamhighCD44(—); Gate 2: EpCamhighCD44(+); Gate 3: EpCam(low/CD44high; Gate 4: EpCam(-)CD44high.

ALDH specific isoforms are highly expressed in PCa clinical specimens

Our findings and results by others [18, 28] concurrently indicate that ALDH activity is associated with high tumorigenicity in PCa. We therefore investigated the expression of selected ALDH specific isoform genes in PCa, benign prostatic hyperplasia (BPH), and normal tissues.

We found high expression of all tested ALDH isoforms in all PCa tissues (Figure 3A, left panel). Expression of these genes was then comparatively evaluated in PCa, BPH, and normal prostate tissues. ALDH3A1 was the only isoform that showed significantly lower gene expression in PCa as compared to BPH (Figure 3A). On the other hand, we observed a trend towards higher expression of ALDH1A1, 4A1, and 9A1 in PCa as compared to BPH and normal tissues (Supplementary Table 3 and Figure 3A). More importantly, ALDH1A3, ALDH7A1, and ALDH18A1 isoforms were expressed to significantly higher extents in PCa tissues as compared to BPH specimens and normal tissues pool (Supplementary Table 3 and Figure 3A). Remarkably, the same trends were observed when we used selected populations of PCa samples, which were found to be positive for the PCa specific TMPRSS2-ERG gene fusion [32] (Supplementary Table 3).

Expression of ALDH isoforms genes was then comparatively analyzed in PCa with different Gleason scores. Interestingly, expression of ALDH1A1 was up-regulated in high as compared to low grade cancers (G5-6 VS G8-9; p=0.03; Figure 3B). The other isoforms did not show any differential expression in high, as opposed to lower grade cancers (data not shown). Overall, these findings might suggest

different contributions of ALDH specific isoforms to prostate cancer initiation and progression.

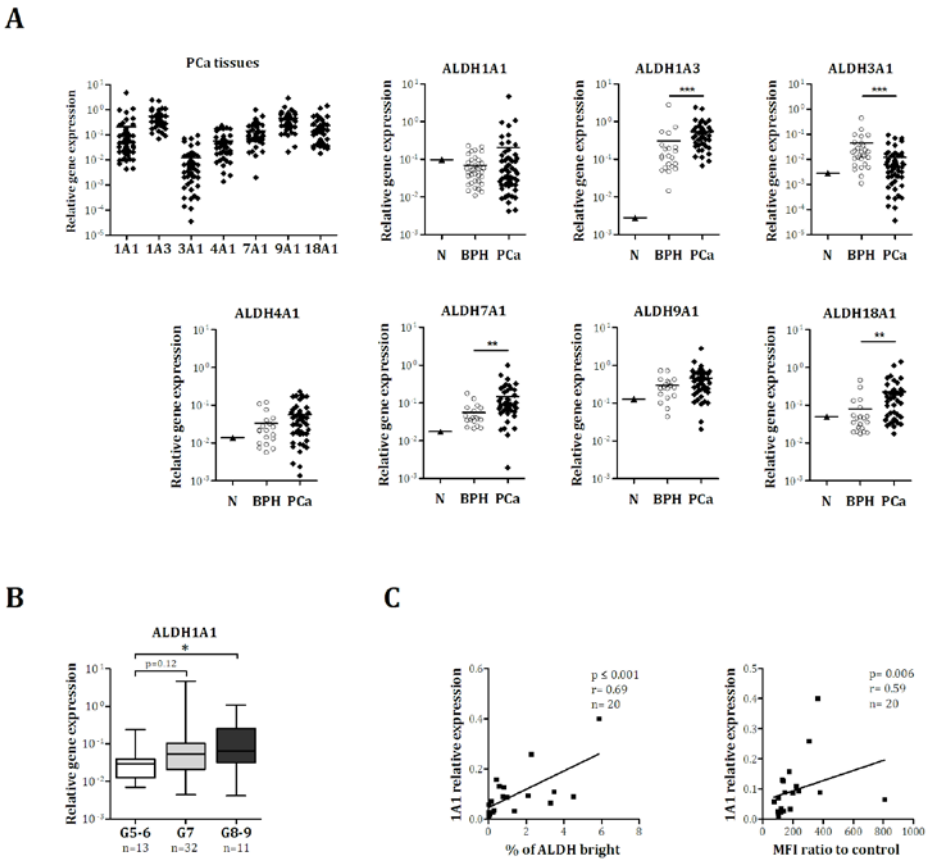

Figure 3: Comparative expression of ALDH specific isoforms in PCa and BPH samples.

A. Expression of genes encoding for ALDH1A1, ALDH1A3, ALDH3A1, ALDH4A1, ALDH7A1, ALDH9A1, and ALDH18A1 isoforms in: clinical PCa specimens (left panel) and in clinical PCa specimens (♦) as compared to BPH samples (○) (other panels). Expression levels are reported as relative values as compared to the housekeeping gene GAPDH. P values and number of samples are reported in Table S3. As a control, cDNA from a normal prostate pool (N) was tested (▲). Mean values (—) are indicated for each group.

B. PCa samples were divided into 3 groups according to their Gleason grade (Gleason 5 to 9) and expression of ALDH1A1 was compared using Mann Whitney test. Horizontal lines represent the medians, while outer limits of the whiskers show the minimal and maximal values.

C. Spearman test was used to analyse the correlation between ALDH1A1 (1A1) gene expression and percentage of ALDHbright cells (left panel) or MFI ratio to control (right panel) in PCa surgical samples. P values lower than 0.05 were considered statistically significant (p≤0.05:*; p≤0.01: **; p≤0.001: ***).

ALDEFLUOR™ reagent is generally thought to act as a substrate for ALDH1A1 specific isoform [32]. In breast cancer, however, aldefluor-dependent ALDH activity has been attributed to ALDH1A3 isoform [33]. Interestingly, in PCa clinical specimens, ALDH1A1 was the only isoform gene, whose expression correlated with levels of ALDH activity (cfr Figure 1B) as detected in the same tissues (Figure 3C). This suggests that, in PCa specimens, ALDH1A1 is the main isoform contributing to measurable ALDH activity.

Localization and expression of ALDH1A1 and ALDH7A1 in prostate tissues

Overall, our findings may suggest an involvement of ALDH specific isoforms in PCa pathogenesis. In particular, ALDH1A1 gene expression appears to be detectable to high extents in tumors of higher grade and to correlate with enzymatic activity. We therefore investigated the expression of ALDH1A1 in sections of benign or cancerous prostatic tissues, using a specific antibody recognizing this isoform. As internal control, we used an antibody recognizing ALDH7A1 isoform, whose expression is increased in PCa, but does not correlate with enzymatic activity.

In BPH, strong ALDH1A1 expression was clearly restricted to cells belonging to the basal epithelial compartment. For ALDH7A1 however, we observed a diffuse staining pattern in high percentages of both basal and luminal cells. Accordingly, we found diffuse ALDH7A1 positivity in high percentages of epithelial cells in PCa samples. In contrast, ALDH1A1 staining was restricted to few strongly positive PCa cells. Representative stainings are shown in Figure 4A-D.

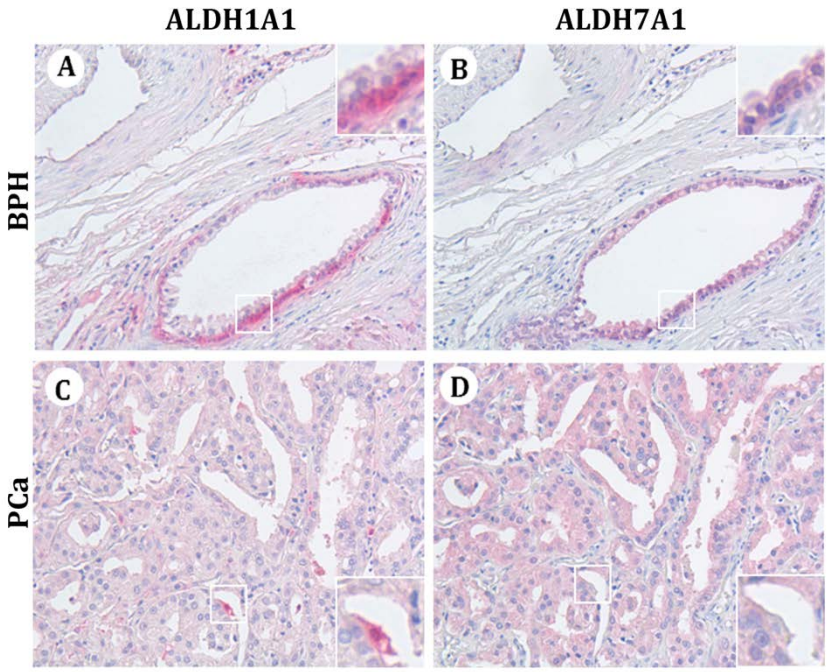

Figure 4: Differential expression and localization of ALDH1A1 and ALDH7A1 proteins in prostate tissues.

