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RESOLUTION OF CELLULAR HETEROGENEITY IN HUMAN PROSTATE CANCERS: IMPLICATIONS FOR DIAGNOSIS AND TREATMENT.

Norman J Maitland[1*], Fiona M Frame[1], Jayant K Rane[1], Holger H Erb[1], John R Packer[1], Leanne K Archer[1] and Davide Pellacani[1,2]

1 Cancer Research Unit, Dept of Biology, University of York, York, YO607SD, United Kingdom

2 Current address: Terry Fox Laboratory, BC Cancer Agency, 675 West 10th Avenue, Vancouver, BC V5Z 1L3, Canada

*To whom correspondence should be directed

Telephone: +441904328700 Email: n.j.maitland@york.ac.uk

Summary

Prostate cancers have a justified reputation as one of the most heterogeneous human tumours. Indeed, there are some who consider that advanced and castration resistant prostate cancers are incurable, as a direct result of this heterogeneity. However, tumour heterogeneity can be defined in different ways. To a clinician, prostate cancer is a number of different diseases, the treatments for which remain equally heterogeneous and uncertain. To the pathologist, the histopathological appearances of the tumours are notoriously heterogeneous. Indeed, the genius of Donald Gleason in the 1960's was to devise a classification system designed to take into account the heterogeneity of the tumours both individually, and in the whole-prostate context. To the cell biologist, a prostate tumour consists of multiple epithelial cell types, inter-mingled with various fibroblasts, neuroendocrine cells, endothelial cells, macrophages and lymphocytes, all of which interact to influence treatment responses in a patient-specific manner. Finally, genetic analyses of prostate cancers have been compromised by the variable gene rearrangements and paucity of activating mutations observed, even in large numbers of patient tumours with consistent clinical diagnoses and/or outcomes. Research into familial susceptibility has even generated the least tractable outcome of such studies: the genetic loci are of low penetrance and are of course heterogeneous. By fractionating the tumor (and patient matched non-malignant tissues) heterogeneity can be resolved, revealing homogeneous markers of patient outcomes.

Prostate tumour 'cells' as a therapeutic target

Based on the Gleason histological system devised in the 1960s (1), imperfect conclusions about prostate cancer patient outcome can be made. The treatment of the tumor, despite its frequency, has yet to be optimized. Studies designed to decide the best early stage treatment such as ProTect (2) have provided an indication, but with a long time scale required to produce a decision. It is however, quite clear from another long-term study, by ERSPC (3) that early intervention, based on screening for prostate-specific antigen (PSA) can have an impact on death rates, with the enduring risk of over-treatment in the absence of good molecular markers for aggressive disease (4). However, when primary prostate cancer has escaped from blockade of male sex hormone responses, the full impact of heterogeneity can be seen. The mean survival times, despite new generation drugs like abiraterone, apalutamide and enzalutamide, designed to manipulate androgen responsiveness (5), and optimized taxane treatments which targets dividing cells (6), remains stubbornly at about 2 years (7). Indeed, many established treatments from other tumor types have only limited efficacy against advanced castration-resistant prostate cancer (CRPC) (8).

There are few simplistic explanations for this apparent resistance. Prostate cancer has, historically been considered to be a slow-growing tumor, yet the cancer cells cycle at about the same rates as those from other tumors. A more likely explanation comes from the observation that prostate cancers seem to have a relatively high intrinsic apoptotic rate: resulting in a *net* slower increase in tumor bulk and cell numbers (9). If this is indeed the case, then there is little justification based on slower cell divisions for the observed resistance to drugs like etoposide and docetaxel, which interfere with the processes of genome and cell duplication respectively.

We are now firmly in the era of novel, targeted therapies for many cancers (including the prostate), where genomic information is rapidly translated into specific reagents such as antibodies and interfering RNA, which can eliminate the activity of particular enzymatic targets. The issue of heterogeneity directly impinges on the use of these targeted reagents. As discussed below, given the degree of polymorphism in man, and the different types of heterogeneity seen in cancers, can one highly targeted drug ever be designed to treat all patients with even a specific grade of a specific cancer?

Cellular heterogeneities within a prostate cancer

To a generation of cell biologists brought up on established cell lines, which could be exploited in controlled growth conditions to model disease, the sheer heterogeneity of cell phenotypes present within a single cancer can appear rather daunting. Such mixtures of cells are only too familiar to prostate histopathologists, who have successively refined the original Gleason grading scheme (10). Increasingly multicellular laboratory studies are now revealing the true roles played by the many cell types which affect the behavior of the primary tumor cells (secretory epithelial cells for an adenocarcinoma like prostate cancer). The representations in Figure 1 illustrates the many cell types to be found within the prostatic normal and tumor microenvironments (11).

The fate of the epithelial cells is intimately linked to the presence of the ancillary cells, a situation which is recapitulated when the primary tumor successfully migrates to a primary and secondary metastatic site. Such tumor spread is frequently associated with epithelial to mesenchymal transition, a transitory state in which a frank epithelial morphology, and the gene expression profile of the cancer cells, changes to one resembling a mesenchymal (stem) cell (12). Under these conditions a further degree of (morphological) heterogeneity is observed within what is essentially the same tumor cell at the genomic level.

One interesting feature of the tumor microenvironment is the extent to which the tumor epithelium defines the phenotype of the ancillary cells i.e. the development of a tumor is marked by co-evolution of the primary transformed cell (which we presume to be the epithelial cells in prostate) perhaps with genetic and epigenetic changes in the associated mesenchyme (13) (14). This hypothesis has resulted in the definition of carcinoma associated fibroblasts (CAFs) as a discrete cell type which can be isolated from tumor areas. Such cells have a more stem cell-like phenotype, and are capable of inducing a carcinogenic phenotype in benign epithelial cultures from prostate such as BPH1, which usually form non-malignant cystic growths when grafted in combination with fibroblasts from normal areas of the prostate (15,16). The mechanism by which the benign cultures become frankly malignant in xenografts is likely to be complex, but there is good evidence to suggest that the TGFbeta signaling axis plays a key role(17).

Tracking heterogeneity in a low mutation tumor such as prostatic adenocarcinoma.

Traditionally cancer has been considered as a process driven by mutation in critical genes, and an increasing frequency of mutation detection as the tumor develops, including the development of resistance to multiple therapies. This certainly seems to be the case with a number of common tumor types such as small cell lung cancer (18), but prostate cancer belongs to a low mutation group (19) (illustrated in Figure 2). Recent next generation sequencing (NGS) of whole prostate tumor biopsy DNA has confirmed this, while confirming the dogma that later stage cancers (after treatment failures) carry a higher mutagenic burden (20-22) compared to treatment naïve tumours. One paradox in this data is the relative prevalence of both IDH1 and TMPRSS2-ERG lesions in early vs late cancers (22). If the latter develop from the former, then the mutation states should be preserved, as shown in recent genomic sequencing studies of multiply and sequentially biopsied tumours (23,24). This is not the case, particularly as IDH1 mutations are undetectable in relapsed cancers (22), which argues either for (i) independent clonal origins for the relapses – probably not correct as specific mutations are shared with the primary tumour, or (ii) that IDH1 mutation confers a transient advantage to the early stage cancers, and that the relapse originates in a less mutated progenitor cell, which makes up a small proportion of the original tumor mass.

In terms of cancer cell survival, mutation can be considered as a poor evolutionary step, particularly in a changing microenvironment manipulated by successive treatments. Whilst such non-reversible changes are observed in genes such as androgen receptor (AR) after therapy to block hormone responses in prostate cancers (25), there is increasing evidence that several tumor types achieve treatment resistance and the evolution of a fatal metastatic phenotype by means of epigenetic rather than mutagenic changes. A good recent example is the paucity of 'metastatic' mutations in malignant pancreatic adenocarcinoma, where the tumor evolution is apparently mediated by genomic methylation changes (26).

Cell Fate decisions in multicellular prostate tissues

Most studies have reflected on the required 'oncogenic changes' required to produce the loss of growth and positional control which characterize human cancers, and frequently used available cell lines (27) to define these changes and their downstream effects. As illustrated in Figure 1, a cancer in man is a complex community of different cell types

which can interact with one another in positive and negative ways. However it is possible to generalize about cellular changes in prostate cancers as shown in Table 1.

Table 1: Some essential characteristics of Human Prostate Cancers

	Tumour property (primary prostate cancers)
1	Primary prostate cancers largely consist of replicating cells with an aberrant luminal phenotype (AR+, PSA secreting, changed energy requirements) whereas in normal tissues luminal cells rarely divide.
2	Primary cancers have lost more than 99% of basal cells compared to normal prostate, where there is (in man) a contiguous basal layer lining prostatic glands.
3	Basement membrane, which provides a barrier between the stromal and epithelial compartments has all but disappeared in higher grade primary cancers.
4	The stromal compartment in cancers has a novel more embryonal phenotype (see above) termed 'reactive stroma'.
5	The intratumoral immune cell components, which include both lymphocytes and macrophages, undergo a distinct phenotypic switch.

When studied at gene expression or genomic level using mixed cell biopsies of tissues, contributions from minor populations are masked, and gene expression from the most transcriptionally active cells (secretory luminal cells), which can be up to 3 logs higher than in more quiescent cells, dominates most gene expression signatures. Of course, each biopsy (and patient) has variable proportions of the constituent cells which simply produces 'noise' in the final analyses and reduced statistical significance for the various markers. A good example of this was the TCGA attempt using a combination of mutations, DNA methylation (at promoters), copy number alterations, mRNA expression (including gene fusions), microRNA expression and protein expression to cluster mixed cell populations from 333 primary prostate cancers, which left about 26% of tumors unclassifiable even into 7 subtypes (22). Similarly, the expression signature of highly secretory immune cells also contaminates many 'tumor signatures' in blood DNA classifications (28), whilst stromal cell content provides the best indicator of malignancy in many colon cancer biopsy studies (29). Such empirical findings may be useful in diagnosis, but the lack of a tumor cell component does not help in our *understanding* of key carcinogenic changes.

The one certainty in such analyses of gene expression, when cancer biopsies are compared to normal, is the predominance of luminal cell markers – since there are extremely small numbers of basal cells in prostate cancers. Indeed, the loss of expression of the strongly basal cell marker TP63 is a commonly used diagnostic aid to distinguish benign from malignant prostate disease (30).

