Identification of a Cancer Stem Cell Enriched Side Population Using Hoechst 33342 Based Isolation

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Cancer Stem Cell Theory

Differentiated cells are generally short lived and derive from a small pool of long lived stem cells (SC) that are present through out the lifetime of the organism. They are required for tissue development, replacement and repair. Their longevity, due partially to their quiescent nature, makes them susceptible to the accumulation of genetic mutations and this phenomenon is partly responsible for their implication in the initiation and propagation of cancer. The stochastic model of carcinogenesis implies that any cell, including those which are terminally differentiated, can potentially be tumourigenic and proliferate in an uncontrolled way to produce a tumour with a homogenous phenotype (1, 2). However, most solid tumours are heterogeneous, suggesting that they either arise from multiple tumour initiating cells or that they arise from a single cell with the potential to give rise to multiple different cell types. The cancer stem cell (CSC) hypothesis states that within a given tumour there exists a small population of cells with the capacity to behave like stem cells i.e. to exhibit the properties of self renewal and to demonstrate the ability of the cellular progeny produced by daughter cells to differentiate and produce a tumour with a heterogeneous phenotype.

The SC attributes of self renewal combined with their intrinsic potential for growth and survival give them a particular relevance in the field of oncology and oncogenesis (2, 3). This may be of cardinal importance in one of the areas which defines malignancy: the ability of a cancer cell to migrate to a distant secondary site where it survives, implants, propagates and grows as a metastasis. Bonnet and Dick are credited with the first description of a CSC, publishing their discovery of a $CD34^{+ve}$ / $CD38^{-ve}$ tumourigenic cell in acute myeloid leukaemia. The phenotype resulted in leukaemic blast formation when engrafted in immune-deficient mice and self renewal was demonstrated by secondary xeno-transplantation of the AML cell (4). Rare populations of carcinogenic cells have now also been identified in solid malignancies of the breast (5)l and brain (6) and there is accumulating evidence to suggest CSC existence in tumours of the kidney (7, 8), liver (9), colon (10, 11), pancreas (12), prostate (13, 14) and bladder (15).

It is possible that CSCs arise from the direct mutation of normal SCs. It is well known that several genetic mutations are required in an individual cancer cell type to initiate and promote invasive cancer behaviour

and it is therefore reasonable to propose that this biological event would occur at the most primitive cellular level, allowing the necessary time for the evolution of the malignant phenotype to occur. Such a process may not be possible during the relatively short life span of the terminally-differentiated cell population. However, this proposition is far from being a proven certainty. There is also evidence suggesting that the Transient Amplifying cell Population (TAP) may gain mutations which enable expansion and survival of a migratory cell population, which, despite having only limited self-renewal capabilities, may contribute significantly to generating malignant cells in large numbers by virtue of their enhanced replicative potential (16, 17). A third hypothesis is that TAP cells undergo a process of de-differentiation, whereby they regain properties of self-renewal, a feature which is combined with an augmented capacity for cellular amplification and expansion of numerical cell mass (18, 19).

Ordinary stem and cancer stem cell populations have a greater proliferative, or potentially tumourigenic capability than the ordinary differentiated "marker negative" cells. However, the strict definition of exactly what constitutes a CSC is still a matter of uncertainty. One specific definition is that it is a cell which is able to reconstitute identical cancerous tumours indefinitely by in vivo transplantation of xenografts in serial individuals (20). If this strict definition is applied then no there has never been a true demonstration of the CSC in solid organs as there is no published evidence that this feat has ever been achieved and currently it would be extremely difficult to do so given the limitations of the techniques currently available. There are a number of reasons why this methodology is so challenging, not least because of the requirement for cell-cell cross talk and critical and site dependent nature of host stromal interaction. If a single cell phenotype was isolated and injected into the orthotopic organ site of a recipient host, its survival would depend significantly on the individual host's cells ability to support and maintain the various critical stages of tumourigenesis. A number of factors play in to this, notably the epithelial and stromal compatibility at the host site and in live hosts, the immune response to the injection of foreign tissue. These factors raise doubts as to whether the resultant tumour would have features which were identical to those of the parent population of cells and whether they were truly representative of the primary tissue / tumour.

Tang *et al* proposed that some form of compromise is required in defining the SC and CSC (20). This group suggested that SCs and CSCs should be purified prospectively and that "irrelevant" cells should be removed by lineage selection. Theoretically this would prevent the possibility of plasticity / trans-differentiation and it would prevent the inaccurate isolation of so called "SCs". This, for example, would require removing both cells of blood and fibroblast lineage and their HSC and mesenchymal SCs respectively. The putative SCs would then need to demonstrate their established and fundamental intrinsic SC properties. *In vivo* tumourigenicity would then ultimately be required to ensure the populations were enriched for SC and CSC characteristics (20). However, this notion raises the question of the interdependence of the CSC on its host environment and begs the question of whether a CSC arising from a specific organ has the ability to survive and propagate in the long term when it is isolated from intercommunication by other epithelial / stromal cells and their milieu.

Problems Identifying CSC

Adult SCs are rare. It is estimated that only 1 in 10,000 to 15,000 cells in human bone marrow are of haemopoietic SC (HSC) lineage (21) and they may be even more scarce in solid tissues. Therefore, proving the existence of a tissue-specific SC can be a long and difficult process. The candidate SCs are required to demonstrate their ability to self-renew and subsequently to differentiate into cells that are phenotypically characteristic of that particular tissue. This replenishment process has successfully been demonstrated by the HSCs, which were able to fully reconstitute the entire haemopoietic system of mice that had previously been lethally irradiated (22, 23). However, recapitulating this process in solid tumours remains problematic.

Following the successes made in the isolation of SC's in haematological malignancies there has been a great deal of research aimed at identifying and isolating CSCs from solid tumours. Adult stem cells have been described in many normal solid tissues including the brain, liver, muscle, breast, skin, gut, kidney and the genito-urinary tract and like stem cells in the haemopoietic system; they are rare by comparison with the number of amplifying and terminally differentiated cells of the organ. As with adult stem cells, the tumour initiating cell or cancer stem cell is thought to be rare relative to the number of non-stem cancer cells constituting an individual tumour. Notwithstanding this fact, CSC's are thought to be present in

significant numbers in a tumour and yet it has not been possible in any of the published experiments to date to take a single CSC and induce tumour formation and propagation in isolation. Many researchers have utilised phenotypic markers thought to be associated with a stem cell-like phenotype and there have been successes in isolating sub-populations of cells. However, the evidence shows clearly that whilst these populations are enriched for tumour initiating cells, they don't contain pure populations of CSC's. Examples of this approach include the use of the markers $CD34^{+ve}$ and $CD38^{-ve}$ in Acute Myeloid Leukaemia, CD133 positivity in colon, brain and prostate cancers and $CD44^{high}$, $CD24^{low}/Lin^{-ve}$ in breast cancer.

Functional Isolation Using Hoechst 33342

The SP was first described in the bone marrow and muscle of various species, including rodents and humans (24, 25). It defines a population of cells that are able to actively pump the cytotoxic dye Hoechst 33342 out of the cell. Hoechst 33342 is a dye which binds to the AT rich regions of the minor groove in DNA and its fluorescent intensity is dependent on the DNA content, chromatin structure and the position of the cell within the cell cycle. Although the uptake of Hoechst 33342 and other lipophilic dyes is universal, the ability to efflux the dye actively appears to be restricted to a subset of cells. These can be identified and isolated on the basis that they don't take up the dye and the 33342 validation process can be taken a step further because this efflux process can be blocked. The mechanism depends on the activation of the ABCG2 transporter system and this can be inhibited by the use of specific isoforms of the calcium channel blocker, Verapamil hydrochloride.