Immunohistochemical (IHC) analysis of the expression of ALDH1A1 and ALDH7A1 in adjacent sections of a BPH sample (panels A and B, respectively) and a PCa sample (panels C and D, respectively). White rectangles indicate areas which are enlarged and shown within inserts. Magnification: x 200.

ALDH1A1 expression and localization in a large panel of BPH and multiple stage PCa tissues

Considering the association of ALDH1A1 with ALDH activity, its differential expression in high grade PCa, and its specific location, we thought to gain more insights into its potential clinical relevance. We thus investigated the expression of ALDH1A1 protein in a series of BPH and multi-stage PCa specimens included in two tissue microarrays (TMA).

Number and characteristics of samples comprised in the TMAs are recapitulated on Table S4. First, we used a prostate progression TMA comprising 290 evaluable samples including BPH, prostatic intraepithelial neoplasia (PIN), early stages PCa (T1a/b), radical prostatectomy specimens (RP), and castration-resistant PCa (CR) samples. Importantly, we observed a significantly more frequently detectable ALDH1A1 positivity in RP ($p= 0.02$) and CR ($p= 0.01$) PCa as compared to

BPH. Likewise, ALDH1A1 protein was significantly more frequently expressed in RP and CR samples (p= 0.04 and p= 0.02 respectively; Figure 5A left panel and Supplementary Table 4) as compared to low stage (T1a/b) samples. Moreover, using ALDH1A1 scoring, the same trends were observed by comparing RP and CR with BPH (p= 0.01 and p= 0.003 respectively; Figure 5A right panel). However, we did not find any significant difference, in terms of percentages of ALDH1A1$^{(+)}$ samples or global score in samples with different Gleason scores or Ki67 levels (data not shown). ALDH1A1 location, in a large series of prostate tissues, might provide additional insights to hypothesize ALDH1A1 putative role in PCa. In BPH, we found a high percentage of ALDH1A1(—) specimens (Supplementary Table 4 and representative sample on Figure 5B). However, when present, positivity was restricted to few cells confined within the basal layer, confirming our previous findings on large sections (Figure 5C). In PIN samples, ALDH1A1 positivity was rare (Supplementary Table 4). Nevertheless, in the few positive samples, ALDH1A1 expression was strongly specific to some PIN lesions (Supplementary Table 4 and Figure 5E). Interestingly, in RP PCa samples, positivity was significantly more frequent and specifically detectable in cancer glands (Figure 6F-G). In CR samples, ALDH1A1 expression showed large variations, ranging from fully negative tumors (Figure 5H), to focal, or diffuse positivity (Figure 5I). Notably, we frequently found ALDH1A1 (+) cells in the peri-epithelial stroma, as illustrated in Figure 5D.

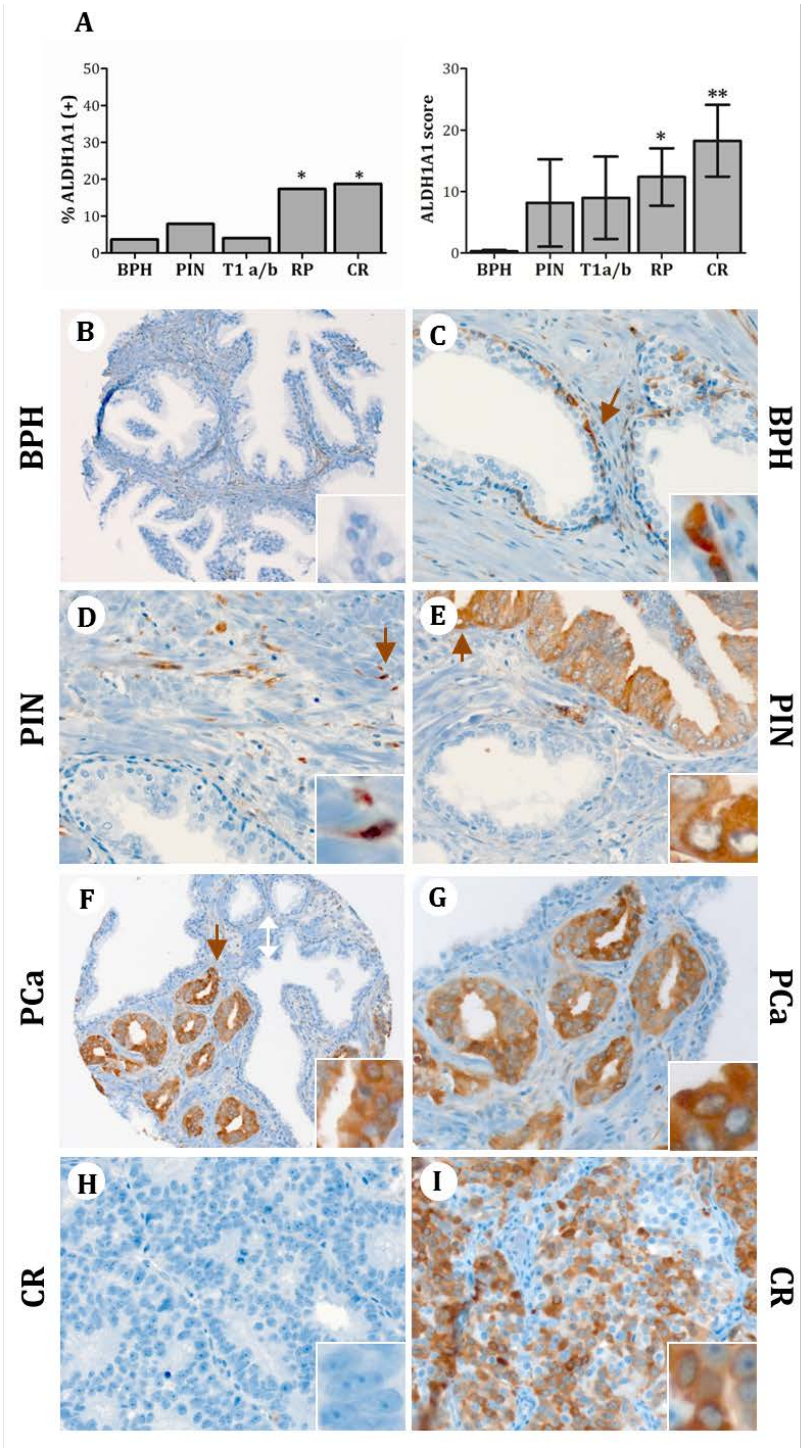

Figure 5: Expression of ALDH1A1 protein on a large cohort of benign and cancerous prostate tissue

A. ALDH1A1 is expressed more frequently (left panel) and to higher extents (right panel) in high grade PCa (RP and CR) as compared BPH and low stage PCa (T1a/b). P values lower than 0.05 were considered statistically significant ($p \leq 0.05$:*).

B to I. Representative IHC analysis of ALDH1A1 expression on a panel of different prostate tissues.
B: Negative BPH glands.
C. BPH glands exhibiting few positive basal cells.
D. Interstitial stromal cells showing some positivity.
E. Positive PIN lesions.
F and **G.** Positive cancer glands (brown arrow) and negative benign glands (right arrow).
H. Negative CR sample.
I. CR sample with diffuse positivity. Brown arrows indicate positive areas enlarged and shown in inserts.

B and F: x200.

ALDH1A1 expression is a predictor of poor prognosis in "hormone-therapy naive" patients

To evaluate the clinical potential of ALDH1A1 as a prognostic marker in PCa, we examined ALDH1A1 expression on another TMA, including "hormone therapy naïve" and castration-resistant samples with complete follow-up data (Castration resistance TMA). To assess overall survival of patients with ALDH1A1 positive or negative tumors, Kaplan-Meier survival curves were constructed and compared using log-rank (Mantel Cox) tests. In the "hormone therapy naïve" patients set, the median survival (MS) was 34 months for patients with ALDH1A1 positive tumors, while patients with ALDH1A1 negative tumors were characterized by a MS of 56 months. Thus, in these patients, ALDH1A1 positivity was strongly associated with poor clinical outcome (p=0.02; Figure 6A).