The heterogeneity is also frequently ignored when the origins of castration-resistant prostate cancers, and the ultimately fatal neuroendocrine form of the disease (31) are posited. In a homogeneous tumor model, there is a requirement for trans-differentiation of the luminal cancer cells into CRPC and NE tumours, which can be readily achieved by selection and treatment of cell lines such as LNCaP (32,33). However the molecular tags of the relatively rare mutations in prostate cancer tells another story. Since it is unlikely that cancers can repair or lose mutations, why as discussed earlier, do some mutations in early treatment naïve cancers, fail to make it into the CRPC form in the same cancers? There are a number of mutations (trunk or founder mutations) which are conserved – indicative of a common cellular origin. We have proposed that all prostate cancers contain a population of largely quiescent stem-like or progenitor cells which are resistant to most treatments and provide a mutated reservoir for the emergence of new cancer clones when the post treatment microenvironment selects for a new variant cell type (34) (Figure 3). Since many of the experiments are carried out *in vitro*, it has been argued that this is a cell culture artifact. However in man, an EORTC study (35) from 12 years ago, provided strong evidence that the cells which regrow after androgen ablation are pre-existing in the tumor population, by comparing relapse rates in 2 cohorts of patients, one given immediate anti-androgen receptor drugs, and a second, where the treatment was delayed until tumour progression was seen. Realistically, if the treatment is inducing luminal cancer cells to transdifferentiate, then the treated cohort would show higher and faster relapse. This was not observed in the trial - as illustrated in Figure 4.

Small Non-coding RNAs as controlling factors of gene expression in prostate epithelial differentiation?

To establish the factors which determine cell phenotype in the prostate, we have developed a fractionation procedure for prostate tissues (36), which enables both purification and ultimate culture of all cell types, with the exception of culturing both normal and cancer luminal cells. To identify the genes whose expression changes

marked the epithelial differentiation process, we employed a pairwise analysis of total gene expression patterns, focusing on benign rather than malignant samples, since the benign epithelium had more consistent expression profiles(37). We reasoned that genes with a regulatory role would change expression in a regular manner as clusters. The published data (38) revealed that genes showing a co-ordinate regulation during differentiation formed 4 distinct and non-overlapping sets (Figure 5). Overall the regulatory networks with the highest significance in the transition from the most primitive stem like epithelium to basal cells committed to differentiation into luminal cells were generic 'tissue developmental events' but more specifically retinoic acid and ROCK2 signalling. Upon the terminal differentiation to luminal cells, all gene expression patterns are dominated by androgen signaling. But what controls the switch from the basal compartment (where retinoic acid signaling predominates) to luminal cells? One clue might come from the regulation of prostatic transglutaminase(hPTG), which belongs to one of the distinct 4 transcriptional co-regulation groups (39). In the upstream control sequences of hPTG, as in many other related genes scattered throughout the genome, the binding sites for the retinoic acid receptor (RAR/RXR), are in close apposition on the genome with those for AR. However the RAR/RXR sites are constitutively occupied in the more committed basal cells, but not in the stem cell compartment. In contrast, AR must bind ligand in the cytoplasm before translocating into the nucleus to occupy its binding sites. The relative proportion of occupied binding sites defines the cell fate decision. The provision of activated ligand from retinol for RAR/RXR is likely to come as a paracrine interaction from the stem/progenitor cells which constitutively express both retinol and aldehyde dehydrogenases, but very low receptor levels. These AR-RAR dually regulated genes mark the transition, but do not effect it – a function assigned for example to numerous transcription factors, such as a master controller like MYOD in muscle development (40).

But how is the simultaneous activation of transcription factors achieved in prostate? Hormones clearly provide part of the explanation, but in mammalian development, small non-coding RNAs play a central role (41). To investigate this possibility, we analysed the expression patterns of microRNAs in the fractionated epithelial cell types. As shown in Figure 6, the highest expression levels were seen in the least differentiated stem/progenitor cells, with a consistently progressive loss of expression towards the committed basal cells. When compared to other cell-type specific miRNA expression patterns in online databases, the stem cell pattern most closely matched that from human embryonic stem cells (42).

However, reflecting the prostatic origin of the cells, the benign SC miRNA expression pattern also matched that of total miRNA in castration-resistant prostate cancers (42). This agrees with a commonly observed SC-like mRNA gene expression pattern seen in CRPC (43). But can any of these miRNAs act to control the differentiation-regulating transcription factors?

Many algorithms have been developed to identify the genes whose expression is modulated by a specific miRNA. However, these are based on the theoretical presence of miRNA recognition sites within the genome. The application of this analysis will confirm a miRNA target gene, but takes no account of whether the gene in question is expressed either in the target tissue, or indeed the cell type of interest within that tissue. A classical example of 'phantom' gene expression control (by miRNA 143/145) was demonstrated in mouse colon (44). To address this, a specialized algorithm, which related *actual* mRNA expression in the prostate epithelium to the miRNA expression patterns, was applied (45). This strategy identified miRNA 548-3p as a master controller of the transcription factors implicated in our earlier studies (RXR, VDR, GR, TAZ, SRF, HSF1 from (42). miRNA 548-3p was consistently overexpressed in the stem-progenitor population but not expressed in the more committed basal cells. When expression was engineered in the committed basal cells, the result was an increase in the stem-like population in cell cultures, based both on increased colony forming (biological) and the upregulated expression of a set of stem/progenitor genes such as CD49b and f (integrins alpha 2 and 6 respectively) (42).

During development of the expression algorithm, the data was also aligned according to gene ontology functions in the differentiating epithelium. The most significant term was DNA repair/radiation resistance, with miR99a/100 as the most relevant miRNA. Previous studies had shown that the radiosensitivity of prostate cell lines (DU145>PC3>22RV1>LNCaP) was inversely related to the expression of miR99a/100 . We had also demonstrated that the stem-like population displayed a markedly higher resistance to ionising radiation (46), and showed that this was a consequence of the highly condensed nature of the chromatin in the SC. The induction of increased stemness by introduction of miR548-3p into CB cells also resulted in an increased radiation-resistance in the population. This aligned well with online data, which associated endogenous miR548-3p expression in patients' tumors with a poorer prognosis for prostate cancer patients (42).

In a similar manner, when the lower expression of miR99a/100 in SC was engineered by introduction of miR inhibitors into the CB populations and various established prostate cell lines, an increased cell survival, assayed by colony forming activity was observed. In this case, there was no accompanying change in the differentiated state of the cells (as seen with miR548-3p) assayed by a lack of expression of stem cell (NFkappaB, ID2, PROM1, SOX2) or EMT (VIM, CHDH1, FN1) markers. Systematic elimination of potential miR99a/100 target genes, based on our algorithm of prostate epithelial mRNA expression patterns, then identified 2 SMARC genes (A5 and D1), which had already been assigned a role in DNA damage repair, where their core function was to affect chromatin condensation levels, and to recruit BRCA1 and RAD51 DNA damage repair proteins to radiation-induced lesions (47). In keeping with a cytoprotective property, upregulation of these SMARC genes was seen within 3 minutes of prostate epithelial cell irradiation.

There is also evidence that cytoprotection can be enhanced by glucocorticoids (GC), and when SMARC levels were quantified after dexamethasone treatment, they were also upregulated by GC. Whilst this provided a mechanistic proof, it also implies a potential controversy in the treatment of prostate cancer patients, where glucocorticoids are historically given to improve patient wellbeing. This implies that the addition of GC to a treatment protocol for radiotherapy would be likely to compromise the effectiveness of the treatment. To test this hypothesis, we used the GC response inhibitor mifpristerone which resulted in an increased sensitivity to radiotherapy, whilst downregulating SMARC expression (47). Thus an understanding of the basic biology of miRNA epigenetic control of cellular differentiation, using fractionated cells from patient biopsies could ultimately influence prostate cancer treatment in patients.

Methylation of CpG sites distal from 'CpG islands' as a genomic control of differentiation and carcinogenesis in the prostate.

As discussed earlier, prostate cancers have a particularly low rate of carcinogenic mutations. Recent studies of pancreatic cancers (which have an inherently higher mutagenic rate than PCa) showed that epigenetic changes such as differential genomic methylation could define the transition from organ confined to malignant cancers (26). The ability of methylation-editing enzymes to change global methylation of CpG and other susceptible sites has also played a role in mammalian tissue differentiation (48). In earlier comparisons of focused methylation within the promoters of target genes, we

showed that the epigenetic modifications in established cell lines formed a distinct cluster, separate from that in primary cell cultures, and independent of pathology (49,50).

To analyse cell-specific methylation patterns, freshly disaggregated tissues were fractionated as previously described to generate a basal fraction, a luminal cell fraction and stromal cells (as shown in figure 8A). Using the relatively conserved tissues from BPH (37), rather than cancers, we had already shown that even in primary cultures, distinctive and functional changes in gene expression were induced *in vitro* (Figure 7B). However such changes were limited, compared to those seen in a total gene expression comparison between multiple primary epithelial cell cultures, and benign (BPH1) or malignant (PC3) prostate epithelial cell lines (Figure 7C).

After DNA purification from homogeneous cell fractions (of< 1000 cells), assayed by both RT-PCR and FACS for cancer and differentiation markers (see Table 2), each DNA extract was divided, and one half was subjected to bisulphite conversion before sequencing of both cell populations {Pellacani et al, in press, BJC 2018}

When subjected to hierarchical clustering, analysis of 4 matched human normal: cancer (defined as a patient with a majority of Gleason Grade 4 pathology in the biopsy) pairs of samples (i.e. 6 cell populations resulting in 12 datasets per patient). Some samples from patient 4 were lost during processing and only those with a high purity were processed.

