INVESTIGATING THE CANCER STEM CELL HYPOTHESIS IN CANINE TUMOURS

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DECLARATION

In accordance with the regulations of the University, I declare that this thesis has been completed entirely by myself, and that the work presented is my own, except where acknowledgement has been made in the text.

This work has not been submitted for any other degree or professional qualification.

Thalia Blacking

1 August 2010

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ABSTRACT

The cancer stem cell hypothesis has recently re-emerged as a compelling paradigm for the development and progression of neoplastic disease. The hypothesis proposes that a specific subset of "cancer stem cells" (CSC), believed to share many features with normal stem cells, is exclusively responsible for maintaining tumour growth and driving progression. If the CSC hypothesis applies, it may require re-evaluation of the clinical approach to neoplasia. Spontaneous cancer in the domestic dog represents a significant welfare problem, with dogs developing many tumours strongly reminiscent of those affecting humans. This study sought to investigate whether cells with characteristics of CSC are identifiable in canine cancer.

Assays to identify, isolate and characterise CSC were adapted to the canine system, and cancer cell lines and spontaneous tumours of diverse origin evaluated for the presence of candidate populations. Whilst analysis of surface expression patterns did not identify specific subpopulations within canine cancer cell lines, these were detectable in cells derived directly from primary tumours. Assays for stem cell-associated drug resistance mechanisms could also be used to identify subsets of putative canine CSC. Formation of "tumourspheres" by canine cancer cell lines was found to be highly density-dependent, so a potentially unreliable method of isolating CSC. Expression of the cell surface glycoprotein CD44 was associated with cellular proliferation status, although it may not represent a stable canine CSC marker. The NFκB survival pathway, associated with apoptosis resistance of some putative CSC, was constitutively active in canine cancer cell lines; suppression using specific inhibitors could reduce cell viability, indicating that this may represent a rational therapeutic target.

Overall, these studies demonstrated that CSC assays may be adapted to the canine model system, although they require rigorous interrogation to distinguish apparent CSC attributes from basic biological properties. Cell lines have provided a stable background upon which to optimise assays, but appear less likely to demonstrate discrete CSC subpopulations. Putative CSC subsets may be more readily identifiable

within heterogeneous primary tumour cells. The application of some of these adapted assays within a clinical setting may enable further characterisation of individual patients' tumours, and inform therapeutic regimes for improved treatment outcomes.

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ABBREVIATIONS USED IN TEXT OF THESIS

ABC	ATP-binding cassette
ABCG2	ATP-binding cassette, Subfamily G, Member 2
ALDH / ALDH1-A1	Aldehyde dehydrogenase / Isoform 1-A1
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
AP	Alkaline phosphatase
APC	Adenomatous Polyposis Coli tumour suppressor protein
APS	Ammonium persulphate
ATP	Adenosine triphosphate
B27	B27 neural cell growth supplement
BAA	Bodipy-Aminoacetate
BAAA	Bodipy-Aminoacetaldehyde
BCRP	Breast cancer resistance protein
bFGF	Basic fibroblastic growth factor
BM	Bone marrow
BMMNC	Bone marrow mononuclear cells
BMSC	Bone marrow-derived stromal cells
bp	Base pair
CD	Cluster of differentiation
CD44s	CD44 standard form
CD44v	CD44 variant forms
CLL	Chronic lymphocytic leukaemia
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukaemia
CMP	Common myeloid progenitor
CO_2	Carbon dioxide
CSC	Cancer stem cell/s
DAPI	4',6-Diamidino-2-phenylindole dihydrocloride
DCV	Vybrant DyeCycle Violet
ddH_2O	Double distilled water
DEAB	Diethylamino-benzaldehyde
DIG	Digoxigenin
DMEM	Dulbecco's modified Eagle's medium
DMEM-F12	DMEM / Hams's F12 nutrient mixture
DMEM-HG	High glucose (4.5g/dl) DMEM
DMEM-LG	Low glucose (1g/dl) DMEM
DMSO	Dimethyl sulphoxide

DNA	Deoxyribonucleic acid
ds	Double stranded
DTT	Dithiothrietol
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetraacetic acid
EGF/R	Epidermal growth factor / receptor
EMSA	Electrophoretic mobility shift assay
ESC	Embryonic stem cell
ESA / EpCAM	Epithelial surface antigen / Epithelial cell adhesion molecule
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FGFR	Fibroblastic growth factor receptor
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
g	Relative centrifugal force
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GMP	Granulocyte monocyte precursor
HA	Hyaluronate
HBSS	Hank's buffered salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFSA	Hospital for Small Animals
Ho	Hoechst 33342
HSA	Haemangiosarcoma
HSC	Haematopoietic stem cell
IκB	Inhibitor of κB
IC_{50}	Inhibitory concentration – 50% toxicity
IKK	IκB kinase
ISNI	InSolution NFκB activation inhibitor
IU	International units
KCl	Potassium chloride
LSC	Leukaemia stem cell
MACS	Magnetic-activated cell sorting
MC	Methylcellulose
MDR	Multidrug resistance
MEP	Megakaryocyte-erythroid precursor
MFI	Mean fluorescence intensity
$MgCl_2$	Magnesium chloride
MM	Multiple myeloma

MSC	Mesenchymal stem cell
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NaF	Sodium fluoride
N2	Bottenstein's N2 neural cell growth supplement
NFκB	Nuclear factor kappa-light-chain enhancer of B cells
NOD/SCID	Non-obese diabetic/severe combined immunodeficient
NP40	Nonidet-P40 (octylphenoxypolyethoxyethanol)
PAGE	Polyacrylamide gel electrophoresis
PB	Peripheral blood
PBMNC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBST-IF	PBS with 0.05% w/v Tween 20
PBST-WB	PBS with 0.1% w/v Tween 20
PCNA	Proliferating cell nuclear antigen
PDGF/R	Platelet-derived growth factor
PE / RPE	Phycoerythrin
PeCy5	Phycoerythrin-Cyanine5
Pen-Strep	Penicillin-Streptomycin
PFA	Paraformaldehyde
P-gP	P-glycoprotein
PI	Propidium iodide
PI3K	Phosphoinositide-3 kinase
PMT	Photomultiplier tube
PTEN	Phosphatase and tensin homolog
R(D)SVS	Royal (Dick) School of Veterinary Studies
RHD	Rel homology domain
Rho123	Rhodamine 123
RNA / mRNA	Ribonucleic acid / messenger RNA
RNAse	Ribonuclease
RT-PCR	Reverse transcriptase polymerase chain reaction
SCID	Severe combined immunodeficient
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
SL-IC	SCID leukaemia-initiating cell
SP	Side population
SSC	Side scatter

STAT3	Signal transducer and activator of transcription 3
TBE	Tris/borate/EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TGFβ	Transforming growth factor-beta
TK / TKI	Tyrosine kinase / Tyrosine kinase inhibitor
TPC	Therapeutic plasma concentration
TSC	Tissue stem cell
UV	Ultraviolet
VEGF/R	Vascular endothelial growth factor / receptor
WBC	White blood cell
WDL	Wedelolactone
w/v	Weight for volume
w/w	Weight for weight

CHAPTER 1

THE CANCER STEM CELL HYPOTHESIS -

THEORY, EVIDENCE AND IMPLICATIONS

Introduction

The concept of the "tumour stem cell" has existed in one form or another for many decades. However, over the past fifteen years, this idea has crystallised into a subject of intense interest, as well as considerable debate. The cancer stem cell hypothesis proposes that a specific subset of cells within a tumour is exclusively responsible for sustaining its growth, acting in a manner similar to the stem cells in normal tissues. If this is the case, it could have profound implications for cancer therapy.

Here is presented the theory underlying, and some of the key evidence in support of, the cancer stem cell hypothesis. Mechanisms and markers which may identify the putative cancer stem cell (CSC) are examined, and the controversies surrounding the hypothesis discussed. Also explored is the concept that, where the CSC hypothesis is applicable, it may require re-evaluation of the clinical approach to neoplastic disease.

Stem Cells and Cancer Stem Cells

In normal adult mammalian tissues, a balance exists between the loss and the production of cells which maintains the steady state of normal tissue renewal. The rate of turnover varies according to the tissue in question, with some being constantly renewed (*e.g.* epithelia and cells of the haematopoietic system), and some formed during development and thereafter showing little regenerative capacity (*e.g.* neural tissue) under physiological conditions.

Within a given tissue, a hierarchy exists within the cell population in terms of the ability to divide and provide new cells for tissue renewal. At the apex of this hierarchy is the Tissue Stem Cell (TSC, also Adult Stem Cell), which can produce all the cells of the tissue in question. This TSC gives rise to more committed precursor or "transit-amplifying" cells, which are more numerous and cycle more frequently than the stem cells, but are restricted in terms of the cell types they can produce. The majority of cells are terminally differentiated and have tissue-specific functions, but

do not have the ability to divide or differentiate (Figure 1) (Weissman, 2000; Reya *et al.*, 2001; Pardal *et al.*, 2003; Sell, 2004).

This process of *determination*, by which progeny at different levels in the hierarchy acquire the differentiated properties of the functional tissue, but gradually lose the ability to proliferate and become restricted in their potential, remains poorly understood. Sell describes the structure of a tissue as analogous to a tree, with the scarce pluripotent TSC represented by the trunk, the progenitor/transit amplifying cells the branches, and the numerous differentiated tissue cells the leaves; the falling of the leaves is likened to the process of apoptosis, whereby proliferation is balanced by controlled loss (Sell, 2004).

Embryonic stem cells (ESC) are described as "totipotent" – that is, their progeny can go on to form any cell type in the developed individual. Somatic or adult stem cells, i.e. those present in formed tissues, have a more restricted differentiation pattern and are termed "multi-" or "pluripotent" (these terms are frequently used interchangeably). The germinal stem cell, which produces the sex cells (oocytes / spermatozoa) is the third kind of normal stem cell (Sell, 2004). These rare cells share the two defining properties which have become accepted as definitive of stem cells – firstly, they have the capacity for unlimited self-renewal, and secondly, they are capable of multilineage differentiation to generate the mature cells of a particular tissue (Reya *et al.*, 2001). Stem cells may self renew by dividing symmetrically (producing two identical stem cells) or asymmetrically (producing an identical stem cell and a more differentiated progeny cell) (Figure 2). By virtue of this unlimited self-renewal capacity, a stem cell can continue to divide (though often infrequently and interspersed by periods of quiescence) for the lifetime of the host.

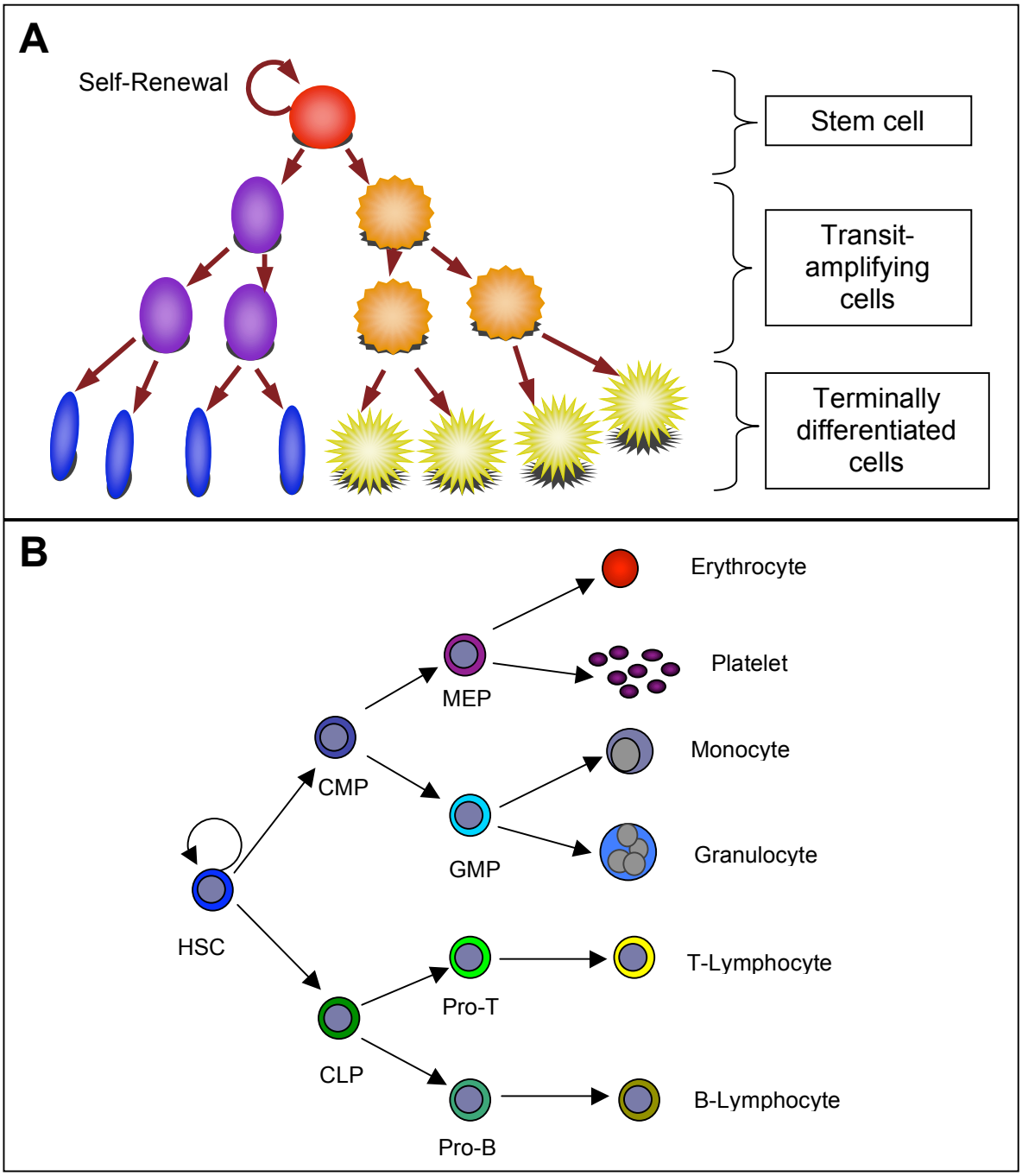


Figure 1 – Hierarchical organisation of normal tissue - schematic.
A - Stem cells exist at the apex of a cellular hierarchy in normal tissues. Successive differentiation through a variable number of transit amplifying stages results in the generation of terminally differentiated cells with tissue-specific function.
B – Hierarchical tissue organisation, exemplified in the haematopoietic system. Schematic illustrating critical stages of lineage divergence. As well as self-renewing, haematopoietic stem cells (HSC) may produce common myeloid or lymphoid progenitors (CMP / CLP), which in turn give rise to the precursors of the respective terminally differentiated cell types. MEP – megakaryocyte-erythroid precursor; GMP – granulocyte-macrophage precursor; Pro-T – Pro-T cell; Pro-B – Pro-B cell.

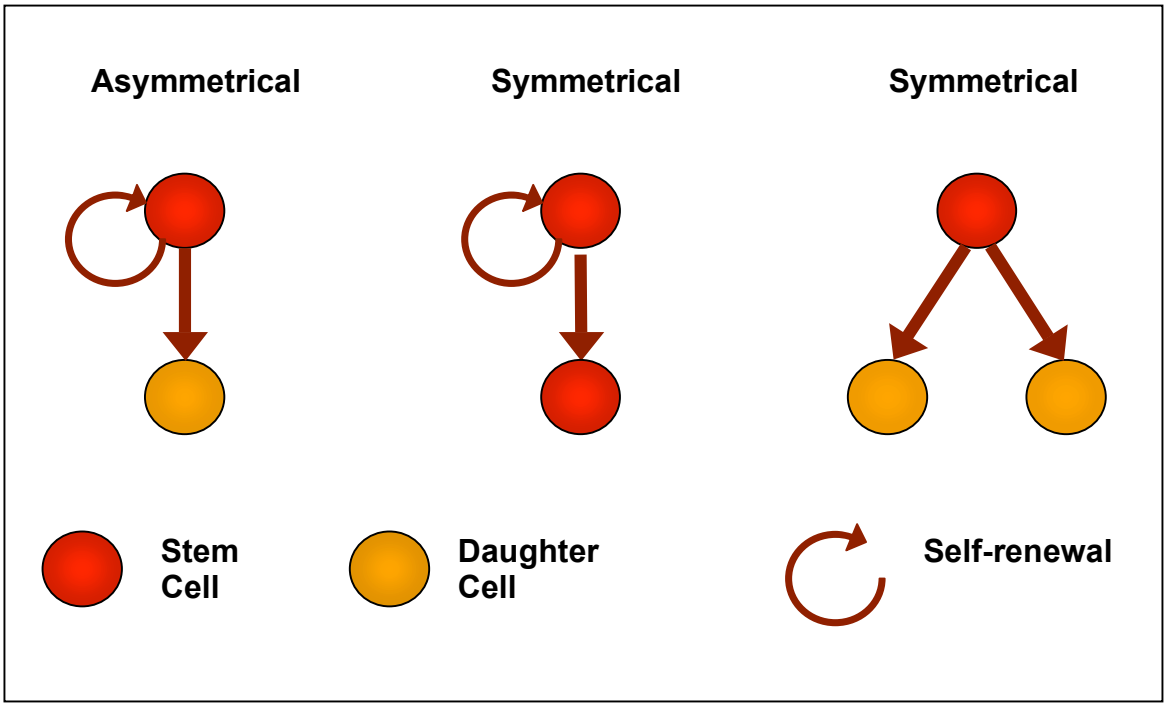


Figure 2 – Stem cell division patterns. Asymmetrical division gives rise to an identical stem cell (self-renewal) and a more differentiated daughter cell, maintaining the stem cell pool. Symmetrical division may lead to formation of two identical stem cells (self-renewal), increasing the stem cell pool, or two more differentiated progeny, with consequent depletion of the stem cell component.

Cancer can be viewed as the acquisition by a cell/tissue of 6 fundamental properties (Hanahan and Weinberg, 2000) -

- Self-sufficiency in growth signals
- Insensitivity to growth-inhibitory (antigrowth) signals
- Evasion of apoptosis
- Limitless replicative potential
- Sustained angiogenesis
- Tissue invasion and metastasis

This has classically been viewed as a multistep process, whereby cells initially accrue mutations enabling proliferation in an unregulated manner, and subsequently those allowing invasion of surrounding tissues and spread to distant sites (Hanahan

and Weinberg, 2000). Early or premalignant manifestations are recognised for many cancers, where cells display several characteristics of the later malignant neoplasm but act in a less aggressive fashion until further transformation allows growth and spread, such as carcinoma-in-situ, or early colonic polyps seen in Familial Adenomatous Polyposis (Leedham *et al.*, 2005).

However, many phenomena recognised in cancer *in vivo* and *in vitro* have been hard to reconcile with this notion of one cell which, having acquired sufficient mutations, may proliferate to create multiple identical clones of itself. These include the observations that *in vitro,* a limited proportion of cells will demonstrate the capacity to proliferate to form clones, and that *in vivo* models frequently require inoculums of many thousands to millions of cells to induce tumour formation (Bruce and Van Der Gaag, 1963; Hamburger and Salmon, 1977; Griffin and Lowenberg, 1986). This heterogeneity amongst populations of genetically identical (albeit abnormal) cancer cells is also apparent histologically, particularly in more "well-differentiated" tumours, which often have a well-defined structural microanatomy that includes all the elements of the corresponding normal tissue.

The stochastic or "clonal evolution" model ascribes heterogeneity in the cancer cell population to ongoing genetic / genomic instability, combined with the effects of the microenvironment, leading to aberrant replication and the generation of multiple cancer cell phenotypes (Nowell, 1976; Clarke *et al.*, 2006; Dick, 2008). It holds that all cells within a tumour are competent for tumourigenesis – that any cell has the potential to proliferate and give rise to more cancer cells - but that the probability of an individual cell achieving this is limited – hence "stochastic". Another way in which heterogeneity might arise within a tumour is if the normal pyramidal process of proliferation and differentiation becomes deranged, such that a hierarchy of cells is produced, but in a dysregulated manner. This is the basis of the cancer stem cell hypothesis (Reya *et al.*, 2001) (Figure 3, Figure 4).

The hypothesis postulates that a specific subset of cells exists within a tumour, which have the capacity both to self-renew and to differentiate into all the different cell

types which make up the tumour. The progeny of these "cancer stem cells" (CSC) have more restricted potential – as such, in the same way as normal tissues are derived through successive differentiation and amplification from the stem cell component, the tumour is ultimately maintained by the CSC (Clarke *et al.*, 2006) (Figure 4).

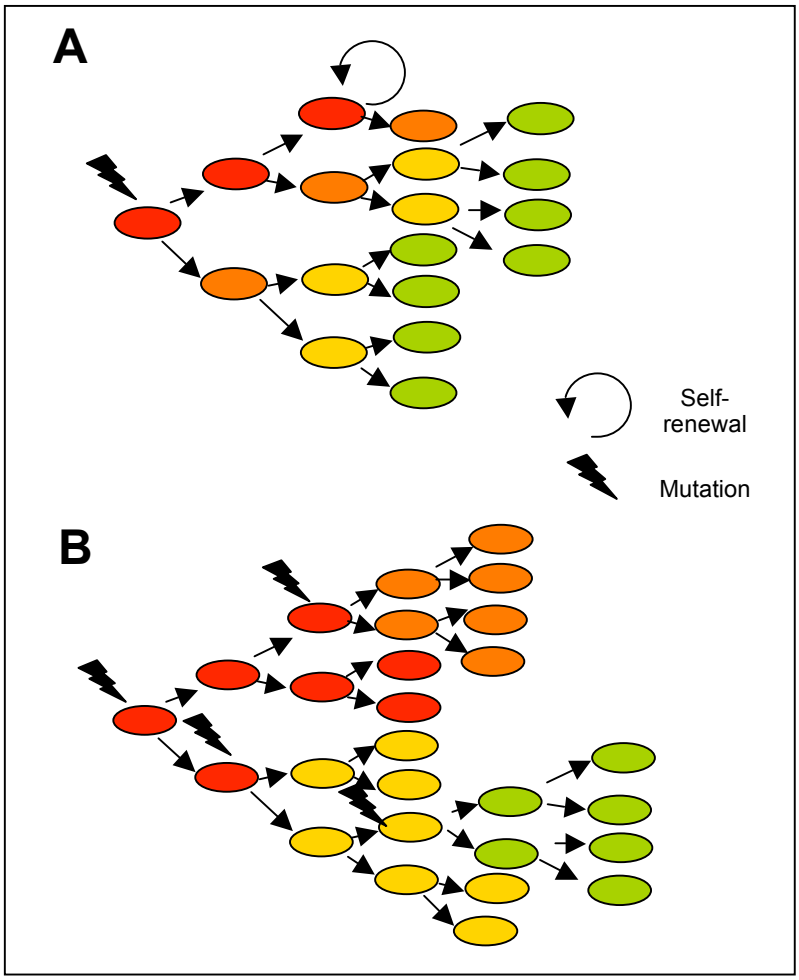


Figure 3 – Models of tumour heterogeneity
A – Cancer stem cell paradigm - CSC (red), through successive differentiation steps, give rise to all of the different cell types within the tumour, and may also self-renew to produce other CSC. Progeny cells have more restricted potential. CSC are thus biologically distinct from the majority of tumour cells and sustain tumour growth and progression. These inherent properties may be predicted, allowing the identification and prospective isolation of tumourigenic cells.
B – Clonal evolution paradigm – Heterogeneity arises through the acquisition of additional mutations (genetic / epigenetic) under the influence of intrinsic and extrinsic factors such as genetic instability, the environment or drug therapy. This results in distinct tumour cell populations based on the prevailing selective pressures. All cells may be inherently tumourigenic, but behaviour is stochastic and cannot be accurately predicted.

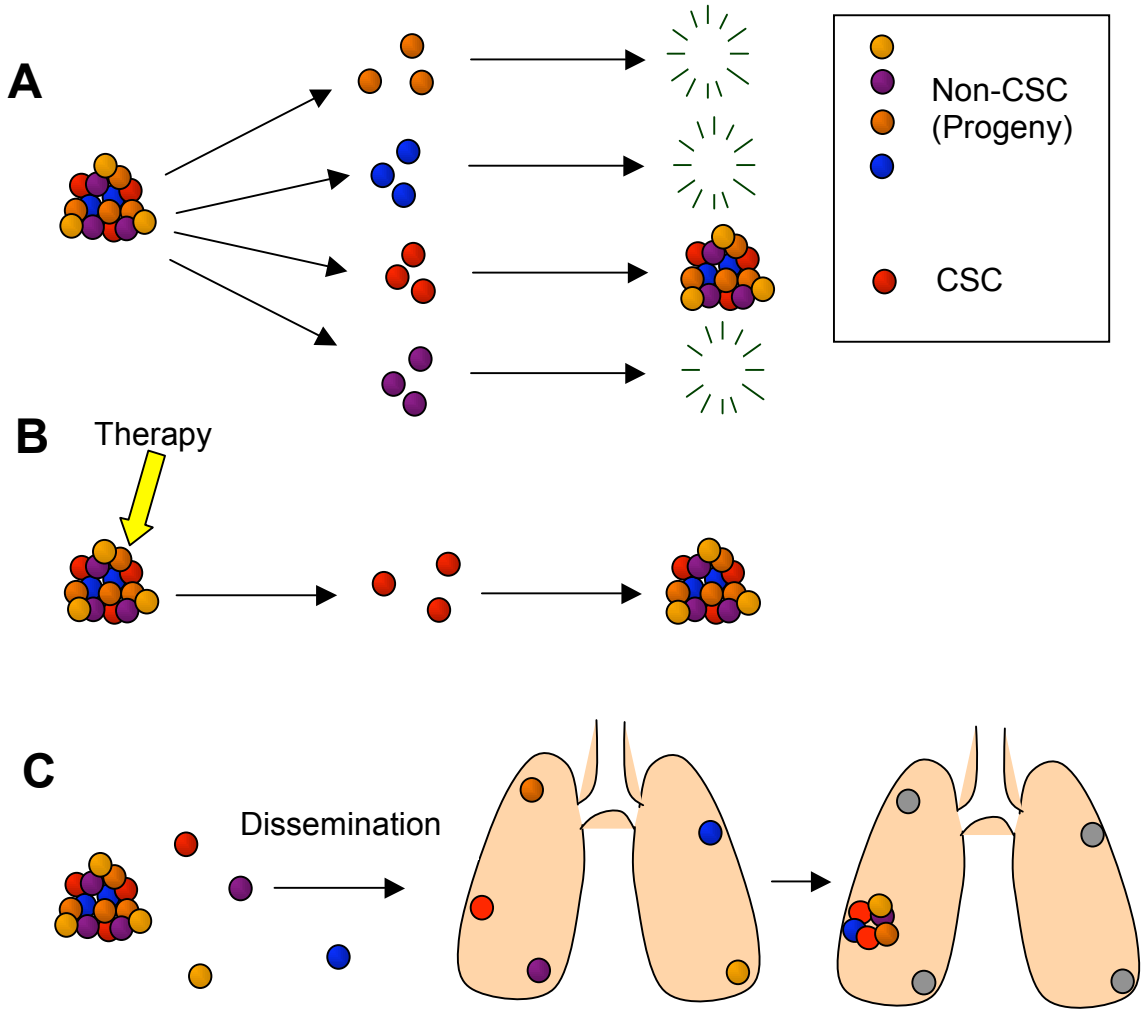


Figure 4 – The CSC hypothesis can account for observations of tumour behaviour
A – When dissociated and plated *in vitro*, some cancer cells do not form colonies; when transplanted *in vivo*, a large number of cancer cells must be inoculated in order to form a tumour. The CSC model predicts that only CSC, and not more differentiated progeny, have the unlimited self-renewal potential and multilineage differentiation capacity required to propagate the malignancy.
B – Even after apparent elimination of a tumour, disease may recur following cancer therapy. The CSC model predicts that this is due to the survival of CSC, which if not eradicated may recapitulate disease, leading to relapse.
C – Not all cells which disseminate from the primary tumour will form active metastases, but metastases show the cellular heterogeneity seen within the primary tumour. The CSC model predicts that dissemination of CSC - but not restricted progeny - to distant sites may result in the formation of secondary tumours with phenotypic characteristics of the primary tumour.

Origins of the Cancer Stem Cell Hypothesis

The idea that cancer results from the abnormal growth of primitive cells with extensive differentiation potential is not new. As far back as the 1800s, the pathologists Virchow, Cohnheim, Durante and Beard contributed to the formulation of the "Embryonal Rest" hypothesis, proposing that cancer arises from embryonic cells which become displaced during development and persist in adult tissues (Sell, 2004; Huntly and Gilliland, 2005). Experiments with both haematopoietic and solid malignancies between the 1960s and the 1980s demonstrated that, *in vitro* and *in vivo,* only a small percentage of cells isolated from a tumour was clonogenic, i.e within a tumour there was variability in the ability of cells to proliferate (Bruce and Van Der Gaag, 1963; Hamburger and Salmon, 1977; Griffin and Lowenberg, 1986; Huntly and Gilliland, 2005).

If heterogeneity amongst tumour cells results from the "hijacking" of normal differentiation processes, clear parallels can be drawn between the behaviour of normal tissue stem cells and the putative CSC. The degree to which the cell types within a tumour may be remarkably reminiscent of those in the normal system is epitomised in teratocarcinoma. The presence of strikingly "normal" tissues, such as formed teeth, hair, brain, muscle, skin, eyes, bone and intestine, within teratocarcinomas suggests that these tumours arise from abnormalities affecting a multi- or totipotent cell (Sell and Pierce, 1994; Reya *et al.*, 2001; Huntly and Gilliland, 2005).

Several prominent investigators in the 1970s and 1980s, notably Van Potter, Barry Pierce and Stewart Sell, argued that this (and by extension other tumours including carcinomas) might be brought about through superimposition of malignancy upon normal stem cells, with resultant "maturation arrest" creating a caricature of the normal tissue (Potter, 1978; Potter, 1987; Sell and Pierce, 1994).

Supporting this were observations made in many tumour types that cancer cells could show characteristics of more than one lineage, suggesting that transformation had

occurred in a multipotential cell (Sabbath *et al.*, 1985; Gorai *et al.*, 1997; Hellmen *et al.*, 2000). Investigations were restricted, however, by a limited understanding of the normal stem cell hierarchy. More recently, advances in the field of cell biology have renewed interest in stem cells and their properties, and facilitated more detailed examination both of their regenerative potential and how they might be involved in carcinogenesis (Weissman, 2000; Pardal *et al.*, 2003).

Evidence for Existence of the Cancer Stem Cell

Haematopoietic stem cells and leukaemia stem cells

The normal haematopoietic stem cell (HSC) is probably the best-defined of the adult TSC, although still not fully characterised (Figure 1B). As well as lacking the cell surface markers associated with specific, terminally differentiated haematopoietic lineages (Lin^-), these cells express the cell surface marker CD34, but not CD38 (expression of which occurs later in the differentiation process). HSC were first demonstrated by their ability to reconstitute the bone marrow of lethally irradiated mice (Till and McCullough, 1961). Mirroring this work, experiments using severe combined immunodeficient (SCID) mice, which lack B and T cells, indicated that normal HSC become engrafted in the bone marrow and could proliferate to recapitulate mature human haematopoietic lineages (Baum *et al.*, 1992; Vormoor *et al.*, 1994).

Work by John Dick and colleagues demonstrated that transplantation of these mice with cells from human haematopoietic malignancies could be performed in the same way. SCID mice injected with cells from human acute lymphoblastic leukaemia (ALL) developed disease with a similar course to that seen in man (Kamel-Reid *et al.*, 1989), and similar recapitulation of human disease was seen for chronic phase and blast crisis chronic myeloid leukaemia (CML), and for acute myeloid leukaemia (AML) (Lapidot *et al.*, 1994; Sirard *et al.*, 1996). Significantly, however, it was found that when cells from human AML were fractionated according to cell surface marker expression, inoculation of the $CD34^+CD38^-$ subset produced disease similar

to that of the unsorted cells. Moreover, $CD34^{+}CD38^{+}$ cells and $CD34^{-}$ cells did not produce leukaemia in the recipient. The frequency of these "SCID Leukaemia-Initiating Cells" (SL-IC) was estimated as 1 in 250000 total cells by limiting dilution assay (Lapidot *et al.*, 1994).

However, the SCID mouse model had a number of limitations, precluding definitive demonstration of tumour recapitulation properties, and it was possible to show these similarities between the SL-IC and normal HSC for only one leukaemia subtype. It was only after the development of the non-obese diabetic (NOD)/SCID mouse, which has additional immune response deficits (e.g. lack of Natural Killer cell activity and complement activation), that the engraftment experiments could be refined to show that SL-ICs had properties which met the definition of CSC. One of the problems with the SCID model had been the need to transplant large numbers of host cells to ensure engraftment in the recipient. It was demonstrated that 10 to 20 times fewer cells were required in the NOD-SCID mice to achieve the same level of engraftment (Bonnet and Dick, 1997).

Experiments using the NOD/SCID model system showed that, for a range of AML subtypes, both unfractionated bone marrow samples and purified $CD34^{+}CD38^{-}$ cells could reproduce the phenotype of the original human tumour in the recipient animal. This was seen even when the $CD34^{+}$ purified fraction represented a tiny (0.2%) proportion of blast cells in the human tumour. In the NOD/SCID recipient mouse, the heterogeneous human-derived blast population (with CD38 expressed by almost all cells, even when transplanted cells had been $CD38^{-}$) seemed to evoke normal haematopoietic differentiation.

Cells could be further transplanted into a secondary recipient, once again recapitulating the tumour; self-renewal was inferred by calculations suggesting that the SL-IC population must have expanded 30-fold. Thus, the $CD34^{+}CD38^{-}$ fraction specifically possessed both self-renewal and multilineage differentiation capacity. These findings were compelling evidence that for AML, transformation had occurred at the stage of the primitive stem cell, and that the heterogeneous leukaemic

population represented the differentiated but proliferation-restricted progeny of this cell, in the manner of the parent-progeny relationships seen for normal HSC (Bonnet and Dick, 1997).

A similarly hierarchical organisation, with a distinct, leukaemogenic stem cell (LSC) population, has since been reported for many different forms of haematological malignancy (Cozzio *et al.*, 2003; Jamieson *et al.*, 2004; Passegue *et al.*, 2004). As well as demonstration that tumourigenicity is held within specific sorted cellular fractions, some studies have introduced specific gene mutations into isolated haematopoietic cell types, and shown that only certain of these are capable of producing disease. For example, Passegue *et al.* (2004) showed that in a murine model of CML, inactivation of the transcription factor JunB in HSC, but not more committed cells, resulted in a myeloproliferative disorder with marked expansion of the granulocyte progenitor compartment and progression to blast crisis. Only transplantation of JunB-deficient HSC, and not myeloid precursors, could recapitulate the disease in immunosuppressed recipients (Passegue *et al.*, 2004). Although the function of JunB in myeloid neoplasia is not yet fully understood, more recent work has indicated that in the normal situation it acts to limit the proliferation of HSC whilst not affecting self-renewal potential, thus controlling the production of myeloid progenitors (Santaguida *et al.*, 2009). Therefore, whilst the malignancy manifests itself in this myeloid compartment, it originates from a lesion occurring more proximally in the developmental hierarchy.

Cancer stem cells in solid tumours

The extension of the CSC hypothesis to solid tumours has been more challenging experimentally. Probably the most significant factor in the demonstration of hierarchical organisation within haematopoietic malignancies has been an appreciation of that within the normal system, which is more clearly defined than that in most solid tissues. Importantly, cell surface markers for normal stem cells and their more differentiated progeny have been identified, facilitating the isolation and comparison of distinct populations.

For many solid tissues, understanding of the normal relationships between progenitors and progeny remains more limited, and assays which reliably identify stem cells have yet to be developed. A further obstacle is the physical nature of the tissues. Not only are cells generally larger and more fragile than blood cells, but often less accessible – for example, whilst blood or bone marrow sampling is likely to occur on multiple occasions during the course of a haematological disorder, solid tumour tissue is often available only at the time of surgery (and/or biopsy). This introduces either the requirement for immediate dissociation and analysis, which may be technically challenging as well as deleterious to viability, or initial expansion in culture, which may lead to a less accurate representation of cellular composition. In either case, the complex architecture and interplay between the various elements (including stromal and vascular components) of the mass, as well as its relationship to surrounding normal tissue, is unlikely to be well represented.

One technique which has become central to many studies investigating CSC in solid tumours is the "Neurosphere" assay. The system was developed and first reported in 1992 by Reynolds *et al,* as a means of propagating normal neural stem cells. They found that, under serum-free, low-density culture conditions and supplemented with epidermal growth factor (EGF), stem and progenitor populations of embryonic rat striatal cells could proliferate as clonal, spheroid clusters (neurospheres) whereas most (>99%) cells died (Reynolds *et al.*, 1992; Reynolds and Weiss, 1992).

Not only did neurosphere cells express the neuroepithelial stem cell marker nestin, but they had the capacity to differentiate into distinct populations expressing neuron-specific enolase (NSE) and glial fibrillary acidic protein (GFAP), markers for the neuronal and astrocytic lineages respectively. Dissociation of spheres and re-seeding led to generation of secondary spheres, at greater frequency than that seen when using unfractionated cells. Thus, the assay conditions and presence of EGF seemed to select for multipotent stem cells, defined by their capacity for self-renewal and multilineage differentiation (Reynolds *et al.*, 1992). Critically, the technique

presented a means by which primitive progenitors might be isolated, identified and propagated in culture.

As well as contributing towards the characterisation of normal (and by extension cancerous) neural stem cells, the neurosphere technique has been widely adapted as a means of enriching putative stem and progenitor cells from a wide variety of normal tissues and tumours. The ability to propagate a putative CSC population as "tumourspheres" for further characterisation is particularly significant where tumour tissues might only be accessible on a single occasion. The assay has limitations, and observations must be carefully interpreted (Reynolds and Rietze, 2005; Singec *et al.*, 2006) – nonetheless, along with flow cytometry, it has provided the basis for many reports of CSC identification.

Between 2000 and 2003, three separate reports from Eric Holland and colleagues reported that neural progenitor cells underwent transformation (increased proliferation (Fults *et al.*, 2002), tumour formation (Holland *et al.*, 2000; Dai *et al.*, 2001)) more readily than more differentiated cell types, in experiments inducing overexpression of the proto-oncogene Myc, the oncogenes Ras and Akt, and platelet derived growth factor (PDGF). It was also reported for the first time that using the neurosphere technique, cells could be derived from human brain tumours which showed self-renewal and multilineage differentiation capacity but were transcriptionally distinct from normal neurosphere-forming clones (through expression of mediators of the Notch signalling pathway, associated with cell fate determination and self-renewal) (Ignatova *et al.*, 2002).

2003 saw publication of the first evidence for the hypothesis that, within a solid tumour, all of the tumourigenic potential might be held by a fraction of the cells (Al-Hajj *et al.*, 2003). Al-Hajj *et al.* used flow cytometry to separate cells from human primary and metastatic breast carcinomas according to the expression of cell surface markers. For all but one tumour, cells in the $CD44^{+}CD24^{-/low}Lineage^{-}$ fraction required much lower inoculums (ten to fifty times fewer cells) than the unsorted population to form tumours in immunosuppressed mice. Tumourigenicity

was further enhanced by additionally selecting for those cells expressing high levels of ESA (epithelial surface antigen, epCAM).

Significantly, $CD24^{+}Lineage^{-}$ cells were unable to form tumours except in one subject. $CD44^{+}CD24^{-/low}$ $Lineage^{-}$ cells could be serially passaged in mice, forming tumours from which further $CD44^{+}CD24^{-/low}Lineage^{-}$ cells could be isolated (i.e. self-renewal capacity), as could be the other nontumorigenic cell populations found in the original tumour (i.e. multilineage differentiation) (Al-Hajj *et al.*, 2003). The ability to demonstrate these two cardinal CSC properties *in vivo* was considered a breakthrough in the demonstration of the CSC hypothesis, and this remains one of the most frequently cited experimental reports in the field. Critical also was the fact that these cells had been *prospectively* identified – confirming the concept that it might be possible to accurately predict the identity of the solid tumour CSC, making it a relevant clinical target.

Much of the earliest published work on prospective isolation of tumourigenic CSC relates to brain and breast cancer. The isolation was reported of brain tumour stem cells from a number of human nervous system cancers including astrocytomas, glioblastoma multiforme, medulloblastomas and ependymomas. The cells formed neurosphere clones in serum-free culture in the presence of mitogenic growth factors, and showed multipotent differentiation capacity when placed back into adherent culture, with the ability to form all of the lineages seen in the initial tumour (Ignatova *et al.*, 2002; Hemmati *et al.*, 2003; Singh *et al.*, 2003; Galli *et al.*, 2004; Singh *et al.*, 2004).

Expression of the foetal neuronal stem cell marker CD133 was also associated with these putative CSC populations (Hemmati *et al.*, 2003; Singh *et al.*, 2003; Singh *et al.*, 2004). The *in vivo* ability of neurosphere (Hemmati *et al.*, 2003; Galli *et al.*, 2004) or $CD133^{+}$ cells (Singh *et al.*, 2004) to recapitulate the primary tumour mass was tested by subcutaneous and/or intracranial (orthotopic) transplantation in rodents. The resulting xenografts showed striking similarities to the tumours from

which the progenitors were derived, albeit with a "peculiar histomorphology" in some cases (Galli *et al.*, 2004).

CSC with transplantable tumourigenic capacity were also isolated from human breast cancers according to their ability to form spheroid clones *in vitro*. A low-adherence, clonal density serum-free *in vitro* culture system had been developed in the laboratories of Max Wicha, and could be used to propagate "mammospheres", enriched in mammary stem/progenitor cells, from normal human mammary tissue. As with the neurosphere assay, most primary mammary epithelial cells died under these conditions, but a few generated spheroidal colonies of cells. These were capable of self-renewal (passage), and of differentiation into the three cellular lineages seen in adult mammary tissue (Dontu *et al.*, 2003).

Extending this, Ponti *et al* described derivation of mammosphere-forming cells from human primary breast tumours and the well-characterised human luminal breast carcinoma cell line MCF-7, which could be serially passaged *in vitro*. Cells within mammospheres were reported as having a predominantly $CD44^{+}CD24^{Low/-}$ surface expression pattern, consistent with the findings of Al-Hajj *et al*, and also to express the embryonic stem cell-associated transcription factor Oct4. Isolated $CD44^{+}CD24^{Low/-}$ cells formed xenografts in immunocompromised mice, phenotypically similar to the human breast tumours from which they originated (Ponti *et al.*, 2005).

Similarly, the identification of candidate $CD44^{+}\alpha_2\beta_1$-$integrin^{High}$ normal prostate stem cells (Collins *et al.*, 2001) facilitated the purification of a population of putative prostatic CSC. These cells also expressed the brain tumour stem cell-associated surface marker CD133 (Richardson *et al.*, 2004). Although it had been observed in many of the brain cancer reports that the frequency of putative CSC appeared to positively correlate with the aggressiveness of the tumour, no association was found between the frequency of prostatic CSC and Gleason score (by which primary prostate tumours are graded) or metastatic status (Collins *et al.*, 2005).

Since these initial reports, putative CSC populations have been reported for primary cells and cell lines representing a wide range of solid tumours, including lung, liver, pancreatic, gastric, colon, nasopharyngeal, head and neck, ovarian and endometrial cancers, osteosarcoma and melanoma (Chu *et al.*, 1997; Bapat *et al.*, 2005; Fang *et al.*, 2005; Gibbs *et al.*, 2005; Dalerba *et al.*, 2007; Harper *et al.*, 2007; Li *et al.*, 2007; Ma *et al.*, 2007; Monzani *et al.*, 2007; O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007; Atsumi *et al.*, 2008; Fujii *et al.*, 2008; Chan *et al.*, 2009; Hubbard *et al.*, 2009).

Prospective Isolation – How can you tell it's a Cancer Stem Cell?

Surface phenotype

A wide variety of surface markers, singly or in combination, has been used to isolate putative CSC from tumour cells and cell lines. These include CD34, CD44, CD49, CD117, CD123 and CD133 (Bonnet and Dick, 1997; Singh *et al.*, 2004; Collins *et al.*, 2005; Jin *et al.*, 2006; Suetsugu *et al.*, 2006; Li *et al.*, 2007; Ma *et al.*, 2007; Monzani *et al.*, 2007; Chiou *et al.*, 2008; Hong *et al.*, 2008; Hurt *et al.*, 2008; Yang and Chang, 2008; Zhang *et al.*, 2008a; Jin *et al.*, 2009; Takaishi *et al.*, 2009). Other putative CSC subsets are characterised by their lack of expression of certain markers (such as CD38 for AML stem cells, or CD138 for those of multiple myeloma) (Bonnet and Dick, 1997; Matsui *et al.*, 2004). Indeed, although the seminal breast cancer work done by Al-Hajj is frequently cited as evidence of CD44's status as a putative CSC marker, the report shows that $CD44^{+}CD24^{+}$ cells did not show enhanced tumourigenicity – indeed, many specimens were predominantly $CD44^{+}$ - suggesting that CD24 expression level is more significant in this model (Al-Hajj *et al.*, 2003).

Markers tested for a given tumour have frequently been selected based on their ability to isolate tumourigenic fractions in other tumour types. CD133, for example, has been reported to enrich for CSC in tumours from a variety of tissues including colon, lung, liver and melanoma (Ma *et al.*, 2007; Monzani *et al.*, 2007; O'Brien *et al.*, 2007; Chen *et al.*, 2008), although prior to early brain tumour stem cell reports it

was recognised primarily as a marker of primitive haematopoietic, endothelial and neural cells (Handgretinger *et al.*, 2003; Hristov and Weber, 2004; Hess *et al.*, 2006).

The utility of CD133 expression as a CSC marker is a subject of some debate, particularly as detection method appears to have considerable bearing upon results (Bidlingmaier *et al.*, 2008; Smith *et al.*, 2008; Boegl and Prinz, 2009), as does glycosylation status of the protein. Indeed, whilst data from two groups, published simultaneously in 2007, described isolation of tumourigenic $CD133^+$ colon CSC (O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007), other investigators reported soon after this that they had found $CD133^-$ cells to show similar activity to the $CD133^+$ fraction (Shmelkov *et al.*, 2008). This is critical – arguably, to substantiate the CSC hypothesis (by confirming hierarchical organisation within a tumour), it is as important to show that specific cells are *not* tumourigenic as to show that others are.

It is becoming clear that there is unlikely to be a universal CSC marker. Significantly, the function of some surface markers employed in CSC isolation, including CD133 and CD34, remains unclear. Also, it is not always clear why a given expression pattern should be associated with more tumourigenic cells – for example, although a $CD24^{low/-}$ phenotype is reported for many putative CSC populations, CD24 has been associated with enhanced proliferation, motility and invasion of cells in breast cancer as well as other solid and haematopoietic tumours (Baumann *et al.*, 2005; Lim and Oh, 2005; Kim *et al.*, 2007).

Conversely, tumourigenic pancreatic and colorectal carcinoma cells are described which express high levels of CD24 (Li *et al.*, 2007; Vermeulen and al., 2008), and work by Shipitsin *et al* suggested that the $CD24^+$ cells of some human breast tumours were genetically distinct from (rather than the progeny of) $CD44^+$ cells (Shipitsin *et al.*, 2007). Thus, it appears that CSC markers may vary, not only between tumours of diverse origin, but possibly also between individual types and/or subtypes (or even individual tumours) derived from the same tissue. This will necessitate testing with a wide range of candidate markers to establish and refine the surface phenotype of putative CSC.

Certain biological associations can inform these choices – for example, CD44, whilst not a defined epithelial stem cell marker, is generally expressed more heavily in the basal layers of epithelia (including prostatic and breast), where self-renewing cells are located (Alho and Underhill, 1989; Abbasi *et al.*, 1993; Collins *et al.*, 2001). Similarly, whilst CD34 is expressed in haematopoietic and endothelial tissues (Baum *et al.*, 1992; Asahara *et al.*, 1997), it seems unlikely that it would identify stem (or any other) cells of mesenchymal or epithelial origin, as it is not associated with these cell types at any stage of differentiation. Nonetheless, cancer is a disorder characterised by cellular mutation, and derangements of normal surface phenotype are likely.

Functional assays

As well as providing a platform for analysis of surface phenotype, flow cytometry is widely used to investigate the utility of functional stem cell assays for detecting CSC. The most frequently described of these is a technique which assesses efflux of Hoechst 33342 through demonstration of a "side population".

The side population (SP) phenotype was first identified in haematopoietic stem cells – when incubated with the fluorescent substrate Hoechst 33342, a minority subset of murine bone marrow mononuclear cells (BMMNC) were able to efflux the dye, and showed low Hoechst fluorescence at flow cytometry (Goodell *et al.*, 1996). These Hoechst-dull cells, representing approximately 0.05% of whole murine BMMNC, fell to the side of the bright-staining main population of cells and were predominantly positive for the murine HSC marker Sca-1 but negative for markers of more differentiated cells. When isolated by FACS, cells in the SP were enriched for repopulating capacity; intriguingly, the cells were negative for the classical HSC marker CD34, and have thus been postulated to represent an even more primitive $CD34^-$ population of HSC (Goodell *et al.*, 1996).

Subsequently, the technique was used to identify putative normal stem cell populations in a variety of tissue types. The dye-efflux SP phenotype for

haematopoietic and several other tissue progenitor cells has been associated both *in vitro* and *in vivo* with expression of the ATP binding cassette (ABC) transporter ABCG2 (Zhou *et al.*, 2001; Scharenberg *et al.*, 2002), thought to facilitate these cells' long lifespan by allowing them to efflux toxic substances. Critically, this membrane transporter protein also plays an important role in multiple drug resistance in cancer (Bunting, 2002; Doyle and Ross, 2003).

In 2004,Kondo *et al* described the presence of an SP phenotype in several cancer cell lines upon Hoechst efflux analysis, including the well-characterised rat C6 glioma and human MCF-7 breast carcinoma. The SP fraction (0.4%) of C6 glioma was increased in serum-free neurosphere culture, showed multilineage differentiation capacity and was more tumourigenic than non-SP cells (Kondo *et al.*, 2004). The same year, Hirschmann-Jax *et al* reported an SP phenotype in 5/5 human neuroblastoma cell lines and 15/23 (65%) of primary human neuroblastomas, as well as a variety of human tumour cell lines including those of breast, colon, ovary and brain (Hirschmann-Jax *et al.*, 2004). The technique has since been used to isolate SP cells from many tumours and cancer cell lines, and variously reported to enrich for cells showing CSC-associated attributes such as surface marker expression, drug resistance and tumourigenicity (Wang *et al.*, 2007; Chua *et al.*, 2008; Engelmann *et al.*, 2008; Matsui *et al.*, 2008; Wu and Alman, 2008).

The demonstration of this phenotype in a subpopulation of tumour cells is consistent with the concept of a CSC fraction, with stem cell-associated properties, which may escape from cytotoxic challenge and mediate relapse after chemotherapy. Other assays developed to identify HSC based on resistance mechanisms, such as expression of the enzyme aldehyde dehydrogenase (ALDH), have been employed to identify putative CSC populations in cancer (Pearce *et al.*, 2005; Ginestier *et al.*, 2007; Matsui *et al.*, 2008, Preffer and Dombkowski, 2009).

Which Cell is the Target for Transformation?

The term "cancer stem cell" to describe a cell with the functional capacity to maintain tumour growth has led to considerable confusion within the CSC field. For

a cell to be considered a stem cell, it must have two cardinal properties – the ability to self renew, and the ability to give rise to all of the cell types within the relevant tissue. This also applies to CSC. This does not, however, equate to an assertion that the CSC must have arisen from the transformation of a normal stem cell. Although alternative nomenclature has been suggested, including terms such as "Tumour-Initiating Cell" and "Tumour-Propagating Cell", these have not necessarily helped to resolve this misconception (Clarke *et al.*, 2006; Jordan, 2009).

An improved understanding of how stem cells are arranged within normal tissues has enabled experiments using targetted expression / deletion strategies, to determine the cell in which mutations initially arise. Region-specific p53 mutation implicated neural stem and/or transit-amplifying populations in the subventricular zone of the brain as the source of glioma precursors in a murine model – whilst not in itself conveying a growth advantage, the mutation was permissive for further oncogenic mutations in affected cells (Wang *et al.*, 2009).

Similarly, facilitated by identification of a normal $LGR5^{+}$ stem cell compartment at the crypt base in normal colon, it was found that knockout of the APC tumour suppressor in these cells (but not more differentiated transit-amplifying cells) resulted in rapidly expanding adenomas (Barker *et al.*, 2009). Nonetheless, as even proponents of the hypothesis will point out, in a fully developed tumour, further mutations are likely to occur. Thus, the CSC maintaining the tumour will not necessarily be the same cell that sustained the initial oncogenic "hit" (Clarke *et al.*, 2006).

As discussed, many of the attributes of normal stem cells make them attractive candidates for malignant transformation into CSC. They are "pre-programmed" for self-renewal and multilineage differentiation. They persist and continue to divide for the lifetime of the host, allowing them more opportunity to accrue transforming mutations in tissues where turnover is rapid, such as epithelia. In many cases, isolated putative CSC are phenotypically similar (e.g. expression of cell surface markers) to the corresponding normal tissue stem cell. The functional attributes

which might enable putative CSC to persist after therapy, such as drug efflux capacity and (more speculatively) quiescence, are features of many normal stem cell populations (Reya *et al.*, 2001).

At the same time, multipotent stem cells represent a small minority of cells within a tissue. An individual stem cell would be a very small target population for the accumulation of sufficient mutations to confer a neoplastic phenotype. Also, pathways controlling normal stem cell function, such as the Wnt-β-catenin pathway, show specific mutations in many cancers (Reya *et al.*, 2003; Reya and Clevers, 2005) – this would perhaps be unexpected if normal stem cell processes had simply been co-opted during tumourigenesis. Moreover, normal stem cell resistance mechanisms such as enhanced DNA repair capacity, quiescence or resistance to xenotoxic substances, might be expected to protect them from acquiring mutations, with their downstream progeny more susceptible.

Polyak and Hahn propose 3 models for the origin of CSC (Polyak and Hahn, 2006) (Figure 5):

1) A mutation causes dysregulation of asymmetric division in a tissue stem cell (TSC), and is passed on to all progeny. Progression to full transformation occurs in this progeny population as further mutations are acquired.
2) The TSC itself acquires mutations sufficient for malignancy, and passes these on to all progeny.
3) The transit-amplifying cells or more differentiated progeny accrue mutations leading to dedifferentiation and acquisition of stem-cell like properties; TSCs themselves are not involved.

Evidence exists to support all of these mechanisms as a potential source of CSC.

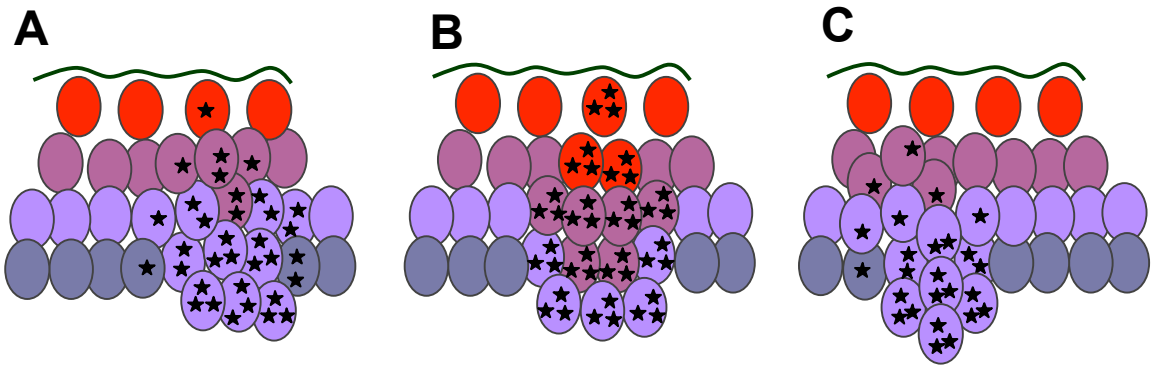


Figure 5 – Origin of the CSC
A – Initial mutation arises in tissue stem cell and is passed on to progeny; acquisition of further mutations by progeny leads to full neoplastic transformation.
B – All of the mutations required for neoplastic transformation occur in tissue stem cell, and are passed on to progeny.
C – Initial and subsequent mutations, including the capacity for unlimited self-renewal, arise in more differentiated cell types - tissue stem cells are not involved.

(Adapted from Polyak and Hahn, 2006 *Nature Medicine*, **12**, 296-300).

For example, both primitive HSC and committed myeloid progenitors produce a transplantable murine model of AML when transduced with a Mixed Lineage Leukaemia (*MLL*) fusion gene. Moreover, the phenotypic characteristics were identical in leukaemias derived from transplanted HSCs, common myeloid progenitors (CMP) or granulocyte-macrophage progenitors (GMP), suggesting that transformation could occur in both primitive or more committed cells (Cozzio *et al.*, 2003). Similarly, whilst development of malignant gliomas *in vivo* occurred more readily upon induction of autocrine PDGF signalling in neural progenitors, induction in differentiated astrocytes also induced tumour formation in mice, enhanced by loss of function of the *Ink-4a* tumour suppressor locus (Dai *et al.*, 2001).

By contrast, mutation of the CEBPA transcription factor (seen in 7-10% of human AML) in a murine model appears to have the HSC as the target for transformation. This produces a "pre-leukaemic" state whereby cells show slight increases in proliferative capacity. However, in this leukaemia subtype, the leukaemia stem cell

(LSC) arises downstream in the myeloid compartment, although it has a specific expression profile ($CD117^+$, $Sca1^-$, $Mac1^+$) which is distinct from either HSC or myeloid progenitors (Kirstetter *et al.*, 2008; Bereshchenko *et al.*, 2009).

A preleukaemic cell was also reported in elegant studies of childhood B-cell ALL in a pair of monochorionic twins, one of whom had developed leukaemia as the result of a chromosomal translocation generating an abnormal fusion protein (TEL-AML1). Low numbers of TEL-AML1-positive cells were also detected in the peripheral blood of the normal twin. The disease is associated with production of an aberrant $CD34^+CD38^{-/low}CD19^+$ cancer-propagating clone – again, low numbers of cells with this phenotype could be detected in the normal twin, although analysis of V-D-J recombination status indicated that the cell was at an earlier stage of differentiation than the LSC observed for the leukaemic twin. Collectively, the results of this study suggested that the initial fusion mutation had arisen at HSC or pro-B cell stage, leading to a preleukaemic cell, with progression to disease in the leukaemic twin after additional loss of the normal TEL allele (Hong *et al.*, 2008).

Although the "dedifferentiation" of a committed cell into one with more primitive, stem-like properties might seem an unlikely event, it has been shown in the fruit fly *Drosophila melanogaster* that cells during early development can be induced to revert to germinal stem cells (Kai and Spradling, 2004). Moreover, rather than full reversion to a TSC, the cardinal property to be acquired by a more committed cell, if it is to function as a CSC, is self renewal – as will be discussed further, *de novo* alterations affecting self renewal capacity are frequently observed in cancer. For example, β-catenin is the downstream effector protein of Wnt signalling, associated with self-renewal and proliferation. In CML, increased nuclear β-catenin was reported in myeloid progenitors as the disease advanced to blast crisis, whilst levels in HSCs remained stable. *In vitro* self-renewal capacity was demonstrated both by GMPs from leukaemic patients, and by normal GMPs with forced β-catenin expression, suggesting that disease might originate in a more committed cell if it acquired stem cell-like attributes (Jamieson *et al.*, 2004).

It has also been proposed that, rather than unlimited self-renewal capacity being conferred by gain-of-function mutations, it may in fact be a "default" pathway (seen, for example, in most single-celled organisms). Thus, if tissue specialisation relies on a balance between self-renewal, differentiation and cell death, any cell in which apoptosis is prevented or differentiation is blocked (the effects of many of the mutations seen in cancer) could act as a self-renewing CSC (Passegue *et al.*, 2003).

Observations made in cancer may contribute to understanding of normal stem cell hierarchies. For example, one hallmark feature of CML is the Philadelphia chromosome – this truncated chromosome 22 results from a reciprocal translocation with chromosome 9, and produces the BCR-ABL fusion protein. The protein occurs in multiple haematopoietic lineages in CML, suggesting that the translocation event arises in a HSC (Daley, 2004; Jamieson *et al.*, 2004). Studies of normal haematopoiesis have recently suggested that there exists an even more primitive population of precursors within the bone marrow, the putative "haemangioblast", which gives rise to both haematopoietic and endothelial lineages (Schatteman and Awad, 2004). Consistent with this, demonstration of BCR-ABL expression in *endothelial* cells from a CML patient may point to the mutational event having occurred in a cell preceding the HSC in the haematological hierarchy, with greater differentiation potential (Gunsilius *et al.*, 2000).

Potential role of Mesenchymal Stem Cells in cancer

Adult mesenchymal stem cells (MSC) are widespread in normal bone marrow (hence the alternative terminology "bone marrow-derived stromal cells", BMSC); they have also been isolated from other tissues, including adipose tissue. These cells show remarkable plasticity, with multilineage potential including adipogenic, chondrogenic and osteogenic differentiation capacity (Pittenger *et al.*, 1999). They also have the ability to mobilise and migrate in the circulation to distant sites, in response to tissue stress including chronic inflammation and injury, where they may promote repair processes including angiogenesis (Lapidot *et al.*, 2005). These cells do not express telomerase and show limited self-renewal *in vitro* (Zimmermann *et*

al., 2003), undergoing replicative senescence after multiple passage (Baxter *et al.*, 2004), although some evidence suggests that they may be susceptible to transformation in long-term culture, resulting in immortalisation (Rubio *et al.*, 2005; Izadpanah *et al.*, 2008).

MSC show a number of similarities with tumour cells, including an "undifferentiated" phenotype, proangiogenic influence and a propensity for migration. The complex, co-ordinating mechanisms involved in the recruitment of MSC to distant sites are also reported to influence the migration and metastasis of cancer cells. For example, interactions between the CXCR4 cell surface receptor and site-specific secretion of its ligand SDF-1 are thought to influence MSC trafficking (Chamberlain *et al*., 2007). As CXCR4 may be expressed on the surface of cancer cells, and SDF-1 is highly expressed in tissues including lymph node, lung, liver and bone, this could explain the propensity for metastatic spread to these organs (Ceradini and Gurtner, 2005; Kucia *et al.*, 2005).

In a murine breast cancer xenograft model, the motility, invasion and metastatic ability of human MDA-MB-231 cells was enhanced by MSC, through interaction of the CCR5 receptor on the tumour cells with its chemokine ligand CCL5, secreted by the MSC (Karnoub *et al.*, 2007). Data published by Galie *et al* showed that the mesenchymal tumour cell component of some carcinomas had a common molecular signature with MSC, and co-implantation of cancer cells with MSC favoured tumour growth in syngeneic rodents - it was postulated that this was the result of proangiogenic effects (Galie *et al.*, 2008).

Moreover, studies have suggested that MSC recruited to tumours may actively participate in the neoplastic process. In a murine model of *Helicobacter pylori*-induced gastric carcinoma, it was found that MSC migrating to the tumour site from bone marrow could contribute towards the abnormal mucosa. It was demonstrated that, rather than a cell fusion event occurring between MSC and gastric tumour cells, the MSC acquired a gastric mucosal phenotype with upregulation of epithelial

markers (Houghton *et al.*, 2004). Thus, it appears that for some tumours, recruitment of these multipotent stem cells may play a role in progression.

Signalling mechanisms regulating CSC function

Many of the signalling pathways and genetic mechanisms dysregulated in cancer are involved with the regulation of normal stem cell function. *HOX* transcription factors, the Wnt-βcatenin, Notch and Sonic Hedgehog (SHh) pathways, and the Polycomb gene *Bmi-1* are important in fate determination, self-renewal and other stem cell properties. Consistent with this, alterations in expression have been reported in putative CSC populations of tumours of both the haematopoietic system and solid tissues (Taipale and Beachy, 2001; Lessard and Sauvageau, 2003; Pardal *et al.*, 2003; Behbod and Rosen, 2005; Huntly and Gilliland, 2005; Zhao *et al.*, 2009).

Bmi-1

Bmi-1 is a member of the Polycomb group of transcriptional repressor proteins, which acts through the *ink-4a* locus to downregulate the tumour suppressors encoded there – $p16^{INK4a}$ and $p19^{ARF}$. Bmi-1 overexpression in mouse embryonic fibroblasts leads to their immortalisation, and in co-operation with *ras* can cause neoplastic transformation (Jacobs *et al.*, 1999). Correspondingly, loss of expression is associated with reduced proliferative capacity, in both normal haematopoietic precursors derived from foetal liver cells, and leukaemic stem cells in a mouse model of acute myeloid leukaemia (Lessard and Sauvageau, 2003). Bmi-$1^{-/-}$ leukaemias were not transplantable into secondary recipients, although this capacity could be rescued by introduction of a retroviral *Bmi-1* provirus. Interestingly, this Bmi-1-mediated rescue was also seen in Bmi-$1^{-/-}$ clones with defects in the expression of $p16^{INK4a}$ and $p19^{ARF}$, indicating additional pathways through which the molecule exerts its effects (Lessard and Sauvageau, 2003).

Glinsky *et al* investigated the role of Bmi-1 in human prostate cancer using microarray analysis; elevation in expression was reported for all tested cancer cell lines, with more metastatic tumour types showing greater increases. An 11-gene

signature, associated with Bmi-1 function in normal stem cells, was expressed in 11 different types of cancer, and consistently predicted metastasis and poor prognosis (Glinsky *et al.*, 2005).

Notch

The four Notch transmembrane receptors found in mammals are activated by their ligands Delta and Jagged, as well as other members of the DSL (Delta, Serrate and Lag-3) family. Activation of the pathway results in transcription of target genes, associated with processes such as cell fate determination during development, and self-renewal in adult tissues (Weng and Aster, 2004).

Notch signalling has been demonstrated as oncogenic in mouse models of T-cell Acute Lymphoblastic Leukaemia (T-ALL) (Hoemann *et al.*, 2000), and can collaborate with the c-neu/erbB2 oncogene in the development of mammary tumours (Dievart *et al.*, 1999). Dontu *et al* showed that activation of Notch signalling promoted self-renewal and proliferation of normal mammary stem/progenitor cells cultured in mammospheres, but had no effect on fully committed mammary epithelial cells, suggesting that it exerts its oncogenic potential at the progenitor level (Dontu *et al.*, 2004).

Microarray analysis of $CD34^{+}CD38^{-}$ LSC from AML has indicated overexpression of *Jagged-2*, with inhibition of *Jagged* and *Notch* signalling reducing LSC growth in colony forming assays (Gal *et al.*, 2006). Interestingly, loss-of-function Notch pathway mutations are also reported to contribute to neoplastic transformation, demonstrating that the role of Notch-activated gene expression is context dependent (Nicolas *et al.*, 2003). For example, in one study of cortical glial tumours, *Jagged-2* was expressed by neurospheres derived from normal, but not malignant, tissue (Ignatova *et al.*, 2002).

Wnt and β-catenin

One of the signalling transduction processes most frequently associated with putative CSC populations is Wnt-β-catenin. Wnt signalling influences cell migration and

developmental patterning, proliferation and survival, through the binding of β-catenin to the LEF/Tcf transcription factors and activation of downstream genes (Wodarz and Nusse, 1998; Taipale and Beachy, 2001).

β-catenin, which has a very short half-life, is normally sequestered in the cytoplasm by a "destruction complex" which phosphorylates the protein, targetting it for ubiquitin-mediated degradation by the proteasome. The destruction complex comprises four proteins including the APC tumour suppressor, mutation of which is a critical factor in human colon cancer. The binding of Wnt proteins to their Frizzled cell surface receptors inhibits this multiprotein complex, permitting cytoplasmic accumulation of β-catenin and translocation to the nucleus to activate gene expression (Taipale and Beachy, 2001; Reya *et al.*, 2003; Reya and Clevers, 2005). Overactivation of the pathway with increased nuclear β-catenin has been reported in haematological and solid malignancies, including those of intestine, prostate, ovary, brain and skin (Pardal *et al.*, 2003).

Overexpression of β-catenin in transgenic murine HSCs is reported to increase their self-renewal capacity (Reya *et al.*, 2003). Similarly, the pathway has been implicated in the self-renewal of LSC – for example, the progenitor population of CML shows increased β-catenin levels, and self renewal of these cells is inhibited *in vitro* by enforced expression of the Wnt signalling antagonist Axin (Jamieson *et al.*, 2004). Moreover, inhibition of β-catenin in CML progenitors *in vivo* prevented transplantation of the disease to secondary murine recipients (Hu *et al.*, 2009). The authors postulated that the survival and self renewal capabilities conferred by Wnt signalling might contribute to resistance of some CML LSC to the tyrosine kinase inhibitor imatinib mesylate.

For a number of solid tissues including breast and intestinal epithelia, aberrant Wnt signalling has been demonstrated preferentially to induce tumourigenesis in progenitor populations. For example, in conditional knockout experiments in a murine model of colon cancer, targetted deletion of APC in $LGR5^+$ intestinal crypt stem cells resulted initially in β-catenin accumulation within these cells, and was

associated with increased proliferation. Within days, foci of β-catenin^{High} cells were observed within the transit-amplifying compartment, leading to the formation of β-catenin^{High} microadenomas, although LGR5-positive cells remained confined to the crypt base suggesting maintenance of a developmental hierarchy (Barker *et al.*, 2009). Interactions between Wnt-β-catenin signalling and the Notch pathway (Fre *et al.*, 2009), and also the CSC-associated cell surface receptor CD44 (Zeilstra *et al.*, 2008), have also been implicated in early intestinal tumourigenesis.

Wnt signalling has also been reported to mediate drug resistance in cancer and putative CSC populations. Activation of Wnt signalling in human hepatocellular carcinoma (HCC) was reported to induce resistance to 5-Fluorouracil (5-FU) *in vitro*. Moreover, a subpopulation of cells, which express the cell surface marker OV6, can be isolated from primary human HCC and cell lines, and shows enhanced Wnt signalling as well as increased tumourigenicity and reduced sensitivity to chemotherapy. Inhibition of β-catenin decreases the proportion of these putative progenitors and increases their drug sensitivity, suggesting that Wnt plays a role in the self-renewal and survival of OV6^{+} putative HCC progenitor cells (Yang *et al.*, 2008).

Markers / regulators of pluripotency – Oct4 and Nanog

Upregulation of tissue-specific "stemness" indicators is reported for many CSC populations. For example, nestin is an intermediate filament protein and a marker for neuroepithelial precursors (Lendahl *et al.*, 1990); increased expression has been reported in undifferentiated normal and brain tumour-derived neurospheres (Reynolds *et al.*, 1992; Hemmati *et al.*, 2003; Singh *et al.*, 2003), as well as putative melanoma CSC (Na *et al.*, 2009) – the latter is consistent with the origin of melanocytes in the embryonic neural crest.

More controversial has been the identification of CSC based on expression of the embryonic stem cell-associated transcription factors Nanog and Oct4 (also Oct 3/4 or POU5F1). Critical to self-renewal and pluripotency of ESC (Chambers and Smith, 2004; Loh *et al.*, 2006; Liang *et al.*, 2008; Torres and Watt, 2008), they are also

expressed in germ cells, but this is strongly suppressed in more differentiated cell types by promoter hypermethylation (Lengner *et al.*, 2007; Cantz *et al.*, 2008).

The expression of Oct4 by a putative CSC population was first reported by Ponti *et al,* in their study of breast cancer-derived mammosphere cells (Ponti *et al.*, 2005). Gibbs *et al* derived "sarcospheres" from osteosarcomas and chondrosarcomas, which showed upregulation of both Oct4 and Nanog, which reduced if tumourspheres were allowed to differentiate in standard serum-contaning, adherent culture (Gibbs *et al.*, 2005). Demonstration of Oct4 and Nanog expression has since been used to substantiate reports of putative CSC isolation in numerous studies.

Expression of both was associated with outcome and survival time in oral cancer, with Nanog proving the worse prognostic indicator of the two (Chiou *et al.*, 2008). Bourguignon *et al* use immunoprecipitation to demonstrate interactions between Nanog and the stem cell-associated marker CD44 and its ligand hyaluronan (Bourguignon *et al.*, 2008). Webster *et al* found nuclear Oct4 expression in all examined canine tumours (29 tumours, 11 tumour types) using immunohistochemistry (Webster *et al.*, 2007), and Wilson *et al* reported upregulation of both Nanog and Oct4 on tumoursphere populations derived from canine osteosarcoma (Wilson *et al.*, 2008).

More recently, considerable debate has arisen over the expression of these proteins, particularly Oct4, in adult stem or CSC populations. A cell with preexisting pluripotency / self-renewal capability is an attractive candidate for transformation to a CSC. However, their role in normal adult TSC remains controversial (Berg and Goodell, 2007). A recent comprehensive study involving both conditional knockout and eGFP reporter models demonstrated that Oct4 is not required for somatic stem cell pluripotency (Lengner *et al.*, 2007).

Oct4 was previously detected in mixed teratomas and embryonal carcinomas formed in murine models upon implantation of ESC, and in certain types of germ cell tumour (Gidekel *et al.*, 2003; Looijenga *et al.*, 2003). Notably, however, in both of

these studies (published prior to the rush of interest in Oct4 as a potential CSC marker) almost every non-germ cell tumour evaluated was Oct4-negative (in a tissue microarray performed by Looijenga *et al*, examining over 3600 tumours of over 100 types, only two lung tumours and one kidney tumour tested positive for Oct4 (Looijenga *et al.*, 2003)). It is possible that, in considering entire tumour cell populations rather than those enriched for putative CSC, expression by a minority subset was not detected.

However, a number of factors suggest a high likelihood of false positives when interrogating samples for these "stemness markers". These include the transcription of non-functional pseudogenes (Looijenga *et al.*, 2003; Suo *et al.*, 2005; Liedtke *et al.*, 2007; Liedtke *et al.*, 2008), and splice variants with cytoplasmic localisation which are therefore unlikely to have relevant transcription factor activity (Takeda *et al.*, 1992; Liedtke *et al.*, 2008). Immunological detection may also pick up other members of the POU gene family due to protein sequence homology, particularly where polyclonal antibodies are employed (Lengner *et al.*, 2007). Cantz *et al.* demonstrated elegantly that overzealous processing of immunofluorescence images can create apparent staining from non-specific and background signal (Cantz *et al.*, 2008). Moreover, at mRNA level, it is likely that there is a background level of RT-PCR signal which does not represent true transcription factor function (Lengner *et al.*, 2007; Cantz *et al.*, 2008).

Evaluation of some of the evidence for Oct4 or Nanog expression as markers of CSC reveals inconsistencies, which may reflect the confounding factors outlined above. For example, in the study by Gibbs *et al.*, protein is detected by both Western blot and immunohistochemistry in cell lines that show no mRNA signal at RT-PCR. Moreover, Oct4 localisation in some tumourspheres shown cannot be identified, but appears to be pancellular rather than nuclear (Gibbs *et al.*, 2005).

Despite "irrational exuberance" (Berg and Goodell, 2007) in some of the reporting with regards these proteins, Hochedlinger *et al* demonstrated in an adult mouse model that ectopic Oct4 expression in somatic tissues could induce dysplasia, with

progenitor cell expansion and increased β-catenin expression (Hochedlinger *et al.*, 2005). The significance of expression has been further emphasised recently by groundbreaking reports that introduction of Oct4 and Nanog expression in terminally differentiated cell types, in combination with other mediators such as Sox2, can generate "induced pluripotent stem cells" (iPS cells) (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007; Yu *et al.*, 2007). Thus, the potential role for Oct4 and Nanog in cellular transformation warrants investigation. However, it has become clear that apparent expression must be analysed critically, preferably using a multimodal approach including confirmation of nuclear localisation, and using appropriate controls.

Telomeres, telomerase and cancer stem cells

When grown in culture, the phenomenon of *senescence* limits the replicative potential of cells, in that eventually the cells will stop dividing (Hanahan and Weinberg, 2000). This is partly governed by the gradual loss of the protective telomeres, tandem repeats of a 6 bp sequence which are present at the ends of chromosomes. DNA polymerases cannot fully replicate the 3' end of the DNA strand, such that upon each cell division there is a loss of 50-100 bp at each end of every chromosome. The telomeres buffer this loss but, as they are of finite length, they gradually become shorter through the life of a dividing cell – this protective mechanism means that the cell will be directed to senescence or apoptosis after a certain number of divisions (Argyle and Nasir, 2003; Ju and Rudolph, 2006).

This "end replication problem" is mitigated by the expression of of *Telomerase*, an enzyme which catalyses the addition of telomeric repeat sequences on to the ends of chromosomes. High levels of telomerase are characteristic of embryonic stem cells (Thomson *et al.*, 1998); low levels have been reported in adult stem cells from a variety of tissues, although telomerase activity in these populations is more controversial and cannot be considered a stem cell marker *per se* (as upregulation may occur in tissues where cells undergo rapid expansion) (Hiyama and Hiyama, 2007).

Telomerase is also expressed in over 80% of human cancers, and is presumed to confer unlimited cell cycling ability. Paradoxically, many tumours show foreshortened telomeres. A two-step process has been proposed, whereby early telomere shortening promotes chromosomal instability and mutation, and then telomerase activity stabilises the telomeres to allow uncontrolled replication. In most haematological malignancies, short telomeres and telomerase activity are detected; telomerase activity between twice and 50 times that of normal haematopoietic precursors has been reported (Ju and Rudolph, 2006).