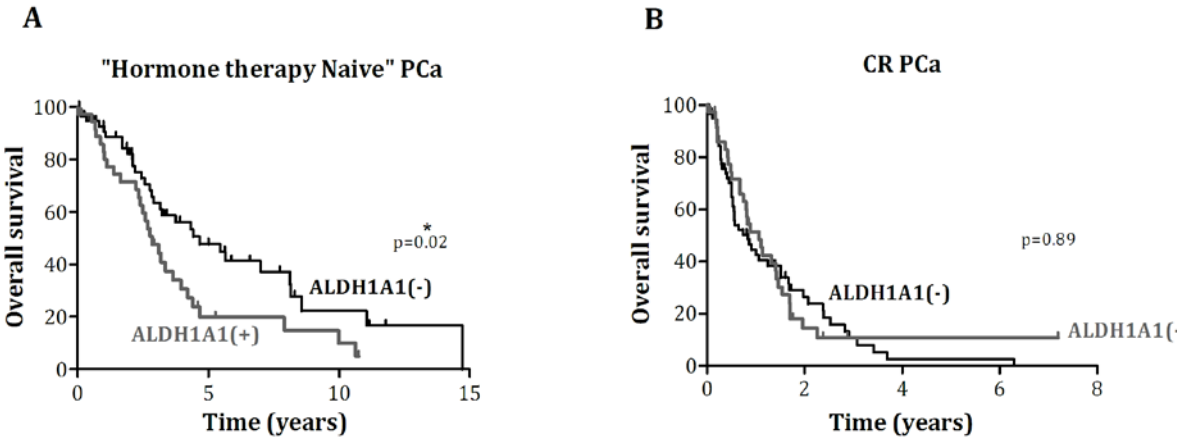

Figure 6: ALDH1A1 expression is predictive of poor prognosis in "hormone therapy naïve" patients.

Kaplan-Meier curves of patient overall survival according to ALDH1A1 positivity and negativity. Log rank (Mantel Cox) tests were used to compare ALDH1A1 positive and negative tumors in "hormone therapy naïve" patients (A) and castration-resistant patients (B). P≤0.05 were considered statistically significant (*).

In the castration resistant set however, MS for patients with ALDH1A1 positive or negative tumors were similar (13 months and 10 months, respectively) and no correlation could be observed between ALDH1A1 expression and patients overall survival (Figure 6B; p= 0.89).

Discussion

Tumor-initiating cells (TIC) are generally considered to be rare cells, exquisitely responsible for tumor initiation and transplantation [3]. TIC are therefore thought to be accountable for treatments failures, since current therapies aim at targeting the bulk of cancer cells but not specifically these cells [4]. In this respect, TIC might have a crucial clinical relevance and TIC quantification and gene signature may be of prognostic significance [34-36]. Nevertheless, identification and characterization of these cells are limited by their expected low frequency and by the lack of reliable surface markers. As an alternative method, high ALDH activity has been proposed to select TIC in several solid tumors including breast [16] and, more recently, prostate cancer [18]. However, published studies mainly rely on the use of established PCa cell lines to investigate properties displayed by $ALDH^{bright}$ cells and functional proof of their existence in fresh PCa surgical specimens is still lacking.

In this study, we have successfully identified and quantified $ALDH^{bright}$ populations present within cells freshly isolated from a series of PCa clinical specimens. To our knowledge, this is the first study reporting the identification of cells with high functional ALDH activity in fresh PCa specimens whereas previous studies have mainly focused on protein expression analysis [18, 37]. Our results reveal a heterogeneous prevalence of $ALDH^{bright}$ cells in PCa patients, with a trend toward higher percentages in higher histological grade cancers. Notably, $ALDH^{bright}$ cells can be detected with relatively high frequency in most PCa samples, which appears to contradict the expected rarity of TIC. Yet selecting for high ALDH activity might enrich in TIC since we and other have shown its association with high

tumorigenic capacity *in vivo* [18, 28]. Importantly, we found that increased ALDH activity detected in PCa surgical specimens, as well as in PCa cell lines (data not shown), mainly resides in cells highly positive for EpCAM and Trop2. Notably, these markers, known to be specific for epithelial cells, have both been described as putative TIC markers [13, 31]. In an effort to elucidate the phenotype of $ALDH^{bright}$ cells in PCa, we also tested whether cells with increased activity were co-expressing CD44, a mostly basal-specific and proposed TIC marker. Notably, even if the majority of cells was positive for CD44, heterogeneous CD44 (+) and (-) phenotypes could be observed within the $ALDH^{bright}$ population.

Several groups have already assessed properties displayed by $ALDH^{bright}$ cells but whether these cells are associated with stemness properties, is still debated [18, 19, 28]. In our hands, $ALDH^{bright}$ cells from Du145 cell line did not display increased stemness-associated *in vitro* functional properties. More importantly, however, in agreement with data by others [18, 28], this cell population was associated with increased tumorigenicity *in vivo*.

To date, nineteen ALDH isoforms have been identified in the human genome, potentially contributing to the activity of the enzyme [14]. Among these isoforms, ALDH1A1 is generally believed to be the main isoform contributing to ALDH activity, as measured by the aldefluor™ assay [32]. However, evidence supporting the contribution of other isoforms in solid tumors has recently been proposed. ALDH1A3 rather than ALDH1A1 has been indeed identified as the main isoform involved in ALDH activity and as a strong predictor of metastasis in breast cancer [33]. Moreover, ALDH7A1 has recently been shown to contribute to ALDH activity of a PC3-derived cell line and to be involved in bone metastasis formation in prostate

cancer [38]. Here, we have tested expression of seven ALDH isoforms, previously shown to be implicated in aldefluor-dependent ALDH activity and in cancer initiation (reviewed in [32]). Interestingly, except for ALDH3A1, we observed a trend towards higher expression of the majority of ALDH isoforms in PCa as compared to BPH and normal samples. In particular, ALDH1A3, ALDH7A1, and ALDH18A1 were significantly more expressed in PCa as compared to BPH. Notably, ALDH1A1 gene was not significantly more expressed in PCa as compared to BPH ($p= 0.12$) but we could observe a significantly higher expression of this isoform in high as compared to lower grade PCa. More importantly, ALDH1A1 was the sole isoform whose gene expression was correlating with high ALDH activity detected in the same patients. Finally, testing an antibody specifically recognizing ALDH1A1 on large prostate sections, we observed that ALDH1A1 positivity was restricted to cells within the basal layer of the benign prostate.

These results led us to investigate localization and expression of ALDH1A1 at protein level, in a large cohort of benign and cancerous samples. In the two TMAs tested, we found a minor proportion of BPH samples exhibiting ALDH1A1 positivity. Interestingly, in the few positive samples, positivity was restricted to a few cells confined within the basal layer of the epithelium, in agreement with a recent study [37]. Notably, ALDH1A1 was significantly more frequently and more highly expressed in samples derived from RP or CR as compared to early stage PCa or BPH samples. These results may support a putative role of ALDH1A1 in prostate tumorigenesis. Moreover, in PCa samples, ALDH1A1 expression was detected in luminal cancer cells and showed broad variation, ranging from full negativity, to focal, diffuse, or strong positivity. These different expression patterns highlight the

remarkable inter-patients heterogeneity characterizing PCa. In this context, it is likely that ALDH1A1 expression and its putative role might be patient-dependent. Notably, we also observed intra-tissue heterogeneity with concomitant presence of negative and positive glands within the same samples.

Convincingly, when we assessed the potential use of ALDH1A1 as a prognostic marker in the untreated cohort, we found a significant correlation between positivity and poor patient outcome ($p= 0.02$). Similar results were previously found in a study published by Li and colleagues, evaluating a smaller and less diversified patients' cohort [37]. This indicates that, at least in these patients, ALDH1A1 might be involved in PCa progression and have prognostic value. Notably, ALDH1A1 has recently been shown to mark a population of castration-resistant prostate cancer cells (CRPC) which survives castration and is responsible for tumor re-growth [12, 39]. Yet we did not find any significant correlation between ALDH1A1 positivity and patients' outcome in patients with castration-resistant disease.

Here, we provide novel evidence demonstrating the existence of cells with high ALDH functional activity freshly derived from PCa samples. Detection at mRNA, protein, and functional levels suggests that ALDH may be involved in PCa progression. However, this involvement might be specific for sub-groups of patients as suggested by the high variability observed between patients. Whether $ALDH^{bright}$ cells might mark TIC populations in PCa primary tumors still has to be clearly demonstrated. Yet these studies are still hampered by the lack of appropriate experimental models and the difficulties inherent with the generation of primary cultures and xenografts derived from PCa primary tissues [40]. Additionally, we clearly show that ALDH1A1 has prognostic value in "hormone-therapy naïve"

patients. This might have implications for the identification of patients at high risk for progression to castration-resistant disease.