Table 2: Selection and phenotype monitoring of cell fractions from fresh human prostate tissues

Cell type	Selection markers	Phenotype markers (RNA)	Phenotype Markers (protein)
Normal Basal Cells	EPCAM+/CD24-/ CD49f+	KRT5/14+; TP63+ ITGA6+/ECAM+	CK5/14++
Normal Luminal Cells	EPCAM+/CD24+/ CD49f-	KRT8+/CD24+ EPCAM+	AR++/CK8+
Normal Stromal cells	EPCAM-/CD24-/ CD49f-	VIM+	VIM+/AR+
Cancer Basal Cells	EPCAM+/CD24-/ CD49f+	AMACR+/KRT5/14+; TP63+/-	CK5+: CK14+

		ITGA6+/ECAM+	
Cancer Luminal Cells	EPCAM+/CD24+/ CD49f-	AMACR++/KRT8+; ECAM+	AR++, CK8+
Cancer Stromal Cells	EPCAM-/CD24-/ CD49f-	VIM+	AR+

Initial hierarchical clustering of the patients based on their different CpG methylation profiles revealed an enhanced ability to distinguish between patients, regardless of pathology. In any multipatient study therefore, this signature could mask underlying pathogenic-related changes. Secondly, once individual signatures were removed, a strong cell-type specific methylation pattern emerged. This could clearly distinguish stromal from both epithelial populations, where signatures were closely related. When the epithelial comparisons were made within each patient (an example of which is shown in Figure 8A), linking the methylated CpG to adjacent genes and their functions, a number of gene ontology terms emerged as significant. Most changes were concerned with basal to luminal differentiation processes, which was the original aim of the study. For example, differentially methylated regions (DMRs) in a comparison of luminal and basal cells from normal prostate epithelium enriched for more than 500 terms. The hypermethylated set of gene ontology (GO) terms included many linked to prostate development or epithelial stem cell regulation. The hypomethylated DMRs were enriched for completely distinct GO terms principally related to androgen receptor signalling and responses to cytokines.

Unlike many previous studies which focussed on the previously determined 'CpG islands' clustered close to gene transcriptional start sites (51), we found that the most significantly altered sites were located at genomic locations more than half of which fell *outside* of known CpG islands, shores or shelves, and >70% were >5 kb distant from annotated transcriptional start sites. Thus, the differentially hypermethylated regions were enriched at loci previously defined as enhancers which were defined by three characteristic properties within the genomic sequence databases such as ENCODE: (i) Evolutionary conservation of sequence and location relative to the gene whose expression is under enhancer control (ii) Binding sites for known transcription factors defined by chromatin immunoprecipitation (ChIP) and (iii) The presence of 'open' chromatin delineated by DNAaseI hypersensitivity. The principal hypomethylated loci included extragenic repetitive sequences such as LINE and LTR, but not SINE

repetitions. For example, hypermethylated enhancers were highly enriched for TFBSs of *TP63*, *TP53* and *NF1*, and hypomethylated DMRs for *FOXA1*, *p65-NFkB* and *GATA3*. A reassuring differential methylation was also seen for 2 well established 'epigenetically controlled' genes GSTP1 and CCDC8, although this was characteristic of a basal to luminal change rather than a cancer-specific hypermethylation, within 5kB of the published transcriptional start sites i.e aligned as before with promoters rather than enhancers (52).

It was clear from the data that, at the level of CpG methylation, there were relatively few cancer-specific methylation changes between the basal cell compartments of normal and malignant tissues (Figure 8A). However, in the luminal compartment, the differentially methylated enhancer sequences detected were enriched for those affecting the expression of genes from the PRC2 complex such as EZH2 and SUZ12, previously shown to be overexpressed in prostate cancer (53,54), and the appropriate gene ontology terms such as metabolic processes, epithelial development and most notably cell proliferation. This agrees with the major physiological change between normal and cancer luminal cells: normal luminal cells are terminally differentiated and rarely divide, whereas the luminal-like cells in prostate cancers are characterized by uncontrolled cell division.

Because the dataset included patient-matched normal:cancer samples, after further elimination of cell type-specific changes, we were able to assess cancer-specific changes in the different cell phenotypes on a patient-by-patient basis. However it should be noted that many of these were also present in a comparison of cancer luminal and cancer basal cells (differentiation) – the cancer significance was emphasised by the absence of such changes in a normal luminal-normal basal comparison. Since luminal cells were the principal constituent of the cancer epithelial populations, we next sought to identify cancer-specific methylation sites in comparisons of normal and malignant luminal cells. The elimination of the approximately 50% basal cell content from the 'normal' samples ensured that we did not rediscover any new upregulated AR stimulated luminal markers in these comparisons.

Since our original discovery sample number was low (Pellacani et al, 2018 in press), we sought to apply the cancer luminal signature to a larger number of cancers from The Cancer Genome Atlas (TCGA) database which contains 50 PCa samples with matched normal counterparts, 452 additional PCa samples without normal counterparts, and 1 metastatic PCa sample. Unfortunately the TCGA data was generated with older array technology, so the sequence data was converted to 100bp bins, to align the two data

sources. In the TCGA database, 255 array probes overlapped the 1472 DMRs we showed by sequencing to be differentially methylated in the cancer luminal to normal luminal comparison. When used to analyse the matched pairs in TCGA, the differentially methylated regions distinguished 50 cancer from matched patient normal tissues with close to 100% efficiency (TPR = 0.92, TNR = 0.92, Chi-squared test p-value = 2.4×10^{-16}) as shown in Figure 8B (Taken from Pellacani et al, 2018, in press).

Using this reasoning, since we had eliminated normal elements, the same analysis was carried out with all 553 cancer samples in the TCGA dataset, resulting in a similar outcome, with one cluster highly enriched in normal samples (Chi-squared test p-value = 1.7×10^{-39}). Intriguingly, this clustering also appeared to divide the PCa samples into two main groups, according to their CpG methylation differences from the normal samples. Exclusive analysis of the cancer samples confirmed this clustering pattern and showed that one cluster was significantly enriched for samples with extra-prostatic extensions (pT3 or pT4 in the TNM classification, with a Chi-squared test p-value < 0.005) in the absence of any significant differences in Gleason score (Chi-squared test p-value >0.1) (Figure 8C).

The requirement to use an extensive panel of differentially methylated regions (DMRs) to achieve classification, is probably an indicator of the multifactorial and diverse mechanisms required to achieve cancer cell malignancy. However, using as few as 17 DMRs we were able to differentiate cancer from normal luminal cell in data from mixed cell biopsies in TCGA with a 92% effectiveness (Figures 8D&E), indicating that further more selective analysis could result in an epigenetic differentiation of the elusive 'tiger' prostate cancers which require immediate aggressive treatment from the 'pussycats', which are best treated by continuous monitoring rather than invasive oncological procedures. Since this was achieved in routine biopsies of intra-prostatic early stage disease, implies that the fate of a prostate cancer is programmed into its genome, not by mutation but by epigenetic means at a relatively early stage, and that 'grade progression' to a more malignant state, may exist (55), but is probably relatively rare (56).

Is Epigenetic change the smart reaction to changing microenvironments in Prostate Cancers?

Our primary aims in this research were to dissect potential epigenetic mechanisms of gene expression control, and to distinguish the changes related to cellular differentiation, from those with a mechanistic importance in carcinogenic change. In contrast to most other studies, our target materials were cells taken directly from prostate cancer patients, employing the ultimate control: cells of the same lineages from normal regions of the same patients' prostates. We had previously shown a distinct

difference in the histone modification profiles, related to chromatin condensation in different cell populations, which could mark more undifferentiated cells. However, these chromatin marks were largely independent of the pathology in the prostate. On a primary screen, the same was largely true for global changes in miRNA expression: which were first identified as differentiation-linked epigenetic controls (57). However, as shown above, a number of cancer-specific changes were related to DNA damage and repair by extracting actual gene expression levels, and matching these to the inverse of changes in miRNA in the same cell populations. Both the ability of mi534 (overexpressed in the prostate SC population) to preserve a stem-like state which was resistant to radiation treatment and the radio-protective activity of low levels of miR99a/100 via the activation of SMARCs provide e a rationale for the survival of a stem-like population after patient radiotherapy. The deleterious effects of glucocorticoid administration, which is clinically acceptable, may have to be reconsidered based on their ability to similarly activate SMARCs.

The overall higher levels of miRNA expression and the existence of 'poised' or bivalent chromatin in the stem-like cells, argues for an epithelial population within both normal and cancer populations which is capable of rapid reaction to a change in microenvironment, after cancer treatment for example. The emergence of a stem-like miRNA signature in the CD133+/a2b1 high/CD44+ population, based on homology with not only human embryonic stem cells but also and surprisingly CRPC tissues, indicates that advanced prostate cancers retain this flexibility, which can only be enhanced by the increased frequency of mutations in DNA damage repair genes in CRPC. The epigenetic ability to rapidly switch phenotype to a treatment resistant cell type, which permits establishment of permanent resistance after subsequent mutation and selection, is entirely consistent with the development of drug resistance in advanced prostate cancers. Since more cells contain the stem-like miRNA pattern in the most advanced cancers, then the possibilities for resistant development is increased as the tumour successively escapes from treatments. This would be consistent with a trans-differentiation model in CRPC drug resistance, whereas the lower content of stem-like cells in treatment naïve tumours would argue for a stem/progenitor origin of the resistant population.

Conclusion

Prostate cancers are multicellular and complex in composition. In scientific attempts to simplify analysis, we have adopted a reductionist approach, using established and well

characterized cell lines. It is clear that with current comprehensive gene expression analyses, these basic cell line tools, established for more than 40 years in the laboratory, no longer represent the detail in the actual tumours. The more we push their relevance to drug response and tumorigenesis, the more apparent this becomes i.e. apart from well characterized hormone responses, translation from lab to bedside has been only moderately successful, and has not extended lifespans more than a few months in most cases.

In fact, the mere act of primary culture induces epigenetic changes which alter the expression of key genes, even in benign prostate tissues, that show much less inter-patient variability compared to cancers (37). Recent attempts to classify prostate cancers according to clinical outcome, on the basis of both genomic and transcriptome changes have disappointed, even when biopsies of tumor tissues were available. For example, in the most comprehensive TCGA survey (22) between a quarter and a third of all cancers were 'unclassified' even after multiparametric analysis.

The data we have presented in this article argues that there is an inherent 'patient specific' pattern of variation, shown here at the epigenetic CpG methylation level. On top of this there is the cellular composition variation between tumors, not only at the level of epithelial cell types, but also the degree of stromal cell, and immune cell content between patients. This cannot be truly represented, even in mouse models of the disease, unless we can break heterogeneity, by sub-fractionating fresh tumors into their component parts. Since most TCGA data has been generated from mixed cell populations, there is now a strong argument in favor of adopting a single cell (or small homogeneous cell number) approach to all new tumor cell analyses, in order to make genomics truly relevant for clinical application.

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Figure and Table legends

Figure 1 Multicellular nature of normal and Malignant human prostate tissues

A: Multicellular nature of a normal prostate acinus. B: Complex multicellular microenvironment of a malignant prostate gland

Figure 2: Prostate cancers contain fewer biologically active mutations than many other common human tumors

Figure 3: Origins of tumor relapses in prostate cancers

Models are based on transdifferentiation (A) or regeneration from a common cancer stem/progenitor cell (B).