Although increased telomerase activity has been reported in some putative CSC populations in comparison to non-CSC (for example, in the SP cells of some oesophageal carcinoma cell lines, or glioblastoma multiforme-derived neurospheres – (Lee *et al.*, 2006; Zhang *et al.*, 2008b), a definitive association is not well established. It has been suggested that if CSC are more quiescent cells, the activity of mechanisms to preserve telomere length is less critical than in more rapidly dividing cells.

However, the novel inhibitor of telomerase activity GRN136L (Imetelstat) is reported to show activity against putative CSC of pancreatic and prostatic cancer *in vitro*, and breast cancer and glioblastoma both *in vitro* and *in vivo*. This is significant, as it suggests that telomerase inhibition may prove a useful strategy for targetting resistant tumourigenic subpopulations (Hochreiter *et al.*, 2006; Bhagwandin and Shay, 2009; Marian *et al.*, 2010a; Marian *et al.*, 2010b).

CSC, Metastasis and the Epithelial-Mesenchymal Transition

The most lethal effects of neoplastic disorders frequently relate to the capacity of cancer cells to migrate to distant sites and form secondary tumours. The cellular heterogeneity of these metastases often reflects that seen in the primary tumour. However, disseminated cells may remain detectable in remote tissues without showing further development (Reya *et al.*, 2001). According to the CSC hypothesis, only CSC have the capacity to propagate malignancy, manifesting as local invasion

over short distances, and metastatic spread for longer migrations. Non-CSC may disseminate and become lodged in tissues, but will not form active metastases (Brabletz *et al.*, 2005).

Although one study of CSC in breast cancer found that the prevalence of the putative $CD44^{+}CD24^{-/low}$ tumour stem cell phenotype in breast tumours did not correlate with tumour progression or prognosis, it *was* associated with a tendency for distant metastasis upon recurrence (Abraham *et al.*, 2005). The presence of disseminated tumour cells in breast cancer can be detected with immunohistochemistry for cytokeratins (CK). Balic *et al* assessed CK+ bone marrow samples from 50 early breast cancer patients and found that all specimens had detectable $CD44^{+}CD24^{-/low}$ cells, with prevalence (33-100%) much greater than that seen in primary tumour masses, again associating the putative breast CSC population with a tendency toward metastasis (Balic *et al.*, 2006).

The "Epithelial-Mesenchymal Transition" (EMT) has attracted recent interest as a mechanism by which CSC may be associated with metastasis. This is a normal process during embryonic development, whereby epithelial cells lose polarity and adopt a motile phenotype (accompanied by characteristic changes, particularly downregulation of the cell adhesion molecule e-cadherin), allowing migration in the extracellular environment (Shook and Keller, 2003). There appear to be multiple parallels between this conversion between cellular states as it occurs in embryogenesis, and that observed during progression of carcinomas (Thiery, 2002).

It is proposed that EMT enables carcinoma cells to escape the confines of the normally structured epithelial environment, facilitating local invasion and also breach of the basement membrane, leading to intravasation and distant dissemination. At distant sites, extravasation may be followed by the reverse process of mesenchymal-epithelial transision (MET), leading to the formation of secondary carcinomas (Figure 6). Many signalling pathways which influence EMT, including Wnt-βcatenin, transforming growth factor-β (TGFβ) and those initiated by receptor tyrosine kinases EGFR and FGFR, are dysregulated in cancer (Thiery, 2002).

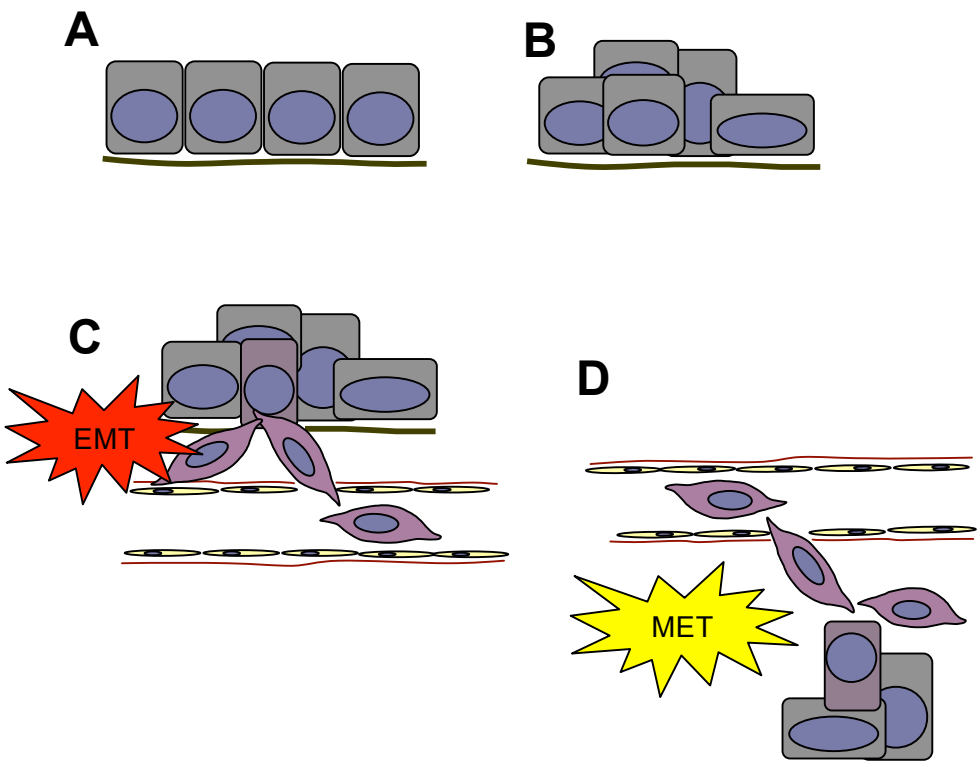


Figure 6 – Proposed role of epithelial-mesenchymal transition in carcinoma metastasis
A – Normal epithelial organisation with intact basement membrane
B – Dysplasia with local proliferation leads to adenoma formation and thence carcinoma *in situ*, but basement membrane remains intact.
C – EMT facilitates breaching of the basement membrane – local invasion – and subsequent intravasation into blood vessels or lymphatics.
D – At distant sites, extravasation followed by mesenchymal-epithelial transition (MET) results in formation of secondary carcinoma.

(Adapted from Thiery, 2002 *Nature Reviews – Cancer*, **2**, 442-454).

Some recent evidence has suggested that EMT may generate CSC (Mani *et al.*, 2008; Hennessy *et al.*, 2009; Santisteban *et al.*, 2009). Mani *et al* demonstrated upregulation of mesenchymal markers in normal and neoplastic mammary cells with the putative CSC phenotype $CD44^{High}CD24^{Low/-}$. Immortalised normal (HMLE) or transformed (HMLER) mammary epithelial cells were exposed to TGFβ, or the EMT-inducing transcription factors Snail or Twist overexpressed. This resulted in a predominantly $CD44^{High}CD24^{Low/-}$ phenotype, the ability to form mammospheres,

and (for HMLER) increased tumourigenicity. The acquisition of these properties is proposed as a mechanism by which CSC arise in epithelial tumours, and may explain their greater propensity to give rise to metastasis (Mani *et al.*, 2008).

It is significant, however, that this is a highly manipulated model. The neoplastic HMLER cells investigated had been artificially transformed by insertion of the vRas oncogene into HMLE cells; interestingly, "HMLEN" cells, transformed using the Her2/Neu oncogene, showed similar phenotypic changes and the ability to form mammospheres when induced to EMT, but were no more tumourigenic than the control cells. Moreover, HMLER control cells also induced tumours, albeit with less efficiency than HMLER-EMT cells. Lesions produced by both were similar squamous metaplasias, suggesting that induction of EMT was not necessarily associated with an invasive breast tumour phenotype. Whilst suggesting a mechanism which may result in the generation of cells with enhanced metastatic capability, it is less clear why or if EMT should confer the cardinal CSC properties of self renewal or multilineage potential *per se* (Mani *et al.*, 2008). Further work may shed more light on a role for EMT in the origin of CSC.

CSC Controversies and Alternative Hypotheses

Although there is now considerable evidence to support the concept of CSC, there has been a great deal of debate about both their existence and their clinical relevance. Not unexpectedly, some of this has arisen from the rather overzealous interpretation of data evident in some reports of putative CSC. However, there are also more fundamental difficulties in reconciling the idea of a cancer cell hierarchy with experimental evidence, particularly for solid tumours.

As discussed above, a number of different features may suggest that a cancer cell is a CSC (Figure 7). However, the working definition of CSC is, "those cells within a tumour exhibiting unlimited self-renewal capacity, and which can give rise to all of the various cell types making up the parental tumour upon serial transplantation in immunocompromised recipients". Importantly, this is a *functional* definition (self renewal and multilineage differentiation capacity), and not one based on any other anticipated stem cell feature such as surface phenotype. As previously discussed, although it may be the case for some tumours, the CSC is not necessarily a transformed stem cell.

The generally accepted "gold standard" method of defining CSC is serial transplantation in laboratory animals, in order to demonstrate self-renewal (passage) and the recapitulation of the tumour. This relies heavily upon the assumption that the formation of a tumour xenograft is influenced only by the nature of the implanted cells. However, the potent effects imparted by the environment or "niche" in which a cell exists cannot be disregarded when considering the evolution of a tumour (Mueller and Fusenig, 2004; Bjerkvig *et al.*, 2005; Polyak and Hahn, 2006). In normal tissues, the stem cell niche provides important extracellular controls and cues additional to the stem cells' own intrinsic program.

Similarly, tumour cells interact with surrounding stroma in a reciprocal manner, and can influence the stroma such that it is more conducive to tumour growth (Mueller and Fusenig, 2004; Perry and Li, 2007). Significantly, the inability of a cell to

Figure 7 – Summary of biological features which may be associated with CSC and used in their identification or isolation.

survive or proliferate when inoculated as a xenograft (particularly where this is not orthotopic, i.e. at the natural tumour site) may not accurately reflect its potential in the original tumour, where it is surrounded by a network of supportive cells, and subject to local cell-cell interactions (Hill, 2006; Adams and Strasser, 2008). Several authors raised the possibility that "CSC" markers may merely enrich for a population of cells with these functional capabilities – i.e. the ability to survive in a foreign host (Hill, 2006; Fillmore and Kuperwasser, 2007; Adams and Strasser, 2008).

A short report by Kelly *et al* demonstrated that unsorted inoculums of as few as 10 cells could reliably reproduce murine lymphoma in histocompatible, non-irradiated mice; indeed, in 3 of 8 recipients injected with a just single cell, lymphoma also developed (Kelly *et al.*, 2007). Overall, results suggested that, far from the idea of a "rare cancer stem cell", a large proportion of cells in some tumours could act as CSC. The authors postulated that the small proportion of leukaemia-initiating cells found in, for example, AML, may reflect the ability of human cells to survive in a murine environment. Proponents of the CSC hypothesis were eager to point out that, despite the fact that it had frequently been emphasised in reports, rarity is not a defining feature of CSC (Kennedy *et al.*, 2007). Nonetheless, the report establishes that the particular transplantation model used in a set of experiments is likely to have profound effects on apparent tumourigenicity of cancer cells (Kelly *et al.*, 2007).

This was further emphasised by a seminal study on human melanoma from the laboratory of Sean Morrison. It was reported that, in NOD/SCID murine recipients, tumour formation was inefficient and required the inoculation of hundreds of thousands of cells. In stark contrast to this, in more immunosuppressed hosts (NOD/SCID/Il2Rg$^{-/-}$ or "NOG" mice, which also lack the Interleukin-2 gamma receptor), efficiency was several orders of magnitude greater – indeed, single unsorted melanoma cells could form tumours (Quintana *et al.*, 2008).

This also had particular significance as the first report of solid tumour formation by a single cell (Quintana *et al.*, 2008). All published putative CSC markers, whilst enriching for tumourigenicity, still required the inoculation of hundreds to thousands

of cells to produce a tumour. Furthermore, close inspection of much tumourigenicity data suggests the influence of undefined variables or inhibitory effects (Kern and Shibata, 2007). For example, in reports of colon cancer stem cells, sorted $CD133^+$ cells produced tumours more efficiently than unfractionated cells, even when the actual number of $CD133^+$ cells within the unsorted inoculum was greater (O'Brien *et al.*, 2007). Similar numerical discrepancies are found in many reports of putative CSC, and draw attention to the need to question carefully why a cell may appear "tumourigenic" or "non-tumourigenic" (Hill, 2006; Kern and Shibata, 2007).

One good example of this was the demonstration by Taussig *et al* that the CD38 antibody used in flow cytometric fractionation of AML samples had a profound inhibitory effect on engraftment in immunosuppressed mice. Inhibition was Fc receptor-mediated and could be overcome using specific immunosuppressive antibodies. This not only permitted engraftment, but also demonstrated LSC activity in the $CD34^+CD38^+$ fraction, which would otherwise have been considered "non tumourigenic" (Taussig *et al.*, 2008). If treatment strategies are to be based upon the premise that certain cells within a tumour have distinct characteristics or are more significant to disease progression, it is critical that these distinctions do not arise artefactually.

Thus, there are limitations when using xenograft tumour models to infer that only a specific subset of tumour cells may propagate disease, and that all of the other cells are incapable of doing so. The major alternative hypothesis of clonal evolution proposes that tumourigenic cells may continually evolve, based on selective pressures, leading to genetically and epigenetically distinct subsets which are not necessarily hierarchically organised. Described initially in 1979 by Nowell, the model thus ascribes the heterogeneity and progression of tumours to the inherent instability of cancer cells, rather than the influence of a stable dominant subpopulation (Nowell, 1976) (Figure 3B).

As well as being a foundation of the CSC hypothesis, the observation that some cancer cells are more tumourigenic than others is also consistent with the clonal

evolution model (Nowell, 1976). Notably, differences between subpopulations do not always appear to arise as a result of a stable, intrinsic parent-progeny relationship between CSC and the rest of the tumour (Shackleton *et al.*, 2009). For example, studies of heterogeneous cellular subpopulations breast cancer cells demonstrated that, whilst $CD44^{+}CD24^{low/-}$ cells did show more "stemness"-associated characteristics and correlated with indicators of prognosis, $CD24^{+}$ cells were not only prevalent in distant metastases in drug-refractory patients but also showed a distinct genetic signature. This suggested that they represented a divergent subpopulation, rather than simply the terminally differentiated progeny of the $CD44^{+}$ cells (Shipitsin *et al.*, 2007).

Whilst the CSC paradigm does seem to apply to some malignancies, in others clonal evolution or a combination of processes may more closely account for tumour behaviour. Importantly, evidence from both haematological and solid tumours has indicated the potential for clonal evolution of tumourigenic CSC subsets (Barabe *et al.*, 2007; Hermann *et al.*, 2007). Thus, it is becoming apparent that it may be overly simplistic to consider a single cancer stem cell phenotype, without accounting for the continued evolutionary changes that are likely to occur within the life history of any individual tumour (Shackleton *et al.*, 2009) (Figure 8).

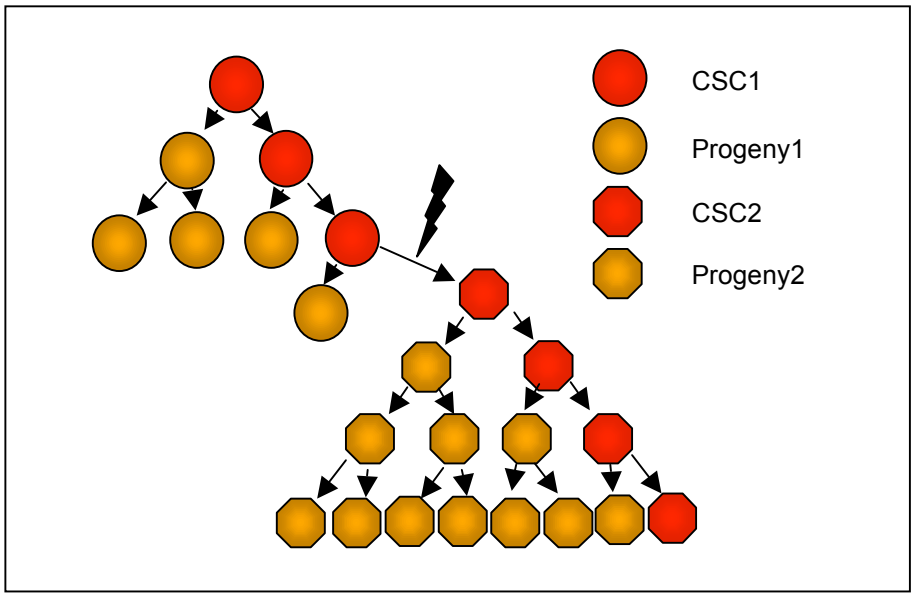


Figure 8 – Tumour progression may involve features of both the CSC and clonal evolution paradigms. The original tumourigenic CSC (CSC1) which gives rise to restricted, nontumourigenic progeny (Progeny1) may undergo further mutation under the influence of intrinsic or extrinsic influences (such as drug therapy), leading to the generation of a distinct, self-renewing tumourigenic CSC (CSC2). This in turn gives rise to distinct progeny (Progeny2). If these mutated CSC have a growth advantage they may become dominant within the tumour.
Therapy based on targetting prospectively identified CSC1 may be evaded by CSC2.

Clinical Relevance of the CSC Hypothesis

If a population of CSC is responsible for the propagation of a tumour, these cells must be eliminated to effect a cure. Conventional cancer chemotherapy has focussed on eradication of the tumour mass, based on the principle that rapidly dividing cells are preferentially eliminated – this targets cancer cells, but unfortunately also other tissues with a high cell turnover (such as hair, gut epithelium and blood), resulting in some of the unpleasant side effects associated with treatment. However, it is not only this dose-limiting morbidity which results in treatment failure, as some cancer cells are inherently resistant to the effects of anticancer agents.

The CSC hypothesis can account for the occurrence of relapse after therapy, sometimes years after apparent eradication of disease. Cancer cells with stem cell

characteristics might be expected to be more resistant than other tissue cells – putative CSC may be more quiescent cells (Holyoake *et al.*, 1999), and often display characteristics conferring a survival advantage, such as the activation of pro-survival signalling pathways or expression of multidrug transporter proteins. The hypothesis also suggests that for successful cancer treatment, if the mutated clone is to be eradicated within an individual, the priority target cell population must be the CSC. This is likely to require changes to strategies currently used in the clinical setting (Reya *et al.*, 2001; Sell, 2004; Behbod and Rosen, 2005; Misaghian *et al.*, 2009) (Figure 4B).

Isolated populations of putative CSC have shown increased resistance to the effects of conventional anticancer strategies such as chemotherapy and ionising radiation (Baumann *et al.*, 2008; Chua *et al.*, 2008; Diehn *et al.*, 2009; Hirsch *et al.*, 2009; Tanei *et al.*, 2009). However, investigations have also revealed agents which may selectively inhibit the CSC fraction. The development of assays for prospectively isolating and propagating CSC has facilitated large-scale drug screening; in some cases, substances not previously considered as anticancer agents have shown a remarkable activity against these specific subpopulations (Guzman *et al.*, 2005; Diamandis *et al.*, 2007; Gupta *et al.*, 2009b; Zhou *et al.*, 2009b).

Promising strategies for directed targetting include inhibition of stem cell-specific mechanisms such as self-renewal pathways, and immunological techniques using monoclonal antibodies to specific cell surface markers. Interference with the support imparted by the niche may also render CSC more susceptible to eradication (Hideshima *et al.*, 2002; Studebaker *et al.*, 2008). Differentiation therapy using agents such as retinoic acid, whereby CSC might be forced down a symmetrical division pathway to produce two daughter cells, thus depriving a tumour of self-renewal potential, has been suggested as a logical CSC-directed strategy. However, whilst this is effective in a majority of cases of human acute promyelocytic leukaemia, responses in other malignancies have been variable (Sell, 2004; Lotem and Sachs, 2006).

The simplest interpretation of the CSC hypothesis suggests a "dandelion root" analogy, such that a tumour will be successfully eradicated if the CSC fraction is destroyed. However, mathematical models and experimental data suggest that targetting only CSC may not be sufficient to eliminate disease, particularly if these show any evolutionary plasticity in the face of challenge (Kern and Shibata, 2007). A successful strategy will probably require simultaneous eradication of both CSC and bulk tumour components (Dingli and Michor, 2006). Indeed, marked synergistic effects between conventional and CSC-selective agents have been demonstrated, often permitting considerable reductions in dose of individual cytotoxic drugs (Guzman *et al.*, 2002; Zhou *et al.*, 2008; Zhou *et al.*, 2009b).

Thus, the CSC hypothesis introduces the possibility that it may be possible to overcome the two major obstacles to successful cancer therapy – patient morbidity, and relapse after treatment. This will require precise identification and complete elimination of cells with potential to form new tumours – simply reducing the number of residual cancer-propagating cells is unlikely to prove clinically beneficial if these may then repopulate the tumour. However, evidence suggests that a greater understanding of the role of CSC in neoplasia, facilitated by refinements in detection techniques and assays, will enable the development of improved therapeutic strategies and increase the likelihood of achieving complete and enduring clinical remissions.

Comparative Oncology – Spontaneous Cancer in the Dog as a Disease Model

Spontaneous cancer is the major cause of non-age-related death in the domestic dog, with one in three pet dogs developing cancer during their lifetime. Dogs develop a wide range of spontaneous neoplasms, many of which mirror those seen in humans in terms of disease course, histology, biological behaviour, and response to treatment. Notably, some canine tumours, such as complex mammary carcinomas, show more similarity to human disease than those occurring in other species (Hellmen *et al.*, 2000; Munson and Moresco, 2007).

The publication of the canine genome in 2005 demonstrated considerable homology between canine and human sequences – approximately the same number of genes have been identified in both species, and most are 1:1 orthologues. Significantly, overall homology is greater than that between human and mouse (Lindblad-Toh *et al.*, 2005; Karlsson and Lindblad-Toh, 2008). Although still less well characterised than the murine system, other aspects of canine cell biology appear to be more representative of the situation in humans. For example, the telomeres of mice are many times longer than those of humans, limiting their utility in models of normal and neoplastic telomere dynamics; by contrast, canine telomeres are of comparable length (Wright and Shay, 2000; Nasir *et al.*, 2001). With improvement in diagnostic modalities, it has become clear that there are also multiple parallels on a molecular and genetic level between canine and human cancers. Similar alterations, disease markers and therapeutic targets are observed in both species (Sagartz *et al.*, 1996; Argyle and Nasir, 2003; Boomkens *et al.*, 2004; Dickerson *et al.*, 2005; Jubala *et al.*, 2005; Wilkerson *et al.*, 2005; Alvarez *et al.*, 2007; Kisseberth *et al.*, 2007; Gelain *et al.*, 2008; Rivera *et al.*, 2009).

The use of laboratory animals in cancer research, whilst seen by most in the field as a necessary and inevitable means of investigation, is a major welfare concern. In many respects, spontaneous cancer in the dog is a more representative model of human disease than experimentally-induced tumours in laboratory rodents or primates (Kimmelman and Nalbantoglu, 2007; Munson and Moresco, 2007; Paoloni and Khanna, 2008). The growth of human tumour xenografts in immunosuppressed animals will give little insight into the influence of the immune system on the pathogenesis of cancer. Artificial introduction of mutations to produce models of disease in a controlled laboratory setting frequently results in rapid development of tumours with a predictable and specific pathogenesis. By contrast, spontaneous canine tumours arise in animals living in the same environment as humans (therefore subject to similar diverse influences), with gradual acquisition of mutations, leading to the development of more comparable, naturally heterogeneous tumours (Rutteman *et al.*, 1988; Augustin-Voss *et al.*, 1990; Hellmen *et al.*, 2000; Kirpensteijn *et al.*, 2002; Zacchetti *et al.*, 2003; Webster *et al.*, 2007; Karlsson and Lindblad-Toh, 2008;

Paoloni and Khanna, 2008). Although some breeds show predisposition to certain cancers (and despite the recently highlighted problems associated with intensive breeding to achieve pedigree standards) the pet dog population overall is outbred, with a far more diverse genetic background than inbred laboratory rodent strains.

The comparative study of disease processes occurring in humans and pet dogs is of mutual benefit. Most of the treatment modalities used in human oncology have been adapted successfully to treat canine patients; similarly, clinical trials in dogs have informed developments in the human field. With the continued adaptation and use of investigative techniques in the canine system, comparative oncology can provide insights into cancer biology, which may improve understanding of disease and aid development of new therapies, in both dogs and humans (Guzman *et al.*, 2007; London *et al.*, 2009).

Data obtained from the study of several tumour types has implicated cells with stem-like characteristics in the progression of some canine malignancies. Analysis of expression patterns in canine haemangiosarcoma (Lamerato-Kozicki *et al.*, 2006), and mammary spindle cell carcinomas and osteosarcomas (Hellmen *et al.*, 2000), suggested that disease had originated from a transformed precursor cell. Using assays adapted from those used in the study of human tumours, such as tumoursphere formation and the demonstration of tumourigenicity in immunosuppressed mice, putative CSC populations have been reported for canine osteosarcoma, mammary carcinoma, glioblastoma and acute myeloid leukaemia (Guzman *et al.*, 2007; Wilson *et al.*, 2008; Cocola *et al.*, 2009; Stoica *et al.*, 2009).

Cancer Stem Cells in Canine Cancer - Hypothesis and Research Questions

The following studies sought to interrogate the validity of the cancer stem cell model in the context of canine neoplasia.

HYPOTHESIS – Canine neoplasms are organised as cellular hierarchies, maintained by specific subpopulations of self-renewing cancer stem cells, which have the ability to give rise to all of the cell types within the tumour.

Primary research questions were formulated as follows, in order to test this hypothesis.

1) Is there evidence cells with stem cell-like properties play a role in the pathogenesis of canine cancer? Can these be prospectively isolated?

The study aimed to evaluate canine cancer cell lines and spontaneous tumours for evidence of putative CSC populations. This was to be accomplished by adapting assays used in seminal studies of CSC in other species, specifically flow cytometry (using assays for both expression pattern and functional capacity) and tumoursphere culture.

2) What are the characteristics of the cells within these subpopulations? Do they fulfil CSC criteria?

Candidate subpopulations were to be analysed for phenotypic and functional characteristics, to support their definition as CSC. These include:

Phenotypic - Expression of markers associated with undifferentiated cells

Functional - Existence as a specific, stable subpopulation
Self-renewal potential
Multilineage differentiation potential

3) Are CSC clinically relevant? Do they show enhanced resistance mechanisms or survival capacity?

Isolated subpopulations were to be assessed for sensitivity to commonly used anticancer agents, to determine whether this might underlie evasion of conventional therapy or relapse.

4) What mechanisms underlie resistance of CSC? Are there means by which they can be eliminated along with non-CSC, or preferentially targetted?

Mechanisms by which stem cell-like cancer cells might evade therapy, and the potential for specific inhibition of CSC subpopulations, were to be investigated.

CHAPTER 2

MATERIALS AND METHODS

Details are given of techniques and experimental procedures used throughout the course of investigations. General laboratory reagents and buffers were obtained from Sigma-Aldrich (Poole, UK) unless otherwise stated.

Cell Culture - Cell Lines

Canine cell lines assessed in the course of this work were the adherent cultures CML10 melanoma, D17 osteosarcoma, J3T glioma, REM134 mammary carcinoma and SB haemangiosarcoma, and the suspension culture 3132 B-cell lymphoma. For some experiments, the feline adherent mammary carcinoma cell line Cat-MT, and the human adherent mammary carcinoma cell line MCF-7 were also evaluated. Details of source and culture conditions are given in Table 1.

All cell culture manipulations were performed within a Class 2 Biological Safety Cabinet. Culture media, foetal calf serum (FCS), Trypsin-EDTA and supplements were obtained from Gibco / Invitrogen (Paisley, UK) unless otherwise stated. Penicillin-Streptomycin (10000IU/ml-10mg/ml) was obtained from Sigma-Aldrich. Culture flasks and plates were obtained from Nunc (Rochester, NY, USA) and TPP (Trasadingen, Switzerland). All cell lines were cultured at 37°c in a humidified atmosphere with 5% CO_2.

Culture media

DMEM-HG – Dulbecco's modified Eagle's medium - 4.5g/l d-glucose, L-glutamine, sodium pyruvate (Gibco 41966) + 10% FCS + 1% Pen-Strep

DMEM-LG - Dulbecco's modified Eagle's medium - 1g/l d-glucose, Glutamax-I, sodium pyruvate (Gibco 21885) + 10% FCS + 1% Pen-Strep

RPMI1640/FCS/PS- Roswell Park Memorial Institute-1640 medium - L-glutamine, 25mM HEPES (Gibco 52400) + 10% FCS + 1% Pen-Strep

EBM-2 / EGM-2 – Endothelial Basal Medium-2 with EGM-2 SingleQuots (Lonza, Basel, Switzerland)

Cell Line	Origin	Source	Culture medium
CML10	Canine melanoma	Courtesy of Prof. Lauren Wolfe, Auburn University, AL, USA	DMEM-LG
D17	Canine osteosarcoma	American Type Culture Collection, Manassas VA, USA (#CCL-183)	DMEM-LG
J3T	Canine glioma	Courtesy of Micheal Behrens, Translational Genomics Research Institute (TGen), Phoenix AZ, USA	DMEM-HG
REM134	Canine mammary carcinoma	Courtesy of Prof. Roderick Else, University of Edinburgh (Else, 1982; Norval, 1984 a, b)	DMEM-HG
SB	Canine haemangiosarcoma (cutaneous)	Courtesy of Prof. Stuart Helfand, University of Wisconsin, Madison WI, USA (Akhtar, 2004)	EBM-2 / EGM-2
3132	Canine B-cell lymphoma	Prof. David Argyle, University of Edinburgh (Strandstrom, 1979)	RPMI1640 /FCS/PS
CatMT	Feline mammary carcinoma	Courtesy of Prof. Roderick Else, University of Edinburgh	DMEM-HG
MCF-7	Human mammary carcinoma	European Collection of Cell Cultures (#86012803)	DMEM-HG

Table 1 – Origin and source of cell lines used in experiments, and growth media used for culture.

Thawing cryopreserved cells

Cryopreserved cells were retrieved from storage in liquid nitrogen and held on dry ice until placed into culture. Vials were thawed rapidly until only a single ice crystal remained, and the contents transferred to a 50ml conical centrifuge tube. Complete medium was added dropwise with swirling to double the volume of the suspension, and this process repeated until the total volume within the tube was at least 20ml. Cells were washed twice in complete medium with gentle centrifugation (250*g*), resuspended in 7.5ml complete medium and transferred to a vented-cap T25 flask for culture. Flasks were incubated until subculture (80-90% confluence). After 48 hours in culture, medium was aspirated, the cells washed with PBS and fresh medium added to the flask. In general, for slowly-growing cells, culture medium was changed in this manner every 4-5 days until confluence.

Subculture – Adherent cells

Complete medium and PBS were prewarmed to 37°C. Medium was aspirated from the monolayer and cells washed twice with PBS. After aspirating PBS, the appropriate volume of 0.25% Trypsin-EDTA (T25 flask – 1ml, T75 flask – 2ml, T150 – 3ml) was added and the flask rocked to coat the monolayer. Excess trypsin was aspirated and the flask placed in the incubator. Tapping of the flask to loosen cells was avoided to reduce clumping. Once cells had become detached, complete medium (T25 – 5ml, T75 – 5-10ml, T150 – 10ml) was used to wash the cells from the base of the flask. Cell counts were performed at this stage as required. Cells were subcultured at 80-90% confluence (generally twice weekly), using a split ratio guided by initial assessment of population growth or seeding absolute cell numbers as required for assays.

Subculture – Suspension cultures

Cells could be counted directly from suspension cultures allowing the appropriate fraction or number of cells to be subcultured. Cells for subculture were washed once

in complete medium, and seeded as required.

Cell counting

Cell counts were performed using a haemocytometer (improved Neubauer ruling – central square volume $1x10^{-4}$ ml; grid volume $9x10^{-4}$ ml). 10µl cell suspension mixed with 10µl 0.4% trypan blue (Sigma-Aldrich) was pipetted into each side of the counting chamber. Cells excluding trypan blue were counted within the central square of each side of the chamber, and the cell count calculated as

$$\text{Number of cells/ml suspension} = (\text{Count 1} + \text{Count 2}) \times 10^4$$

For very low cell numbers, cells were resuspended in a small volume (0.5-1ml) for counting, and/or cells within the large grid were counted, with the calculation amended accordingly. Counts were performed in triplicate for assays where cell count represented the endpoint result.

For counts of primary haematopoietic cells (performed after Ficoll density gradient centrifugation), White Cell Diluting Fluid (crystal violet 1% w/v, glacial acetic acid 2% v/v) rather than trypan blue was used in a 1:1 ratio with the cell suspension, to lyse residual erythrocytes and stain leukocyte nuclei.

Cryopreservation

Cells were washed and resuspended in complete medium at approximately $6x10^6$/ml. Freezing medium (90% FCS, 10% dimethylsulphoxide (DMSO)) was freshly prepared. Both freezing medium and cells were held on ice during preparation to minimise the toxic effects of DMSO. An equal volume of freezing medium was added to the cell suspension dropwise with swirling to achieve a final concentration of 5% DMSO. Aliquots of 1ml (approximately $3x10^6$ cells) were made in cryovials and placed in a precooled (4°C) cryopreservation canister (Mister Frosty, Nalgene, Nunc), which was immediately transferred to -70°C. After at least several hours

(allowing for cooling at -1°C/minute within the cryopreservation canister) vials were transferred to liquid nitrogen for long-term storage).

Primary Cells

Solid tissue

Solid tissue was harvested into DMEM-LG. Samples were minced finely using scissors and further with a scalpel and forceps, and washed twice with DMEM-LG in 50ml conical tubes. Samples were either resuspended at this stage in DMEM-LG and transferred into T25 culture flasks (4-5 flasks from a 0.5cm^3 block of tissue) or resuspended in 10ml 0.25% Trypsin-EDTA and incubated at 37°C for 15 minutes with occasional agitation to facilitate dissociation, followed by a further wash in 20ml DMEM-LG and transfer into T25 culture flasks. Culture flasks were either untreated or precoated with 1% Fibronectin in distilled water (2ml per T25 culture flask and allowed to dry). Larger tissue pieces were initially plated along with dissociated cells.

After 48 hours, flasks were examined for adherent cells and checked for signs of bacterial contamination. If contamination was heavy, flasks were discarded. Where it was suspected, medium was removed, the base of the flask washed twice with PBS-1% Pen-Strep, and fresh medium added. Solid tissue pieces were generally lost from the flask at this stage.

Medium was changed twice weekly and replaced with 50% fresh:50% conditioned medium (growth medium from flasks centrifuged at 400g to remove debris) after gently washing adherent cells twice with PBS. At 80-90% confluence, cells were transferred to larger (T75) culture flasks after dissociation of the monolayer using 0.05% Trypsin-EDTA, and then subcultured as necessary. 50% conditioned medium was used for medium changes and plating at subculture up to 5-7 passages. Where numbers permitted, cells were cryopreserved at each passage up to 20 passages.

kt-osa1-5 are populations of adherent cells derived by the author from the excised primary osteosarcoma of an eight-year-old female entire Rottweiler (see Chapter 3). Cells were cultured in DMEM / 10% FCS / 1% Pen-Strep. kt-osa1, kt-osa4 and kt-osa5 were subcultured repeatedly and maintained as continuous cell lines. kt-osa2 and kt-osa3 populations were expanded in adherent culture for assays but not carried forward to subculture.

Isolation of bone marrow mononuclear cells (BMMNC)

Bone marrow was obtained immediately post-mortem from animals euthanased for unrelated reasons. Where aspiration was not possible due to post-mortem circulatory stasis, the proximal humerus was split at the intertubercular groove using an osteotome, and marrow extracted with a sterile spoon. Marrow (2-10ml) was collected into heparinised 50ml conical tubes and diluted 1:1 with PBS at room temperature. Mononuclear cells were isolated by Ficoll-Paque (Amersham Biosciences / GE Healthcare, Little Chalfont, UK) density gradient centrifugation, with cells collected from the interface washed twice in PBS, passed through a 40µm cell strainer and counted in white cell diluting fluid using a haemocytometer. Cells were resuspended in the appropriate medium or buffer for flow cytometric analysis, or in DMEM-LG for culture of bone marrow-derived stromal cells.

Bone marrow-derived stromal cell culture

BMMNC were isolated from whole canine bone marrow as described. Cells were plated in DMEM-LG in uncoated T25 or T75 culture flasks. Although adherent cells were obtained using the seeding densities of 1.6-5.4 x 10^4/cm^2 recommended by Kadiyala *et al* (Kadiyala *et al.*, 1997), yields were improved at higher inocula (1.5 x 10^5 cells/cm^2 (Kamishina *et al.*, 2006)).

Medium was changed twice weekly and replaced with 50% fresh:50% conditioned medium after gently washing adherent cells twice with PBS. At 80-90% confluence, cells were subcultured after dissociation of the monolayer using 0.05% Trypsin-

EDTA. After two or three passages, cells arrested and showed no further growth.

Isolation of peripheral blood mononuclear cells (PBMNC)

Whole blood (2-5ml) was collected into heparinised glass tubes and diluted 1:1 with PBS at room temperature. Mononuclear cells were isolated by Ficoll density gradient centrifugation and resuspended in the appropriate medium or buffer for flow cytometric analysis.

Flow Cytometry

Fluorochrome-conjugated antibodies were obtained from BD Biosciences (San Jose, CA, USA), Biolegend (San Diego, CA, USA) and eBioscience (San Diego, CA, USA) – Table 2. Staining was performed in conical 15ml polypropylene centrifuge tubes (TPP, Trasadingen, Switzerland) or round-bottomed 5ml polystyrene tubes (BD Falcon, Bedford, MA, USA).

Antibody (Cat #)	**Specificity**	**Clone**	**Isotype**	**Fluorophore**	**Manufacturer**
CD24 (311103)	Human	ML5	Mouse IgG2aκ	FITC	BioLegend
Iso-CD24 (400209)			Mouse IgG2aκ	FITC	BioLegend
CD34 (559369)	Canine	1H6	Mouse IgG1κ	PE	BD Biosciences
Iso-CD34 (556650)			Mouse IgG1κ	PE	BD Biosciences
CD44 (100307)	Mouse / Human	IM7	Rat IgG2bκ	PE	BioLegend
Iso-CD44 (400607)			Rat IgG2bκ	PE	BioLegend
CD117 (559879)	Human	YB5.B8	Mouse IgG1κ	PeCy5	BD Biosciences
Iso-CD117 (550618)			Mouse IgG1κ	PeCy5	BD Biosciences
CD133 (17-1331)	Mouse	13A4	Rat IgG1κ	APC	eBioscience
Iso-CD133 (17-4301)			Rat IgG1κ	APC	eBioscience

Table 2 - Fluorochrome-conjugated antibodies used for flow cytometry.
FITC – Fluorescein Isothiocyanate; PE – R-Phycoerythrin; APC – Allophycocyanin. PeCy5 is a tandem conjugate combining R-Phycoerythrin and a cyanine dye.

Antibody	**Concentration mg/ml**	**Recommended / 10^6 cells / 100µl**	**µl stock/ 10µl staining volume**
CD24	Not provided	"Test" †	"Test" †
CD34	0.5	≤ 1µg	2
CD44	0.2	≤ 0.25µg	1.25
CD117	0.2	n/a	1.25
CD133	0.2	≤ 0.125µg	0.625

Table 3- Concentration of fluorochrome-conjugated antibodies
† Staining volume 20µl where "test" concentration predetermined by manufacturer. Optimal staining concentration for antibodies were determined for each cell line by initial titration experiments.

Protocol for antibody titration

Directly-conjugated antibodies (maintained at 4°C / wet ice, protected from light)
Facs-DMEM - DMEM (Gibco 41966) + 2% FCS + 0.1% Sodium Azide
Facs-PBS - For antibody dilutions : PBS + 1% FCS + 0.1% Sodium Azide
Propidium Iodide (PI) – Dead cell discriminator - 200μg/ml in ddH_2O

Doubling dilutions of test antibody and concentration-matched isotype control antibody were made in Facs-PBS, with manufacturers' recommended concentration as the initial dilution (Tables 3 & 4). Cells were harvested in complete medium, counted by trypan blue exclusion and centrifuged at 300*g*, 4ºc for 5 minutes. Supernatant was decanted and cells resuspended in Facs-DMEM at 1 x 10^6/ml. Aliquots of cell suspension were made, with samples for each test concentration, isotype-matched controls and at least one unstained sample. Samples were centrifuged at 4ºc, 250 *g* for 5 mins and resuspended in 100μl cold Facs-DMEM.

10μl antibody / appropriate isotype control dilution was added (see Table 4). Tubes were flicked gently to mix and placed on ice, in the dark. After 30-45 minutes, copious cold Facs-DMEM was added to each sample and cells washed by centrifugation at 4ºc , 250*g*, for 5 minutes. Samples were resuspended in 300μl cold Facs-DMEM, and held on ice, in the dark, pending flow cytometry. PI was added just prior to flow cytometry at 2μg/ml for dead cell discrimination if required.

Optimal concentration of test antibody was determined for each cell line, such that the fluorescence of the concentration-matched isotype control sample was equivalent to that of the unstained aliquot.

Antibody/10^6 cells (10μl)	**Dilution**
0.25μg	3μl stock solution + 21μl PBS^+ (**1**)
0.125μg	12.5μl dilution **1** + 12.5μl PBS^+ (**2**)
0.0625μg	12.5μl dilution **2** + 12.5μl PBS^+ (**3**)

plus isotype controls at same dilutions – **4, 5, 6**

Table 4 - Example of dilution series for antibody titration - PE Anti-CD44 (P44 1-6)

Flow cytometers and software used for acquisition are shown in Table 5.

Cytometer	Acquisition Software	Application
FACSCalibur	CellQuestPro	Surface marker analysis Rhodamine efflux Aldefluor fluorescence DNA content analysis Cell cycle analysis (PI)
FACSAria	FACSDiva	FACS (Cell Sorting) Hoechst efflux analysis DCV efflux analysis Aldefluor analysis
LSRII	FACSDiva	Hoechst efflux analysis Simultaneous cell cycle (DAPI) / surface marker analysis

Table 5 – Cytometers and acquisition software used for flow cytometric analysis (all BD Biosciences).

Post-acquisition analysis was performed using FlowJo (Treestar, Ashland, OR, USA).

Antibody staining protocol

Surface staining experiments were performed as for antibody titration protocol, using optimised concentrations for antibody in the test sample. Control samples (cells incubated with equivalent concentration of isotype-matched control antibody / unstained cells) were evaluated for every experiment. At least 100000 events were acquired for analysis.

Cell sorting

Sort-DMEM : DMEM (Gibco 41966) + 2% FCS + 2% Pen-Strep
Collect-DMEM : DMEM (Gibco 41966) + 20% FCS + 2% Pen-Strep

For cell sorting, sodium azide was omitted from the staining medium to reduce

inhibitory effects on cell growth. Pen-Strep was added to medium as a biocidal agent. The concentration of FCS in the collecting medium was increased such that, when diluted with sorted cells+sheath fluid, it would approximate that of the cells' growth medium (10%).

Cells were harvested in complete medium, passed twice through a 40μm cell strainer, counted by trypan blue exclusion and resuspended at $1x10^6$/ml in Sort-DMEM. The number of cells required for staining/sorting was calculated using the anticipated percentage of total cells. 1.5x this number of cells (to allow for cell losses) was aliquotted into a 15ml conical tube; aliquots of $1x10^6$ cells were made for Unstained and Isotype control samples. Cells were centrifuged and resuspended in ice-cold Sort-DMEM at $1x10^6$ cells/100μl.

Antibody concentrations were determined from previous titrations. Isotype control antibody was diluted in Facs-PBS and added in a 10μl staining volume. Test antibody was added directly to the cell suspension at the appropriate corresponding concentration. Samples were incubated on ice, in the dark, for 30-45 minutes. Cells were washed with copious ice-cold Sort-DMEM and resuspended in ice-cold Sort-DMEM according to cell number:

Isotype / Unstained – Resuspend in 300μl
Test – Resuspend at up to $1x10^7$ cells/ml

Samples were held on ice, in the dark, pending FACS. Cells were collected into tubes containing Collect-DMEM (at least 2ml for each $1x10^6$ cells to be isolated).

Fixation of antibody-stained cells for flow cytometry

PBS-Fix – PBS + 1% Paraformaldehyde (PFA) (Sigma-Aldrich) + 0.1% Sodium Azide

Cells were prepared and incubated with antibody as for antibody staining protocol.

After washing with copious Facs-DMEM and centrifugation (5 minutes, 4°C, 300*g*) supernatant was decanted and the cell pellet flicked gently to resuspend in residual fluid.

0.5ml ice-cold PBS-Fix was added to each tube and samples were incubated on ice, in the dark, for at least 15 minutes. Cells were centrifuged (5 minutes, 4°C, 300*g*), resuspended in 0.5ml PBS-Fix and stored at 4°C, in the dark, for up to 2 weeks pending flow cytometry. If cells showed a tendency to clump, they were resuspended in PBS for storage and passed through a 40μm cell strainer prior to analysis. Just prior to flow cytometry, cells were washed twice with 2ml ice-cold PBS and resuspended in 300μl PBS for analysis.

N.B. As PFA fixation causes the death of all cells, use of PI as an indicator of viability is not feasible for fixed samples.

Cell Cycle / DNA content analysis

Ribonuclease A (RNAse A) (Sigma-Aldrich) - 100μg/ml in ddH_20
Propidium Iodide (PI)- 50μg/ml in PBS *or*
4',6-Diamidino-2-phenylindole dihydrocloride (DAPI) (Sigma-Aldrich) - 2μg/ml in PBS

Cells were harvested, washed and resuspended in cold PBS and counted using trypan blue exclusion. Aliquots of equal cell number (for comparison between samples) were placed into 15cm conical tubes. Samples were centrifuged (5 minutes, 4°C, 300*g*) and resuspended in 1ml cold PBS. 3ml ice-cold (-20°C) absolute ethanol (i.e. final concentration 70-75%) was added dropwise while vortexing, to minimise cell clumping. Samples were placed on ice for at least 30 minutes and were stored at this stage for up to two weeks prior to staining and cytometry.

Cells were washed twice with PBS – samples were centrifuged at 800*g*, with supernatant carefully decanted leaving a greater than normal amount of residual fluid

for the first wash, to reduce cell losses (cells become lighter and more flocculant upon storage in ethanol). For PI staining (binds dsRNA), 50µl of 100µg/ml RNAse A was added; samples were flicked gently to mix, and incubated at 37°C for 15 minutes. 200µl PI was added for a final concentration of 40µg/ml. For DAPI staining, cells were resuspended in 250µl PBS, and 250µl DAPI added to each tube for a final concentration of 1µg/ml. RNAse was not required, as DAPI does not bind RNA. Samples were analysed by flow cytometry. At least 25000 events were acquired for analysis.

Low-density, Serum-free Culture for Tumourspheres

N2/MC medium: DMEM/Ham's F12 base medium (Sigma-Aldrich)
0.8% Methylcellulose (Sigma-Aldrich)
N2 supplement (Invitrogen) - (Bottenstein and Sato, 1979)
Insulin 5µg/ml
Transferrin 100µg/ml
Putrescine 100µg/ml
Progesterone 20µg/ml
Sodium selenite 30µg/ml
Recombinant human epidermal growth factor (Invitrogen) 5ng/ml
Recombinant human basic fibroblastic growth factor (Invitrogen) 5ng/ml

Cells were harvested as for subculture, and live cells counted by trypan blue exclusion. For three wells of a six-well plate, $2.4x10^5$ cells were washed in PBS and resuspended in 4ml N2/MC (60000 cells/ml), to allow for volume losses associated with manipulating the viscous medium.

1ml N2/MC was placed into each of three wells of an UltraLow Attachment 6-well plate (Corning Life Sciences, Corning NY, USA) and 1ml cell suspension added to each well to give a final concentration of 60000 cells in 2ml N2/MC per well. Plates were incubated at 37°C / 5% CO_2 / 100% humidity. 12µl epidermal growth factor (EGF 100µg/ml in ddH_2O) and 12µl basic fibroblastic growth factor (bFGF 100µg/ml in ddH_2O) were added every 48 hours. Cells were monitored for sphere formation, and passaged at 7-14 day intervals, depending on the rate of proliferation and sphere growth.

Tumoursphere passage protocol

2ml PBS was added to each well of tumourspheres and the plate rocked to aid in aspiration of viscous medium. Cells and medium were aspirated from the wells, transferred to 15ml conical centrifuge tubes and the base of the well washed with a

further 1ml PBS to collect any residual spheres. Tumourspheres were washed gently (1000rpm, 5 minutes) using at least an equal volume of PBS. Supernatant was aspirated carefully to avoid disturbing the cell pellet. Cells were resuspended in 0.5ml 0.05% Trypsin-EDTA and incubated at 37°c for 10-15 minutes, with occasional agitation. 0.5ml complete adherent culture medium was added and the suspension triturated gently 20 times using a pipette, avoiding the creation of bubbles.

To reduce the persistence of cellular aggregates after tumoursphere dissociation, the cell suspension was passed twice through a 40μm cell strainer (BD Falcon). Live cells were counted using trypan blue exclusion. For assays such as flow cytometry and drug sensitivity, cell numbers were adjusted and resuspended appropriately at this stage for further use. For passage, an aliquot was taken, washed in PBS and resuspended at 60000 cells/ml in N2/MC for replating. Tumourspheres between 5th and 10th passage were used for assays.

Transferring tumourspheres to adherent culture

2ml PBS was added to each well of tumourspheres and the plate rocked to aid in aspiration of viscous medium. Cells and medium were aspirated from the wells, transferred to 15ml conical centrifuge tubes and the base of the well washed with 1ml PBS to collect any residual spheres. Tumourspheres were washed gently (200*g*, 5 minutes) using at least an equal volume of PBS. Supernatant was aspirated carefully to avoid disturbing the cell pellet. Cells were resuspended in 7.5ml adherent culture medium and transferred to T25 culture flasks.

Chemosensitivity Assays

Cells were assessed for sensitivities to different chemotherapy drugs over a range of concentrations to include those achieved in plasma within a clinical setting.

Drug (Manufacturer)	TPC (µg/ml)	FW	TPC (µM)	Reference
Doxorubicin (Pharmacia / Pfizer)	0.006 - 0.09	543.52	0.011 – 0.160	Regenthal *et al*, 1999
Mitoxantrone (Baxter Healthcare)	0.63 - 0.95 *	444.481	1.42 - 2.14	Canal *et al*, 1993
Carboplatin (Bristol-Myers Squibb)	10-25	371.25	26.9 - 67.3	Regenthal *et al*, 1999
Cyclophosphamide (Baxter Healthcare)	10-25	279.1	35.8 – 89.6	Regenthal *et al*, 1999
Vincristine (Hospira UK)	0.001-0.02†	824.96	0.001-0.02 0.002-0.01†	Van den Berg *et al*, 1982; Sethi *et al*, 1981

Table 6 – Therapeutic plasma concentrations (TPC) for humans of commonly-used chemotherapy drugs (*Mitoxantrone at 15mg/m^2 dose; †Approximate values derived from pharmacokinetic data).

Cells were harvested, counted, resuspended in complete medium and plated in black 96-well culture plates at 50µl / 5x10^3 cells per well. Plates were incubated at 37ºc, 5% CO_2. After 24 hours, drug dilutions were made up in complete medium at 2x final concentration, with volume-matched vehicle controls where appropriate, and 50µl of each dilution added to triplicate wells. 50µl of medium only was added to triplicate wells as a control. 50µl of medium only was added to the outer wells of each plate, which were excluded from the assay. Plates were incubated at 37°C, 5% CO_2 for a further 72 hours.

The CellTiterGlo ATP luminescence cell viability assay (Promega, Madison WI, USA) was used according to the manufacturer's protocol. This uses a 96-well plate format and determines ATP content (a measure of cellular metabolic activity) in treated wells as compared to untreated controls, which shows good correlation with cell viability (Bosanquet, 1993; Andreotti *et al.*, 1995; Cree *et al.*, 1995).

Briefly, reagents and plates were equilibrated to room temperature (for 2 hours and 30 minutes respectively), and 100µl of CellTiterGlo reagent added to each test well. After 2 minutes on an orbital plate shaker and 10 minutes incubation/equilibration at room temperature, luminescence was measured using a microplate reader (Victor^3 Wallac 1420 Multilabel Counter – PerkinElmer, Waltham MA USA)

Viability was calculated as a percentage of the control wells and dose-response curves fitted using nonlinear regression with GraphPad Prism 5.0b (GraphPad Software, San Diego CA, USA). Where comparisons were being made between drug sensitivities of different cell populations, best-fit values for IC_{50} were compared using an extra-sum-of-squares F test ($p < 0.05$).

Western Blotting

Preparation of cell pellets

To prepare cell pellets for lysis, medium was discarded from 80-90% confluent monolayer and cells washed with ice-cold PBS. Cells were harvested by scraping into 1ml ice-cold PBS, transferred to chilled (4°C) Eppendorf tubes and placed on ice. For suspension cultures, cells were washed twice with ice-cold PBS, resuspended in 1ml ice-cold PBS, transferred to chilled (4°C) Eppendorf tubes and placed on ice. Samples were spun for 5 minutes / 200*g* in a precooled (4°C) microcentrifuge. Supernatant was carefully aspirated. Samples could be lysed at this stage, or pellets snap frozen in liquid nitrogen, and stored at -70°C pending lysis.

Cell lysis

Nonidet-P40 (NP40) Lysis Buffer -	0.1% NP40	5mM dithiothrietol (DTT)
	150mM KCl	25mM HEPES pH 7.4
	50mM NaF	

Buffer was dispensed into aliquots (450µl) and stored at -20°C.

10x Protease inhibitor cocktail – cOmplete Mini (Roche, Mannheim, Germany) - one tablet dissolved in 1ml ddH_2O.
Solution was dispensed into aliquots (50µl) and stored at -20°C.

At each use, one aliquot of each was thawed, and 1 volume of 10x protease inhibitor cocktail added to 9 volumes NP40 lysis buffer.

All manipulations were performed on ice. Approximately twice the pellet volume of freshly-prepared lysis buffer/protease inhibitor mix was added to each sample, and the suspension mixed by pipetting. For frozen pellets, after adding lysis buffer/protease inhibitor the pellet was allowed to thaw on wet ice prior to mixing. Samples were held on ice for 15 minutes and then centrifuged for 15 minutes at

16000*g* / 4°C. The supernatant (cell lysate) was aspirated into a fresh, chilled (4°C) Eppendorf tube. Protein concentration was determined using the Bradford assay, and cell lysates snap frozen in liquid nitrogen and stored at -70°C.

Bradford Assay for protein quantification

Bradford Reagent (Quick Start™, Bio-Rad)
Protein Standards - Bovine gamma globulin 2mg/ml stock (Bio-Rad) and dilutions

Bradford reagent was allowed to equilibrate to room temperature, and protein standards prepared by serial dilution in ddH_2O.
For each cell lysate to be tested, 20µl of a 1:10 dilution was prepared in ddH_2O.

For each standard and each lysate to be tested, 250µl of Bradford reagent was added to triplicate wells of a 96-well plate, and 5µl of protein solution added. Samples were gently agitated on an orbital plate shaker to mix and allowed to stand at room temperature for 5-10 minutes. Absorbance at 595nm was determined for each well using a microplate reader. Mean absorbance for standards was calculated and plotted to produce a calibration curve, from which protein concentration of tested lysates was determined.

Western Blotting - reagents / materials

1.5M Tris pH 8.8
1M Tris pH 6.8
10% w/v sodium dodecyl sulphate (SDS) (Sigma-Aldrich) in ddH_2O
10% w/v ammonium persulphate (APS) (Sigma-Aldrich) in ddH_2O
N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma-Aldrich)
30% w/v acrylamide / 0.8% bis-acrylamide mix (ProtoGel, National Diagnostics, Hull, UK)
Washing buffer – PBST-WB (PBS + 0.1% Tween 20)
Blocking solution – PBST-WB + 5% w/v skimmed milk powder

ECL reagent (Amersham ECL, GE Healthcare, Chalfont St Giles, UK)
Prestained molecular weight marker [Prestained SDS-PAGE Standards, Broad range (Bio-Rad) / Full Range Rainbow Molecular Weight Marker (GE Healthcare)]
Nitrocellulose membrane (Amersham Hybond ECL, GE Healthcare)
Radiographic film (Amersham Hyperfilm-ECL, GE Healthcare)

Buffers

Running buffer (pH 8.3)- 25mM Tris
190mM glycine
0.1% SDS

Transfer buffer (pH ≥8) - 25mM Tris
190mM glycine
20% v/v methanol

2x Loading buffer (pH 6.8) - 100mM Tris pH 6.8
4% SDS
0.2% bromophenol blue
20% w/v glycerol
200mM DTT (add prior to loading)

Polyacrylamide gel preparation

10% resolving polyacrylamide gels were prepared (based on anticipated protein product size of 15-100kDa), along with 5% stacking gels, on the day of electrophoresis. All components other than APS and TEMED were mixed for resolving and stacking gels as detailed in Table 7, and plates and casting equipment prepared.

APS and TEMED were added to the resolving gel components to initiate polymerisation immediately before casting. After mixing by inversion, the gel was cast between glass plates; ddH_2O was overlaid to inhibit the formation of bubbles.

Once set (approximately 15 minutes) the water was decanted, and APS and TEMED added to the stacking gel components. This was overlaid on the resolving gel and a comb placed for sample separation. Once set, the comb was removed and wells washed with ddH_2O in preparation for addition of samples. Apparatus was set up for electrophoresis with the prepared gel and sufficient running buffer.

Component	For 10% Acrylamide Resolving Gel (ml)	For 5% Acrylamide Stacking Gel (ml)
ddH_2O	1.9	2.1
30% Acrylamide mix	1.7	0.5
1.5M Tris (pH 8.8)	1.3	n/a
1.0M Tris (pH 6.8)	n/a	0.38
10% SDS	0.05	0.03
10% APS	0.05	0.03
TEMED	0.002	0.002

Table 7 - Preparation of polyacrylamide gels. Volumes shown are to make up 5ml and 3ml of resolving and stacking gel, respectively.

Sample preparation

Cell lysates were allowed to thaw on ice. Based on the predetermined protein concentration, the volume of cell lysate for required protein quantity per well was calculated. This was transferred to a fresh Eppendorf tube and mixed 1:1 with 2x loading buffer. Samples were denatured at 95-98°C in a heat block for 3 minutes before loading. A molecular weight marker (volume as recommended by manufacturer) was loaded into at least one well of each gel.

Sample separation and transfer

Samples were run at 180V at room temperature for 45 minutes, or until loading buffer had reached the end of the gel. Gels were removed from electrophoresis apparatus and separated from glass plates. Gels were layered with nitrocellulose membrane between sheets of blotting paper and sponges, which had been presoaked in transfer buffer, and a glass rod rolled over the assembly to exclude bubbles. This

was placed into a transfer cassette. Samples were transferred overnight at 20mA at room temperature, with stirring.

Blocking and primary antibody incubation

Unconjugated primary antibodies were obtained from Abcam (Cambridge, UK), Dako (Glostrup, Denmark) and Novus Biologicals (Littleton, CO, USA). All washes were performed at room temperature with gentle agitation.

Membranes (blots) were removed from the transfer assembly and washed for 1 minute in PBST-WB. Blots were incubated with Ponceau S stain (0.1% w/v in 5% v/v acetic acid) at this stage to ensure the presence of protein. After staining, blots were washed 2-3 times in PBST-WB and then blocked in blocking solution for 30 minutes at room temperature.

Primary antibodies were diluted in blocking solution, initially according to the manufacturer's recommendation and thereafter to optimised concentrations (Table 8). Blots were incubated with primary antibody at 4°C overnight in a humidified chamber.

Antibody	Clonality / Isotype	Manufacturer	Species Specificity	Dilution
ABCG2 Clone BXP-21	Mouse Monoclonal	Abcam	Human	1:200
c-Kit	Rabbit Polyclonal	Dako	Human	1:200
Oct4	Rabbit Polyclonal	Abcam	Human	1:200
Nanog	Rabbit Polyclonal	Abcam	Human / Mouse	1:200
β-actin Clone AC-15	Mouse Monoclonal	Abcam	Xenopus; cross-reactivity incl Human / Mouse / Canine	1:5000 – 1:10000
GAPDH	Rabbit Polyclonal	Novus Biologicals	Bovine; cross-reactivity incl Human / Mouse	1:100 – 1:200

Table 8 – Primary antibodies for western blotting / immunofluorescence

Secondary antibody incubation

Blots were washed for 3 x 5minutes in PBST-WB. Horseradish peroxidase- (HRP-) conjugated secondary antibodies were obtained from Dako (Swine anti-Rabbit-HRP; Rabbit anti-Mouse-HRP). The appropriate secondary antibody was diluted 1:1000 in blocking solution and applied to the membrane. After incubation at room temperature for 1 hour, blots were washed for 3 x 5 minutes in PBST-WB.

Chemiluminescent detection

ECL reagent was prepared according to the manufacturer's instructions and applied to blots. After 1-2 minutes, excess reagent was drained and blots transferred to a Saran wrap folder secured within a film cassette. In a darkroom, radiographic film was loaded into the cassette and allowed to expose for 2-20 minutes depending on signal strength. After developing, the molecular weight marker sizes were labelled on to the radiographic film to allow determination of protein band size.
Alternatively, after draining excess reagent, chemiluminescent signal was visualised using a ChemiDoc XRS imaging system equipped with QuantityOne software (Bio-Rad); a corresponding image was captured indicating the position of the molecular weight marker.

Loading control

Loading controls were performed to check for even protein loading between wells where comparisons were to be made between expression levels in different samples. Blots were washed for 3 x 5minutes in PBST-WB. Anti-human β-actin antibody was diluted in blocking solution and applied to the membrane. After incubating for 2h at room temperature, washing, incubation with secondary swine anti-mouse-HRP and chemiluminescent detection were performed as described.

Immunofluorescence

Primary antibodies for immunofluorescence analysis are detailed in Table 8. Antibodies were tested initially at the manufacturer's recommended concentration and thereafter at optimised dilutions. For each cell type and marker to be assessed, sufficient coverslips / slides were prepared for controls as well as at least two test samples (Table 9).

Sample	Primary Antibody	Secondary Antibody
Test	Yes	Yes
Autofluorescence control	No (Blocking solution only)	No (Blocking solution only)
Secondary-only control – Non-specific binding	No (Blocking solution only)	Yes

Table 9 – Test and control samples prepared to assess expression of each marker, by a given cell type, using immunofluorescent staining

Coverslip preparation for adherent cell cultures

13mm diameter coverslips (VWR International, West Chester, PA) were prepared by acid-washing prior to use in immunofluorescence experiments. 4ml concentrated hydrochloric acid was added to 25ml distilled water, and coverslips washed in the solution on an orbital shaker. After 1 hour, coverslips were rinsed thoroughly with distilled water (approximately 20 changes), and then washed for a further 30 minutes in sterile distilled water. Water was decanted and replaced with absolute ethanol (molecular biology grade). Coverslips were stored under absolute ethanol at 4°C until required.

Preparation of adherent cells

Cells were grown in complete medium in 3.5cm or 10cm culture plates into which had been placed the required number of acid-washed coverslips (see protocol for coverslip preparation). Once cells had grown to 70-80% confluence, the medium

was removed and the monolayer and coverslips washed twice with ice-cold PBS. Coverslips were transferred using forceps to glass Petri dishes for fixation, ensuring that they remained "cell side up".

Preparation of tumoursphere cells

Passage 5-10 tumourspheres were harvested and washed once in PBS as for passage. The cell pellet was resuspended in 1ml cold PBS and held on ice pending cytospin preparation.
100µl aliquots of tumoursphere suspension were transferred onto charged slides (Snowcoat X-tra Adhesive, Surgipath, Richmond IL) at 300rpm (3 minutes) using a Shandon Cytospin 2 (Fisher Scientific, Loughborough, UK). Slides were transferred immediately to glass Coplin jars for fixation/permeabilisation.

Fixation and permeabilisation of samples

Washing buffer – PBST-IF (PBS + 0.05% Tween 20)
All washes were performed at room temperature with gentle agitation.

1) Methanol - Acetone

Slides or coverslips in glass containers were fixed in ice-cold (-20°C) methanol inside a -20°C chest freezer. After 5 minutes, methanol was decanted and replaced with ice-cold (-20°C) acetone for 5-10 minutes to permeabilise cells (permeabilisation times were optimised, with longer incubations used where necessary for nuclear antigens). Slides or coverslips were washed in PBST-IF (3 x 5 minutes) prior to blocking and antibody staining.

2) PFA - TritonX-100

4% PFA was prepared freshly from 12% frozen stocks by thawing and diluting with PBS. Slides or coverslips were fixed in 4% PFA at room temperature for 10

minutes, washed twice in PBS and then permeabilised with 0.1% v/v TritonX-100 (Sigma-Aldrich) in PBS for 5-10 minutes at room temperature (permeabilisation times were optimised, with longer incubations used where necessary for nuclear antigens). Samples were washed with PBST-IF (2 x 5 minutes) prior to blocking and antibody staining.

Blocking and antibody staining

Blocking buffer – PBST + 2% goat serum
DAPI nuclear counterstain – 0.2µg/ml in PBS (1:50000 dilution of 5µg/µl stock)
Mowiol 4-88 mounting medium (Calbiochem/Merck KGaA, Darmstadt, Germany)

Samples were blocked in blocking buffer for 1 hour at room temperature. Primary antibody was diluted in blocking solution (Tables 8 & 9). Samples were incubated overnight with primary antibody (or blocking solution only for secondary only / autofluorescence controls) at 4°C in a humidified chamber, and washed 2x 5min with PBST.

All subsequent manipulations were carried out protected from light. Fluorescein Isothiocyanate- (FITC-) conjugated secondary antibodies were obtained from Abcam, Cambridge, UK (goat anti-mouse-FITC) and Jackson Immunoresearch Laboratories, West Grove, PA (goat anti-mouse-FITC). The appropriate secondary antibody was diluted 1:200 – 1:1000 in blocking solution and applied to samples (or blocking solution only for autofluorescence controls). After incubation in a darkened humidified chamber at room temperature for 1 hour, slides / coverslips were washed for 5 minutes in PBST-IF followed by 5 minutes incubation at room temperature with DAPI nuclear counterstain.

Samples were washed 2 x 5minutes with PBST, mounted using Mowiol (samples on coverslips were inverted onto glass slides; samples on slides were covered with 22mm square coverslips) and allowed to harden at 4°C prior to examination by fluorescence microscopy.

Microscopy

Photomicrographs were captured with a Leitz Fluovert microscope (Leitz, Wetzlar, Germany) equipped with a Nikon Coolpix 4500 camera, or a Zeiss Axiovert 40 microscope (Carl Zeiss, Jena, Germany) equipped with a cooled CCD camera and Axiovision software. Fluorescent images were captured using a Zeiss Axiovert 40 microscope and all post-acquisition image processing was performed using Axiovision software (Carl Zeiss, Jena, Germany).

CHAPTER 3

USE OF FLOW CYTOMETRIC TECHNIQUES TO IDENTIFY CANCER STEM CELLS IN CANINE TUMOURS

INTRODUCTION

As well as the cardinal properties of self-renewal and multilineage differentiation capacity, cancer stem cells might be expected to share features with normal tissue stem cells, in terms of both phenotype and behaviour. These include:

- Unlimited self-renewal capacity - give rise to multiple progeny.
- Multilineage differentiation capacity - reconstitute the tissue from which they are derived.
- Persist in tissues within a supportive microenvironment ("niche").
- Enhanced resistance to toxic agents.

It seems logical that some of the markers used to identify these specialised properties in normal stem cells may be applicable to the search for subpopulations in cancer.

Flow cytometry is used in almost every piece of published research describing the identification or isolation of cancer stem cells. Flow cytometry is a fast and quantitative means of analysing and sorting cells at an individual level, and allows separation according to multiple criteria simultaneously. This makes it ideal for defining heterogeneity amongst populations of cells (Baumgarth and Roederer, 2000; Shapiro, 2003). Moreover, using multiple parameters it can identify very small subpopulations precisely, enabling recognition of potentially rare stem cells (Preffer and Dombkowski, 2009; Tarnok *et al.*, 2010). Flow cytometry is an effective technique for immunophenotyping cells derived from both solid and haematological canine malignancies (Greenlee *et al.*, 1987; Vernau and Moore, 1999; McDonough and Moore, 2000; Culmsee *et al.*, 2001; Weiss, 2001; Akhtar *et al.*, 2004; Jubala *et al.*, 2005; Wilkerson *et al.*, 2005).

The use of flow cytometry to identify cancer stem cells can be broadly divided into two categories – definition according to surface marker phenotype, and demonstration of stem cell-associated functions.

Identification of Cancer Stem Cells using Cell Surface Markers

The list of cell surface antigens used to identify putative cancer stem cells is extensive. In some cases, these are well-established markers of normal stem and progenitor cells (for example, CD34 in the haematopoietic system). For others (e.g. CD133, CD44) the situation is less clear – although they have received considerable attention as cancer stem cell markers for a wide variety of tumour types, they have not been definitively associated with repopulating ability in many of the corresponding non-neoplastic tissues.

The cell surface antigens chosen for this study have been associated with the cancer stem cell fraction of multiple tumour types, by many investigators.

CD34 is a cell surface sialomucin and is one of the most important markers of primitive haematopoietic cells. Although its function has not been fully elucidated, progressive downregulation occurs during differentiation, and the $CD34^+$ fraction of bone marrow has been shown to be highly enriched (although not exclusively responsible) for repopulating activity in multiple species including human, mouse and dog (Sutherland *et al.*, 1990; Brown *et al.*, 1991; Baum *et al.*, 1992; McSweeney *et al.*, 1998; Suter *et al.*, 2004). In seminal studies of leukaemic stem cells (LSC) in acute myeloid leukaemia, the $CD34^+CD38^-$ fraction was shown to be more tumourigenic than the $CD34^-CD38^+$ fraction, and the marker has since been used to isolate LSC in other haematological malignancies (Lapidot *et al.*, 1994; Bonnet and Dick, 1997; Cox *et al.*, 2004; Hong *et al.*, 2008). CD34 is also a marker of endothelial progenitor cells (Hristov and Weber, 2004; Rustemeyer *et al.*, 2006). Expression in some canine leukaemias (McSweeney *et al.*, 1998; Vernau and Moore, 1999; Wilkerson *et al.*, 2005; Gelain *et al.*, 2008) and haemangiosarcomas (Lamerato-Kozicki *et al.*, 2006) is reported.

CD44 is a near-ubiquitously expressed cell surface transmembrane glycoprotein whose major ligand is hyaluronate . It is involved in cell-cell and cell-matrix adhesion and so plays roles in organ structure, cell homing and migration. As well

as the standard form, multiple variants exist as a result of alternative exon splicing and post-translational modification, and these are preferentially expressed by haematopoietic cells (Sneath and Mangham, 1998; Goodison *et al.*, 1999). Aberrant expression of CD44, particularly the variant isotypes, occurs in many types of cancer, particularly in association with invasion and metastasis (Goodison and Tarin, 1998; Herrera-Gayol and Jothy, 1999a; Marhaba and Zoller, 2004). CD44 was first associated with cancer stem cells when the $CD44^{+}CD24^{low/-}$ fraction of human breast tumours was shown to harbour most of their tumourigenic potential (Al-Hajj *et al.*, 2003). It has since been used for prospective CSC isolation in a variety of tumour types (Collins *et al.*, 2005; Jin *et al.*, 2006; Li *et al.*, 2007; Prince *et al.*, 2007; Honeth *et al.*, 2008; Yang and Chang, 2008; Shimada *et al.*, 2009; Takaishi *et al.*, 2009).

CD24 is a glycosylphosphatidylinositol-linked cell surface protein expressed by haematopoietic cells and some neuronal and epithelial tissues (Baumann *et al.*, 2005; Lim and Oh, 2005). Following the demonstration by Al-Hajj *et al* that the tumourigenicity of $CD44^{+}$ breast cancer cells lay within the $CD24^{Low/-}$ fraction (Al-Hajj *et al.*, 2003), the markers have been used in combination to isolate putative $CD44^{+}CD24^{low/-}$ cancer stem cells from multiple tumours and cancer cell lines. It has been suggested that higher levels of CD24 expression are associated with more differentiated cell types, such as luminal breast cancers (Sheridan *et al.*, 2006; Fillmore and Kuperwasser, 2007). In pancreatic cancer, however, $CD24^{+}$ fractions were more tumourigenic than $CD24^{-}$ cells (Li *et al.*, 2007); similarly, a study of colon cancer stem cells found that CD24 expression correlated more closely with clonogenicity than CD44 (Vermeulen and al., 2008). Moreover, the molecule is associated in its own right with tumour progression, invasive and metastatic properties (Baumann *et al.*, 2005; Kim *et al.*, 2007; Shipitsin *et al.*, 2007).

The tyrosine kinase receptor ***CD117 (c-Kit)*** (Yarden *et al.*, 1987) is expressed by certain subsets of normal haematopoietic progenitor cells (Ogawa *et al.*, 1991; Okada *et al.*, 1991; Huss *et al.*, 1995; Niemeyer *et al.*, 2001), and also some more committed cells including mast cells, melanocytes, ductal mammary epithelium and

some neural subsets (Crosier *et al.*, 1993; Matsuda *et al.*, 1993; London *et al.*, 1996; Morini *et al.*, 2004). Its ligand is stem cell factor (SCF, also known as steel factor, Kit ligand and mast cell growth factor). Ligand binding leads to activation of downstream signalling cascades with roles in cell growth and differentiation (Nocka *et al.*, 1990; Williams *et al.*, 1990; Tsai *et al.*, 1991; Shull *et al.*, 1992; Schuening *et al.*, 1993). Along with other tyrosine kinases such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR), dysregulation of CD117 may occur in cancer through overexpression, mutation, chromosomal translocation or autocrine activation (due to production of ligand by tumour cells or stroma). Alterations of expression are reported for multiple human cancers (Wang *et al.*, 1989; Ikeda *et al.*, 1991; Lerner *et al.*, 1991; Sekido *et al.*, 1991; Strohmeyer *et al.*, 1991; Natali *et al.*, 1992; Turner *et al.*, 1992; Rygaard *et al.*, 1993; Toyota *et al.*, 1993; Satzger *et al.*, 2008; Mansuroglu *et al.*, 2009), as well as mast cell tumours, haemangiosarcomas and gastrointestinal stromal tumours in the dog (London *et al.*, 1996; London *et al.*, 1999; Fosmire *et al.*, 2004; Morini *et al.*, 2004; Lamerato-Kozicki *et al.*, 2006). Putative $CD117^+$ cancer stem cell populations have been reported for acute myeloid leukaemias, oral squamous cell and ovarian carcinomas (Chiou *et al.*, 2008; Kirstetter *et al.*, 2008; Zhang *et al.*, 2008a). Tyrosine kinase receptors have become the focus of much attention since the advent of small molecule and monoclonal antibody cancer therapies, which allow more precise targetting of the cells which express them (Gleixner *et al.*, 2007; London *et al.*, 2009; Yan *et al.*, 2009; Zhang *et al.*, 2009a).

CD133 (Prominin-1) is a pentaspan transmembrane glycoprotein, originally recognised on $CD34^+$ haematopoietic stem cells; neural stem cells and endothelial progenitors are also $CD133^+$ (Uchida *et al.*, 2000; Kobari *et al.*, 2001; Handgretinger *et al.*, 2003; Salven *et al.*, 2003; Pfenninger *et al.*, 2007). CD133 has been extensively associated in the literature with putative cancer stem cell populations, in multiple tumour types including brain, lung, prostate, hepatocellular, stomach and colorectal cancers, melanoma and leukaemia (Singh *et al.*, 2004; Collins *et al.*, 2005; Monzani *et al.*, 2007; O'Brien *et al.*, 2007; Chen *et al.*, 2008; Ma *et al.*, 2008; Smith

et al., 2008; Cox *et al.*, 2009). The function of the protein has not been determined, and its status as a cancer stem cell marker remains controversial (Kern and Shibata, 2007; Bidlingmaier *et al.*, 2008; LaBarge and Bissell, 2008). It has been suggested that in some cases, detectable expression may reflect angiogenesis and the presence of endothelial or other supportive cells rather than the tumour cells themselves (Kelly *et al.*, 2007; Adams and Strasser, 2008). In addition, tumourigenic activity exists in both the $CD133^+$ and $CD133^-$ fractions of glioblastomas and colon cancers, each showing distinct molecular and behavioural characteristics (Beier *et al.*, 2007; Shmelkov *et al.*, 2008). It appears that the molecule may be more widely expressed on normal epithelia than previously recognised (LaBarge and Bissell, 2008). Definitive identification of expression is confounded by practical issues, such as the orientation of tissues within histological sections, and variable glycosylation status of epitopes. Available monoclonal antibodies detect one of two major epitopes, AC133 and AC141, which may not necessarily produce concordant staining patterns (Bidlingmaier *et al.*, 2008).

Alternatives to Flow Cytometry for Surface Marker-based Isolation - MACS

Magnetic cell sorting (MACS), using commercially available beads conjugated to CD133 and CD34 antibodies, has become popular as a means of isolating small populations of putative stem or cancer stem cells, as it permits larger samples to be processed at greater speed than can be achieved with fluorescence activated cell sorting (FACS). This leads, however, to cells being "blindly" selected - non-specific binding may occur due to unrelated processes which increase cellular adhesion within the separation column, and cannot be distinguished from specific immunoreactivity (by contrast with flow cytometry, where non-specific binding and dead cells can be readily discriminated). Thus, without subsequent verification of the purity of the separated fraction(s) by flow cytometry, it is an unreliable method of isolation. This is particularly germane when studying cells from a species other than that to which the antibody was raised, or when target cells constitute a rare-event population. The technique was therefore not used in this study.