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Supplementary tables

TABLE S1. Characteristics of PCa patients (n=71)		
		N patients (%)
Stage		
	pT2a	6 (8.5%)
	pT2b	2 (2.8%)
	pT2c	38 (53.5%)
	pT3a	14 (19.7%)
	pT3b	10 (14.1%)
	NA	1 (1.4%)
Gleason score		
	5-6	17 (23.9%)
	7	41 (57.8%)
	8	8 (11.3%)
	9	5 (7.0%)
Nodal status		
	N0	55 (77.5%)
	Nx	4 (5.6%)
	N1	5 (7.0%)
	NA	7 (9.9%)

NA: not available

Table S2: *In vivo* tumorigenic capacity

Cell subset	Cell number	Tumor incidence
ALDH bright	10^4	4/4 (100%)
	10^3	5/5 (100%)
	10^2	7/7(100%)
ALDH low	10^4	4/4 (100%)
	10^3	4/5 (80%)
	10^2	0/5 (0%)
Unsorted	10^4	3/3(100%)
	10^3	4/4 (100%)
	10^2	5/6 (83%)

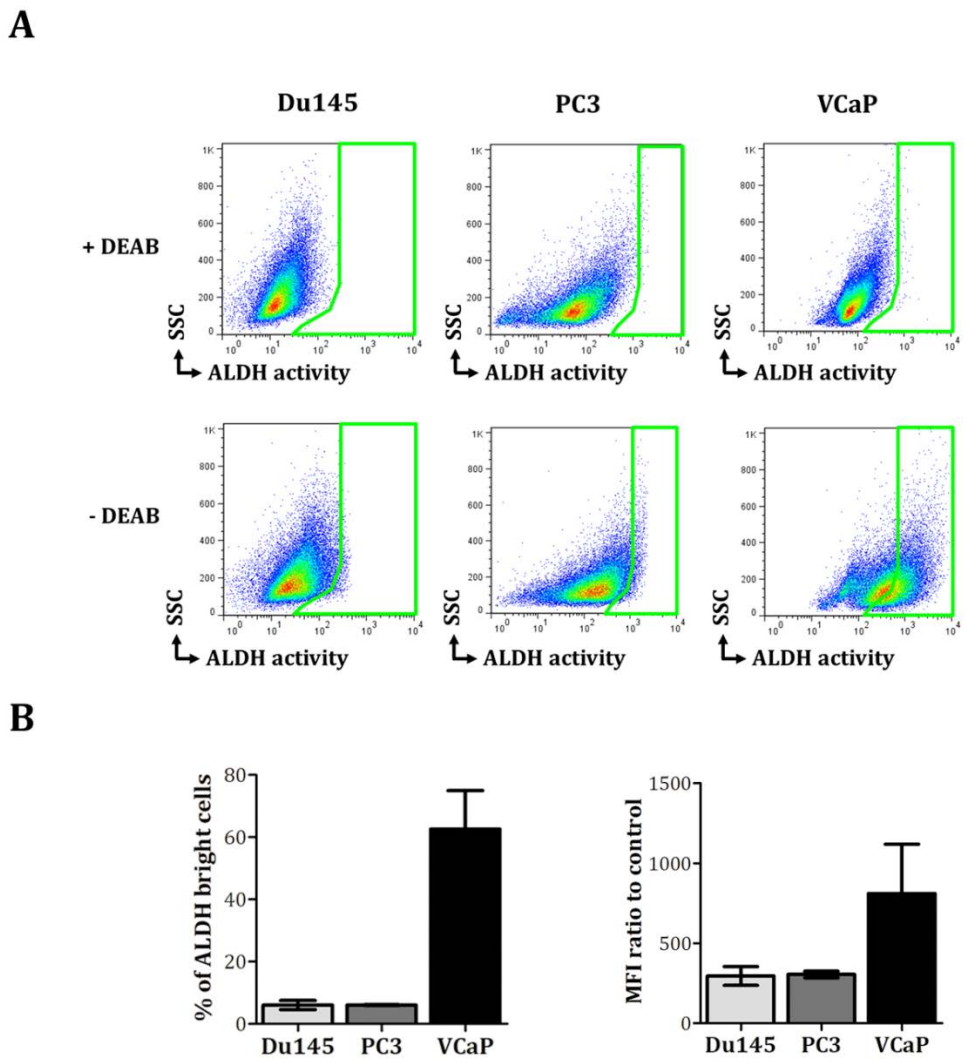
Table S3: Analysis of the expression of ALDH specific isoforms in BPH VS PCa

Gene	n BPH	All PCa (n)	P	PCa TMPRSS2-ERG+(n)	P
ALDH1A1	35	56	0.12	41	0.16
ALDH1A3	21	41	0.0001	27	0.0005
ALDH3A1	28	55	0.008	40	0.0003
ALDH4A1	19	45	0.13	30	0.26
ALDH7A1	18	45	0.001	30	0.003
ALDH9A1	19	43	0.13	28	0.07
ALDH18A1	20	41	0.001	28	0.003

Table S4: Immunohistochemical analysis of ALDH1A1 expression on TMAs

	Total Interpretable	Negative	Positive	Average score
	n (%)	n (%)	n (%)	
Progression TMA				
BPH	54 (100%)	52 (96%)	2 (4%)	0.3
PIN	38 (100%)	35 (92%)	3 (8%)	8.2
T1a/b	49 (100%)	47 (96%)	2 (4%)	9.0
RP (pT2-pT3b)	69 (100%)	57 (83%)	12 (17%)	12.4
CR	80 (100%)	65 (82%)	15 (18%)	18.3
Castration resistance TMA				
BPH	11 (100%)	10 (91%)	1 (9%)	1.7
UT	100 (100%)	60 (60%)	40 (40%)	40.5
CR	107 (100%)	67 (63%)	40 (37%)	33.7

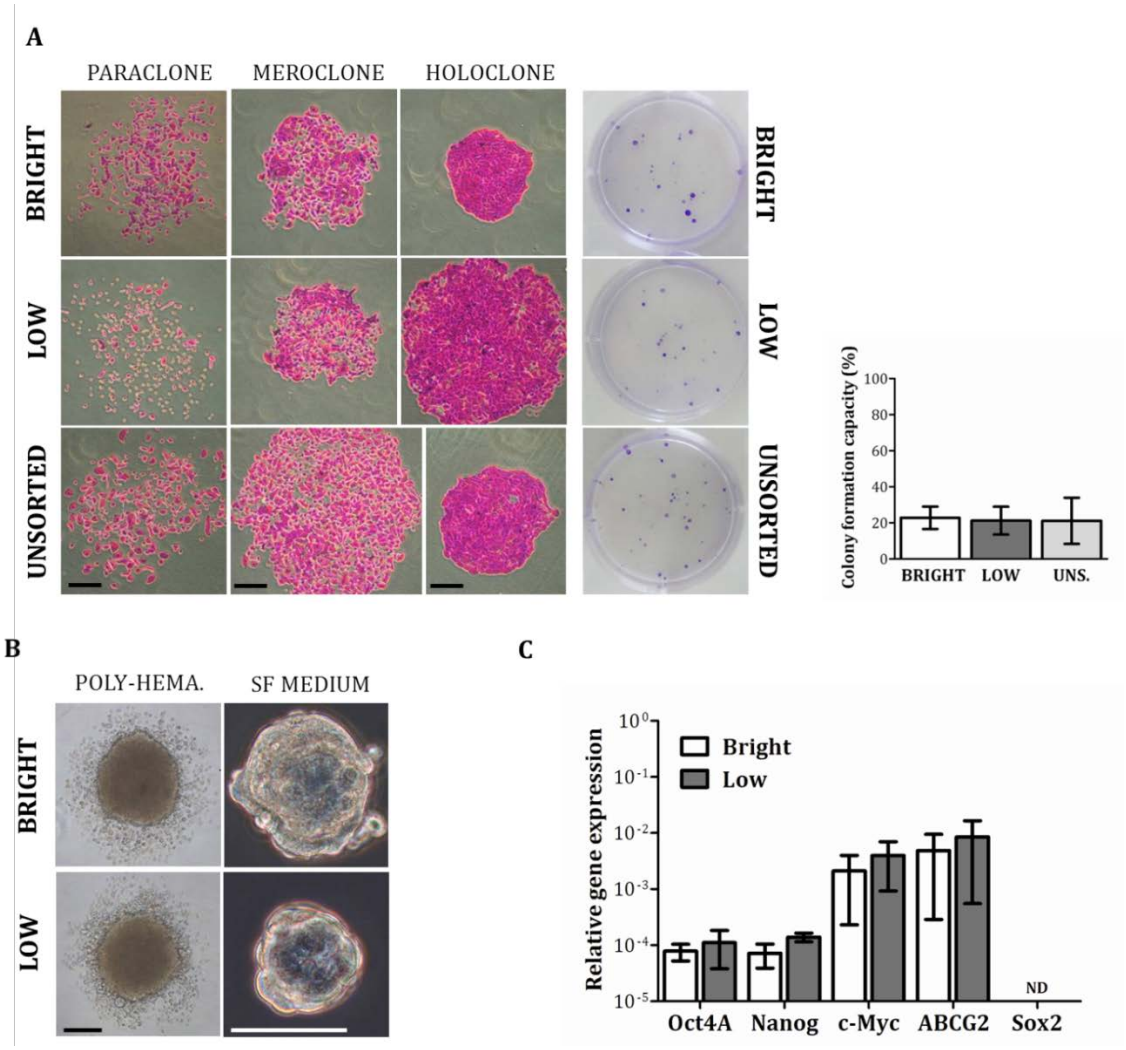
Supplementary Figures



Supplementary Figure S1: Detection of ALDH bright populations in Du145, PC3 and VCaP PCa cell lines.