Figure 4: Similar relapse rates for prostate cancers after immediate and deferred androgen blockade

(A) Shows a selection model where mutations in the AR are induced or highly selected compared to a re-differentiation model from a stem-like progenitor. Original trial data showing identical relapse rates (B) from Studer et al, 2006.

Figure 5: Four non-overlapping sets of genes are co-expressed during differentiation of prostate epithelial cells

Data from Rane et al Stem Cell Reports 2014

Figure 6: Patterns of miRNA expression in fractionated cells from multiple prostate biopsies

Note the overall high levels of miRNA in the SC compartments where the expression profile is most closely related to that found in human embryonic stem cells and total CRPC miRNA patterns of expression. In keeping with the differentiation-linked functions of miRNAs a principal component analysis was only able to align miRNA content with epithelial cell type, and not prostate pathology (normal, benign or malignant)

Figure 7: Fractionation strategy for primary human prostate tissues

Multiple biopsy strategy and fractionation from radical prostatectomy tissues (A) Gene expression changes (B) induced in benign prostate epithelial cell cultures, relative to tissues – data from Rane et al, 2016 (C) Gene expression differences between cell lines and primary cell cultures of prostate epithelial cells (unpublished data from Leanne Archer).

Figure 8: Epigenetic segregation of normal and malignant tissues after cell fractionation to establish homogeneous patterns of genomic methylation

Gene ontology differences derived from CpG methylation measurements of fractionated cells from a single patient (A). Values for each transition refer to the number of DMRs which vary between each cell population. Clustering analysis of differentially methylated sequences using data from TCGA can distinguish cancer from normal cells with high efficiency (B). Application of the DMR data can also segregate malignant from organ-confined prostate cancers (TCGA data) independently of the gleason grade of the original cancer (C) and (D/E). A 17 locus signature can be generated to distinguish normal from cancer tissues based solely on the pattern of DMRs.

Data taken from Davide Pellacani et al, British Journal of Cancer, 2018 (in press)

Table 1: Essential properties of Human Prostate Cancers

Table 2: Selection and phenotype monitoring of cell fractions from fresh human prostate tissues

Figure 1

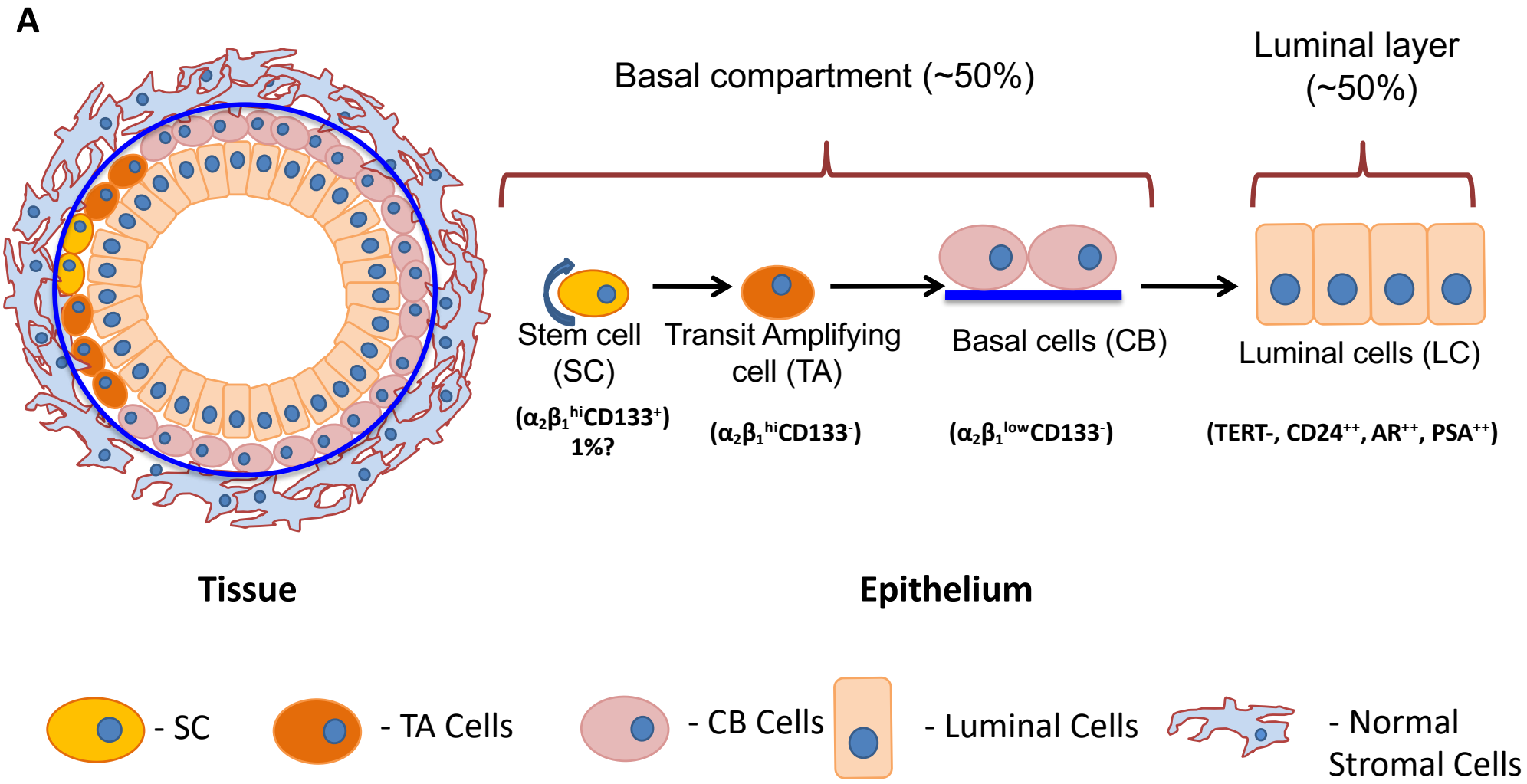

A
Basal compartment (~50%)
Luminal layer (~50%)
Stem cell (SC)
Transit Amplifying cell (TA)
Basal cells (CB)
Luminal cells (LC)
(α2β1hiCD133+) 1%?
(α2β1hiCD133-)
(α2β1lowCD133-)
(TERT-, CD24++, AR++, PSA++)
Tissue
Epithelium
- SC
- TA Cells
- CB Cells
- Luminal Cells
- Normal Stromal Cells

Figure 1

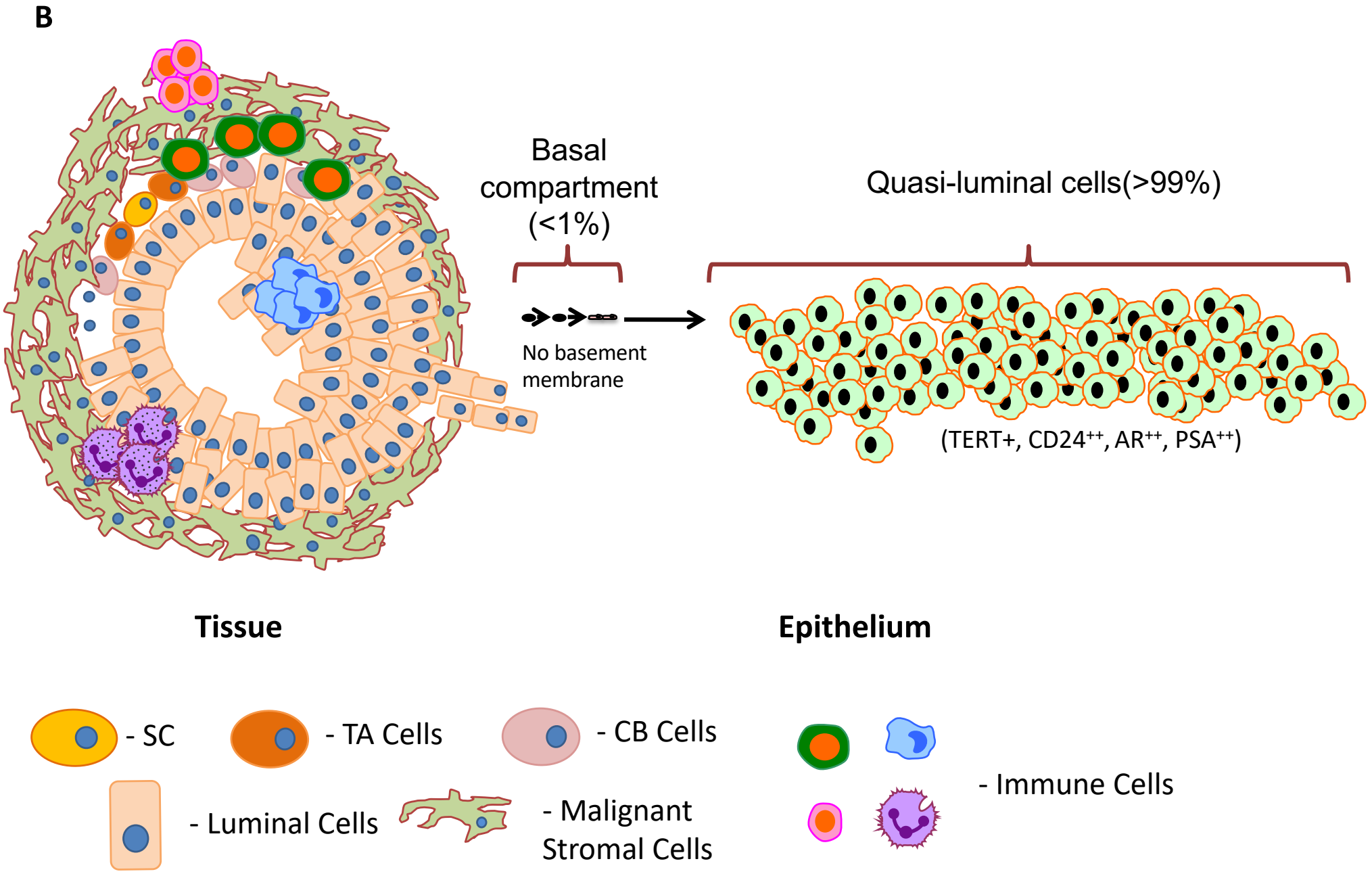

B
Basal compartment (<1%)
Quasi-luminal cells(>99%)
No basement membrane
(TERT+, CD24++, AR++, PSA++)
Tissue
Epithelium
- SC
- TA Cells
- CB Cells
- Luminal Cells
- Malignant Stromal Cells
- Immune Cells