Identification of Cancer Stem Cells using Functional Assays

Efflux of fluorescent substrates

Normal haematopoietic stem cells express membrane transporter proteins, which may play a protective role by enabling these cells to efflux substances which would otherwise prove toxic to the cells (Zhou *et al.*, 2001; Bunting, 2002; Hadnagy *et al.*, 2006). This property may be exploited in order to identify these cells - those capable of pumping out specific fluorescent substrates will appear dull compared to the main population of cells when analysed using flow cytometry or fluorescence microscopy. Fluorescent dyes used for this purpose include Rhodamine123, a substrate of P-Glycoprotein (P-gP) (Spangrude and Johnson, 1990; Chaudhary and Roninson, 1991), and Hoechst 33342, a substrate of ABCG2 (also known as breast cancer resistance protein, BCRP) (Scharenberg *et al.*, 2002).

P-gP and ABCG2 are members of the ATP-Binding Cassette (ABC) superfamily, a large group of proteins with a common ability to bind ATP as an energy source for the transport of molecules across the cell membrane (Bunting, 2002). Expression of ABC transporters by neoplastic cells has long been recognised as a major problem in clinical cancer therapy, affording multidrug resistance (MDR) – that is, the ability for tumour cells to evade not only the cytotoxic effects of a particular drug or class of drugs, but also those of otherwise unrelated chemotherapeutics, through the expression of a single protein (Licht *et al.*, 1994; Wuchter *et al.*, 2000; Gottesman *et al.*, 2002; Doyle and Ross, 2003; Leonard *et al.*, 2003). Thus, it might be expected that assays for dye efflux properties should be appropriate to identify subpopulations of cancer cells with stem cell-like properties, which may persist after therapy.

Hoechst 33342 exclusion : the Side Population assay

In 1996, Margaret Goodell *et al* described a method for flow cytometric isolation of progenitor-like cells from murine bone marrow, by virtue of their ability to exclude the fluorescent dye Hoechst 33342. The "Side Population" (SP) cells of Hoechst^{low}

fluorescence can be seen as a small (0.05% in murine bone marrow) population of cells to the left of the majority G_0-G_1 population on a blue vs. red profile (Goodell *et al.*, 1996). The SP follows a characteristic arc, due to an increase in the ratio of blue:red emission for this subset, rather than tracing directly from the main population to the plot origin. Human and murine bone marrow SP cells are predominantly $CD34^-Lineage^-$, *i.e.* showing limited correlation with populations isolated according to expression of the classic HSC marker CD34. However, the enrichment of engraftment / reconstitution potential within isolated SP fractions is comparable with that achieved by fractionation according to surface marker expression (Goodell *et al.*, 1996; Goodell *et al.*, 1997; Challen and Little, 2006). The technique has been adapted to identify cells with repopulating ability in a number of normal tissues.

Whilst ABCG2 has been demonstrated as a molecular determinant of the SP phenotype (Zhou *et al.*, 2001), there is some overlap between the roles of different membrane transporters, and it appears that other characteristics may influence cells' presence in the side population (Naylor *et al.*, 2005). For example, in human umbilical cord blood (Alt *et al.*, 2009) and murine embryonic stem cells (Zhou *et al.*, 2001) ABCG2 expression correlates poorly with the SP phenotype, and ABCG2 knockout mice demonstrate normal haematopoiesis (Zhou *et al.*, 2001). Evidence obtained through gene-knockout experiments in mice also suggests that other mechanisms may lead to the appearance of this characteristic profile (Jonker *et al.*, 2005).

Although originally defined as a means of isolating normal haematopoietic repopulating cells from murine bone marrow, side population analysis has become a commonly used method to identify putative cancer stem cells. The side population of numerous malignancies and cancer cell lines has been shown to possess properties such as colony formation, stem cell-associated gene expression, tumoursphere-forming capacity, chemo-/radioresistance and tumourigenicity, exceeding those of the main population (Hirschmann-Jax *et al.*, 2004; Kondo *et al.*, 2004; Wang *et al.*, 2007; Zhou *et al.*, 2007; Addla *et al.*, 2008; Engelmann *et al.*, 2008; Loebinger *et al.*,

2008; Bleau *et al.*, 2009; Fukuda *et al.*, 2009). The size of the SP fraction identified within many tumours and cell lines is variable, sometimes considerably greater than that in corresponding normal tissues; one study showed a range of 4 - 37% in human neuroblastoma cell lines and 0.8 - 51% in primary neuroblastomas (Hirschmann-Jax *et al.*, 2004). Also, the shape of the profile is less consistent than that of the standard SP profile seen for murine BMMNC.

For this reason, it is important that the side population is defined not only by its appearance, but by its reduction when ABC transporter function is inhibited using drugs such as verapamil, reserpine, or Fumitremorgin-C (Eaker *et al.*, 2004). C6 rat glioma cells cultured with platelet-derived growth factor (PDGF) showed a SP-like appearance on a flow cytometric dot plot, but this was not blocked by verapamil – the experimenters accepted that this was therefore "not a *bona fide* side population" (Kondo *et al.*, 2004). The assay itself is very sensitive and requires careful titration with regards cell type and species, in order to ascertain optimal staining concentrations and times (Goodell *et al.*, 1997) – for example, a larger than expected SP may actually represent *under*staining of the majority population. A review of the literature revealed only two published reports describing use of the Hoechst 33342 exclusion assay with canine cells, in the analysis of dental pulp and of normal liver (Iohara *et al.*, 2006; Arends *et al.*, 2009).

As with every CSC assay, the underlying biology of the phenomenon is not fully understood, leading to some uncertainties in interpretation of results. Proliferation status may affect dye efflux capacity, such that it can identify quiescent cells (such as HSC in bone marrow) but may less readily discriminate between actively cycling cells (Spangrude and Johnson, 1990; Uchida *et al.*, 2004; Stingl *et al.*, 2006). In studies of prostatic cancer cell lines and a xenograft tumour, Patrawala *et al* found that whilst the SP showed enhanced tumourigenicity, this did not correlate with ABCG2 expression - $ABCG2^-$ cells had tumourigenic capacity equivalent to $ABCG2^+$ cells, and also expressed certain stem cell-associated genes not seen in the $ABCG2^+$ subset. The incomplete overlap between ABCG2-expressing cells and SP

was attributed in this report to differences in cell cycling time (Patrawala *et al.*, 2005).

Studies of normal murine mammary epithelial cells found poor correlation between SP and stem cell identity / repopulating ability as defined by cell surface markers (Shackleton *et al.*, 2006; Stingl *et al.*, 2006). Similarly, overlap between Hoechst-effluxing and surface marker-defined CSC populations in cancer may be limited (Fan *et al.*, 2006), and in some cancers there is no association between side population and CSC properties (Lichtenauer *et al.*, 2008).

It has been pointed out that, since Hoechst 33342 can be toxic to some cells (Fried *et al.*, 1982; Durand and Olive, 2001), enhanced replicative capacity in a Ho^{low} subpopulation is perhaps not surprising, and may be secondary to the nature of the assay itself rather than a feature of the cells isolated *per se* (Hadnagy *et al.*, 2006; Hill, 2006). Zheng *et al* demonstrated that growth and clone formation of C6 glioma cells incubated in the presence of Hoechst was reduced and that this in itself could account for differential behaviour of side *vs* main population cells in tumourigenicity assays (Zheng *et al.*, 2007). Conversely, other investigators have shown equivalent growth or viability in cells which retain and those which efflux Hoechst (Murase *et al.*, 2009; Zhang *et al.*, 2009b)

Thus, the appearance of a SP is a useful guideline for stem-like properties, but is not reliable when considered alone. As not all SP cells are stem cells (Triel *et al.*, 2004; Addla *et al.*, 2008) (and as with any assay purporting to identify stem or cancer stem cells), it is important to validate any isolated SP cells using phenotypic markers and/or functional analysis for stem cell-like properties. This relies on an appreciation of the expected phenotype of a stem or progenitor cell in the tissue under scrutiny. It is also important to recognise that the growth kinetics and characteristics of cell lines are likely to be different from primary tissues in terms of expected SP (Zheng *et al.*, 2007).

For optimal excitation, analysis of Hoechst 33342 (*ex* 350nm, *em* 461nm) requires an ultraviolet laser. In addition to these being available only on high-end flow cytometers, high power UV radiation has greater potential to cause DNA damage, and thus be deleterious to cell viability. Simpson *et al* showed that the presence of a side population, albeit with a less defined profile, may be determined using a violet laser (Simpson *et al.*, 2006). In addition, Vybrant®DyeCycle™Violet, a fluorescent substrate used for cell cycle analysis of viable cells, can also identify the SP in haematopoietic cells and may prove more suitable for live cell isolation (Telford *et al.*, 2007).

Rhodamine 123 efflux

Rhodamine 123 is another fluorescent substrate used to identify subsets of bone marrow cells enriched for haematopoietic stem cell activity (Visser and de Vries, 1988; Spangrude and Johnson, 1990; Baum *et al.*, 1992; Phillips *et al.*, 1992; Zijlmans *et al.*, 1995; Bertoncello and Williams, 2004). Rhodamine 123 efflux has been demonstrated in canine haematopoietic progenitors (Niemeyer *et al.*, 2001; Suter *et al.*, 2004; Wijewardana *et al.*, 2007). This capability is afforded by P-glycoprotein (P-gp), an ABC membrane transporter pump encoded by the MDR1 gene (Steingold *et al.*, 1998; Zhou *et al.*, 2001). In addition, point mutations in ABCG2 [specifically, substitution of threonine for arginine at amino acid 482 – $ABCG2^{482T}$] alters substrate specificity to permit Rhodamine 123 efflux (Honjo *et al.*, 2001; Robey *et al.*, 2003; Alqawi *et al.*, 2004).

Rhodamine efflux analysis has been used less widely than side population analysis in the identification of putative CSC populations (Monzani *et al.*, 2007; Wu *et al.*, 2007). Nonetheless, it seems logical that it might similarly demonstrate the existence of subpopulations of stem-like cells with enhanced drug efflux / resistance capacity. Moreover, its suitability for the analysis of canine cells has been demonstrated, and logistically it has a major advantage over Hoechst 33342 in that its fluorescence characteristics (*ex* 488nm *em* 520nm) permit analysis using standard benchtop flow cytometers.

Activity of intracellular enzymes – Aldehyde Dehydrogenase

Identification of haematopoietic cells expressing high levels of aldehyde dehydrogenase, ALDH, is now a recognised flow cytometric method of identifying human HSC (Kastan *et al.*, 1990; Storms *et al.*, 1999; Armstrong *et al.*, 2004; Hess *et al.*, 2004; Christ *et al.*, 2007); in fact, ALDH expression correlates better with repopulating ability than side population in the human system (the converse is true for murine HSC) (Pearce and Bonnet, 2007)(K. Samuel, University of Edinburgh - *pers. comm.*). This enzyme imparts the resistance shown by hHSC to alkylating agents such as cyclophosphamide (Gordon *et al.*, 1985; Sahovic *et al.*, 1988).

The Aldefluor (Stem Cell Technologies, Grenoble, France) assay is available as a commercial kit, and detects high levels of the isoenzyme ALDH-1A1. Optimised to identify human HSC, the assay has been reported to discriminate candidate stem cell populations within other tissues including breast, brain and colon, and to identify endothelial and mesenchymal progenitors (Gentry *et al.*, 2007; Ginestier *et al.*, 2007; Huang *et al.*, 2009b). Cells from other species, including dog, have also been investigated with results suggesting that the assay may be applicable in diverse model systems (Fiordalisi *et al.*, 2005). The fluorescent assay substrate, Bodipy-Aminoacetaldehyde (BAAA), can diffuse freely across plasma membranes - in the presence of ALDH-1A1 it is converted to Bodipy-Aminoacetate (BAA), a polar molecule which is retained within the cell. ALDH-positive cells can be identified as a bright population which is diminished in the presence of a specific inhibitor, diethylamino-benzaldehyde (DEAB). Normal human haematopoietic progenitors appear as an $ALDH^{bright}$ $Side\text{-}Scatter^{low}$ subset (Storms *et al.*, 1999).

As with membrane transporter-mediated efflux, intracellular detoxification mechanisms allowing evasion of the effects of chemotherapy drugs might be expected to identify persistent cancer stem cells. Aldehyde dehydrogenase expression has been used to identify putative CSC in malignancies including colon, prostate, breast and lung tumours, acute myeloid leukaemia and multiple myeloma (Pearce *et al.*, 2005; Ginestier *et al.*, 2007; Matsui *et al.*, 2008; Carpentino *et al.*,

2009; Huang *et al.*, 2009b; Jiang *et al.*, 2009; Li *et al.*, 2010; Morimoto *et al.*, 2009; Tanei *et al.*, 2009), and in some cases has been demonstrated as a negative prognostic indicator (Ginestier *et al.*, 2007; Jiang *et al.*, 2009; Li *et al.*, 2010; Morimoto *et al.*, 2009).

This study sought to apply these flow cytometric techniques to the canine model system, to identify candidate canine CSC subpopulations.

MATERIALS AND METHODS

Cell Surface Phenotyping

Immunophenotyping was performed using fluorophore-conjugated test antibodies using a direct (one-stage, no secondary antibody) technique, as described in Materials and Methods chapter. For each cell line and marker, optimal antibody concentration was determined by titration. Antibody specificity, fluorophore and source are detailed in Materials and Methods chapter (Table 2).

Hoechst 33342 Efflux / Side Population Analysis

Hoechst 33342 (Sigma) – 1mg/ml (1µg/µl) in ddH_20
Hoechst-DMEM – DMEM + 2% FCS + 10mM HEPES
Hoechst-HBSS – HBSS + 2% FCS + 10mM HEPES
Verapamil (Sigma) – 5mM (100x) in absolute ethanol

Cells were harvested in complete medium, counted, centrifuged and resuspended in prewarmed (37°C) Hoechst-DMEM at $1x10^6$ cells/ml. Aliquots of $1x10^6$ cells were placed in 15ml conical centrifuge tubes, with at least 2 aliquots (test + control) per sample. 50µM Verapamil (5µl of 100x stock) was added to control samples and all tubes preincubated at 37°C waterbath for 10-15 minutes. Hoechst 33342 was added at 5µg/ml* (5µl of 1mg/ml stock) to all samples. Samples were incubated at 37°C, with regular mixing, for 90 minutes*.

All tubes were placed on ice immediately after incubation; cells were centrifuged at 300*g* / 4°C / 5 minutes, resuspended in 1ml ice-cold Hoechst-HBSS and held on ice pending flow cytometry. PI was added just prior to flow cytometry at 2µg/ml for dead cell discrimination. At least 50000 - 100000 events were aquired for analysis except where, as stated, this was precluded by low cell numbers

* Standard conditions from Goodell *et al.* (Goodell *et al.*, 1996)

Conditions were optimised for each cell line by varying Hoechst concentration and incubation time..

DyeCycle Violet staining protocol

(Modified from Telford *et al* (Telford *et al.*, 2007))
Vybrant DyeCycle Violet 5mM solution in ddH_2O (Molecular Probes / Invitrogen)

As for Hoechst 33342 analysis with some modifications:
Cells were suspended in Hoechst-HBSS at 1 x 10^6/ml and prewarmed at 37°C +/- verapamil (10-15 minutes). DyeCycle Violet (DCV) was added to 10µM from 5mM stock – i.e. 2µl added to 1ml cell suspension. (Resolution is possible at 5µM - but Telford *et al* report more reproducible at this higher concentration). After 90minutes incubation at 37°C, samples were centrifuged at 300*g*, 4°C for 5 minutes and resuspended at 1x10^6 cells/ml in cold Hoechst-HBSS. PI (2µg/ml) was added just prior to flow cytometry for dead cell discrimination.

† Telford *et al* stained aliquots of 5x10^6 cells/ml. Due to limited available cell numbers and to allow comparison with Hoechst 33342 we used 1x10^6/ml.

Rhodamine Efflux Analysis

Rhodamine 123 (Sigma) - 1mg/ml in absolute ethanol
PBS-Rho – PBS + 2% FCS

Cells were harvested in complete medium, counted by trypan blue exclusion and resuspended at 1x10^6/ml in PBS-Rho. Two aliquots of 1x10^6 cells were labelled "Test" and "Control" and placed on ice. Rhodamine 123 was diluted 1:50 in PBS to 20µg/ml (200x) and added to cells at a final concentration of 0.1µg/ml (5µl per ml of cell suspension). Cells were placed in a waterbath at 37°C, in the dark, for 30 minutes (loading) and then washed twice with 2ml of ice-cold PBS-Rho.

Control samples were resuspended in 1ml ice-cold PBS-Rho and placed on ice, in the dark, for 40-60 minutes. Test samples were resuspended in 2ml PBS-Rho and placed in a waterbath at 37°C, in the dark, for 40-60 minutes (efflux).

All samples were centrifuged at 4°C, 300*g* for 5 minutes, resuspended in 300µl ice-cold PBS-Rho, and held on ice, in the dark, pending analysis by flow cytometry. PI was added just prior to flow cytometry at 2µg/ml for dead cell discrimination if required. At least 100000 events were acquired for analysis.

Aldefluor Staining Protocol for ALDH Expression

Aldefluor Test Kit (Aldagen, Stem Cell Technologies, Grenoble, France)
Comprises Aldefluor substrate, diethylamino-benzaldehyde (DEAB) inhibitor, Aldefluor buffer

Cells were harvested in complete medium, counted, centrifuged and resuspended in Aldefluor buffer‡ at $1x10^6$ cells/ml. Two tubes were labelled "Test" and "Control". 1ml cell suspension was placed in "test" tube. 5µl DEAB inhibitor was placed in "Control" tube and the lid replaced tightly.

5µl (1.5µM)‡ Aldefluor substrate was added to cell suspension in "Test" tube and mixed. Immediately, 0.5ml of the mixture was withdrawn and placed in the "Control" tube. Tubes were incubated at 37°C, in the dark, for 30-60 minutes (not exceeding 60 minutes). After incubation, samples were placed on ice and centrifuged at 4°C, 300*g* for 5 minutes. The supernatant was discarded and cells resuspended in 500µl fresh ice-cold Aldefluor buffer, and held on ice pending flow cytometry. PI was added just prior to flow cytometry at 2µg/ml for dead cell discrimination. At least 100000 events were acquired.

‡ Fiordalisi Modification (Fiordalisi *et al.*, 2005)
0.1% Sodium Azide / 50µM Verapamil added to Aldefluor buffer.
Concentration of Aldefluor substrate increased to 4.8µM

Western blots, derivation of kt-osa1-5 from a spontaneous canine osteosarcoma and isolation of normal canine BMMNC and leukaemic canine BMMNC / PBMNC were performed as described in Materials and Methods chapter.

RESULTS

Analysis of Surface Marker Expression on Canine Cancer Cell Lines

Each of the canine cancer cell lines was assessed for expression of the stem cell-associated surface markers CD34, CD44, CD117 and CD133 and CD24. Of the antibodies used, CD34 (anti-canine, clone 1H6) is species-specific (McSweeney *et al.*, 1998) and CD44 (anti-mouse/human, clone IM7) has confirmed canine cross-reactivity (Neame and Isacke, 1993; Sandmaier *et al.*, 1998). The use of the antibodies to CD133 (anti-mouse, clone 13A4) and CD117 (anti-human, clone YB5.B8) has been reported in the immunophenotyping of canine cancer cells (London *et al.*, 1999; Lamerato-Kozicki *et al.*, 2006).

Binding of the CD34, CD117 and CD133 antibodies to canine cells was confirmed using normal canine bone marrow mononuclear cells (BMMNC). Staining pattern and relative position of stained cells on light scatter (Forward Scatter FSC *vs* Side Scatter SSC) are shown in Figures 1 and 2. Positive cells represented 0.8-1%, 0.1% and 0.2% of BMMNC, respectively. Unstained and isotype-matched controls supported specificity of antibody binding. The majority of $CD34^{+}$ and $CD133^{+}$ cells were SSC^{Low}, indicating relatively low cellular complexity, and of these the majority were also FSC^{Low} (FSC is an indicator of relative cell size) - haematopoietic stem/progenitor cells are predominantly recognised as $FSC^{Low}SSC^{Low}$ cells. $CD117^{+}$ cells were distributed more evenly across the scatter profile, which may reflect expression of the antigen on more differentiated cellular subsets.

For all of the canine cancer cell lines, and for the feline mammary carcinoma cell line CatMT, staining for CD34, CD117 and CD133 was negative. Figure 3 gives an example of how antibody binding was assessed. Live cells were selected for analysis using the scatter profile, followed by assessment of fluorescence for test sample, unstained cells (to define the baseline for intrinsic cellular fluorescence, i.e. autofluorescence) and isotype-matched control antibody (to assess levels of non-

specific binding). None of the cell lines showed binding of test antibody beyond isotype control levels.

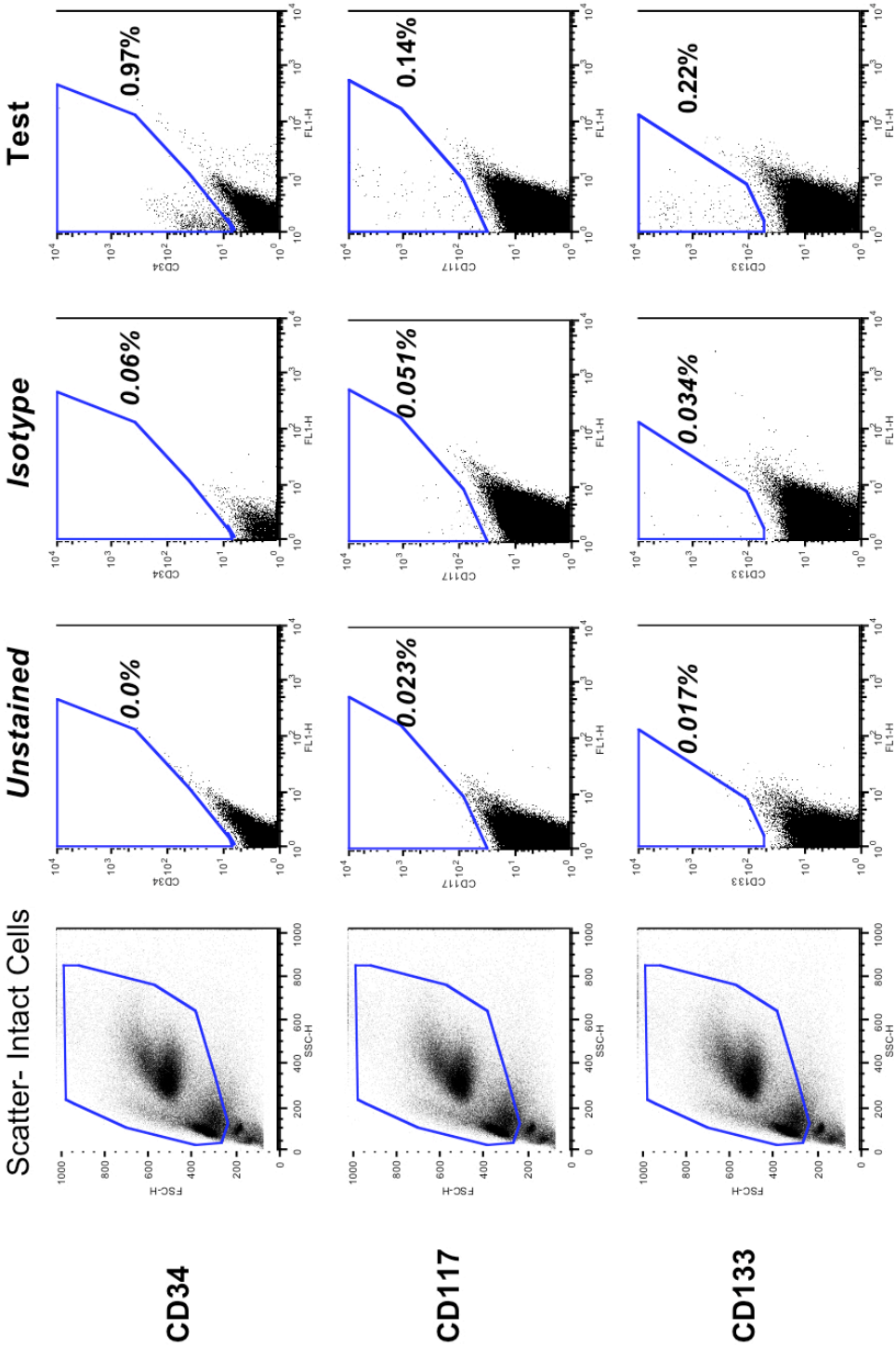


Figure 1 - Staining of normal canine BMMNC with CD34-PE (top), CD117-PECy5 (middle) and CD133-APC (bottom). Far left plots show gating strategy to select only intact ("live") cells for analysis and exclude erythrocytes. Autofluorescence (unstained) and non-specific binding (isotype) controls are illustrated for comparison. At least 1x10e6 events acquired for each sample.

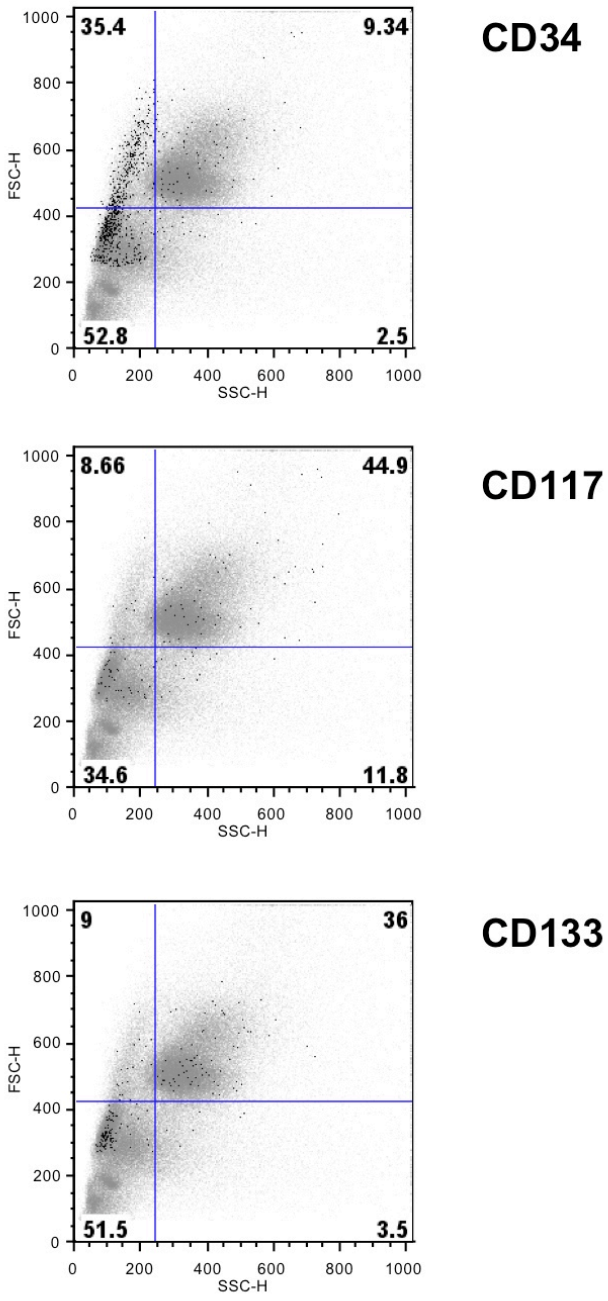


Figure 2 - Plots to show relative position of normal canine BMMNC stained for CD34 (top), CD117 (middle) and CD133 (bottom) on light scatter profile. Cells positive for each marker are shown in black, overlaid on to total acquired cells. Figures indicate percentage of stained cells in each quadrant of plot. For CD34 and CD133, the majority of cells are SSC-low, and most of these are also FSC-low. $CD117^{+}$ cells are distributed more evenly across the scatter profile.

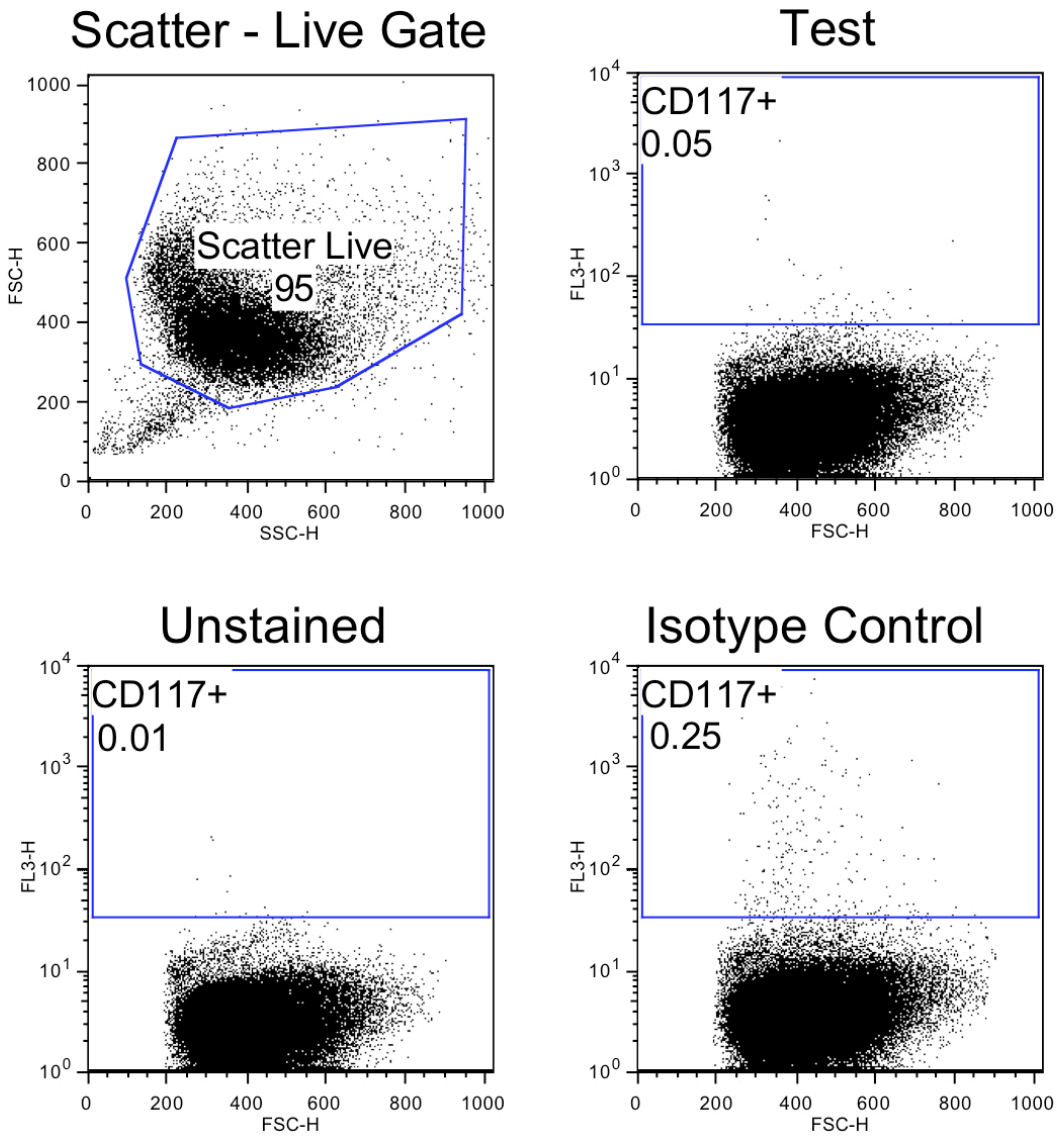


Figure 3 - Example of standard gating strategy used to determine surface expression by canine cancer cell lines.
Figure shows assessment of CD117 expression for 3132 canine lymphoma cell line.

Top left - Dead cells and debris are excluded from analysis using light scatter characteristics (FSC vs SSC profile). Staining on test sample (*top right*) is negative, as assessed by comparison of percentage positive cells with unstained (*bottom left*) and isotype control (*bottom right*) samples.

Staining for CD24 was also negative, or equivalent to that seen with isotype-matched controls, in all tested cell lines. The antibody to CD24 showed a tendency towards non-specific binding, with an affinity to dead cells for some cell lines - apparent weak positive staining on a small population of cells was lost when dead cells and debris were excluded from the analysis (using the FSC-SSC profile +/- addition of propidium iodide as a dead cell discriminator) (Figure 4). Although the 3132 B-cell lymphoma cell line showed strong CD24 staining, this was matched by the isotype control despite serial titration / increased levels of blocking protein; this suggests that these cells have an affinity for this class of antibody (Figure 5). It is likely that an alternative antibody will be required for investigation of this marker with canine cells.

All canine cancer cell lines tested were $CD44^{+}$. For all of the cell lines, positivity was manifest as a shift in the entire population along the fluorescence axis rather than segregation of a specific subpopulation of stained cells. As shown in Figure 6, whilst the level of expression (CD44 staining brightness) varied between cell types, for all of the cell lines the frequency of positive cells was >97%, indicating expression on almost all cells. The human MCF-7 mammary carcinoma cell line demonstrated a lower frequency of positive staining for CD44 (48.9%), with a greater proportion of cells staining positive for CD24 (73.7%), in agreement with published findings (Sheridan *et al.*, 2006; Fillmore and Kuperwasser, 2008) (Figure 7).

Thus, for the canine cancer cell lines examined, specific subpopulations of cells expressing CSC-associated markers were not identified. Where antibody staining was positive (e.g. CD44), it led to an increase in the fluorescence of the whole population, rather than on a specific subset of positive cells.

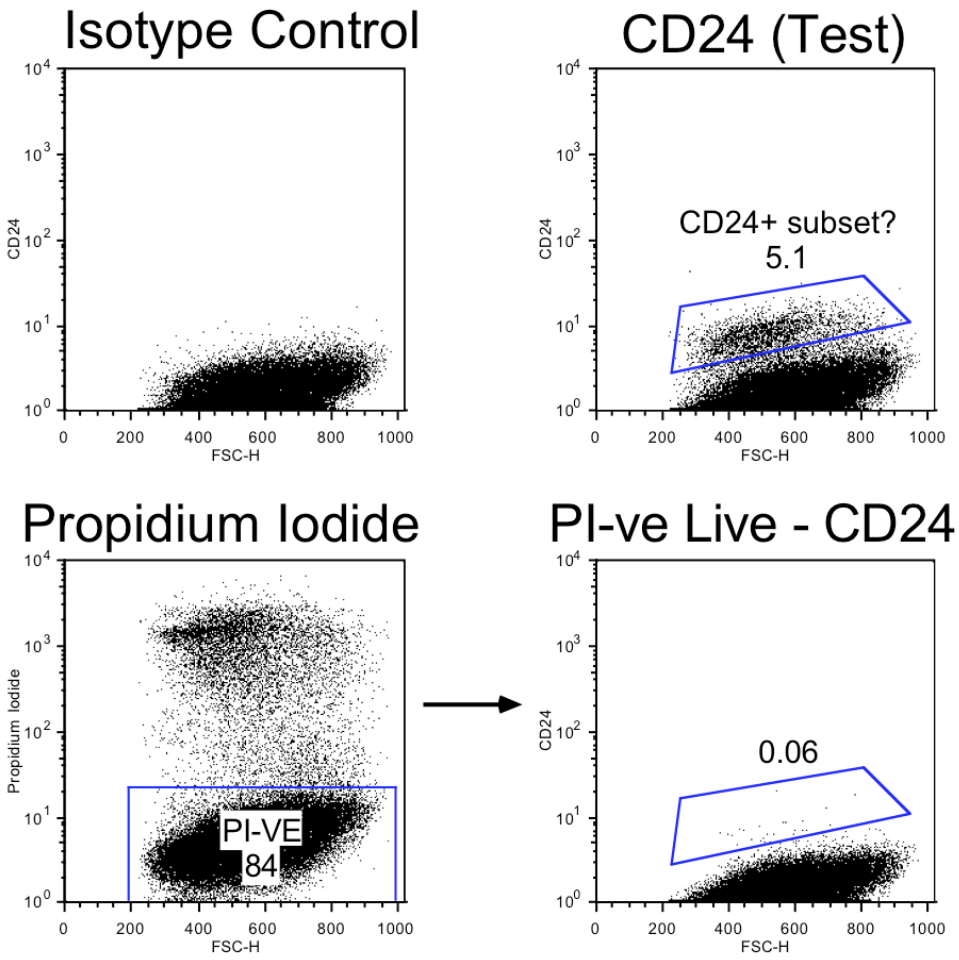


Figure 4 - Small population of REM134 canine mammary carcinoma cells showing apparent positive staining for CD24 compared to isotype control (upper panels). However, this is lost when dead cells are excluded from the analysis using propidium iodide (lower panels).

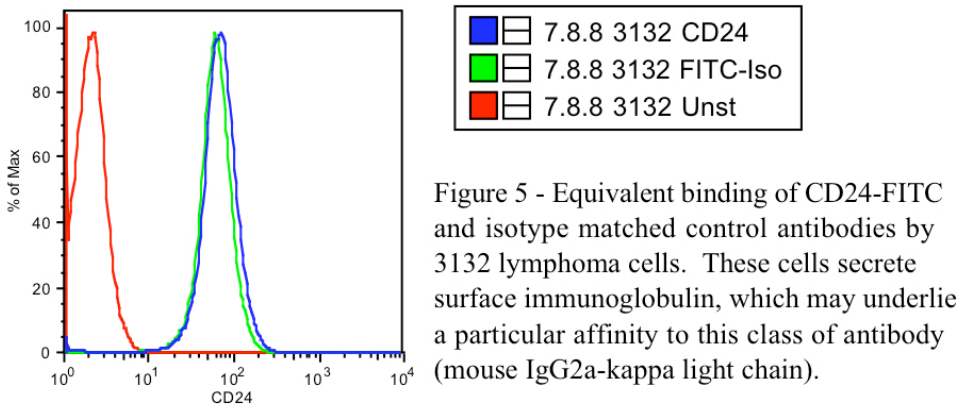


Figure 5 - Equivalent binding of CD24-FITC and isotype matched control antibodies by 3132 lymphoma cells. These cells secrete surface immunoglobulin, which may underlie a particular affinity to this class of antibody (mouse IgG2a-kappa light chain).

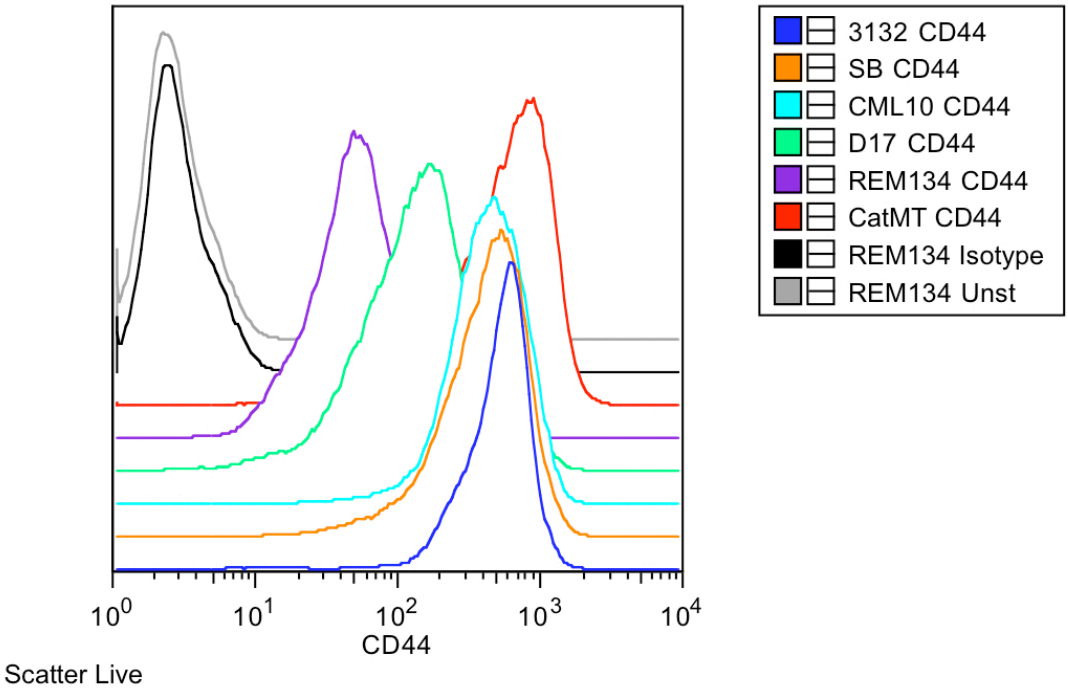
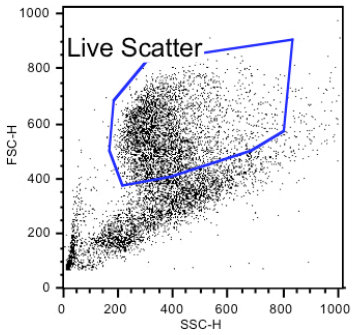


Figure 6 - (Above) Overlay of histograms illustrating CD44 surface expression by canine (and feline CatMT) cell lines. Representative unstained and isotype control samples (REM134) are also shown.

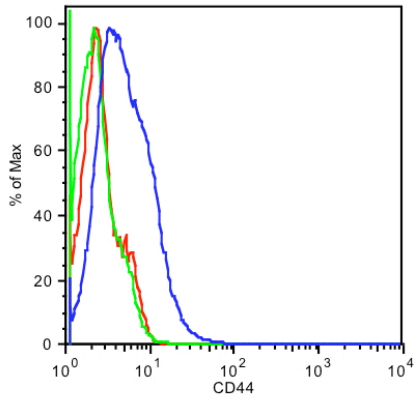
(Below) - Table to show percentage of CD44-positive cells for each cell line, as compared to the relevant isotype control sample. All lines are >97% CD44+ve.

Cell Line	CD44 Positive (%)
3132 CD44	99.177
SB CD44	99.58
CML10 CD44	99.764
D17 CD44	98.334
REM134 CD44	97.372
CatMT CD44	99.276

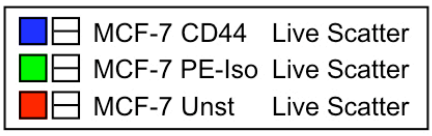


Ungated
MCF-7 Unst+PI

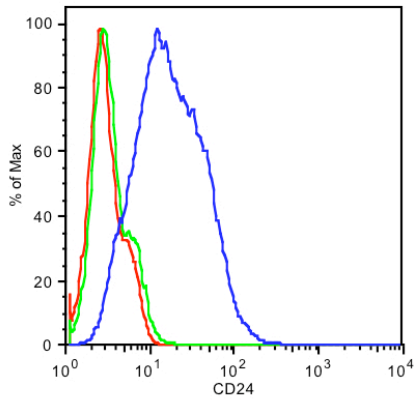
Figure 7 - Surface expression of CD44 and CD24 for human MCF-7 mammary carcinoma cells.
Left - Live gate strategy as determined using light scatter and confirmed with PI dead cell discriminator.
Below - Histograms representing level of CD44 (upper) and CD24 (lower) staining in comparison with unstained and relevant isotype control samples.
MCF-7 cell line show an increased frequency of CD24+, and fewer CD44+, cells as compared to tested canine cancer cell lines.



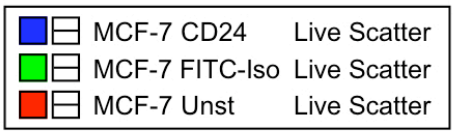
Live Scatter



Sample	%Positive CD44
MCF-7 CD44	48.992



Live Scatter

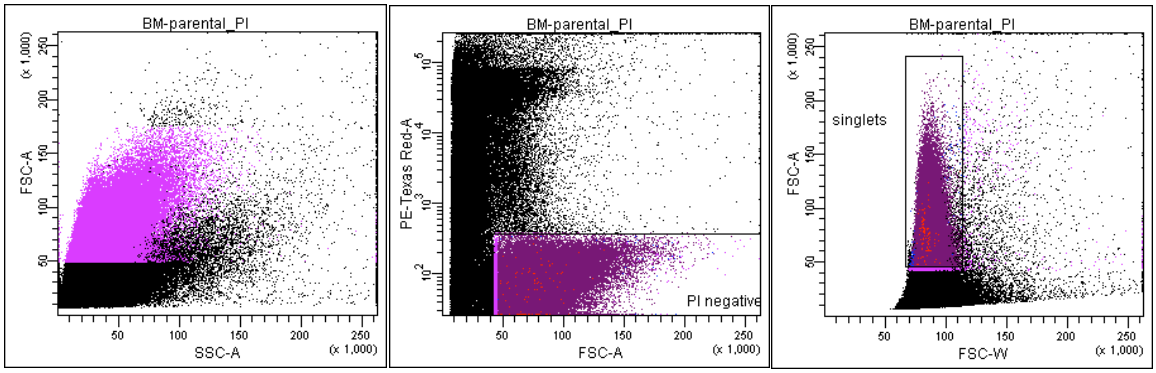


Sample	%Positive CD24
MCF-7 CD24	73.713

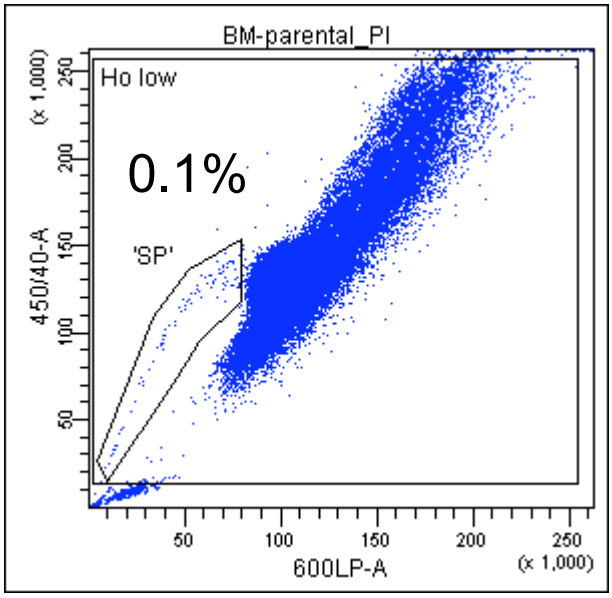
Demonstration of a Hoechst-effluxing Side Population within Canine Cancer Cell Lines

Normal canine BMMNC were tested for the presence of a side population profile, according to the protocol of Goodell *et al* (Goodell *et al.*, 1996). Although the expected profile could be demonstrated, with a SP of 0.084 – 0.1% of live cells (Figure 8), this was inconsistent. In particular, the response to the addition of verapamil was unpredictable, and did not reproducibly lead to reduction or loss of the SP – on one occasion, the frequency of cells in the SP gate was greater, and the profile more defined, in the presence of verapamil than in the test sample. The human MCF-7 breast carcinoma cell line was examined for the presence of a SP – this was seen reproducibly, representing 0.3 – 0.8% of live cells (Figure 9). Canine cancer cell lines were analysed for Hoechst 33342 efflux, with titration of both substrate concentration and incubation time to optimise assay conditions (Table 1). All of the cell lines showed a candidate SP, diminished or lost in the presence of verapamil, on at least one occasion. In the protocol as developed by Goodell *et al* to define repopulating cells in murine BMMNC, and in most subsequent reports of SP analysis, cells are incubated for 90 minutes at 5µg/ml Hoechst ("standard conditions"). 3132 lymphoma cells showed best definition of SP under these conditions, having 3.2% of cells within the gate compared to 0.3% for the verapamil control - a candidate SP (0.9%) was also visualised after 60 minutes' incubation at this concentration of Hoechst (Table 2).

REM134 canine mammary carcinoma cells showed best definition of SP (2.41% of live single cells) at 2.5µg/µl Hoechst, with a slightly longer incubation (120 minutes) (Table 3, Figure 10). Indeed, incubation under standard conditions gave a greater percentage of cells in the SP gate of the verapamil control (0.87%) than in the test sample (0.51%). Increased incubation times were associated with increased cell death (as determined by uptake of propidium iodide by intact cells) – at 120 minutes, cell death was greater than 20% for both 2.5 and 5µg/ml Hoechst, increasing to 50.9% at 180 minutes / 5µg/ml Hoechst.



Light Scatter Profile: Select Intact Cells → Propidium Iodide: Select PI-ve Live Cells → Signal Area *vs* Width: Select Single Cells



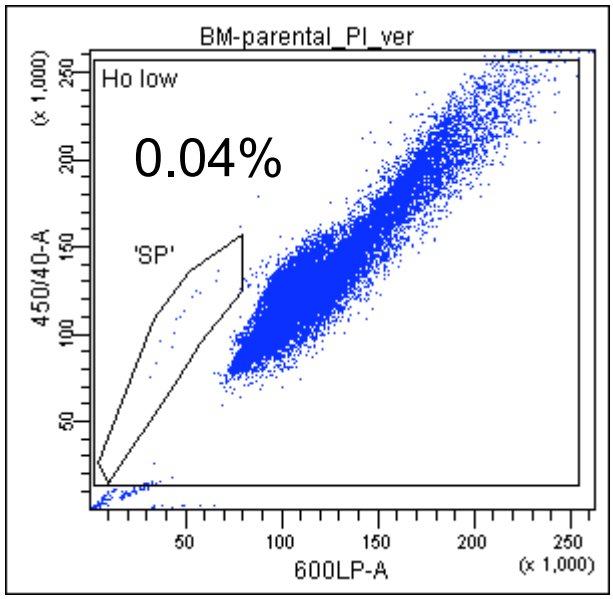
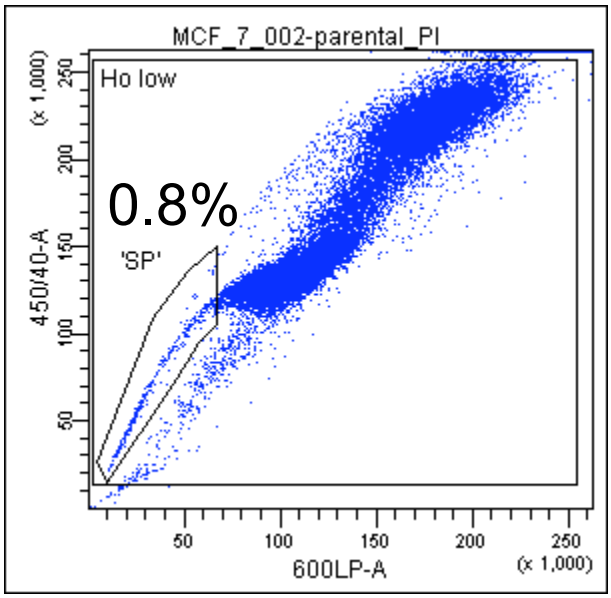


Figure 8 – Hoechst 33342 efflux analysis identifies a side population in normal canine bone marrow mononuclear cells.

Top panels – Standard gating strategy used to select only intact, live single cells for analysis. Sufficient events were acquired for at least 100000 PI^{-} cells.

Upper Left – Test sample – a defined side population represents 0.1% of live single cells.

Lower Left – Control sample - side population is reduced in the presence of the ABC transporter inhibitor verapamil.



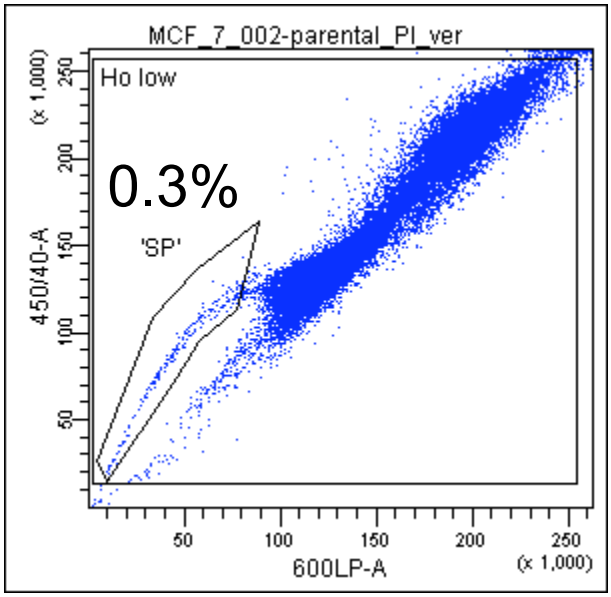


Figure 9 – Hoechst 33342 efflux analysis of human MCF-7 mammary carcinoma cell line demonstrates a side population.

Upper Left – Test sample – SP of 0.8%. The appearance of an SP for this cell line was reproducible, representing between 0.3% and 0.8% of live single cells.

Lower Left – SP is reduced in the presence of verapamil inhibitor.

Cell Line	Origin	Hoechst (µg/ml)	Time (min)
3132	Haematopoietic	5	90
REM134	Epithelial	2.5	120
CML10	Neurectodermal	5	120
SB	Endothelial	5	120
D17	Mesenchymal	5	90
CatMT	Epithelial	5	60

Table 1 – Optimal conditions for SP discrimination for canine, and feline CatMT, cancer cell lines

Hoechst (µg/ml)	Time (min)	**TEST** SP (%)	**TEST** Dead (%)	**VERAP** SP (%)	**VERAP** Dead (%)	**Comments**
5	60	**0.9**	9.2	**0.0**	9.7	SP present, reduced with verapamil
5	90	**3.2**	14.5	**0.3**	12.8	SP present, reduced with verapamil
5	120	0.0	20.1	0.0	38.9	No SP. Increased cell death
2.5	90	22.0	17.6	9.6	10.5	Many cells in SP region
2.5	120	8.4	17.9	5.7	16.7	Many cells in SP region

Table 2 – Summary of titration of assay conditions for SP analysis of 3132 canine lymphoma cells. This was one of the cell lines chosen for further investigation (see text and figure 15). Subsequent assays were performed at 5µg/µl Hoechst / 90 minutes incubation, for optimal differentiation between test sample and verapamil control.

Hoechst (µg/ml)	Time (min)	**TEST** SP (%)	**TEST** Dead (%)	**VERAP** SP (%)	**VERAP** Dead (%)	**Comments**
5	60	**1.96**	11.4	**0.42**	10.8	Poorly-defined profile
5	90	0.51	18.5	0.87	15.7	SP increases with verapamil
5	120	**0.55**	26.7	**0.27**	24.0	Poorly-defined profile
5	180	**3.58**	50.9	**1.17**	32.0	Streak to origin. Cell death ++
2.5	90	**0.88**	14.4	**0.34**	16.6	Poorly-defined profile
2.5	120	**2.41**	23.2	**0.2**	18.6	**Best definition of SP profile**
10	90	0.12	17.2	0.24	23.5	SP increases with verapamil

Table 3 – Summary of titration of Hoechst efflux by REM134 canine mammary carcinoma cells. The size of the SP, where seen, is variable (0.55 – 2.41% of live cells). Cell death increases with incubation time.

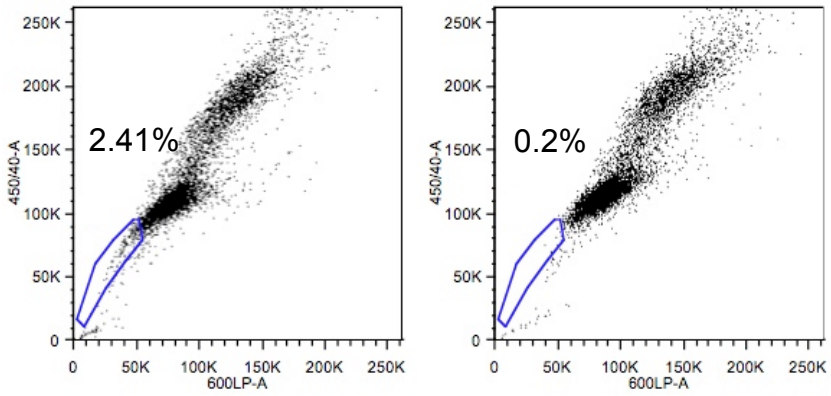


Figure 10 – SP profile of REM134 cells at 2.5µg/ml Hoechst / 120 minutes incubation (*left*), reduced by verapamil (*right*). Similar profiles were seen at 5µg/ml / 60 minutes and 5µg/ml / 180 minutes. These results were replicated on only one subsequent occasion despite repeated attempts, and never when the assay was scaled up for cell sorting.

The CML10 melanoma and SB haemangiosarcoma cell lines showed optimum resolution of SP, compared to corresponding verapamil controls, at 5µg/ml Hoechst / 120 minutes incubation. For both of these cell lines, the Hoechst staining profile was unusual, with two distinct populations each giving rise to a candidate SP arm (Figure 11). On a standard Hoechst efflux profile for normal bone marrow, the SP originates from the G_0G_1 population; the G_2/M population appears twice as far from the plot origin on the linear scale, as its cells contain twice the amount of DNA. The profiles seen for CML10 and SB may therefore indicate a Hoechst-effluxing SP within both G_0G_1 and G_2/M populations. Alternatively, the unusual profiles may be indicative of aneuploidy within these cell lines, whereby the upper population includes G_0/G_1 cells with increased DNA content. CML10 showed a similar, but less defined, SP profile after 90 minutes' incubation with 5 or 10µg/ml Hoechst, but at 2.5µg/ml, the verapamil control had a greater proportion of cells in the SP region (1.66%) than the test sample (0.018%). SB showed this "reversal" of profiles at all other tested timepoints / Hoechst concentrations.

D17 osteosarcoma cells showed a SP only under standard conditions, representing 9.2% of live single cells; although substantial compared to the SP of normal bone marrow, this was reduced by verapamil (5.6%), and greater frequencies of SP have been reported for other cancer cell lines. However, this was not replicated when the cells were retested using the same assay conditions – cells were present in the SP gate for the control but not the test sample (Figure 12). This inconsistency between experiments was a frequent and frustrating finding when testing canine cells using this assay.

As well as reversal of expected profiles, verapamil in some cases caused the entire population to shift from its location in the test sample, complicating interpretation. Gates to define the SP for each test sample are set according to its loss in the corresponding verapamil sample, and so should be similarly located in relation to the main (non SP) population for accurate quantification. This may indicate that other ABCG2 inhibitory drugs may be more suitable for use in this assay for analysis of

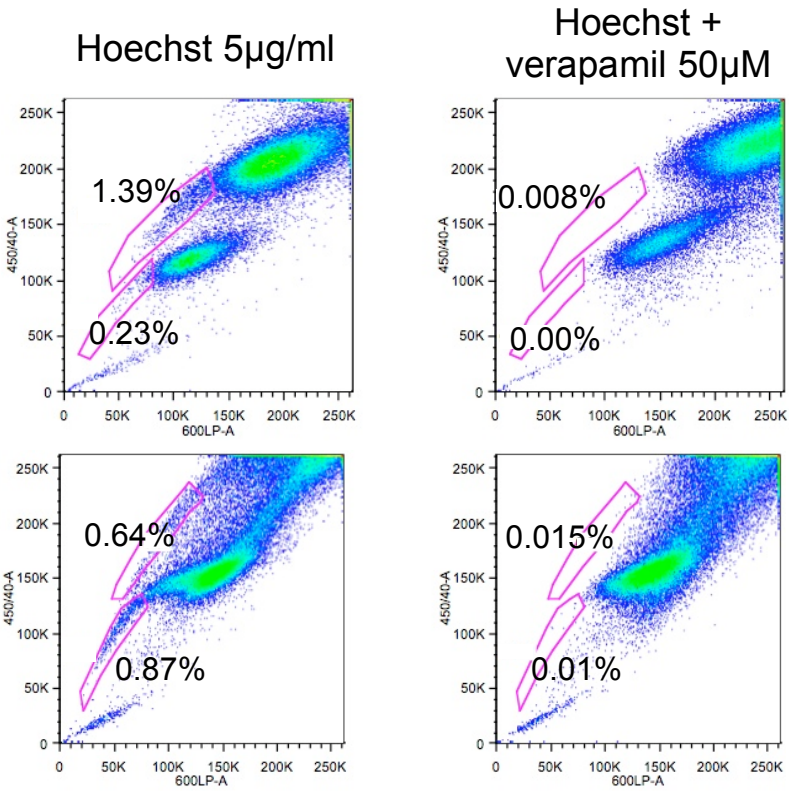


Figure 11 – Hoechst Efflux analysis of CML10 canine melanoma (*upper*) and SB canine haemangiosarcoma (*lower*) cell lines.
Incubation time is 120 minutes.
Although both lines showed best definition of SP compared to verapamil control under these assay conditions, an unusual double profile gave rise to two candidate SP arms.

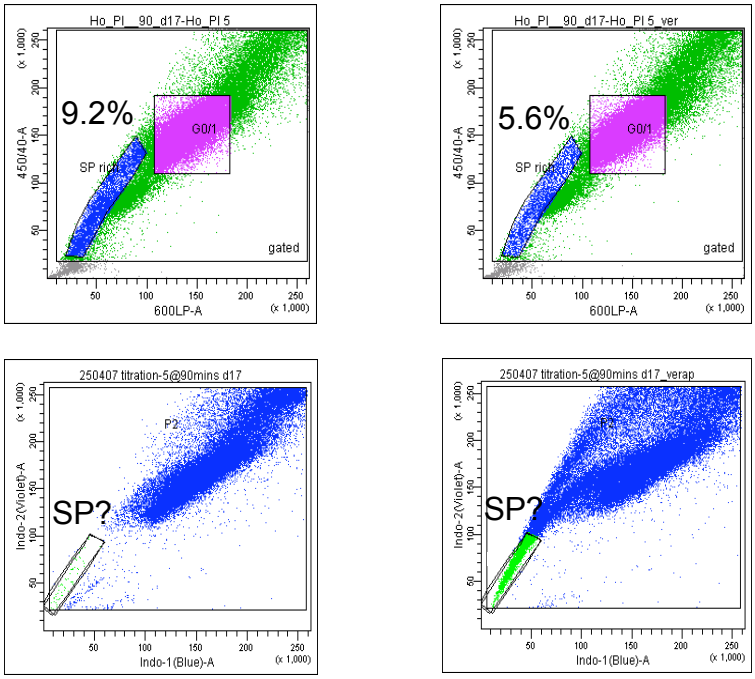


Figure 12 – Inconsistent Hoechst staining profile for D17 canine osteosarcoma cells tested on separate occasions.
Hoechst 5µg/ml, 90 minutes incubation.
Upper panels – Numerous cells in SP region; population reduced by verapamil.
Lower panels – No defined SP in test sample, but verapamil control has many cells in SP region.

canine cells. However, the appearance of the SP itself in the test sample, despite optimisation of the protocol for each cell line, was also erratic.

Intriguingly, the assay appeared to identify SP cells in the feline mammary carcinoma cell line CatMT reasonably consistently, showing more systematic changes in the SP size with varying substrate concentration and incubation time. These feline cells appeared sensitive to the toxicity of Hoechst, particularly in combination with verapamil. At 10µg/ml Hoechst and 90 minutes' incubation, the proportion of non-viable (propidium iodide-positive) cells in the test and verapamil control samples was 35.8% and 84.0%, respectively, as compared to 5.6% and 5.8% at 2.5µg/ml, and 10.4% and 14.3% at 5µg/ml (Figure 13, Table 4).

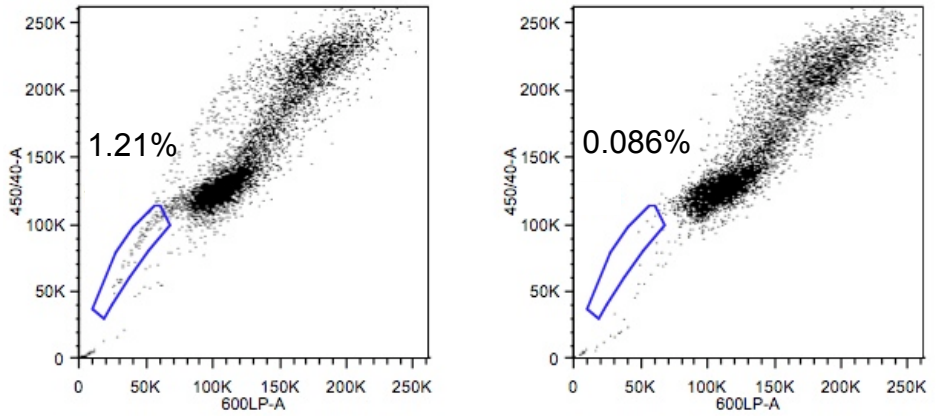


Figure 13 – Side population of CatMT feline mammary carcinoma cell at 2.5µg/ml Hoechst / 60 minutes incubation. Although SP varied between samples, profile and population size were much more consistent than those seen with canine cells.

Hoechst (µg/ml)	Time (min)	**TEST** SP (%)	**TEST** Dead (%)	**VERAP** SP (%)	**VERAP** Dead (%)	**Comments**
5	60	**1.21**	4.7	**0.086**	8.0	**Best definition of SP profile**
5	90	**0.85**	10.4	**0.083**	14.3	Good profile
5	120	**0.34**	13.6	**0.096**	19.5	Less defined profile
5	180	**0.9**	25.2	**0.03**	65.7	Less defined, increased cell death
2.5	90	**0.82**	5.6	**0.39**	5.8	Good profile
2.5	120	**0.91**	9.0	**0.018**	9.2	Good profile
10	90	0.013	35.8	0.015	84.0	Cell death++ esp. with verapamil

Table 4 - Summary of titration of Hoechst efflux by CatMT feline mammary carcinoma cells. Most samples show a side population which is reduced in the presence of verapamil. SP represents 0.82 – 1.21% of live cells depending on assay conditions. Higher concentrations of Hoechst, especially when combined with longer incubations, lead to increased cell death.

DyeCycle Violet identifies an SP-like profile within canine BMMNC

DyeCycleViolet (DCV) was tested as an alternative substrate for canine bone marrow, 3132 lymphoma and MCF-7. The bone marrow showed a SP of 0.12%, reduced by verapamil (Figure 14). Although this was less well defined than the 0.08 – 0.1% population detected with Hoechst (Figure 8), this suggests that DCV may have potential as an alternative substrate for SP analysis in canine cells. The test was performed using cryopreserved BMMNC, and live cells represented 46.9% of total events; in the sample stained in parallel with Hoechst 33342, viability was 27.9% of total cells. DCV may prove a less toxic alternative to Hoechst for side population

analysis – further work is required to evaluate this. However, 3132 cells showed no SP profile, although this had been detected with Hoechst. Moreover, whilst MCF-7 showed an apparent SP of 1.3% (more frequent than the Hoechst SP of 0.3-0.8%, Figure 9), this was present in the verapamil sample also (1.26%) (data not shown).

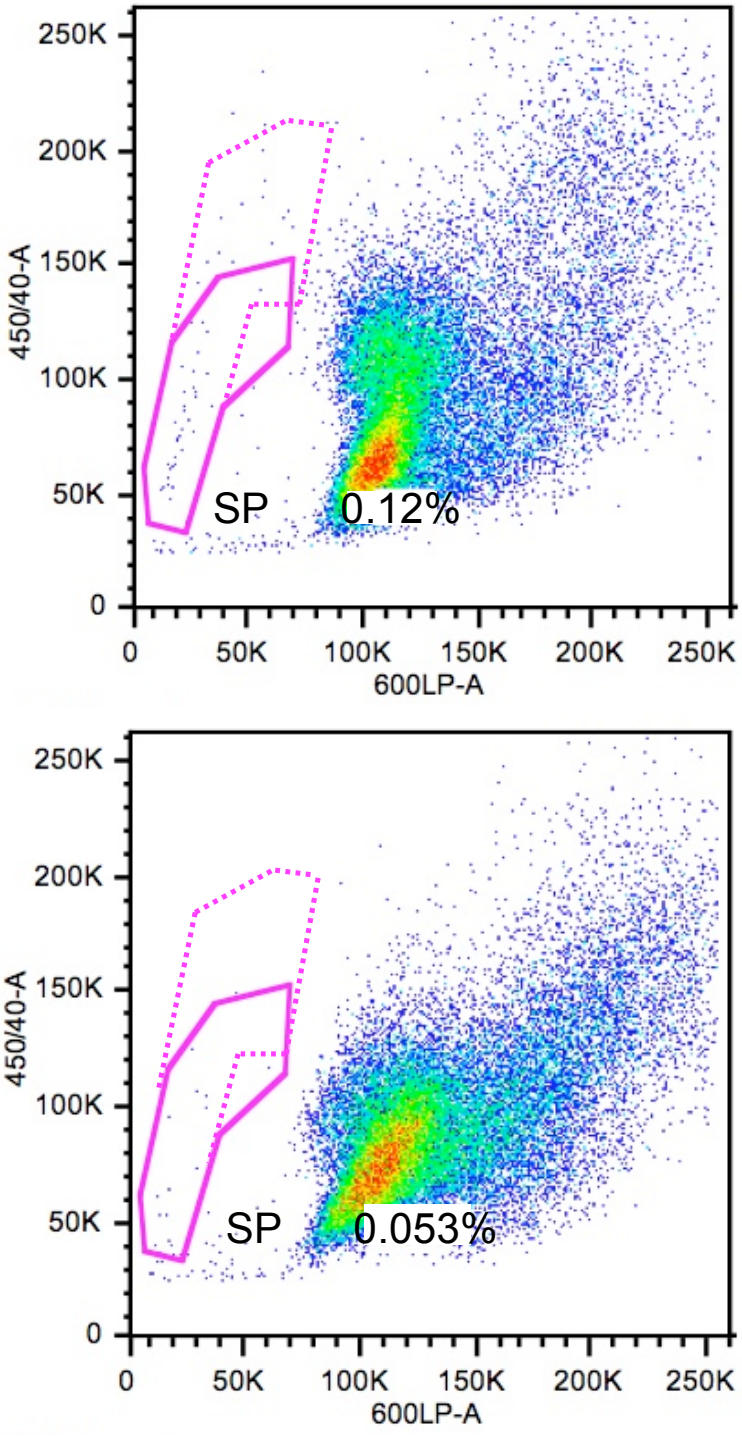


Figure 14 - Vybrant DyeCycle Violet (DCV) identifies an SP-like population in normal canine BMMNC (*top*), which is reduced in the presence of verapamil (*bottom*).

62000 events acquired for test sample (46.9% intact PI-ve).

Gates have been placed conservatively due to the low cell numbers - the SP may in fact be larger than that shown (dotted line).

The 3132 lymphoma and REM134 mammary carcinoma cell lines were selected for further investigation of the side population. The former was chosen as the assay was originally developed to identify progenitors within populations of haematopoietic cells, the latter as its use has been described in the study of both normal breast and breast cancer stem cells in other species (Alvi *et al.*, 2003; Dontu *et al.*, 2003; Hirschmann-Jax *et al.*, 2004; Kondo *et al.*, 2004; Patrawala *et al.*, 2005; Zhou *et al.*, 2007; Engelmann *et al.*, 2008; Tanaka *et al.*, 2009).

Hoechst staining of 3132 cells could be visualised using not only ultraviolet (355nm, BD LSRII) but also violet (407nm, BD FACSAria) excitation. The latter profile was less defined, requiring comparison with the verapamil control to accurately define the SP cells – however, when collected and reanalysed using the 355nm laser, the sorted SP cells occupied the correct position on a standard UV excitation plot (Figure 15). This is significant, as it shows that SP analysis of canine cells may be performed using cytometers equipped with violet lasers, more generally available on both analytic and sorting cytometers.

Sorted side population and main population / unfractionated cells were to be compared for characteristics such as surface phenotype, growth characteristics and drug sensitivity. The assay was scaled up accordingly, increasing the number of cells incubated to yield sufficient SP cells for further analysis. However, despite multiple attempts for both cell lines, defined and reproducible SP profiles were not obtained, even when key experimental conditions such as passage number and cellular confluence were comparable. Thus, whilst a SP may be identified within canine bone marrow and cancer cell lines, the assay produces inconsistent results - these are difficult to replicate, particularly with large scale incubations.

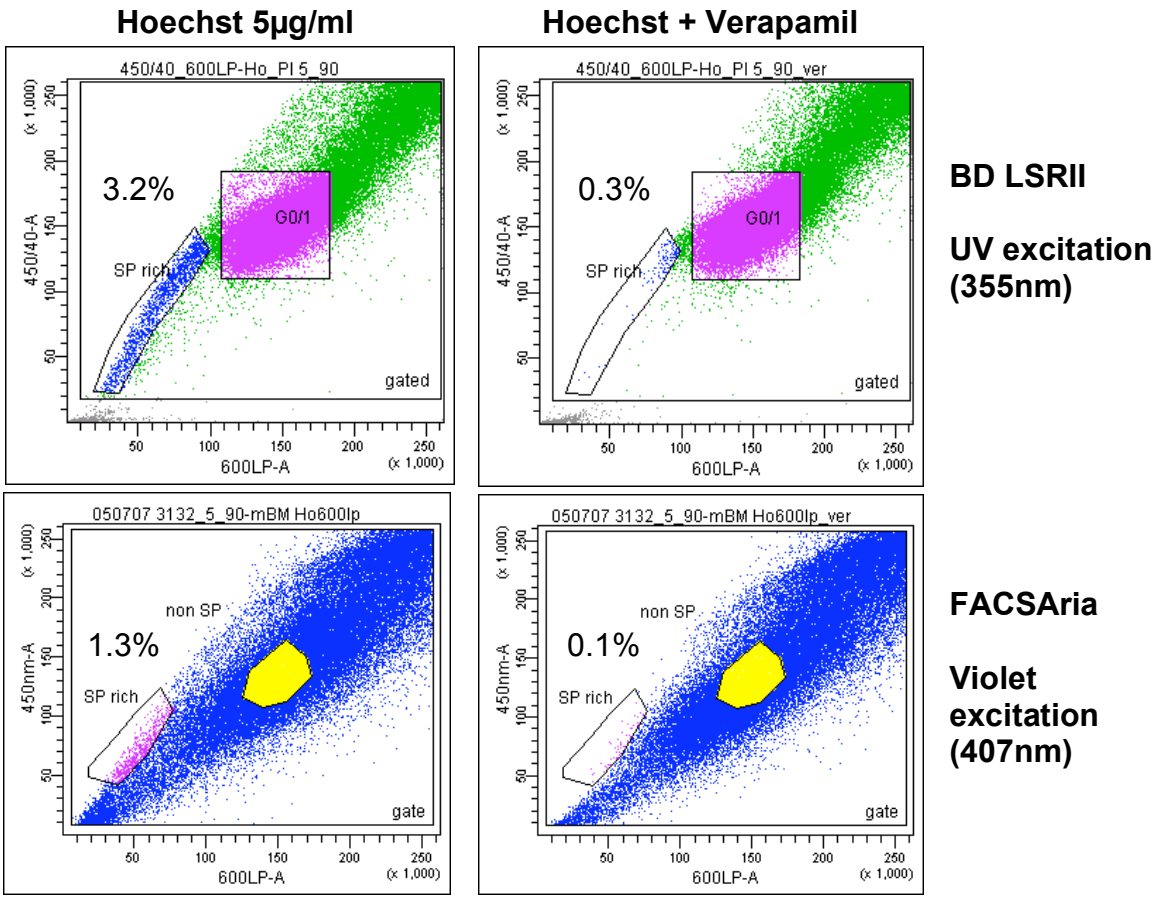
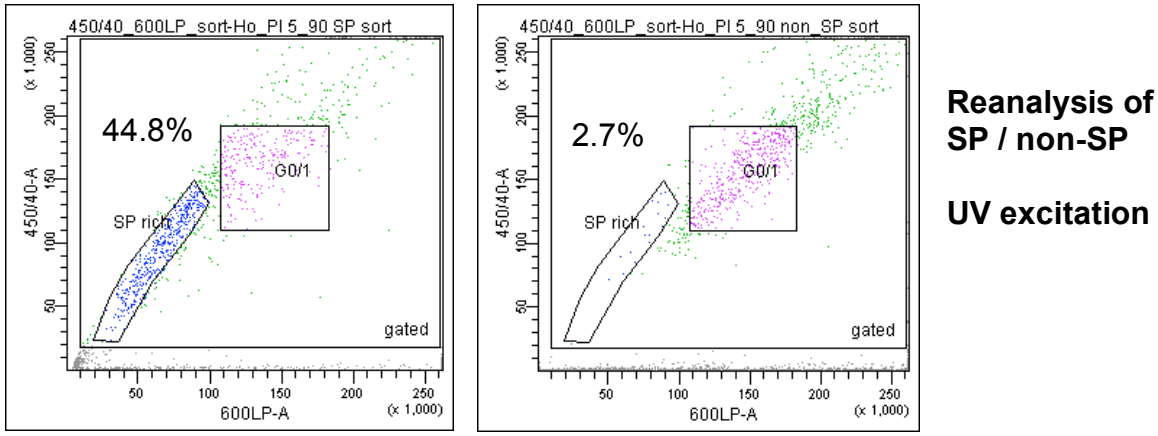


Figure 15 – Side population analysis of 3132 lymphoma cells – Hoechst 5µg/ml / 90 minutes

Above, Upper Panels – Ultraviolet (355nm) excitation – SP-like profile
Above, Lower Panels – Violet (407nm) excitation – SP profile defined using verapamil control. SP and representative non-SP populations gated as shown and isolated.