A. Representative flow cytometry analysis of the 3 PCa cell lines tested for ALDH activity, using the ALDEFLUOR™ assay system. As a negative control, ALDH specific inhibitor, DEAB, was used to established baseline fluorescence (top panel: + DEAB). Cells with high ALDH activity (ALDH bright) are detected in the 3 tested cell lines (green gate; down panel: - DEAB). Dead cells were excluded for all analysis.

B. Data are represented as means ± SD of 3 independent experiments. As an indicator of ALDH activity, percentages of ALDH bright cells (left panel) or mean fluorescence intensity (MFI) as compared to control are represented.



Supplementary Figure S2: In vitro characteristics displayed by ALDH bright and low populations derived from Du145 cell line.

A. Colony formation capacity. ALDH bright, low, and unsorted populations are able to initiate 3 types of clones including holoclones (left: panel of pictures) and display similar colony formation capacity (right). Data are represented as means ± SD of 4 independent experiments **B.** Both ALDH bright and low cells are able to grow as 3D spheroids, on poly-hema plates (left) or using serum-free medium (right). **C.** Expression of stemness-associated genes in ALDH bright and low cells. Expression levels are reported as relative values as compared to the housekeeping gene GAPDH. Data are represented as means ± SD of 3 independent experiments.

Supplementary methods

Evaluation of colony formation capacity and morphology

ALDH-derived populations were washed with PBS and counted using trypan blue exclusion method. After serial dilution, cells were then plated (10 cells/ml) in petri dishes in triplicates. After ten days, cells were washed in PBS, fixed in paraformaldehyde 4% and stained with violet crystal for 10 minutes. Finally, cells were washed to remove exceeding violet crystal, colonies were counted and morphology was observed under microscope (microscope brand)

Evaluation of sphere formation ability

Immediately after sorting, ALDH-derived populations were cultured in serum-free medium (DMEM, EGF 20 ng/ml, bFGF 10 ng/ml, insulin 5 µg/ml, BSA 0.4%). Cells were plated at a "clonal" density (1000 cells/ml), to favour spheres formation. After 7 days, morphology of the structures was observed under microscope. In the other hand, cell subsets were also cultured on plates pre-coated with a 50µg/ml poly-2-hydroxyethyl methacrylate (polyHEMA) layer, preventing cells adhesion to the bottom and thus promoting spheres formation. After 6 days of culture, morphology was observed under microscope.

Discussion and Outlook

I. Summary and Discussion

The notion that tumour initiation and heterogeneity might be driven by small population of tumour-initiating cells (TIC) has gained high significance since the pioneering identification of TIC in leukaemia [1]. Ever since, the identification of TIC in solid tumours has been the aim of a worldwide research effort. In prostate cancer, however, demonstration of the presence and identification of TIC have been hampered by a lack of consistent *in vitro* and *in vivo* models. Further studies are therefore required to establish relevant models and to provide evidence for the existence of TIC in PCa.

1. Inadequacy of current models and phenotypic plasticity

One classic approach to identify putative TIC is represented by the use of flow cytometry to isolate candidate cell populations expressing a combination of surface markers. In **Chapter I**, we therefore investigated the expression of TIC putative markers in commonly used PCa cell lines and cells derived from freshly excised PCa specimens. The expression of published markers was neither reliably detectable in cell lines, nor in clinical PCa specimens. In particular, cell lines did not reflect the range of prostate cancer cell phenotypes, which is a probable consequence of the selective pressure inherent with long-term culture. Additionally, cells derived from PCa specimens were characterised by a remarkable inter and intra-patients heterogeneity. Using these primary PCa samples would offer a unique opportunity to identify TIC in PCa but it requires adequate *in vitro* and *in vivo* approaches. With this in mind, we attempted to generate short-term primary cultures which could recapitulate PCa cell heterogeneity. Preliminary characterisation experiments

suggest that it might be possible but that additional research efforts are required to define optimal culture conditions favouring luminal cells growth.

PCa is characterised by a remarkable genetic intra- and inter-tumour heterogeneity illustrated by various chromosomal aberrations. The most recent examples of chromosomal rearrangements in PCa are those which activate members of the ETS transcription factors family [2]. Among these rearrangements, the most common one results in a TMPRSS2-ERG fusion gene, which is frequently found in ~50% of the PCa patients [3]. Our results and results published by others [4] suggest that the culture conditions we used in our experiments, do not support the maintenance and growth of these specific TMPRSS2-ERG (+) cells. It has indeed been suggested that current culture conditions might not support the survival of the most genetically aberrant cancer cells *in vitro* [5]. Thus, since all cells are competing with each other for survival and growth, the perspective of generating cultures reflecting PCa phenotype heterogeneity remains unfulfilled and is probably still unrealistic.

Additionally, similarly to a wide variety of other cells, TIC appear to be exposed to dynamic phenotypic changes [6]. This phenotypic plasticity is highly dependent on their micro-environmental niche (adjacent cells and extracellular matrix), which control them via a range of signals [7–9]. In this context, it is also likely that primary cultures might turn out to be oversimplified and might fail to reflect the complexity of tumour pathophysiology. The endogenous microenvironment may also have positive or negative effects on the tumorigenic properties of cancer cells. Within this framework, a new method has recently been developed based on co-grafting of neonatal mouse urogenital mesenchyme along with human primary PCa samples [10].

Altogether, these observations highlight the scarcity and the inadequacy of models systems to study PCa and more particularly prostate cancer TIC. Yet, while eagerly expecting the successful generation of innovative models, we have attempted to optimally exploit currently available models and assays.

2. Looking for a putative stemness signature in PCa

The use of surface markers to identify TIC has important limitations. In **Chapter II**, we used an alternative method aiming at identifying cancer cells potentially endowed with stem-like characteristics. We investigated the expression of five genes previously shown to characterise pluripotent embryonic stem cells. In particular, we expected that this gene signature might identify rare populations of cells and might be more present in aggressive cancers. Unexpectedly, we found high expression of the 5 genes of interest in PCa cell lines and primary specimens. Notably, they were also expressed at high levels in BPH samples, suggesting that they might not be associated with cancer features. Among these factors, Klf4 gene was nevertheless significantly more expressed in PCa as compared to BPH. Based on these findings, we assessed the specific expression of Klf4 at protein level. Confirming our findings at RNA level, we found high levels of Klf4 protein in both BPH and PCa specimens. Nevertheless, localisation of Klf4 was both nuclear and cytoplasmic in BPH but rather cytoplasmic only in PCa samples. This peculiar location could be explained by the expression of Klf4α, a Klf4 specific isoform devoid of nuclear localisation signal and therefore located in cellular cytoplasm. Thus, our findings reveal that Klf4 is expressed in a majority of differentiated cells and argue against the use of this specific marker to identify rare cells with stem characteristics.

Our work suggests that specific variants of these transcription factors, possibly characterised by altered functions, and escaping current qRT-PCR methods, might be predominantly expressed in PCa cells.

Interestingly, we had chosen to investigate these 5 transcription factors, based on the seminal work from Yamanaka and colleagues [11] . To successfully induce pluripotency in differentiated fibroblasts, they used overexpression of Oct4, Sox2, Myc, and Klf4 factors and further selection with Nanog. Subsequently, an important research effort has been made to reduce the number of reprogramming factors, thus avoiding safety issues inherent with the use of potential oncogenes. As a result of this effort, Klf4 was removed from the reprogramming factors in several studies and appears to not be necessary to confer pluripotency [12,13]. Therefore, the link between Klf4 and stemness is currently highly debated. Indeed, our results and results from others suggest that Klf4 is highly expressed in differentiated cells from various normal and cancerous tissues [14–16].

The idea to investigate stemness-signatures in cancer still sounds attractive, but relies on an accurate identification of genes associated with stem characteristics. In this context, embryonic and development-associated genes still represent appealing candidates. Recently, such genes signatures have been convincingly shown to be associated with aggressive cancers and poor outcome in PCa [17,18].

Besides their significance for the CSC concept, our data also pave the way for additional studies aimed at obtaining mechanistic insights into the regulation of Klf4 gene expression in the prostate. On the other hand, they raise the issue of the functional significance of differentially spliced cytoplasmic Klf4 in PCa.

3. Characterisation and clinical relevance of ALDH^{bright} populations in PCa

In view of the limited and controversial effectiveness of phenotypic markers in the identification of TIC in PCa, we thought to alternatively investigate a functional property characterising TIC in various other tissues. High aldehyde dehydrogenase (ALDH) activity has been shown to identify normal stem cells and CSC/TIC in tissues such as breast or colon [19,20]. Yet, until recently [21], this property had never been investigated in prostate cancer. In **Chapter III**, we therefore investigated presence, properties, and clinical relevance of cells with high ALDH activity (ALDH^{bright} cells) in PCa.