Figure 2

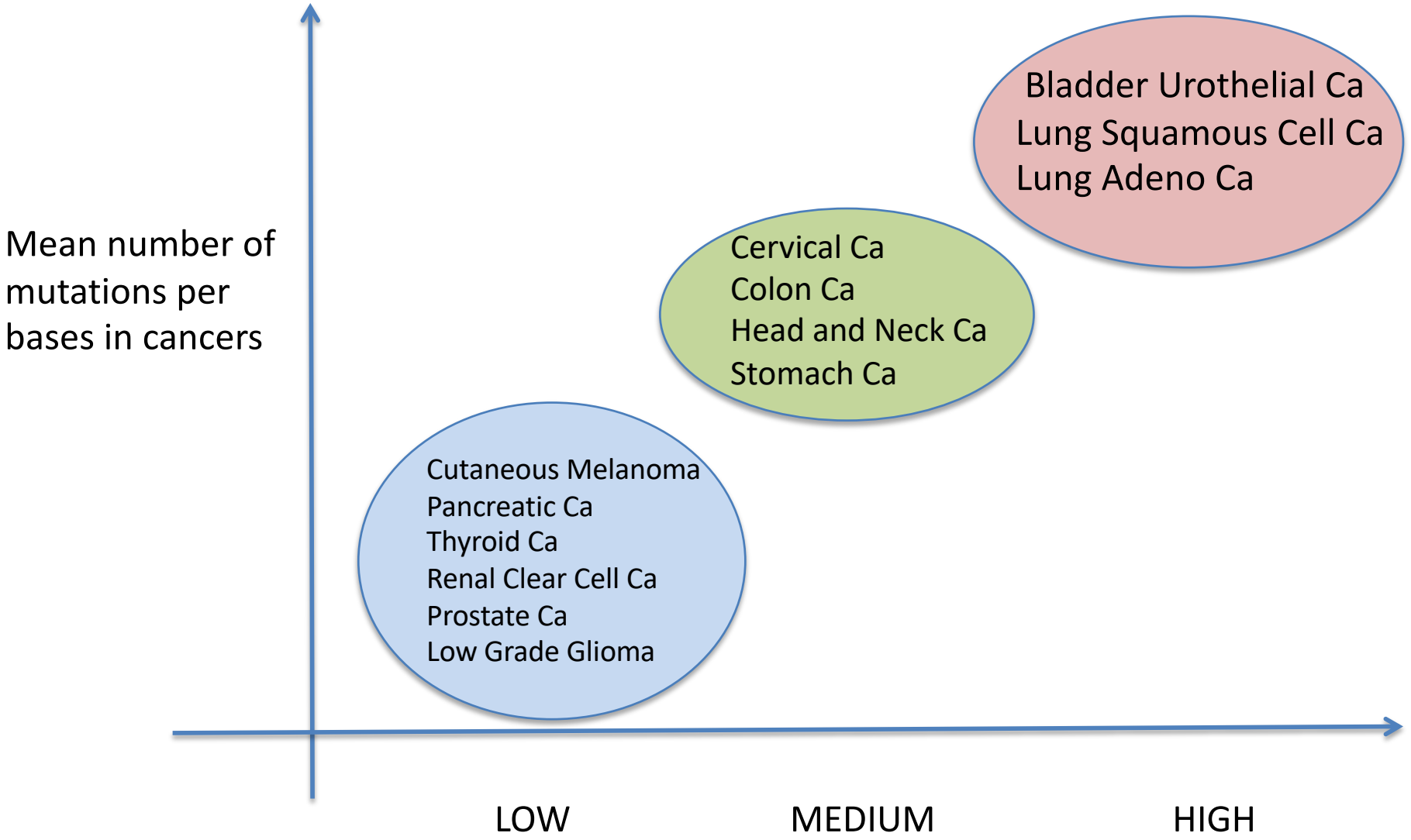
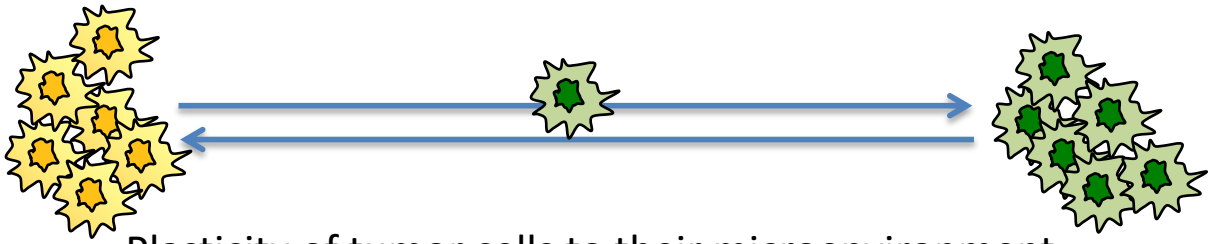

Mean number of mutations per bases in cancers
Bladder Urothelial Ca
Lung Squamous Cell Ca
Lung Adeno Ca
Cervical Ca
Colon Ca
Head and Neck Ca
Stomach Ca
Cutaneous Melanoma
Pancreatic Ca
Thyroid Ca
Renal Clear Cell Ca
Prostate Ca
Low Grade Glioma
LOW
MEDIUM
HIGH

Figure 3

A

Plasticity Model



Plasticity of tumor cells to their microenvironment
(Location and presence of selective drug)
Development of further drug-induced and selected mutations

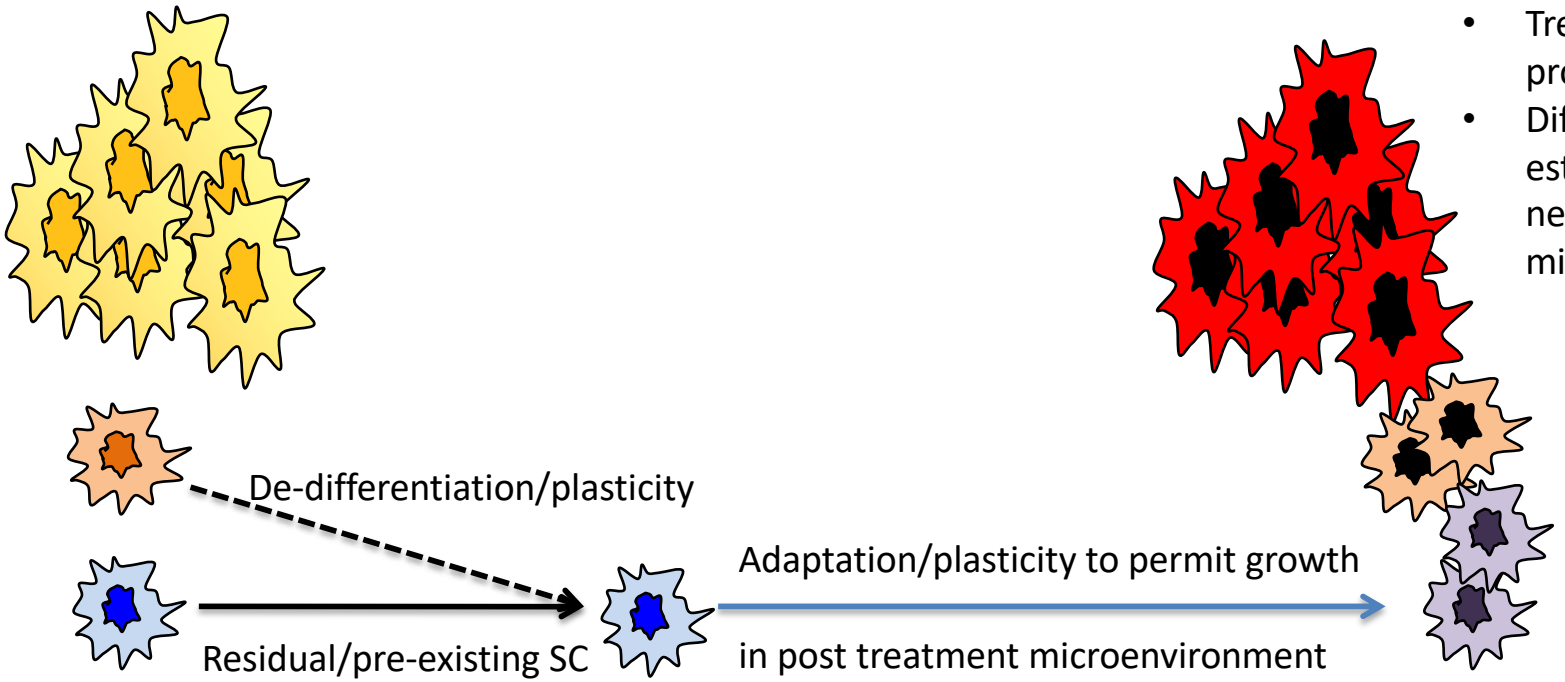
Predictions:

- No pre-existing resistant cells
- Treatment induces or selects for resistant cells
- Mature cancer cell trans-differentiates into mature resistant cancer cells.