Cells with the ability to efflux lipophilic dyes can therefore be characterised and sorted by their fluorescent profile using FACS based methods in to Hoechst high and Hoechst low. The FACs profile of the cells which actively efflux Hoechst 33342 has a characteristic appearance and has been termed the ''side population'' or SP (Figure 1). This SP has been shown to have many of the functional properties of stem cells within their tissue of origin and furthermore, the cells contained therein have the capacity for trans-differentiation. Although the molecular basis for the assay remains uncertain, the efflux mechanism appears to be distinct, arising from ATP-binding cassette transporters ABCG1a/b (Mdr1a/b) and ABCG2 (Bcrp1)

(26). Investigation of these transporter cassettes in mice showed that they each play a role in defining the SP and interestingly their contribution to the SP varies from tissue to tissue. Jonker *et al* (27) looked at the effect of single (Bcrp1, Mdr1a or Mdr1b) or triple knockout mice (Bcrp1/Mdr1a/Mdr1b) on the SP profiles from Breast or bone marrow tissue. Unlike the bone marrow, in which only Bcrp1 gave rise to the SP, both Bcrp and Mdr1 contributed to the formation of an SP from breast epithelial cells. Although the exact dye efflux pathway for each tissue is currently unknown the Hoechst dye efflux assay has been applied successfully to cellular isolation of an SP from solid organ tissues such as prostate (13, 14), kidney (8), bladder (15) and breast (28), allowing the isolation of a stem cell enriched population, providing cells exhibiting the properties of proliferation, differentiation and self-renewal when grown in co-culture systems with mouse fibroblasts (29).

History of the Hoechst 33342 assay

The side population was originally seen during a study by Goodell *et al* (24) reported in 1996 following a study of HSC cell cycle distribution of bone marrow cells. Examining the fluorescent emissions at 2 wavelengths (675nm and 450nm) it was noted that a distinct population representing <0.1% of the total cell number, appeared on the FACS plot. These cells were characterised as small cells, they expressed the stem cell marker Sca1 and they were $lin^{neg/low}$. Functionally, the cells were enriched over 1000 fold for cells able to repopulate the bone marrow of lethally irradiated mice.

A functional purification of stem cells in haemopoietic research has used the active efflux of two cytotoxic dyes Rhodamine 123 and Hoechst 33342 as a measure of the stem cell's capacity for self-preservation within a cytotoxic environment (24, 30). The cells which demonstrated the capacity to decrease the intra-nuclear concentration of the dyes displayed the capacity to form colonies with long-term survival in secondary recipients, further demonstrating the combined stem cell qualities of proliferation, differentiation and self-renewal (31, 32).

Based on this methodology the Hoechst 33342 FACS based dye efflux pathway has been used to define a stem cell enriched population from human bone marrow. The bone marrow cells are sorted according to

their Hoechst 33342 status, which, when viewed on a dot plot of Hoechst Blue vs. Hoechst Red, are low in fluorescence signal at both wavelengths. The grouping of this population on the FACS plot gives the appearance of the cells arising from the side of the main body of the Hoechst 33342 retaining cell population. This characteristic appearance has given rise to the sub-population being known as the "side population" or SP. The efflux of Hoechst 33342 via ABCG2 is effectively blocked by adding the calcium channel blocking drug verapamil. This action eradicates the SP by maintaining the Hoechst 33342 intra-cellular concentration and shifting the constituent cells into the main Hoechst Red^{high} / $Blue^{high}$ cell body. The main Hoechst Red^{high} / $Blue^{high}$ cell population display the lineage marker of differentiated haemopoietic cells, Lin^{+ve}. By contrast, the SP, which represents 0.1% of whole bone marrow, displays the stem cell marker Sca-1 whilst being $CD34^{-ve}$ and Lin^{-ve}.

The cell cycle status of the SP is consistent with the stem cell theory in that it contains a high proportion of cells displaying a quiescent G_0 cell cycle status. Furthermore, the stem cell properties of differentiation, proliferation and self-renewal are demonstrated functionally by transplanting SP cells, which will reconstitute the bone marrow and peripheral blood of previously lethally irradiated mice. Repeating the transplantation using bone marrow SP cells from these bone marrow replenished mice into a further set of lethally irradiated murine recipients again gives further successful haemopoietic repopulation and radioprotection (24, 33). Additional analysis by this same group of researchers found that the SP was $CD34^{-ve}$, which differs from the previously defined HSC $CD34^{+ve}$ phenotype (25, 34).

Hoechst 33342 dye efflux FACS based assay has been applied to solid organ tissue including lung, liver, intestine(35), breast (36, 37), prostate (13, 14), kidney (8) and bladder (15). These studies have shown that a validated SP can be isolated from human epithelia and by so doing, have shown that it is not only bone marrow which contains cells with stem–like characteristics. The epithelial derived tissues also demonstrate an ABCG2 / Brcp1 Hoechst 33342 efflux pathway, with the SP FACS based derivation being negated by the use of verapamil (Figure 1). SP cells also display stem cell plasticity, as illustrated by a muscle derived SP being able to trans-differentiate along haemopoietic lineages (38).

The Hoechst 33342 dye efflux assay has been successfully translated to isolate verapamil sensitive populations from the genito-urinary tract. This was first demonstrated in prostatic tissue (13) but has since been shown to be a feature of normal and malignant Renal (8) and bladder epithelia (15). The prostate epithelial SP, which expressed the epithelial antigen BerEP4, displayed the postulated prostate stem cell adhesion molecule α2β1 integrin found in the basal cells of the prostate acinus, which is known to be involved closely in the development of metastatic behaviour *in vitro* (39-42) and the preferential binding of prostate cancer cells to human bone marrow endothelia (43). Purification and verification of the isolated cells as prostatic epithelial cells was confirmed by their negative expression of the haemopoietic lineage (lymphocyte) antigen CD45 (13, 44, 45).

Drug pumps

The cell's ability to efflux Hoechst 33342 arises as a consequence of its use of the p-glycoprotein related adenosine tri-phosphate (ATP) binding cassette (ABC) transporter superfamily, particularly the ABCG2 linked mechanism. This group of trans-membrane proteins are active in cellular metabolism and trans-membrane transport of organic anions, lipids and iron. HSC's also express p-glycoproteins, which are encoded by the multi-drug resistance gene MDR-1, with enhanced expression of the gene leading to proliferation of the stem cell population, myeloproliferative syndrome, leukoplasia and splenomegaly (46, 47).