In established PCa cell lines, ALDH^{bright} populations were heterogeneously detectable. In the cell lines under investigation, ALDH^{bright} cells were not convincingly associated with enhanced stem characteristics as compared to cells exhibiting low ALDH activity (ALDH^{low}). Yet, the gold standard property being the tumour propagation in immunodeficient mice, we tested their tumorigenic capacity *in vivo*. ALDH^{bright} cells did show a higher tumorigenicity *in vivo* as compared to ALDH^{low} cells, consistent with the results recently published by van den Hoogen and colleagues [21].

While we were performing this study, other groups published their own studies investigating the use of high ALDH activity to select for TIC in prostate cancer [22]. Importantly, all these studies were performed using established cells lines as a model and it is likely that their outcome might be cell line and assay-dependent. For example, we and others [23] failed to show improved stem-like properties in the cells lines we tested, while other groups did find an association between stem

properties and ALDH^{bright} populations [21,22]. These discrepancies again highlight the lack of consistent models and the limitations of established cell lines. Furthermore, in published studies, self-renewal capacity of the cells, an essential property characterising stem cells, was never investigated.

Moreover, while other groups have investigated the expression of **ALDH specific proteins** in PCa, we have chosen to assess **functional ALDH populations** (i. e identification of cells with high ALDH activity) in primary PCa specimens. We identified ALDH^{bright} subsets in all tested samples with a heterogeneous prevalence across PCa patients. To assess clinical relevance of ALDH^{bright} populations, we tested the expression of ALDH1A1 isoform, whose expression was correlating with ALDH activity. ALDH1A1 protein was more expressed in high grade cancers as compared to low grade and benign samples. Yet *in vivo* tumorigenicity of cells derived from primary PCa samples should be tested to formally prove that ALDH1A1 might be a marker of TIC in PCa, Importantly, in higher grade cancers, we found a high percentage of samples fully negative for ALDH1A1. This suggests that even if ALDH1A1 might mark TIC populations, it may not be considered as a universal marker in PCa.

In conclusion, even if ALDH^{bright} populations appear to be associated with high tumorigenicity in cell lines, they represent a heterogeneous but relatively frequently detectable population in PCa clinical specimens. Remarkably, ALDH ″brightness" is significantly correlated with ALDH1A1 whose expression is significantly higher in advanced PCa correlating with poor survival in untreated patients.

II. Conclusions: aim of the thesis

Here, we critically investigated the consistence of several models to study TIC in human PCa (**Chapter I**). In addition, we assessed characteristics and relevance of putative stem-like populations in PCa (**Chapter II and III**). Our data highlight the complexity of the TIC concept and the urgent need for more accurate assays. Our findings might also provide an interesting paradigm for the use of combined methods to identify TIC in PCa. Taken together, these data pave the way for further studies aiming at identifying TIC in human PCa.

III. Challenges and Perspectives

Cancer stem cells: The root of all evil?
The Economist; September 2008

Hope for a new therapeutic cellular target

The Cancer stem cell concept has revolutionised the way we think about cancer and offered promises for important developments in the diagnosis and treatment of this disease. Besides the scientific community, this notion has spread to the general public, as illustrated by an increasing number of articles in traditional media (The Economist, BBC news, etc…).

Yet, since it has first been proposed, the CSC concept has raised controversies and debates in the scientific community. The most convincing evidence for CSC and hierarchical organisation of tumours comes from studies of haematological malignancies. In that context, these findings have already been adapted to clinical practice in patients with leukaemia.

Towards a universal model of tumour heterogeneity…?

The leukaemia model has provided a paradigm for other cancers and a significant amount of work has been done to prove its significance in solid tumours. Yet solid tissues are more challenging than haematological malignancies since they require dissociation into single cells and manipulations which can affect cell viability and behaviour. It is therefore likely that current methods are not well adapted to the transfer of the CSC concept in solid malignancies. In consequence, evidence for the presence of CSC in human solid tumours has been rare. Moreover, it is probable that not all tumours are hierarchically organised and that the CSC model might not be universally applicable [24].

One crime…several culprits

Blaming a rare population of cells for exclusively being responsible of a disease as complex as cancer might be simplistic. Tumour heterogeneity and maintenance might be fuelled by cancer stem cells but the whole process surely occurs under the influence of other cell types such as surrounding stromal cells. Once again, this highlights the urgent need for assays which better reflect pathophysiological conditions.

In addition, increasing evidence suggest that different TIC populations might be present within the same tumour, adding complexity to the concept [25].

The case of prostate cancer

Regarding solid tumours complexity, prostate cancer is surely no exception to the rule. PCa indeed represents a paradigm for clinical and genetic heterogeneity in cancers. Besides, studies of TIC in PCa have been considerably hampered by the relative incapacity of PCa cells to be propagated *in vitro* and *in vivo* and no tumor-initiating study using primary human prostate cancer cells has yet been published. One will need to adapt investigations in function of each distinct disease, which are probably initiated by different types of TIC.

Thus, formal proof of the significance of the CSC model in PCa will require the development of novel methods and technologies.

A long road towards cure

Even if the road towards cure is long, the concept opens a theoretical framework on which new therapeutic approaches could be elaborated. Once reliably identified, the challenge will be to specifically kill CSC without affecting normal stem cells since they may share many properties. Therefore, a considerable research effort has to be made to find differences between these two cell populations in order to define reliable therapeutic window. Therapeutic options could include specific killing of the cells but also differentiation therapy aiming at promoting a phenotype more sensitive to current treatments. Considering the role of the microenvironment and the co-presence of distinct TIC populations, combinatorial approaches might be necessary for successful treatment.

Hope coming from mouse models

Recently, several experimental studies have provided initial evidence for the existence of CSC in solid tumours and have shown by lineage tracing that CSC arise *de novo* during tumour formation in intact organs ([26–28]and see Annexe). Even if it is still unknown how well mouse CSC recapitulate their human counterparts, these studies provide a paradigm to validate the CSC model.

In my opinion, combination of mouse model studies paralleled by studies performed in humans will clearly help to unravel and validate one of the most controversial theories in the cancer research field.

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“Anybody who has been seriously engaged in scientific work of any kind realizes that over the entrance to the gates of the temple of science are written the words: 'You must have faith.'”

Max Planck

WORDS OF WISDOM

About the CSC concept

"Maybe its greatest utility is just a better perspective to look at the cancers. You have stem cell haters and stem cell lovers, and both groups are just entirely too dogmatic"
William Matsui (Johns Hopkins University)

"Cells within a tumour are not irrevocably in stem-cell and non-stem cell states."
Robert Weinberg (MIT, Boston)

"Whatever we're defining as a cancer stem cell is not very stable, so the frequency and phenotype may be changing."
Craig Jordan (University of Rochester)

"If you really want to understand cancer, you need to take time to improve the assay, rather than taking one-size-fits all approach."
Sean Morrison (University of Texas Southwestern Medical Center)

About the cell of origin and TIC in prostate cancer

"Right now the most logical conclusion is that there is a least one cell of origin for human prostate cancer, and we certainly have not ruled out that there may be others"
Owen N. Witte (UCLA)

"No one has yet published a tumor-initiating study with primary human prostate cancer cells"
Michael M. Shen (Columbia University, NYC)

FORUM: Cancer

Resolving the stem-cell debate

New research backs the contentious idea that solid tumours are not masses of equivalent cells, but instead contain cancer stem cells that support tumour maintenance. Here, two experts provide complementary views on the findings and on the implications for potential therapies. SEE LETTERS P.522 & P.527

THE PAPERS IN BRIEF

- Evidence that cancer stem cells spawn more highly differentiated (non-stem) cells in solid tumours has relied almost entirely on analyses of tumours formed by human cancer cells injected into mice that have compromised immune systems.
- Chen *et al.*[1] (page 522) and Driessens *et al.*[2] (page 527), together with Schepers *et al.*[3] (writing in *Science*), traced individual cells in intact tumours as the tumours developed from non-cancerous cells in mice*.
- The studies identify specific cell subsets that act as cancer stem cells in brain, skin and intestinal tumours, with one of the reports[1] indicating that targeting such cells may improve therapeutic outcome.
- The papers also describe how different cell subpopulations emerge and evolve as a tumour grows and in response to anticancer treatment.

Meet the parents

RICHARD J. GILBERTSON

Despite decades of research, one-third of patients with cancer die within five years of diagnosis. Therefore, it is not surprising that any concepts offering a sea change in the way we think about and treat cancer garner enormous attention and resources. One such concept is the cancer stem cell (CSC) hypothesis, which suggests that cancers are organized into aberrant cell hierarchies in which 'differentiated' daughter cells that have limited capacity to proliferate are produced by a subset of parent CSCs that replicate indefinitely (Fig. 1).