Figure 3

B

Hierarchical or Stem Cell Model



Predictions:

- Resistant Stem-like cells
- Treatment results in progressive changes to SC
- Differentiation hierarchy is re-established (dependent in new post-treatment microenvironment)

Figure 4

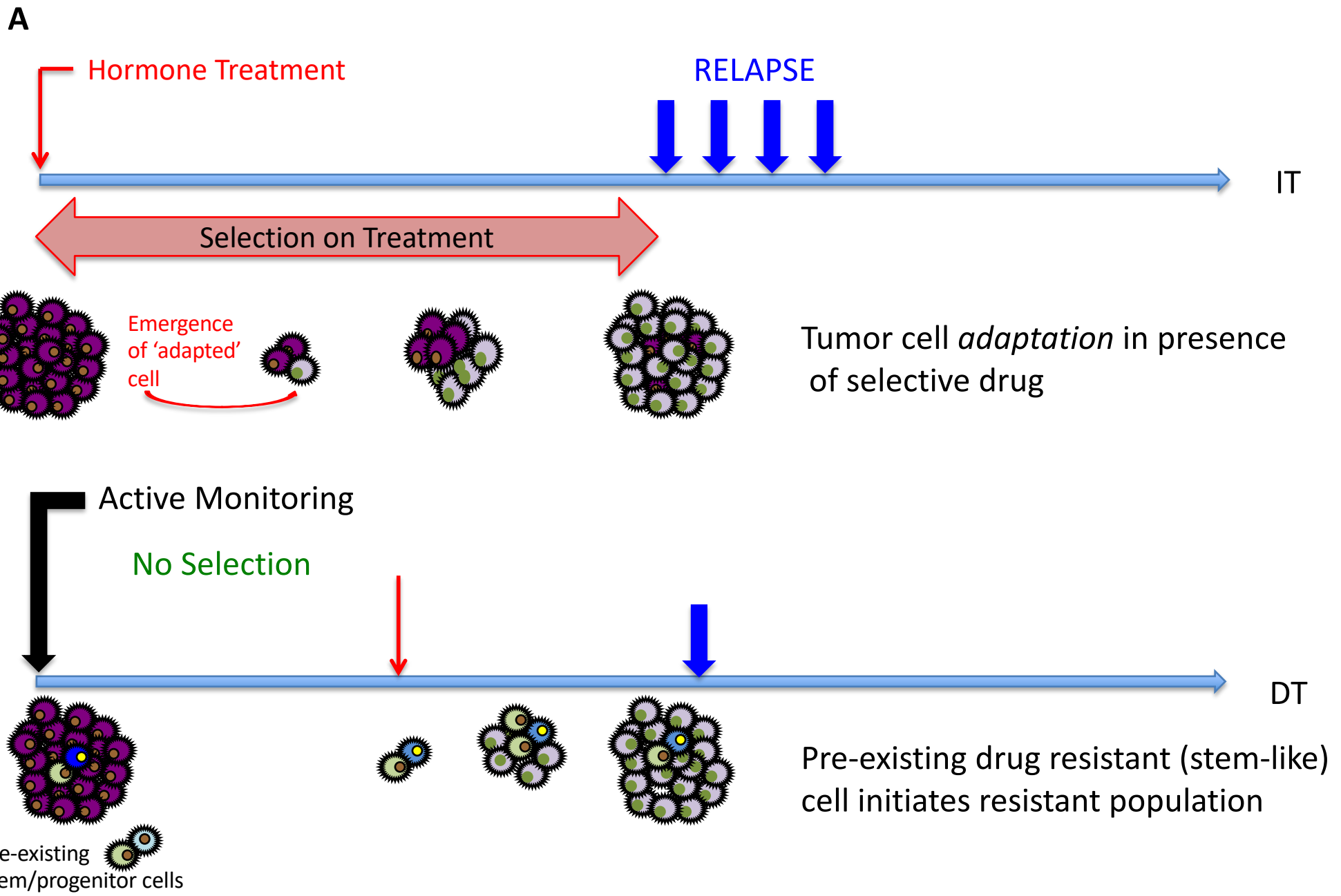
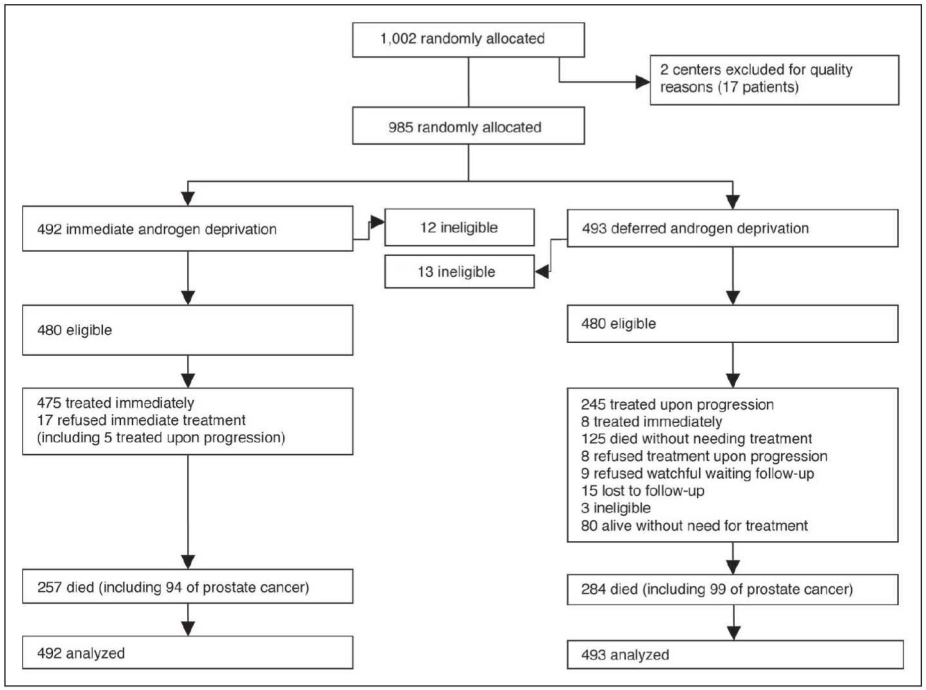
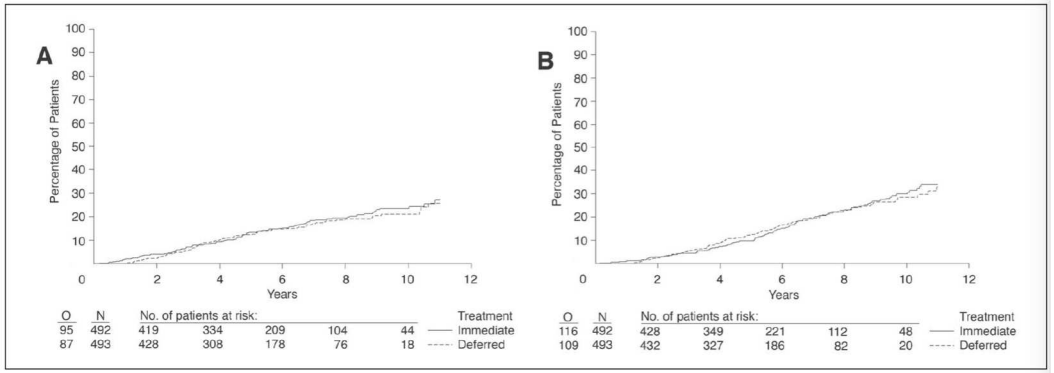

A
Hormone Treatment
RELAPSE
IT
Selection on Treatment
Emergence
of 'adapted'
cell
Tumor cell *adaptation* in presence
of selective drug
Active Monitoring
No Selection
DT
Pre-existing drug resistant (stem-like)
cell initiates resistant population
Pre-existing
Stem/progenitor cells

Figure 4

B





Cumulative incidence of and time from study entry (A) to symptomatic progression of hormone refractory disease after immediate or deferred androgen deprivation and (B) to objective progression of hormone refractory disease after immediate or deferred androgen deprivation.

Figure 5

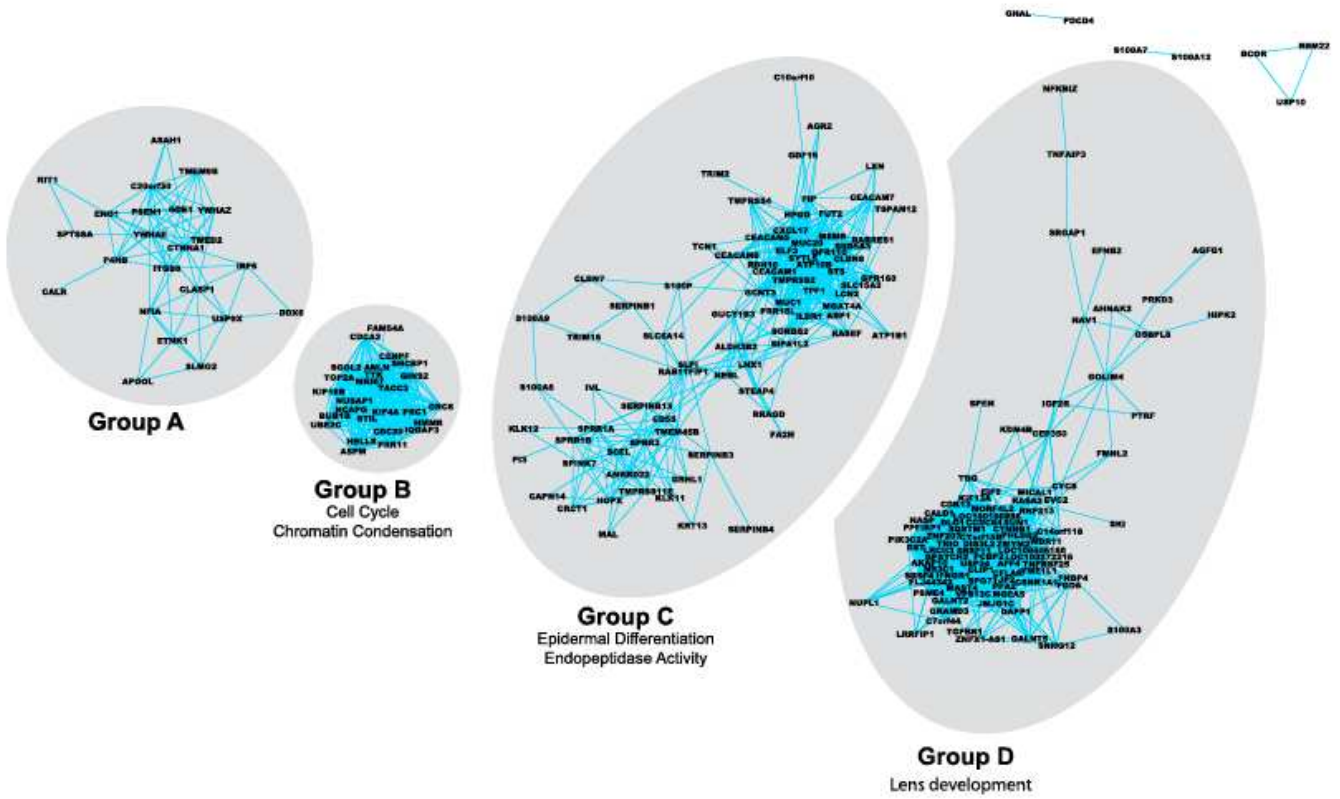

Group A
Group B
Cell Cycle
Chromatin Condensation
Group C
Epidermal Differentiation
Endopeptidase Activity
Group D
Lens development