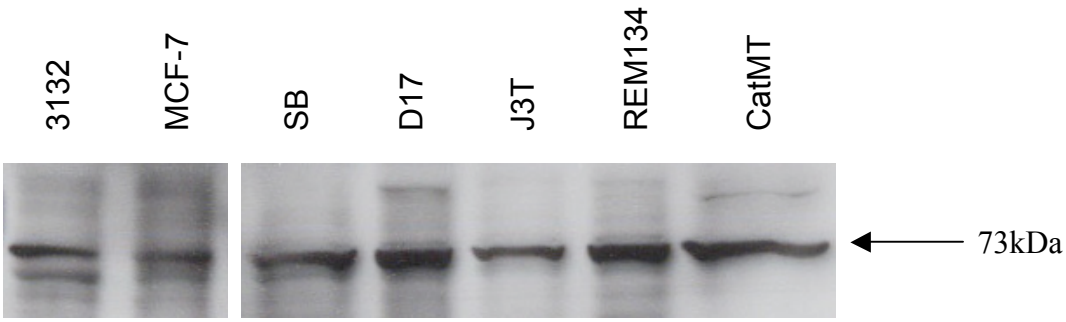
Below – Re-analysis of sorted fractions. *Left* – SP; *Right* – non-SP.
SP (sorted using violet excitation profile) enriched in appropriate gate on ultraviolet plot (44.8%) as compared to non-SP population (2.7%).



ABCG2 expression by canine cancer cell lines

In light of the SP profiles seen intermittently for each canine cancer cell line, cell lysates were analysed for expression of the ABCG2 drug transporter shown to be responsible for the phenomenon in many cell types. D17, REM134, 3132 and SB, as well as J3T canine glioma and the feline CatMT and human MCF-7 mammary cancer cell lines showed a band of the predicted size (73kDa) on Western blot analysis (Figure 16).

Figure 16 – Western blot demonstrating ABCG2 expression by cancer cell lines. Band of predicted size (73kDa) seen for canine cell lines, as well as human MCF-7 mammary carcinoma and feline CatMT mammary carcinoma. 20µg protein per lane.



Rhodamine 123 Identifies an Effluxing Population Within Canine BMMNC, but not 3132 Lymphoma Cells

Normal canine bone marrow was analysed for the efflux of Rhodamine 123. 13.2 – 14.7% of live cells showed a fluorescence shift in test as compared to control samples (which are placed on ice after Rho123 loading to inhibit ABC transporter activity), suggesting efflux capacity (Figure 17a). This is a substantial population; other investigators have found that analysis of Rho123 efflux may detect committed progenitor cells. Backgating analysis of these cells showed them to originate predominantly from a $FSC^{low}SSC^{low}$ position on the total bone marrow scatter plot (Figure 17b).

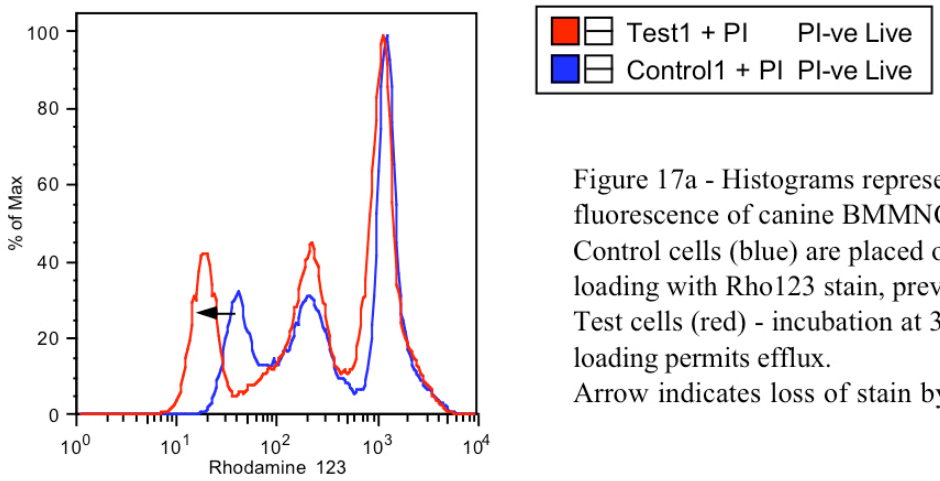


Figure 17a - Histograms representing Rho123 fluorescence of canine BMMNC.
Control cells (blue) are placed on ice after loading with Rho123 stain, preventing efflux.
Test cells (red) - incubation at 37oC after loading permits efflux.
Arrow indicates loss of stain by test sample.

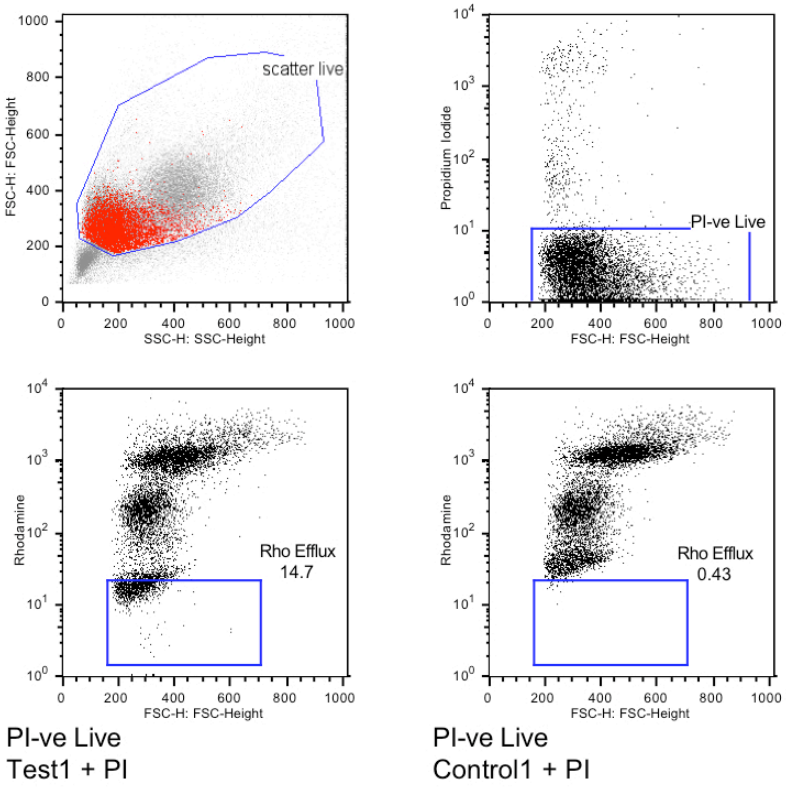
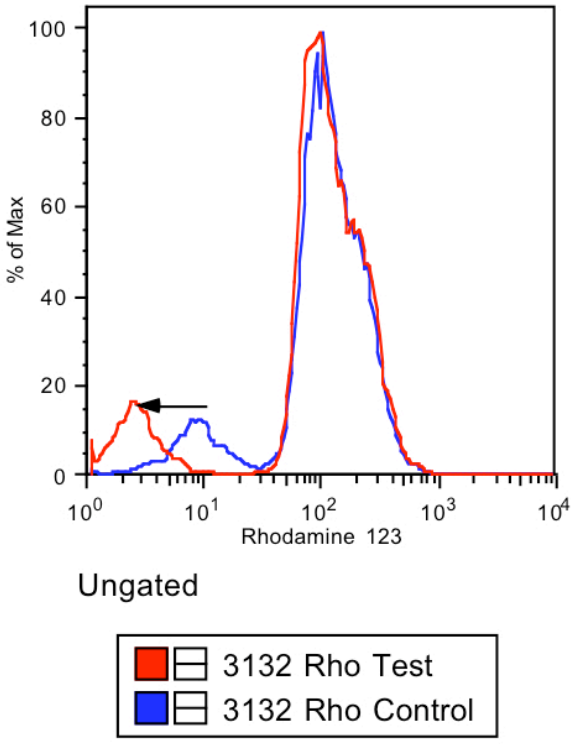


Figure 17b - Dotplots showing gating strategy (upper) and Rhodamine fluorescence (lower) for canine BMMNC. Lower left - test; lower right - control.

When backgated onto light scatter plot, Rho-effluxing cells are predominantly low-FSC, low-SSC (overlay in red).

As Rhodamine123 efflux is an established technique for examination of haematopoietic cells, 3132 lymphoma cells were selected for investigation using this assay. Analysis of ungated (total) cells defined a Rho^{low} subpopulation - when test samples were compared with controls, the peak fluorescence of this subset was seen to shift, suggesting efflux (Figure 18a). However, upon gating to exclude dead cells, using the scatter profile and/or the dead cell discriminator propidium iodide, this population was lost from the analysis (Figure 18b). This indicates that for 3132 canine lymphoma cells, apparent Rhodamine123 exclusion occurs due to reduced uptake of stain amongst non-viable cells rather than *bona fide* efflux by a population of stem-like cells. The further loss of stain shown by this subset in test samples may be a result of diffusion kinetics; leakage of stain due to loss of membrane integrity is likely to occur more slowly in controls, as they are held on ice after the initial loading phase.

Figure 18a - Histogram of rhodamine fluorescence for 3132 canine lymphoma cells. With ungated cells, an apparent Rho-Low subpopulation shows loss of fluorescence (arrow) by test sample (red) compared with control (blue), suggesting efflux.



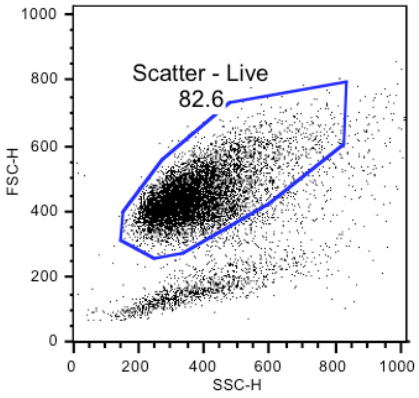
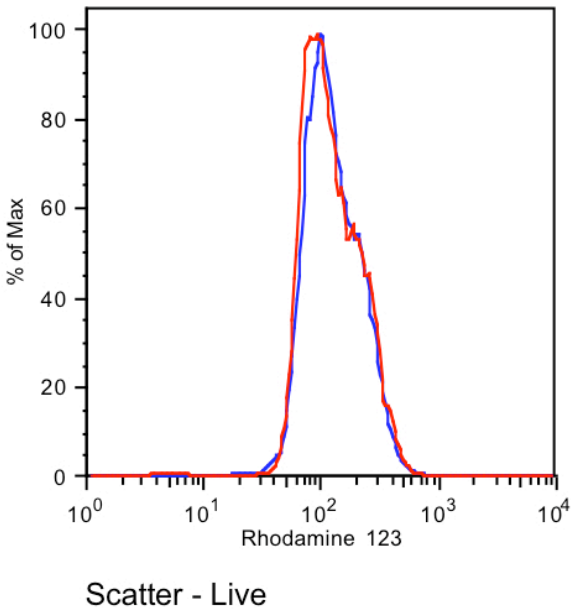


Figure 18b - Only intact / live cells included in analysis based on scatter profile gate (above), leading to loss of Rho-low subpopulation. Similar results achieved when PI used as dead cell discriminator.



Assessment of Aldehyde Dehydrogenase Activity in Canine Cancer Cell Lines

The commercial Aldefluor kit is optimised for the detection of human haematopoietic stem and progenitor cells, which appear on a plot of side scatter *vs* ALDH (Aldefluor) fluorescence as a discrete $ALDH^{Bright}SSC^{Low}$ population. Fiordalisi *et al* reported that a subpopulation of $ALDH^{Bright}SSC^{Low}$ cells was detectable in canine bone marrow using a slightly modified protocol - 0.1% sodium azide and 50μM verapamil were added to all buffers and the Aldefluor substrate concentration was increased to 4.8μM (hereafter referred to as "Fiordalisi modification") (Fiordalisi *et al.*, 2005). Normal canine BMMNC were analysed using both the modified and the manufacturer's standard protocol. The assay was performed on several different occasions, with BMMNC from different normal individuals.

A candidate $ALDH^{Bright}SSC^{Low}$ HSC population was not identified under either set of assay conditions (Figure 19). Using the standard protocol, a slightly wider variation in the ALDH fluorescence of granulocytic myeloid cells was noted in the test sample compared to the control sample, although maximal brightness was comparable. A similar shift in the fluorescence of committed cells has been noted for murine bone marrow (Pearce and Bonnet, 2007). There were few distinctions between test and control sample profiles using the Fiordalisi modification, which did not enhance detection of putative HSC, nor did exclusion of myeloid cells from the analysis. This suggests that Aldefluor may not prove to be a reliable technique for identification of normal canine HSC.

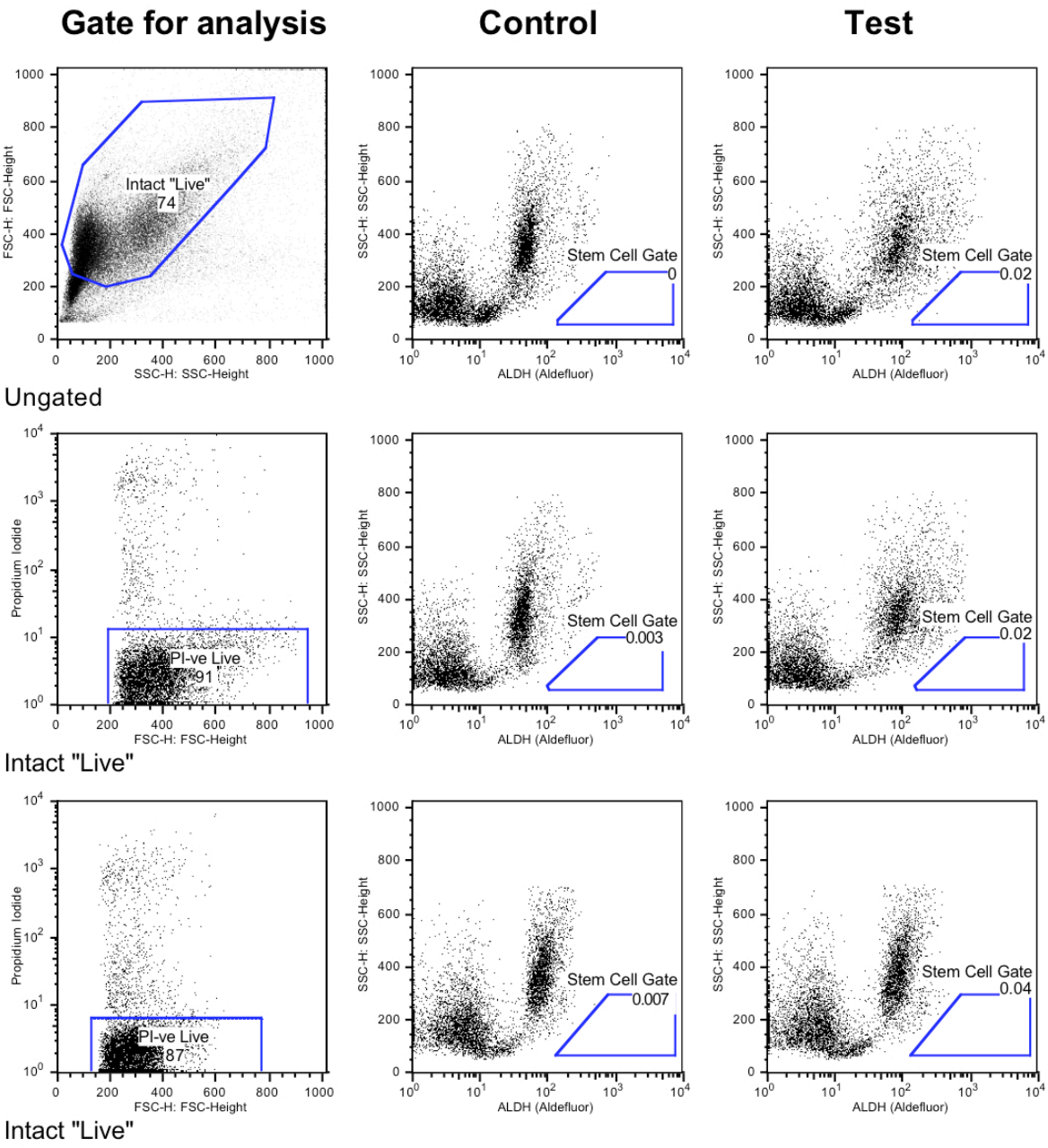


Figure 19 - Analysis of normal canine BMMNC using Aldefluor.
Top/Middle row - Standard protocol; Bottom row - Fiordalisi modification.

Top row - Cells gated as "live" according to scatter profile; standard protocol. No candidate HSC population is seen in the stem cell gate. Middle row - Addition of propidium iodide as a dead cell discriminator does not enhance the appearance of a candidate HSC population. Bottom row - Fiordalisi modification does not enhance the appearance of a candidate HSC population, although produces less variability in ALDH fluorescence of myeloid cell population.

Where used in the cancer stem cell literature, the assay seeks to identify ALDH^{Bright} cells lost in the presence of DEAB, with less emphasis placed on light scatter properties (as these may be less characteristic than those of HSC). Nonetheless, none of the canine cancer cell lines investigated for this study demonstrated ALDH^{Bright} populations when examined using either the standard protocol or the Fiordalisi modification (Figure 20).

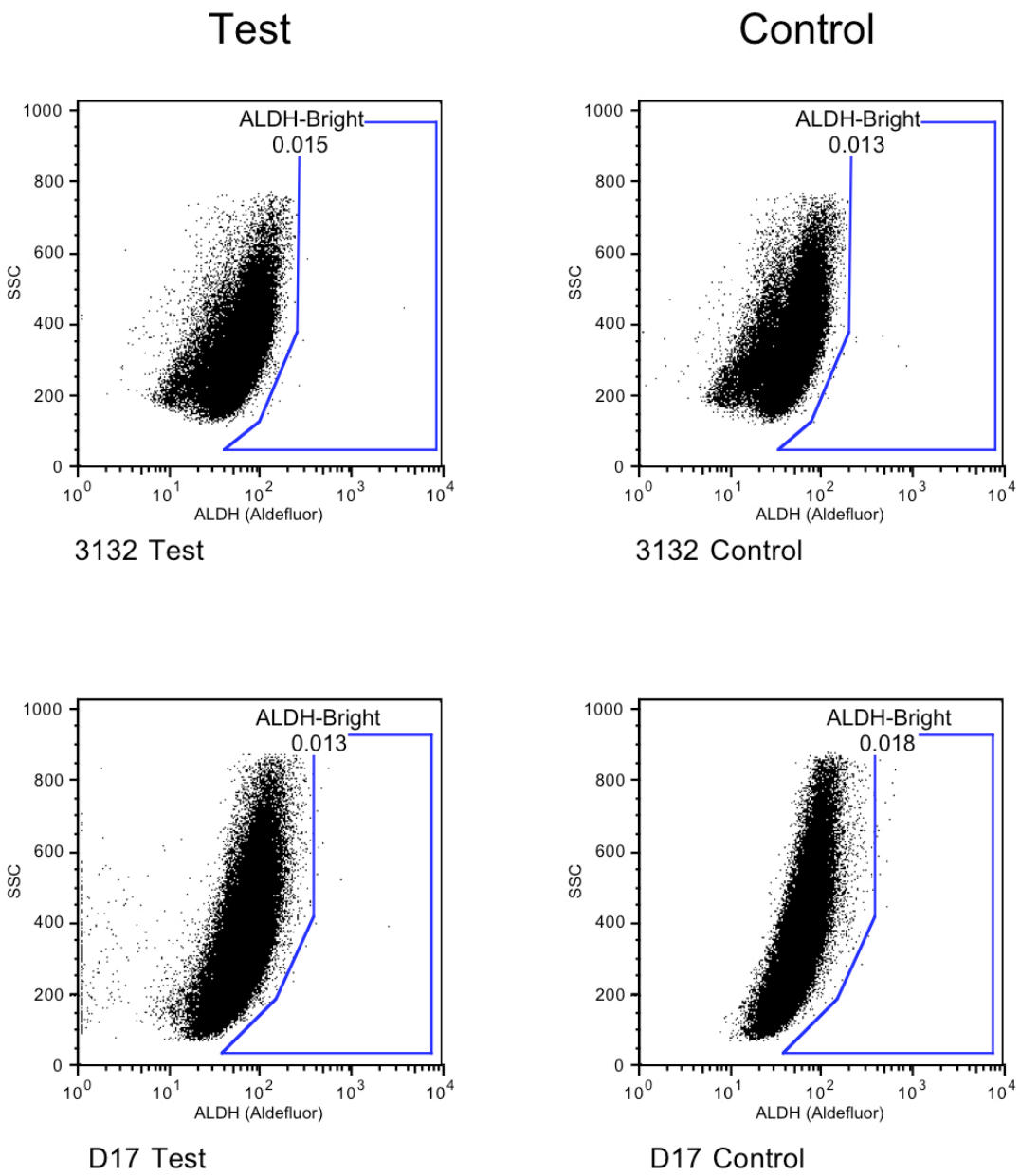


Figure 20 - Representative Aldefluor Test (left) and DEAB Control (right) plots for established canine cancer cell lines. No ALDH-Bright subpopulations detected. Upper - 3132 Lymphoma; Lower - D17 osteosarcoma

Evaluation of CSC-Associated Phenotypes in Spontaneous Canine Neoplasia

i) Primary Osteosarcoma

An eight year old female entire Rottweiler was presented to the Hospital for Small Animals, Royal (Dick) School of Veterinary Studies, for investigation of a right hindlimb lameness of 2 weeks' duration, refractory to analgesia with the non-steroidal anti-inflammatory drug meloxicam. Radiography and computed tomography were suggestive of osteosarcoma, which was confirmed histologically by bone biopsy. The tumour was excised by hindquarter amputation – no chemotherapy was given prior to surgery. Although preoperative clinical staging was not suggestive of metastatic disease, the dog developed neurological signs within a few weeks of surgery and was euthanased on the grounds of suspected brain metastasis and poor quality of life.

Cells obtained from this tumour were expanded in culture, yielding five flasks of adherent cells (kt-osa 1-5), three of which were subcultured and maintained as cell lines by repeated passage (kt-osa1, kt-osa4 and kt-osa5). The cells displayed marked morphological heterogeneity, not only between but also within populations (Figure 21 (i)-(iii)). Cells migrating directly from tumour tissue, and also following subculture, displayed a mixture of morphologies including epithelioid, fusiform, palisading fibroblastic and multinucleate. Large, atypical cells were seen occasionally amongst all isolates (Figure 21 (ii)). A tetraploid population observed at DNA analysis – Figure 22 – could reflect these multinucleate cells, but may indicate chromosomal aneuploidy; karyotypic analysis has not been performed. Each population was derived from the total proliferating cells in each flask, without selection or cloning, to try and preserve the heterogeneity observed at initial derivation. However, upon repeated subculture each line displayed a reduction in heterogeneity, with one morphology predominating (Figure 21 (iii)). When kt-osa1, kt-osa4 and kt-osa5 cells were cultured in low-density, serum-free conditions with epidermal growth factor (EGF) and basic fibroblastic growth factor (bFGF), tumoursphere formation was seen for all three populations (Chapter 4).

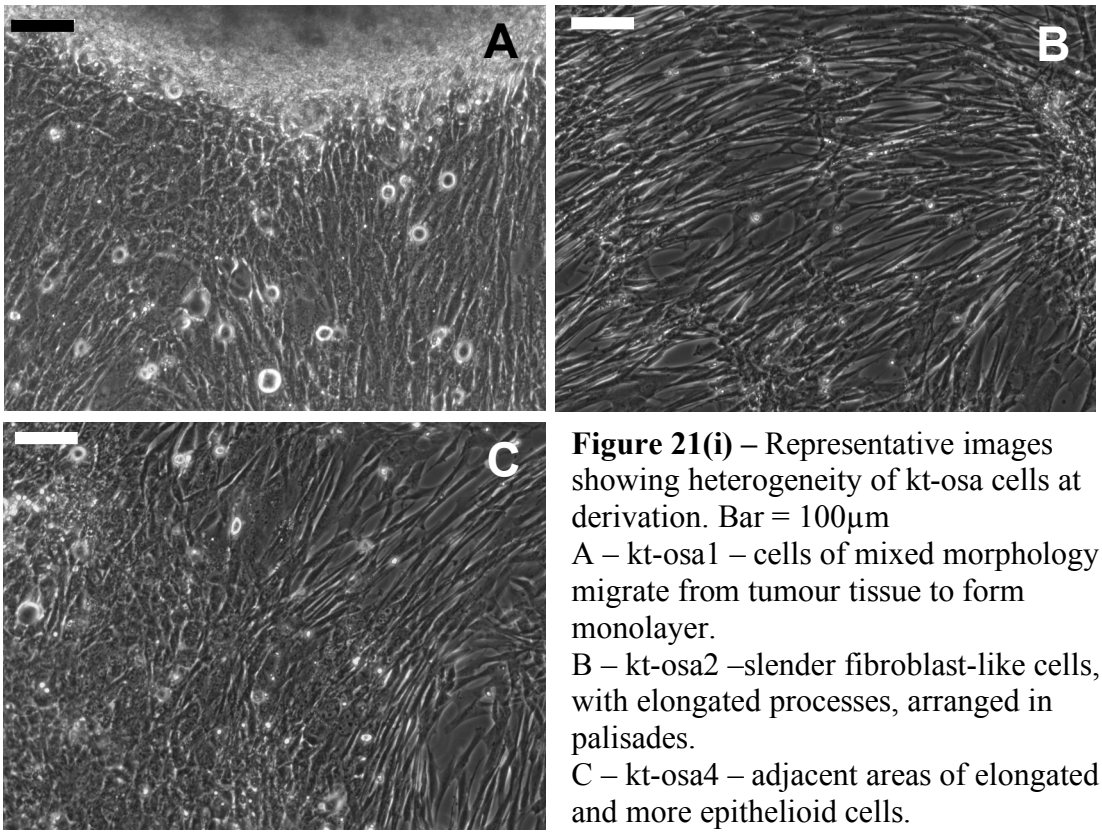


Figure 21(i) – Representative images showing heterogeneity of kt-osa cells at derivation. Bar = 100µm
A – kt-osa1 – cells of mixed morphology migrate from tumour tissue to form monolayer.
B – kt-osa2 –slender fibroblast-like cells, with elongated processes, arranged in palisades.
C – kt-osa4 – adjacent areas of elongated and more epithelioid cells.

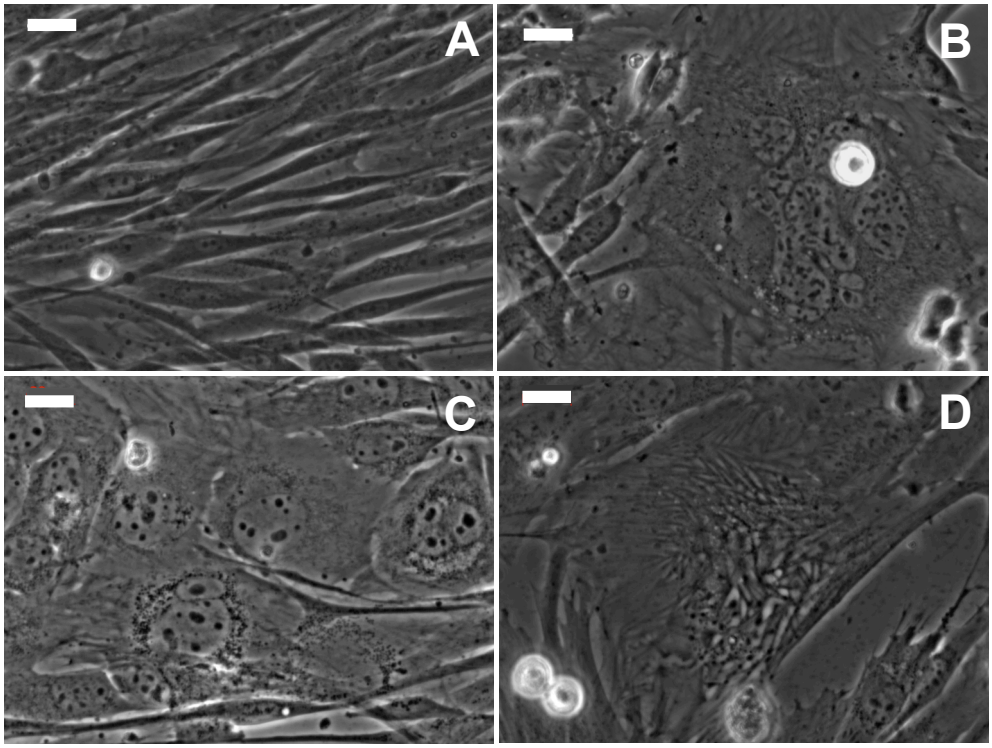


Figure 21(ii) – Variation in cellular morphology in kt-osa populations at derivation.
A – kt-osa4 – slender fusiform / spindle cells. B – kt-osa4 – large multinucleate cell.
C – kt-osa1 – large round cells with prominent nucleoli and perinuclear granules
D – kt-osa5 – large atypical cell. Bar = 20 µm

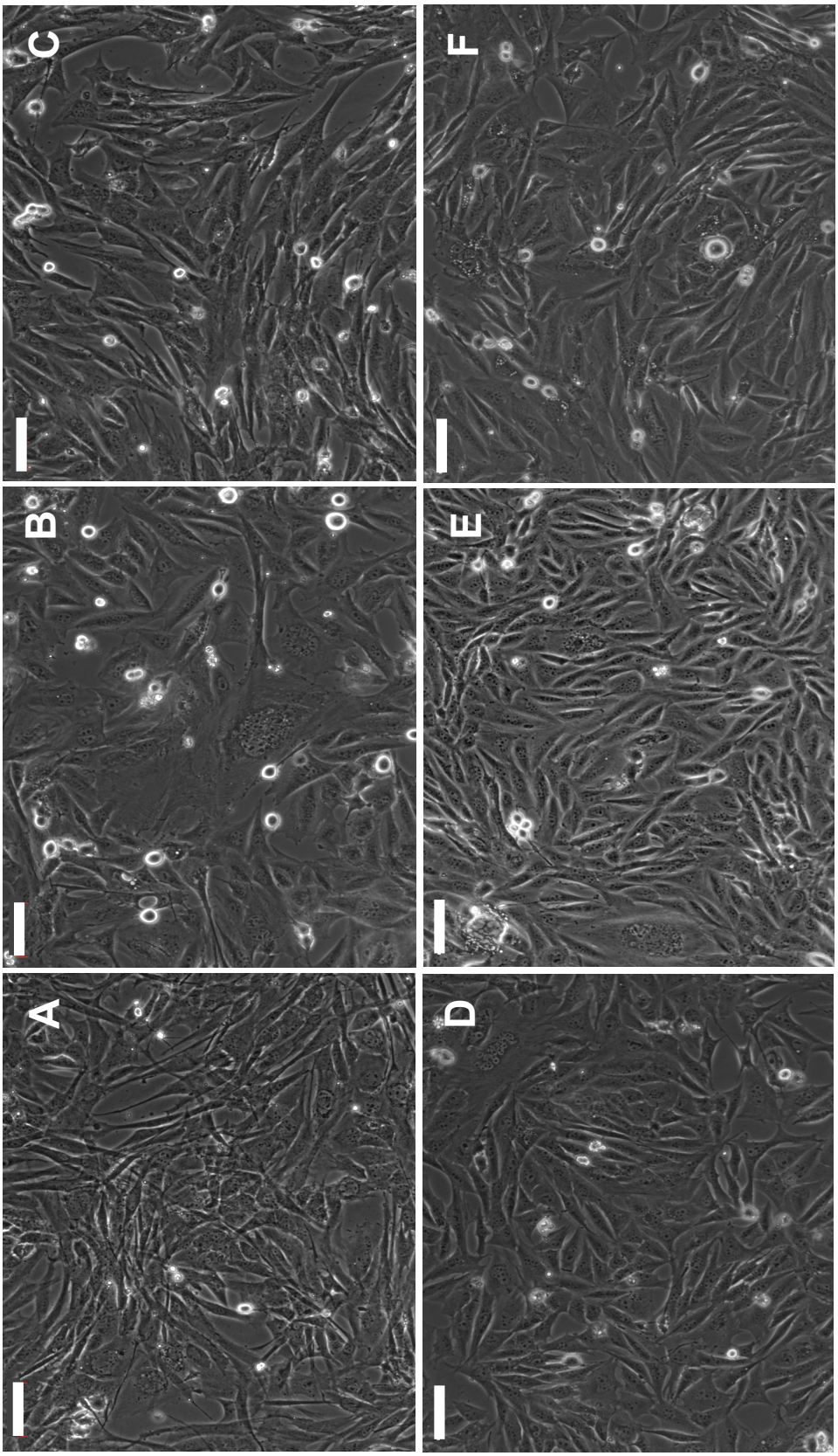


Figure 21(iii) – Reduced morphological heterogeneity and more orderly arrangement of kt-osa monolayers with repeated subculture *in vitro*.
A – kt-osa1 (passage 5); B – kt-osa4 (passage 5); C – kt-osa5 (passage 3).
D – kt-osa1 (passage 12); E – kt-osa4 (passage 13); F – kt-osa5 (passage 10).
Bar = 100 µm

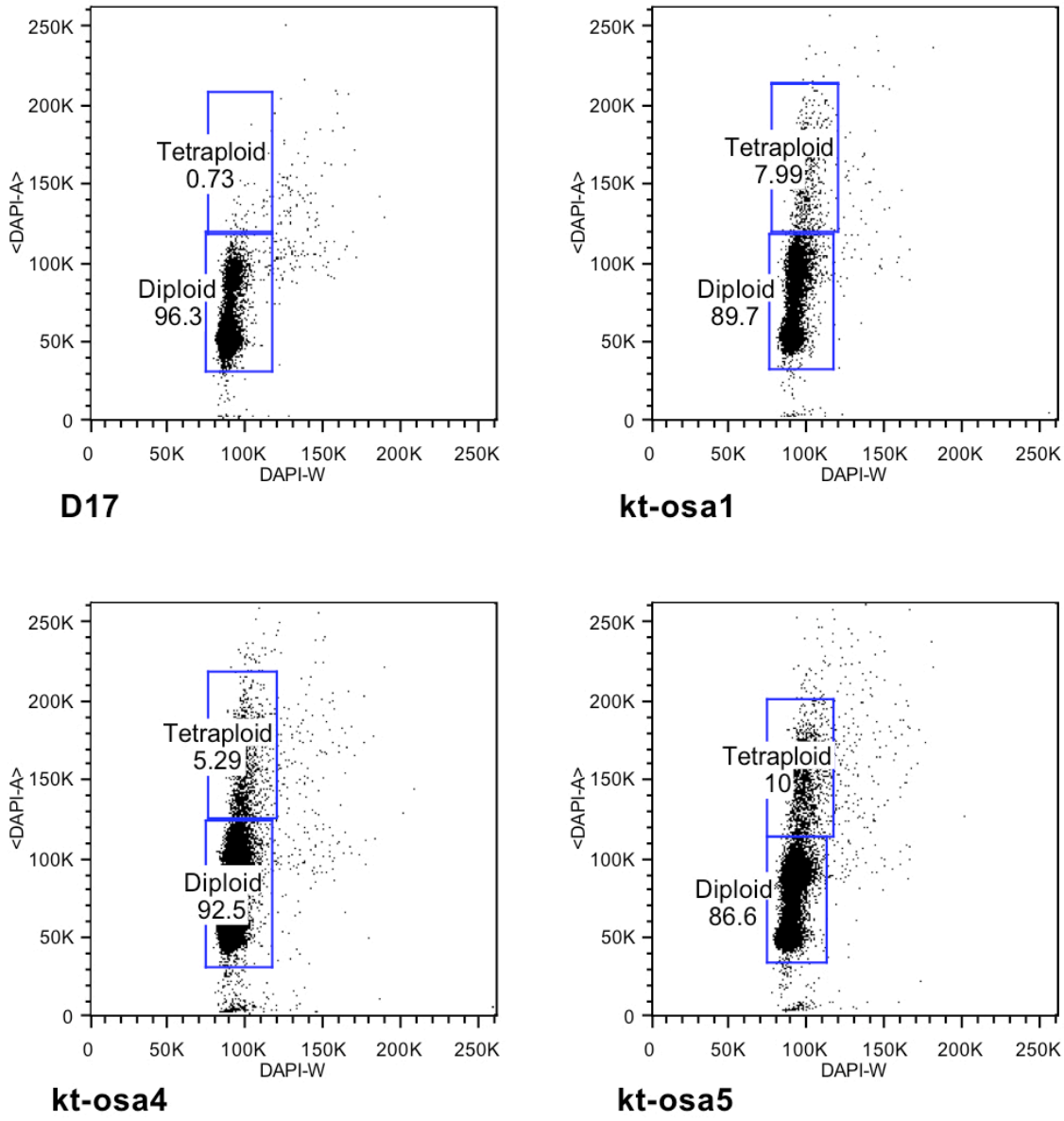


Figure 22 - DAPI-stained DNA content of canine osteosarcoma cell lines.
D17 are predominantly diploid, consistent with the reported karyotype (2n=78).
A significant proportion of kt-osa1, kt-osa4 and kt-osa5 cells show increased DNA content suggestive of DNA tetraploidy.

Cell surface markers

All tested populations derived from the primary canine osteosarcoma were predominantly (>97%) positive for CD44, both at initial expansion in adherent culture and after subculture for 26 passages. The pattern of expression – an overall fluorescence shift of the bell-shaped histogram – mirrored that seen in the established canine cancer cell lines (Figure 23).

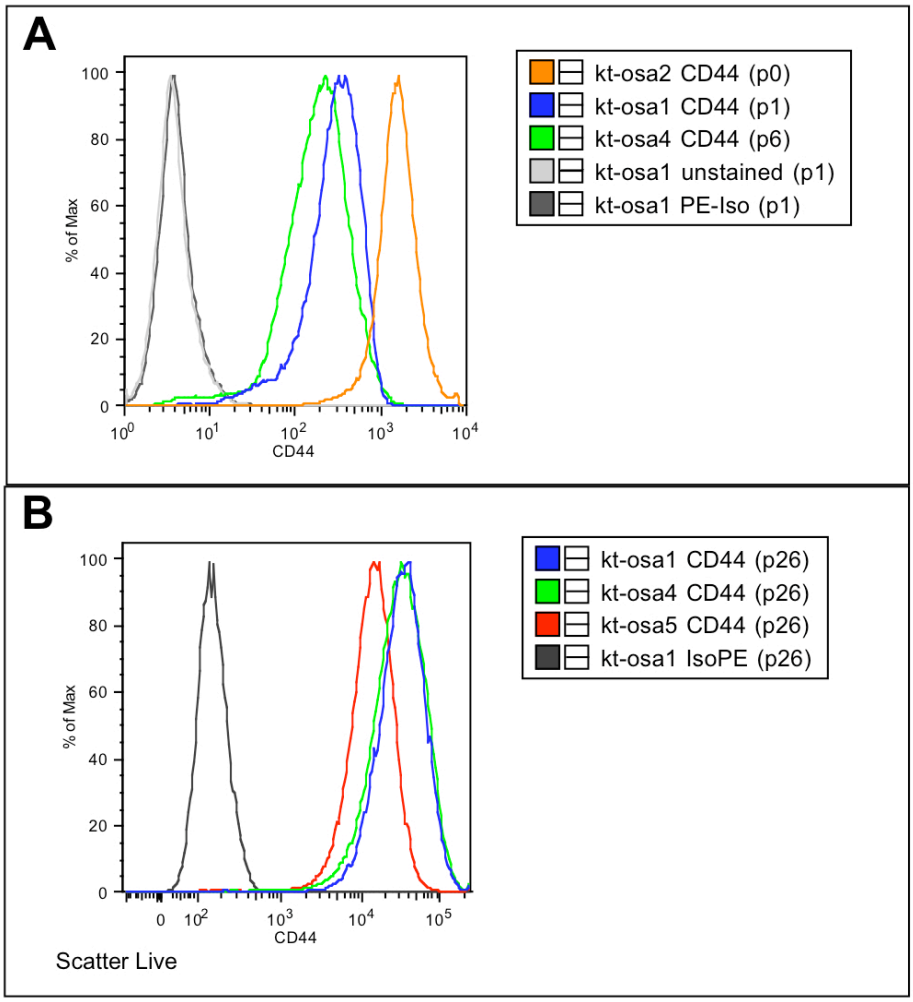


Figure 23 - Histograms representing expression of CD44 on kt-osa populations derived from canine osteosarcoma, at early (A) and later (B) passage.
Passage number is indicated in brackets.
Representative unstained / isotype control samples (kt-osa1) shown for comparison.

When examined at early passage (subculture 1-2) for expression of CD133, a small subpopulation (0.034%) of kt-osa1 cells demonstrated positive staining compared to the isotype-matched control (0.007%) (Figure 24). Cell numbers in both cases were low (35 positive cells *vs* 7 for isotype; ≥100000 cells analysed per sample). Nonetheless, four passages later this positive staining had reduced to less than the non-specific binding seen in the isotype control sample (0.005% *vs* 0.011% respectively). kt-osa4 (tested at passage 6) and kt-osa3 cells (which were never subcultured) were negative for this marker.

A subpopulation of the three cell lines kt-osa1, kt-osa4 and kt-osa5 stained positive for CD117 (c-kit) (Figure 25) as compared to unstained or isotype control samples. When analysed against a blank fluorescence channel (FL-1) to increase the sensitivity of detection (by revealing staining otherwise masked in a histogram by cellular autofluorescence), the proportions of positive cells were 1.25% (kt-osa1), 0.31% (kt-osa4) and 0.035% (kt-osa5). Intriguingly, it was found that when cells were retested for expression of this marker only 4-5 passages later, the level of staining had reduced markedly such that kt-osa1 showed only 0.2% positive cells, and kt-osa4 was now CD117-negative.

Thus, CD44 was expressed throughout each of the populations derived from this tumour - the staining pattern did not appear to be affected by repeated subculture *in vitro* - but, as for cell lines, the marker did not identify heterogeneous cellular subpopulations. Conversely, staining for CD117 and CD133 was restricted to a small fraction of cells amongst some, but not all, of the populations. The identification of small, positive-staining populations in these cells contrasted with the observations for the established cancer cell lines, where staining of discrete cellular subsets was never seen. However, these small populations diminished over time in culture, in parallel with a conspicuous loss of morphological heterogeneity.

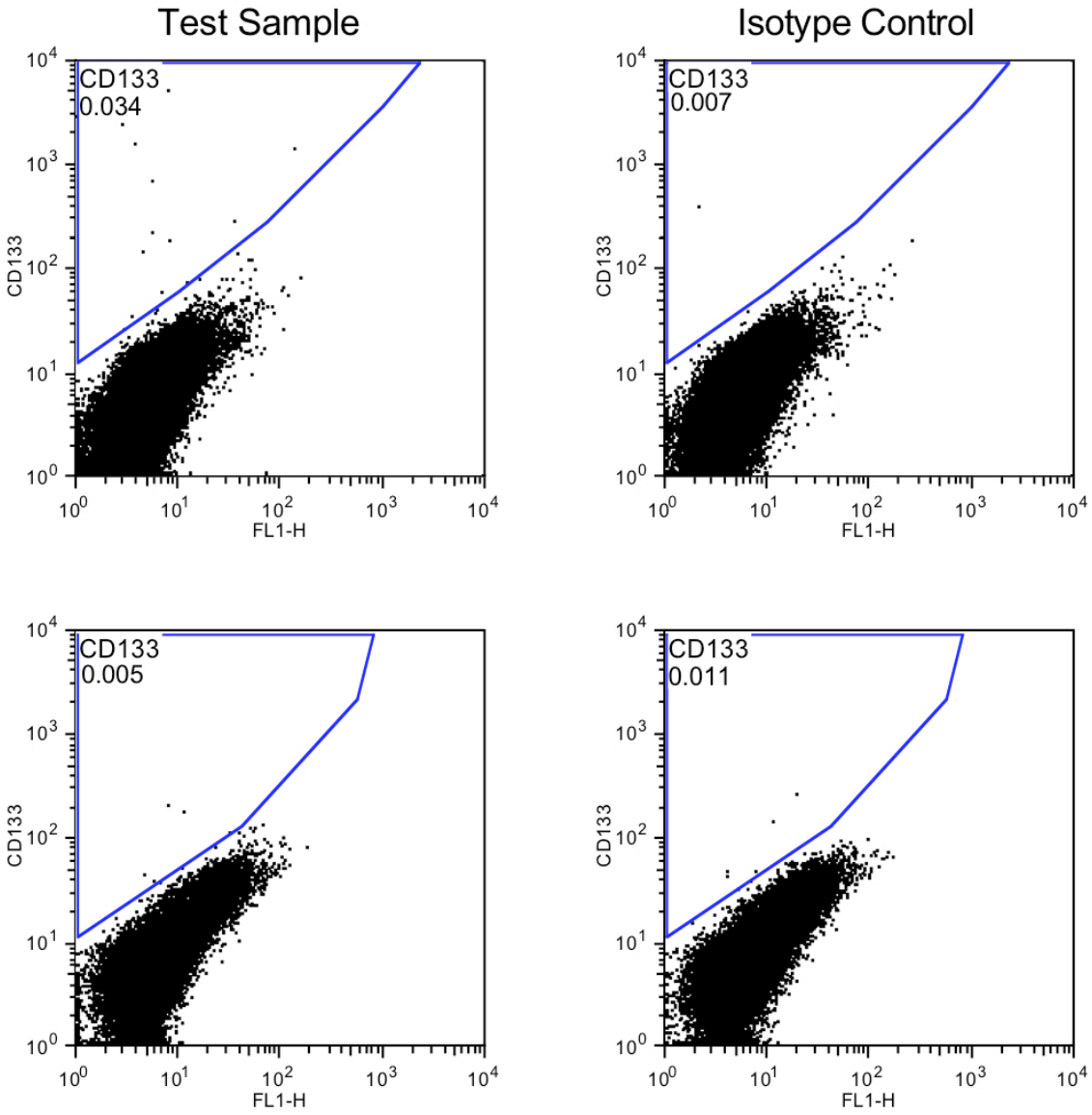


Figure 24 - Loss of small CD133+ subpopulation within kt-osa1 canine osteosarcoma cells.
Above - Positive staining at initial subculture (left) exceeds non-specific binding by isotype control antibody (right).
Below - at passage 6, nonspecific binding in isotype control sample (right) exceeds test antibody binding (left).

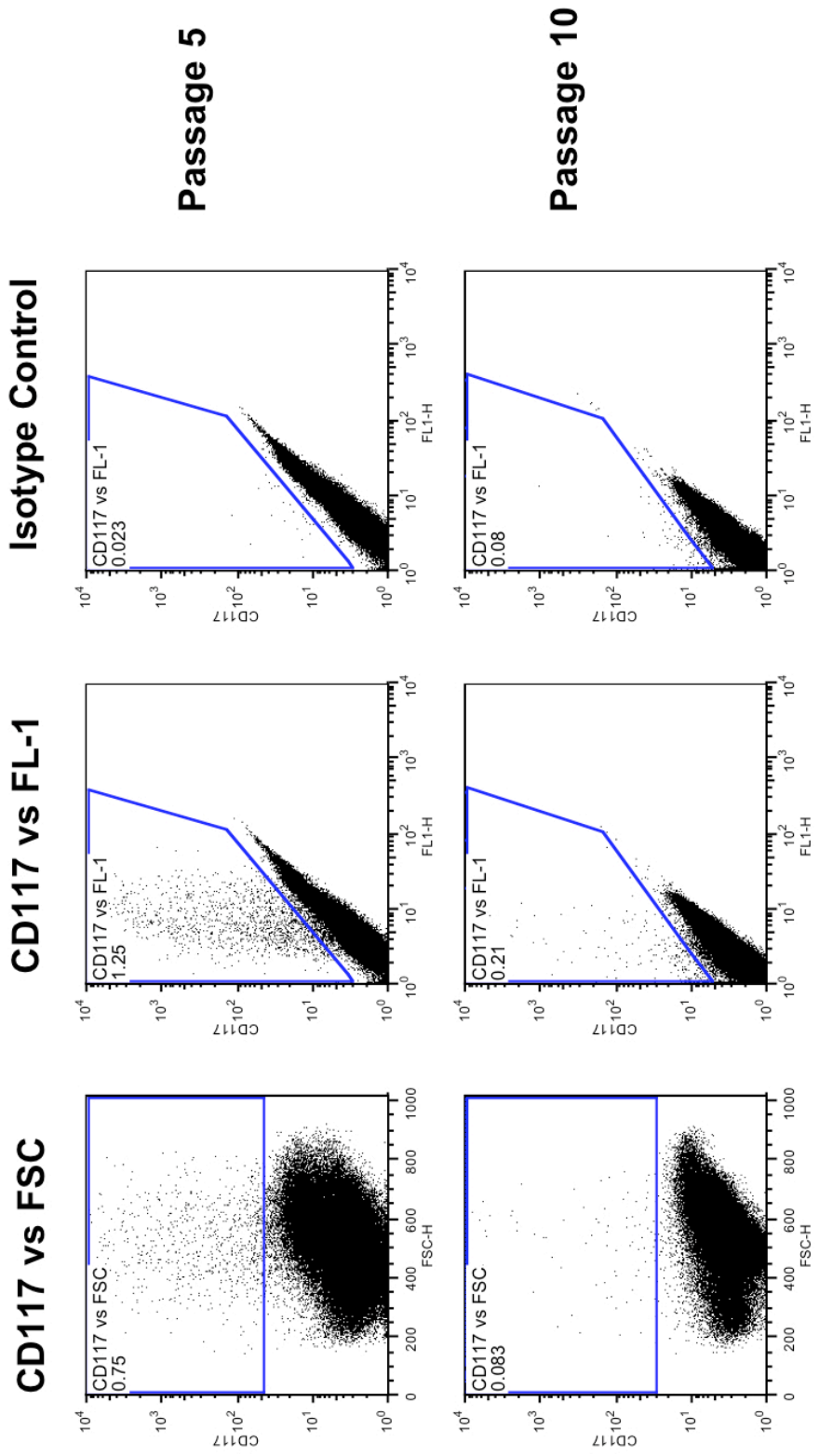


Figure 25 - kt-osa1 osteosarcoma derived cells - CD117 expression analysed in relation to forward scatter and blank (FL-1) channel. Relevant isotype control samples shown for comparison.
Upper plots - CD117 expression at passage 5; Lower plots - CD117 expression at passage 10

Functional assays

kt-osa1, kt-osa4 and kt-osa5 were tested for Hoechst efflux capacity. A side population was seen for kt-osa1 - as with the established cancer cell lines tested previously, the profile was highly variable – when tested on a single occasion, the SP of one sample represented 0.38% of live single cells, and that of another 1.95%, despite cells being obtained from the same culture. However, cells in the SP region for both samples were considerably more frequent than in the corresponding verapamil control (0.014%) (Figure 26). The latter two cell lines did not demonstrate a SP.

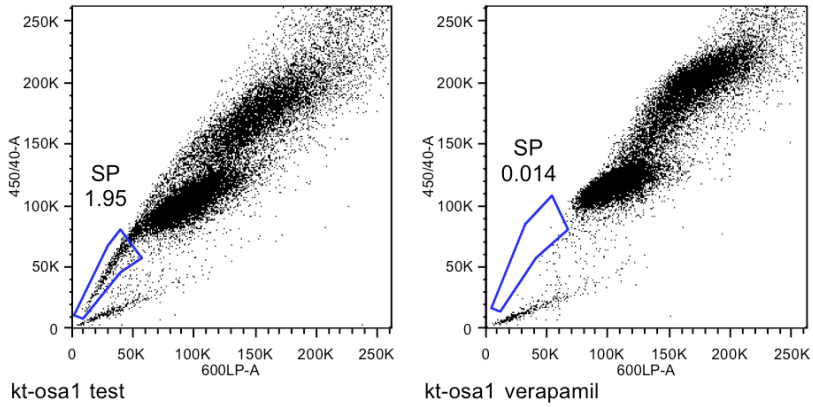


Figure 26 - Hoechst efflux analysis of kt-osa1 canine osteosarcoma cells. Upper plot - Test sample; Lower plot - Control sample (+50µM verapamil)

When the three populations were tested for the ability to efflux Rhodamine, all showed a minority population with reduced fluorescence in both test and control samples after gating for live cells based on light scatter characteristics (Figure 27). Histogram analysis suggested efflux for kt-osa1 and kt-osa4 – for the former, the Rho^{low} population was considerably larger for the test sample as compared to the control; for the latter, the Rho^{low} test sample showed reduced fluorescence. However, upon addition of propidium iodide and analysis of PI^{-} cells only, the Rho^{low} subpopulation was lost in all cases, indicating that, as with the 3132 cell line, these were non-viable cells. The Rho^{low} cells were distributed throughout the FSC*vs*SSC plot – this further emphasises the importance of stringent dead cell

discrimination when using this assay in canine cells to avoid artefacts, particularly when looking for rare-event populations.

Nonetheless, both kt-osa1 and kt-osa4 showed an overall fluorescence shift by all cells in the test sample as compared to the control (Figure 27). This may indicate the expression of multidrug transporters such as P-glycoprotein or ABCG2. This assay was performed when all cell lines had been subcultured at least ten times (kt-osa1 – passage 12-13; kt-osa4 – passage 13-14; kt-osa5 – passage 10-11).

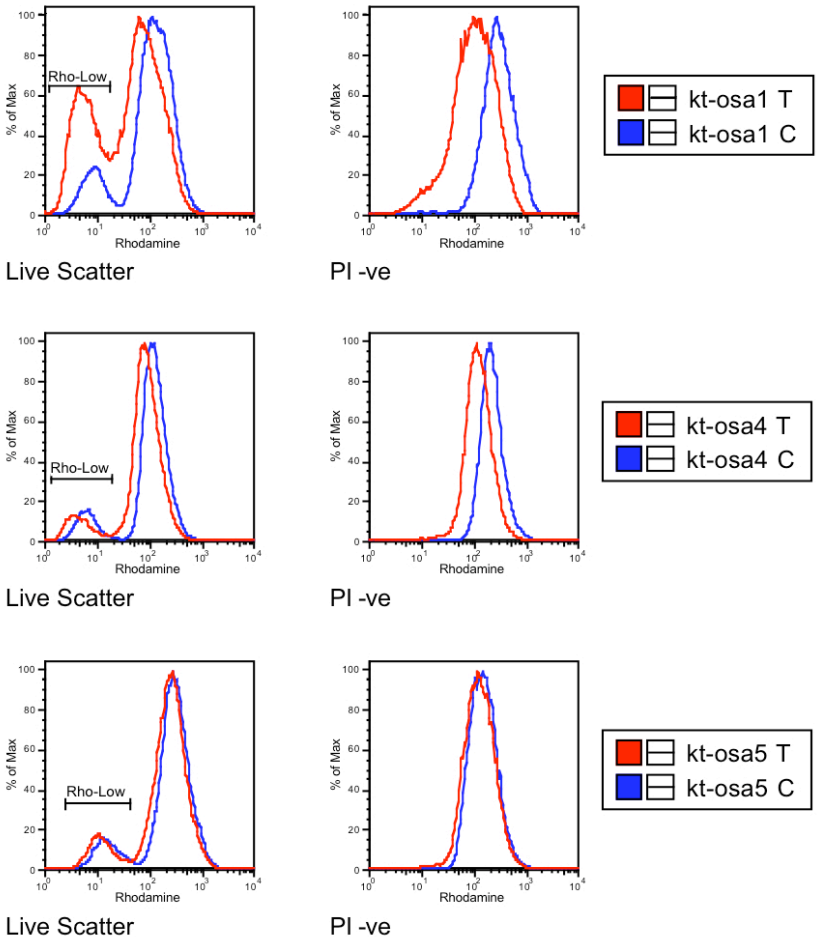


Figure 27 - kt-osa1, kt-osa4 and kt-osa5 tested for efflux of Rhodamine 123. Histograms representing level of fluorescence. C=Control, T=Test sample.
Left plots - Analysis of cells within "Live" gate based on light scatter profile. All three populations show a subset of cells with reduced fluorescence ("Rho-low") compared to main population.
Right plots - Exclusion of dead cells by gating out PI+ve cells results in loss of Rho-low subset. kt-osa1 and kt-osa4 test samples show loss of fluorescence, suggesting efflux of Rho123.

When examined using Aldefluor, kt-osa1 and kt-osa4 also showed a small subpopulation of $ALDH^{Bright}$ cells, absent from the DEAB control, at early passage. For kt-osa1, this represented 0.06% of live cells at subculture 2; at subculture 3 this had reduced to 0.01% (Figure 28a). For kt-osa4, the 0.08% of $ALDH^{bright}$ cells seen at subculture 3 had been all but lost four passages later (Figure 28b). It is acknowledged that, for both populations, the fluorescence of cells identified as "ALDH positive" was weak in comparison to that shown by HSC when the assay is used to evaluate human haematopoietic cells, and may not represent genuine expression. Nonetheless, no differential between control and test samples had been seen for any of the established cancer cell lines, and so the visualisation of these slightly brighter cells in the test sample for kt-osa1 and kt-osa4 was notable.

A summary of the cancer stem cell-associated properties shown by kt-osa populations is shown in Table 5.

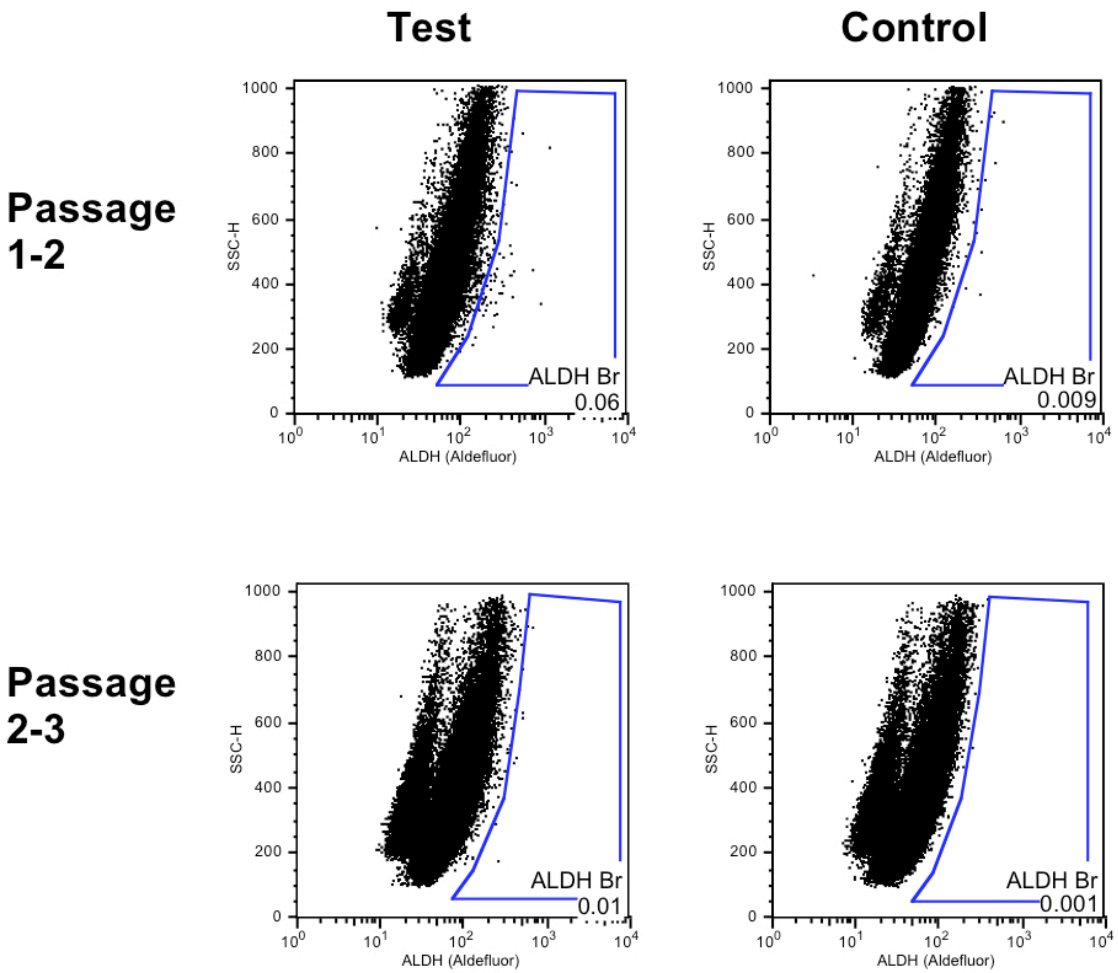


Figure 28a - kt-osa1 canine osteosarcoma cells - Aldefluor assay. (Upper) - Early passage - test sample suggests a small proportion of weakly ALDH-positive cells compared with DEAB control. (Lower) - Subsequent passage - reduced ALDH-positive population.

Sample	ALDH-Bright
kt-osa1 test p1-2	0.06
kt-osa1 control p1-2	0.009
kt-osa1 test p2-3	0.01
kt-osa1 control p2-3	0.001

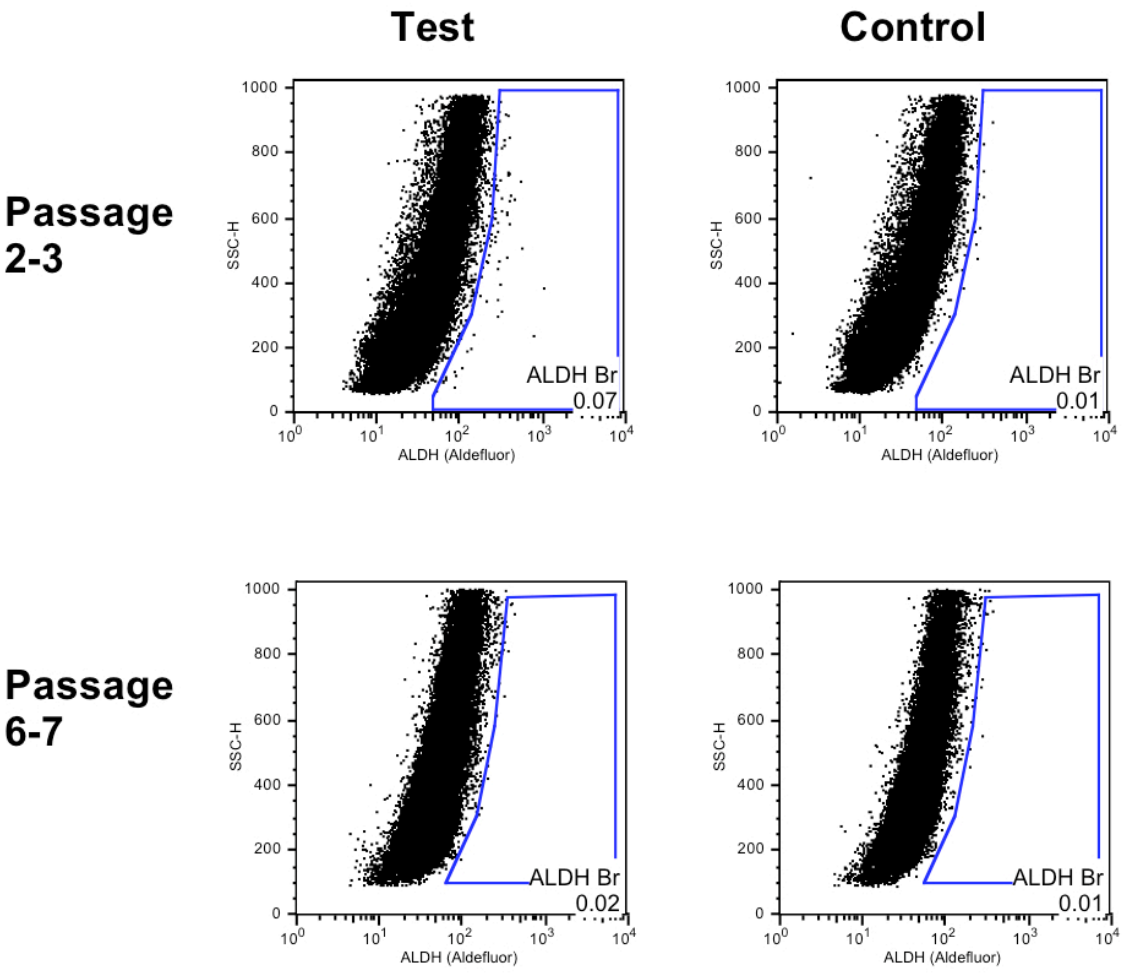


Figure 28b - kt-osa4 canine osteosarcoma cells - Aldefluor assay.
(Upper) - Early passage - test sample suggests small proportion of weakly ALDH-positive cells.
(Lower) - Later passage - reduced ALDH-positive population.

Sample	ALDH-Bright
kt-osa4 test p2-3	0.07
kt-osa4 control p2-3	0.01
kt-osa4 test p6-7	0.02
kt-osa4 control p6-7	0.01

Cells	$CD44^+$	$CD133^+$	$CD117^+$	Hoechst SP	Rho 123 Efflux	ALDH-Bright	Sphere Formation
kt-osa1	98.0% (p1) 99.6% (p26)	0.034% (p1) -ve (p5)	1.25% (p5) 0.21% (p10)	Present (p16)	All cells (p12)	0.06%? (p1) 0.01%? (p2)	Yes
kt-osa2	99.5% (p0)	n/d	-ve (p0)	n/d	n/d	n/d	n/d
kt-osa3	n/d	-ve (p0)	n/d	n/d	n/d	n/d	n/d
kt-osa4	97.2% (p6) 99.1% (p26)	-ve (p6)	0.3% (p6) -ve (p10)	Absent (p17)	All cells (p13)	0.08%? (p2) -ve (p6)	Yes
kt-osa5	99.0% (p26)	n/d	0.04% (p8)	Absent (p14)	Absent (p10)	n/d	Yes

Table 5 – Summary of cancer stem cell-associated properties shown by kt-osa populations. n/d – assay not performed with this population; p=passage number.

ii) Acute Lymphoblastic Leukaemia

A four month old male entire Hovawart was presented to the HFSA, R(D)SVS as systemically unwell with polyuria/polydipsia, hypercalcaemia, thrombocytopenia and leukocytosis ($22x10^9$/l), largely composed of large, fragile lymphoblasts. A diagnosis of acute lymphoblastic leukaemia was made based on cytological examination of peripheral blood and bone marrow smears . Within 48h of presentation the total white cell count had fallen to $2x10^9$/l. Mononuclear cells were isolated from a bone marrow aspirate (BMMNC) and peripheral blood (PBMNC) obtained as part of the clinical staging process at initial examination, and analysed using flow cytometry.

A CHOP chemotherapy protocol (cyclophosphamide / doxorubicin / vincristine / prednisolone) was instituted; the puppy's owners requested a minimally invasive clinical approach and no further bone marrow aspirates were taken. PBMNC were isolated for re-evaluation at eight and nine weeks after treatment began. Unfortunately, after an initial good response to therapy, the puppy suffered a clinical relapse and was euthanased at the owners' request on grounds of poor prognosis and quality of life.

Cell surface markers

In common with many other studies of leukaemia and leukaemic stem cells (LSC), CD34 was positive on a subpopulation of both PBMNC (0.14%) and BMMNC (0.37-0.44%). Interestingly, and contrasting with the findings in normal bone marrow where $CD34^+$ cells tended to be $FSC^{Low}SSC^{Low}$, these cells showed much more varied light scatter properties, which may indicate increased cell size and cellular complexity associated with abnormal blast cells and/or aberrant surface marker expression associated with the disease process (Figure 29). By contrast with some reports of human paediatric ALL, CD133 was negative (Cox *et al.*, 2009), as was CD117 (data not shown).

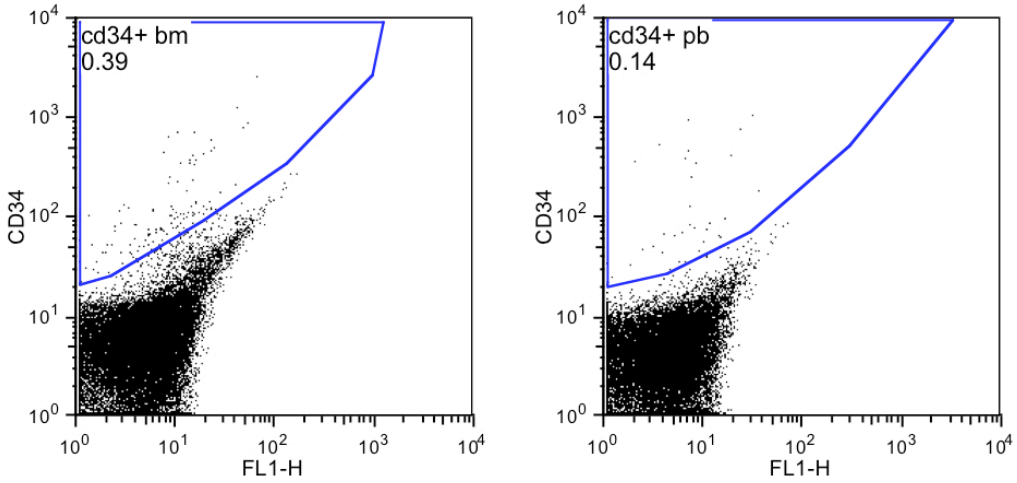
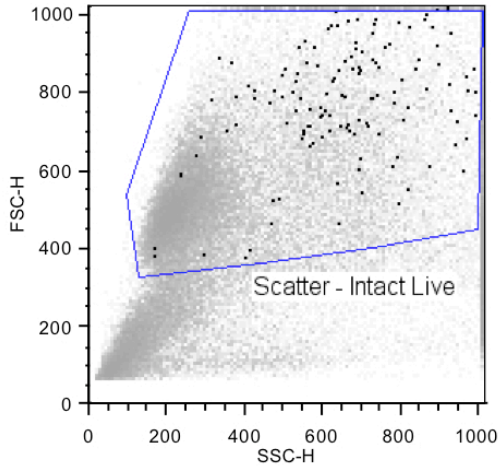


Figure 29 - Canine ALL BMMNC (above left) and PBMNC (above right) - CD34 staining. Below - Overlay of plots showing relative position on light scatter profile (grey) of CD34+ BMMNC (black). ALL CD34+ cells are more widely distributed across scatter profile than CD34+ population of normal bone marrow (shown in Figure 2).



Functional assays - ALDH activity

BMMNC were analysed for ALDH activity, using the standard protocol and the Fiordalisi modification. An $ALDH^{Bright}$ population, representing 1.83% of live BMMNC, was identified under standard assay conditions (Figure 30a). By contrast with the $ALDH^{Bright}SSC^{Low}$ cells expected in normal bone marrow, these cells were $SSC^{Med\text{-}High}$ – possibly reflecting, as with the unexpected distribution of $CD34^{+}$ cells, increased nuclear complexity associated with large, abnormal blasts.

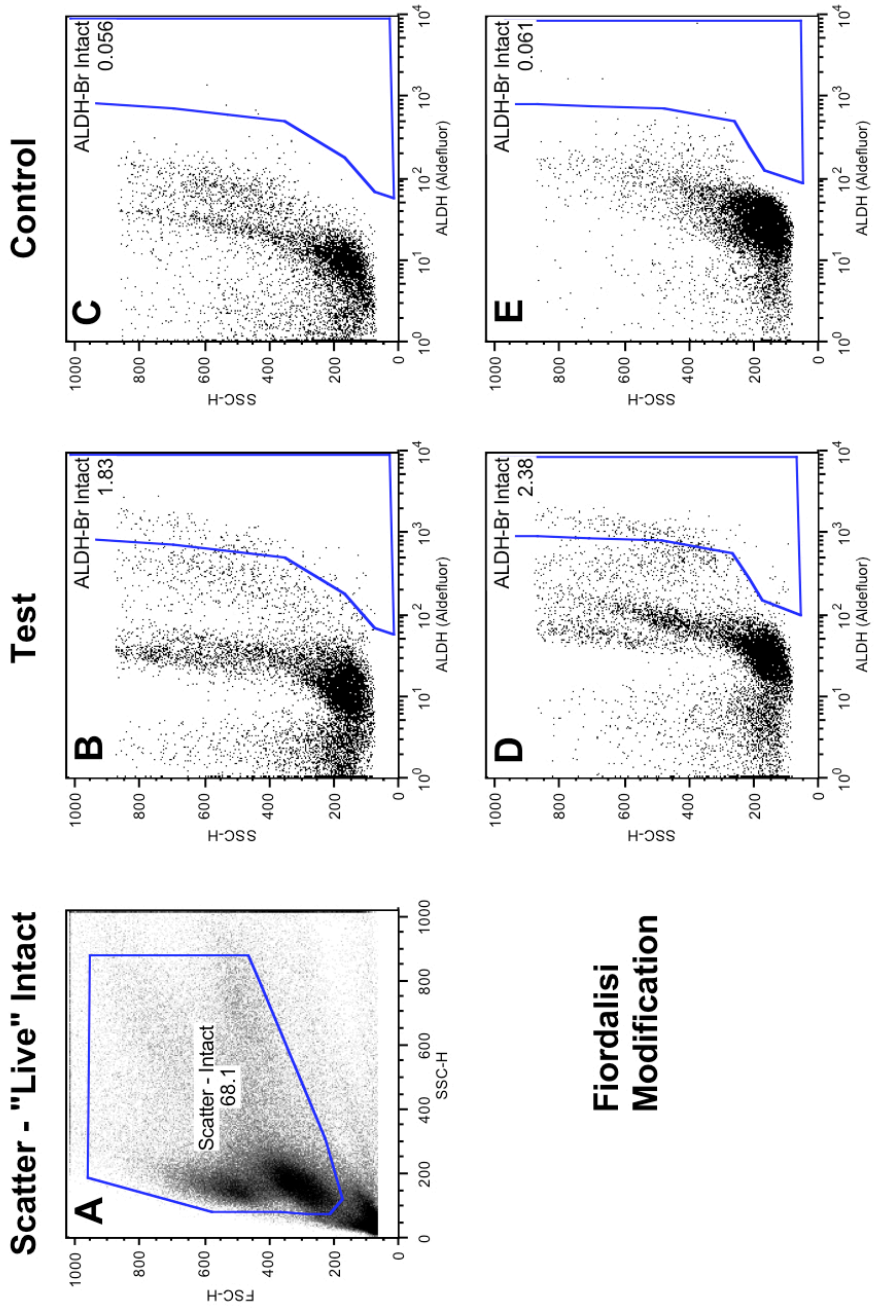


Figure 30a - Canine ALL BMMNC - ALDH analysis.
A - Representative scatter plot showing 'live' analysis gate
SSC vs Aldefluor plots obtained with standard protocol (B, C) and Fiordalisi modification (D, E).
ALDH-bright population, diminished in DEAB control sample, visualised using both protocols.

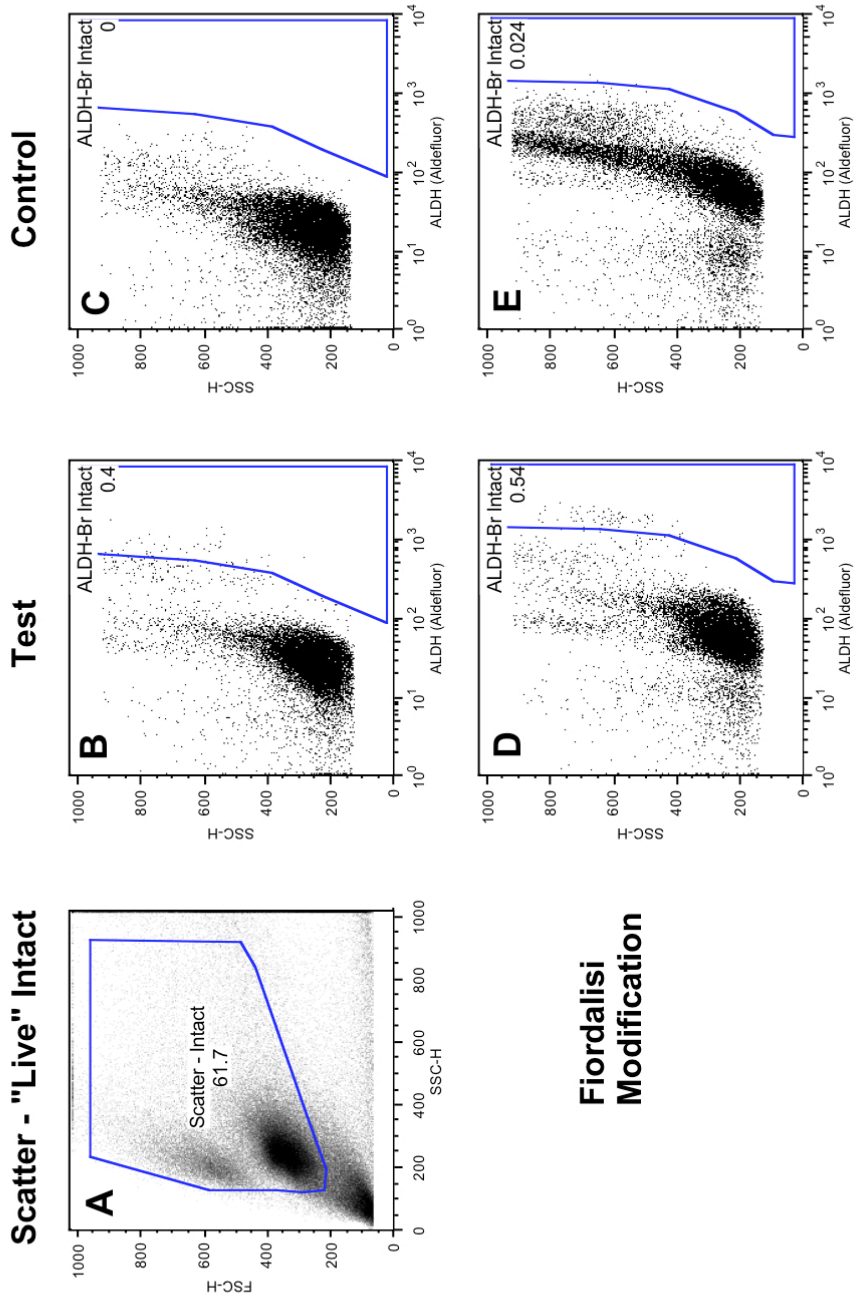


Figure 30b - Canine ALL PBMNC - ALDH analysis.
A - Representative scatter plot showing 'live' analysis gate
SSC vs Aldefluor plots obtained with standard protocol (B, C) and Fiordalisi modification (D, E).
ALDH-bright population, diminished in DEAB control sample, visualised using both protocols.