Until now, evidence for the existence of CSCs has been controversial, but the hypothesis is extremely attractive because it provides a conceptual framework on which new therapeutic approaches could be built: any drug capable of killing CSCs would, in theory, be curative. Now, three independent studies of mouse models of brain[1], skin[2] and intestinal[3] tumours provide the first evidence that CSCs do exist and arise *de novo* during tumour formation in intact organs.

Lineage tracing is a technique that allows permanent *in vivo* fluorescent marking of stem cells and their progeny. This method has been used previously[4] to identify intestinal stem cells, which give rise to the various cell types that make up intestinal epithelial tissue in mice. The same study also revealed that when a gene encoding the protein APC is deleted, these stem cells generate benign tumours (intestinal adenomas).

To test whether the tumours were maintained by CSCs, Schepers *et al.*[3] used a lineage-tracing strategy involving intestinal stem cells in which *APC* had been knocked out, so that cells randomly adopted one of four fluorescent tagging colours when the mice were given a low dose of the drug tamoxifen. Initial tamoxifen dosing often generated single-colour 'clonal' adenomas, indicating that they typically originated from single intestinal stem cells. Remarkably, a subsequent dose of tamoxifen switched the colour of individual cells in the adenomas, and the progeny of these newly coloured cells (which included differentiated tumour cells) went on to populate the tumour, pinpointing their parents as CSCs.

> ***"Cancer stem cells repopulate the cancer when the bulk of the tumour is wiped out by anticancer drugs."***

Similar observations were made by Driessens *et al.*[2] in a mouse model of a benign skin tumour (papilloma). Using lineage tracing of individual papilloma cells, the authors observed great variability in the cells' proliferative potential, with only 20% of them being able to generate daughters that populated large swathes of tumour.

The studies by Schepers *et al.* and Driessens *et al.* provide elegant demonstrations of stem-cell activity in intact tumours. But adenomas and papillomas are benign tumours, not cancers. The cells in these tumours are organized in much the same way as the corresponding normal tissue, and so it is not surprising that these benign tumours contain cell hierarchies that approximate to normality.

A key question, therefore, is whether cell hierarchies driven by CSCs exist in the invasive malignant tumours that kill patients. With this in mind, Driessens *et al.* also analysed a mouse model of squamous skin cancer. The researchers found that, in comparison with papillomas, the malignant tumours contained much higher numbers of long-term replicating cancer cells that showed little evidence of cell differentiation. This raises the possibility that cancers slip from hierarchical organization into relative anarchy as they progress from the benign to the malignant state.

So what is the evidence that malignant tumours contain CSCs? Chen *et al.*[1] provide compelling data that glioblastomas (the deadliest brain tumours) are organized hierarchically. Using a clever combination of 'suicide-gene' technology that selectively killed glioblastoma CSCs, and antitumour drugs that eliminate the bulk of dividing cancer cells, they show that CSCs repopulate the cancer when the bulk of the tumour is wiped out by anticancer drugs (Fig. 1a). Targeting both CSCs and their daughter cells with a combination of suicide-gene targeting and anticancer drugs, the authors dramatically impeded the growth of glioblastomas *in vivo*.

The three papers represent an important new chapter in the debate over CSCs. They introduce us for the first time to these cells in their native habitats and provide the first hard evidence that such cells are a legitimate therapeutic target. The next steps will include determining how well mouse CSCs recapitulate their human counterparts, and how best to destroy these for the benefit of patients.

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Stemming tumour evolution

TREVOR A. GRAHAM

Cancer formation is an evolutionary process: repeated rounds of mutation and selection lead to outgrowth of the fittest mutant clones and transform normal healthy cells into cancer cells[5]. The identification of CSCs as a restricted population of cells responsible for the maintenance of tumours suggests that these are the cells that have the inherent ability to propagate mutations throughout a tumour and to drive cancer evolution: CSCs are the 'movers and shakers' of carcinogenesis. Selective killing of such cells is therefore an appealing therapeutic prospect. Indeed, Chen *et al.*[1] observed a near-twofold reduction in the density of brain tumours in mice when they combined standard anticancer drugs with selective killing of CSCs, compared with standard agents alone.

But should the primary goal of cancer therapy now be to kill the CSC 'root' of the tumour? This is akin to asking if we may safely ignore the non-stem-cell population of tumour cells. It is conceivable that selected mutations in non-stem cells will cause them to revert to a stem-cell-like state and so contribute to tumour evolution. Furthermore, non-stem cells in tumours may revert to such a stem-cell-like state even in the absence of mutation[6]. If that is the case, then selectively killing a CSC population may vacate a niche within the tumour, opening it up to occupancy by a rival population of cells. Trying instead to limit 'stemness' — perhaps by modifying the microenvironment that supports stem cells in tumours[7] — may prove a more effective therapeutic strategy than simply eradicating CSCs.

"The results place competition between tumour cells at the centre of cancer evolution."

Driessens *et al.* and Chen *et al.* show that the cellular organization of the early (pre-cancer) skin and intestinal tumours are caricatures of their normal organs, and are composed of both stem cells and non-stem cells. The presence of non-stem cells may represent a brake on tumour evolution: such cells not only consume the limited resources available, but also may be evolutionary dead ends, in the sense that (unlike the stem cells) they have only limited potential for growth. Interestingly, Driessens *et al.* observed that progression to cancer in benign skin tumours was associated with an expansion of the CSC population and a decrease in the production of non-stem cells. This suggests that tumour evolution enriches the CSC population.

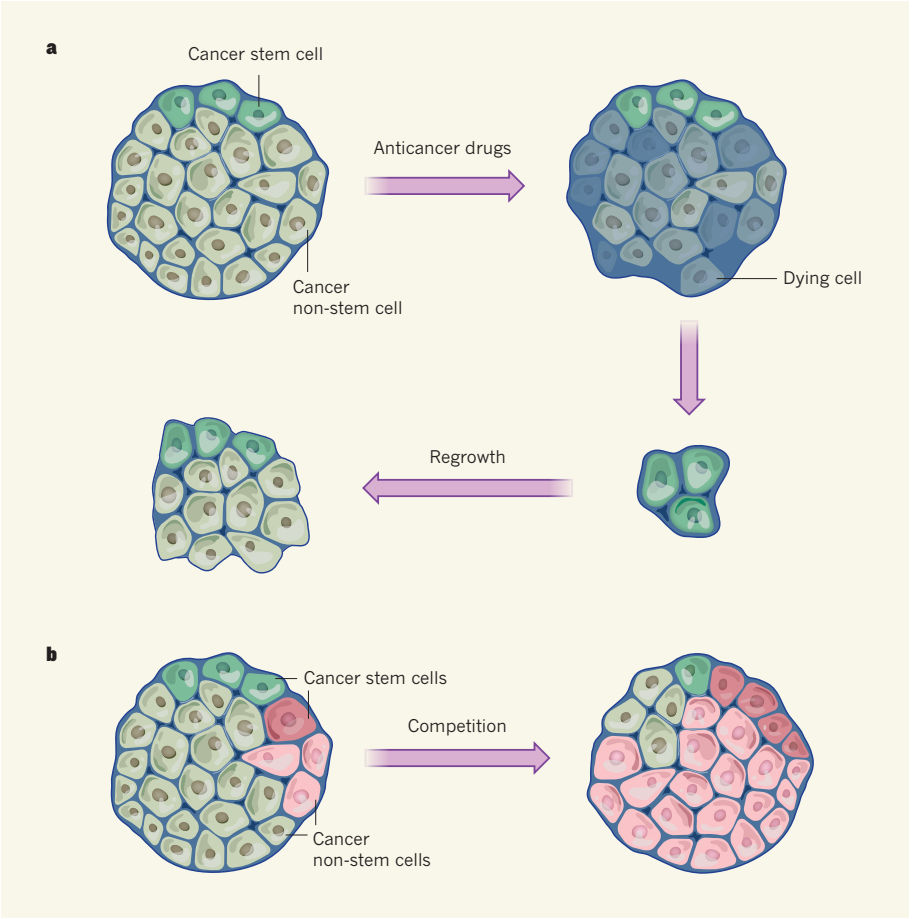

Figure 1 | Not all cells in a tumour are equal. Chen *et al.*[1], Driessens *et al.*[2] and Schepers *et al.*[3] show that brain, skin and intestinal tumours include cancer stem cells (CSCs) that self-renew and that produce other, more-differentiated (non-stem) cells that constitute the bulk of the tumour-cell population. **a**, Chen and colleagues' results indicate that, although current anticancer drugs can wipe out most of the dividing non-stem cells, surviving CSCs can repopulate the tumour. Therefore, targeting both CSCs and the dividing cells would be required for complete tumour eradication. **b**, Driessens *et al.* report that CSCs continuously compete with each other for a place in the tumour, and that the winners' daughter cells predominate. Red and green colours indicate different clonal populations, each one originally derived from an individual CSC.

Thus, designing therapies that prevent increases in stemness may be a means to restrict tumour progression to cancer.