Figure 6

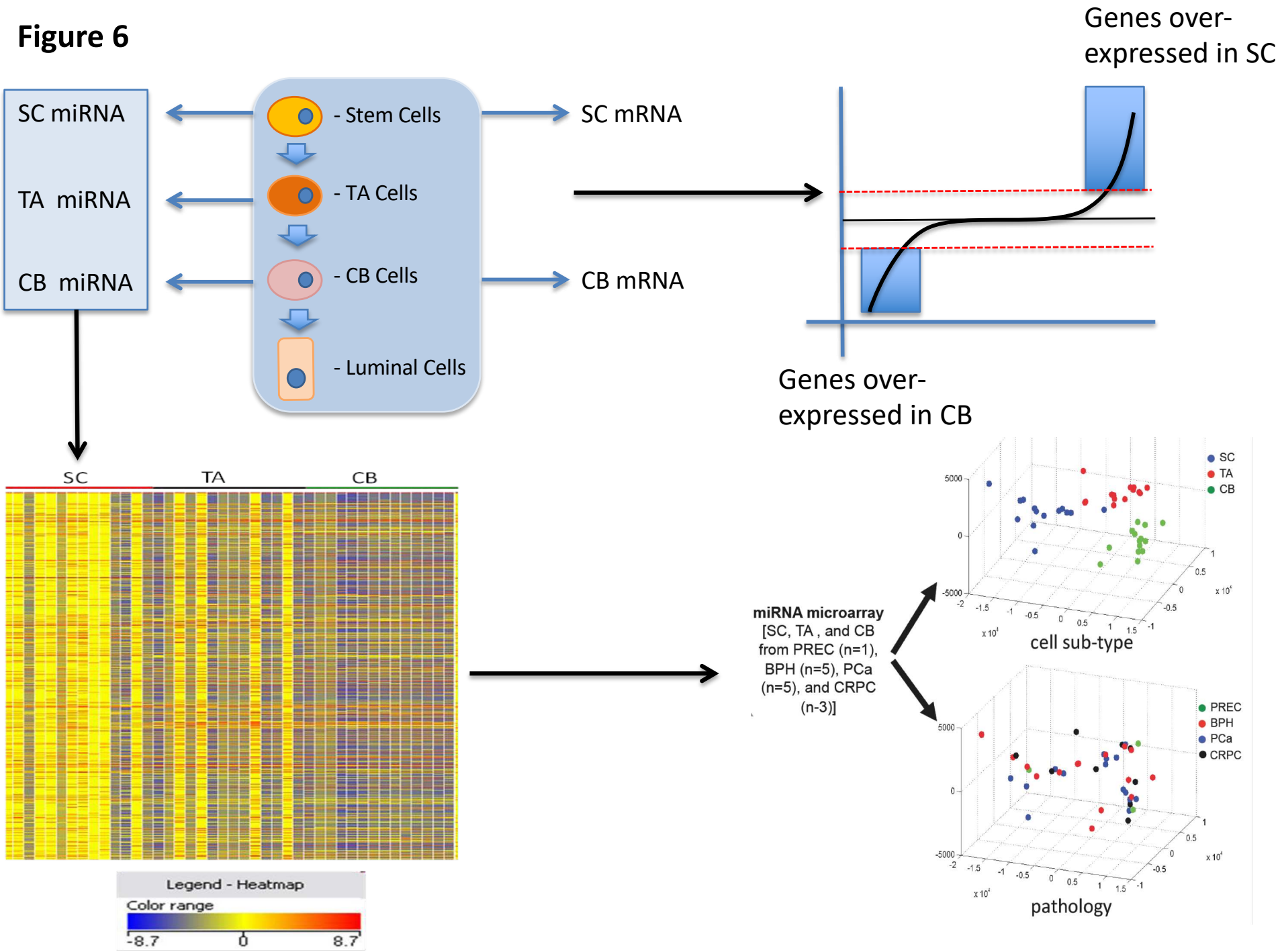

SC miRNA
TA miRNA
CB miRNA
- Stem Cells
- TA Cells
- CB Cells
- Luminal Cells
SC mRNA
CB mRNA
Genes over-expressed in SC
Genes over-expressed in CB
SC
TA
CB
Legend - Heatmap
Color range
-8.7
0
8.7
miRNA microarray [SC, TA , and CB from PREC (n=1), BPH (n=5), PCa (n=5), and CRPC (n-3)]
SC
TA
CB
cell sub-type
PREC
BPH
PCa
CRPC
pathology

Figure 7

A

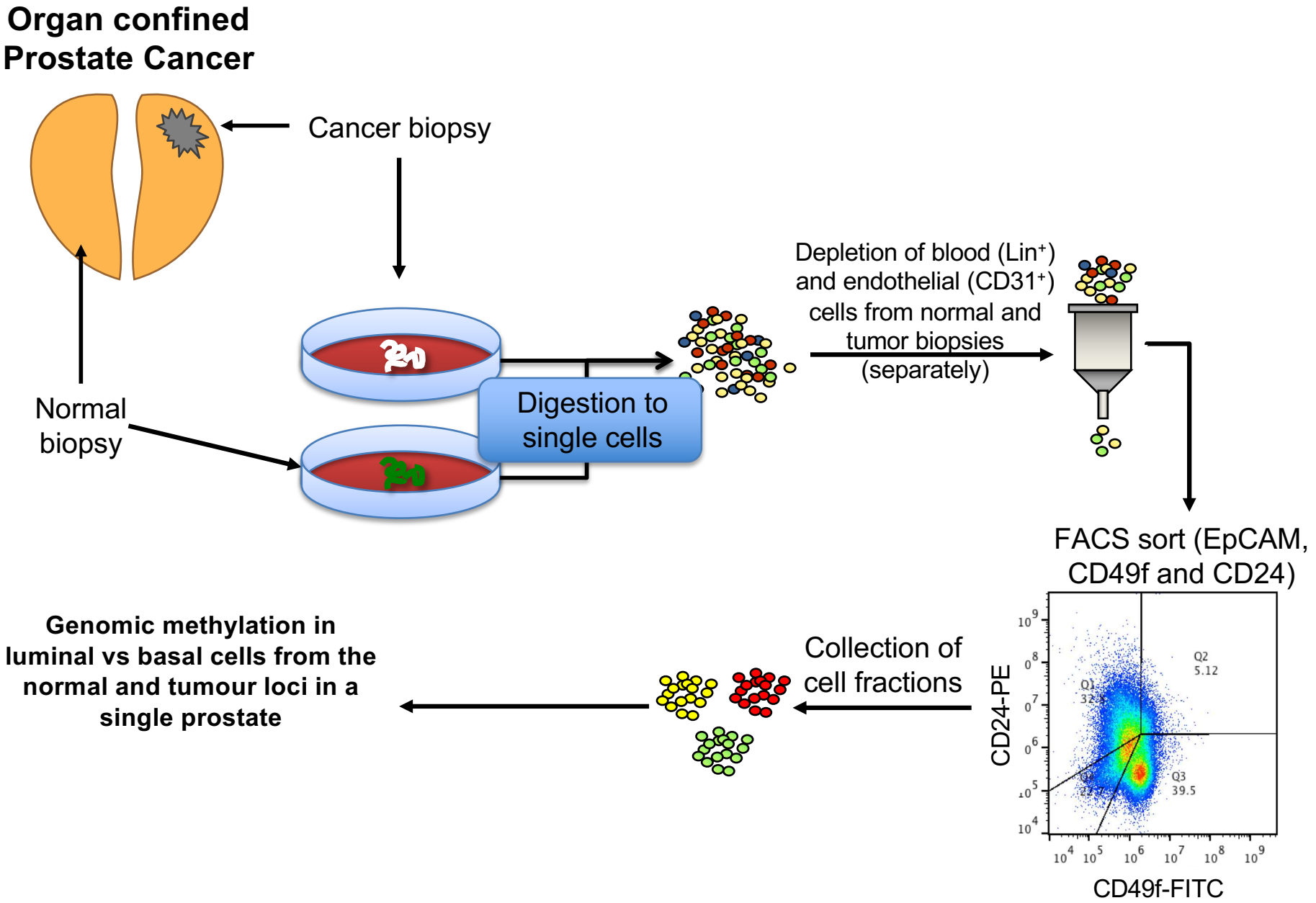
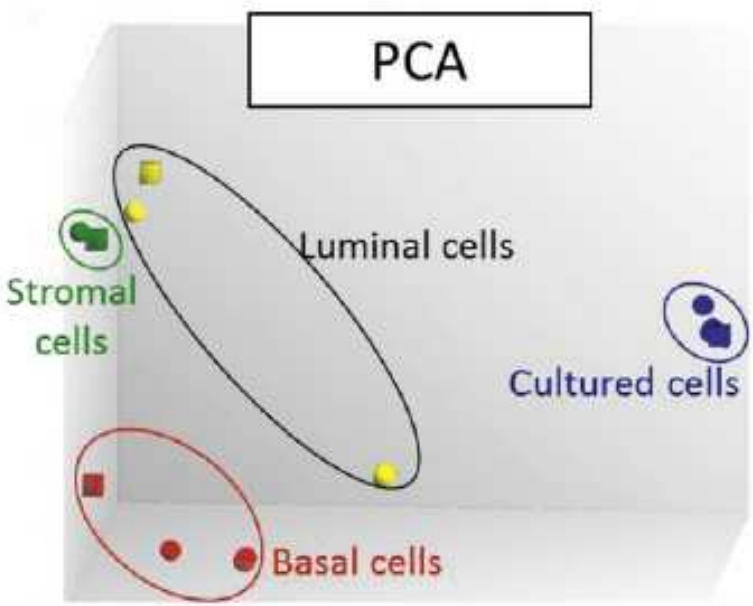

Organ confined
Prostate Cancer
Cancer biopsy
Normal
biopsy
Digestion to
single cells
Depletion of blood (Lin⁺)
and endothelial (CD31⁺)
cells from normal and
tumor biopsies
(separately)
FACS sort (EpCAM,
CD49f and CD24)
CD24-PE
CD49f-FITC
Q1
32.8
Q2
5.12
Q3
39.5
Collection of
cell fractions
Genomic methylation in
luminal vs basal cells from the
normal and tumour loci in a
single prostate