Gates were set conservatively when quantifying this ALDH^{Bright} population, to reduce the likelihood of including normal myeloid cells (in light of their slightly wider distribution in normal bone marrow test samples). As such, the contribution of normal cells to the observed ALDH^{Bright} population cannot be ruled out in the absence of more detailed cell surface phenotyping. However, the cells identified were at least a log decade brighter than the brightest cells in the control sample, a greater differential than was seen for normal bone marrow. Moreover, the ALDH^{Bright} population (2.38%) was also detected using the Fiordalisi modification (for which normal myeloid cells showed no variation in fluorescence between test and control samples). Although smaller than that seen within BMMNC, this ALDH^{Bright} population was also visualised upon examination of PBMNC (standard = 0.4%, Fiordalisi = 0.54%) (Figure 30b).

Gating of specific populations within the intact cell gate suggested that non-myeloid / lymphocytic cells also expressed ALDH. Upon exclusion of granulocytic myeloid cells from the analysis, 0.6% of cells in the test sample showed increased ALDH fluorescence compared to the corresponding control (Figure 31). Again, simultaneous analysis of cell surface phenotype would be required to substantiate these observations, as cells cannot be reliably identified on the basis of light scatter properties alone. However, these results suggest that cellular subpopulations expressing ALDH may be identified within BMMNC and PBMNC in canine ALL.

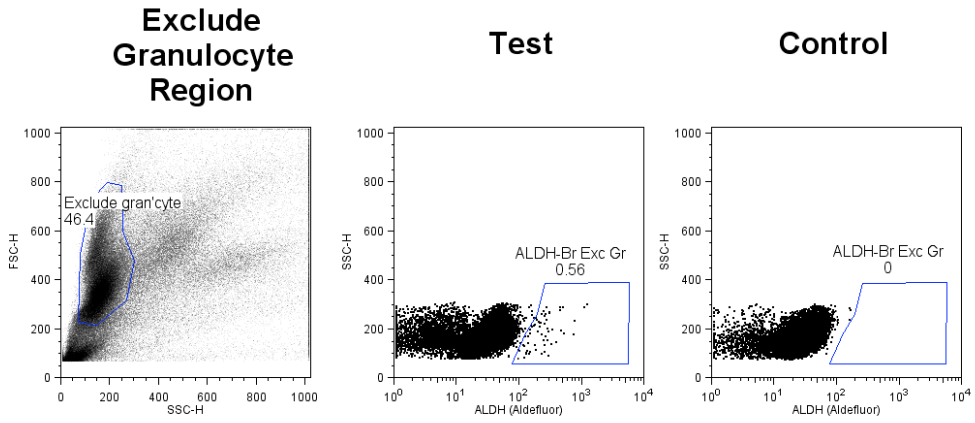


Figure 31 - Canine ALL BMMNC - ALDH analysis excluding granulocyte / myeloid region.

Eight weeks after therapy began, PBMNC were re-examined using the standard protocol. Having shown an initial good response to therapy, the puppy had begun to show signs of relapse, including an increased serum ionised calcium level. Once again, an $ALDH^{Bright}SSC^{Med\text{-}High}$ population was clearly defined, and was larger than at previous evaluation (3.54% of live cells). A subset of cells in the lymphoid region of the light scatter profile also demonstrated One week later, the animal was showing further clinical signs of deterioration, with a dramatic reduction in total white blood cell count. Notably, the proportion of live intact PBMNC on the scatter profile had reduced considerably (10-20% as compared to 50-70% at initial evaluation), most likely the result of increasing cellular fragility.

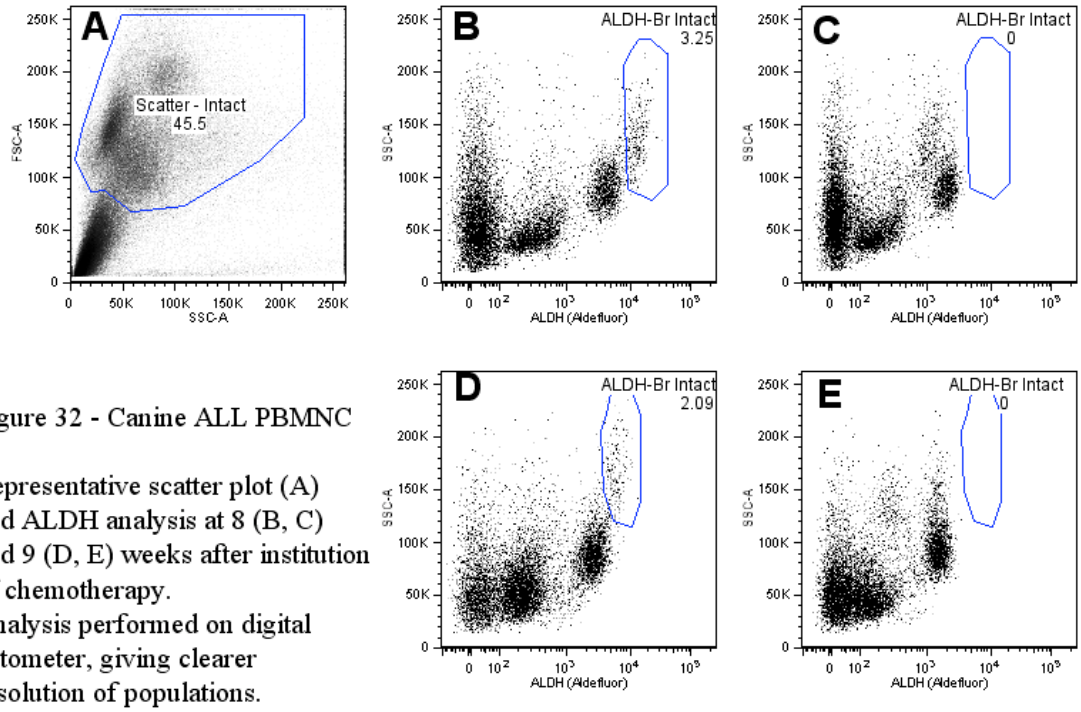


Figure 32 - Canine ALL PBMNC

Representative scatter plot (A) and ALDH analysis at 8 (B, C) and 9 (D, E) weeks after institution of chemotherapy.
Analysis performed on digital cytometer, giving clearer resolution of populations.

On this occasion, the $ALDH^{Bright}SSC^{Med\text{-}High}$ PBMNC population (2.09%) was isolated by FACS (Figure 32); the cells were assessed for *in vitro* chemosensitivity to the alkylating agent cyclophosphamide, compared to the main population of cells (Figure 33). Overall viability was poor, as evidenced by the low luminescence

values obtained. The $ALDH^{Br}SSC^{Med\text{-}High}$ cells showed greater viability; however, when survival of each population was assessed as a proportion of the corresponding untreated control cells, $ALDH^{Br}SSC^{Med\text{-}High}$ cells and main population cells showed similar responses to the drug. Although therapeutic plasma levels of cyclophosphamide in dogs are not well documented, the drug was tested over a range of concentrations to incorporate levels achieved in humans at therapeutic doses (35.8 - 89.6μM) (Regenthal *et al.*, 1999). However, there was very little response by either population to cyclophosphamide at concentrations below 100μM.

Functional assays - Hoechst 33342 efflux

PBMNC were also analysed for Hoechst efflux capacity. However, staining was poor in the vast majority (97%) of intact cells, which appeared towards the origin of the Hoechst blue *vs* red plot, and no defined side population could be visualised (Figure 34). It is unclear why these cells did not successfully stain with Hoechst. This may have arisen as a consequence of substrate toxicity and cell fragility, or interference with Hoechst interaction by the drugs being administered as chemotherapy. It is also possible that Hoechst efflux capacity was a feature of most cells in this aggressive haematopoietic malignancy.

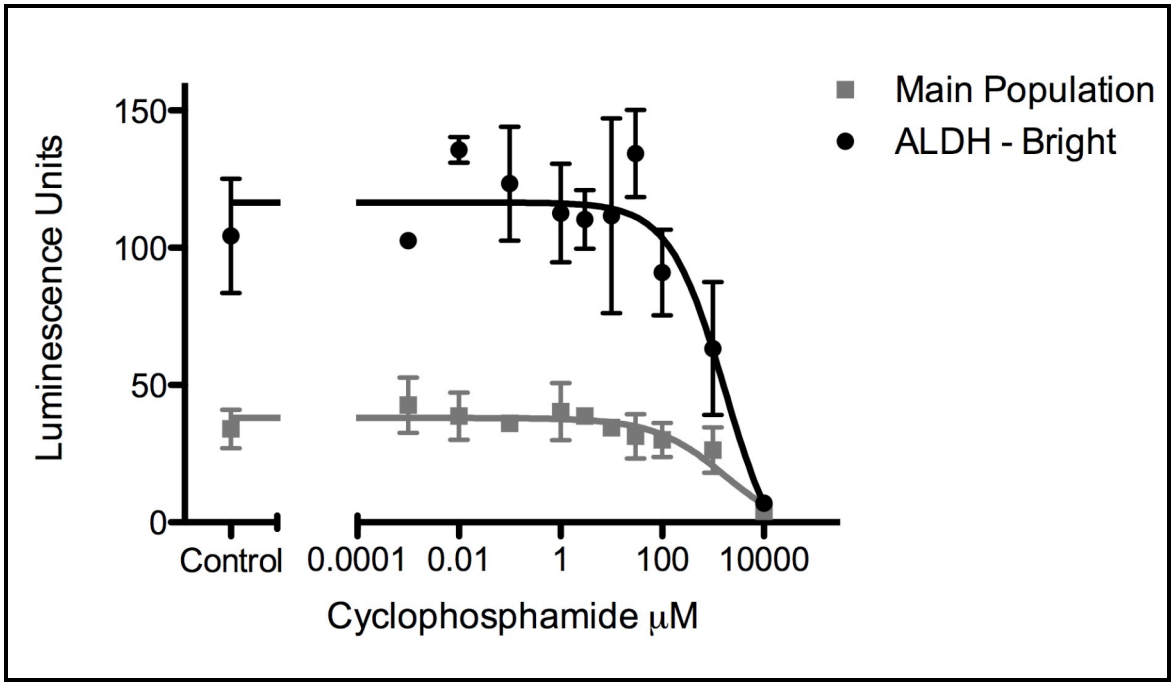
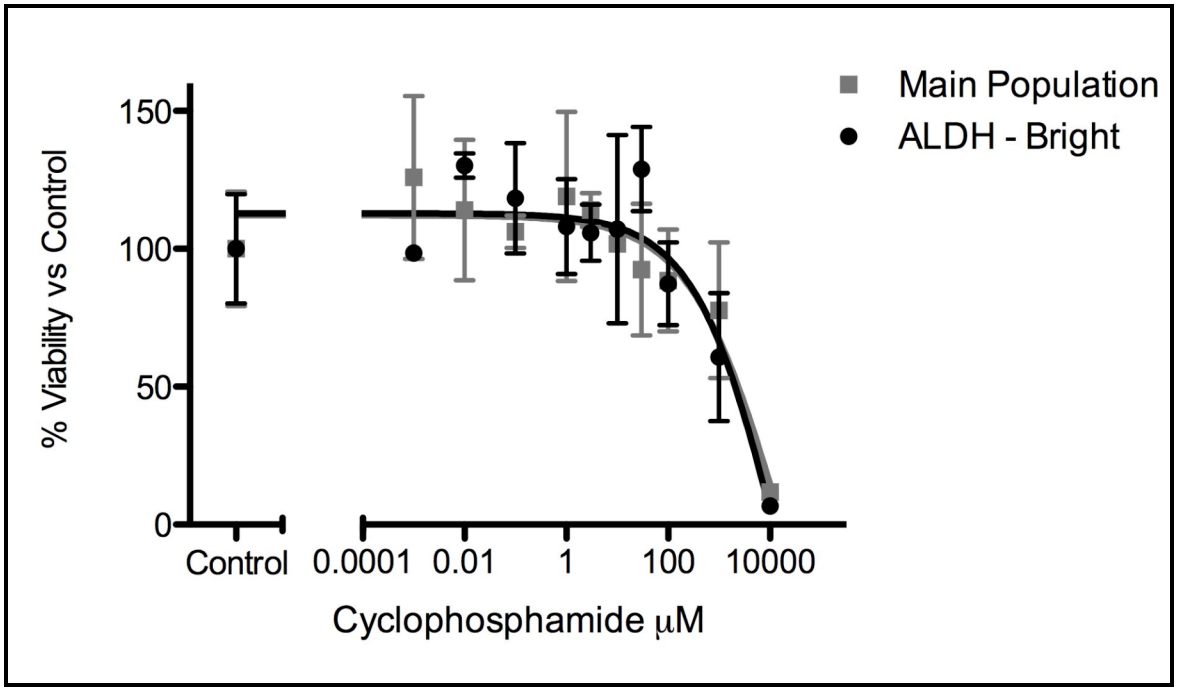


Figure 33 – Canine ALL PBMNC sorted for ALDH fluorescence and treated with cyclophosphamide.
Above – Absolute cell viability as measured by ATP luminescence.
Below – Relative cell viability as a proportion of corresponding untreated control cells.



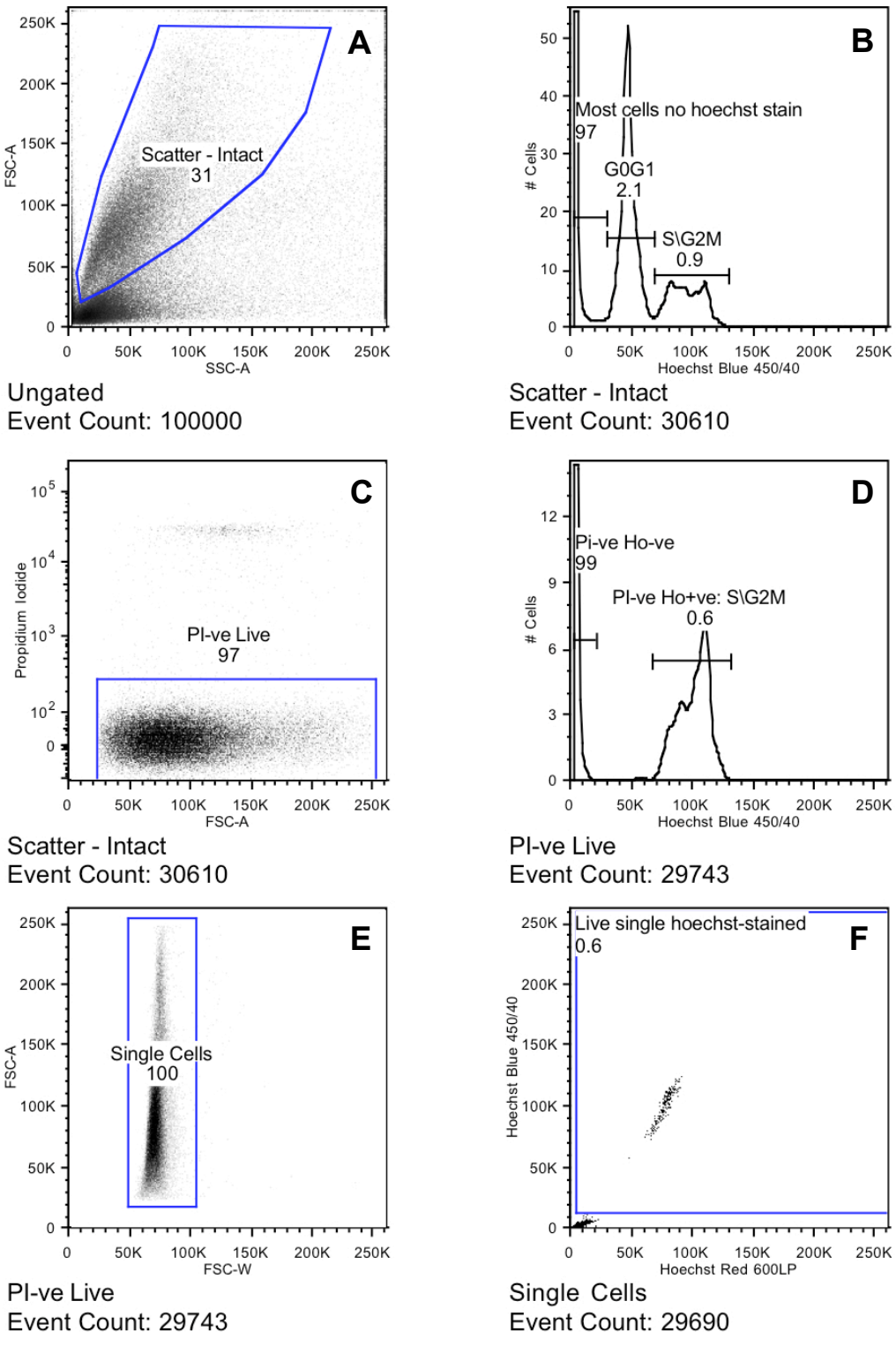


Figure 34 – Hoechst efflux analysis of canine ALL PBMNC.
A, B – Hoechst staining profile for intact cells : uptake by only 3% of cells
C, D – Selection of only live PI-ve cells – loss of Hoechst-stained G_0/G_1 population.
E, F – Intact, live single cells selected for analysis. Only 0.58% have taken up dye and appear in expected position on standard Hoechst blue-red plot.

DISCUSSION

The aim of this study was to identify candidate cancer stem cell populations in canine tumours. A rapidly evolving field of interest, the CSC hypothesis could have significant implications for the clinical approach to canine neoplasia. It was hoped that optimisation of published techniques using canine cancer cell lines would allow evaluation of markers for their utility in CSC identification, and their functional significance in differentially-expressing populations.

Cellular subpopulations within canine cancer cell lines

None of the canine cancer cell lines demonstrated stable subpopulations of cells expressing CD24, CD34, CD117 or CD133. Conversely, in each line CD44 was expressed by almost all cells. These cell surface markers selected for this study are among those cited most commonly when using flow cytometry to isolate CSC in other model systems, such as human and murine. However, others such as CD20, CD123, ESA (epCAM) and integrins are also used (Guzman *et al.*, 2001; Collins *et al.*, 2005; Fang *et al.*, 2005; Li *et al.*, 2007; Matsui *et al.*, 2008; Moshaver *et al.*, 2008; Jin *et al.*, 2009; Munz *et al.*, 2009), and it is possible that investigation of alternative antigens would identify positive subpopulations within these cell lines. For example, it has been proposed that lymphoma may arise from malignant transformation of germinal centre B-cells (Kuppers, 2005; Klein and Dalla-Favera, 2008), whose expression pattern is CD19+ CD20+ CD38+ CD77+ , and so these markers could be investigated for 3132.

Nevertheless, the finding that cell lines tended to display an “all or none” staining pattern, with an overall shift by all cells for positive staining, is not unexpected. Cell lines are ostensibly clonal populations, which may originate from single cancer cells depending on the experimental procedures used in their derivation. There is an inevitable loss of the parental tumour’s heterogeneity with repeated passage in culture, as dominant populations outgrow others that proliferate less rapidly.

Stable heterogeneity within cell cultures may exist as the result of an equilibrium between individual clones, whereby the relative proportions are stable with respect to each other (Poste *et al.*, 1981; Ye *et al.*, 2005). However, not only might this equilibrium be altered by interventions which preferentially select for the survival and growth of certain clones (Poste *et al.*, 1981), cellular expression patterns may be affected by both cell-intrinsic and extrinsic factors, including basic determinants such as culture medium and growth substrate (Luo *et al.*, 2006; Stockholm *et al.*, 2007). It has been suggested, and seems logical, that in a continuous cell line maintained using normal culture techniques, a small subpopulation of *bona fide* cancer stem cells would be rapidly overwhelmed by the growth of the majority population within a few passages (Zheng *et al.*, 2007). Thus it would be expected that, within a cell line, a CSC population must represent a substantial fraction (or show a higher growth rate than non-CSC) to avoid being lost.

In many cases within the CSC literature, cellular subpopulations in cell lines have been "derived" through flow cytometric gating, separating cells according to expression level even where there is no clear demarcation between positive *vs* negative or low *vs* high expression (Sheridan *et al.*, 2006; Atsumi *et al.*, 2008; Fillmore and Kuperwasser, 2008). This technique was used in a later study to investigate the significance of CD44 expression in canine cancer cell lines (Chapter 5). Where cells express more than one candidate marker, multicolour analysis enables simultaneous examination such that cells with different combinations of surface marker may be isolated and assessed for CSC properties. Although this was intended for this study, it was not feasible as only one of the cell surface markers (CD44) was stably expressed on any of the cell lines.

It is recognised that cellular proliferation status may influence functional assays such as Hoechst 33342 and Rhodamine 123 efflux. These have been described most extensively as methods to mark out haematopoietic stem cells from bone marrow, in which environment these cells are more likely to be slow-cycling or quiescent (Spangrude and Johnson, 1990; Bertoncello and Williams, 2004; Uchida *et al.*, 2004; Stingl *et al.*, 2006). The preservation of stable quiescence is less likely within the *in*

vitro environment, and this may explain why these assays did not produce consistent results.

Hoechst 33342 may itself be used as a DNA-binding dye for cell cycle analysis, but in the context of efflux analysis, the SP cells definitively do not appear as part of any stage of the classic cell cycle profile (as they lie between the plot origin and the G_0G_1 population). Simultaneous staining with Hoechst and another indicator of cell cycle status may demonstrate whether there is an association between side population and particular stages of the cell cycle (Benchaouir *et al.*, 2004; Stingl *et al.*, 2006; Kamohara *et al.*, 2008; Oates *et al.*, 2009). Substrates such as Pyronin-Y (which gives a measure of RNA content, and can thus be used to identify quiescent cells) may be suitable for analysis of live cells. Alternatively, the SP cells could be collected, fixed immediately, and analysed using standard cell cycle reagents with non-overlapping fluorescence spectra, such as propidium iodide.

The expression of ABCG2 in itself did not seem sufficient to ensure the consistent presence of a SP profile, as protein was detected in all of the canine cell lines tested. As the relative proportions of proliferating cells will change in a cell culture during exponential growth, this might influence the presence or magnitude of a SP (Masters and Stacey, 2007). It is an acknowledged limitation of the presented Hoechst efflux data that, when experiments with a given line were repeated, cells were tested at different (if comparable) levels of passage. This variation may have contributed to the failure to observe SP on some occasions. Stringent passage protocol - with cells tested at the same passage and the same level of confluence on every occasion - may give more consistent results, and should be investigated.

Nevertheless, that the feline Cat-MT and human MCF-7 mammary carcinoma cell lines reproducibly demonstrated a SP, whilst this was only intermittently seen for each of the canine cell lines, suggests that this may not be a reliable assay for the evaluation of canine cells. The preliminary results obtained from feline cells using the Hoechst efflux protocol are encouraging and warrant further investigation. Inconsistency in results obtained through Hoechst efflux analysis of cells other than

murine bone marrow is not unique to this study (G. McLachlan, M. Waterfall, University of Edinburgh; J. Mountford; University of Glasgow – *pers. comm.*), and this is reflected in published data. For example, although many studies describe the existence of a side population within MCF-7 cells, its size varies enormously (0.2 – 7.5%) between reports (Kondo *et al.*, 2004; Patrawala *et al.*, 2005; Zhou *et al.*, 2007; Engelmann *et al.*, 2008; Liu *et al.*, 2008; Steiniger *et al.*, 2008; Yin *et al.*, 2008; Tanaka *et al.*, 2009).

Although analysis of the canine cell lines did not identify any stable CSC subpopulations, experiments did help to highlight important aspects of protocol when looking for potentially rare cellular subsets, particularly when adapting assays to the canine system:

- Controls must be adequate, particularly when using antibodies raised to epitopes of other species. Of those used in this study, only CD34 was raised to canine protein, and CD44 had documented canine crossreactivity. Testing against normal bone marrow allowed confirmation of reactivity. Negative controls (isotype / unstained) are important particularly to rule out non-specific interactions, which might easily be interpreted as true positive binding (e.g. with magnetic bead sorting), and autofluorescence.
- Dead cell discrimination was similarly important. For example, $CD24^{low/-}$ cells are frequently associated with CSC-like attributes such as enhanced tumourigenicity. In this study, had cells been fractionated on this basis, the "$CD24^{+}$" cells would have performed poorly in assays when compared to negative cells, as a result of non-specific binding by the antibody to non-viable cells. Propidium iodide allows identification of dead/necrotic cells, but not those in earlier stages of apoptosis – the latter may be achieved through addition of other agents such as Annexin V (Vermes *et al.*, 1995).
- Sufficient events must be collected to rule out the influence of biological noise, particularly where positive cells are only weakly fluorescent, do not appear as a "population" with regards light scatter, or where positive cell numbers are very small.

- Protocol (e.g. antibody concentrations, gating and instrument settings) should be consistent between experiments. Flow cytometry is a powerful technique but, as with all fluorescence-based modalities, the absolute brightness shown by an individual cell is affected by multiple factors – comparisons should be consistent to prevent misinterpretation. Results should be reproducible.

Unlike more "precious" primary tumour cells, cell lines provide a readily available and relatively consistent (and, for some cell types/species, well characterised) system, and are thus invaluable for the adaptation and optimisation of assays. They provide a reproducible biological model with which to determine the cellular behaviour associated with a given phenotype. Experiments to investigate the implications of CD44 expression on canine cancer cells are described in Chapter 5.

Cellular subpopulations identified within primary canine tumour cells

By contrast with the cell lines, cells from both of the spontaneous tumours examined in this study demonstrated small subpopulations of cells with CSC-associated phenotypes. In the case of the primary osteosarcoma, cells were obtained on a single occasion and expanded in culture for assessment. Whilst small subpopulations of cells expressing markers such as CD117 and CD133 were identified soon after derivation, these were gradually lost upon successive passage. It was striking that these changes took place within a relatively short time – for example, CD117 expression by kt-osa1 reduced by over 80% between passages 5 and 10, and expression by kt-osa4 was lost completely. That this occurred in parallel with a marked reduction in morphological variation suggests that one cell type within each population had become dominant at the expense of other minority subsets, or that the conditions of *in vitro* culture directly inhibited these expression patterns. Thus, over time in culture, each cell line seems to have become less representative of the heterogeneous composition of the primary tumour.

The Rhodamine 123 efflux demonstrated by kt-osa1 and kt-osa4 warrants further evaluation of expression of drug resistance proteins, such as P-glycoprotein or

ABCG2^{482T}, by these cells (Steingold *et al.*, 1998; Doyle and Ross, 2003; Robey *et al.*, 2003). It is possible that, had this been evaluated at derivation, a smaller proportion of cells would have shown Rho efflux capacity, and that these had become dominant within the culture at the time of testing (kt-osa5 cells did not efflux Rho, so this was not a feature of all cells within the tumour). If the overall Rho efflux shown by these populations was due to outgrowth of a membrane transporter-expressing clone, this was not a cell concurrently expressing the other CSC-associated markers CD117, CD133 or ALDH.

Notably, a variety of CSC-associated phenotypes were identified amongst these cells using flow cytometry, by contrast with the established canine cancer cell lines; in addition, a proportion of kt-osa1, kt-osa4 and kt-osa5 cells were capable of forming tumourspheres when plated in low-density, serum-free culture (described in Chapter 4). Subpopulations identified by different methods represented different proportions of the parental population, indicating incomplete overlap between phenotypes. This could be assessed using simultaneous analysis of cell surface and functional markers.

The results obtained through investigation of the kt-osa populations raise some interesting points. It is clear that such investigations are best performed soon after derivation of primary cells to ensure that small subpopulations do not become overwhelmed, or stem cell-associated expression patterns suppressed, in culture. Cells were not cloned, in an attempt to preserve as much heterogeneity as possible for analysis. The derivation and characterisation of clones originating from single cells might allow longer term maintenance in culture of individual phenotypes. Equally, however, this introduces an element of selection (in terms of which cells survive the cloning process), again resulting in a skewed representation of tumour composition. Additionally, cloning prevents the interactions between heterogeneous cell types that will occur in the parental tumour tissue and may support CSC survival. Thus, whilst both techniques have a role in evaluation of CSC phenotypes in solid tumours, overall it is likely that the most representative picture of phenotypic composition, and so greatest likelihood of identifying CSC subsets, will be obtained by analysing cells at derivation.

If cancer cell lines are maintained by cancer stem cells, it is also clear that those maintaining kt-osa1, kt-osa4 and kt-osa5 were *not* those expressing the CSC-associated markers CD117 or CD133 soon after derivation. Given the significance ascribed to these markers in the CSC literature, this may not accurately reflect the situation within the primary tumour. It is also interesting that the five populations of cells derived from this canine osteosarcoma each demonstrated distinct stem cell-associated expression patterns and cellular functions, despite having originated from a small ($1cm^3$) piece of tumour tissue.

Unlike solid tumours, where cells are generally only available at the time of surgical resection or biopsy unless expanded *in vitro*, it is possible to evaluate primary cells from bloodborne malignancies *ex vivo* throughout the course of disease. This has the obvious advantages of permitting more frequent examination and reducing the likelihood of culture-induced artefact. Also, cells isolated from blood or bone marrow are more likely to represent the neoplasm as a whole than those derived from a piece of tissue taken from one region of a heterogeneous solid tumour. If subjects are undergoing treatment, clearly this may result in changes to cellular characteristics or to the relative proportions of heterogeneous subsets over time. In the context of the CSC hypothesis, this information could be valuable for defining resistant cellular phenotypes, and modifying therapeutic interventions accordingly.

The Aldefluor assay has been shown to identify tumourigenic subpopulations in a variety of malignancies, both solid and haematopoietic (Pearce *et al.*, 2005; Ginestier *et al.*, 2007; Matsui *et al.*, 2008; Carpentino *et al.*, 2009; Huang *et al.*, 2009b; Jiang *et al.*, 2009; Li *et al.*, 2010; Tanei *et al.*, 2009); rationale for use of the assay is stronger in the latter, as the expression of ALDH1A1 by a subset of normal human haematopoietic repopulating cells has been more fully characterised (Kastan *et al.*, 1990; Storms *et al.*, 1999; Armstrong *et al.*, 2004; Hess *et al.*, 2004; Christ *et al.*, 2007). The appearance of an $ALDH^{Bright}$ population in the BMMNC of a dog with acute lymphoblastic leukaemia could represent a candidate leukaemic stem cell population, and changes in the proportion of this population during treatment with a

chemotherapy protocol incorporating the alkylating agent cyclophosphamide may reflect this.

There is only one report available of this assay being used in the canine system, to identify normal canine HSC (Fiordalisi *et al.*, 2005). The study was presented as an abstract and $ALDH^{Bright}SSC^{low}$ cells were not fully characterised. Conversely, in the work presented here, Aldefluor did not identify a candidate $ALDH^{Bright}SSC^{Low}$ HSC population within normal canine BMMNC, when used according to either the manufacturer's protocol or that detailed by Fiordalisi *et al*.

The principle behind the Aldefluor assay is oxidation by intracellular ALDH of the BAAA substrate, and specifically the ALDH1-A1 isoform expressed by normal human HSC (for which the system is optimised). ALDH1 and ALDH3 have been detected using this assay in human tumour cell lines. However, not all members of the ALDH superfamily are capable of oxidising the substrate, and indeed some cell lines which express ALDH1-A1 do not convert BAAA (Foster *et al.*, 2003 / manufacturers' data). Thus, the failure to detect an $ALDH^{Bright}SSC^{Low}$ population within normal canine BMMNC using this commercial assay does not necessarily rule out the presence of ALDH activity within normal canine HSC, although it does appear that Aldefluor may not provide a reliable means by which to isolate these cells. Further investigation of this could include assessment of ALDH expression at mRNA (RT-PCR) or protein (immunoblot) level, or spectrophotometric measurement of enzyme activity, in canine haematopoietic progenitors isolated on the basis of other features such as CD34 expression (Niemeyer *et al.*, 2001) or rhodamine efflux (Wijewardana *et al.*, 2007). Similarly, although the ALDH fluorescence of the "positive" cells for kt-osa1 and kt-osa4 was weak, it remains possible that these cells expressed a canine isoform with poor enzymatic activity towards BAAA.

By contrast, the $ALDH^{Bright}SSC^{Med\text{-}High}$ cells identified within the BMMNC and PBMNC of a dog with ALL were readily visualised as considerably brighter than the main population of cells. Although induction of ALDH expression in cancer cell

populations by exposure to alkylating agents *in vitro* is reported (Sreerama and Sladek, 1994), at the point where these cells were first evaluated, this dog was chemotherapy-naïve. It is reported that, in comparison to that shown by normal haematopoietic cells, ALDH activity in tumour cells is high (Sreerama and Sladek, 1997). Upregulation of ALDH - from levels undetectable in normal BMMNC to those producing the bright fluorescence seen at Aldefluor analysis of ALL mononuclear cells - may have been a part of the disease process.

Although the viability of sorted $ALDH^{Bright}SSC^{Med\text{-}High}$ cells from this case of leukaemia was greater than that of the main population, these cells did not demonstrate enhanced resistance to cyclophosphamide *in vitro*. However, the active metabolite of cyclophosphamide, 4-hydroxycyclophosphamide, is produced through hepatic metabolism of the prodrug *in vivo*. It is therefore unlikely that the use of cyclophosphamide in this assay was representative. 4-hydroxyperoxycyclophosphamide (4-HC) may be used as an alternative in cell culture assays, as it is converted to 4-hydroxycyclophosphamide *in vitro* (Teicher *et al.*, 1996), and would have more accurately demonstrated whether these cells showed resistance to alkylating agents. An alternative possibility is that exposure of all cells *in vivo* to cyclophosphamide had selected for non-resistant cells prior to testing *in vitro*.

Unfortunately, the dog succumbed to disease before these $ALDH^{Bright}SSC^{Med\text{-}High}$ cells could be fully characterised, limiting the conclusions which may be drawn from the data. Clone-forming capacity of the $ALDH^{Bright}SSC^{Med\text{-}High}$ population was not demonstrated. Also, the leukaemic animal examined was juvenile (4-6 months old) - no normal bone marrow from animals of a comparable age was examined, and so the possibility that the profile seen is age-related rather than a feature of disease cannot be excluded.

Nonetheless, it is tempting to speculate that, as the leukaemic $CD34^{+}$ subset showed increased FSC / SSC in comparison with those of normal bone marrow, these cells could have been part of the $ALDH^{Bright}SSC^{Med\text{-}High}$ population, which was similarly

distributed across the scatter profile. Ideally, CD34 and Aldefluor would have been evaluated simultaneously, to see whether positive populations correlated, and might represent a leukaemic stem cell subset. Combined surface marker staining / ALDH detection would also have allowed more accurate segregation of populations, to further characterise both the $ALDH^{Bright}SSC^{Med\text{-}High}$ cells and also the SSC^{Low} subset with increased Aldefluor fluorescence (identified when the myeloid region was excluded from analysis).

Although for normal haematopoietic cells there is incomplete overlap between subsets identified using ALDH, Hoechst efflux and CD34 (Goodell *et al.*, 1996; Goodell *et al.*, 1997; Pearce and Bonnet, 2007; Addla *et al.*, 2008), both ALDH and side population analyses can be used to enrich drug resistant $CD138^{-}$ multiple myeloma stem cells (Matsui *et al.*, 2008). To this author's knowledge, there are no reports describing ALDH activity in subsets of canine neoplastic cells. If this proves to be a valid assay for detecting subsets of cancer cells with drug detoxifying properties, there could be important clinical ramifications in terms of selected treatment protocol. Moreover, the assay is reasonably straightforward (compared, for example, to the Hoechst 33342 efflux protocol) and requires only a standard 488nm laser, available on most cytometers. Significantly, the population was seen not only in bone marrow but also peripheral blood, and so testing or ongoing assessment need involve procedures no more invasive to the patient than blood sampling involved in the standard monitoring of cancer chemotherapy.

In conclusion, this study demonstrates that published protocols to investigate the CSC hypothesis may be adapted for use in the canine model system. Cancer cell lines provide a stable background for adaptation and optimisation of these assays for canine cells, allowing investigation of the implications of specific markers or expression patterns. However, stable, discrete cellular subpopulations are less readily identified. Conversely, analysis of primary tumour cells presents practical challenges, but these are more likely to demonstrate stable heterogeneous subpopulations, some of which may represent CSC.

Whether the result of a specific CSC population, heterogeneous clonal evolution, or a combination of processes, resistance to anticancer agents and persistence of cancer cells after therapy are major clinical problems. The ultimate aim in cancer stem cell investigations is to identify, and find ways of specifically targetting, cells which may be responsible for driving disease progression or causing relapse after treatment. Flow cytometry may provide a rapid and readily accessible means of characterising and monitoring clinical disease. If subpopulations can be isolated from spontaneous tumours, *in vitro* susceptibility testing may inform more individualised treatment regimes (Twentyman *et al.*, 1989; Bosanquet, 1993), to increase the likelihood of clinical efficacy and reduce the more generalised adverse effects of antiproliferative agents. The assays described in this study thus warrant further investigation within the clinical setting.

CHAPTER 4

TUMOURSPHERE CULTURE TO ISOLATE CANINE CANCER STEM CELLS

INTRODUCTION

Identifying Cancer Stem Cells in Solid Tumours

Normal stem cells exist at the apex of a hierarchy within tissues. This progresses, through a variable number of transit-amplifying steps, to the terminally differentiated cells making up the majority of the tissue (Weissman *et al.*, 2001). This results in phenotypic and functional heterogeneity such that, in order to characterise them specifically, stem cells must be separated from the bulk population. The scarcity of normal tissue stem cells thus imposes a challenge – that is, how to obtain sufficient numbers of cells from a tissue with which to perform assays.

If phenotypic and/or functional markers have been characterised, these may be used for isolation – however, there is likely to be overlap between surface marker profiles of cells at successive stages of differentiation, as this represents a continuum (as manifest by the haematopoietic system). Ideally, stem cells could be isolated and propagated in culture, allowing the generation of stable populations to compare with the progenitor and terminally differentiated components of a tissue. However, this relies upon the ability to maintain the cells in an undifferentiated state rather than permitting their development along any of their programmed lineages.

As well as permitting further investigation of the biology and regenerative potential of normal stem cells, techniques to isolate self-renewing, multipotent cells should provide a platform for investigating the role of CSC in tumours. The differentiation pathways of the haematopoietic system, and the progression from haematopoietic stem cells (HSC) through divergent lineages to produce fully differentiated effector cells, are reasonably well characterised (Weissman *et al.*, 2001). This has been central to the investigation of putative CSC in haematological malignancies. Assays widely used to identify normal HSC, such as expression of CD34 and efflux of Hoechst 33342, have been extended to identify subsets of stem-like cells responsible for tumour maintenance. Equally, appreciation of the normal haematopoietic hierarchies, and associated surface phenotypes, has provided compelling evidence

for CSC originating in more committed lineages (Bonnet and Dick, 1997; Cozzio *et al.*, 2003; Jamieson *et al.*, 2004; Matsui *et al.*, 2004; Bonnet, 2005; Taussig *et al.*, 2005; Gal *et al.*, 2006; Nishida *et al.*, 2009).

The extension of the cancer stem cell hypothesis to solid tumours has been more challenging experimentally, not least because for many, the identity of the corresponding normal tissue stem cell and its progression through to terminally differentiated cells has yet to be fully understood. Reliable methods to isolate normal, and consequently malignant, tissue stem cells are less well developed. Another problem is the physical nature of the tissues. Cells from solid tissues and tumours are often larger and more fragile than blood cells (and less accessible for sampling), and creation of viable single-cell suspensions is challenging.

Moreover, the biology of solid tumours is less well understood than the haematological malignancies, which more often follow a well-recognised, lineage-specific course. Solid tumours are often markedly heterogeneous, displaying multiple phenotypes within a single mass. As well as intrinsic interactions between malignant cells, the stroma and microenvironment are critical in the development of this heterogeneity - this is complicated further by involvement of cells from other tissues through angiogenesis, inflammation and, more recently recognised, the recruitment of mesenchymal stem cells or endothelial progenitors to sites of neoplastic growth (Hiscox and Jiang, 1997; Houghton *et al.*, 2004; Mueller and Fusenig, 2004; Nolan *et al.*, 2007).

Propagating Solid Tissue Stem Cells – the Neurosphere Assay

The ability to isolate and propagate normal neural stem cells *in vitro* using the "neurosphere" technique represented a breakthrough in cell culture. Reynolds *et al* demonstrated that in the absence of serum and the presence of a mitogenic growth factor (epidermal growth factor, EGF), single cells with self-renewal and multilineage differentiation capacity proliferated as clusters in which the progeny showed similar stem cell-like properties. The "unique observation" of spheroidal

growth was not seen in serum-containing cultures, in which cells grew in a flat monolayer (Reynolds *et al.*, 1992; Reynolds and Weiss, 1992).

Reynolds and Weiss derived neurospheres from Embryonic Day 14 mouse striata, and performed clonal and population analyses in order to define more accurately the proportion of cells capable of self-renewal and multilineage differentiation. In initial cultures, approximately 1% of cells were capable of sphere formation. However, when these were dissociated, up to 49% of cells could form secondary spheres, demonstrating a considerable enrichment of growth factor-responsive precursors (putative neural stem or progenitor cells) (Reynolds and Weiss, 1996).

Secondary spheres were examined at each stage to assess their ability to differentiate into neurons, astrocytes and oligodendrocytes, confirming multipotency and further supporting the concept that the sphere-forming, EGF-responsive precursors were indeed stem cells. The authors showed that the growth pattern was maintained over multiple passages and, therefore, that these cells were capable of generating large numbers of progeny (Reynolds and Weiss, 1996).

The authors have been careful to encourage a degree of parsimony in the interpretation of their findings – not least in their recognition that spheres may arise from and comprise not only stem but also more differentiated progenitor cells, particularly at early passages (Reynolds and Rietze, 2005). Nonetheless, the assay has been adopted in the search for stem cell populations in a wide variety of normal solid tissues, including breast, prostate and retinal epithelium (Tropepe *et al.*, 2000; Dontu *et al.*, 2003; Xin *et al.*, 2007).

Tumoursphere Culture to Identify Cancer Stem Cells in Solid Tumours

CSC are postulated to be capable of self-renewal and differentiation along diverse lineage pathways, and (more speculatively) to have improved capacity for survival in less permissive environments. As such, the neurosphere assay has been widely adapted by cancer cell biologists in the hope that it might allow isolation of primitive

stem-like cancer cells from solid tumours (Figure 1). Candidate CSC populations have now been described for many tumour types on this basis. "Tumourspheres" have been derived from both primary tumours and tumour cell lines, and sphere-forming cells analysed for properties including expression of surface markers, transcription factor activity, resistance to drugs, and enhanced tumourigenicity (Ignatova *et al.*, 2002; Hemmati *et al.*, 2003; Singh *et al.*, 2003; Fang *et al.*, 2005; Gibbs *et al.*, 2005; Fujii *et al.*, 2007; Harper *et al.*, 2007; Ricci-Vitiani *et al.*, 2007; Fillmore and Kuperwasser, 2008; Wilson *et al.*, 2008; Bisson and Prowse, 2009; Stoica *et al.*, 2009).

Tumoursphere populations have been shown to express many stem cell-associated markers at levels exceeding those shown by corresponding non-sphere-forming cells. These include cell surface molecules such as CD133, CD117, CD44 and integrins (Singh *et al.*, 2003; Collins *et al.*, 2005; Chiou *et al.*, 2008; Zhang *et al.*, 2008a), transcription factors such as Oct4 and Nanog (Gibbs *et al.*, 2005; Ponti *et al.*, 2005), and membrane transporter proteins such as ABCG2 (Bisson and Prowse, 2009; Bleau *et al.*, 2009). For example, upregulation of the normal neural stem cell marker Nestin is widely used to demonstrate expansion of a stem cell compartment in normal neurosphere culture, and this is also seen in brain malignancies (Hemmati *et al.*, 2003; Singh *et al.*, 2003; Galli *et al.*, 2004; Dell'Albani, 2008).

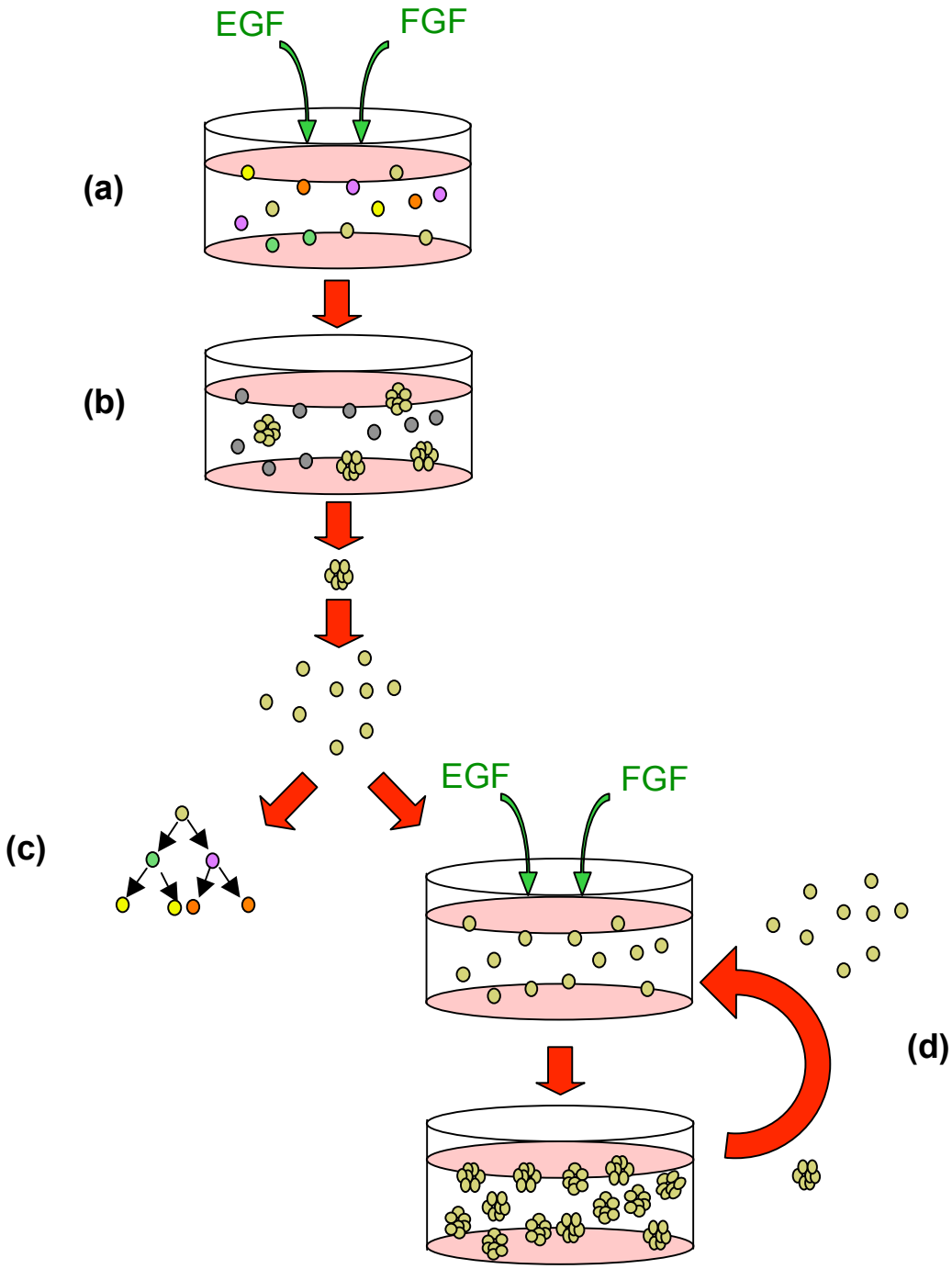


Figure 1 – Principle of the tumoursphere assay for CSC identification and propagation
(a) Dissociated cells cultured in serum-free conditions supplemented with growth factors.
(b) Putative CSC, but not differentiated cells, proliferate as clonal spheroid colonies.
(c) Tumoursphere-forming cells show capacity to differentiate into all cell types seen within parental tumour or cell line.
(d) Tumoursphere-forming cells may be dissociated and placed back into culture, and self-renew to form further tumoursphere colonies.

Potential advantages of tumoursphere culture techniques

Work published by Lee *et al* in 2006 suggested that cells from primary glioblastomas cultured in serum-free neurosphere conditions were, in fact, *more* representative of the tumour than were those propagated using conventional methods. They also found that they remained more stable over time in terms of growth pattern, telomerase activity, genomic and karyotypic stability, colony formation and tumourigenicity. The sphere-derived tumours were histologically more infiltrative (hence more similar to primary glioblastomas) than those from standard glioma cell lines such as U87MG. The results suggested that the expression of "glioblastoma-defining genes" is lost upon serial passage in serum-containing conditions, and that serum-free spheroid cultures reflect the original tumour more faithfully (Lee *et al.*, 2006). Similarly, De Witt Hamer *et al* examined gene expression profiles in primary and cultured human glioblastoma, and reported that the correlation between sphere cultures and their parental tumours was greater than that for corresponding adherent cultures (De Witt Hamer *et al.*, 2008).

A major benefit in using the sphere technique to isolate putative CSC is the ability to propagate them continuously in culture, allowing not only detailed analysis of their properties but also enabling large-scale drug screening. Enhanced activity against the tumoursphere cells, as compared to the corresponding adherent (putatively more differentiated) counterparts, may allow identification of compounds which selectively target CSC populations (Diamandis *et al.*, 2007).

Potential problems associated with the tumoursphere technique

Several features of neurosphere, and by extension tumoursphere, culture systems complicate the interpretation of the data acquired. Transit-amplifying progenitor populations, as well as *bona fide* stem cells, may survive and proliferate - Reynolds and Rietze advise that spheres between passages 5-10 should be used in assays, to allow the senescence of these more limited progenitors and reduce their influence on results (Reynolds and Rietze, 2005). Nonetheless it is recognised that some degree

of differentiation, and also spontaneous apoptosis, does occur within spheres, so they cannot be viewed as a totally "pure population" of stem cell-like cells.

Additionally, spheres show a tendency to clump and coalesce in culture, even when components such as methylcellulose are added to the medium to increase its viscosity. This makes precise analysis of clonality difficult, as it cannot be inferred that every sphere originated from a single cell. An elegant study by Singec and colleages in 2006 used timelapse microscopy to show the fusion of neurospheres, and also cocultures of cells labelled with either EGFP or β-galactosidase to demonstrate that most spheres plated at "clonal density" (5 cells/μl) comprised the progeny of more than one cell. Indeed, even at densities as low as 0.5 cells/μl, much lower than those used in most published tumoursphere experiments, over 40% of spheres were polyclonal (Singec *et al.*, 2006).

Staying round or going flat? Adherent cultures of brain tumour stem cells

A recent report by Pollard and colleagues described the culture of putative glioma CSC using adherent, rather than suspension, culture conditions. Cells from primary tumours were grown in neural stem cell medium on laminin-coated tissue culture plastic, and proliferated as a monolayer, expressing markers associated with neural stem cells. Additionally, they showed fewer signs of differentiation and apoptosis than parallel neurosphere cultures, and more readily established cell lines. The authors postulated that improved access to growth factors was responsible for many of these apparent improvements to the neurosphere culture method, and that increased uniformity and easier manipulation of cells represent a significant advance with regards not only propagation but also utility for screening drugs which may target CSC (Pollard *et al.*, 2009b). However, there remain several questions as to the validity of this technique, not least that of the identity of the homogeneous population of cells selected and propagated – as pointed out in a not-unexpected response from Reynolds and Vescovi,

"Lack of differentiation markers in a given cell is in no way equated to being a stem

cell. Without evidence demonstrating that each cell cultured under these conditions (or the progeny of these cells) is able to exhibit cancer stem cell characteristics (i.e., extensive self-renewal, generation large number of progeny, multilineage differentiation capability, and tumor formation), the assessment that the culture contains a homogeneous cancer stem cell population is unfounded." (Reynolds and Vescovi, 2009)

Pollard and colleagues, whilst defending their findings on the most part, do concede in their reply to this critique that, "additional clonal data would be valuable" (Pollard *et al.*, 2009a).

Interpretation of Data Gathered from Tumoursphere Culture

Within the published literature, there is a great degree of variability in the culture conditions used to derive spheres (Chaichana *et al.*, 2006). Whilst DMEM:F12 is commonly employed as a base medium (Reynolds and Weiss, 1992; Singh *et al.*, 2003), commercial serum-free formulations or conditioned media are also used by some investigators (Dontu *et al.*, 2003; Collins *et al.*, 2005; Sansone *et al.*, 2007), with or without the addition of methylcellulose or agar to increase viscosity (Kukekov *et al.*, 1999; Ignatova *et al.*, 2002; Gibbs *et al.*, 2005). Serum-replacement supplements such as Bottenstein's N2 (Bottenstein and Sato, 1979; Reynolds and Weiss, 1992; Gibbs *et al.*, 2005) or B27 (Hemmati *et al.*, 2003; Sansone *et al.*, 2007) are also variously used, as are additional growth factors (such as epidermal growth factor (Reynolds *et al.*, 1992; Dontu *et al.*, 2004), basic fibroblastic growth factor (Hemmati *et al.*, 2003; Galli *et al.*, 2004), leukaemia inhibitory factor (Hemmati *et al.*, 2003; Singh *et al.*, 2003), stem cell factor [KIT] (Collins *et al.*, 2005), neuronal survival factor (Singh *et al.*, 2003)) at a range of concentrations and in an assortment of combinations. Indeed, in some instances serum itself is used in the culture medium (Kukekov *et al.*, 1999; Fang *et al.*, 2005). Different substrates are described, with some experiments employing standard culture vessels, and others those coated with substances such as poly-l-ornithine (Reynolds and Weiss, 1992), or commercial low-attachment plates (Gibbs *et al.*, 2005). As addressed by Singec *et*

al, the cell density used to afford "clonal" conditions also varies between reports (Singec *et al.*, 2006).

The result of this diversity is that it is often rather difficult to pinpoint the criteria being used by investigators to define "tumourspheres", or what then indicates that they represent a stem cell population (Chaichana *et al.*, 2006). A good example of this is the recent report by Stoica and colleagues of a canine glioblastoma whose cells grow as nonattached "neurospheres" in both serum-free and serum-containing cultures (Stoica *et al.*, 2009). When compared to adherent cells, both populations of canine glioblastoma neurospheres show increased expression of CD133 (although the experimenters use an anti-mouse antibody with no isotype control, such that nonspecific binding cannot be ruled out). However, clonogenicity of both sphere-forming and adherent cells was found to be 100%, and all cell types produced tumours in nude mice, with no evidence of enhanced tumourigenicity amongst either neurosphere population. The decisive factors by which the neurospheres are designated the "cancer stem cells" are therefore unclear (Stoica *et al.*, 2009).

The percentage of cells with clone-forming (sphere-forming) capacity is interpreted as an indicator of self-renewal. Zheng *et al* concluded from clonal and population analysis of the C6 rat glioma cell line that it is "mainly composed of cancer stem cells", owing to the fact that almost 100% of cells formed spheres, and that primary and secondary spheres were equally tumourigenic. They reported reduced clone-forming ability in serum-free medium, with cells becoming quiescent until serum-supplemented, at which point further expansion occurred. Thus, culture conditions appeared to exert a significant effect on the apparent self-renewal capacity and "stem cell" behaviour (Zheng *et al.*, 2007). Conversely, whilst Kondo *et al* also showed the effects of different culture conditions / growth factors on sphere-forming capability of C6 cells, they estimated the CSC population as only 0.4%, based on Hoechst efflux (Kondo *et al.*, 2004).

The surface glycoprotein CD133 is often referred to as a "stem cell marker", and has been shown to be expressed more heavily in sphere cultures than corresponding

adherent cells for a variety of normal tissues and tumours, including those of brain, colon, lung, prostate, and malignant melanoma (Singh *et al.*, 2003; Collins *et al.*, 2005; Monzani *et al.*, 2007; Ricci-Vitiani *et al.*, 2007; Tirino *et al.*, 2009). By contrast, however, Shmelkov *et al* found that $CD133^+$ and $CD133^-$ metastatic colon cancer cells formed spheres in culture, and that $CD133^-$ cells expressed higher levels of CD44, another stem cell-associated marker (Shmelkov *et al.*, 2008). In another report examining primary and secondary glioblastomas, both sphere-forming $CD133^+$ and adherently-growing $CD133^-$ cells were similarly tumourigenic (Beier *et al.*, 2007). These are just a few examples of some of the conflicting data within the tumoursphere literature.

Thus, formation of spheres by cells *in vitro* must be critically assessed in each instance, and backed up with further evidence for (cancer) stem cell identity, such as multipotentiality, self-renewal and expression of appropriate markers.

The growth of tumour cells as multicellular spheroids is not a new concept, although investigations previously had concentrated on how such three-dimensional cultures might more closely mimic the behaviour of cells *in vivo* rather than on any propensity to enrich for stem or progenitor cell populations (Freyer and Sutherland, 1980; Durand, 1981; Toburen, 1981; Olive and Durand, 1985; Desoize and Jardillier, 2000; Alajati *et al.*, 2008). Nonetheless, Durand and Olive point out that, depending on the growth conditions, "virtually any proliferating cell can be induced to grow as multicell spheroids." (Durand and Olive, 2001) This is particularly relevant when, as has been seen so frequently in recent CSC literature, the "ability to form spheres" is reported as a direct indicator of a CSC population. Moreover, there is evidence that some degree of resistance to apoptosis and hence certain chemotherapeutic drugs, combined with impaired penetration of drugs into spheres, contributes to drug resistance in three-dimensional cultures regardless of any intrinsic CSC-like properties (Durand, 1981; Toburen, 1981; Olive and Durand, 1985; Kerbel *et al.*, 1994; Olive and Durand, 1994; Olive *et al.*, 1997; Weaver *et al.*, 2002).

It has been shown previously that, like human osteosarcoma, the canine osteosarcoma cell line D17 produces spheroidal colonies in serum-free, low-adherence, growth factor-supplemented conditions (Wilson *et al.*, 2008). The proportion of cells which could form these "sarcospheres" was estimated as 0.2 – 0.5%. Spheres showed increased immunostaining for the embryonic stem cell-associated markers Oct4, Nanog and STAT3, and adherent cultures greater expression of Stro-1, a cell surface marker which might represent more differentiated cells (although it may be associated also with mesenchymal stem cells, and with preosteoblasts) (Simmons and Torok-Storb, 1991; Gronthos *et al.*, 1999; Gibbs *et al.*, 2005; Chamberlain *et al.*, 2007; Wilson *et al.*, 2008).

If the propagation of canine cancer cells in low-density, serum-free culture selects for the growth of CSC, this would not only allow propagation and detailed characterisation of tumourigenic cells, but could also provide a platform for drug screening, to identify CSC-selective agents. Following on from the work of Wilson *et al*, this study aimed to determine whether canine cancer cell lines derived from diverse solid tumours are capable of forming tumourspheres, and whether these represent the expansion of a specific subpopulation of CSC. Clonal and population analyses were performed to determine the frequency of sphere-forming cells within the parental population, and tumourspheres were assessed for the expression of CSC-associated markers and resistance to commonly-used chemotherapy drugs.

MATERIALS AND METHODS

Tumoursphere Culture

Growth medium and standard conditions for low-density, serum-free tumoursphere culture are described in Materials and Methods chapter.

For some cell lines, the effects of additional or alternative growth factor supplementation were assessed:
-B27 supplement (Invitrogen) – Substituted for N2 supplement (B27/MC) or 1:1 with B27 (N2-B27/MC) in growth medium.
-Recombinant canine vascular endothelial growth factor (VEGF) (R&D Systems, Minneapolis MN, USA); 10µg/ml in PBS. Added with EGF and bFGF at 5-15ng/ml (1-3µl/well) every 48h.

Tumoursphere passage protocol

Wilson *et al* describe passage of D17 tumourspheres as follows: formed spheres were transferred to adherent conditions, the cells grown as a monolayer and then dissociated, and seeded once again into sphere culture (Wilson *et al.*, 2008). However, it was considered that this methodology might not be expected to *enrich* for a putative stem cell-like population with repeated passage - the normal differentiated cell population might once again expand upon transfer to permissive culture conditions, re-establishing the cellular identity of the parental line. An alternative passage protocol, described in Materials and Methods chapter, was devised whereby formed spheres at 7-14 days in vitro (d.i.v.) were dissociated to single cells, counted and plated directly back into tumoursphere culture conditions.

Limiting Dilution Assay for Population Analysis of Tumourspheres

For analysis of the relationship between cell density and tumoursphere formation in bulk culture, D17 and SB cells were resuspended in N2/MC medium at $6x10^4$ cells/ml as for standard tumoursphere culture. Serial doubling or tripling dilutions of this suspension were made in N2/MC. 1ml of each suspension was added to 1ml of N2/MC medium in triplicate wells of UltraLow Attachment 6-well plates and cells incubated as for tumoursphere culture (37°C, 5% CO_2).

12µl EGF, 12µl bFGF ± 2µl VEGF (SB only) were added every 48 hours. After 7 days in culture, tumourspheres were harvested and the number of cells in each well counted by trypan blue exclusion. Where low cell numbers precluded counting of individual wells, contents were pooled and the mean cell number/well calculated.

Limiting Dilution Assay for Clonal Analysis of Tumourspheres

Single cell suspensions were created of confluent adherent monolayers and/or tumourspheres. Viable cells were quantified using trypan blue, and cells resuspended at 1×10^6 cells/ml in N2 medium, with or without methylcellulose depending on the assay.

Dilutions of the cell suspension were made to achieve the required final cell number for 200µl per well. Cell suspensions were dispensed into the innermost 32 wells of UltraLow Attachment 96-well plates (Corning), with PBS only in the outer wells, to reduce possible plate-edge effects.

Plates were incubated at 37°c, 5% CO_2, 100% humidity. After 72h, growth factors were added (1.2ng EGF, 1.2ng bFGF, ±VEGF). Wells were scored for the presence and number of spheres after approximately 7 and 14 days in vitro.

Flow cytometric assessment of surface expression, Western blotting, immunofluorescence and chemosensitivity assays were performed as described in Materials and Methods chapter.

RESULTS

Canine Cancer Cells can Proliferate as Spheres in Serum-Free, Low-Adherence, Growth Factor-Supplemented Conditions

Canine cancer cell lines D17 osteosarcoma, SB haemangiosarcoma, REM134 mammary carcinoma and CML10 melanoma were cultured on a low-adherence substrate in N2/MC medium, in the presence of EGF and bFGF ("standard conditions"). Clusters of cells began to form within 24-48 hours of plating, and expanded over time as compact tumourspheres (Figure 2). With continued expansion, tumourspheres began to coalesce, forming larger spheres and often extensive, irregular aggregates (Figure 3). When tumourspheres were transferred to standard (uncoated) tissue culture plates in serum-containing medium, they attached to the substrate, the resultant adherent cells once again forming a monolayer with morphology similar to that of the original cell line (Figure 4).

Cell numbers yielded under standard conditions permitted repeated subculture of D17 and REM134 tumourspheres beyond 5 passsages, and these lines were therefore used in most assays. Rather than expansion in adherent conditions at each passage using the method of Wilson *et al* (Wilson *et al.*, 2008), tumourspheres were subcultured using the adapted sphere passage protocol to maximise enrichment of any putative CSC population without expansion of adherent (putatively more differentiated) cells. Proliferation of CML10 and SB cells was more limited, and often poor after subculture. All cell lines other than REM134 sporadically failed to proliferate under tumoursphere conditions, either at first plating or at subculture. Cells would remain apparently quiescent, gradually becoming dense and granular and failing to proliferate.

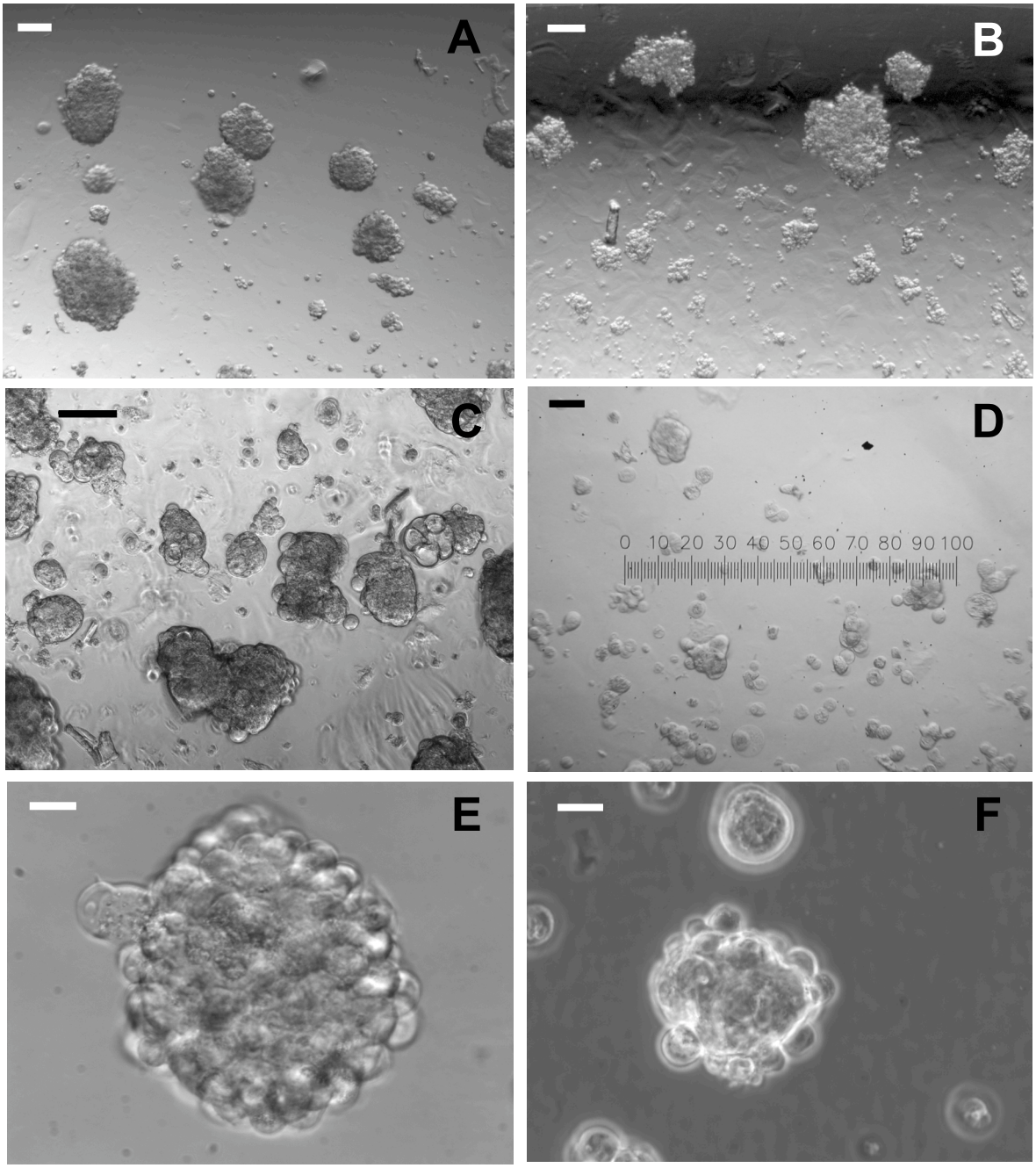


Figure 2 – Tumoursphere formation by canine cancer cell lines.
A – D17 osteosarcoma (8 days in vitro)
B – SB haemangiosarcoma (14 d.i.v.)
C – REM134 mammary carcinoma (8 d.i.v.)
D - CML10 melanoma (11 d.i.v.).
[A – D: Bar = 100µm]
E – D17 (8 d.i.v.)
F – REM134 (4 d.i.v.)
[E, F: Bar = 20µm]

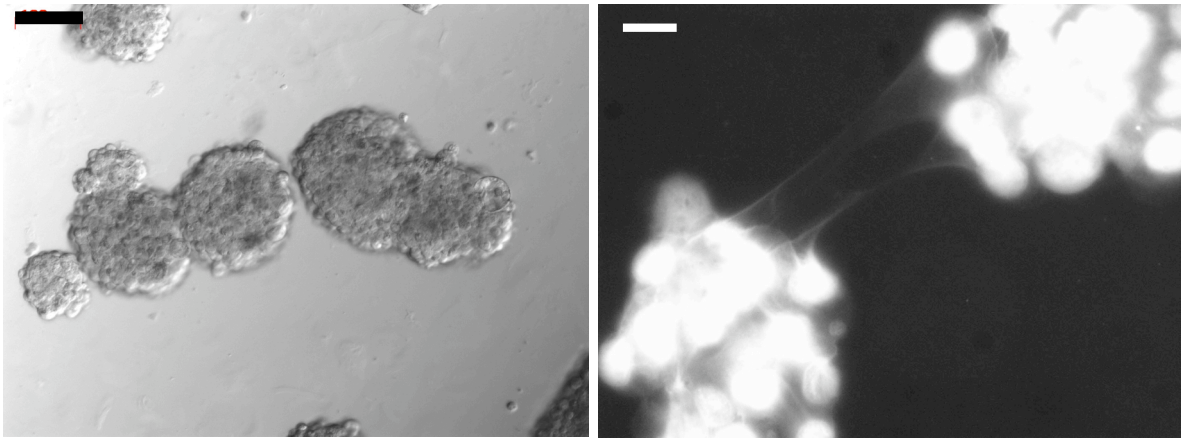


Figure 3 – *(Left)* D17 tumourspheres at 8 d.i.v. showing tendency towards fusion and coalescence (Bar 100µm). (*Right*) Cellular connections between D17 spheres, noted as an incidental finding at immunofluorescence microscopy. The positive fluorescence signal is a DAPI nuclear counterstain (Bar 20µm).
These images emphasise that counting tumourspheres in bulk culture is likely to be an unsatisfactory means to quantify the proportion of tumoursphere-forming cells.

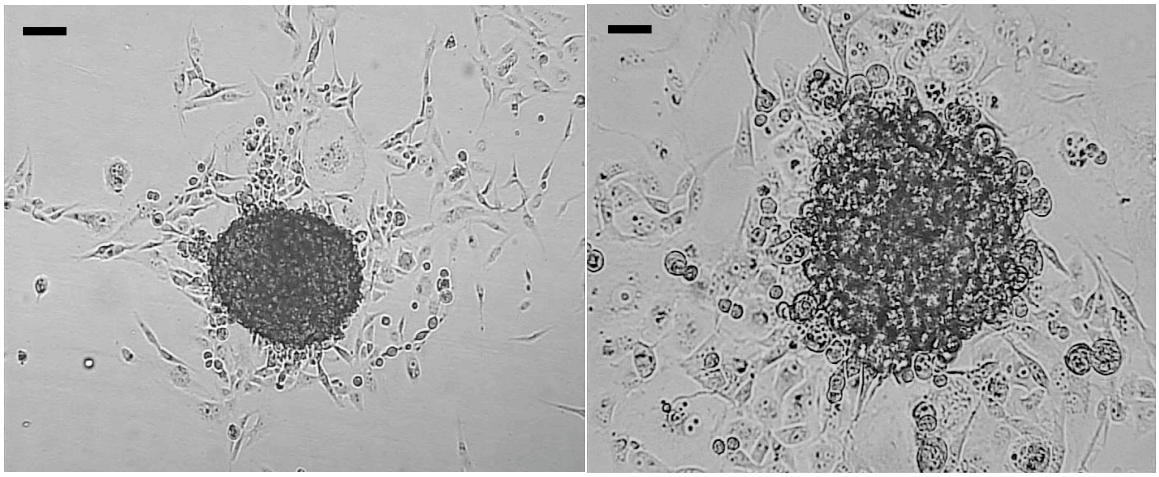


Figure 4 – D17 sphere after transfer to standard serum-containing culture conditions, demonstrating outgrowth of adherent cells. Left – Bar = 100µm; Right – Bar = 50µm.

Determinants of Tumoursphere Formation

Some investigators have suggested that, rather than selection of a pre-existing stem cell population, addition of mitogenic growth factors or use of specialized media may directly alter expression pattern, and thus be responsible for some of the phenotypic changes associated with sphere formation (Cicero *et al.*, 2009). Equally, although the addition of serum within classical neurosphere cultures leads to multilineage differentiation, and its absence promotes stem and progenitor cell self-renewal, it is unclear whether or not the differentiation of CSC similarly relies on the presence of serum.

Although these questions were not extensively addressed as part of this study, it was observed that one of the significant factors in producing the canine tumoursphere phenotype was the low-adherence substrate. D17 cells, plated under standard tumoursphere conditions but without additional growth factor supplementation, formed expanding tumourspheres (Figure 5). Moreover, when D17 or SB cells were plated in N2 medium on conventional (uncoated) tissue culture vessels, the cells attached to the substrate and proliferated as a monolayer despite the absence of serum.

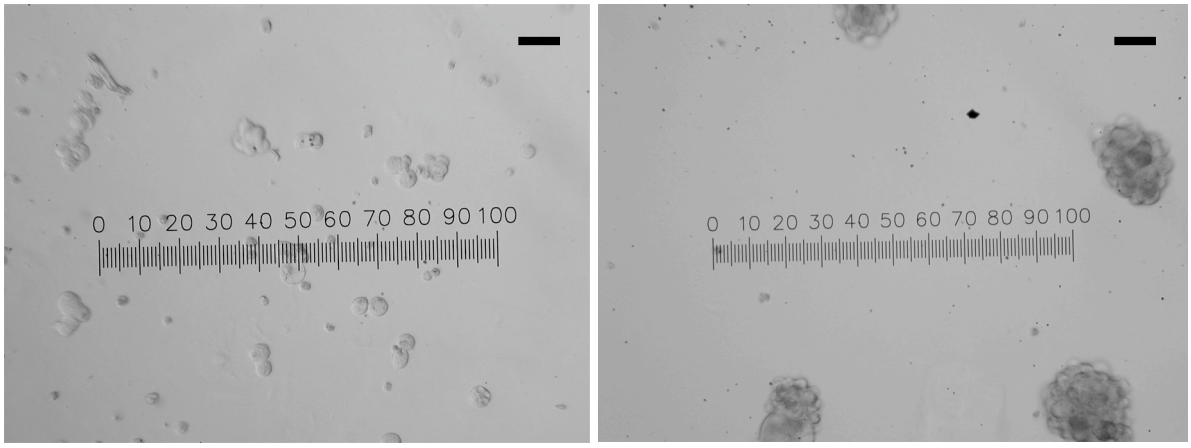


Figure 5 – D17 tumourspheres forming with no EGF or bFGF supplementation. Cells were still growing well as tumourspheres at 11 d.i.v. – i.e. D17 can form tumourspheres in the absence of supplementary growth factors.
Left – 3 d.i.v.; *Right* – 11 d.i.v.
Bar = 100µm

Conversely, attachment of D17 tumourspheres to the substrate, with outgrowth of adherent cells, was observed even under low-adherence conditions when the spheroids became large and settled to the bases of the wells (Figure 6). Thus, the absence of serum from culture medium does not necessarily preclude the adherent growth of canine cancer cells, and absence of growth factor supplementation does not preclude the formation or expansion of tumourspheres.

The addition of the mitogenic growth factors bFGF and EGF did appear to have direct effects on cellular phenotype. D17 cells were cultured in conventional, serum-containing adherent conditions and supplemented with growth factors at concentrations equivalent to those employed in sphere culture ("D17 AdherentGF"). Cell morphology was markedly altered, with cells developing along "cords" rather than as a smooth monolayer, often with pronounced spindle-like processes, and showing a tendency to clump into tumoursphere-like aggregates (Figure 7). With increasing confluence, these spheres tended to detach from the substrate and became suspended in the medium, suggesting a predominance of intercellular adhesion over that to the culture substrate.