Zhou *et al* examined the Hoechst 33342 and Rhodamine 123 dye efflux pathways. They examined whether the ABC transporters encoded by the MDR1 genes Mdr1a/b effluxed Hoechst 33342 and found that these gates actively removed rhodamine 123, but not by Hoechst 33342, a function which is blocked by the MDR-1 transport inhibitor reserpine (26). The murine pathway for efflux of Hoechst 33342 was found to be ABCG2, which is the human homologue of the breast cancer resistance protein gene BCRP1 and can be inhibited by specific isoforms of verapamil. This finding and the stem cell like behaviour of the SP has subsequently been confirmed by other groups (48-50). The role of this cellular mechanism and specifically its augmented expression in the putative stem population is currently unknown but it is interesting to hypothesise that the ABCG2 pathway may represent a form of primitive defence in stem cells by providing

the cell with the capacity to efflux potentially harmful substances, thereby giving the cell a survival advantage. This in turn may allow the host tissue to survive adverse conditions or stimuli and replenish differentiated tissue following an injury or metabolic insult (51-53). This notion is of particular significance in the field of clinical oncological practice and particularly from the point of view of chemo and even radio-resistance. It has been shown that various types of ATP-binding cassette (ABC) transporters, including those encoded by the multidrug-resistant (MDR) gene 1, the MDR protein (MRP), and the breast cancer-resistant protein 1 (BCRP1), contribute to drug resistance in many cancers by pumping the drugs out of the cell (54). These transporters are also expressed by many kinds of stem cell. BCRP1, for example, pumps out the Hoechst 33342, identifying an unlabeled side population (SP), which is enriched for stem cells (24, 26, 53, 55). It is a possibility that the commonly observed clinical response to the administration of cytotoxic chemotherapy, which is one characterised by an initial treatment response, followed thereafter by relapse, is consistent with the toxin damaging the susceptible differentiated and rapidly amplifying population of cells whilst having a restricted effect on the stem enriched population, leaving it viable and fully able to reconstitute the cell mass.

SP in Cancer

There has been considerable interest in the isolation of the putative “cancer stem” or “tumour initiating” cell (TIC). Characterisation of such a TIC would provide invaluable knowledge regarding carcinogenesis and the mechanisms underpinning its subsequent natural history. It would also provide critically important new information enabling novel biomarker development and the potential for initiating and testing new approaches to therapy directed at the root of the cancer. Whilst this remains a worthy aspiration for all researchers in this field, the “Holy Grail” of the single marker for CSCs in solid tumours remains elusive. In the absence of such a marker, alternatives are currently under study and prominent amongst these is the SP isolation method utilising the Hoechst 33342 dye efflux assay.

Malignant side populations were first isolated from the bone marrow of patients with acute myeloid leukaemia (AML). The SP was shown to be enriched for tumourigenic cells. SP’s have since been identified in various solid tumours, including neuroblastoma (56), melanoma (Grichnik J 2006) Glioma

(57), renal (8), prostate (13, 14), bladder (15), breast (28, 29), thyroid (58), Lung (59) Gut (60), Liver (61) and Ovarian (62) carcinoma and in mesenchymal (63) tumours. The proportion of cells within the SP varies from tumour to tumour, ranging between 0.5% in prostate cancer (14) to over 10% in mesenchymal neoplasms (63). It has also been suggested by Wu *et al* that the size of the SP isolated from tumours is proportional to the tumour aggressiveness.

Characterisation of the SP from various solid tumours has shown that the SP is enriched for cells with stem-like properties. Physically these are small and have little cytoplasm or endoplasmic reticulum. Cell cycle studies have shown that the SP contains a population of quiescent cells, which correlates with the increased number of cells within the SP expressing cell checkpoint markers such as p21 and p27 and a reduction of cells expressing the proliferative marker Ki-67. They are also enriched for cells expressing surface based stem cell markers. This is demonstrated in Figure 2 and table 1 where the phenotypic characteristics of SP and non-SP cells from Renal and Prostate cancer are illustrated. In both SP's there is enrichment for stem cell markers such as b-catenin, Notch, Musashi-1 and CD133 and for lineage specific markers commensurate with an undifferentiated phenotype.

Due to the lack of markers which are specific for adult stem cells and their associated malignancies, functional cellular characterisation is often the definitive approach most utilised for defining a stem cell. Stem cells, although normally quiescent, are able to undergo asymmetric division, resulting in self renewal, with the daughter progeny going on to undergo enhanced proliferation and differentiation whilst the "mother" stem cell returns to its niche. This asymmetric divisional process enables the basic stem cell to restore tissue after cell loss through injury or following apoptosis related regression e.g. as seen in the differentiated epithelial components of the prostate following androgen withdrawal. The "gold standard" for some researchers in demonstrating self renewal and proliferation is the serial passage of tumours in nude mice. Data from several independent groups using this approach have shown that by comparison with the non-SP or with whole tumour populations, SP cells isolated from tumours in the liver (61), lung (59), stomach (60) or nasopharynx (64) had a far greater ability to survive and grow than cells from the non-SP fraction. It has also been shown using mesenchymal neoplasms that whilst both the SP and the non-SP can

both generate primary tumours, only the SP cells are able to be passaged serially. This suggests that whilst the non-SP contains a transiently amplifying cell type which is able to initiate a tumour its life span is limited and it is not able to undergo serial passage *in vivo*. It has also been shown that the serial tumours from SP primary contain both an SP and non-SP when re-analysed with the Hoechst 33342 assay following serial passage (8). This phenomenon is not reported in non-SP cellular fractions from tumours.

Self renewal of SP cells has been confirmed by studies examining the ability of SP and non-SP cells in culture to maintain their SP phenotype. Kondo *et al* (57), and Hirschmann-Jax *et al* (56) have shown that only the SP can maintain and give rise to an SP and non-SP, a feature which was not observed in the non-SP. However there appear to be contradictory reports by both Benchaouir *et al* (65) and Addla *et al* (8) who have shown that both the SP and non-SP can generate an SP after culture. Interestingly in the case of renal tissue, Addla *et al* showed that only the SP fraction could give rise to a quiescent SP, whereas the SP generated from the non-SP were all in cycle, with a large proportion of cells in G_1. This suggests that in the renal tissue the only the SP can undergo self renewal and generate a quiescent stem like cell and that the SP generated from the non-SP are more likely to comprise a population of transiently amplifying cells (TAP) expressing a highly drug resistant phenotype. It is noteworthy that in clinical practice RCC is a highly chemo-resistant tumour. The findings of Addla *et al* (8) also fit the reported phenotypic data from their SP isolation experiments, which show that the SP is a heterogeneous population containing the most primitive cancer stem cells along with early transiently amplifying cells.

In vitro studies have also shown that the SP is enriched for cells with enhanced proliferative abilities which are able to undergo differentiation to produce multiple cell types. Using a Matrigel matrix to suspend the SP and non-SP cells within a 3D matrix both Brown *et al* and Addla *et al* showed that both SP and non-SP cells were able to proliferate and produce sphere like structures (figure3). Like the *in vivo* studies, not all the cells were able to induce colony formation, with the SP containing a greater number of sphere forming cells than the non-SP. However there are marked differences in the structure of the spheres formed by cells from the SP and the non-SP. The non-SP cells form solid balls of cells composed of cells with a similar phenotype, highly suggestive of clonal expansion. In comparison the SP cells induced hollow spheroids

comprised of multiple cell layers, which contained cells of multiple phenotypes, and often displayed a branching differentiation features. The enhanced proliferative capacity of cells within the SP, the maintenance of the G_0 quiescent SP phenotype and their ability to form differentiated structures within both *in vitro* and *in vivo* assays supports the hypothesis that the SP from tumours contain the tumour initiating cells or cancer stem cells.

Cellular heterogeneity and the SP

Characterisation of the cells within the SP suggests that the population is enriched for immature, poorly differentiated and highly tumourigenic cells. The cells seen in this fraction are generally of a more primitive phenotype than those from the non-SP. This has been confirmed by both SP gene expression profiling and by the expression of putative stem cell markers. Characterisation of the SP isolated from both malignant human prostate and renal tissue have shown that the SP is enriched for cells with a stem cell phenotype but results show clearly that it is not a homogeneous population.