Figure 7

B


PCA
Luminal cells
Stromal cells
Cultured cells
Basal cells

C

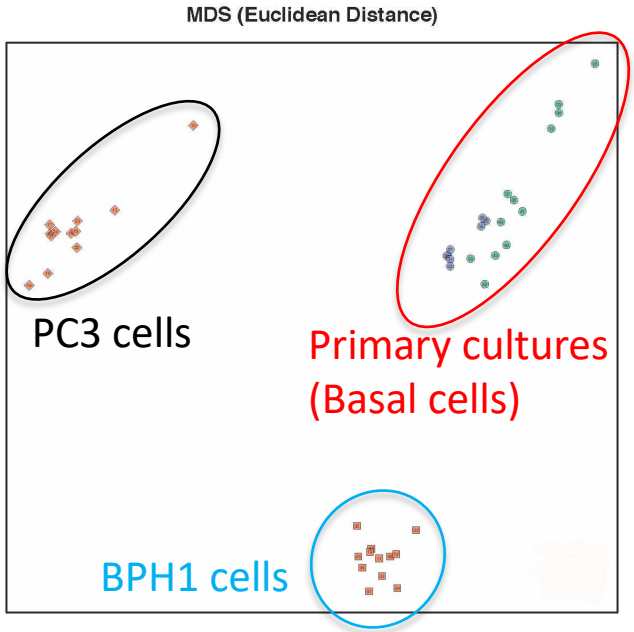

MDS (Euclidean Distance)
PC3 cells
Primary cultures (Basal cells)
BPH1 cells

Figure 8

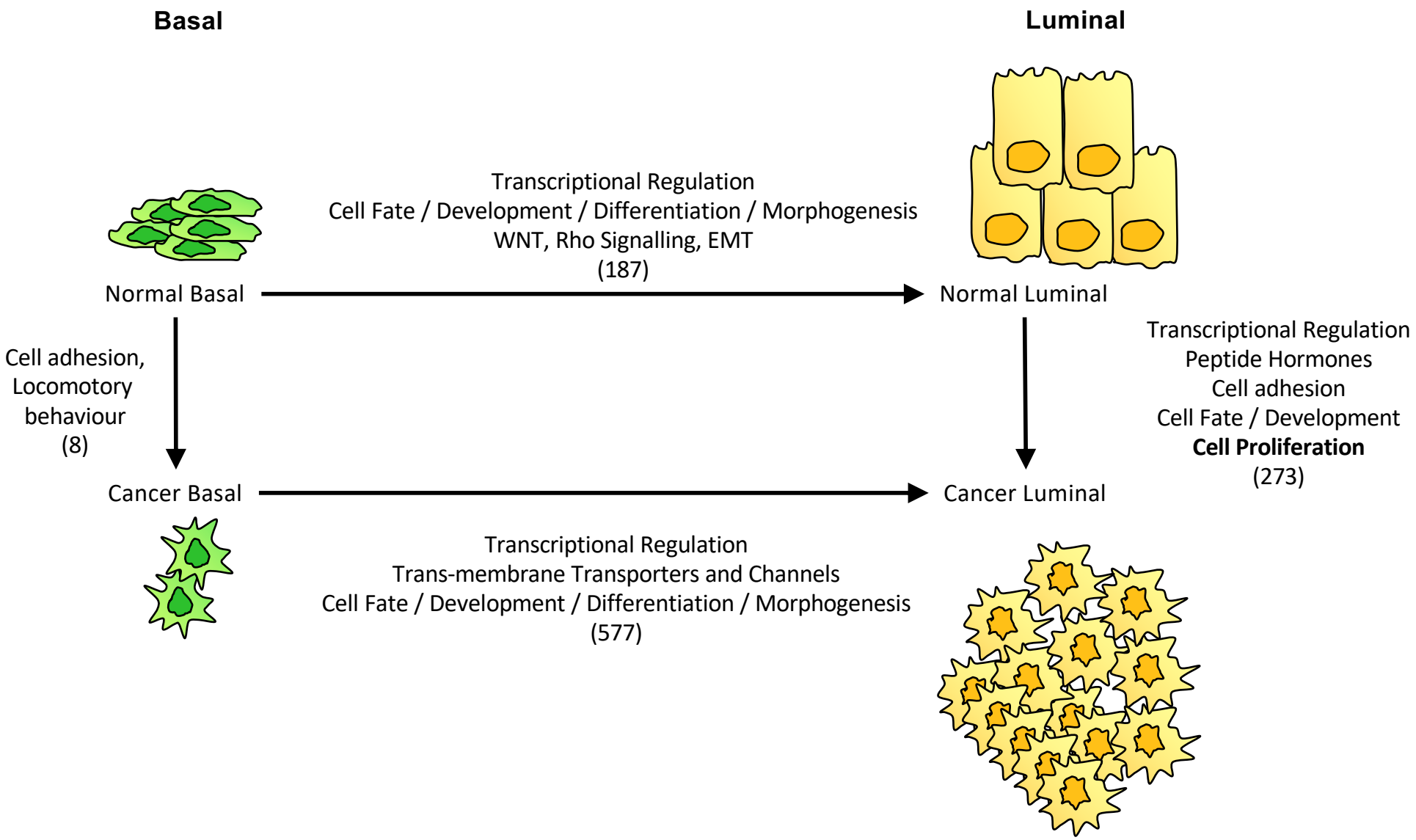
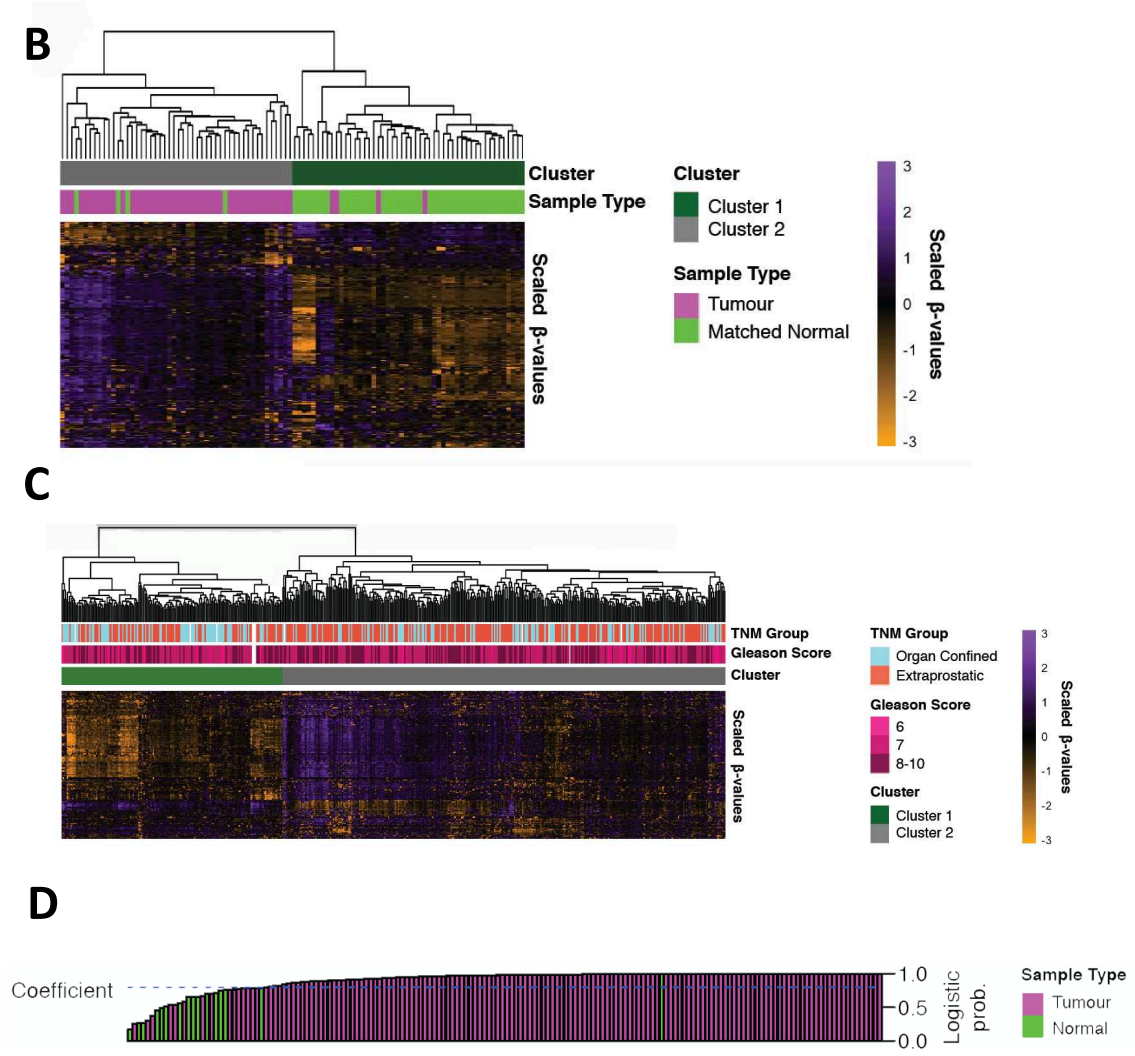

A
Basal
Luminal
Transcriptional Regulation
Cell Fate / Development / Differentiation / Morphogenesis
WNT, Rho Signalling, EMT
(187)
Normal Basal
Normal Luminal
Cell adhesion,
Locomotory
behaviour
(8)
Transcriptional Regulation
Peptide Hormones
Cell adhesion
Cell Fate / Development
Cell Proliferation
(273)
Cancer Basal
Cancer Luminal
Transcriptional Regulation
Trans-membrane Transporters and Channels
Cell Fate / Development / Differentiation / Morphogenesis
(577)

Figure 8


B
Cluster
Sample Type
Scaled β-values
Cluster
Cluster 1
Cluster 2
Sample Type
Tumour
Matched Normal
3
2
1
0
-1
-2
-3
Scaled β-values
C
TNM Group
Gleason Score
Cluster
Scaled β-values
TNM Group
Organ Confined
Extraprostatic
Gleason Score
6
7
8-10
Cluster
Cluster 1
Cluster 2
D
Coefficient
1.0
0.5
0.0
Logistic prob.
Sample Type
Tumour
Normal
E
AUC = 0.92
prob. cutoff = 0.8
True positive rate
False positive rate
0.0
0.2
0.4
0.6
0.8
1.0