A common model of cancer evolution involves sequential waves of clonal expansion, each triggered by a new mutation[5]. Remarkably, Driessens and colleagues' results are at odds with this model. The authors found neutral competition between CSCs; that is, every CSC within a tumour is equally likely to clonally expand or die off, probably even in the absence of new mutations (Fig. 1b). Their observations suggest that clonal expansion is a continuous process in tumours, not a rarity driven by a new, selectively advantageous mutation. And the results place competition between tumour cells at the centre of cancer evolution. In this context, mutations that simply drive proliferation may be of less importance than previously thought, whereas mutations that slightly tip the balance of competition to favour one CSC over another — perhaps by improving survival, promoting self-renewal or monopolizing limited resources — might be the ones that are highly selected in tumours .

The main take-home message from the three studies is that cells are organized hierarchically within tumours; all tumour cells are not equal. Understanding how these cellular hierarchies shape carcinogenesis, and exploiting them to change the course of tumour evolution, holds promise for effective treatment. ■

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To the *Prostate Gang*:
Chantal: Thanks for choosing me, trusting me and immediately making me feel comfortable.
Malte *Urologie Rieken*: our "collaboration" will hopefully continue in the best bars of NYC.
Ema: whatever you are working on now (who knows…?), I won't forget our PCR (and party…) sessions. Keep on going! DAJE!!!!!

To my parents and family: Merci de m'avoir soutenue et encouragée lorsque je doutais de moi.

To the *3 other fantastic*: Nano, Sandrasch and Silvietta
We are the living proof that an italian-french-portuguese team is the best combination ever. Merci! Grazie! Obrigada! I will be waiting for you in NY for the next match.

To the 2008 PhD *grand Cru*: Wikilele, Piccins, and Sinanovitch.
It has been 5 intense years full of important moments shared with you. It wouldn't have been the same without you. I already miss you!

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Last but not least: A well-deserved thanks to the Paddy Reilly's for providing a (almost) trustful place to relieve my PhD stress.

Curriculum Vitae

Personal information

Surname(s) / First name(s)	**LE MAGNEN CLEMENTINE**
Address	✉ Lothringerstrasse, 23. 4056 BASEL (SWITZERLAND)
Telephone(s)	☎ 0041-612652376 (work) Mobile 0041-767447862
E-mail(s)	lemagnenc@uhbs.ch
Nationality	French
Date of birth	04.12.1984
Gender	Female

Work experience

02.01.2008 – 21.11.2012

PhD student

Identification, properties, and clinical significance of putative stem-like populations in prostate cancer.

- Clinical specimens processing (blood, tissue digestion and analysis)
- Cell culture (cell lines and primary cells)
- FACS analysis, PCR, RT-PCR
- Various *in vitro* assays (clonogenic, spheres, etc.), *in vivo* tumorigenicity assay

Institute for surgical research and hospital management (ICFS), Oncology group, University hospital Basel (Switzerland)

OCTOBER 2006 – JULY 2007

Master thesis training period

Post-transcriptional silencing of Beet necrotic yellow vein virus RNA-3: Effects on viral replication and systemic infection.

- Molecular biology (Northern blot, PCR, cloning...)
- Phytovirus infection and agroinfiltration

IBMP, Institut de Biologie Moléculaire des Plantes (CNRS), Strasbourg, France.

JULY 2006

Training period

Hepatitis C research - Molecular biology techniques, notions of cell culture

Tibotec BVBA, Mechelen, Belgium

SUMMER 2005/2004/2003

Lab technician/office clerk/clinical samples delivery

Laboratory of Anatomy-Pathology Itten-Petters, Mulhouse, France

Education and training

JULY 2007 — **Master degree** in Biology of micro-organisms, Speciality in Virology.

University Louis Pasteur (ULP), Strasbourg, France.

JULY 2005 — **Bachelor degree** in General biology - ULP, Strasbourg, France

JULY 2004 — **Two year university degree (DEUG)** in General biology - ULP, Strasbourg, France

JULY 2002 — **High school leaving certificate**, Speciality in Sciences (Baccalauréat S)

Lycée Montaigne, Mulhouse, France.

Personal skills

Language(s)	French (mother tongue), English (advanced level, good scientific level), German (basic level)
Computer skills and competences	Ms office, Adobe Photoshop, GraphPad, Corel Draw
Other skills and competences	Enjoy travelling, doing sports (ski, badminton, dance, jogging...)
Driving licence(s)	B

Oral and poster presentations

Poster	**"High aldehyde dehydrogenase activity, mediated by ALDH1A1, is associated with high tumorigenicity and poor clinical outcome in prostate cancer"** 20th meeting of the EAU Section of Urological Research (ESUR)-October 25-27, 2012, Strasbourg, France
Oral presentation	**"High ALDH activity is associated with high tumorigenicity in prostate cancer"** European Cancer Center Basel-Freiburg-Strasbourg, 19th Annual meeting, May 5th, 2012, Freiburg, Germany
Poster	**"High ALDH activity is associated with high tumorigenicity in prostate cancer"** American association of cancer research (AACR) annual meeting- March 31- April 4, 2012- Chicago, Illinois, USA
Poster	**"Is ALDH a valuable cancer stem cell marker in prostate cancer?"** FEBS advanced lecture course on translational cancer research – September 27-October 4, 2011 – Algarve, Portugal
Oral presentation	**"Is ALDH a valuable cancer stem cell marker in prostate cancer?"** 9th World congress on urological research-15-17 September 2011, Innsbruck, Austria
Poster + short oral presentation	**"Looking for a stemness signature in prostate cancer"** 26th Annual European association of urology (EAU) Congress -18-22 March 2011,Vienna, Austria **Price of the best poster presentation of the session**
Poster	**Looking for a stemness signature in prostate cancer** 19th meeting of the EAU Section of Urological Research (ESUR)-October 7-9, 2010, Vilnius, Lithuania
Posters	**"Looking for a stemness signature in prostate cancer"** **"Is ALDH a valuable cancer stem cell marker in prostate?"** Stem cells and Cancer-6th International Heinrich F.C. Behr Symposium-October 3-5, 2010 Heidelberg, Germany
Poster	**"Looking for a stemness signature in prostate cancer"** BioValley Life Sciences Week conference-September 21-24, 2010, Basel, Switzerland
Oral presentation (invited speaker)	**"Looking for a stemness signature in prostate cancer"** Work in progress seminar; Medizinische klinik- July 30th, 2010, Freiburg, Germany
Poster + short oral presentation	**"Characterization of Cancer-Initiating Cells derived from prostate malignancies"** 97. Jahreskongress der Schweizerischen Gesellschaft für Chirurgie (SGC). May 26-28th, 2010, Interlaken, Switzerland.
Oral presentation	**"Looking for a stemness signature in prostate cancer"** European Cancer Center Basel-Freiburg-Strasbourg, 17th Annual meeting, May 7th, 2010, Strasbourg, France
Poster	**"Characterization of cancer stem cells derived from prostate malignancies"** Frontiers in Cancer Stem Cell Research: from basic science towards a cure, December2-4th, 2009, Oslo, Norway.

Poster + short oral presentation

"Characterization of cancer stem cells derived from prostate malignancies"
65. Jahresversammlung der Schweizerischen Gesellschaft für Urologie, September 3-5th, 2009, Lausanne, Switzerland

Poster

"Characterization of cancer stem cells derived from prostate malignancies"
2nd DKFZ-NCI International Conference on stem cells and cancer, October 26-28th, 2008, Heidelberg, Germany.

Publications

MAGE-A10 is a nuclear protein frequently expressed in high percentages of tumor cells in lung, skin, and urothelial malignancies.
Int J Cancer 2011.
Schultz-Thater,E.; Piscuoglio,S.; Iezzi,G.; Le,Magnen C.; Zajac,P.; Carafa,V.; Terracciano,L.; Tornillo,L.; Spagnoli,G.C.

High levels of circulating IL-7 and IL-15 in localized prostate cancers
J Transl Med 2011
C. Mengus, C. Le Magnen, K. Yousef, L. Bubendorf, M. Provenzano, A. Bachmann, M. Heberer, G.C Spagnoli, and S. Wyler

Klf4 transcription factor is expressed in the cytoplasm of prostate cancer cells
European Journal of Cancer 2012
C. Le Magnen, L. Bubendorf, C. Ruiz, I. Zlobec, A. Bachmann, M. Heberer, G.C. Spagnoli, S. Wyler and C. Mengus

In revision:
Characterization and clinical relevance of ALDH^{bright} populations in prostate cancer. ***Clinical Cancer Research***
C. Le Magnen, L. Bubendorf, C. A. Rentsch, C. Mengus, J.R. Gsponer, T. Zellweger, M. Rieken, G. N. Thalmann , M. G. Cecchini , M. Germann A. Bachmann, S. Wyler, M. Heberer, and G.C. Spagnoli