It has been hypothesised that prostate stem cells reside within the basal epithelial cells of the prostate acini and that as such, they should express a basal phenotype characterised by cells which are $CK5^{+ve}$ / $CK14^{+ve}$ and PSA^{-ve} along with putative stem cell markers. Studies by Brown *et al* (14) have shown that whilst the SP is enriched for theses markers (table 1 and Figure 2) the population is heterogeneous and also contains cells of a luminal ($CK8^{+ve}$ / $CK18^{+ve}$, AR^{+ve}, PSA^{+ve}) as well as cells of a basal stem cell phenotype. This observation has also been made in renal carcinoma, where the SP is enriched with cells with a stem cell marker phenotype but they are also co-existent with other cell types within the SP, showing that it is not a homogeneous population (Table 1 and Figure 2). This finding is also supported by data showing that not all SP cells can form either tumours *in vivo* or can initiate spheroid formation *in vitro*. Thus, although the Hoechst 33342 assay is an effective means of isolating stem populations using a functional approach, it is not yet possible to isolate SC or CSC as purely as is necessary for characterisation and experimentation at an individual cellular level. The SP still has significant heterogeneity and it must be further purified to isolate a specific subpopulation of the SP which has greater homogeneity of the functional characteristics of tumour initiating cancer stem cells.

Hoechst 33342 Side Population Fractionation

A further development and refinement of the functional approach using Hoechst 33342 isolation methods is exemplified by studies characterising different subsets of haemopoietic SP cells. These have shown that cells isolated from the lower part of the tail, i.e. those cells with the greatest ability to efflux the Hoechst 33342 dye, are the most primitive cells (Figure 4) (66-68) These studies showed that with progression down the SP tail towards its tip, this distal sub-fraction contained cells with the most active efflux mechanisms. These cells had lost CD34 expression, gained CD133 expression and had great clonogenicity, demonstrating enhanced ability to engraft in murine models. These findings support the hypothesis that by further fractionation and analysis of the distal SP tail, it may be possible to isolate a SP sub-population with a greater enrichment of CSC's or TIC's.

Recently the primitive haemopoietic cell marker CD133 has gained favour as a marker for cancer stem cells from solid tumours. Further evidence relating to this has been sought, combining CD133 with standard Hoechst 33342 SP assays. The purpose of this was twofold, firstly to determine the localisation of $CD133^{+ve}$ cells within the SP profile and secondly, to see whether combining these two approaches would isolate a purer CSC population (figure 5). Using epithelial cells isolated from human prostate tumours it was found that $CD133^{+ve}$ cells were found throughout the Hoechst 33342 profile, i.e. in both the SP and the non-SP. This finding was also a feature of SP isolations from RCC. This would suggest that either CD133 on its own does not isolate a homogenous population of CSCs or that CSCs are present in both SP and non-SP populations. The functional studies outlined above would suggest that it is more likely that the former proposition is correct, as only the SP appears to contain cells with stem like characteristics, leading to doubts relating to the veracity of the notion that CD133 is truly a CSC marker in cancer tissues (69). Even though there was a spread of $CD133^{+ve}$ cells throughout the Hoechst 33342 profile it is interesting to note that within the SP the $CD133^{+ve}$ cells formed a dense subpopulation towards the lower quadrant of the SP, which in the haemopoietic system is occupied by increasingly primitive haemopoietic cells. Phenotypic analysis of this $CD133^{+ve}$ SP from human prostate tissue showed that the incorporation of CD133 in to the Hoechst 33342 dye efflux assay enriched the population further for cells with a stem like phenotype, with

up to 38.84% of the cells expressing the stem cell marker Musashi-1 in comparison with 3% of cells without CD133 enhancement (70). However it is still a mixed population of cells with approximately 20% of cells expressing the terminally differentiated markers CK8 and the androgen receptor (AR). Interestingly there is a subpopulation of cells with even greater ability to efflux Hoechst 33342 below the $CD133^{+ve}$ subpopulation, at the very tip of the SP profile from renal epithelial cells (69). Although the exact phenotype of this population is currently under investigation studies by Umemoto *et al* (71) would suggest that this subpopulation will contain even more primitive cells. They have shown that the subpopulation at the tip of the SP from murine bone marrows, unlike the rest of the SP, where highly enriched for Lin^{-ve}/integrin β3 cells, which have the same capacity for long term haematopoiesis as the classically defined c-Kit^{+ve}/Sca-1^{+ve}/Lin^{-ve} HSC suggesting that the primitive HSC reside within the tip of the SP. Further interrogation of this "distal SP tail" in human prostate tissue has confirmed that there is a definite resident subpopulation of cells with stem cell characteristics and that there are distinct genetic differences between prostate tissue derived from normal prostate, benign prostatic hyperplasia and from prostate cancer (72). This new data is of added importance because it has been undertaken on human cells which have not been subjected to sub-culture and expansion, methods which are known to be associated with genetic drift and subsequent uncertainty regarding the genetic analysis of the final cellular population. These studies have developed a methodology which potentially opens the way for further genetic characterisation of the stem / cancer initiating cell population in solid tumours.

Conclusions and Future Perspectives

The surge in interest in identifying and characterising the tumour initiating cell or cancer stem cell has had the defining ethos of discovering novel diagnostic and prognostic markers and generating new therapeutic approaches based on a better understanding of the signalling pathways for enhanced survival and tumour propagation which are a fundamental properties of these cells. The major hurdle to this process has been the lack of a truly cancer stem cell specific biomarker. The use of a functionally based assay such as the Hoechst 33342 dye efflux assay has provided a useful alternative to the use of putative stem cell surface based markers for the identification of cancer stem / cancer initiating cells. Whilst this technique has been useful in isolating stem cell enriched populations from many solid tumours and cell lines it should be

emphasised that on its own, this technique isolates a population enriched for but not homogeneous for cancer stem cells. There is therefore a need to continue to develop functional assays still further in order to define a pure cancer stem cell population which can then be characterised specifically. The fractionation of the distal tail of the SP is one approach that has been used successfully in haematological SP (66-68), with the distal tail defined by the cell population which most actively effluxes the dye and which appears tocontain the most primitive cells. This approach has now been adapted to solid tissues from the genitor-urinary tract with promising results (72).

In addition to the potential benefits of characterisation, this approach also offers the potential for development of new approaches to therapy by virtue of the interdependence of the CSC / cancer initiating cell on the chemo-resistance mechanisms. Primitive SCs express ATP-binding cassette (ABC) (26) and multidrug resistance (MDR) (47) efflux pumps and they are thus are able to remove toxic metabolites and xenobiotics from the interior of the cell. This effect has been demonstrated in SP cells from both primary cancers and cell lines, which have been shown to possess the ability to efflux cytotoxic agents. Not only does this property enable differential selection for SCs, CSC / cancer-initiating cells, it also focuses on the mechanisms which confer treatment-resistance on this cellular sub-population. This is of fundamental importance clinically. For example, the isolated SP from primary neuroblastoma and a variety of cancer cell lines (bone, brain, breast, kidney, lung and ovary) has been shown to be able to efflux the chemotherapeutic agent Mitoxantrone and ultimately to facilitate survival of the SP following cytotoxic treatment whereas the non-SP cells were unable to proliferate and form colonies following treatment (56). Similar findings were demonstrated in the SP from gastro-intestinal cell lines, which were more resistant to the three chemotherapeutic agents; 5-Fluorouracil; Doxorubicin and Gemcitabine (60). Indeed evidence is available to demonstrating the relative chemoresistance of SCs compared with the differentiated cellular populations (73). This survival advantage is clearly beneficial to the organ or tissue as it is the SC that would survive to repair and repopulate an injured tissue. There are therefore a number of reasons why it is important to continue to strive to identify and characterise the CSC / cancer initiating cell population as this sub-group is likely to represent the population of cells with the greatest ability to survive in adverse conditions, and included in this is the ability to resist modern therapies including cytotoxic chemotherapy

and ionising radiation. Study of the SP, and particularly the distal SP using Hoechst 33342 based methodology is a significant avenue for research in this field.