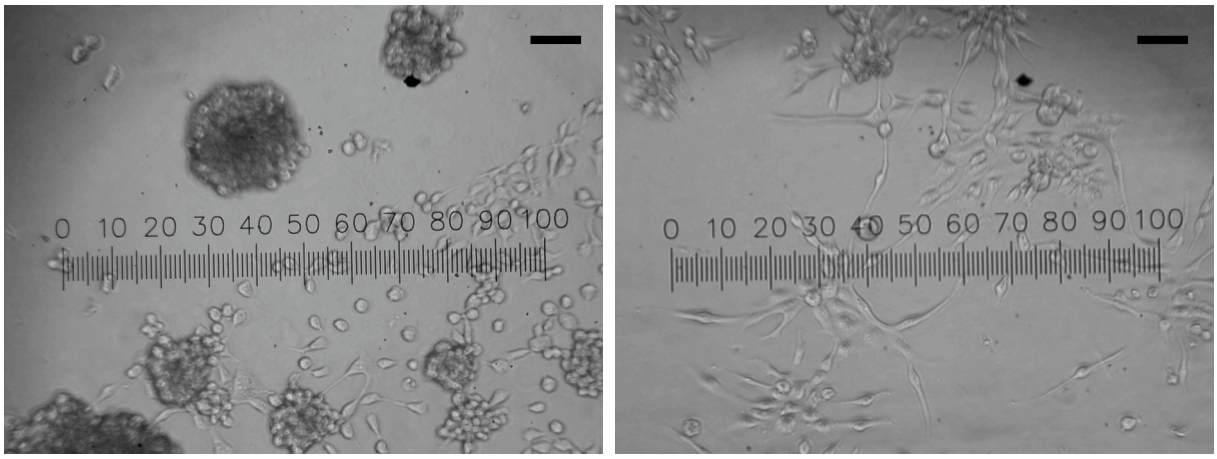


Figure 6 – D17 cells in tumoursphere culture demonstrating attachment and proliferation despite low-adherence substrate. Bar = 100µm

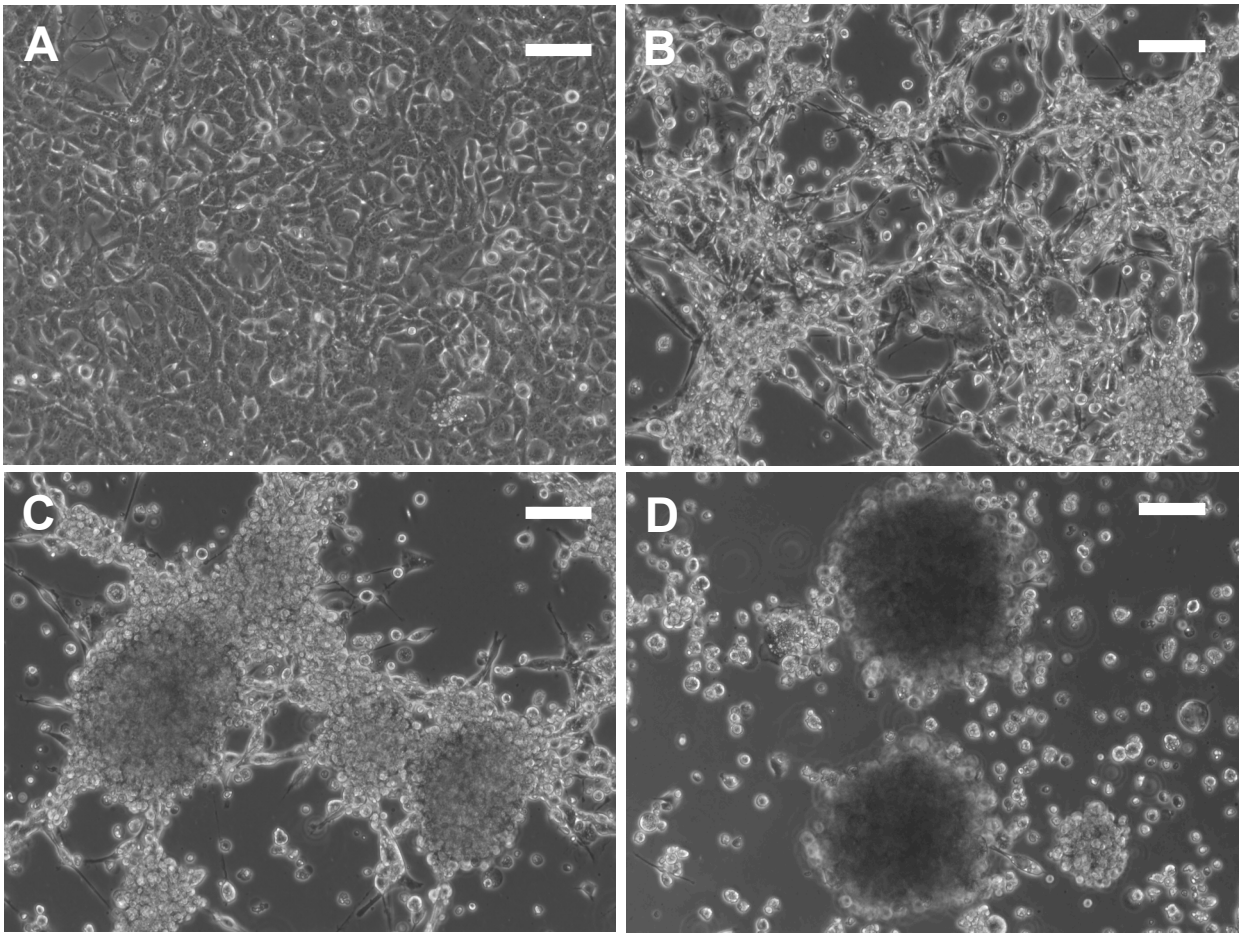


Figure 7 – (A) Standard adherent and (B-D) D17 AdhGF cells. B, C – 8 d.i.v.; D – 11 d.i.v. Note altered morphology and tendency for intercellular adhesion, overcoming that for attachment to substrate such that cells gradually become suspended as floating spheroids – i.e. D17 cells can form tumourspheres in the presence of serum.. Bar = 100µm.

Preliminary studies were conducted to assess the growth of SB haemangiosarcoma and CML10 melanoma cells in alternative serum-free media, or with additional growth factor supplementation. Tumoursphere formation in N2/MC medium was compared with that containing an alternative serum-replacement supplement, B27, and also medium containing both supplements (N2-B27/MC). Although B27 contains slightly lower levels of some N2 constituents - the precise concentrations are not disclosed by the manufacturers - it also contains additional factors not present in N2, and has been used by a number of experimenters in sphere culture (Dontu *et*

al., 2003; Hemmati *et al.*, 2003; Chaichana *et al.*, 2006; Pellegatta *et al.*, 2006; Sansone *et al.*, 2007). Growth of both SB and CML10 was poor in medium supplemented only with B27; spheres formed were fewer, smaller and less compact than in other media, and the cells within these loose clusters appeared granular and poorly viable. Tumourspheres produced by CML10 cells were largest and most numerous in the growth medium containing both supplements (N2-B27/MC). For SB cells, proliferation as tumourspheres in N2-B27/MC was equivalent to that in N2/MC (Figure 8 A-F).

Expression of receptors for vascular endothelial growth factor (VEGF) is reported for both haemangiosarcoma (and specifically SB cells (Akhtar *et al.*, 2004)) and malignant melanoma (Liu *et al.*, 1995). The addition of VEGF at increasing concentrations (5nM, 10nM, 15nM) enhanced growth of SB cells leading to the formation of larger and more numerous tumourspheres, particularly when combined with N2-B27 supplementation. There was a less marked enhancement in the formation of tumourspheres by CML10 cells (Figure 8 G-L). These results suggest that the observation of "tumoursphere formation" by a given cell line is influenced by the selected combination of culture medium, substrate and supplemental growth factors.

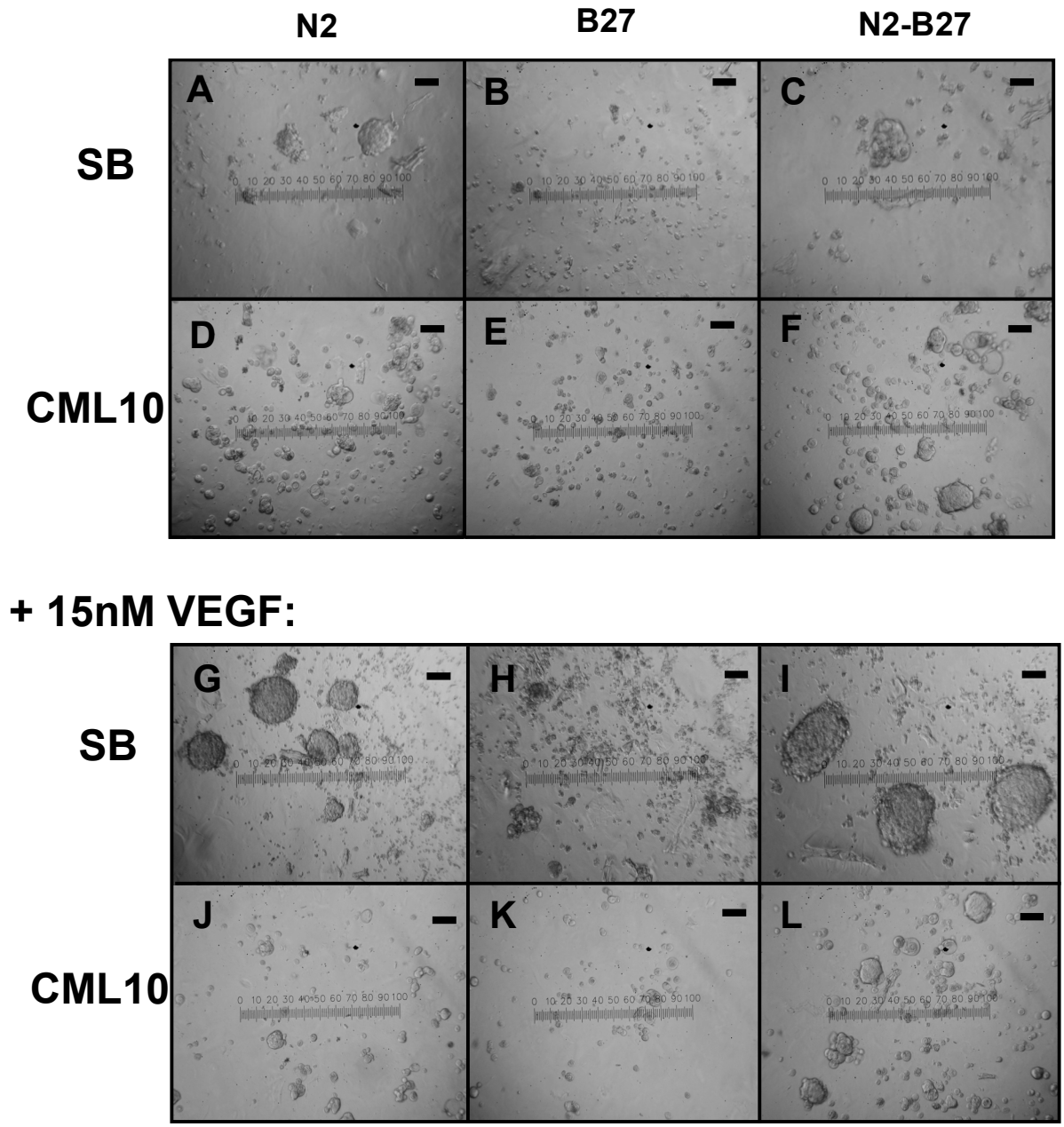


Figure 8 – Tumoursphere formation by SB haemangiosarcoma and CML10 melanoma in response to additional supplementation.
(Upper) – Proliferation in medium containing N2, B27 or a combination of both serum-replacement supplements.
(*Lower*) – Proliferation in alternative media with additional growth factor supplementation (15nM VEGF).
Bar = 100μm

Microscopic Observations and Clonal and Population Analyses suggest that Growth of Canine Cancer Cells as Spheroids results from Cellular Aggregation and not Autonomous Self-Renewal

According to the principle of the neurosphere assay, enrichment of cancer stem and progenitor cells occurs through selection of cells capable of first surviving in serum-free conditions, and their subsequent self-renewal to create clonal spheres of similar cells. If the formation of tumourspheres by cancer cells in low-density conditions represents cell-autonomous self-renewal of individual cells, it should be possible to quantify the proportion of replication-competent cells on a single cell level.

Isolated D17 cells do not form tumourspheres

Clonal analysis was performed in order to assess the ability of individual tumour cells to form tumourspheres. D17 tumourspheres at fifth passage were dissociated and plated into 96-well plates at 30, 3, 1 and 0.3 cells per well. Although small clusters of cells formed in some wells, no tumourspheres developed in any well even at 30 cells/well, and by 9 d.i.v. most cells were shrunken and showed no signs of replication. This was in sharp contrast to the cluster formation seen at 24-48 hours after plating, and the development of large tumourspheres by 7 d.i.v., under standard conditions.

The limiting dilution assay was repeated at densities of 200, 100 and 50 cells per well. Both D17 adherent cells and dissociated tumourspheres (passage 8) were assessed for sphere-forming capacity, in case tumoursphere passage had enriched a population of clonogenic cells. However, for both sphere and adherent cells, sphere formation at low densities was very poor - even at 200 cells per well, any spheroids were small (no more than 2-3 cells in diameter) and not comparable with those seen under bulk culture conditions (Figure 9).

For adherent cells, 13/32 wells showed evidence of proliferation at 200 cells/well (40% positive wells). Tumoursphere cells performed better at this density, with

25/32 wells (78%) showing a cell cluster – this may support the concept of enrichment of a putative tumoursphere-forming CSC population by successive passage, although the effect might also be influenced by incomplete tumoursphere dissociation and the persistence of cellular aggregates, or adaptation of cells to tumoursphere culture conditions during successive passage. However, cluster formation was reduced to 3/32 (9%) at 100 cells/well and 2/32 (6%) at 50 cells/well (Figure 10). This is strongly suggestive of a density-dependent effect, as otherwise it would be expected that there would be a linear relationship between number of cells per well and wells containing spheres, from which could be calculated the minimum number of cells required per well to yield at least one sphere (Singh *et al.*, 2003).

In fact, there appears to be an initial plateau at low cell densities, where very few cells will replicate, followed by an increasing tendency to form spheres as cell density increases (Figure 10). SB cells produced no spheres in any well at either 50, 100 or 200 cells per well upon limiting dilution, even with additional growth factor supplementation (VEGF 10ng/ml).

Most limiting dilution assays were carried out using N2 medium *without* methylcellulose, to permit more accurate manipulation of small fluid volumes. Notably, when D17 cells were plated in methylcellulose-containing medium at 50, 100 and 200 cells per well, no cell replication was seen and no tumourspheres formed, at even the highest density. This observation supports the concept that tumoursphere formation and expansion by D17 cells is promoted by initial cell-cell contact – within the small wells of a 96-well plate, the viscous methylcellulose has more of an immobilising effect than in a 6-well plate, where cells tend to gravitate towards the edges of wells.

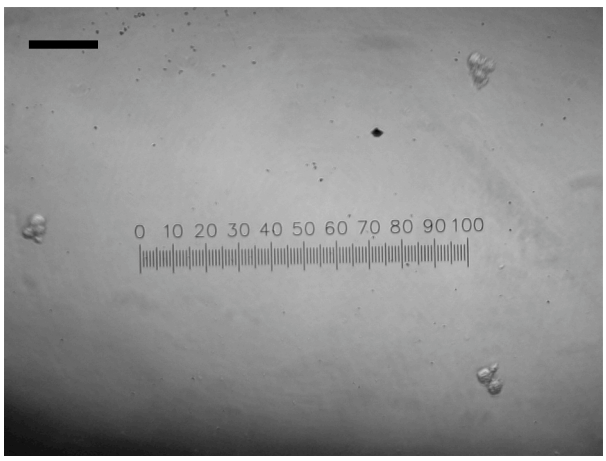


Figure 9 – D17 limiting dilution assay. 200 cells/well in 96 well plate at 7 d.i.v.
No tumourspheres comparable to those in bulk culture were seen in any well.
Bar = 100μm

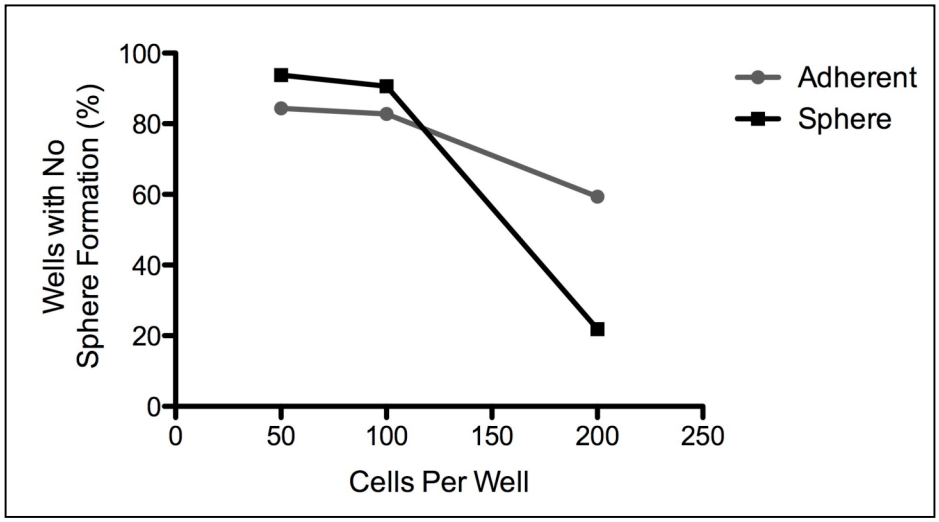


Figure 10 – Non-linear relationship between number of cells plated per well of a 96-well plate and tumoursphere formation, for D17 adherent and tumoursphere cells. *y* axis represents the proportion of wells in which no spheres form, for a given number of cells *per* well (*x* axis). For cell-autonomous and density-independent tumoursphere formation, a linear relationship is expected. This allows linear regression lines to be plotted, with the *x* axis intercept indicating the number of cells/well required to ensure at least one sphere in each well.
Wells were scored after 14 days *in vitro;* any tumourspheres formed were much smaller than those seen in bulk culture after a similar incubation period.

Single REM134 cells do not form spheres comparable to those in bulk cultures, and serial tumoursphere passage does not enrich for sphere formation

REM134 cells, which like D17 readily formed and expanded as tumourspheres under standard conditions, also failed to show comparable proliferative ability in a single cell assay. In order to test the hypothesis that successive passage of spheres would enrich for a cancer stem cell population, dissociated adherent and tumoursphere (passage 6) cells were plated in low-adherence 96-well plates at 1 cell per well. Wells were scored after 24 hours for the presence of single cells, and sphere formation assessed at days 8 and 16 of incubation.

Of 44 single adherent cells, only 4 (9.09%) showed any sign of replication. Of 52 single sphere cells, fewer proliferated, with only 3 spheres (5.77%) at 8 d.i.v. and 2 (3.85%) at 16 d.i.v., one of the spheres scored at the earlier assessment having broken down (Figure 11). Thus, REM134 adherent cells form tumourspheres at the single-cell level, at a frequency which might be expected for a putative CSC

population, but serial passage as tumourspheres does not enrich for sphere-forming capacity. Again, the tumourspheres formed were smaller than the large compact bodies formed in the standard 6-well assay (proposed to originate from single tumour cells) (Figure 12). These results indicate discrepancies between tumoursphere-forming efficiency at the level of single cells *versus* bulk culture, suggesting that cell density exerts a profound effect.

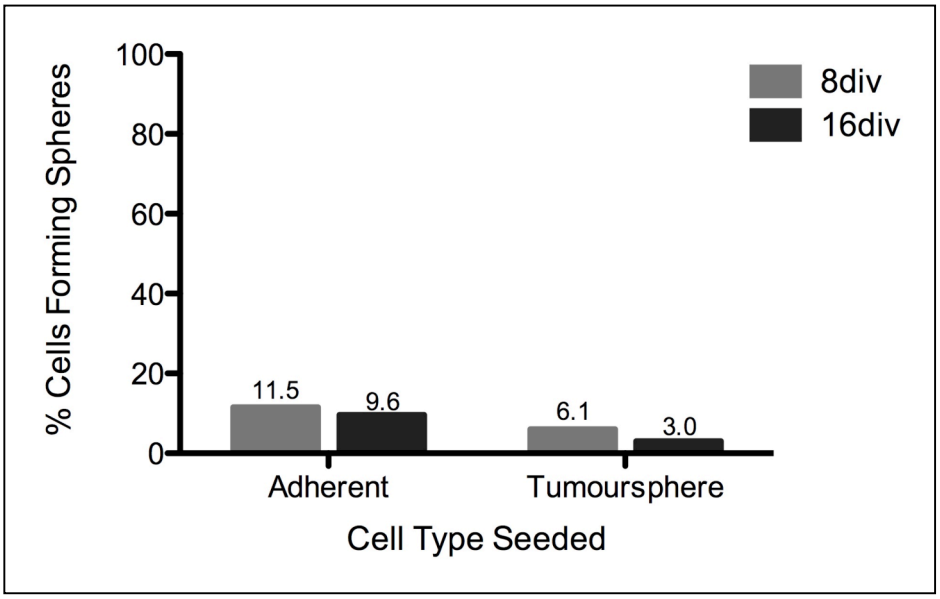


Figure 11 – Tumoursphere formation at the single-cell level by REM134 adherent and tumoursphere cells. Wells confirmed as containing a single cell after 1 day *in vitro*, and scored for tumoursphere formation at 8 and 16 d.i.v..

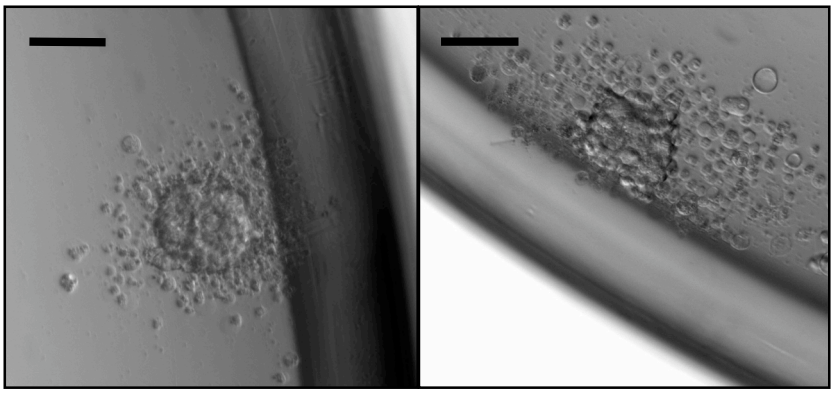


Figure 12 - REM134 tumoursphere formation in single cell assay at 16 d.i.v.
Left – Derived from single adherent cell; *Right* – Derived from single sphere cell
Bar = 100µm

Sphere formation in bulk tumoursphere cultures is density dependent

The influence of cell density on tumoursphere-forming efficiency was further investigated at the population level. D17 and SB haemangiosarcoma cells were plated in the 6-well format, at standard density (6 x 10^4 cells /well / 2ml) and serially diluted cell concentrations. If replication as spheres is a cell-autonomous process, the *proportion* of cells proliferating as spheres is hypothetically the same, and so overall population doubling should show a constant relationship to initial inoculums.

(Population Doubling = 3.32 x [log(cells yielded) – log(cells seeded)])

As seen in Figure 13, this was not the case, this non-linear relationship again indicating a density-dependent effect on cell proliferation. For D17, fewer than 3250-5000 cells per well in tumoursphere culture led to a negative population doubling – that is, overall cell numbers declined – whereas above this density, an overall expansion was seen. For SB, the cutoff was higher at approximately 19000 cells per well.

Population analysis over serial tumoursphere passage at standard density (60000 cells per well) suggested an exponential population expansion, with equivalent proliferation capacity at each subculture. Population doublings were calculated for each passage, and the theoretical total cell yield over time (if all cells had been placed back into culture) determined (Figure 14)**.** Whilst it might be expected that gradual enrichment of a (putatively more slowly-dividing) stem cell-like subpopulation might lead to alterations in growth kinetics, the results suggested that the cells within tumourspheres not only underwent exponential growth over time producing a large number of progeny, but also that the rate of proliferation was reasonably constant.

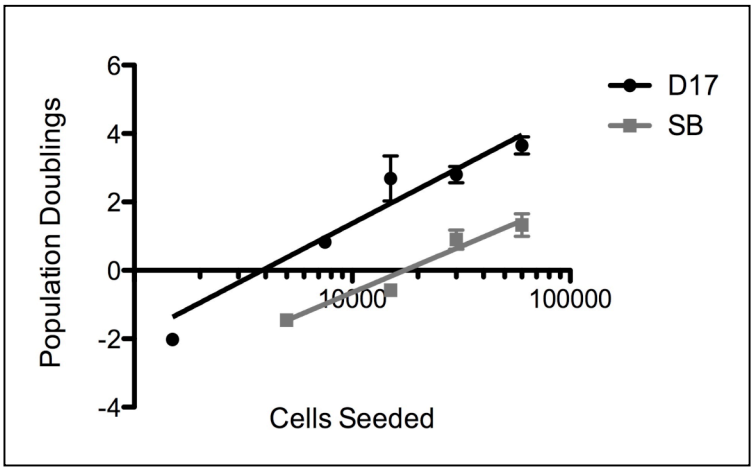


Figure 13 – Density-dependent growth of D17 and SB cells in tumoursphere conditions. Graph shows Log(Cells/Well) *vs* Population Doublings. *x*-axis intercept indicates minimum number of cells required *per* well of a 6-well plate to produce an overall population expansion..

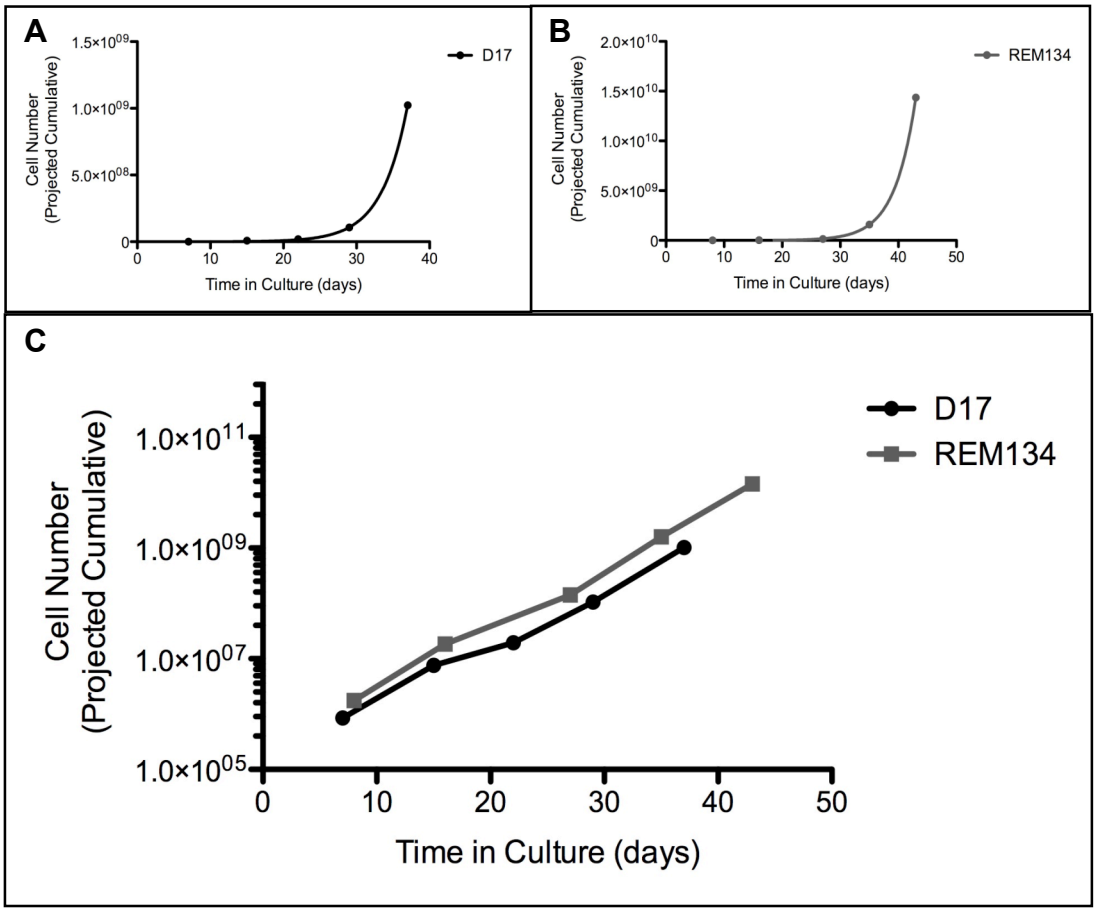


Figure 14 – Exponential growth of D17 and REM134 cells as tumourspheres.
A and B show exponential growth curves for D17 and REM134 cells, respectively, based on estimated cumulative cell number (as if all cells had been cultured at each passage). Population doubling time calculated from each curve = 2.5 days.
C - Plotting Time *vs* Log(Cells) gives a similar straight line for each cell line.

In addition, the degree of expansion suggested that most plated cells were capable of replicating to contribute towards total cell number, at both early and later passages. This was supported by the microscopic observation that for both D17 and REM134, most cells appeared to participate in cluster formation soon after seeding (Figure 15).

Together, these results suggest that sphere formation in canine cancer cell lines, rather than representing the autonomous self-renewal of individual stem-like cells, is the result of cellular aggregation and proliferation at permissive cell densities.

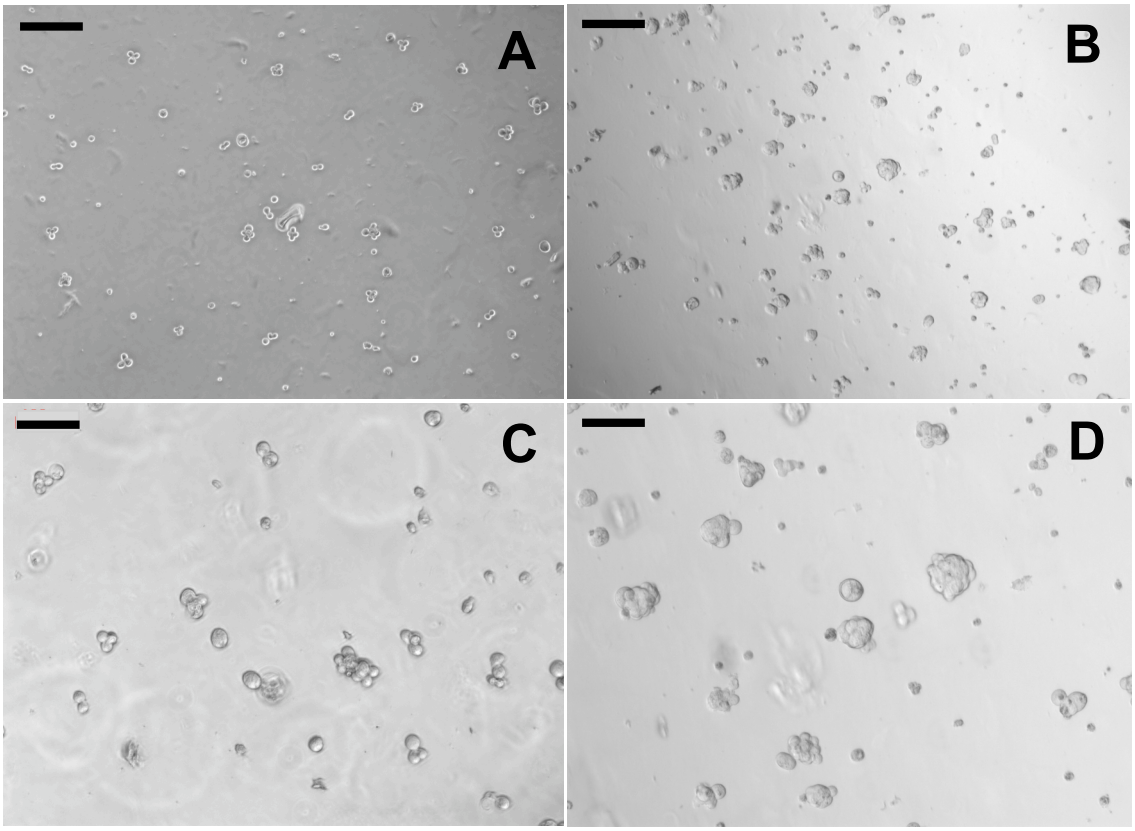


Figure 15 – Cells proliferating as tumourspheres are not rare in bulk culture.

A – D17 cells at 2 d.i.v (phase contrast).
B – REM134 cells at 4 d.i.v. (brightfield).
C – D17 cells at 2 d.i.v. (brightfield).
D – REM134 cells at 4 d.i.v. (brightfield).

All cells seeded at 60000/well. A, B - Bar = 200µm. C, D - Bar = 100µm.

Expression Patterns do not suggest that Spheres formed by Canine Cancer Cell Lines Represent a Specific Stem Cell-Like Population

Flow cytometry

D17 and REM134 tumourspheres were dissociated and analysed by flow cytometry. The FSC *vs* SSC profile of sphere cells was less defined than that of the adherent cells (Figure 16). There was a greater range of cell size (large cells containing pronounced vacuoles were often visible at light microscopy in sphere cultures) and complexity, in part due to the persistence of cellular aggregates despite the suspension having been passed through a 40μm cell strainer prior to analysis. There was more debris and non-viable material present in the sphere cell suspension, probably reflecting not only apoptosis in the cultures, but also the longer period of enzymatic exposure and mechanical trituration required to dissociate the spheres as compared with adherent cultures. Sphere cells showed a greater tendency towards autofluorescence than did adherent cells, necessitating stringent analysis to distinguish antibody binding from artefact.

D17 spheres did not demonstrate significant upregulation of the stem cell-associated markers CD34, CD133 or CD117. Both sphere and adherent cultures were CD34-ve. Whilst the frequency of $CD133^+$ tumoursphere cells (0.59%) was greater than that of adherent cells (0.065%), this was equivalent to the level of binding for the isotype control antibody (0.58%) (Figure 17). Similarly, although 0.2% of tumoursphere cells were positive for CD117, compared to 0.14% of adherent cells, the isotype control antibody stained 0.24% of cells. This demonstrates a tendency of tumoursphere cells to non-specific binding, most likely exaggerated in the presence of cellular aggregates, debris and early apoptosis.

REM134 tumoursphere cells were assessed for expression of the putative mammary CSC marker CD44. The proportion of $CD44^+$ tumoursphere cells (71%) was lower than that of the adherent cells (98%), i.e. *reduced* expression of a stem cell-associated marker on tumoursphere-forming cells for this cell line (Figure 18).

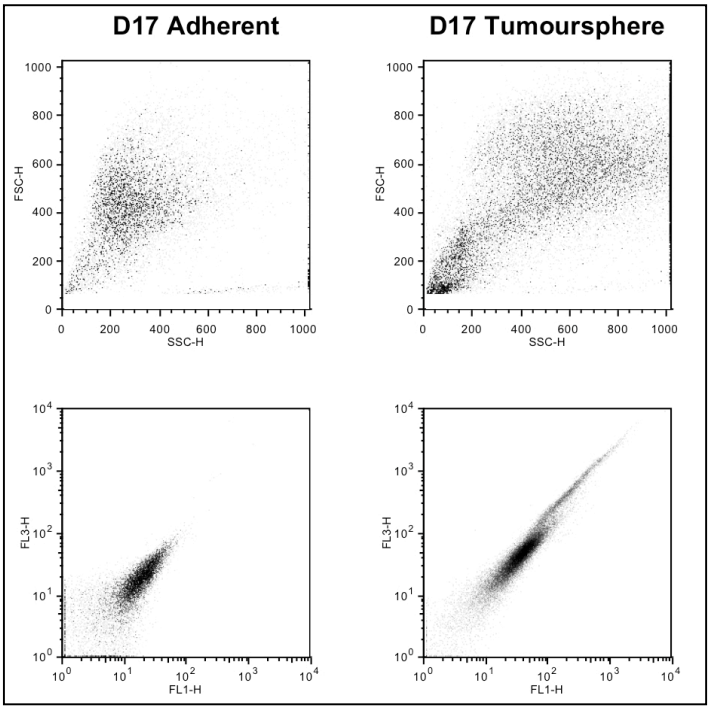


Figure 16 – Tumourspheres show greater variation in size and complexity than adherent cells, as demonstrated by wider distribution on Forward *vs* Side Scatter profile (*Upper plots)*.
Tumoursphere cells show greater inherent fluorescence (autofluorescence) than adherent cells, as demonstrated on bivariate fluorescence plot of unstained cells (*Lower plots*).
Acquisition performed using same instrument settings for both cell types.

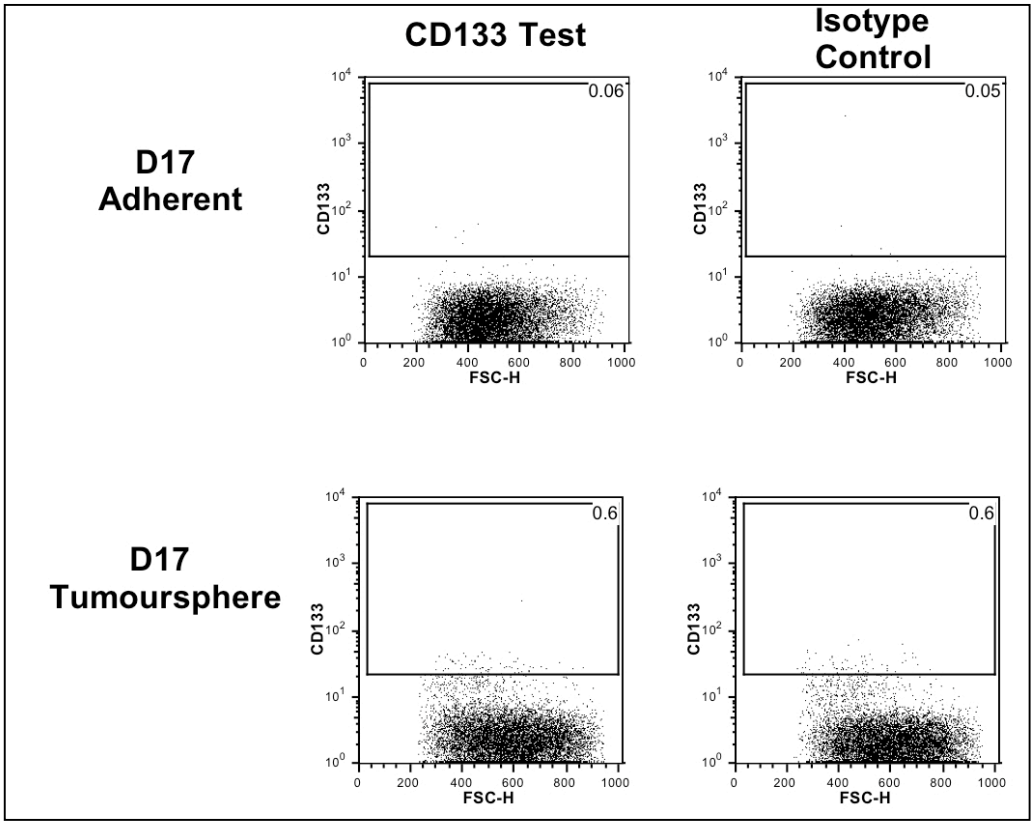


Figure 17 – Representative plots showing assessment of surface staining on tumoursphere cells. D17 tumourspheres (*lower plots*) do not contain an increased frequency of cells positive for the CSC-associated marker CD133, as determined by comparison with isotype-matched control antibody, than D17 adherent cells (*upper plots*).

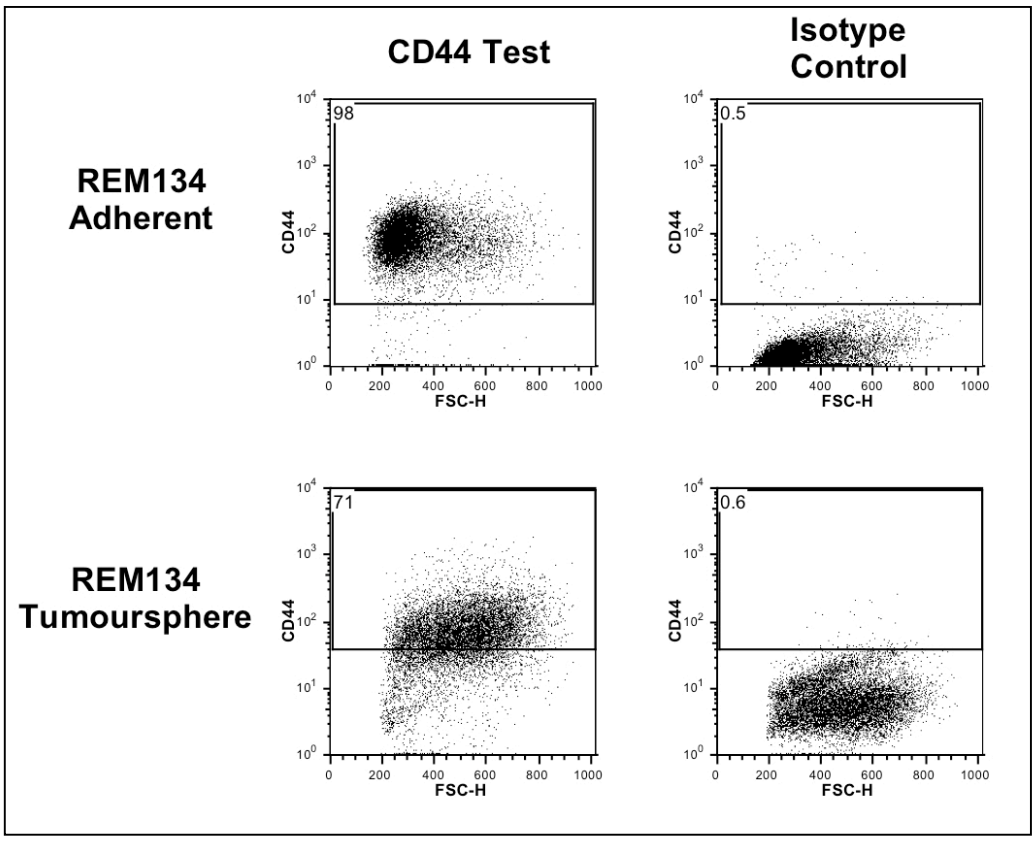


Figure 18 – REM134 tumoursphere cells (*lower plots*) show a reduced frequency of $CD44^{+}$ cells, as determined by comparison with isotype-matched controls, than REM134 adherent cells (*upper plots*). Acquisition performed using same instrument settings for both cell types.

Western Blot

Lysates of D17 adherent, and adherent-GF and tumoursphere cells were examined using Western blotting for expression of Oct4, Nanog and c-Kit. The expected product size for each protein was 43kDa, 39kDa and 120-155kD respectively. Non-specific bands occurred frequently and were generally larger than the expected product size. No candidate protein products for Nanog or c-Kit were seen for any of the populations despite multiple attempts to optimise antibody concentrations (data not shown). When probed for Oct4, a protein product of approximately the predicted size could be detected for all three cell types, although this was not the major band. In comparison to the β-actin loading control, D17 Adh-GF cells or tumourspheres did not appear to show significant upregulation of this product in comparison with

adherent cells (Figure 19). A single band for Oct4, of the predicted size, was detected for adherent bone marrow-derived stromal cells at initial derivation, although not when lysates of passaged cells were tested (Figure 19).

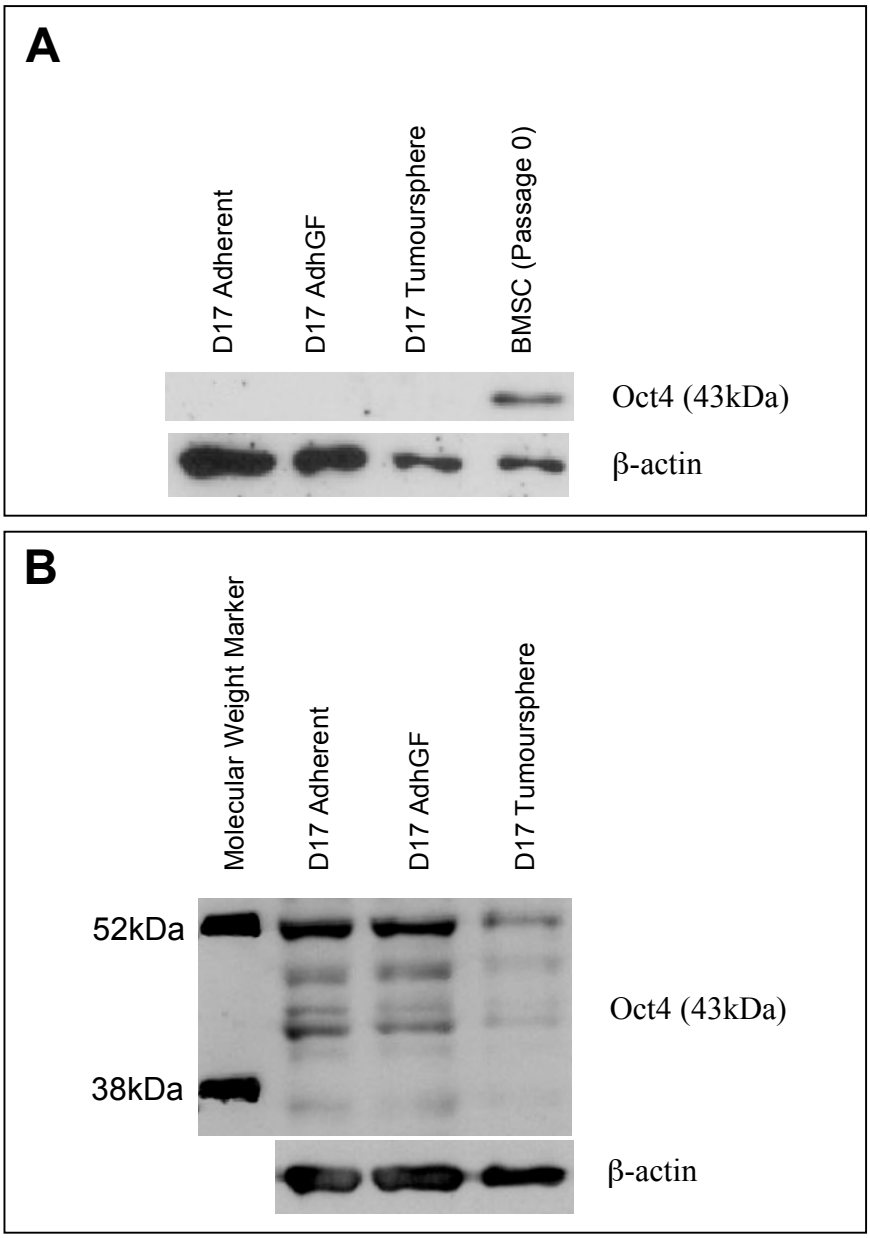


Figure 19 – Western blot analysis for expression of Oct4.
A - Bone marrow-derived stromal cells prior to subculture demonstrate expression; no candidate band seen for D17 adherent, AdhGF or tumoursphere cells at this concentration of primary antibody.
B – Where bands of appropriate size are detected, these are not the major protein band. There is no evidence of increased expression of Oct4 by D17 AdhGF or tumoursphere cells, in comparison to D17 adherent cells.

This data suggests that D17 osteosarcoma cells cultured as tumourspheres do not show increased expression of the stem cell-associated markers Oct4, Nanog or c-Kit at a protein level, nor do the growth factors EGF and bFGF lead to their direct upregulation.

Immunofluorescence

Cytospin preparations of REM134 and D17 tumourspheres were examined using immunofluorescence microscopy for the expression of c-Kit, Oct4 and Nanog. GAPDH and β-actin were employed as positive controls and DAPI as a nuclear counterstain. A methanol/acetone protocol for fixation-permeabilisation was found to be superior to paraformaldehyde/Triton X-100 – penetration of spheroids by antibodies was more homogenous, and background fluorescence reduced. As with flow cytometry, tumourspheres showed increased autofluorescence, particularly if fixation was not followed immediately by antibody staining (Figure 20). Multiple titrations were performed to optimise fixation / permeabilisation and staining protocols.

Bone marrow-derived stromal cells were cultured as mesenchymal stem cells to act as positive control populations expressing Oct4, Nanog and cKit. However, whilst occasional strongly positive-staining cells were detected, corresponding phase contrast images suggested that these were in fact dead or apoptotic cells becoming detached from the monolayer (Figure 21).

Both D17 and REM134 tumourspheres showed positive immunofluorescence with Oct4, comparable to that demonstrated by Wilson *et al.* for D17 spheres (Figure 22). However, examination at higher magnifications showed that staining was confined to the cytoplasm, a defined unstained area in each cell corresponding with the position of the nucleus (Figure 23). Oct4 acts as a nuclear transcription factor, and the cytoplasmic localisation of the protein in the D17 and REM134 tumourspheres therefore does not support its role in maintaining pluripotency and self-renewal capacity within this culture system.

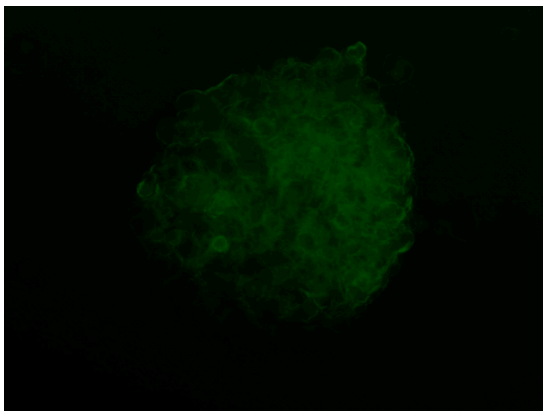


Figure 20 –
D17 sphere demonstrating autofluorescence, having been neither fixed nor stained.
Fluorescent signal was also detectable through DAPI and rhodamine channels.

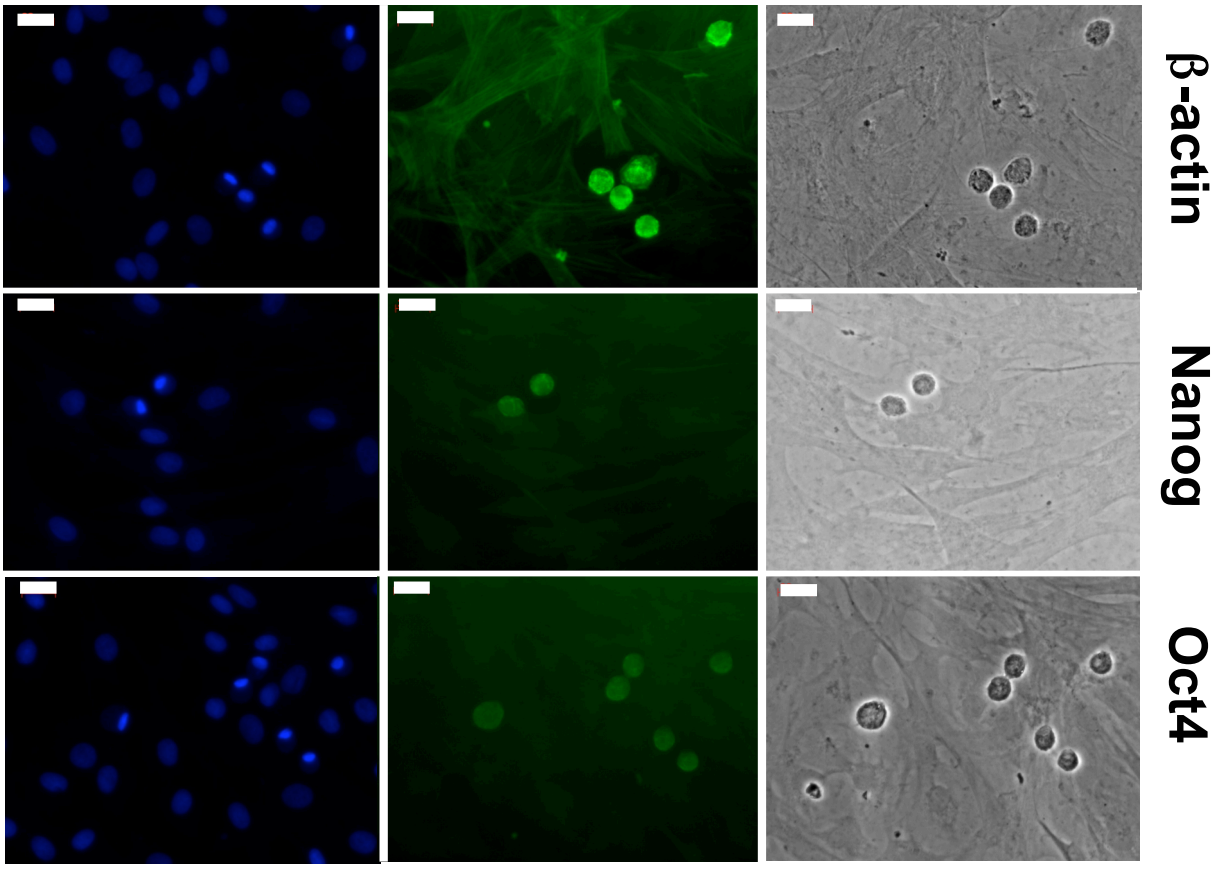


Figure 21 – Immunofluorescent staining of Bone Marrow-derived Stromal Cells demonstrating that apparent positivity (FITC fluorescence, centre panels) for Nanog and Oct4 is the result of non-specific antibody binding to dead or dying cells.
From top - β-actin, Nanog, Oct4.
Note that cells with increased FITC fluorescence in each of the central panels correspond with cells showing nuclear condensation (left panels, DAPI) which are detaching from the monolayer (right panels, phase contrast).
Bar = 20µm.

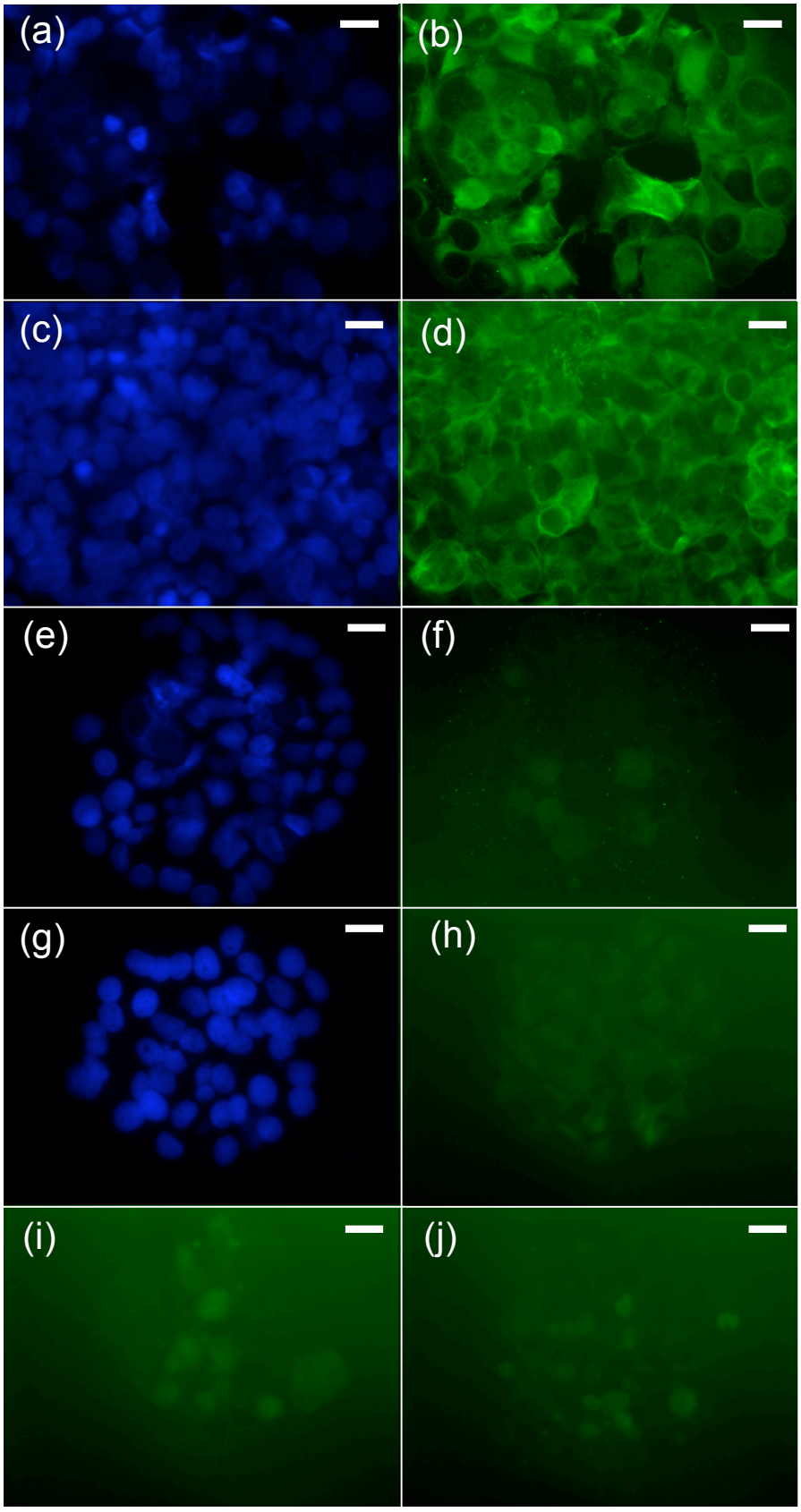


Figure 22 – D17 spheres show positive staining for Oct4. c-Kit and Nanog do not bind above background levels.

(a), (b) – GAPDH
(c), (d) – Oct4
(e), (f) – c-Kit
(g). (h) – Nanog

Left panels show DAPI nuclear counterstain.

(i) – Secondary antibody only negative control
(j) – No antibody autofluorescence negative control

Images have been captured using equal exposures for each fluorochrome. There has been minimal to no postacquisition processing of the raw linear data.

Bar = 20μm

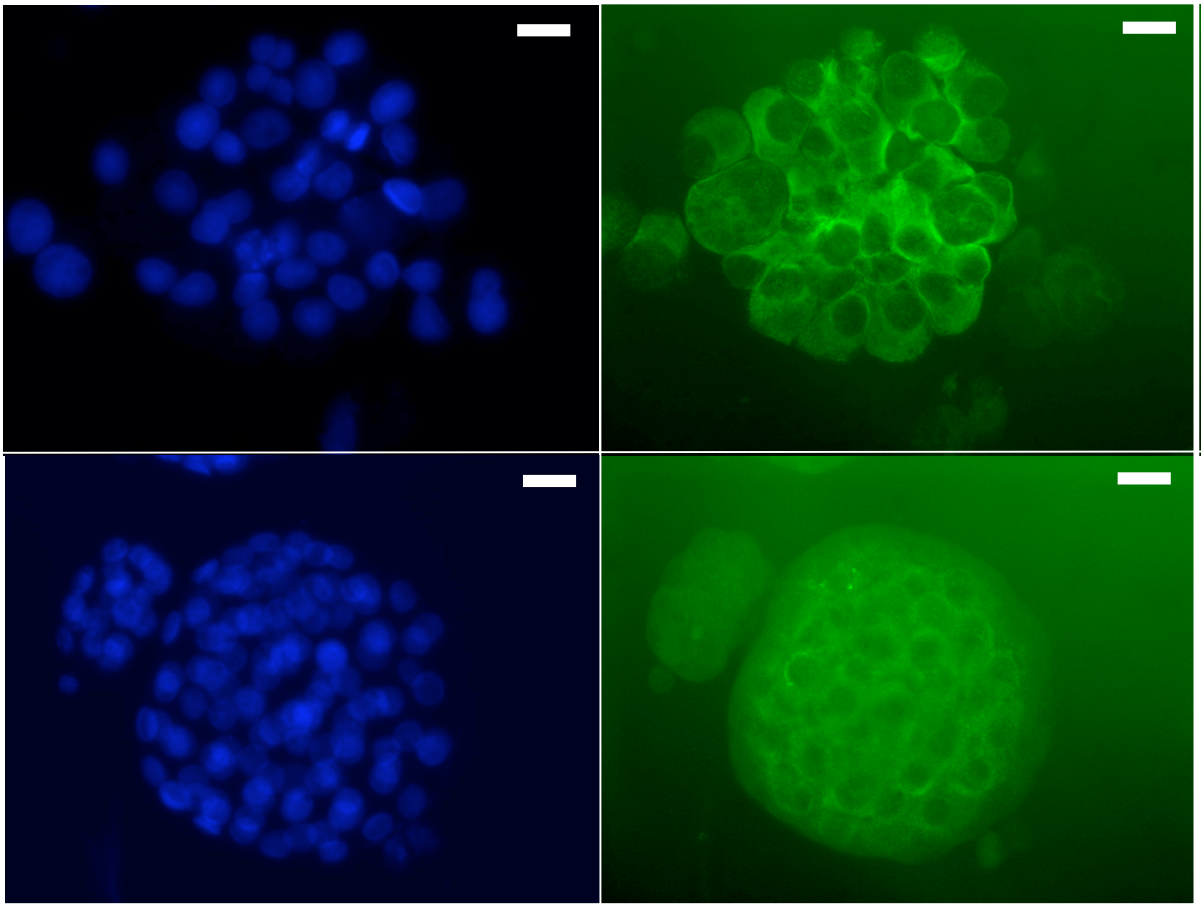


Figure 23 – Immunofluorescent staining proposed to represent expression of pluripotency-associated transcription factors is inappropriately localised in D17 and REM134 tumourspheres.
Upper Panels – DAPI nuclear counterstain (left) and Oct4-FITC staining (right) of D17 tumoursphere. Positive signal is distinctly localised to the cytoplasm, with no nuclear staining.
Lower Panels – DAPI nuclear counterstain (left) and Nanog-FITC staining of REM134 tumoursphere. Positive signal is cytoplasmic, with a peri- but not intranuclear pattern. The FITC image was taken with a long (6 second) exposure, and processed to reduce background and enhance the weak fluorescence for the purposes of this figure.
Bar = 20µm

Although weak staining for Nanog and c-Kit could be visualised in both REM134 and D17 tumourspheres, this was similar to that seen in negative control samples. It was possible, using long exposures and post-acquisition image manipulation, to enhance the fluorescent signal, but this was accompanied by increased background staining, and could be achieved to a similar degree with negative control samples. This indicates nonspecific binding and autofluorescence, rather than specific positive staining (Figure 24, 25). Notably, where Nanog staining was exaggerated in this manner, fluorescence was cytoplasmic/perinuclear, so once again inappropriately localised for a transcription factor (Figure 23).

These results suggest that D17 and REM134 canine tumourspheres do not express the embryonic stem cell-associated nuclear transcription factors Oct4 or Nanog, or the cell surface marker c-Kit (CD117).

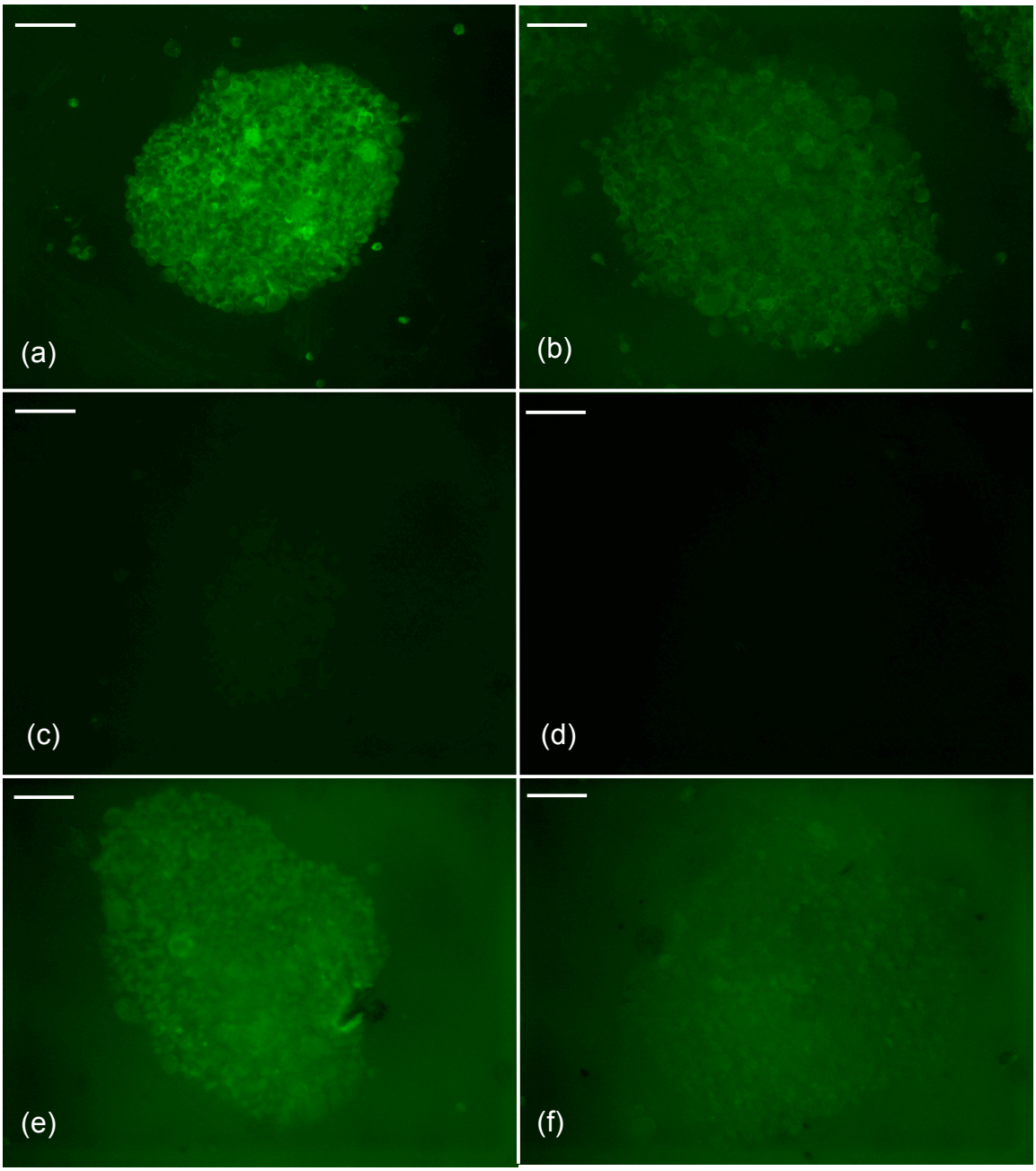


Figure 24 – Autofluorescence and non-specific background signal may contribute to apparent immunofluorescent positivity of D17 spheres if exposure factors are not consistent.
(a) GAPDH [positive control] (b) Oct4 (c) c-Kit and (d) secondary-only [negative control] were exposed for 2 seconds.
(e) and (f) are long (8 seconds) exposures of c-Kit and negative control samples, respectively, leading to the appearance of weak positive staining in both samples.
Bar = 100µm

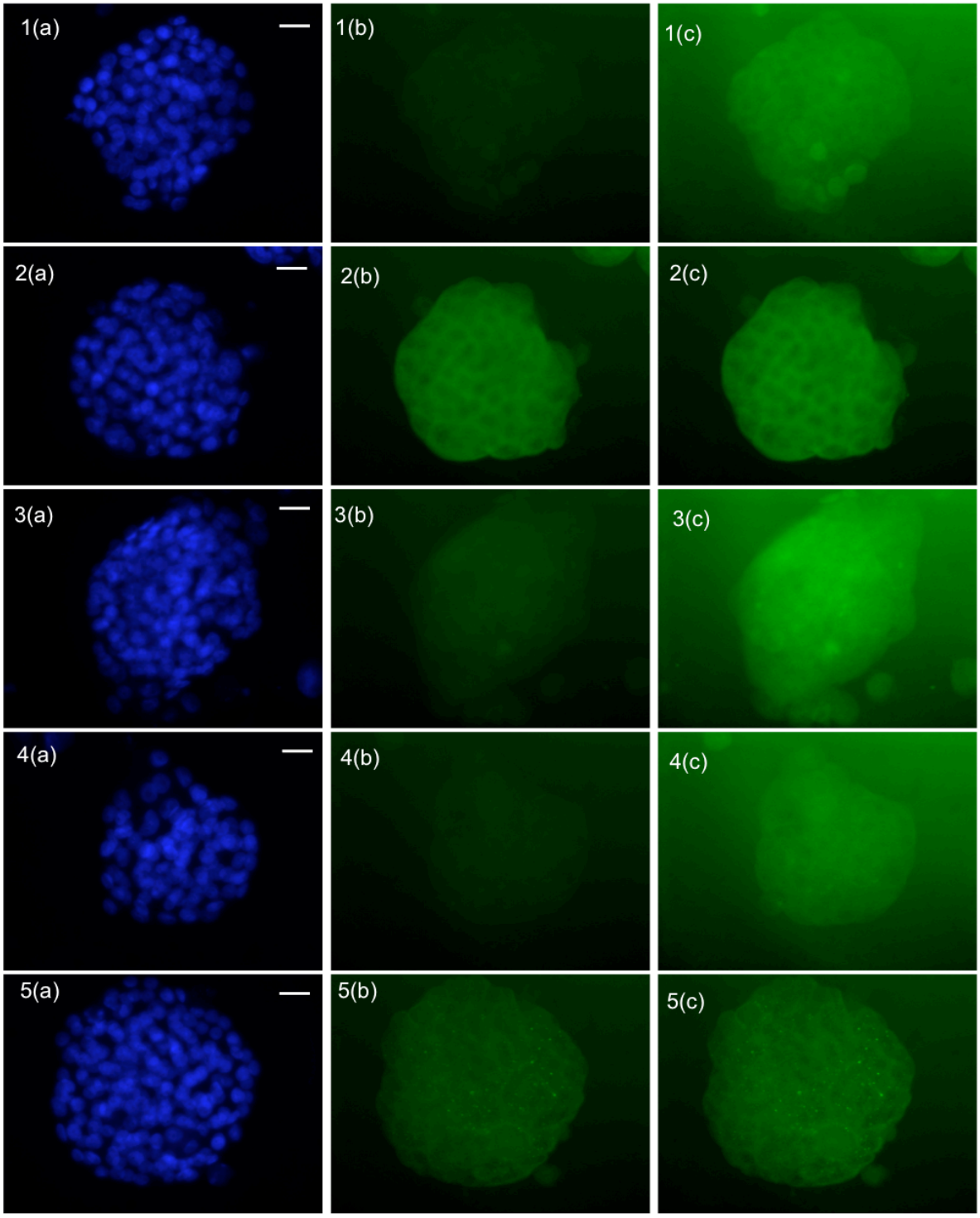


Figure 25 - Immunofluorescence of REM134 tumourspheres (passage 9). Methanol-Acetone fixation / permeabilisation. (a) - DAPI. (b) - FITC, with minimal or no manipulation of raw image. (c) - FITC, image processed using "MinMax" function to enhance weak fluorescence. Note consequent apparent positivity of c-Kit, Nanog and negative secondary-only controls. Bar = 20µm

1 - Secondary only control; 2 - GAPDH positive control; 3 - c-Kit (CD117); 4 - Nanog; 5 - Oct4

Tumourspheres Formed by Canine Cancer Cell Lines Display No Significant Alterations in Sensitivity to Commonly Used Anticancer Drugs

Sensitivity of D17 sphere and adherent cells to conventional chemotherapy drugs was evaluated by an ATP luminescence assay. A cancer stem cell population might be expected to show increased resistance to conventional chemotherapeutic drugs (Diamandis *et al.*, 2007; Todaro *et al.*, 2007; Wright *et al.*, 2008; Zhang *et al.*, 2008a; Zhou *et al.*, 2009a). However, as shown in Figure 26, the drug sensitivity profiles of both cell types were very similar, with no significant differences between the IC_{50} values of D17 sphere and adherent cells for either doxorubicin (adherent 8.66nM, tumoursphere 9.44nM; $p = 0.69$) or mitoxantrone (adherent 63.7nM, tumoursphere 42.2nM; $p = 0.27$). REM134 sphere and adherent cells also showed almost equivalent sensitivity to doxorubicin (adherent 12.6nM, tumoursphere 12.2nM; $p = 0.96$) (Figure 26 C).

Thus, for REM134 and D17 cell lines, culture as tumourspheres in serum-free, growth factor-supplemented conditions does not select for cells with increased resistance to the conventional chemotherapeutic drugs doxorubicin and mitoxantrone.

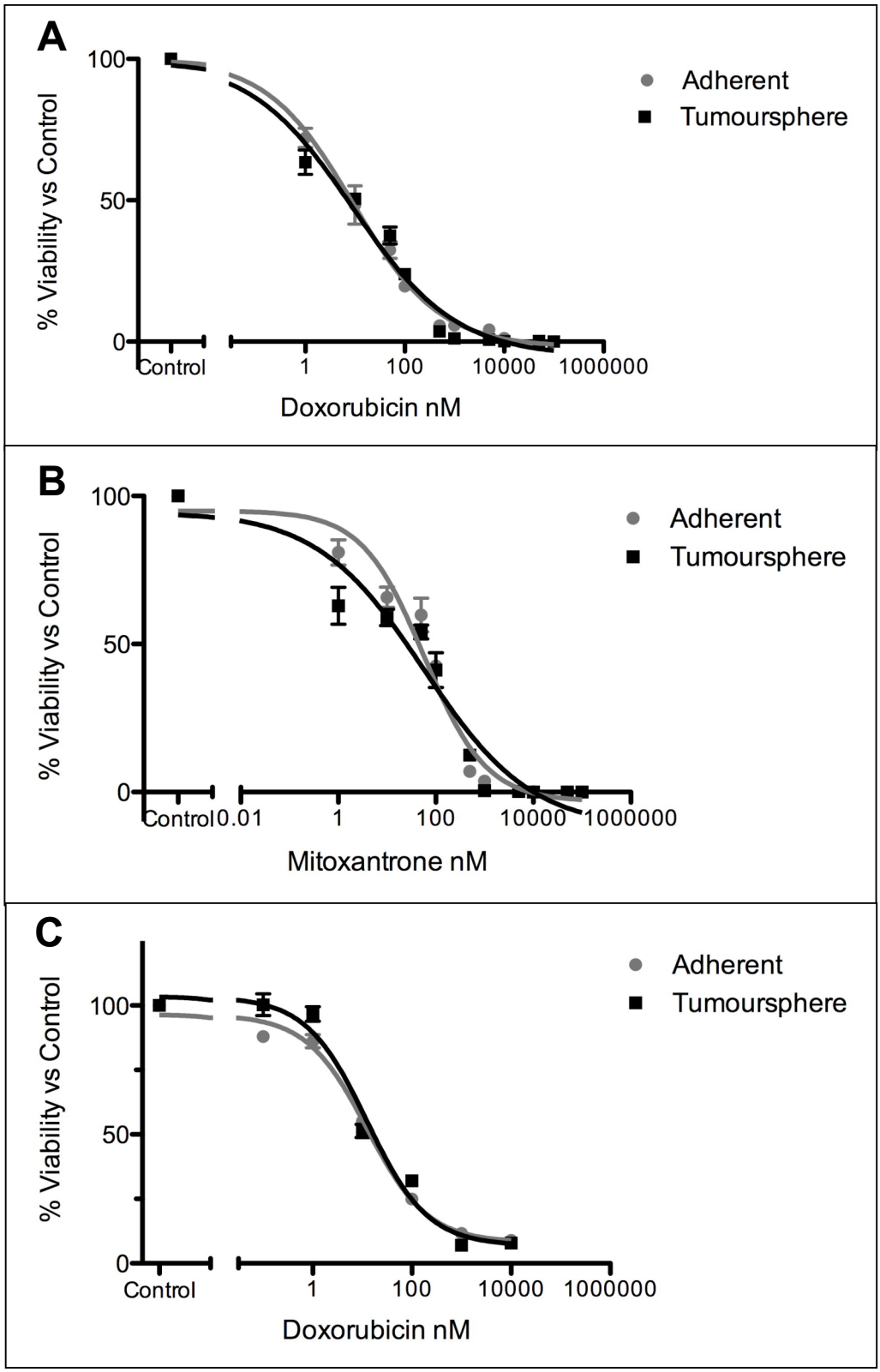


Figure 26 – Viability of D17 adherent and tumoursphere cells in response to increasing concentrations of the conventional cytotoxic drugs doxorubicin (A) and mitoxantrone (B), and REM134 adherent and tumoursphere cells in response to doxorubicin (C).

Distinct Tumoursphere Populations may be Cultured from an Individual Spontaneous Canine Tumour

Cells of kt-osa1, kt-osa4 and kt-osa5, three populations derived from a single spontaneous primary canine osteosarcoma, were cultured in standard tumoursphere conditions after initial expansion in adherent culture. Cells of all three populations formed tumourspheres, although the size and frequency of these varied between the cultures, consistent with the morphological heterogeneity observed both within and between the three cell populations under adherent conditions (Figure 27).

Microscopic observation suggested that a smaller proportion of kt-osa1, kt-osa4 and kt-osa5 cells participated in tumoursphere formation, compared to the more frequent cellular clusters seen when D17 and REM134 were cultured under standard conditions. However, as shown in Figure 28A, fusion of clusters also contributed to the growth of some spheroids. As tumourspheres of kt-osa1 and kt-osa4 populations expanded, a defined pellucid region was observed surrounding the spheroid – the significance of this was not determined, but may indicate secretion by constituent cells, for example of proteinaceous or lipid factors (Figure 28B). Cell numbers yielded at subculture were greatest for kt-osa1 and least for kt-osa4. Tumourspheres could be dissociated and cells seeded back into serum-free conditions for further growth as tumourspheres (Figure 28C).

When tumourspheres were harvested and replated back into conventional, serum-containing conditions, they attached to the substrate with subsequent outgrowth of adherent cells (Figure 29). These showed morphological heterogeneity, with a mixture of elongated fusiform / fibroblastic and more epithelioid cell types, as well as occasional multinucleate and atypical cells, reminiscent of that seen in the parental population. This data suggests that cells derived from primary tumours may be cultured as tumourspheres in low-density, serum-free conditions, and that the cells comprising them may reproduce the morphological heterogeneity of the parental population when placed back into conventional adherent culture.