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Figure Legends

Figure 1: Hoechst 33342 epithelial profiles. Single epithelial cell suspensions were stained with 5μM Hoechst 33342 for 90minutes with or without the SP inhibitor Verapamil hydrochloride 106 (50μM). Cells were analyzed using a Becton Dickinson FACS Vantage SE flow cytometer (FACS), exciting at 357nm and detecting Hoechst Blue with a 424/44 broad pass (BP) filter and Hoechst Red with a 675/20 BP filter (Omega Optical, Brattleboro VT). The SP is outlined and events highlighted in red.

Hoechst 33342 SP profiles from benign and malignant human prostate epithelial cells. The SP is boxed and highlighted in red. The SP accounts for 0.93±0.12% of total benign epithelial population and 0.52±0.11% of total malignant epithelial population

Figure 2: The SP is a heterogeneous population. The barcharts show the phenotypic characterisation of both the SP and the NSP of human renal carcinoma (RCC) and prostate cancer (CaP) using both lineage specific and putative stem cell markers.

Figure 3: The SP is enriched for cells capable of proliferation and differentiation in vitro. A function of stem cells is their ability to undergo enhanced proliferation and differentiation in to the cell types of that organ. The in vitro model of SP cells cultured in the 3D matrix Matrigel in the presence of irradiated feeder cells shows the stem like functional phenotype of proliferation and differentiation to form 3D structures or "spheroids". Spheroids are often hollow spheres containing differentiated cell types and may become branched structures.

Figure 4: Fractionation of the SP isolates increasingly primitive cells. Cells with greater Hoechst 33342 dye efflux ability are more primitive in the human haemopoietic system. Characterisation of the SP shows that as fractions closer to the SP tip have reduced numbers of cells expressing CD34 whilst an increase in numbers of cells expressing the more primitive marker CD133.

Figure 5: Enrichment of stem cell fraction of the SP by combining the Hoechst 33342 dye efflux assay with putative stem cell markers. Combining putative stem cell markers with the SP isolation enriches for cells with a stem cell phenotype in prostate cancer. $CD133^{+ve}$ SP = 0.049±0.052% of all prostate epithelial cells in comparison with the SP=0.52±0.12%

Figure 6: Combining putative stem cell markers with the SP isolation enriches for cells with a stem cell phenotype but the SP is still heterogeneous. Prostate epithelial cells were stained with CD133 and Hoechst 33342. Comparison of the phenotypes of a prostate epithelial SP (green) with a $CD133^{+ve}$ SP (red) shows enrichment for stem cell markers and for the prostate basal markers

Table 1. The SP is a heterogeneous population. Phenotypic characterization of the SP from malignant tissue using both lineage specific and putative stem cell markers has shown that the SP, whilst enriched for cells

with a stem cell phenotype, is a heterogenous population. Table shows examples of the SP and NSP phenotype from human renal carcinoma and prostate cancer.

Figure 1

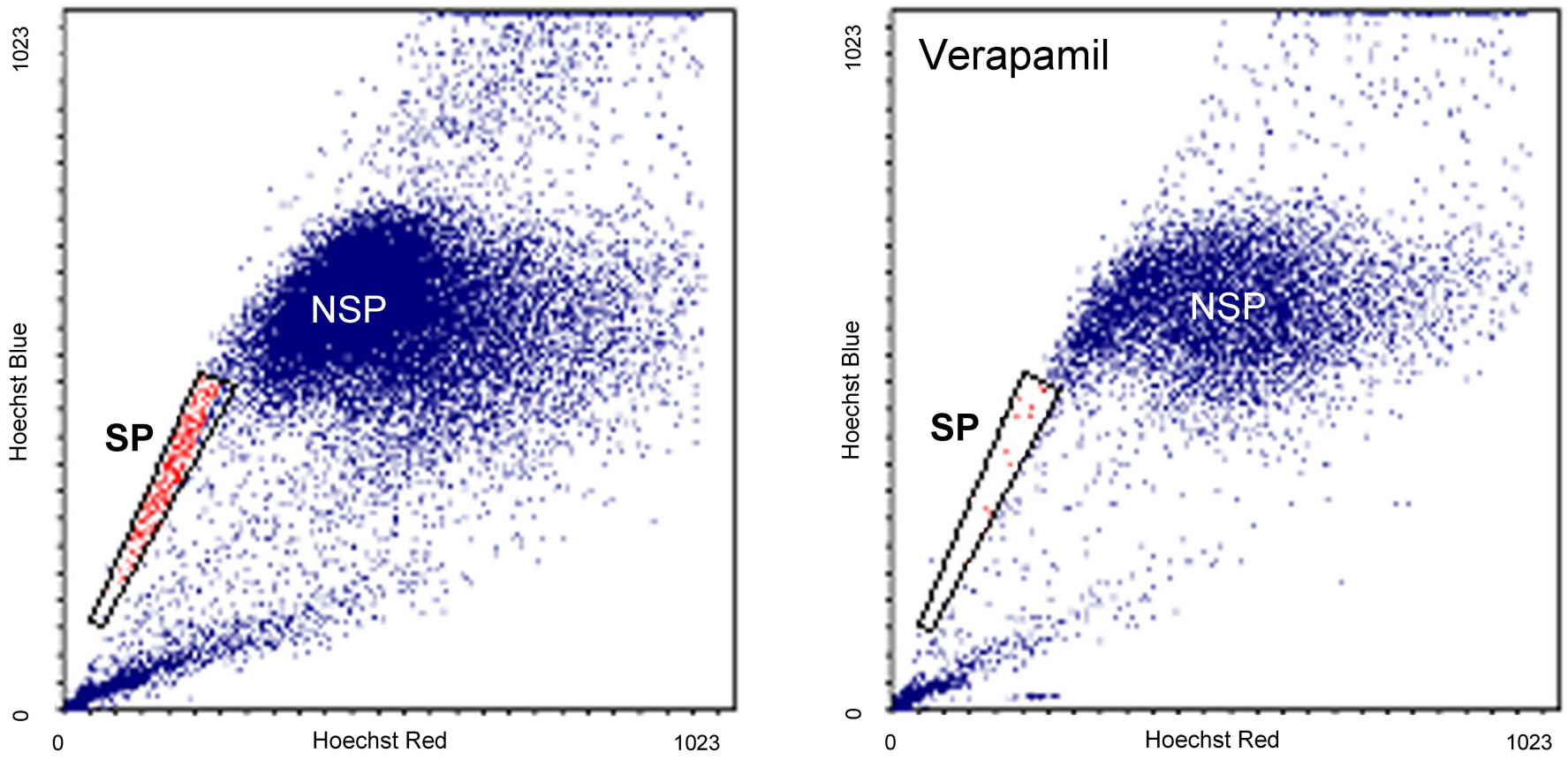
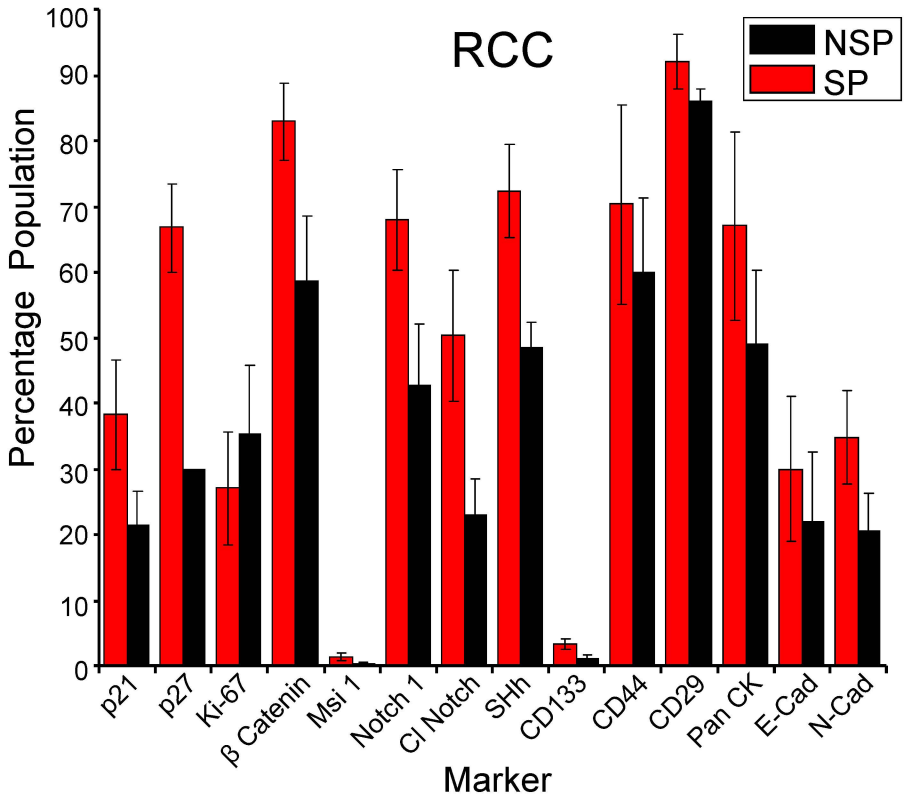

1023
Hoechst Blue
0
NSP
SP
0
Hoechst Red
1023
Verapamil
1023
Hoechst Blue
0
NSP
SP
0
Hoechst Red
1023

Figure 2


RCC
NSP
SP
Percentage Population
100
90
80
70
60
50
40
30
20
10
0
p21
p27
Ki-67
β Catenin
Msi 1
Notch 1
Cl Notch
SHh
CD133
CD44
CD29
Pan CK
E-Cad
N-Cad
Marker

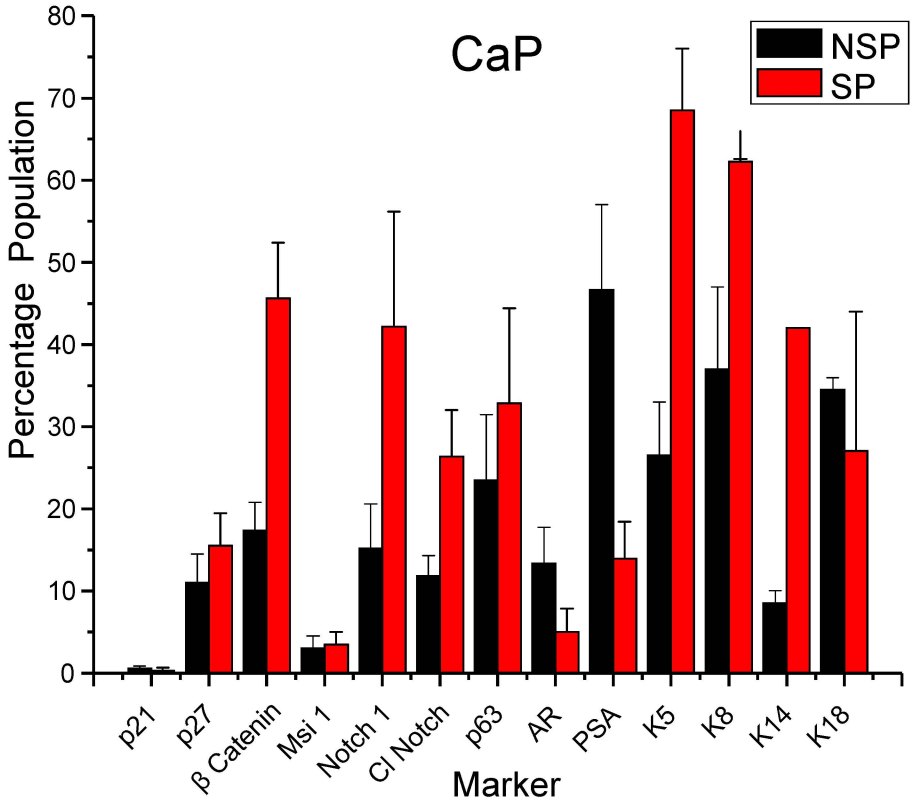
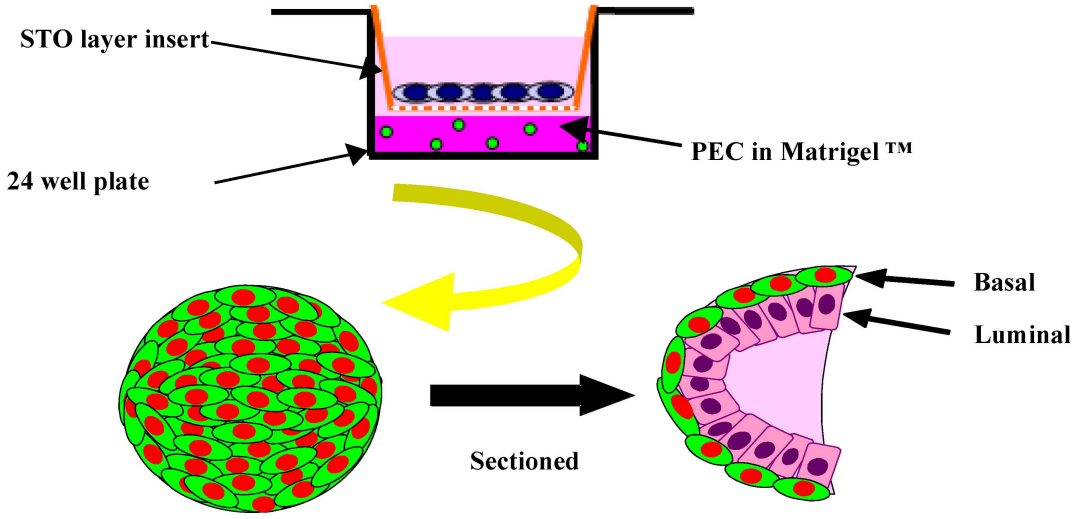

CaP
NSP
SP
Percentage Population
80
70
60
50
40
30
20
10
0
p21
p27
β Catenin
Msi 1
Notch 1
Cl Notch
p63
AR
PSA
K5
K8
K14
K18
Marker