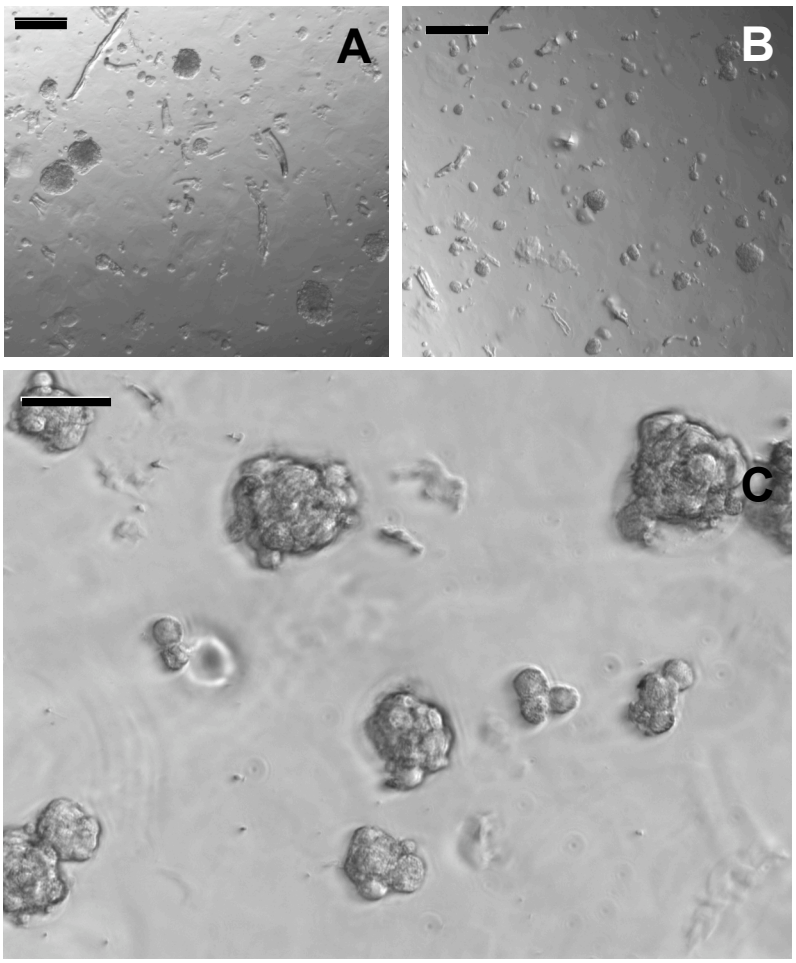


Figure 27 - Tumoursphere formation by kt-osa cells.

A – kt-osa1
B – kt-osa5
Bar = 200µm

C – kt-osa4
Bar = 50µm

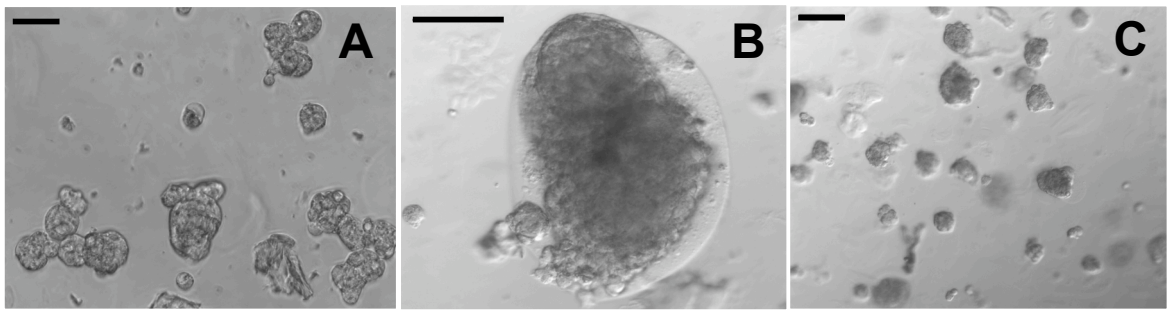


Figure 28 – Tumourspheres formed by kt-osa populations.
A – kt-osa1 tumourspheres showing clustering and coalescence as noted also for continuous cell lines.
B – Pellucid area surrounding a kt-osa1 tumoursphere. This was observed around larger tumourspheres in initial kt-osa1 and kt-osa4 cultures.
C – kt-osa5 tumourspheres at passage 2. kt-osa4 tumoursphere cells failed to proliferate after subculture; kt-osa1 and kt-osa5 tumourspheres were passaged twice prior to growth arrest.
Bar = 100µm.

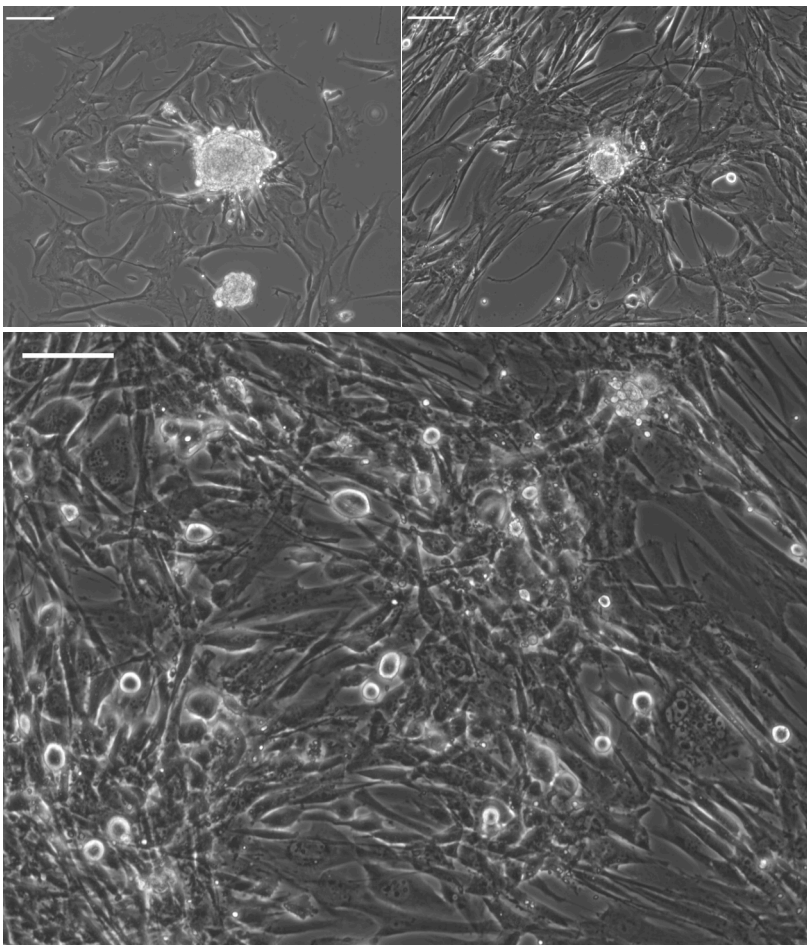


Figure 29 – Transfer of kt-osa4 tumourspheres to conventional serum-containing culture. Outgrowth of adherent cells to form a monolayer with morphological heterogeneity similar to that seen in original adherent culture. Bar = 100μm

Population expansion as tumourspheres, however, was limited for all three populations. Overall cell numbers declined and growth stalled at second (kt-osa4) or third (kt-osa1, kt osa5) passage, precluding further assays or subculture. Further tumoursphere cultures were initiated from each population, at which point kt-osa1, kt-osa4 and kt-osa5 had undergone 11, 12 and 9 subcultures in adherent conditions, respectively. As described in Chapter 3, increased morphological homogeneity and diminution of small cellular subsets within the adherent populations was becoming evident upon repeated passage *in vitro*.

Interestingly, there were some distinctions between these and the tumourspheres cultured sooner after initial derivation of the primary cells. kt-osa4 and kt-osa5 tumourspheres showed greater proliferative capacity, the former producing larger spheroids and the latter more numerous, than kt-osa1 (Figure 30). By contrast with

the tumourspheres cultured soon after derivation of the three populations, at subculture kt-osa5 yielded the greatest cell numbers and kt-osa1 the lowest. No pellucid region was seen surrounding kt-osa1 or kt-osa4 tumourspheres.

Population expansion was again low - overall cell numbers diminished to preclude culture beyond second passage, and no further characterisation of these tumoursphere populations was undertaken. However, these results suggest repeated passage *in vitro* may produce alterations in the nature of derived tumoursphere populations. Thus, information regarding putative CSC subsets within a given tumour may be more representative if obtained through direct tumoursphere culture of primary cells.

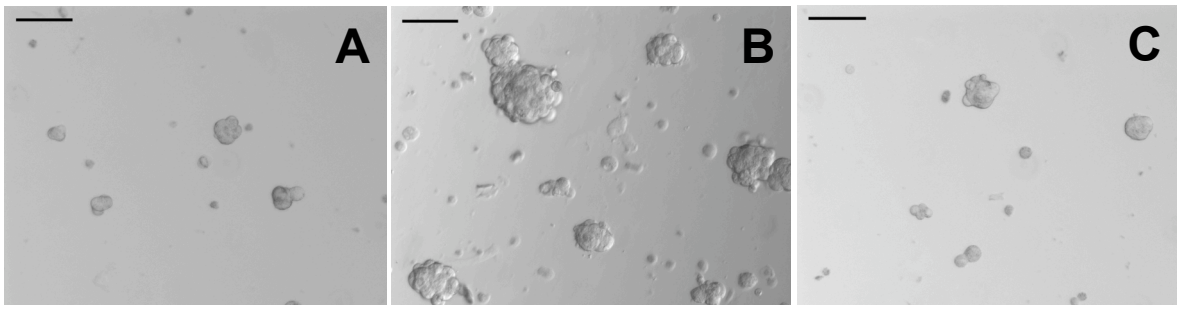


Figure 30 – Tumourspheres derived from kt-osa cells passaged 9-12 times in adherent culture conditions.
A – kt-osa1 (4 days in vitro); B – kt-osa4 (5 d.i.v.); C – kt-osa5 (4 d.i.v.).
kt-osa1 tumourspheres derived from later passage adherent cells failed to proliferate after subculture; kt-osa4 and kt-osa5 grew for a further passage prior to growth arrest.
Bar = 100µm.

DISCUSSION

The neurosphere assay has become a standard protocol in the isolation and expansion of normal neural stem cells. The principle that low-adherence, serum-free culture in the presence of mitogenic growth factors promotes the expansion of progenitor populations and inhibits their differentiation has been extended to other tissue types, such as normal breast and prostate, in order to try and isolate normal tissue stem cells. It is now also widely used by those investigating the stem cell theory of cancer in solid tumours.

Evidence to support the assertion that tumourspheres are composed of stem cells includes surface marker expression as demonstrated by flow cytometry or magnetic cell sorting, mRNA expression by RT-PCR, protein expression by Western blotting or immunoprecipitation, and microscopic techniques such as immunofluorescence or immunocytochemistry. Many studies have shown that sphere cells have altered resistance to cytotoxic agents such as chemotherapy drugs and radiation – where enhanced, this would explain the persistence of CSC after treatment, and where reduced, could identify potential CSC-targeting therapies (Todaro *et al.*, 2007; Baumann *et al.*, 2008; Chen *et al.*, 2008; Fillmore and Kuperwasser, 2008; Zhang *et al.*, 2008a).

In order for a selected population to satisfy the CSC hypothesis, cells must demonstrate two fundamental traits – the capacity for unlimited self-renewal, and the potential to recapitulate all lineages seen within the tumour. Whilst serial transplantation assays to demonstrate tumourigenicity in immunocompromised laboratory animals remain the accepted "gold standard" for both of these conditions (although there are numerous reservations regarding this assertion (Hill, 2006; Fillmore and Kuperwasser, 2007; Kelly *et al.*, 2007; Quintana *et al.*, 2008; Shackleton *et al.*, 2009)), *in vitro* data should at least strongly support the existence of these attributes in any putative cancer stem cell population.

The results in this study suggest that tumoursphere formation by the investigated canine cancer cell lines is dependent (at least partially) on cell-cell interaction, rather than autonomous self-renewal capability of individual cells. Supporting this are the observations that a) any proliferation occurring at low densities resulted in fewer and much smaller clusters of cells than at high densities, and b) at high densities, most cells appear to participate in cluster formation, whereas at low densities, few cells proliferate.

A linear relationship between number of cells seeded *vs* number of spheres formed, which should allow assessment of the frequency of sphere-forming cells by limiting dilution assay (Singh *et al.*, 2003), was not apparent for the canine cancer cell lines examined. This contrasts with reports of tumoursphere formation by cells derived from primary brain and colon tumours, where the proportion of sphere-forming cells remains constant when cell density is altered (Singh *et al.*, 2003; Vermeulen and al., 2008; Xu, 2009).

The large tumourspheres seen for all of the canine cancer cell lines in bulk culture were never observed in the 96-well format. According to the principle of the neurosphere technique, tumoursphere size should also be independent of cell density, as each sphere is supposed to arise from a single cell. Secondary spheres, formed after dissociation and replating of a single sphere, are expected to be identical to those of the initial culture (Reynolds and Weiss, 1996; Dontu *et al.*, 2003; Singh *et al.*, 2003; Zheng *et al.*, 2007), allowing assessment of how many clonal cells are present in a "typical" sphere.

The great disparity between proliferation of D17 and REM134 cells in bulk culture and at the single cell level not only precluded this single sphere analysis, but also suggested that mechanisms of cluster formation and growth in the two situations may not be comparable. The formation of small tumourspheres by a minority of REM134 cells in the single cell assay does suggest that individual cells are capable of proliferation in serum-free conditions. This may indicate a stochastic event due to unrelated processes, such as cell cycle status at the time of plating. It could also be

argued that this may represent self-renewal of a defined CSC population, and that the tumourspheres were many times smaller than those in bulk culture because no fusion could occur with other cells.

However, if each sphere cell represents a stem-like cell as a result of self-renewal of a selected population, spheres should arise more frequently from dissociated spheres than from the parental adherent cells (Reynolds and Weiss, 1996; Singh *et al.*, 2003). This was not the case with any of the cell lines examined here – in particular, fewer REM134 tumoursphere cells showed signs of proliferation in the single cell assay than did adherent cells. Thus, successive passage of REM134 cells as tumourspheres does not appear to lead to enrichment of clonal self-renewing cells.

Clonal and population analyses therefore suggest that derivation of tumourspheres from canine cancer cell lines may not be an appropriate model with which to investigate the CSC hypothesis. Similar inconsistencies are seen in reported data for tumourspheres derived from cell lines. Using single cell analysis of "prostaspheres" derived from LNCaP and C4-2B prostate carcinoma cell lines, Bisson *et al* found that only a small minority of sphere cells generated daughter spheres (Bisson and Prowse, 2009). Of the 1000 cells in a typical C4-2B sphere, only 2 gave rise to spheres upon dissociation. Moreover, the number of LNCaP spheres generated reduced with serial passage. The immunofluorescence data in the study shows an LNCaP sphere with one cell positive for ABCG2, and a C4-2B sphere with two or three $CD133^{+}$ cells. Although the corresponding images of adherent cells are negative for these markers, it is difficult to see how this is consistent with the concept of tumourspheres as an enriched population of self-renewing stem cells (Bisson and Prowse, 2009).

In many ways, it is difficult to reconcile the notion of a population of immortalised, genetically identical cells with the concept that sphere culture should enrich for a pre-existing cancer stem cell subset. This is particularly germane when considering commercial cell lines passaged over many generations or those which have been

clonally derived, less likely to represent the heterogeneity seen within the cells of the parental tumour.

However, distinct cellular phenotypes do seem to be maintained within some continuously cultured cancer cell lines. The phenotype of some clonal cells has been reported to "switch" according to environmental factors and stochastic cell-cell interactions, creating distinct subpopulations with contrasting characteristics (Stockholm *et al.*, 2007). For example, the ARPE-19 cell line (a spontaneously transformed model of human retinal pigment epithelium) shows variability in cellular morphology and differentiation, influenced by aspects of culture protocol including plating density and feeding schedule. Whilst all cells grew in either serum-containing or serum-free media, the medium composition significantly altered expression patterns (Luo *et al.*, 2006).

Similarly, some cells of the rat C6 glioma cell line showed inability to form clones in serum-free conditions (Zheng *et al.*, 2007). Whilst this may suggest enhanced cancer stem cell-like properties in the clone-forming cells, in an immortalised line of genetically identical cells this differential may also be due to epigenetic variation, which can represent fluctuating rather than stable change (Chang *et al.*, 2008; Brock *et al.*, 2009). Indeed, tumourigenicity could be demonstrated in *all* of the C6 cells under serum-containing conditions. Thus, whilst the serum-free medium did not support clone formation in some cells at the time of plating, it did not identify a subpopulation of inherently more tumourigenic cells (Zheng *et al.*, 2007).

The principle of culturing tumorspheres from both primary tumours and cell lines is that cells capable of growing as spheres have increased tumourigenic potential. However, as noted by Singec *et al*, "Any dividing cell from virtually any tissue in serum-free medium on a nonadherent substrate will form floating cell clusters, with intercellular adhesiveness predominating." (Singec *et al.*, 2006) In fact, this has been exploited extensively in cancer cell lines in the creation of 3-d cultures, using manipulations including agitation or low-adherence substrates to induce spheroid

formation (Freyer and Sutherland, 1980; Durand, 1981; Olive *et al.*, 1993; Olive *et al.*, 1997).

As the ready proliferation of cancer cell lines as clusters is a well-recognised phenomenon, it seems vital to ensure that "sphere formation", proposed to represent the properties of an individual specialised cell, is not the result of cellular aggregation. As well as the limiting dilution assays used in this study, timelapse microscopy of cells in culture, or use of expressed cell markers such as green fluorescent protein, can be used to indicate that a sphere represents symmetrical self-renewal of a specific CSC, rather than proliferation facilitated by cell-cell interaction (Singec *et al.*, 2006). The results of the limiting dilution assays for REM134, D17 and SB cells support the latter of these possibilities.

Inference of "stemness" from the tendency of cells to form suspended spheres *per se* appeared to be questionable. For the D17 cell line, this phenotypic variation was strongly influenced by specific culture conditions – on a low adherence substrate, most cells grew as spheres, whereas in equivalent medium but on normal plates, cells attached and proliferated. Moreover, sphere-like aggregates were seen in overconfluent adherent cultures, and towards late passage. Addition of EGF and bFGF to serum-containing D17 cultures seemed to increase the tendency towards intercellular adhesion, and reduce attachment of cells to the culture plate. It is possible that this combination of mitogenic growth factors directly alters the cell-cell and cell-matrix adhesion characteristics of these cells. Indeed, Cicero *et al* showed that sphere-forming cells from murine retinal pigment epithelium were not, as previously suggested, a selected population of *bona fide* retinal stem cells. Rather, dissociation of the tissue and exposure to growth factor-supplemented medium led directly to changes in gene expression, including the upregulation of the neural stem cell marker nestin (Tropepe *et al.*, 2000; Cicero *et al.*, 2009).

The potential for specific culture conditions to affect the sphere-forming behaviour of cancer cell lines was also apparent in the responses of CML10 and SB cell lines to different combinations of serum replacement and concentrations of VEGF. Similar

variation has been noted by other experimenters in response to changes in supplementation (Na *et al.*, 2009). A multiplicity of culture conditions and supplements has been used to derive putative progenitors using the sphere assay (Chaichana *et al.*, 2006). If tumourspheres are to be considered a candidate CSC population, it will therefore be important to determine whether these diverse culture conditions are enhancing tumoursphere formation by the same cells, or whether they are permissive for the growth of distinct cellular subsets.

Neither REM134 nor D17 tumourspheres showed increased expression of the stem cell-associated surface markers CD34, CD133 or CD117 at flow cytometry. Lack of expression of the HSC-associated antigen CD34 is predictable for these solid tumour-derived cell lines. Inasmuch as expression of CD117 and CD133 has been reported for the putative CSC populations of diverse tumours (Singh *et al.*, 2003; Monzani *et al.*, 2007; Ricci-Vitiani *et al.*, 2007; Chen *et al.*, 2008; Smith *et al.*, 2008; Zhang *et al.*, 2008a), increased expression by tumoursphere populations in this study might have further supported the notion that they represented stem-like cells. However, as the markers were not expressed on any of the parental cell lines, it is again perhaps not surprising that they were not demonstrated on the spheres – the assay theoretically enriches for a pre-existing subpopulation – and so in itself this does not preclude the concept that sphere cells might be stem-like.

The changes in the expression of CD44, present almost ubiquitously on REM134 adherent cells and downregulated on tumourspheres, were unexpected. Candidate cancer stem cells exist within the $CD44^+$ compartment in a variety of tumours, including breast, prostate, colon, pancreas, stomach, ovary and HNSCC (Al-Hajj *et al.*, 2003; Collins *et al.*, 2005; Li *et al.*, 2007; O'Brien *et al.*, 2007; Zhang *et al.*, 2008a; Takaishi *et al.*, 2009). Therefore, the reduced frequency of $CD44^+$ cells within REM134 tumourspheres was intriguing, as it was one of the few observations which distinguished tumoursphere from adherent cell populations. The role of CD44 as a potential CSC marker was further investigated in separate experiments (Chapter 5).

The weak 43kDa band detected for the transcription factor Oct4 at western blot of D17 adherent, Adh-GF and tumoursphere lysates did not indicate upregulation in tumoursphere populations or in response to growth factor exposure. Moreover, at immunofluorescence analysis of tumoursphere populations, detected protein was inappropriately localised to the cytoplasm. Expression of Nanog was not demonstrated for adherent, AdhGF or tumoursphere populations by Western blot, or for tumoursphere cells by immunofluorescence. The results of the Oct4 and Nanog Western blot and immunofluorescence analyses seem to contradict those of Wilson *et al.*, where both RNA and protein were reported as being detected in D17 tumoursphere cells.

However, close examination of the RT-PCR results in that study suggest that transcription levels were greater for adherent cell than tumoursphere populations - in particular, D17 tumourspheres show no band for Oct4. Whilst the immunofluorescence images of Wilson *et al.* do seem to show increased staining for both Oct4 and Nanog for tumourspheres, image resolution does not allow any analysis of the subcellular localisation of the proteins (Wilson *et al.*, 2008). It is possible, therefore, that the spheres are demonstrating the cytoplasmic fluorescence observed in this study. High levels of transcription and translation of non-functional pseudogene products is characteristic of both Oct4 and Nanog, which may contribute towards this localisation of fluorescent signal.

It may be significant that the previous report of D17 tumoursphere culture shows a population of cells which has been expanded at each passage in adherent culture, rather than enriched through repeated subculture as spheres – it is possible that changes in gene expression led to upregulation of these markers. Also, as shown by Cantz *et al.*, careful interpretation of immunofluorescence images of tumourspheres is required to avoid falsely enhancing background signal, particularly considering their high levels of autofluorescence and non-specific antibody binding (Cantz *et al.*, 2008).

Canine bone marrow-derived stromal cells were cultured as part of this study to act

as positive control populations, as expression of both Oct4 and Nanog has been reported in MSC of other species (Tondreau *et al.*, 2005; Greco *et al.*, 2007), but the cells were negative by immunofluorescence for both markers, and lysates showed a band for Oct4 only at an early stage after derivation. In fact, this is consistent with reports by other investigators (Lengner *et al.*, 2007; Liu *et al.*, 2009). Liu *et al.* describe expression of Oct4 as being initially low and further reduced upon culture *ex vivo* of human MSC, and Nanog as, "almost undetected even at early passage" (Liu *et al.*, 2009). Indeed, expression of embryonic stem cell-associated proteins such as Oct4 and Nanog in adult stem cell and CSC populations has been called into question by a number of investigators (Berg and Goodell, 2007; Lengner *et al.*, 2007; Zangrossi *et al.*, 2007; Cantz *et al.*, 2008; Liedtke *et al.*, 2008).

Nonetheless, whilst polyclonal antibodies were used to maximise the chances of cross-species detection, and the β-actin and GAPDH controls indicated successful protocol, failure of the Oct4 and Nanog antibodies to bind to canine epitopes cannot be ruled out. Ideally canine embryonic stem cells would be used as a positive control, and also allow comparison of expression level (several studies have shown that a basal level of signal in ostensibly negative samples can confound results (Lengner *et al.*, 2007; Zangrossi *et al.*, 2007; Cantz *et al.*, 2008)). These were not available at the time of the study.

There were no significant differences between the responses of adherent and tumoursphere populations to doxorubicin or mitoxantrone, consistent with the concept that tumourspheres may represent an alternative, culture-induced phenotypic manifestation of the same cell type, rather than a specific CSC population. However, it seems likely that a number of factors may confound assays attempting to compare the drug sensitivities of sphere and adherent cells.

For example, in this study, chemosensitivity assays compared resistance of dissociated sphere and adherent cells. The ATP luminescence system necessitated the use of opaque-walled black plates, which are not available with a low-adherence coating. Although D17 and REM134 spheres had been through multiple passages (8

and 6, respectively) prior to plating, it is likely that once the cells were dissociated and plated on an uncoated substrate they adopted an adherent, rather than tumoursphere, morphology. Once attached, a population of sphere-forming putative stem cells would be expected to begin differentiating to recapitulate the parental cell line. Although other investigators have reported differential sensitivities using this assay, and some enrichment may remain prior to the addition of drug, such methods are unlikely to compare a pure population of sphere cells with a pure population of adherent cells (Todaro *et al.*, 2007; Zhou *et al.*, 2007; Zhou *et al.*, 2009b).

Drug sensitivity testing by MTT assay, which measures cell viability through colourimetric detection on transparent plates, would permit the use of commercial low-adherence 96-well plates for the tumoursphere cells. In this format, however, the assay is confounded by the distinct morphologies of the two cell types being tested. Cells grown as three-dimensional spheroids show changes in sensitivity to multiple anticancer agents including ionising radiation, chemotherapeutic drugs and photodynamic therapy (Freyer and Sutherland, 1980; Durand, 1981; Olive and Durand, 1985; Olive *et al.*, 1993; Olive *et al.*, 1997).

Position within the sphere has some influence on this – external, more rapidly cycling cells are likely to be more sensitive to antiproliferatives, and drugs must diffuse into the spheroid to access the innermost cells (Kansal *et al.*, 2000). Cells at the centre of a sphere are also likely to be relatively hypoxic compared to those at the periphery, and this reduced oxygen tension is well established as a factor in resistance to radiotherapy, (Teicher, 1994; Brown and Wilson, 2004; Vaupel and Mayer, 2005). There is also a "Contact Effect", which leads to profound changes in cell sensitivity even after 3-d cultures have been disaggregated. Alterations to cell shape and polarisation, nuclear shape and chromatin structure are reported to occur in three-dimensional culture systems, influencing signalling and apoptosis resistance mechanisms (Olive and Durand, 1985; Olive and Durand, 1994; Durand and Olive, 2001; Weaver *et al.*, 2002).

Elegant experiments have demonstrated that large 3-d spheroids are more resistant to doxorubicin than smaller spheres or cells in a monolayer, and that disaggregated spheroids retain this enhanced drug resistance. Similarly, even small 3-d spheroids and cells cultured in close contact show increased resistance to ionising radiation for many cell types (Freyer and Sutherland, 1980; Sutherland, 1988).

In the context of the tumoursphere assay, these reports of dramatic changes in cellular resistance due to the architecture of a culture must be acknowledged. It is possible that they could influence some of the apparent changes in sensitivity reported between monolayer cultures and their corresponding tumourspheres in the cancer stem cell literature, particularly for cell lines.

Moreover, one premise of the cancer stem cell hypothesis is that CSC are resistant to cytotoxic treatments as a result of relative quiescence, and the serum-free conditions of tumoursphere culture are likely to encourage slower cell growth. Cells in the centre of 3-d spheroids created by the aggregation of whole cell lines in suspension culture are inherently less proliferative than those at the periphery (Durand and Olive, 2001). This is likely to be the case also in tumoursphere culture.

This variation in cell cycle status is implied by the wider distribution of light scatter (FSC *vs* SSC) noted at flow cytometry for dissociated tumourspheres (growth phase will influence both cell size and nuclear complexity). Less synchronous growth of cells within tumourspheres may also influence results in assays such as serial transplantation. If cells cultured as spheroids are distributed differently through the phases of the cycle, this could have an effect on apparent engraftment potential. Whilst asynchronous proliferation may more closely resemble the situation within a tumour, it must be considered when comparing the behaviour of the cells to other populations (Kansal *et al.*, 2000).

Thus, if a tumoursphere system is to be used to characterise putative cancer stem cells, cellular heterogeneity cannot be disregarded. If, as with neurospheres, they comprise a population of nonidentical cells in terms of viability, stage of

differentiation and growth phase, many variables could account for differential behaviour in assays.

Therefore, whilst tumoursphere culture may be useful for propagating CSC from some populations, further selection and enrichment is probably required in order to gather meaningful data. If comparisons are to be made between CSC and a bulk population, the assay conditions for the two should be comparable – this again advocates either further selection of cells from spheres prior to testing, or direct isolation from bulk cultures after characterisation using the tumoursphere assay, to eliminate the effects of contrasting culture conditions and architecture.

It may be valuable to investigate further methods of maintaining candidate tumour stem cell populations in adherent cultures, as in some respects this could represent a more satisfactory platform to evaluate CSC against their differentiated progeny in drug screens. The cells are exposed more uniformly to insult when arranged as a monolayer, and may be more consistent in terms of their stage of differentiation (Pollard *et al* reported less heterogeneity in adherent glioma stem cells than in corresponding suspension/sphere cultures) (Pollard *et al.*, 2009b). Moreover, cells are more likely to be in a similar phase of growth to their neighbours, avoiding the influence on sensitivity of position in the cell cycle.

The use of three-dimensional spheroids is valuable in demonstrating that cells *in vivo* do not behave as a monolayer. However, as Durand notes, in order to gain the most useful information from this phenomenon, it,

> "makes little sense to acknowledge that 3-d systems contain a multitude of different microenvironments but then simply to look at the net response without knowing which cells respond or why." (Durand and Olive, 2001)

Fluorescent dyes such as Hoechst 33342 differentially diffuse into 3-d cultured spheroids, allowing the location of the cell within the sphere to be identified after disaggregation. Subsequent labelling with fluorescent antibodies to markers of

interest, and simultaneous detection by flow cytometry, could enable the architecture of a heterogeneous sphere to be more accurately determined in terms of the range of cellular phenotypes it comprises, and their distribution within it.

Given the wealth of data already gathered using 3-d culture systems, this could be combined with analysis of tumoursphere-forming putative cancer stem cells. Cells fractionated according to localisation, phenotype, growth phase, or a combination of factors, could be assessed for properties such as drug sensitivity or clonogenicity. Greater understanding of the interactions of heterogeneous cells within a tumoursphere may also provide insight into the arrangement of putative cancer stem cells within the tumour niche.

Tumourspheres derived from heterogeneous primary tumours may be a more appropriate model of cancer stem cells than those derived from cell lines. For all of the tumourspheres derived from the primary canine osteosarcoma in this study, cells had undergone some expansion in serum-containing culture conditions. However, the populations were not derived from cloned cells, and as such demonstrated considerable heterogeneity at early passage, as evidenced by microscopic observation and flow cytometric analyses (Chapter 3). Notably, for the population proliferating most readily in initial tumoursphere culture, kt-osa1, adherent cells had demonstrated a greater frequency of $CD117^+$, side population and $CD133^+$ cells at flow cytometry than the other two populations.

As morphological heterogeneity and surface marker-defined subpopulations of the three adherent populations declined with duration *in vitro*, it is tempting to speculate that this was related to the altered tumoursphere characteristics noted at later passage – for example, kt-osa1 cells losing expression of both CD117 and CD133, and also becoming less proliferative in tumoursphere culture.

Unfortunately, both early- and late-derived spheres failed to proliferate after two or three passages and were not characterised. This could be interpreted as lack of the self-renewal ability which is required to define a CSC in this assay. However, some

investigators have reported that the natural heterogeneity and gene expression patterns of the primary tumour are better preserved using tumoursphere culture, and that irreversible changes occur upon adherent culture in serum-containing conditions. Thus a detrimental effect of the initial expansion of kt-osa populations in serum-containing culture cannot be ruled out. Further studies of tumourspheres derived directly from canine tumours are indicated.

In conclusion, the data presented here emphasise that any model based on the hypothesis that tumourspheres derived from cancer cell lines comprise self-renewing, stem-like cells must be carefully interrogated to ensure that this is indeed the case, rather than a culture artefact. The basic biology of 3-d cultures must be considered, when interpreting apparent differences between these and adherent cells. As elegantly stated by Singec,

> "One must recognize the utility of the neurosphere assay while not going beyond the limits of its sensitivity and specificity; that is, not conferring upon this common tissue culture phenomenon a significance beyond that entitled by its biology… Sphere formation is a useful culturing tool, not a metric." (Singec *et al.*, 2006)

Nonetheless, where genuinely permissive for the growth of specific cellular subsets, tumoursphere culture may be valuable for identifying putative CSC within populations of canine cancer cells, allowing their propagation and further characterisation.

Although cancer cell lines are invaluable investigative tools, it appears less likely that tumoursphere formation will signify proliferation by a stable subpopulation of cells. Direct culture of tumourspheres from primary tissue may be a more representative model for identifying CSC within heterogeneous populations, and warrants further investigation.

CHAPTER 5

INVESTIGATING CD44 AS A POTENTIAL CANCER STEM CELL MARKER IN CANINE TUMOURS

INTRODUCTION

CD44

CD44 is a cell surface transmembrane glycoprotein, highly conserved in mammalian species (Goodison *et al.*, 1999). The gene is located on human chromosome 11, and on chromosome 18 in the dog; homology between the canine and human sequence is reported as 85-90% (greater than that between canine and rodent) (Milde *et al.*, 1994; Sandmaier *et al.*, 1998). Ten constant exons code for the standard form of the protein – alternative splicing of a further ten variant extracellular domain exons, as well as post-translational modification such as glycosylation, produces a number of variant forms (Screaton *et al.*, 1992) – the size of the molecule therefore varies (80-200kDa) (Figure 1). CD44 expression is almost ubiquitous, with tissue-specific factors regulating alternative splicing - the standard form (CD44s) predominates except in haematopoietic cells such as lymphocytes, which express variant forms (CD44v) (Marhaba and Zoller, 2004).

The major ligand of CD44 is hyaluronate (HA) (Aruffo *et al.*, 1990), a polysaccharide molecule ubiquitously expressed on extracellular matrix. CD44 also

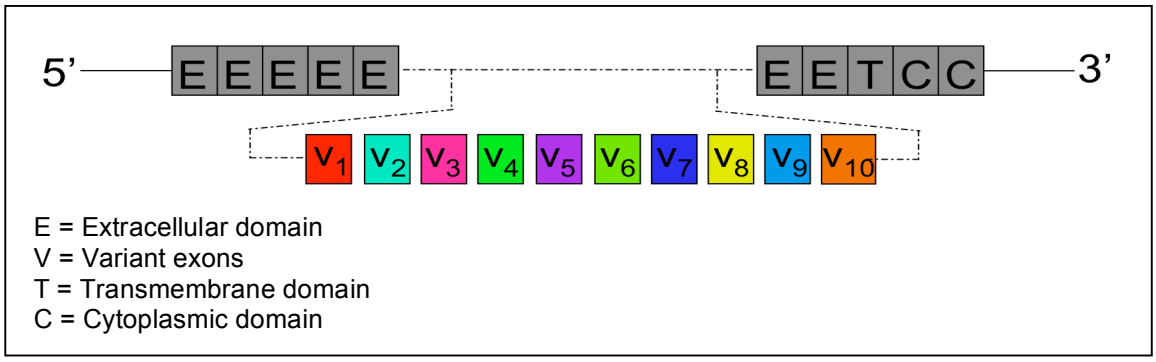


Figure 1 – Schematic diagram showing structure of CD44 gene. Ten constant exons (grey) are transcribed to produce the standard form (CD44s). An additional ten extracellular domain exons may be incorporated by alternative splicing, producing multiple variant isoforms of the protein (CD44v).

binds a number of other extracellular ligands including collagen, fibronectin, osteopontin and selectin. The molecule is thus associated with cell-matrix and cell-cell interactions, with a role in organ structure, cell homing and migration (Sneath and Mangham, 1998; Goodison *et al.*, 1999; Marhaba and Zoller, 2004). As well as its cell adhesion functions, CD44 (and its variant isoforms in particular) has been shown to interact with a range of signal transduction processes. CD44 itself has no catalytic activity, but the extracellular domain has consensus sequences for a wide range of signalling molecules including receptor tyrosine kinases such as EGFR, growth factors such as bFGF and VEGF, and matrix metalloproteases (Marhaba and Zoller, 2004).

CD44 in Cancer

Gunthert *et al* showed in 1991 that transfection of CD44 isoforms with variant exons 6 or 7 could confer metastatic potential to otherwise non-metastatic rat pancreatic carcinoma cells (Gunthert *et al.*, 1991). Altered expression of CD44 has since been reported for many types of malignancy, particularly in association with invasion and metastasis (Sneath and Mangham, 1998; Herrera-Gayol and Jothy, 1999b). Dysregulation of CD44 expression has been reported in canine neoplastic disorders including benign and malignant mammary tumours, malignant melanoma and malignant histiocytosis (Alldinger *et al.*, 1999; Serra *et al.*, 2004; Madrazo *et al.*, 2009; Paltian *et al.*, 2009).

Several studies suggest that cancer progression is more strongly associated with aberrant expression of variant isoforms than with the standard form. It is notable that CD44v isoforms are expressed on normal haematopoietic cells, given the many similarities between the metastatic process and that of lymphocyte migration and homing. Monoclonal antibodies to CD44 have been shown to inhibit infiltration by malignant lymphoma cells in mouse models in a tissue- and isoform-specific manner (Naor *et al.*, 2008). However, expression of CD44v is not the sole form of dysregulation seen in cancer, with some tumours showing altered expression of the standard form (Sneath and Mangham, 1998).

Given its role in cell-cell and cell-matrix interaction, altered CD44 expression may disturb the normal structural organisation within solid tissues. Aberrant expression may also lead to disruption of epithelial-mesenchymal interactions within tissues to facilitate invasion (Goodison *et al.*, 1999), and promote aggregation of cells and subsequent entrapment within capillary networks as part of the metastatic process. CD44-HA binding may lead to morphological changes in the cytoskeleton, mediating processes such as migration and extravasation (Marhaba and Zoller, 2004).

Interaction between CD44 (particularly variant isoforms) and its ligands may lead to suppression of apoptosis, through mechanisms such as the PI3K/Akt survival pathway or activation of anti-apoptotic Bcl family members such as Bcl-2 and Bcl-Xl (Marhaba and Zoller, 2004). Stable transfection of the human colon cancer cell line SW620 with the CD44v3-10 isoform conferred increased resistance to apoptosis induced by etoposide (Lakshman *et al.*, 2004). Another study of CD44-mediated apoptosis resistance investigated the murine colon cancer model C57BL/6J*(APC* $^{min/+}$*)*, which is tumour-prone due to a heterozygous point mutation in the APC tumour suppressor gene. Knockout of the CD44 gene resulted in increased apoptosis at the crypt base, and reduced formation of aberrant crypts. This suggests that the expression of CD44 might enhance cellular survival where apoptosis would otherwise occur in response to altered growth signals (Zeilstra *et al.*, 2008).

CD44 as a Cancer Stem Cell Marker

In seminal experiments published in 2003, cells from human breast tumours (either directly dissociated or passaged in NOD/SCID mice) were depleted of lineage-positive cells, and sorted according to expression of CD44 and CD24 using flow cytometry. It was found that tumourigenicity lay within the the $CD44^{+}CD24^{-/low}$ subset, and could be further enriched by isolating cells within this fraction expressing ESA (EpCAM) (Al-Hajj *et al.*, 2003). However, it is noteworthy that for both

passaged and unpassaged cells, $CD44^+CD24^-$ cells efficiently formed tumours whereas $CD44^+CD24^+$ cells did not, indicating that CD44 in isolation in this model did not significantly enrich for tumourigenicity. For example, where 10^4 passaged cells were injected orthotopically, $CD44^+CD24^{-/low}$ cells produced tumours in 10/10 mice, compared to 0/10 for $CD44^+CD24^+$ (and 3/12 for unsorted cells). Similarly, 11/13 mice injected with primary $CD44^+CD24^{-/low}$ cells developed tumours, compared to only 1/13 for $CD44^+CD24^+$ cells (Al-Hajj *et al.*, 2003).

CD44 expression, either as a single marker or in conjunction with others such as CD24, ESA, CD133 and $\alpha 2\beta 1$-integrin, has since been reported to identify cancer stem cells in a wide variety of primary tumours and cancer cell lines, including those of stomach (Takaishi *et al.*, 2009), colon (Dalerba *et al.*, 2007), pancreas (Li *et al.*, 2007), breast (Ponti *et al.*, 2005; Fillmore and Kuperwasser, 2008), ovary (Zhang *et al.*, 2008a), prostate (Collins *et al.*, 2005) and bladder (Chan *et al.*, 2009), and in acute myeloid leukaemia (Jin *et al.*, 2006), melanoma (Dou *et al.*, 2007) and squamous cell carcinoma (Prince *et al.*, 2007). In a number of studies, an association has been demonstrated between CD44 expression and growth as "tumourspheres" in serum-free culture, with increased expression on tumourspheres or enrichment of tumoursphere-forming capability amongst $CD44^+$ cells (Collins *et al.*, 2005; Fillmore and Kuperwasser, 2008; Hurt *et al.*, 2008; Wright *et al.*, 2008; Zhang *et al.*, 2008a; Takaishi *et al.*, 2009). CD44-HA interaction may promote resistance to chemotherapeutic agents through induction of the P-glycoprotein membrane pump transporter (Bourguignon *et al.*, 2008). Direct targeting of cd44-expressing putative cancer stem cells, with gene knockout strategies or CD44-specific monoclonal antibodies, reduces tumour formation in some murine models of cancer (Jin *et al.*, 2006; Zeilstra *et al.*, 2008).

The aim of this study was to investigate the biological significance of CD44 expression in canine cancer cell lines, to establish whether it might represent a relevant cancer stem cell marker and thus a potential target for therapy.

MATERIALS AND METHODS

Antibody Staining

Antibody staining was performed as described in Materials and Methods chapter. Phycoerythrin-conjugated CD44 rat anti-mouse/human antibody (clone IM7, BioLegend) or isotype-matched control antibody was added at an optimised concentration, as determined by titration for each cell line. Clone IM7 has well-documented canine crossreactivity, and recognises the standard form of CD44 and its variants (Neame and Isacke, 1993; Sandmaier *et al.*, 1998).

For cell sorting, penicillin-streptomycin was substituted for sodium azide to reduce inhibitory effects on cell growth (Sort-DMEM : DMEM / 2% F.C.S. / 2% penicillin-streptomycin). Sorting gates were set at the highest ($CD44^{high}$) and lowest ($CD44^{low}$) extremes of the staining distribution, each collecting 10% of cells within the FSC-SSC live gate.

Simultaneous Surface Marker and Cell Cycle Analysis

Cells were harvested at 65-80% confluence as assessed visually by microscopy, except Cat-MT cells, which had achieved almost 100% confluence at the time of harvest. Cells were prepared and incubated with antibody as described previously. After washing with copious FACS-DMEM, cells were fixed in PBS / 0.5% paraformaldehyde / 0.1% sodium azide for 15 minutes on ice, and resuspended in 1ml ice-cold PBS. 3ml ice-cold (-20°c) absolute ethanol was added dropwise while vortexing, to minimise cell clumping. Samples were held on ice for at least 30 minutes. Cells at this stage could be stored at 4°c, in the dark, for up to 48h pending DNA staining.

DAPI [ex-max 350nm; em-max 461nm] (Sigma) was used for DNA staining to afford minimal spectral overlap with phycoerythrin [ex-max 496nm, 546nm; em-max 578nm]. Cells were washed twice with PBS, resuspended in 250µl PBS, and 250µl

DAPI (2μg/ml in PBS) added to each tube for a final concentration of 1μg/ml. Samples were analysed by flow cytometry, with 10% of cells at each extreme of the CD44 staining distribution gated and analysed for DAPI fluorescence. At least 25000 events were acquired for analysis.

Surface staining, cell sorting and simultaneous CD44-cell cycle analysis were performed, respectively, on FACSCalibur [CellQuestPro software], FACSAria and LSRII [FACSDiva software] cytometers (all BD Biosciences). Post-acquisition analysis was performed using FlowJo (Treestar).

To determine levels of surface antigen expression, relative Mean Fluorescence Intensity (ΔMFI) was calculated by subtracting the MFI of the unstained aliquot from that of the antibody-stained aliquot. For $CD44^{High}$ and $CD44^{Low/-}$ populations this was expressed as a percentage of the ΔMFI of unfractionated cells. For tumourspheres this was expressed as a percentage of the ΔMFI of the corresponding adherent cell population.

Colony Formation Assay

5 x 10^3 cells were collected of each of $CD44^{high}$ and $CD44^{low/-}$ fractions, and of the unstained sample. Cells were washed twice in complete medium and resuspended at 500 cells/ml. For each fraction, 500 cells were plated in each of five 10cm-diameter culture plates, in 7ml of complete medium. Plates were incubated until macroscopic colonies were visible (9 days). Plates were washed twice with PBS, and fixed for 1 hour at room temperature in 4ml absolute methanol. Colonies were stained for 1 hour at room temperature with 70% methanol / 2% crystal violet and rinsed thoroughly with water. Colonies were counted using a ChemiDoc XRS imaging system and Quantity One software (Bio-Rad). Statistical analyses were performed using GraphPad Prism (GraphPad Software). Statistical significance for colony formation assays was calculated using a One-Way ANOVA (Kruskal-Wallace) for all groups and Mann-Whitney U-test for 2 groups ($p = 0.05$).

Tumoursphere Formation Assay

Sorted $CD44^{high}$ and $CD44^{low/-}$ cells were resuspended in N2/MC medium and plated in UltraLow Attachment 6-well plates (Corning) at 60000 cells / 2ml N2/MC per well. Plates were incubated at 37°c in a humidified atmosphere with 5% CO_2. 12µl EGF (100µg/ml) and 12µl bFGF (100µg/ml) were added every 48 hours.

Drug Sensitivity Testing

Sorted $CD44^{high}$ and $CD44^{low/-}$ cells were resuspended in complete medium at $1x10^5$ cells/ml and plated in black 96-well culture plates (Corning) at $5x10^3$ cells per well. A chemosensitivity assay to evaluate the viability of each population in response to increasing concentrations of doxorubicin was performed as described in Materials and Methods chapter. Briefly, after 24 hours' incubation, doxorubicin dilutions were added to triplicate wells. 50µl of medium only was added to triplicate wells as a control. Plates were incubated for a further 72 hours before assessment of viability by ATP luminescence assay. Viability was calculated as a percentage of untreated controls, and dose-response curves fitted using four-parameter nonlinear regression with GraphPad Prism software. Best-fit values for IC_{50} were compared using an extra-sum-of-squares F test ($p < 0.05$).

RESULTS

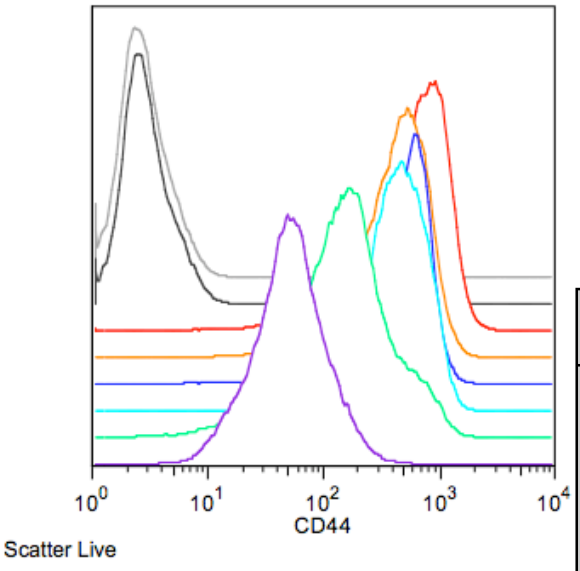
CD44 Expression in Canine Cancer Cell Lines Does Not Identify Positive Subpopulations

Established canine cancer cell lines representing tumours of diverse origin (REM134 mammary carcinoma, D17 osteosarcoma, J3T glioma, SB haemangiosarcoma, 3132 B-cell Non-Hodgkins lymphoma) were assessed for surface expression of CD44. Feline (CatMT) and human (MCF7) mammary carcinoma cell lines were also evaluated. All tested cell lines showed CD44 expression; in all cases, positive staining was manifest as a shift in the entire population along the CD44-PE fluorescence axis, rather than the separation of a defined subpopulation of cells (Figure 2).

Although expression level (as determined by titration of test/control antibodies) varied between the canine and feline cell lines, almost all cells were positive. As shown in Figure 2, the frequency of $CD44^+$ cells, as compared to isotype-matched control antibody, ranged from 97.4% (REM134) to 99.8% (CML10). By contrast, the frequency of $CD44^+$ cells was lower for the human MCF-7 breast cancer cell line, at 49% (Figure 3).

All populations derived from the primary canine osteosarcoma showed a similarly high frequency of $CD44^+$ cells, both at initial expansion prior to subculture (kt-osa2, 99.5% $CD44^+$) and throughout early and later passage (see Figure 4). These populations were not cloned and showed considerable morphological heterogeneity in culture, although this was reduced upon repeated subculture (as discussed in Chapter 3). kt-osa4, tested at passage 6, showed the lowest frequency of $CD44^+$ cells (97.2%); at passage 26, these cells were >99% $CD44^+$.

Thus, CD44 was expressed robustly and near-ubiquitously in all the canine cancer cell populations tested, including those recently derived from a spontaneous tumour, with no discrimination of specific $CD44^+$ subpopulations .



Cell Line	CD44 Positive (%)
REM134	97.37
D17	98.33
CML10	99.76
SB	99.58
3132	99.12
CatMT	99.28

Figure 2 – Overlay of histograms illustrating CD44 surface expression by canine (and feline CatMT) cell lines. Representative unstained and isotype control samples (REM134) are also shown. Table shows frequency of $CD44^+$ cells as compared to corresponding controls for each cell line.

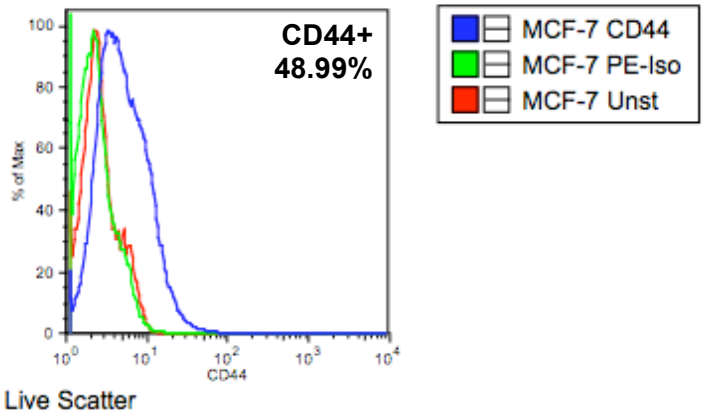
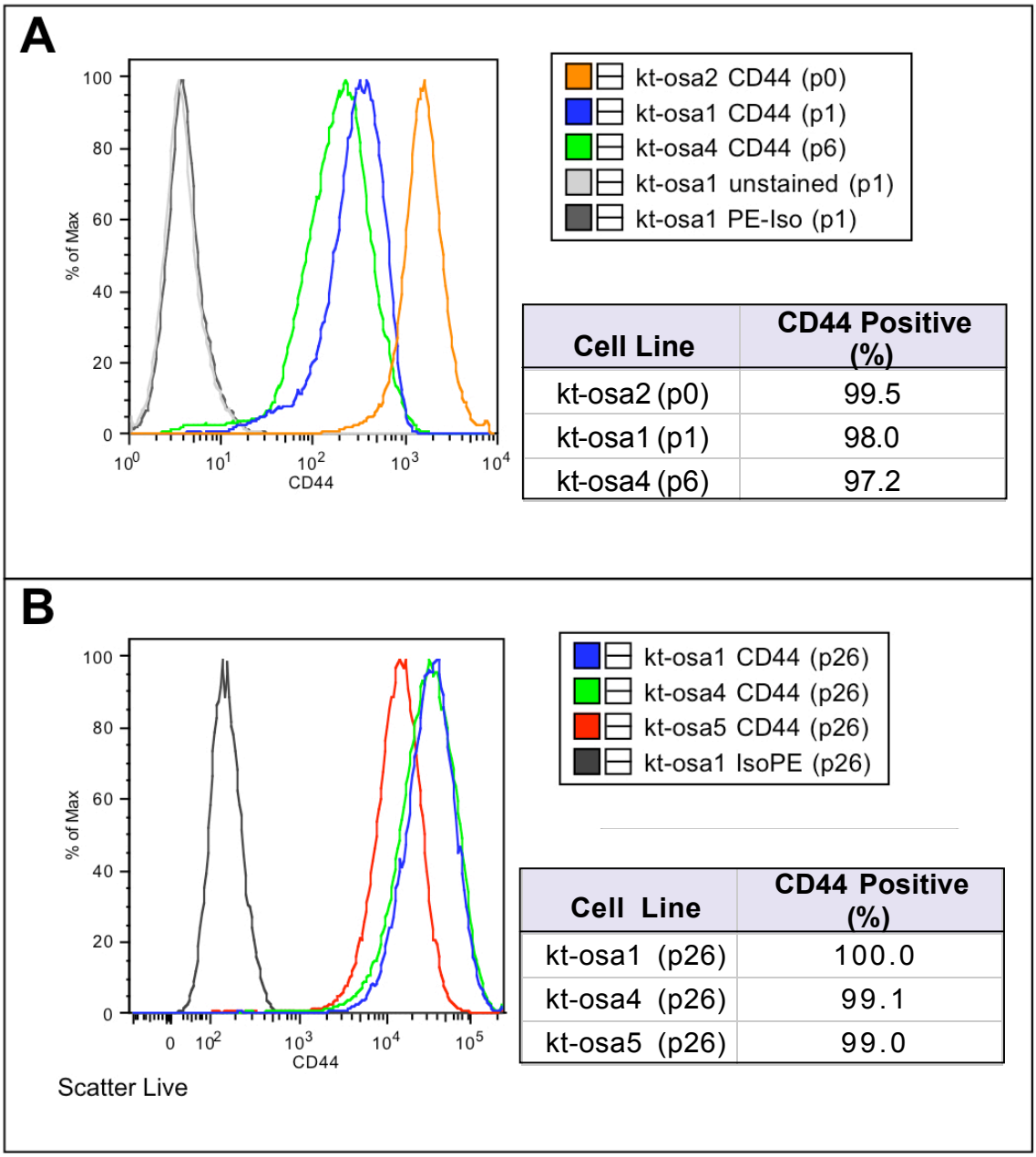


Figure 3 – Histogram of CD44 expression for MCF-7 human mammary carcinoma cell line, demonstrating lower frequency of $CD44^+$ cells than seen for canine cancer cell lines. Relevant isotype and unstained control samples shown for comparison.



Cell Line	CD44 Positive (%)
kt-osa2 (p0)	99.5
kt-osa1 (p1)	98.0
kt-osa4 (p6)	97.2

Cell Line	CD44 Positive (%)
kt-osa1 (p26)	100.0
kt-osa4 (p26)	99.1
kt-osa5 (p26)	99.0

Figure 4 – Histograms showing CD44 expression for kt-osa canine osteosarcoma cell populations and representative control samples, at early (A) and late (B) passage. Tables show frequency of $CD44^{+}$ cells as compared to corresponding controls for each population.

It had been intended to define subsets within the $CD44^{+}$ population using concurrent CD24 staining (mouse anti-human clone ML5). However, the antibody showed a high degree of non-specific binding, with a particular affinity for non-viable cells, risking the artefactual appearance of enhanced proliferative capacity amongst the $CD24^{low}$ population (discussed in Chapter 3). Therefore, in order to determine the possible implications of CD44 expression, cells were isolated from the extremes of the staining distribution using FACS, and subjected to further assays.

Flow Cytometric Sorting of REM134 $CD44^{High}$ and $CD44^{Low/-}$ Fractions

The REM134 canine mammary carcinoma cell line was chosen for investigation because of the significance ascribed to this marker in other studies of breast/mammary cancer and cancer stem cells (Herrera-Gayol and Jothy, 1999a; Al-Hajj *et al.*, 2003; Abraham *et al.*, 2005; Fillmore and Kuperwasser, 2008; Honeth *et al.*, 2008; Wright *et al.*, 2008; Buess *et al.*, 2009; Madrazo *et al.*, 2009; Marangoni *et al.*, 2009; Paltian *et al.*, 2009). This cell line was established from the primary mass of a metastatic canine mammary carcinoma and has been shown to be serially tumourigenic in nude mice, producing poorly differentiated, locally invasive tumours. The cells have an epithelioid morphology, abnormal karyotype (average 130 chromosomes/cell) and are negative for oestrogen and progesterone receptors (Else *et al.*, 1982; Norval *et al.*, 1984b; Norval *et al.*, 1984a).

Cells showing the lowest and highest levels of CD44 expression (each representing 10% of live cells) were collected using FACS (Figure 5A). 90% of $CD44^{Low/-}$ cells and 100% of $CD44^{High}$ cells were positive by comparison with the unstained fraction. The relative Mean Fluorescence Intensity (ΔMFI), an indicator of cell surface antigen expression, was calculated by subtracting the MFI of the unstained aliquot from that of each stained fraction. The magnitude of the ΔMFI of $CD44^{Low/-}$ cells was 14%, and $CD44^{High}$ cells 263%, of that of unfractionated cells (Figure 5B).

Sorted cells were re-analysed to assess purity (Figure 5C). Sorting efficiency was maintained at 94-99% during the procedure – nonetheless, when fractions were

reassessed, a small proportion of cells fell outwith the gates used for collection, despite this being performed immediately after sorting. More marked for $CD44^{High}$ cells, bleaching of the PE fluorophore during FACS is likely to have contributed to this observation. However, the effect was less apparent for the $CD44^{low/-}$ cells, and some ungated cells of this fraction showed increased (rather than reduced) fluorescence. This suggested that CD44 expression may be transient or temporally fluctuating.

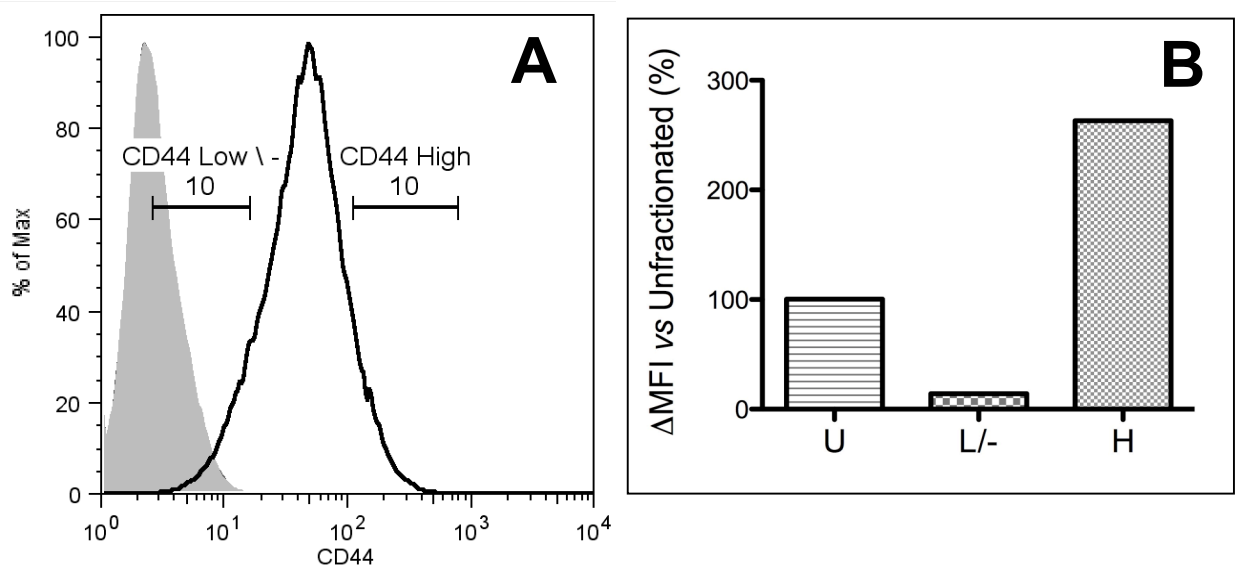
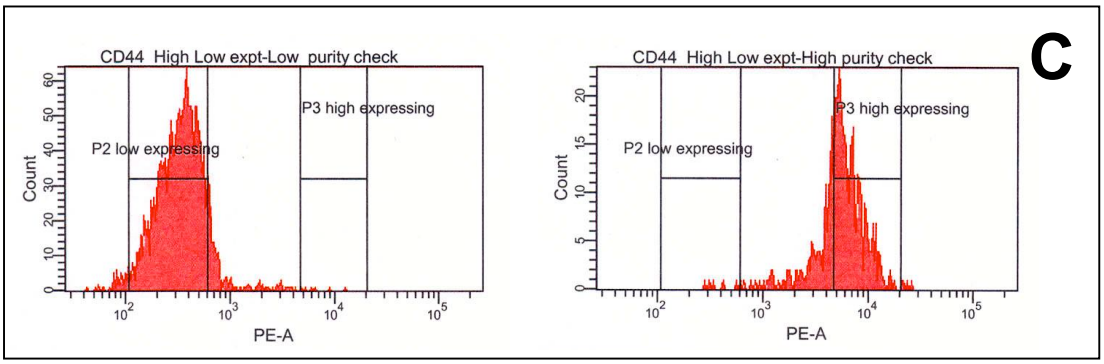


Figure 5 – FACS of REM134 canine mammary carcinoma based on CD44 expression.

A - Histogram of CD44 expression by REM134 cells. Shaded histograms show representative unstained (pale) and isotype control (dark, just seen) samples. Markers indicate placement of gates for FACS of CD44-stained cells.

B – Relative median fluorescence intensities (ΔMFI) for $CD44^{High}$ (H) and $CD44^{Low/-}$ (L/-) fractions, as compared to unfractionated (U) cells.

C - Assessment of purity of $CD44^{Low/-}$ (left) and $CD44^{High}$ (right) fractions. A small proportion of sorted cells consistently fell outwith the gate used for collection, despite high sorting efficiency.



REM134 CD44^{high} Cells Proliferate More Rapidly in Culture than CD44$^{low/-}$ Cells

Equal numbers of sorted CD44^{high} and CD44$^{low/-}$ cells were seeded in culture plates at standard densities (3.5 x 10^4 cells/cm^2). As shown in Figure 6, CD44^{high} cells after 24h in culture had attached to the substrate, were adopting an elongated morphology and showing signs of early proliferation. By contrast, many CD44$^{low/-}$ cells remained small, rounded and unattached at this stage. Although by 96h in vitro, both CD44$^{low/-}$ and CD44^{high} cells were proliferating to form a monolayer, this was sparse for the CD44$^{low/-}$ as compared to the CD44^{High} fraction. Cell counts at 48h suggested that a proportion of CD44$^{Low/-}$ cells had died, with a calculated population doubling (3.32 x [log(cells yielded)-log(cells seeded)]) of -1.27, compared to 1.21 for CD44^{High} cells. Population doublings calculated at 96h were 1.95 for CD44$^{Low/-}$ and 3.68 for CD44^{High} cells, giving a cumulative doubling time of 49h and 26h, respectively (compared with 3.22 doublings / 30h doubling time for the corresponding unfractionated cells).

REM134 CD44^{high} Cells Show Enhanced Colony Formation as Compared to CD44$^{low/-}$ or Unfractionated Cells

Sorted CD44^{high} and CD44^{low} cells were assessed for colony forming ability. An equal number of cells from the unstained sample were collected to control for deleterious effects of the sorting procedure on cell viability. Representative culture plates are shown in Figure 7 and results summarised in Figure 8. Of the three cell types, mean colony formation / 500 cells was greatest for CD44^{High} cells, at 175 ± 16 (p = 0.0091). There was no significant difference between the number of colonies formed by unfractionated cells (74 ± 12) and CD44^{Low} cells (70 ± 15), although subjectively CD44$^{Low/-}$ cells produced smaller colonies (Figure 7B). Together, these results suggest that REM134 CD44^{High} cells show an increased proliferative capacity when compared to both CD44$^{Low/-}$ and unfractionated cells.

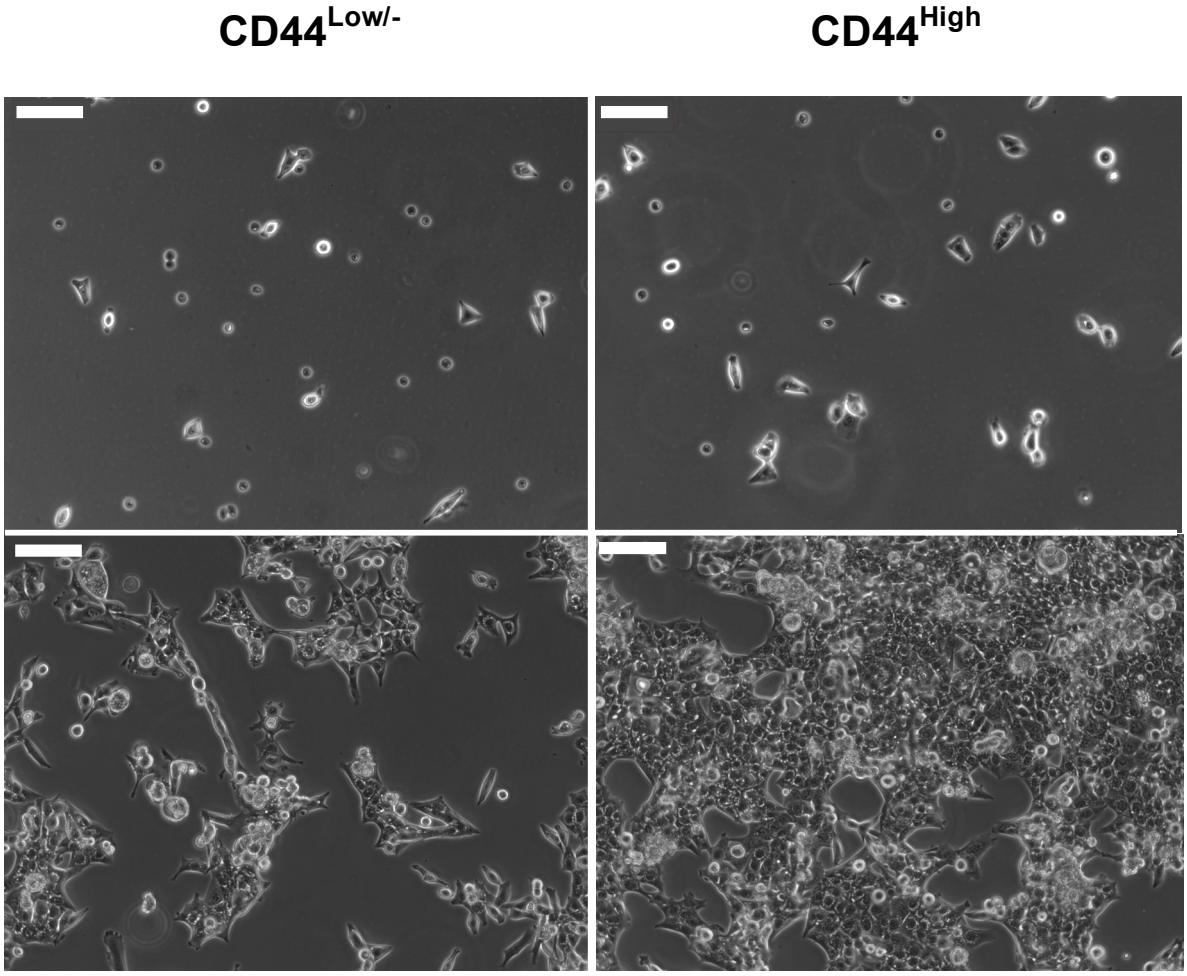


Figure 6 – Adherent growth of REM134 cells sorted for CD44 expression level
Left– $CD44^{low/-}$ cells; *Right*– $CD44^{high}$ cells. Bar = 100µm
Populations plated at equivalent densities to compare growth after sorting.
Representative images showing growth after 24h (*upper*) and 96h (*lower*).

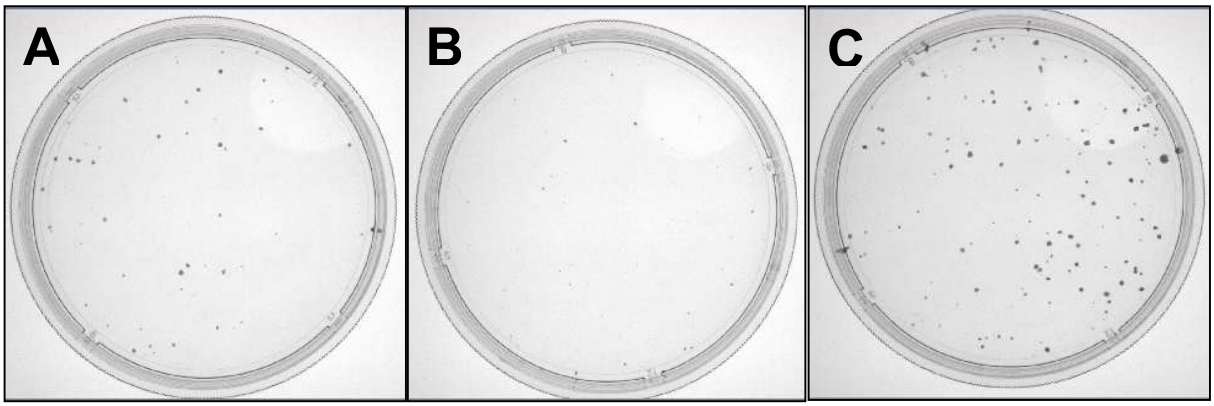
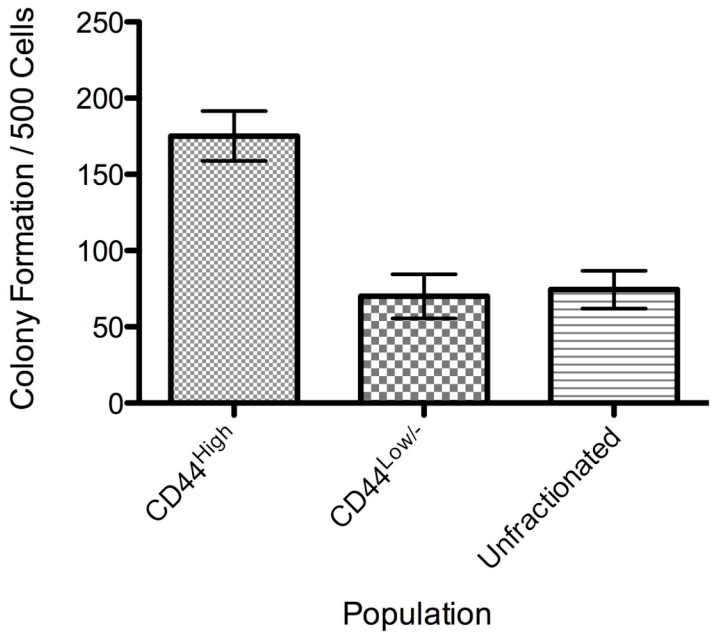


Figure 7 - Representative 10cm-diameter culture dishes showing colony formation by (A) unfractionated, (B) $CD44^{low/-}$ and (C) $CD44^{high}$ REM134 cells.
Mean colony formation / 500 cells was 74 ± 12.5 (unfractionated),70 ± 14.5 ($CD44^{Low/-}$), and 175 ± 16.3 ($CD44^{High}$).



	CD44^{High}	CD44$^{Low/-}$	Unfractionated
Mean	175	70	74
Std. Deviation	16	15	12

Figure 8 – Graph to illustrate increased colony formation by REM134 CD44^{High} cells, as compared to CD44$^{Low/-}$ and unfractionated cells ($p = 0.0091$). There was no significant difference between the colony forming abilities of the latter populations.

REM134 CD44^{high} cells Proliferate as Tumourspheres in Serum-Free Culture, Whereas CD44$^{low/-}$ Cells Do Not

Sorted CD44^{high} and CD44$^{low/-}$ cells were cultured in low-density, serum-free conditions and supplemented with the mitogenic growth factors EGF and bFGF. This "tumoursphere" assay is widely used in the investigation of cancer stem cells, and is proposed to enrich for self-renewing CSC and progenitors within some tumour cell populations and cancer cell lines. The REM134 canine mammary carcinoma cell line shows robust proliferation as spheroidal colonies under these culture conditions; when dissociated and analysed by flow cytometry, the cells comprising these spheroid colonies show reduced expression of CD44 (82.4% CD44^{+}) when compared to the corresponding adherent cell population (97.8% CD44^{+}) (Figure 9). ΔMFI for tumoursphere cells, representing levels of surface antigen expression, was

82.3% that of adherent cells, suggesting downregulation of CD44 on REM134 cells cultured as tumourspheres as well as reduced frequency of positive cells. However, this is partially attributed to an increase in the relative autofluorescence of the cells from the tumourspheres.

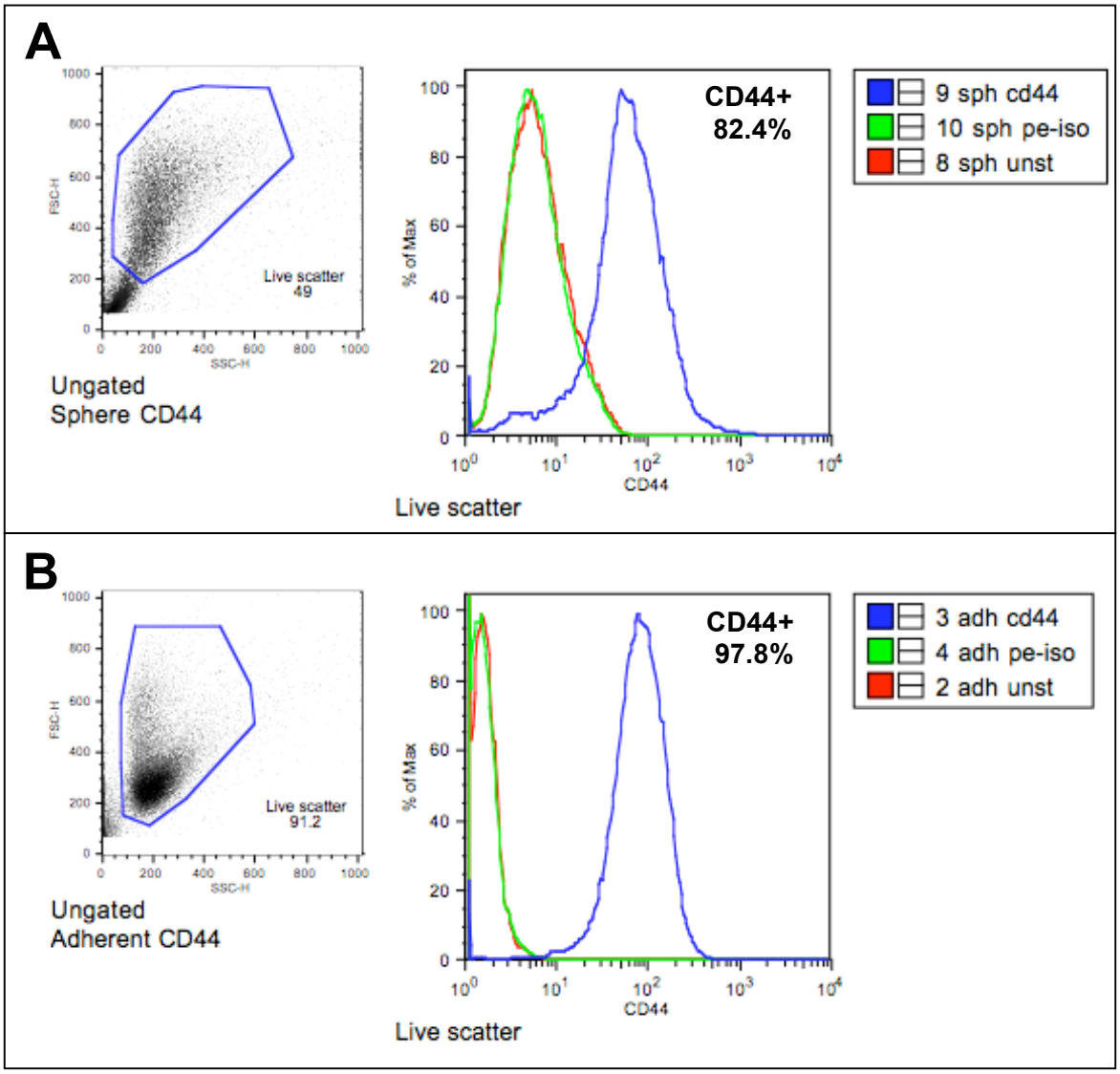


Figure 9 – Frequency of CD44 expression by REM134 canine mammary carcinoma tumourspheres (A) is reduced in comparison with adherent cells (B). Intact "live" gate strategy is shown for each population. Analysis of both populations performed using the same instrument settings. ΔMFI for tumoursphere cells is 82.3% that of corresponding adherent cells.

There was a marked contrast between the growth characteristics of sorted REM134 $CD44^{Low/-}$ and $CD44^{High}$ in serum-free culture (Figure 10). $CD44^{High}$ cells readily expanded as spherical colonies, similar to those seen when total REM134 cells are cultured under these conditions. By contrast, $CD44^{low}$ cells showed no proliferation at all and remained as single cells up to 10 days *in vitro*. When assessed at this stage by trypan blue exclusion, all of the cells within the $CD44^{Low}$ wells stained with the dye, indicating that they were no longer viable.