Figure 3: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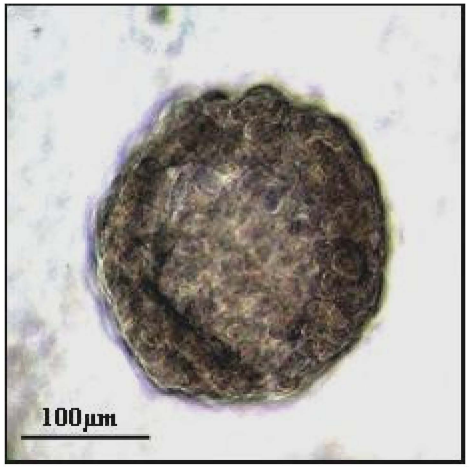
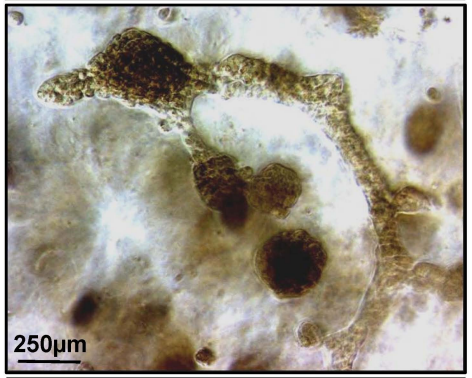
24 well plate
PEC in Matrigel ™
Basal
Luminal
Sectioned


100µm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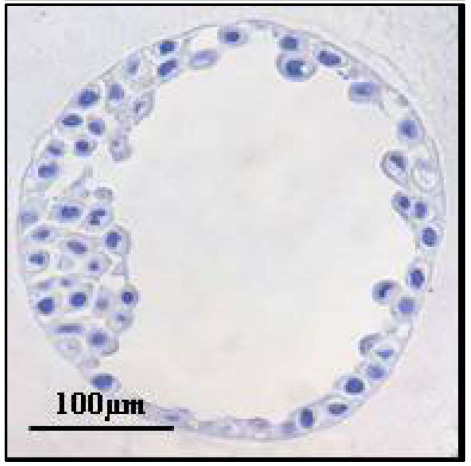
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Figure 4:

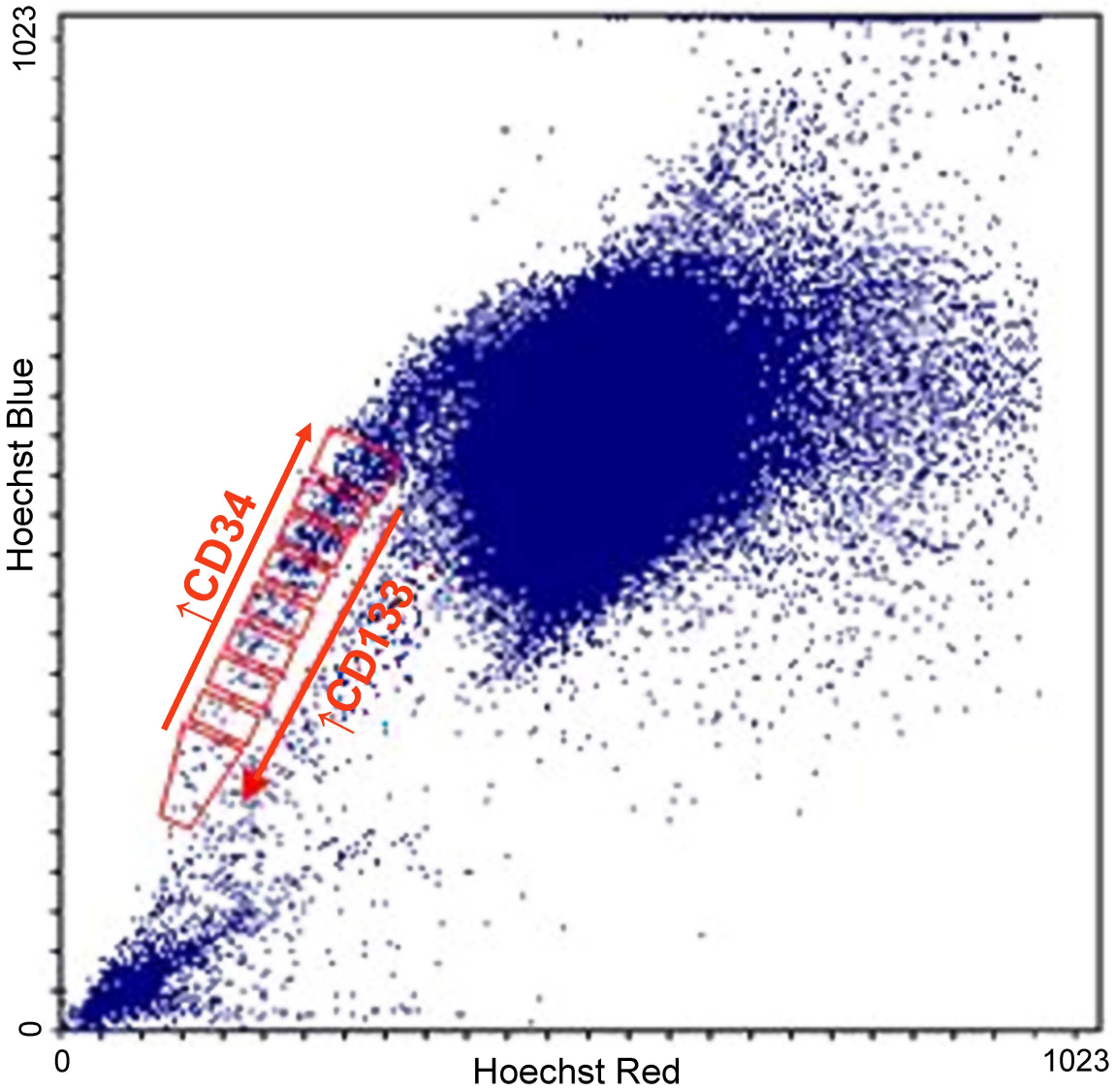


Figure 5:

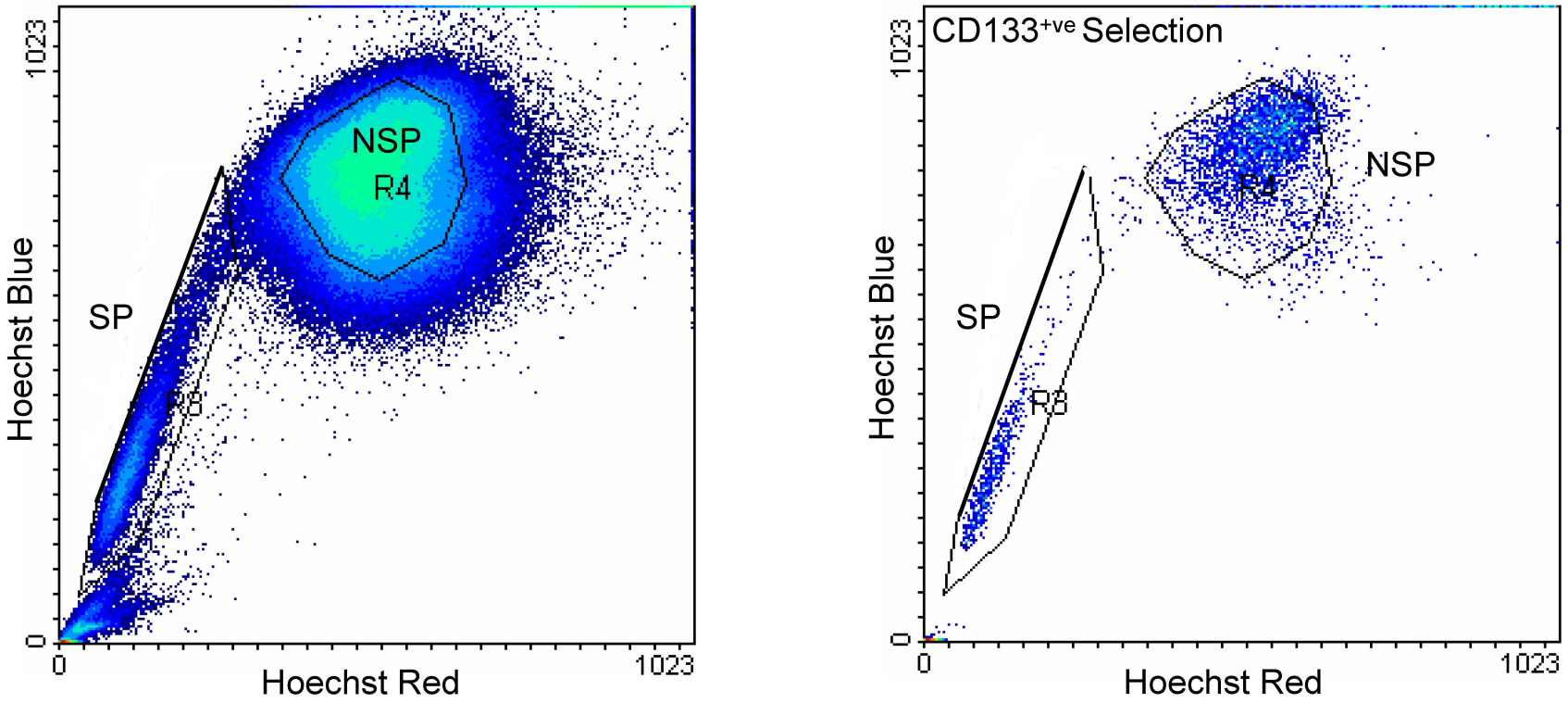
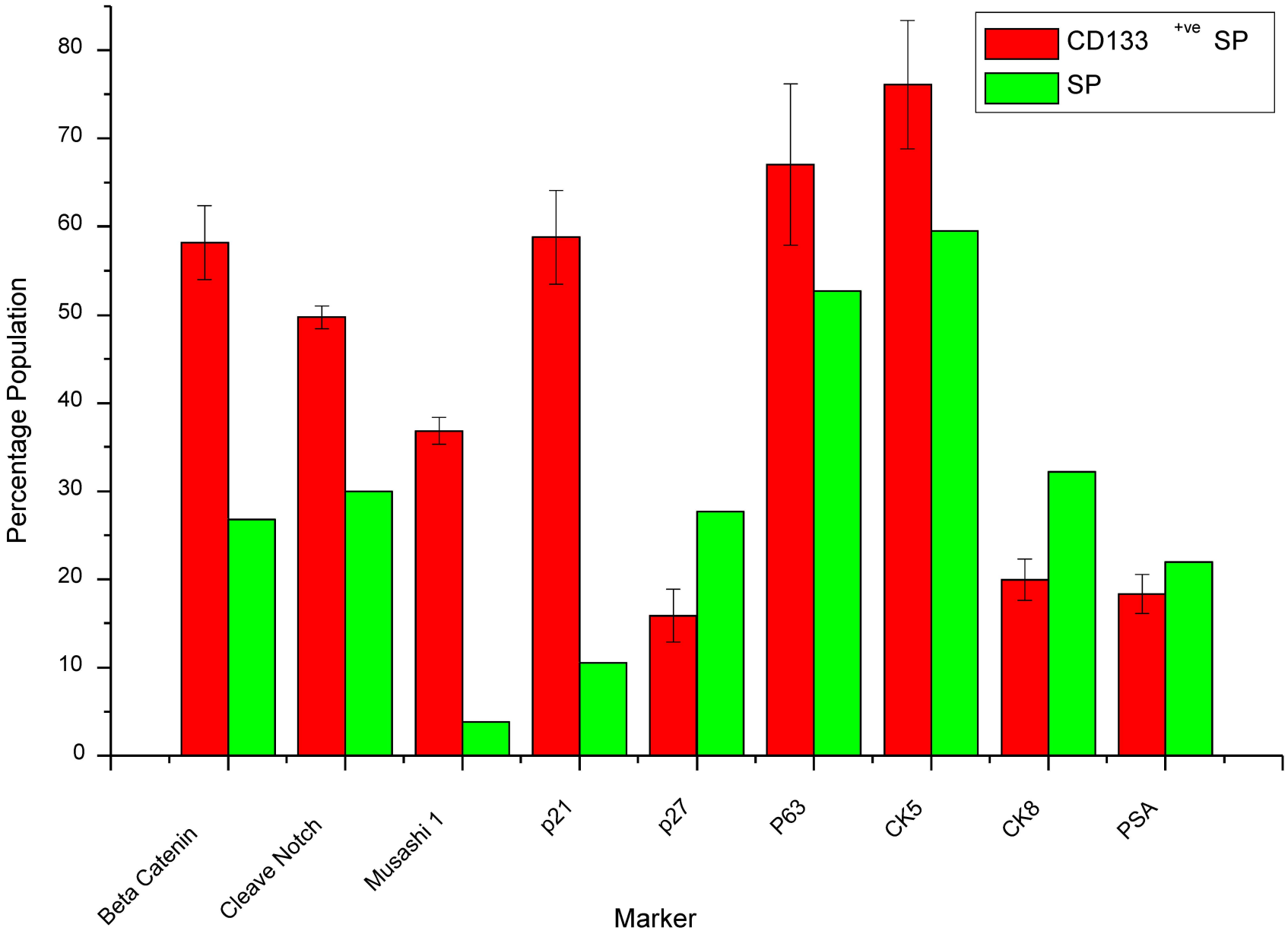

CD133+ve Selection
NSP
R4
SP
R3
Hoechst Blue
Hoechst Red
0
1023

Figure 6


CD133 +ve SP
SP
Percentage Population
0
10
20
30
40
50
60
70
80
Beta Catenin
Cleave Notch
Musashi 1
p21
p27
P63
CK5
CK8
PSA
Marker

Marker	Percentage population ± sem			
	Renal Cell Carcinoma		Prostate Cancer	
	SP	NSP	SP	NSP
p21	38.3±8.4	21.3±5.4	0.3±0.3	0.5±0.3
p27	66.8±6.7	30±7.6)	15.5±4.0	11±3.5
Ki-67	27±8.6	35.3± 10.5		
β Catenin	83±5.9	58.5±9.9	45.6±6.8	17.3±3.5
Msi 1	1.3±0.5	0.3±0.3	3.4±1.6	3±1.5
Notch 1	68±7.7	42.8±9.2	42.2±14.0	15.2±5.4
Cleaved Notch	50.3±9.9	23±5.5	26.3±5.7	11.8±2.5
Sonic Hedgehog	72.3±7.1	48.5±3.9	-	-
CD133	3.3±0.7	1±0.6	-	-
CD44	70.3±15.1	60±11.1	-	-
CD29	92±4.1	86±2	-	-
p63	-	-	32.8±11.6	23.5±8.0
AR	-	-	5±2.9	13.3±4.4
PSA	-	-	13.9±4.6	46.7±10.4
Pan Cytokeratin	67±14.4	49±11.2	-	-
E-Cadherin	30±11.2	21.8±10.9	-	-
N Cadherin	34.8±7.1	20.5±5.9	-	-
K5	-	-	68.5±7.5	26.5±6.5
K8	-	-	62.3±3.8	37±10
K14	-	-	42±0	8.5±1.5
K18	-	-	27±17	34.5±1.5

Table 1. The SP is a heterogeneous population. Phenotypic characterization of the SP from malignant tissue using both lineage specific and putative stem cell markers has shown that the SP, whilst enriched for cells with a stem cell phenotype, is a heterogenous population. Table shows examples of the SP and NSP phenotype from human renal carcinoma and prostate cancer.