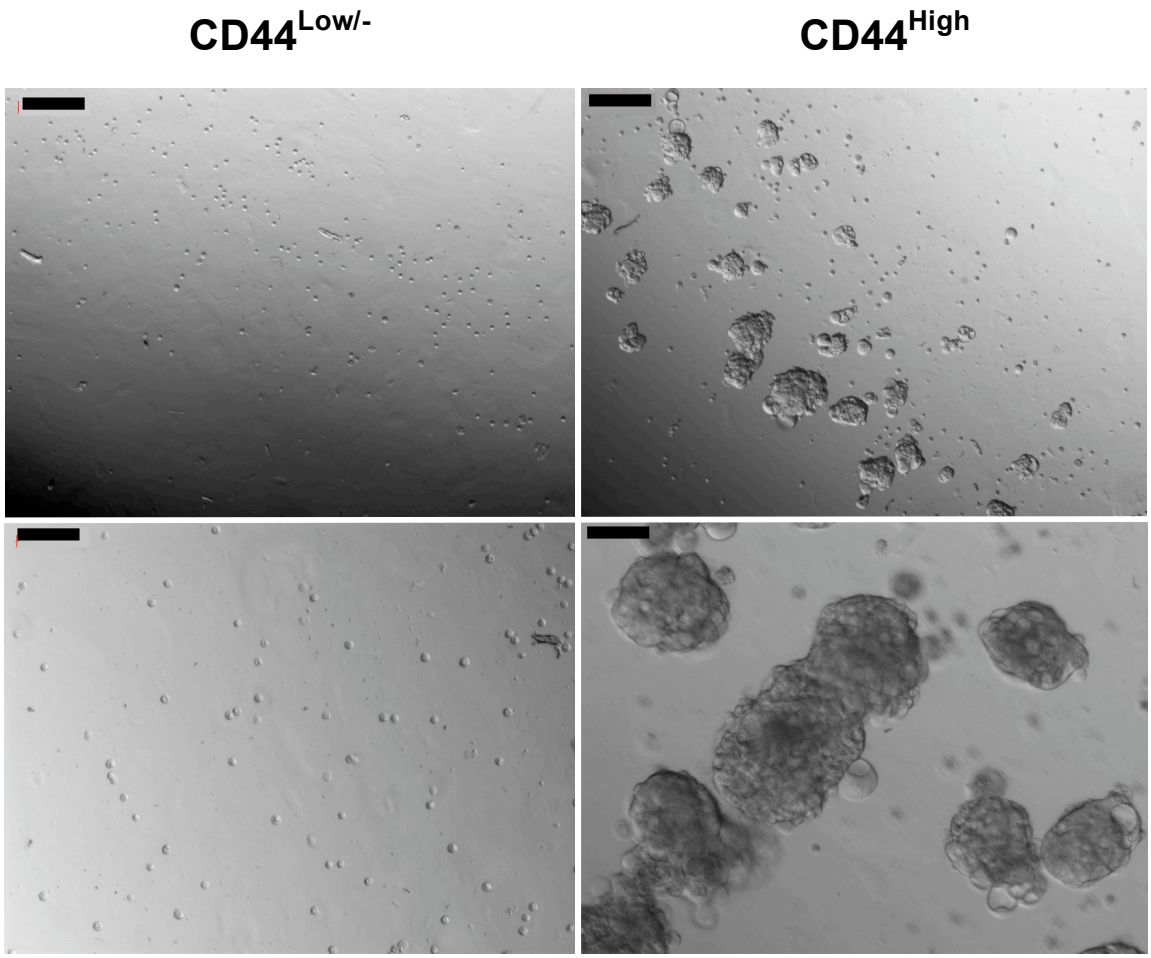


Figure 10 – Tumoursphere formation by sorted REM134 $CD44^{low/-}$ (*left)* and $CD44^{high}$ (*right*) cells.
Representative images showing growth after eight (*upper*) and ten (*lower*) days' culture.
(Upper: 50x objective, Bar = 200µm; Lower: 100x objective, Bar = 100µm)

CD44 Expression by Progeny of REM134 CD44^{high} and CD44$^{low/-}$ Cells Returns Towards the Pattern Shown by Unfractionated Cells

To assess the potential of CD44$^{low/-}$ and CD44^{high} populations, equal numbers of sorted cells were allowed to proliferate under standard adherent culture conditions, and reanalysed by CD44 surface staining after 48h and 96h *in vitro*. At 48h incubation, both CD44^{High} and CD44$^{Low/-}$ populations / progeny retained some of the red fluorescent signal from the initial antibody staining, having a higher baseline fluorescence than cells which had never been treated with antibody (Figure 11). The CD44 molecule has a surface half-life of 15-17h (Neame and Isacke, 1993); signal could also originate from internalised antibody-antigen complexes.

The frequency (% positive cells) (Figure 11A) and surface antigen level (ΔMFI) (Figure 11B) of CD44 expression was compared to that of a corresponding unstained control aliquot, from each population. At 48h, the ΔMFI for progeny of CD44^{High} cells was 154.8% of that of unfractionated cells, whereas that for CD44$^{Low/-}$ cells was 69.7%, demonstrating that surface expression level was maintained by the CD44^{High} cells, but to a lesser extent than at the time of sorting. However, frequency of expression for cells derived from both CD44^{High} (95.0%) and CD44$^{Low/-}$ (97.9%) was similar to that of the unfractionated population (98.7%) The frequency of CD44^{+} cells as compared to the unstained sample was actually slightly lower amongst the progeny of CD44^{High} cells (95.0%) than for the CD44$^{Low/-}$ (97.9%) and unfractionated (98.7%) populations (Figure 11A) despite the fact that at the time of sorting CD44^{High} cells had been 100% CD44^{+} and CD44$^{Low/-}$ cells ≤90% CD44^{+} (Figure 5A).

After 96h *in vitro*, the baseline fluorescence of CD44^{low} cells' progeny was almost equivalent to that of unstained cells, whilst CD44^{high} cells remained slightly brighter. At this stage, the ΔMFI of CD44$^{Low/-}$ cells had increased to 89.9% of that of unfractionated cells; for CD44^{High} cells this remained greater at 159.9%, suggesting that CD44^{High} cells maintained an increased level of surface antigen expression at this timepoint. However, once again, the frequency of CD44^{+} cells within progeny

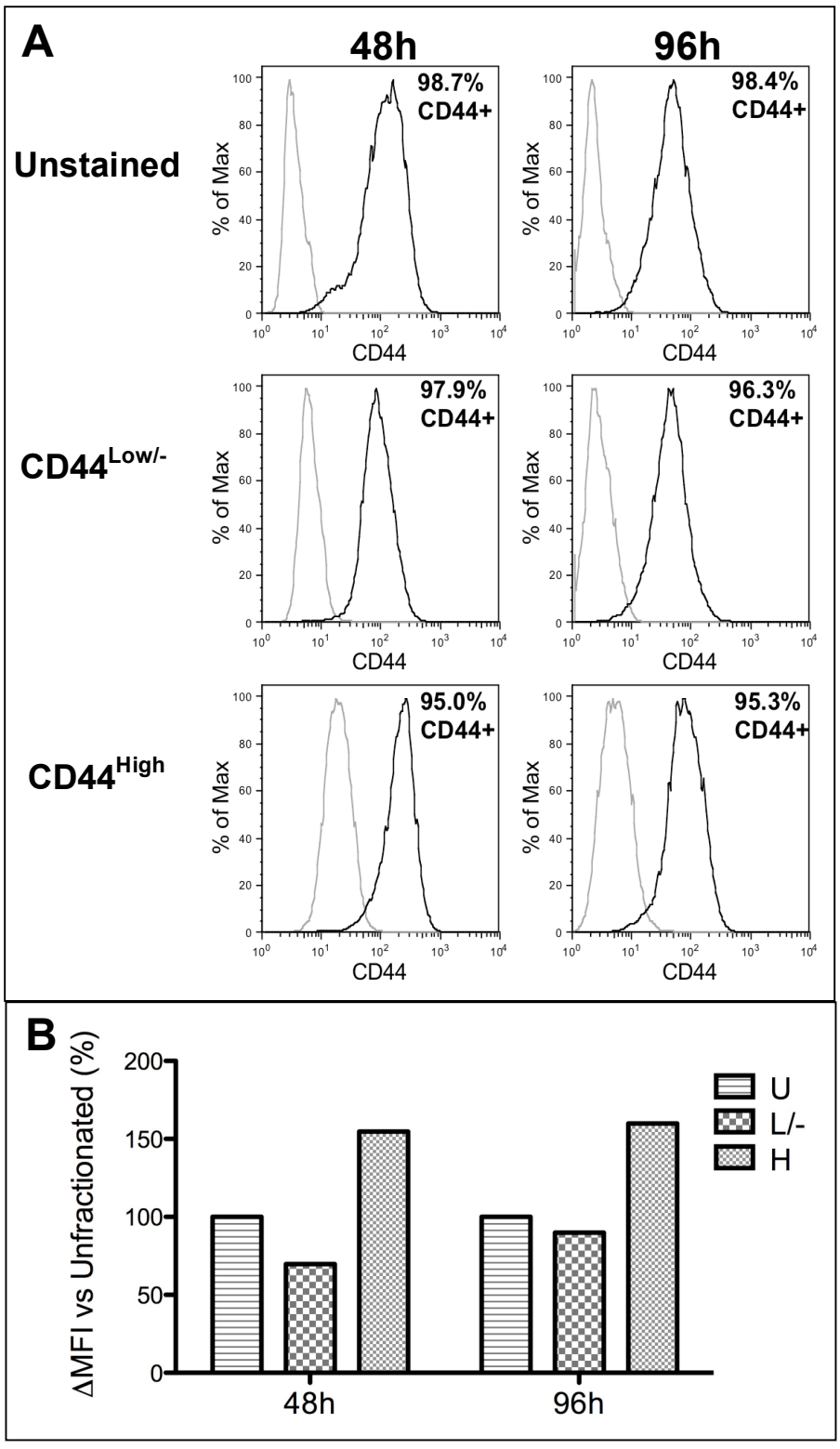


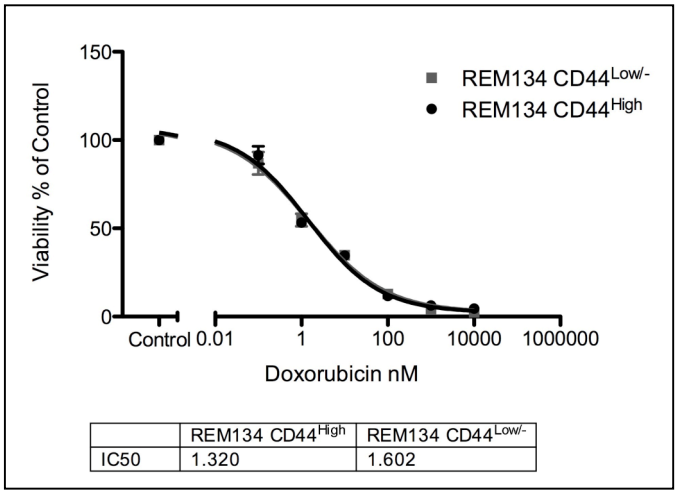
Figure 11 – A - Progeny of REM134 $CD44^{High}$ and $CD44^{Low/-}$ cells show a similar frequency of CD44 to unfractionated cells, when compared with a corresponding unstained aliquot. Black – CD44 stained aliquot; grey – unstained aliquot. B – ΔMFI (surface expression level) for $CD44^{Low/-}$ (L/-) and $CD44^{High}$ (H) progeny (relative to corresponding unstained aliquot), in comparison to progeny of unfractionated cells (U), after 48h and 96h in culture.

of both sorted CD44^{High} (95.3%) and CD44$^{Low/-}$ (96.3%) cells approximated that for unfractionated cells (98.4%), with less difference between the two sorted populations than at 48h (Figure 11).

Thus, whilst the progeny of cells expressing high levels of CD44 show an increased level of surface antigen after 96h in culture, both CD44^{High} and CD44$^{Low/-}$ cells recapitulate the frequency of expression seen within the parental line. Moreover, changes between the time of sorting and after culture suggest a return by both fractions towards the pattern of expression shown by the parental line.

REM134 CD44^{high} and CD44$^{low/-}$ Cells Show Equivalent Sensitivity to Doxorubicin

Sorted CD44$^{low/-}$ and CD44^{high} cells were assessed for sensitivity to the chemotherapy drug doxorubicin. Enhanced resistance to conventional chemotherapeutics has been demonstrated in putative CSC populations (Kruger *et al.*, 2006; Ma *et al.*, 2007; Gupta *et al.*, 2009b; Tanei *et al.*, 2009), including those identified according to CD44 expression (Fillmore and Kuperwasser, 2008; Takaishi *et al.*, 2009). Doxorubicin is an anthracycline antibiotic, which binds to DNA by intercalation to inhibit DNA synthesis, and also acts upon Topoisomerase II, leading to DNA strand scission (Minotti *et al*., 2004; Chun *et al*., 2007). However, the drug sensitivity curves for REM134 CD44^{high} and CD44$^{low/-}$ cells were similar, with no significant differences between the IC_{50} values (CD44$^{low/-}$ = 1.60nM, CD44^{high} 1.32nM; $p = 0.6105$) (Figure 12).

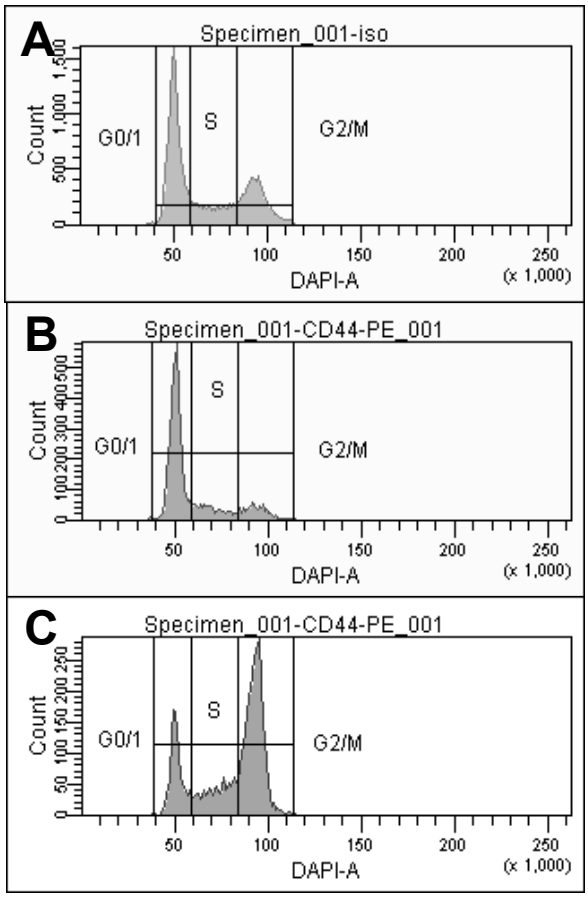


	REM134 CD44^{High}	REM134 CD44$^{Low/-}$
IC50	1.320	1.602

Figure 12 – Sensitivity curves for REM134 CD44^{High} and CD44$^{Low/-}$ cells treated with increasing concentrations of doxorubicin.

CD44 Expression and Cell Cycle Distribution in Canine Cancer Cell Lines

Simultaneous analysis of CD44 and DNA staining was undertaken for REM134 cells, and cell cycle profiles for unfractionated, $CD44^{low/-}$ and $CD44^{high}$ cells compared. There was a marked difference between the cell cycle distributions of $CD44^{low/-}$ and $CD44^{high}$ cells (Figure 13). The majority (70.6%) of $CD44^{low/-}$ cells were in G_0/G_1, with a much smaller proportion (13.0%) in the G_2/M phase of the cycle. The unfractionated sample also showed a preponderance of G_0/G_1 (53.2%) cells, with 28.2% in G_2/M phase. Conversely, 57.6% of $CD44^{high}$ cells were in G_2/M (cell division) phase of the cell cycle, as compared to only 22.2% in the resting/growth G_0/G_1 phase.



D

REM134 Subset	G_0/G_1 (%)	S (%)	G_2/M (%)
A Unfractionated	53.2	18.3	28.2
B $CD44^{Low/-}$	70.6	15.9	13.0
C $CD44^{High}$	22.2	20.1	57.6

E

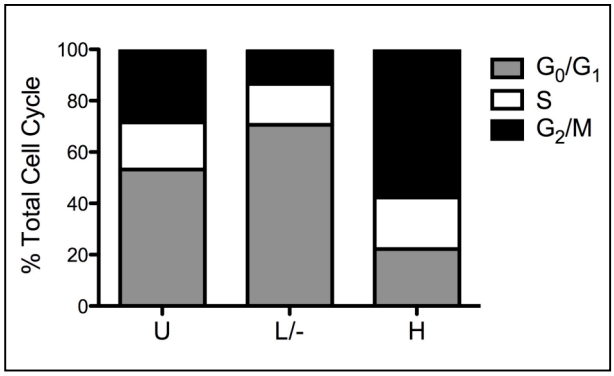


Figure 13 – Cell cycle distribution of unfractionated (A), B- $CD44^{Low/-}$ (B) and $CD44^{High}$ (C) REM134 cells, as assessed by DAPI dsDNA staining.
D – Table showing percentage of cells in each stage of the cell cycle.
E – Graph to illustrate proportion in each phase of the cell cycle for unfractionated (U), $CD44^{Low/-}$ (L/-) and $CD44^{High}$ (H) cells.

Analysis of CD44 expression level and cell cycle distribution was performed for the canine cancer cell lines D17 osteosarcoma and J3T glioma, the canine osteosarcoma-derived populations kt-osa1, kt-osa4 and kt-osa5, and for the feline mammary carcinoma cell line Cat-MT. As shown in figure 14, for all cell types there was a clear contrast between cell cycle profiles of $CD44^{low/-}$ and $CD44^{high}$ populations, with the former consistently demonstrating a greater proportion of cells in G_0/G_1, and the latter the G_2/M phase of the cycle. Data is summarised in Tables 1 and 2 and Figure 15.

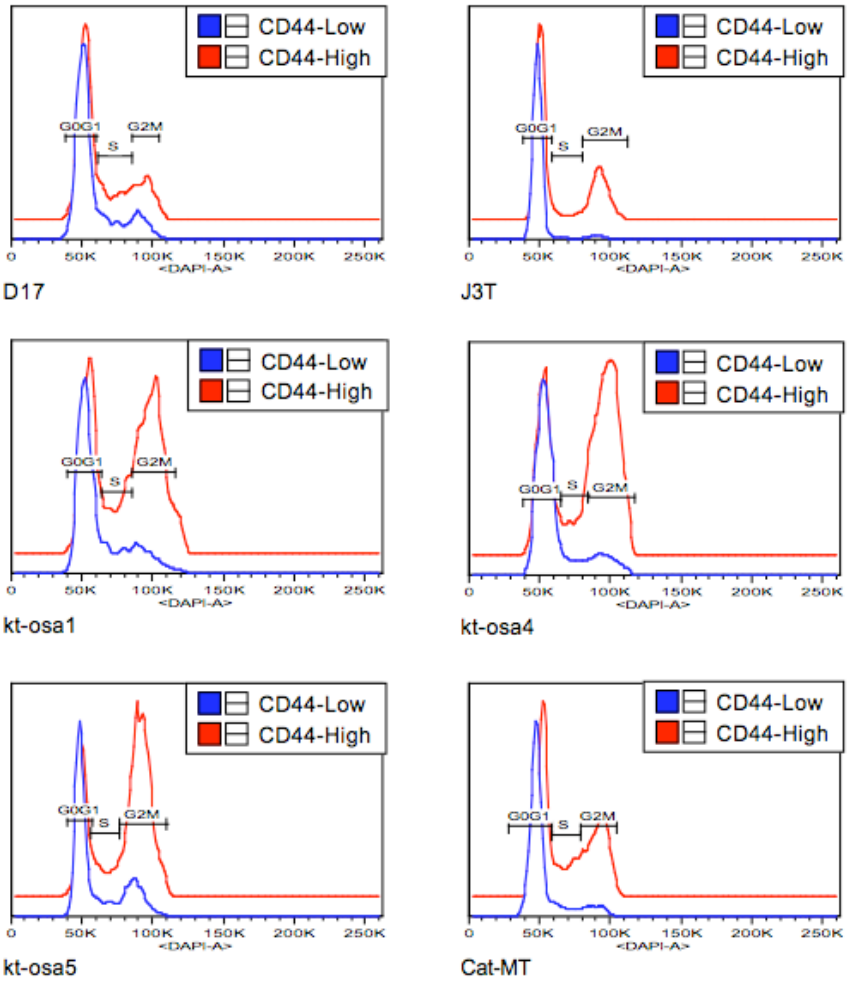


Figure 14 – Cell cycle profiles of $CD44^{Low}$ and $CD44^{High}$ cells for canine, and feline Cat-MT, cancer cell lines. Prevalence of G_2/M-phase cells greater in $CD44^{High}$ (red histogram) than $CD44^{Low}$ (blue histogram) fraction for all cell lines.

Cell Line	$CD44^{Low}$			$CD44^{High}$		
	G_0/G_1 (%)	S (%)	G_2/M (%)	G_0/G_1 (%)	S (%)	G_2/M (%)
REM134	70.6	15.9	13.0	22.2	20.1	57.6
D17	71.2	16.2	10.9	56.6	19.0	23.0
J3T	92.6	2.8	4.3	60.9	6.7	32.2
kt-osa1	64.8	19.1	15.8	28.4	19.6	51.6
kt-osa4	68.9	16.4	14.2	28.9	13.8	57.0
kt-osa5	60.2	19.2	20.0	24.2	17.6	57.7
CatMT	84.7	8.4	5.8	34.2	17.4	48.3

Table 1 – Frequency of cells in each phase of the cell cycle for $CD44^{Low}$ and $CD44^{High}$ fractions of canine cancer cell lines, and feline Cat-MT cells. All cell lines show increased proportion of G_0/G_1 cells in $CD44^{Low}$ fraction, and G_2/M cells in $CD44^{High}$ fraction.

Cell Line	G_0/G_1 $CD44^{Low}$:$CD44^{High}$	G_2/M $CD44^{High}$:$CD44^{Low}$
REM134	3.2	4.4
D17	1.3	2.1
J3T	1.5	7.5
kt-osa1	2.3	3.3
kt-osa4	2.4	4.0
kt-osa5	2.5	2.9
CatMT	2.5	8.3

Table 2 – Ratios demonstrating proportion of cells in G_0/G_1 and G_2/M phases of the cell cycle for $CD44^{Low}$ and $CD44^{High}$ fractions. $CD44^{Low}$ subset contains greater proportion of cells in resting/growth phase; $CD44^{High}$ subset shows increased proportion of dividing cells.

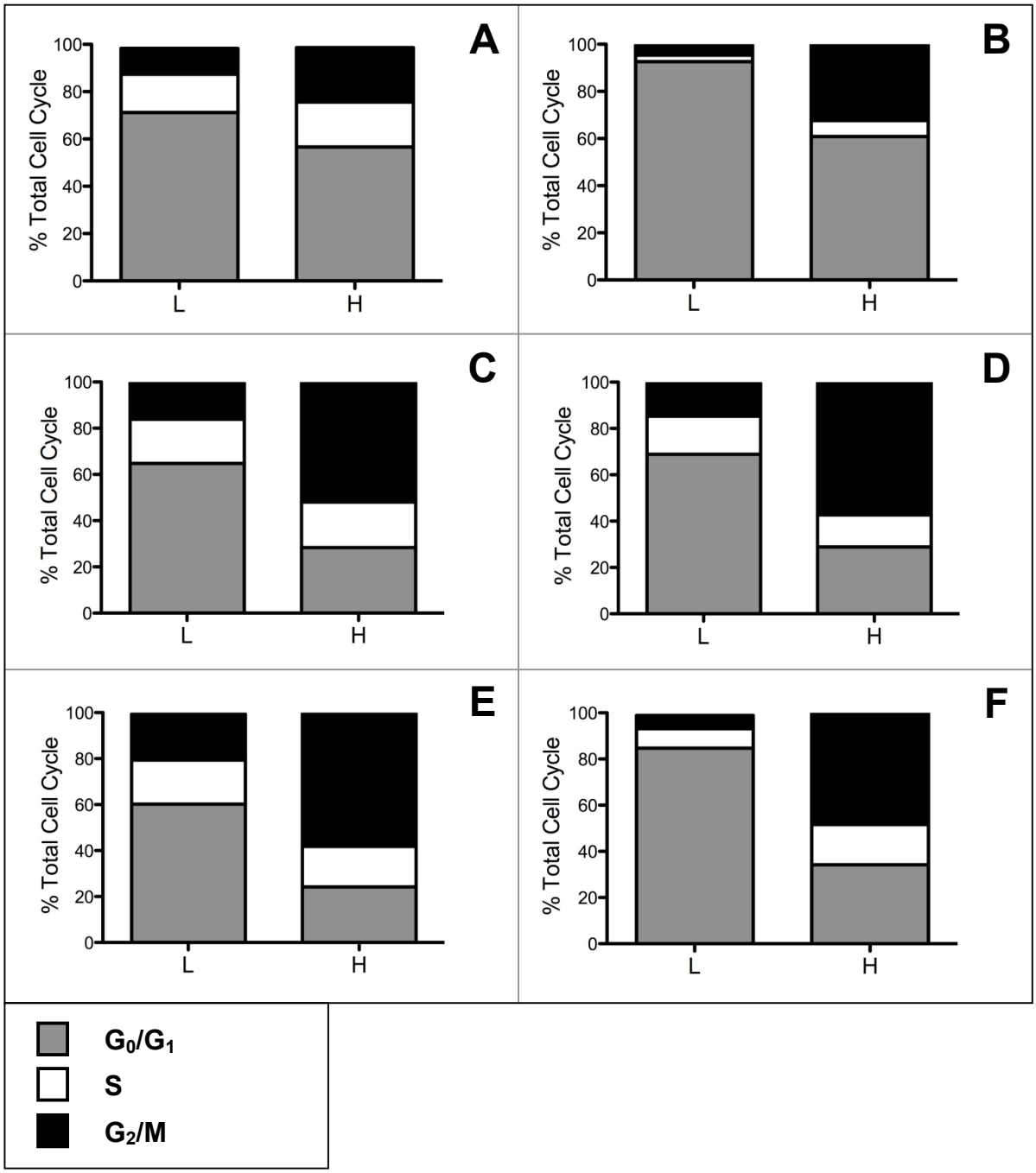


Figure 15 - Barcharts illustrating relative proportions of $CD44^{Low}$ and $CD44^{High}$ cells in phases of the cell cycle. L = $CD44^{Low}$; H = $CD44^{High}$.
A – D17; B – J3T; C – kt-osa1; D - kt-osa4; E – kt-osa5; F – Cat-MT.

For D17, although the $CD44^{High}$ fraction still showed a greater proportion of cells in G_0/G_1 than in G_2/M, it contained more than twice as many cells in the actively dividing phase than the $CD44^{Low/-}$ fraction (ratio G_2/M for $CD44^{High}$:$CD44^{Low/-}$ = 2.1:1). $CD44^{High}$ cells of J3T glioma and CatMT cell lines showed approximately eight times as many cells in this part of the cell cycle than $CD44^{Low/-}$ cells.

In the $CD44^{High}$ fraction of all cell lines other than D17 and J3T, the proportion of cells in G_2/M exceeded that in G_0/G_1. Distortion of the cell cycle profile due to the large G_2/M peak in these fractions precluded accurate determination of the cut-off between S- and G_2/M phase. However, the combined proportion of G_2/M and S-phase cells was always greater in the $CD44^{High}$ fraction. By contrast, in the $CD44^{Low/-}$ fraction of all populations, a majority of cells were in resting/growth phase (Table 2, Figure 15).

These results suggest that in cultured canine cancer cells, CD44 expression is associated with position in the cell cycle, with increased expression levels shown by proliferating cells, and lower levels by those in G_0/G_1 phase.

DISCUSSION

According to the cancer stem cell hypothesis, the elimination of specific cancer stem cell subpopulations with enhanced tumourigenic capacity is critical in preventing disease relapse. In dogs, as in humans, cancer therapy is confounded by the two major problems of relapse and metastatic spread; spontaneous cancer in the dog represents a valuable comparative model of human disease. Notwithstanding the debate surrounding the hypothesis, it is rational to try and identify and characterise markers identifying CSC, or clonally evolved subpopulations, which might possess enhanced malignant capabilities or persist after therapy. CD44 is a candidate for such investigation, as it has documented associations with processes involved in cancer progression, such as invasion, metastasis and drug resistance (East and Hart, 1993; Herrera-Gayol and Jothy, 1999b; Tzankov *et al.*, 2003; Zhang *et al.*, 2003; Marhaba and Zoller, 2004; Abraham *et al.*, 2005; Krause *et al.*, 2006; Sheridan *et al.*, 2006; Shipitsin *et al.*, 2007; Buess *et al.*, 2009).

All of the canine cancer cell populations examined in this study showed CD44 expression on >97% of cells, including those recently derived from a primary tumour. A similar staining pattern was seen in non-neoplastic canine bone marrow-derived stromal cells (data not shown). As the staining pattern did not distinguish a specific subpopulation of $CD44^{+}$ cells, the biological significance of CD44 expression was examined using flow cytometry to separate those cells with the highest surface levels from those with the lowest. It was considered that collecting cells from the extremes of the staining distribution, rather than using gates to bisect it, might reveal more clearly any contrasting characteristics associated with different expression levels.

Our investigations indicated that CD44 expression in cultured canine cells is associated with proliferative status and position in the cell cycle. The proportion of G_2/M cells in the $CD44^{High}$ fraction was consistently greater than that in the $CD44^{Low}$ fraction, which in turn showed a consistently larger proportion of cells in G_0/G_1 phase. Moreover, in five out of seven cell types examined, more of the cells in the

$CD44^{High}$ fraction were in G_2/M phase than in G_0/G_1. For two cell lines (D17, J3T) the proportion of cells in G_0/G_1 was greater than that in G_2/M for both fractions. Although aspects of culture technique, such as the level of confluence at which cells are harvested, may affect the balance between different phases of the cell cycle, the canine cells in this study were analysed at similar stages of exponential growth. It is likely that the growth characteristics of individual cell types influence the relationship between CD44 expression and proliferation.

Nonetheless, for all cell types, there were at least twice (and up to eight times) as many G_2/M cells in the $CD44^{high}$ than in the $CD44^{low/-}$ fraction. Accordingly, REM134 $CD44^{High}$ cells proliferated more readily in culture soon after plating than $CD44^{Low/-}$ cells, and showed superior colony forming ability and tumoursphere formation. In the absence of cell cycle data to account for these differences in biological behaviour, the latter observations in particular could be interpreted as suggestive of CSC identity.

This association of CD44 with proliferative status is consistent with the observed downregulation CD44 observed when REM134 mammary carcinoma cells are cultured as tumourspheres, where proliferation is likely to be less synchronous, and the proportion of quiescent and apoptotic cells is greater than in corresponding monolayer cultures (Kansal *et al.*, 2000). A similar observation was made by Chiou *et al*, who described downregulation of CD44 on tumourspheres derived from high grade human oral squamous cell carcinomas (Chiou *et al.*, 2008).

In the literature, other markers for candidate CSC populations, such as CD133, have been associated with proliferation status in cell lines (Chen *et al.*, 2008; Tirino *et al.*, 2008). Whilst some investigators have reported an association between CD44 expression and cellular proliferation (Alho and Underhill, 1989; Abbasi *et al.*, 1993), others have found only partial (Fukuse *et al.*, 1999) or no concordance (Furuta *et al.*, 1996) between CD44 and the proliferation marker PCNA.

Al-Hajj *et al* showed no difference in cell cycle status between the tumourigenic and non-tumourigenic subpopulations isolated from one of the specimens described in their report on breast cancer stem cells (Al-Hajj *et al.*, 2003). Moreover, Buess *et al* found that expression of M-phase cell cycle genes was actually decreased in the $CD44^{+}CD24^{-}$ fraction of ex-vivo breast tumour cultures as compared to the $CD44^{-}CD24^{+}$ fraction (Buess *et al.*, 2009). Slower cell cycle kinetics were demonstrated in the $CD44^{+}CD24^{-}$ fraction of human breast and prostatic tumour cell lines (Fillmore and Kuperwasser, 2008; Hurt *et al.*, 2008).

According to the cancer stem cell hypothesis, the CSC can give rise to cells with the phenotypic heterogeneity seen in the parental population, whereas non-CSC do not have this capacity. Thus, cells within a tumourigenic subpopulation expressing a CSC-specific marker should be capable of differentiating into non-cancer stem cells negative for that marker, but not vice versa.

$$x^{+} \Rightarrow x^{+} \text{ and } x^{-} \text{ whereas } x^{-} \Rightarrow x^{-}$$

Although REM134 $CD44^{high}$ cells (a greater proportion of which are in the process of active proliferation) demonstrate enhanced colony forming ability when seeded at very low densities, both $CD44^{low/-}$ and $CD44^{high}$ cells return to the frequency distribution seen for unfractionated cells after a short period at standard culture densities. Zheng *et al* demonstrated a similar effect when C6 rat glioma cells were sorted for the putative CSC marker CD133 (Zheng *et al.*, 2007). Although the relative MFI, representative of the level of antigen expression, remained greater for rapidly proliferating $CD44^{High}$ cells, the $CD44^{Low/-}$ fraction gave rise to progeny whose surface expression level increased towards that of the bulk population with time in culture. This was accompanied by increased proliferative potential after initial poor growth. This suggests that the level of antigen expression on the $CD44^{Low/-}$ subpopulation was related to their proliferative status at the time of isolation, rather than to a putatively "more differentiated" non-CSC status. This is further supported by the results from the drug sensitivity assay, where no resistance advantage was associated with high CD44 expression levels.

Unless expression of a given cancer stem cell marker is stable, its relevance in the context of the CSC hypothesis is questionable (Visvader and Lindeman, 2008; Gupta *et al.*, 2009a). If an individual cell may oscillate rapidly between "CSC" and "non-CSC" phenotypes, it is difficult to see how the marker used will reliably identify a specific subpopulation of tumourigenic cells, as opposed to those which are more likely to survive or proliferate at the time of a particular assay.

This applies particularly for cell lines, ostensibly clonal entities, if certain subpopulations are to be defined as the "cancer stem cells" within a putative hierarchy (Kondo *et al.*, 2004; Setoguchi *et al.*, 2004; Patrawala *et al.*, 2005; Hadnagy *et al.*, 2006; Atsumi *et al.*, 2008; Fillmore and Kuperwasser, 2008; Huang *et al.*, 2009a). In a clonal population, non-genetic heterogeneity for a given antigen may arise through fluctuation of surface expression levels around the mean (Chang *et al.*, 2008; Brock *et al.*, 2009). Whilst this may alter the context-dependent fate of a cell, it may not equate to that cell being biologically distinct, in terms of the definitive CSC properties of self-renewal and tissue-specific differentiation potential. It is significant that, as $CD44^{high}$ cells demonstrate such enhanced growth capabilities in *in vitro* assays, these might have translated to produce apparently enhanced tumourigenicity in "gold standard" *in vivo* transplantation assays in immunocompromised rodents, through the effective selection of a population of more actively proliferating cells at a given moment in time.

The results of the assay in which sorted $CD44^{high}$ and $CD44^{low}$ cells were cultured and restained suggest that neither population is necessarily destined to give rise to progeny with a particular proliferative tendency or surface expression pattern, but rather that CD44 may be more heavily expressed by any cell when it is proliferating. Similarly for tumoursphere formation, it seems rational that actively dividing cells might more successfully survive when plated in low-density conditions, whilst those plated during a resting phase might senesce, particularly if cell-cell contact is important for successful proliferation. Put simplistically, a cell undergoing division, which finds itself in low-density culture, is likely to have at least one other cell to

support it soon after plating. As discussed in a balanced review by Shackleton *et al*, it is important to distinguish cellular potential from context-dependent fate when inferring cancer stem cell identity (Shackleton *et al.*, 2009).

As discussed in Chapter 4, the tumoursphere assay was developed for primary neural stem cell culture (Reynolds *et al.*, 1992; Reynolds and Weiss, 1992), and there exist some fundamental reservations regarding its interpretation and validity when applied to other systems, including cancer cell lines (Singec *et al.*, 2006; Zheng *et al.*, 2007; Shipitsin and Polyak, 2008). A marked density-dependence of proliferation is seen for several canine cancer cell lines when grown under these conditions, including REM134, in limiting dilution assays. It has also been observed that many canine cell lines form tumourspheres more readily from subconfluent cultures, still in rapid exponential expansion, than from those closer to confluence.

This suggests that, for some canine cancer cell lines, tumoursphere formation is not truly reflecting cell-autonomous growth by a specific CSC population. As a cell adhesion molecule, it is possible that increased surface expression levels of CD44 contribute to the superior performance of $CD44^{High}$ REM134 cells in assays such as colony or tumoursphere formation, by promoting intercellular interactions.

The transition of individual cells between phases of the cell cycle is likely to occur more rapidly in an immortal cell line, and at more regular intervals, than within a naturally occurring tumour. Whilst *in vitro* manipulations such as subculture and medium changes will also have a significant influence, this does not preclude the value of cell lines for investigating characteristics associated with even transiently expressed phenotypes, which might be more stable in a tumour *in vivo*.

However, it would seem important to evaluate this data parsimoniously, to prevent the inference of "cancer stem-ness" where more basic biological reasons might account for observed cellular behaviour. For example, whilst there were no significant differences between the sensitivities of REM134 $CD44^{high}$ and $CD44^{low}$ cells to doxorubicin, it is not considered to be a cell cycle-specific agent. Where

response to chemotherapeutic drugs is used to assess the sensitivity of a putative CSC population, agents such as plant alkaloids or antimetabolites (Todaro *et al.*, 2007; Fillmore and Kuperwasser, 2008; Zhou *et al.*, 2008; Gupta *et al.*, 2009b) might be expected to show differential cytotoxicity if the CSC marker is associated with a particular phase of the cell cycle.

It is interesting that all cultured canine cells in this study robustly expressed CD44. If CD44-mediated intercellular interactions actively promote proliferation (East and Hart, 1993), the combination of cellular proximity and frequent division within a cell line may sustain overall expression at a level higher than that in the natural situation. A limitation of this study is that all of the cells analysed had undergone at least initial expansion *in vitro*. Further studies are therefore indicated using directly dissociated primary samples, to assess the relevance of CD44 *in vivo*.

Also, whilst studies to evaluate the significance of CD44 expression in canine spontaneous mammary tumours have been inconclusive, these have employed either the IM7 clone used here (which does not discriminate between standard and variant forms) or antibodies which recognise only CD44s (Madrazo *et al.*, 2009; Paltian *et al.*, 2009). In some human tumours including breast carcinomas, CD44 (particularly variant isoforms) has been localised to proliferative areas, such as the colonic crypts or the invasive tumour front (Abbasi *et al.*, 1993; Herrera-Gayol and Jothy, 1999a). Immunohistochemical or transcriptional analysis discriminating CD44s from its variants might provide further insight into the implications of expression in canine cancer.

The results of this study suggest that CD44, if stably expressed by specific cells in spontaneous tumours, might be associated with enhanced proliferative potential, in which case it may yet identify cancer stem or dominant tumour cell populations. If so, it represents a potential therapeutic target. Strategies to disrupt the molecule such as anti-CD44 antibodies could readily be developed and tested *in vitro* on canine cancer cell lines and then primary cells (Jin *et al.*, 2006). Additionally, high levels of CD44 might indicate more rapidly growing tumours and provide valuable prognostic

information. It would be important to distinguish infiltrating leukocytes, likely to express CD44v, from tumour cells, and this could be achieved using flow cytometric gating to exclude $CD45^+$ cells. Examination of other markers in conjunction with CD44, such as CD24 or integrins, might select for a subset of cells with additional tumourigenic attributes, particularly in primary tumours where there is likely to be a greater diversity of cellular phenotypes.

In summary, expression of CD44 by canine cancer cell lines is associated with proliferative status. Although this results in the enrichment within the $CD44^{high}$ fraction of cells showing apparent cancer stem cell properties, transient and fluctuating expression means that CD44 cannot be considered a *bona fide* cancer stem cell marker in the canine cell lines examined here, as it does not distinguish a specific cellular subpopulation. Further studies of CD44 expression, particularly in primary tumours and metastases, should help to define whether CD44 expression identifies CSC or subpopulations of tumour cells with enhanced neoplastic characteristics, and may help to elucidate its role in canine cancer progression and metastasis.

CHAPTER 6

CANCER STEM CELLS AND

DRUG RESISTANCE

INTRODUCTION

Anticancer drug development is a huge industry, with an ever-increasing array of chemotherapy agents available. Within the clinical setting, however, elimination of cancer to produce complete remissions is all too frequently the exception rather than the rule. Conventional cytotoxic agents, still the mainstay of most cancer chemotherapy protocols, target rapidly proliferating cells. Whilst these are highly effective in some cases, drug resistance (both inherent and acquired) frequently leads to eventual treatment failure and relapse. Moreover, off-target effects on susceptible normal tissues such as epithelia, neural and haematopoietic cells result in dose-limiting toxicities, morbidity and mortality.

Recent years have seen the development of an increasing number of targetted therapies, such as small molecule tyrosine kinase inhibitors and monoclonal antibodies, used as single agents or as part of combination chemotherapy regimes. Although these often produce fewer adverse effects, careful patient selection is often required to see clinical benefits, and overall survival times in many cases have shown little improvement (Sandler *et al.*, 2006; Heinemann *et al.*, 2008; Burris, 2009; Yan *et al.*, 2009). Moreover, resistance is seen to these agents also (Stegmeier *et al.*, 2010). For example, the Bcr-Abl tyrosine kinase inhibitor imatinib mesylate (Gleevec, Novartis) produces excellent clinical responses in chronic myeloid leukaemia. However, cells carrying the Bcr-Abl fusion protein persist even in patients showing clinical remission, leading to the prospect of recurrence upon withdrawal of treatment (Holyoake *et al.*, 1999; Bhatia *et al.*, 2003; Misaghian *et al.*, 2009).

Thus the cancer stem cell hypothesis, where applicable, has very important implications for the clinical approach to cancer. The model can account for tumour recurrence despite apparently good initial responses to conventional therapy, but also makes clear that if CSC are responsible for residual disease, they must be eliminated to afford a cure. Moreover, more precise targetting of these cells might help to avoid some of the more generalised adverse effects of antiproliferative agents, reducing

patient morbidity. Even if CSC are more resilient in the face of treatment than the bulk tumour cell population, it may be possible to exploit their inherent resistance mechanisms as a means of selectively eradicating them.

NFκB Signalling as a Resistance Mechanism – and Potential Drug Target

The NFκB (Nuclear Factor kappa-light-chain enhancer of activated B cells) family of transcription factors comprises five proteins - RelA (p65), RelB, c-Rel, NFκB1 (p105/p50) and NFκB2 (p100/p52) – each with a common 300-amino acid Rel homology domain (RHD). NFκB1 and NFκB2 are synthesised as large precursors (p105 and p100, respectively) which are post-translationally cleaved to the active p50 and p52 forms by an ATP-dependent process of polyubiquitinylation and proteasomal degradation (Ghosh *et al.*, 1998; Ghosh and Karin, 2002; Packham, 2008).

NFκB was first characterised in B lymphocytes but is expressed ubiquitously in eukaryotic cells. It is largely held in the cytoplasm in latent (inactive) form by proteins of the IκB family, whose n-terminal ankyrin repeat regions mask the nuclear localisation signal of NFκB. Removal of the IκB-mediated repression allows translocation of NFκB to the nucleus, where it activates target genes. This is achieved through phosphorylation of IκB by the upstream kinase IκB-kinase, IKK, leading to ubiquitin-dependent proteasomal degradation of IκB and the release of NFκB (Ghosh and Karin, 2002; Ravi and Bedi, 2004). Induction of NFκB-mediated signalling therefore does not require new protein synthesis, permitting rapid transmission of signals from the cytoplasm to the nucleus.

NFκB proteins function as homo- and heterodimers, functioning on the most part as transcriptional activators, although p50 and p52 homodimers act as transcriptional repressors. Activation occurs through either the “canonical” or the “alternative” (“noncanonical”) pathway, the former involving primarily RelA-p50 heterodimers and the latter, RelB-p52 heterodimers. Canonical signalling is mediated through activation of the IKK complex, comprising IKKα, IKKβ and IKKγ subunits, of

which IKKβ is most significant in this context. The alternative pathway is activated by IKKα (Figure 1). Target genes are involved in a diverse array of cellular processes such as inflammation and immunoregulation (e.g. IL-2, IL-6, TLR-2), cell cycle progression (e.g. cyclins D1 and D2, c-myc), cell survival (e.g. the anti-apoptotic mediators Bcl-2 and Bcl-XL), angiogenesis (e.g. VEGF, Cox-2) and drug efflux (MDR-1) (Zhou and Kuo, 1997; Lee *et al.*, 1999; Hideshima *et al.*, 2002; Bentires-Alj *et al.*, 2003; Ravi and Bedi, 2004; Keats *et al.*, 2007; Packham, 2008).

Aberrant activation of NFκB signalling occurs in many haematological and solid malignancies, including B- and T-lymphocytic and myeloid leukaemia, Hodgkins and non-Hodgkins lymphoma, multiple myeloma, melanoma, breast, colon and prostate cancers (Rayet and Gelinas, 1999; Greten and Karin, 2004; Keats *et al.*, 2007; Compagno *et al.*, 2009; Lee *et al.*, 2009; Pratt *et al.*, 2009; Zheng *et al.*, 2009). Genetic alterations such as amplification, mutation and chromosomal translocation may promote NFκB or IKK activity, or inhibit the repressive function of IκB proteins, and may affect both canonical and alternative pathways. Additionally, many other signalling pathways dysregulated in cancer, such as those involving Notch, Ras, PTEN/PI3k/Akt, STAT3 and E2F1, interact with NFκB to promote its transcriptional activity (Mayo *et al.*, 1997; Romashkova and Makarov, 1999; Ravi and Bedi, 2004; Barbie *et al.*, 2009; Downward, 2009; Lee *et al.*, 2009; Zheng *et al.*, 2009). Constitutive activation of the pathway may also result from autocrine stimulation of cell surface receptors by growth factors, or the activity of viral oncoproteins such as the Tax protein of Human T-cell Leukaemia virus (HTLV-1) and the EBNA-1 protein of Epstein-Barr virus (Ravi and Bedi, 2004; Packham, 2008).

NFκB signalling in neoplasia promotes uncontrolled cellular growth and proliferation through its activation of antiapoptotic and prosurvival mediators, and its promotion of the cell cycle; proangiogenic effects may also promote invasion and

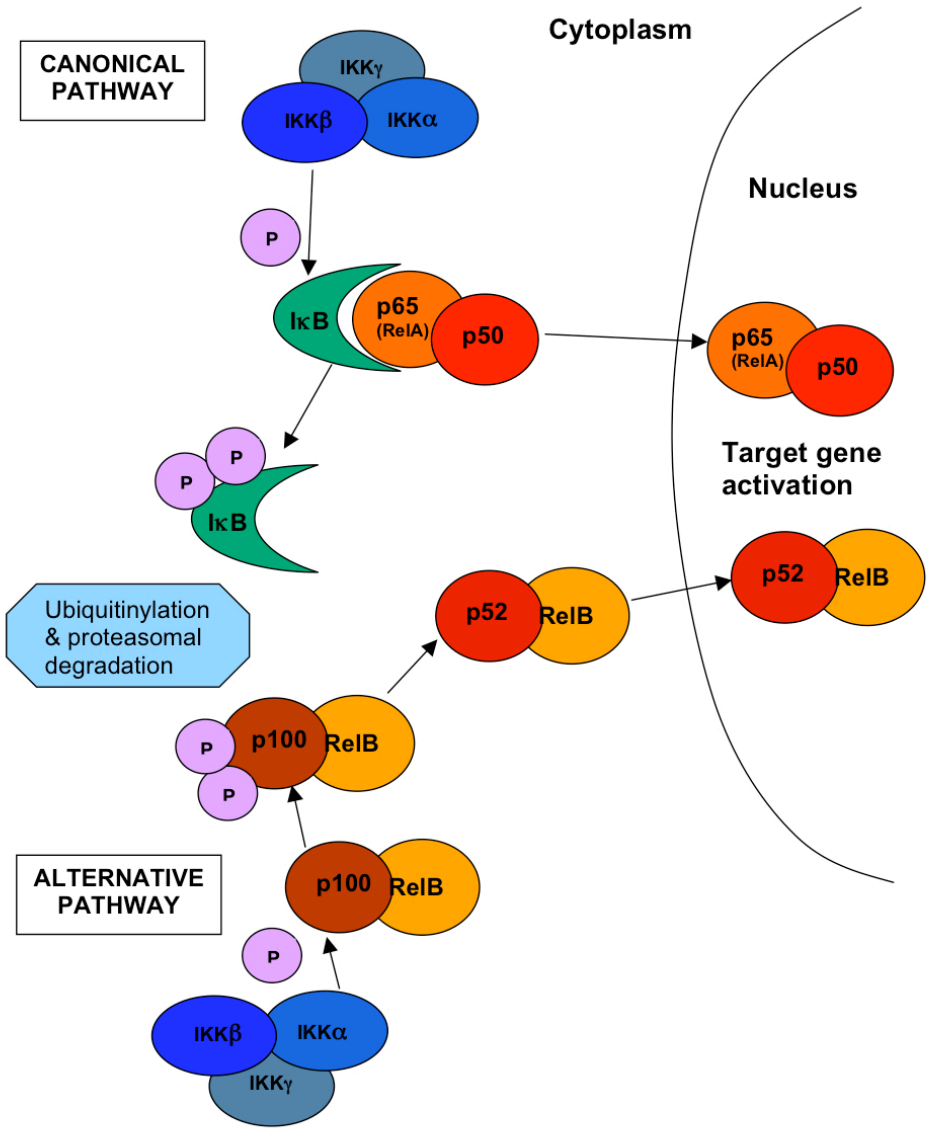


Figure 1 – Canonical and alternative pathways for activation of NFκB-mediated gene transcription.
The canonical pathway involves phosphorylation, with ubiquitinylation and proteasomal degradation, of the IκB repressor by the IKK complex (primarily IKKβ). This releases NFκB (primarily p65/RelA-p50 heterodimers) for translocation to the nucleus and activation of target genes.
The alternative pathway is initiated through IKKα-mediated phosphorylation of p100, with resultant proteasomal degradation to p52. RelB-p52 heterodimers translocate to the nucleus to activate target genes.

metastasis (Rayet and Gelinas, 1999; Darnell, 2002; Greten and Karin, 2004). NFκB also interferes with the activity of the p53 tumour suppressor and upregulates its negative regulator MDM2 (Tergaonkar *et al.*, 2002). Activation of NFκB signalling thus confers increased resistance to the induction of apoptosis by anticancer agents, and may also directly upregulate drug resistance mechanisms such as the MDR1 drug transporter pump (Zhou and Kuo, 1997; Bentires-Alj *et al.*, 2003).

Critically, exposure to chemotherapy drugs including taxanes, vinca alkaloids, anthracyclines and topoisomerase inhibitors may induce NFκB activity. Individual drugs may activate signalling through effects on different points in the pathway – for example, doxorubicin may directly mobilise the IKK complex (Bottero *et al.*, 2003), but can also promote IKK-independent degradation of IκB (Das and White, 1997; Tergaonkar *et al.*, 2003). Thus, induction of NFκB signalling plays a significant role in the development of resistance to chemotherapy (Nakanishi and Toi, 2005).

NFkB Inhibition as a Therapeutic Strategy

The NFκB pathway thus represents a rational therapeutic target, as inhibition may not only slow the progression of disease but also increase the sensitivity of neoplastic cells to proapoptotic therapies. Mechanisms of intervention include specific inhibitors targetting IKK or NFκB, proteasome inhibitors (which prevent the degradation of IκB or p105 / p100 processing) and siRNA knockdown strategies, with specific targets dependent on which arm of the pathway (canonical / alternative / both) is active. For example, in a study of Barrett's Oesophagus cell lines, resistance to radiation-induced apoptosis was circumvented using siRNA specific for RelA, indicating that this resistance was mediated through activation of the canonical pathway (Hormi-Carver *et al.*, 2009). Conversely, the alternative pathway may play a role in some aggressive prostatic cancers, and Xu *et al* reported that siRNA knockdown of RelB decreased the tumourigenicity of PC-3 prostatic carcinoma cells (Xu *et al.*, 2009).

Importantly, however, the complex interactions between different NFκB family members and those between NFκB and other regulatory processes must be considered. For example, canonical NFκB pathway activation is a feature of all multiple myeloma (MM) cell lines - some also show alternative pathway activity. Hideshima *et al* demonstrated that whilst inhibition of either IKKβ (canonical) or IKKα (alternative) inhibited cellular growth, only IKKβ blockade was associated with reduced activation of NFκB (Hideshima *et al.*, 2009a). This suggests IKKα may have alternative targets in this context, or that there is crosstalk between the pathways.

Similarly, the proteasome inhibitor Bortezomib is licensed for use in MM, and has shown good clinical efficacy. Through inhibiting degradation of the IκB repressor, and processing of p105 or p100, the drug may inhibit activity of both the canonical pathway and the alternative pathway (Richardson *et al.*, 2006; Keats *et al.*, 2007; Packham, 2008). Notably, however, the drug produces greater growth inhibition than that achieved through specific NFκB blockade in MM cells. Moreover, recent evidence has suggested that bortezomib may in fact induce activation of canonical NFκB signalling in some MM cell lines and tumour cells, indicating that interference with other (NFκB-independent) mechanisms mediated by the proteasome contributes to its antitumour activity (Hideshima *et al.*, 2002; Nakanishi and Toi, 2005; Richardson *et al.*, 2006; Hideshima *et al.*, 2009b).

Proteasome inhibitors and other suppressors of NFκB signalling may also reverse NFκB-mediated chemoresistance, showing co-operative effects with standard therapies (Mabuchi *et al.*, 2004; Nakanishi and Toi, 2005; Packham, 2008). Critically, as chemotherapy drugs may induce NFκB activation, inhibition of the pathway may offset this to restore sensitivity to their cytotoxic effects (Cusack *et al.*, 2001; Avellino *et al.*, 2005; Nakanishi and Toi, 2005). For example, Cusack *et al* reported that pre-treatment of human colorectal cancer cells with bortezomib inhibited the induction of NFκB by SN-38 (a metabolite of the topoisomerase-1 inhibitor irinotecan), significantly enhancing its growth inhibitory effects *in vitro*. Accordingly, in a xenograft model *in vivo*, irinotecan alone led to 48% reduction in

growth of human colon tumours whereas when combined with bortezomib, the reduction was 94% (Cusack *et al.*, 2001). Inhibition of the pathway may also reduce protective effects of the tumour niche, by blocking NFκB-mediated cytokine release (Hideshima *et al.*, 2002).

It is important to note that, because of the complex interactions between NFκB and other signalling pathways, the consequences of inhibition are context dependent and will not necessarily produce an anticancer effect. For example, in lung cancer cell lines and a murine lung cancer model expressing oncogenic Ras, inhibition of NFκB led to apoptosis, and reduced tumourigenicity (Meylan *et al.*, 2009). By contrast, Dajee *et al* found that in human epidermis (grown as xenografts in murine hosts), blockade of NFκB signalling in combination with oncogenic Ras led to the formation of large tumours (Dajee *et al.*, 2003).

Can NFκB Activity be Exploited to Eradicate CSC?

Data suggests that NFκB activation may occur as an early event preceding malignant transformation, in diverse cell types including mammary epithelium and mesenchymal stem cells (Rayet and Gelinas, 1999; Izadpanah *et al.*, 2008). Certainly, the pre-existence of an anti-apoptotic mechanism within a cell makes it hypothetically an attractive target for transformation, and could also facilitate the persistence of a resistant population of tumourigenic cells.

There is growing interest in the NFκB pathway as a means of selectively targetting cancer stem cells. NFκB interacts with a number of stem cell-associated signalling processes, including Nanog, STAT3 and Wnt/β-catenin (Torres and Watt, 2008; Hideshima *et al.*, 2009a). The $CD34^+$ progenitors of human acute myeloid leukaemia (AML) show constitutively active NFκB signalling – by contrast, unstimulated normal $CD34^+$ haematopoietic progenitors do not express NFκB (Guzman *et al.*, 2001; Majeti *et al.*, 2009).

NFκB inhibition to target LSC in acute myeloid leukaemia

A series of reports from Guzman *et al* provided compelling evidence that NFκB inhibition in AML may selectively target leukaemia stem cells (LSC) whilst sparing normal haematopoietic progenitors. The proteasome inhibitor MG132 induced apoptosis in $CD34^+$ LSC but showed little inhibition of normal $CD34^+$ cells (Guzman *et al.*, 2001). Moreover, a complementary effect was seen with the anthracycline idarubicin – at levels where the individual toxicity of each drug was relatively low, the combination resulted in almost 90% cell kill. Induction of apoptosis was also mediated by upregulation of p53-regulated genes such as Bax and p21 (Guzman *et al.*, 2002). A similar selectivity towards LSC was reported for the IKK inhibitor parthenolide (Guzman *et al.*, 2005), and its orally bioavailable dimethylamino analogue DMAPT (Guzman *et al.*, 2007).

As part of the DMAPT study, the authors assessed the *in vitro* and *in vivo* activity of the drug in spontaneous canine leukaemias (Guzman *et al.*, 2007). Isolated mononuclear cells of seven of eight canine leukaemia samples tested positive for NFκB activation, and exposure to DMAPT *in vitro* reduced both NFκB activity and cell survival. Three dogs with $CD34^+$ (≥40% of PBMNC) spontaneous leukaemia were treated with intravenous and/or oral DMAPT. Results showed reduced numbers of $CD34^+$ progenitors and an increase in circulating neutrophils, suggesting an increase in the proportion of more differentiated cells (although circulating WBC numbers showed no clear reduction). Significantly, immunofluorescent staining of blast cells for NFκB suggested a reduction in expression levels. Although overall clinical outcomes in this study were poor, the dogs were in advanced disease or had extremely high circulating WBC at the point where treatment began ($\leq 96.5 \times 10^9$/l). The reported reduction in the $CD34^+$ progenitor population and expression of NFκB by circulating blasts suggests that concomitant treatment with conventional agents (with activity against the main population of blasts) might prove more successful when treating this extremely aggressive canine disease (Guzman *et al.*, 2007).

NFκB inhibition to target CSC in breast cancer

Studies by Zhou *et al* have shown preferential inhibition of putative breast CSC by NFκB inhibitors (Zhou *et al.*, 2008; Zhou *et al.*, 2009b). Tumoursphere and side population cells derived from the human MCF-7 breast carcinoma cell line showed greater reduction in viability than the bulk (unselected) populations upon treatment *in vitro* with NFκB inhibitors; by contrast, sphere and SP cells were more resistant to the conventional chemotherapy drug paclitaxel than the majority population. Treatment of mice inoculated with MCF-7 cells using a combination of the NFκB inhibitor PDTC and paclitaxel led to greater inhibition of tumour formation than either drug given alone, although it should be noted that the results suggested additive (rather than synergistic) drug effects (Zhou *et al.*, 2008).

Similarly, the small molecule 8-quinolinol (8-Q) showed preferential inhibitory activity against a highly-selected population of putative MCF-7 CSC (tumourspheres derived from SP). Notably, 8-Q-mediated inhibition of NFκB activity was similar for both bulk and sphere cells in this study, suggesting alternative mechanisms of action contribute to the drug's CSC selectivity. *In vivo,* the combined effect of 8-Q and paclitaxel against tumour formation by bulk MCF-7 cells was again greater than that of either drug alone (Zhou *et al.*, 2009b). Thus, it may be possible to exploit the inherent resistance mechanisms of CSC to produce better clinical outcomes.

CSC as a Moving Target – are Stem Cell-Associated Resistance Mechanisms Inherent or Acquired?

Consistent with their long lifespan, normal stem cells show several features which render them inherently less sensitive to toxic insults, such as relative quiescence, resistance to apoptosis, enhanced DNA repair capacity and expression of membrane transporters and intracellular mediators which permit elimination or degradation of toxic substances. If a CSC arises through *bona fide* transformation of a normal stem cell, it might be expected to have similar capabilities (arguably, of course, some of these features might equally be expected to reduce the susceptibility to

transformation in the first place). Where a cancer stem cell has arisen through dedifferentiation of a more committed cell, the situation is less clear, although the hypothesis is not inconsistent with the acquisition of traits conveying a growth advantage, through multistage transformation.

Although these properties are cited to explain their persistence, not all are definitively associated with CSC. For example, quiescence has long been appreciated as a factor in resistance (Weisenthal and Lippman, 1985; Secchi, 1990), with resting G_0 cells recognised as surviving drug and radiation treatment. However, whilst the putative CSC populations in some studies are slow-cycling or show an increased G_0 population (Holyoake *et al.*, 1999; Guzman *et al.*, 2002; Hadnagy *et al.*, 2006; Szotek *et al.*, 2006; Addla *et al.*, 2008; Hurt *et al.*, 2008), others show more rapid proliferation (Loebinger *et al.*, 2008; Moserle *et al.*, 2008; Tirino *et al.*, 2008).

Similarly, whilst expression of membrane transporters such as ABCG2 and P-gP is inherent to some stem cell populations, there is not a direct correlation between expression of these drug efflux pumps and CSC identity, or with the presence of an SP profile (Chaudhary and Roninson, 1991; Zhou *et al.*, 2001; Scharenberg *et al.*, 2002; Triel *et al.*, 2004; Patrawala *et al.*, 2005; Stingl *et al.*, 2006; Addla *et al.*, 2008; Lichtenauer *et al.*, 2008). The acquisition of multidrug resistance following cytotoxic chemotherapy is a well-recognised phenomenon in cancer, and a major clinical problem (Licht *et al.*, 1994; Heenan *et al.*, 1997; Doyle *et al.*, 1998; Steingold *et al.*, 1998; Abolhoda *et al.*, 1999; Wuchter *et al.*, 2000). Classically this has been considered to result from the selection of cells which have undergone genetic changes, such as upregulation of membrane transporters, allowing them to survive drug treatment and repopulate the tumour with resistant clones (Figure 2) (Dean *et al.*, 2005).

Alternatively, CSC which already possess such attributes will be inherently resistant and so capable of both self-renewal and recapitulation of progeny populations. There may be combination of processes at work, whereby CSC evolve further evasive mechanisms in the face of toxic challenge, leading to the development of

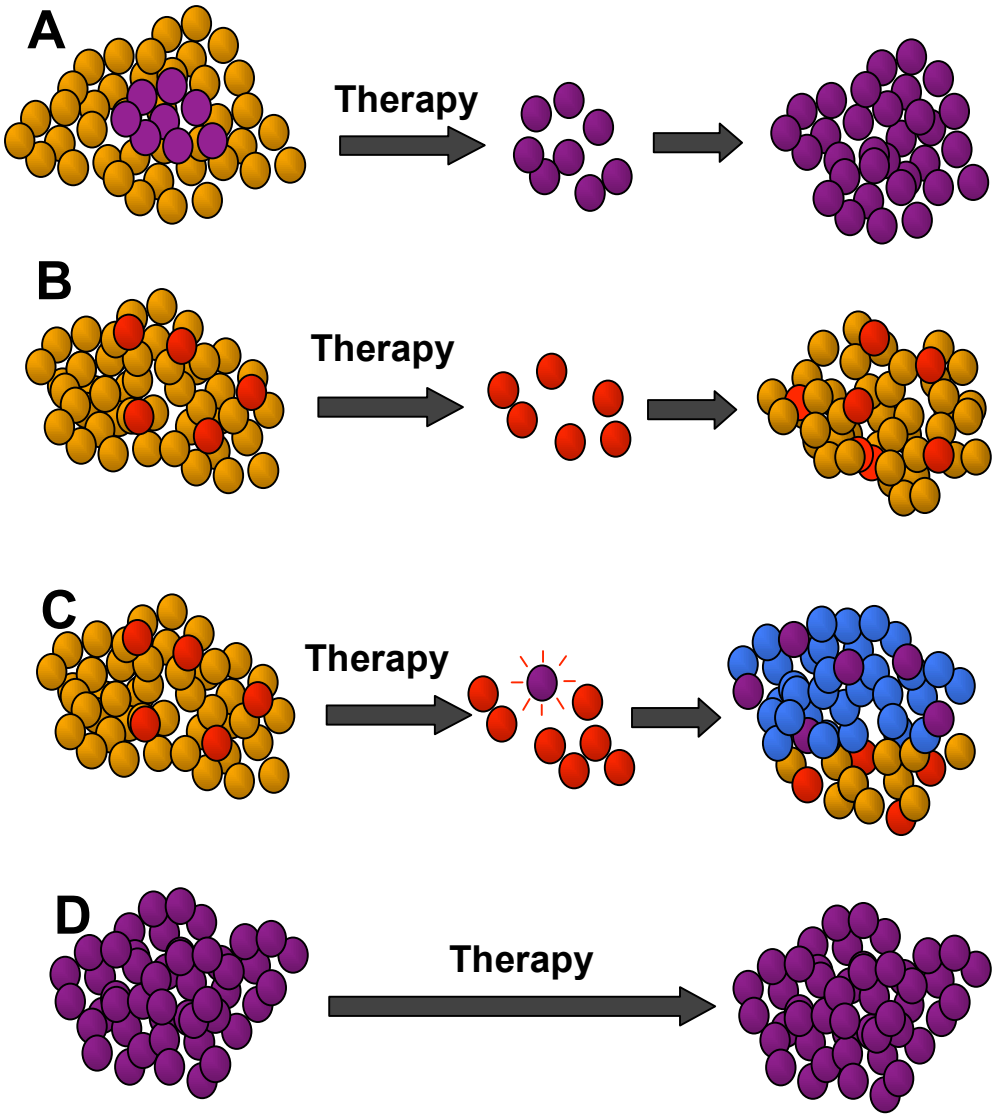


Figure 2 – Models for drug resistance in tumours.
A – Drug therapy selects for cells which have evolved resistance mechanisms such as membrane transporter pumps, which may then repopulate the tumour.
B – Drug therapy eliminates the bulk of tumour cells, but inherently resistant CSC persist and repopulate the tumour.
C – CSC survive therapy and evolve further resistance mechanisms, repopulating the tumour with drug-resistant variant progeny.
D – All cells are inherently resistant (or inherently sensitive). Drug therapy does not select for any particular population.

(Adapted from Dean, Fojo and Bates, 2005 *Nature Reviews Cancer,* **5**, 275-285).

CSC variants – a "moving target". Finally, in some cancers there is little evidence of differential response to chemotherapy drugs. Germ cell tumours tend to show extreme sensitivity to protocols incorporating platinum compounds with good overall response rates (despite being prime candidates for having arisen from stem cells), suggesting that therapy has eliminated the CSC component (Polyak and Hahn, 2006). Conversely, renal cell cancers tend to express P-gP throughout the tumour such that the bulk and any putative CSC are similarly unaffected by treatment (Dean *et al.*, 2005). Interestingly, when van der Pol *et al* investigated drug transporter expression in minimal residual disease in AML, drug treatment had not changed the actual expression level of drug transporters, nor selected for a specific population of cells with increased expression – most cells expressed the relevant protein (van der Pol *et al.*, 2003). This suggests that resistance may be associated with the degree of functional activity of transporters expressed throughout the population, rather than their expression by specific cells.

Drug resistance may be induced *in vitro* through incubation of continuous cell lines in the presence of increasing concentrations of cytotoxic agents (Coley, 2004). The ABCG2 drug transporter, most frequently associated with SP, was first characterised *in vitro* in MCF-7 breast carcinoma cells chronically exposed to doxorubicin (Doyle *et al.*, 1998). Uozurmi *et al* developed three drug resistant variants of the canine GL-1 B-cell lymphoma cell line, through long-term doxorubicin challenge (Uozurmi *et al.*, 2005). Western blot analysis suggested progressive upregulation of a ~180kDa protein, suggested to be canine PgP - the cells showed resistance to both doxorubicin and vincristine. Interestingly, vincristine is generally recognised as being transported by ABCG2 (73kDa) rather than P-gP, and on the Western blots presented with the report, a protein product of between 66 and 97kDa is also apparent (Uozurmi *et al.*, 2005).

It follows, then, that Hoechst SP may be an inducible phenomenon both *in vivo* and *in vitro*. In the literature, there are reports of SP in primary tumour cells isolated at the time of excision (Addla *et al.*, 2008; Wu and Alman, 2008; Zhang *et al.*, 2009b) and at relapse (Hirschmann-Jax *et al.*, 2004), but much less data which examines

whether SP changes through the duration of treatment. Notably, in the study of relapsed tumours, the SP constituted a large proportion (≤51%) of total cells. The side population of neurospheres derived from a PTEN-deficient mouse glioma model was increased by temozolomide treatment *in vitro* (Bleau *et al.*, 2009). The drug produced a similar effect on human glioma cell lines, accompanied by increased numbers of progenitor-like cells. However, this was not affected by ABCG2 knockdown - co-expression of other ABC transporters may therefore have contributed to the response (Chua *et al.*, 2008).

If the goal of therapy based on the CSC hypothesis is to identify and target defined cellular subpopulations, it is important to know whether these will remain constant during the tumour's lifetime, or whether and how they might change – that is, whether or not treatment might induce or modulate the CSC phenotype.

In the following experiments, CSC-associated mechanisms of drug resistance were investigated in canine cancer cells. We sought to establish whether NFκB signalling is active in canine cancer cells, and the effects on cell viability of blocking this with selective inhibitors. As NFκB may promote resistance to apoptosis induced by conventional cytotoxic drugs, which may themselves upregulate NFκB signalling, combinations of NFκB inhibitors and doxorubicin were evaluated for complementary or synergistic anticancer effects (Nakanishi and Toi, 2005). Additionally, cells chronically exposed to doxorubicin were evaluated for altered sensitivity to NFκB inhibition. As interference with the pathway allows selective eradication of putative CSC in some cancers, cellular subpopulations isolated according to CD44 surface expression or tumoursphere formation were tested for differential susceptibility to NFκB inhibitors.

The effect of cytotoxic chemotherapy on CSC phenotype was also explored. Rather than representing a consistent and predictable therapeutic target, CSC may evolve additional survival mechanisms in the face of cytotoxic challenge (Dean *et al.*, 2005; Adams and Strasser, 2008). The SP phenotype has been reported in the context of the CSC hypothesis as a phenomenon associated with a specific pre-existing

subpopulation of stem-like cells. The effects on SP of chronic exposure to chemotherapy were evaluated.

MATERIALS AND METHODS

Cell Viability Assays

Assessment of chemosensitivity / sensitivity to NFκB inhibitors was performed using the standard ATP luminescence protocol previously described (Materials and Methods chapter). Cells were seeded at a density of $5x10^3$ cells/well except where specified (see Results). As well as standard untreated controls, additional vehicle controls were analysed for NFκB inhibitors to identify any inhibitory effects of the DMSO carrier. For each dilution of drug in medium, a volume-equivalent dilution was made of DMSO only. Once inhibitory effects were confirmed as DMSO-independent, a single vehicle control (volume-equivalent to the highest drug concentration) was included on each plate. Results were analysed using Microsoft Excel and GraphPad Prism.

NFκB inhibitors tested were IKK Inhibitor II Wedelolactone (WDL), IKK Inhibitor VII, and InSolution™ NFκB inhibitor (ISNI) (all from Calbiochem / Merck).

Extraction of Nuclear Protein for EMSA Assays

Buffers for nuclear protein extraction

Buffers were prepared and stored at 4°C. At each use, 1 volume of 10x protease inhibitor cocktail [cOmplete Mini (Roche) - one tablet dissolved in 1ml ddH_2O] was added to 9 volumes nuclear extraction buffer.

Buffer 1 - 25mM HEPES
5mM KCl
0.5mM $MgCl_2$
1x protease inhibitor cocktail

Buffer 2 - 25mM HEPES
5mM KCl
0.5mM $MgCl_2$
1% (v/v) NP-40
1x protease inhibitor cocktail

Buffer 3 – 1:1 mixture of Buffer 1 and Buffer 2

Buffer 5 - 25mM HEPES
10% (w/v) Sucrose
350mM NaCl
0.01% (v/v) NP-40
1x protease inhibitor cocktail

Cells were harvested and pelleted as for preparation of cell lysates. After discarding the supernatant, the pellet was resuspended in 200µl of Buffer 1. 200µl of Buffer 2 was added, the suspension mixed gently by pipetting and rotated at 4°C for 15 minutes, followed by centrifugation in a prechilled (4°C) microcentrifuge at 600*g* for 1 minute.

The resultant supernatant (cytoplasmic protein) was transferred to a fresh chilled eppendorf tube. 200µl of Buffer 3 was added to the pellet, followed by gentle mixing and centrifugation at 4°C / 600*g* for 1 minute. Supernatant was discarded and the pellet resuspended in in 250µl of Buffer 5 (including protease inhibitor mix); the suspension was rotated at 4°C for 1 hour followed by centrifugation at 4°C / 16000*g* for 10 minutes. The resultant supernatant (nuclear protein) was transferred to a fresh chilled eppendorf tube. Protein was quantified for cytoplasmic and nuclear protein extracts using the Bradford assay.

Electrophoretic Mobility Shift Assay (EMSA) for NFκB Activity

Double stranded NFκB consensus oligonucleotide was obtained from Promega. EMSA assays were performed using the DIG Gel-Shift Kit 2nd Generation (Roche, Mannheim, Germany) according to the manufacturer's protocol:

End-labelling of oligonucleotide sequences with digoxigenin (DIG)

100ng dsNFκB or control oligonucleotide was incubated at 37°C in labelling buffer, with 0.05mM digoxigenin-11-ddUTP (DIG-ddUTP), 5mM $CoCl_2$ and 20 U/µl terminal transferase, for 15 minutes before placing on ice. 2µl 0.2M EDTA (pH 8.0) was added to stop the reaction. ddH_2O was added to a final concentration of 4ng/µl of labelled oligonucleotide. Labelled oligonucleotides were stored at -20°C.

Gel-shift binding reaction

Samples for the gel shift binding reaction were set up and mixed on ice according to the manufacturer's protocol. Briefly, for each cell line being tested, 5-10µg nuclear extract was mixed in binding buffer with 0.4ng/µl DIG-labelled NFκB oligonucleotide, 1µg/µl poly [d(I-C)] and 0.1µg/µl poly-L-lysine. In addition, for each line, competition control with specific unlabelled probe (addition of 0.1µg/µl unlabelled NFκB oligonucleotide) and supershift (addition of 1µg/µl specific NFκB RelA/p65 antibody – added prior to labelled probe) reactions were performed. Negative control contained no nuclear extract, and for at least one cell line in each experiment, additional specificity control reactions (competition control with non-specific Oct2A oligonucleotide; supershift reaction with non-specific p53 antibody) were performed. Samples were incubated at 15-20°C for 15 minutes and held on ice prior to gel electrophoresis.

Gel electrophoresis and electroblotting

Samples were mixed with 5X Hi-Density TBE sample buffer (TBE running buffer (Invitrogen) with 0.1% bromophenol blue and 15% w/v Ficoll 400), and run on a pre-electrophoresed non-denaturing polyacrylamide gel (Novex Precast 6% DNA Retardation Gel, Invitrogen) in 0.5x TBE running buffer. Samples were transferred to a positively charged nylon membrane (Roche), followed by ultraviolet crosslinking of oligonucleotides at 120mJ/cm^2. The membrane could be dried at this stage, and stored at 2-8°C pending chemiluminescent detection.

Chemiluminescent detection

Washing buffer, maleic acid buffer and detection buffer (DIG Wash and Block Buffer Set, Roche) were prepared according to the manufacturer's instructions. Blocking solution (blocking reagent 10% w/v in maleic acid buffer), antibody solution (Anti-Digoxigenin-AP, 1:10 000 in blocking solution) and CSPD® working

solution (1:100 in detection buffer) (DIG Gel-Shift Kit 2nd Generation, Roche) were prepared freshly according to the manufacturer's instructions. Incubations were performed at 15-25°C with gentle agitation. The membrane was rinsed briefly in washing buffer and incubated in blocking solution for 30min, followed by 30min incubation in antibody solution. The membrane was washed twice (2x15min) in washing buffer and equilibrated for 5min in detection buffer. CSPD working solution was applied and the membrane incubated at 37°C for 10min to enhance the luminescent reaction between CSPD and the alkaline phosphatase antibody conjugate. The membrane was exposed to radiographic film (Hyperfilm ECL, GE Healthcare) for 15-25min at room temperature to visualise the luminescent signal.

Cell Cycle Analysis / Cell Morphology After NFκB Inhibition

D17 and REM134 cells in complete medium were seeded in T25 culture flasks at densities comparable to those used in 96-well plate assays ($1.56x10^4$ cells/cm^2). After 24h, 40μM WDL or 3.5nM ISNI were added and cells incubated for a further 72h. Photomicrographs were captured using an Axiovert 40 CFL microscope, with Axiovision software. Cell fixation, propidium iodide staining and cell cycle analysis were performed as previously described (Materials and Methods chapter).

Drug Combination Assays

The effects of simultaneous treatment with doxorubicin and NFκB pathway inhibitors WDL (REM134) or ISNI (D17) were assessed by cell viability assay. For REM134, the disparity between the range of concentrations over which drug showed activity precluded testing at a constant drug ratio. Responses were tested over a range of doxorubicin concentrations superimposed on a constant concentration of WDL. For D17, the drugs were tested at a constant ratio of 1:10 and 1:100 ISNI:doxorubicin. Relevant medium only, single drug and DMSO vehicle controls were included on each plate.

Development of Drug-Resistant Cell Lines

3132 lymphoma cells and MCF7 human mammary carcinoma cells were cultured in the presence of gradually increasing concentrations of doxorubicin, as described by Coley (Coley, 2004). Cells were plated to achieve 20% confluence after 24h incubation (37°C, 5% CO_2, 100% humidity), at which point doxorubicin and 5μM verapamil were added. At confluence, cells were harvested, washed in complete medium, counted and replated, and drugs added again at 24h / 20% confluence. A population of the parental cells from which the drug-adapted cells were derived was cultured in parallel ("3132Par" / "MCF7Par"), as a control for comparative assays. The doxorubicin concentration was increased once cells had been subcultured at a given concentration for 2-3 passages with no further growth retardation. A population of treated cells was maintained at the previous concentration as a "backup" in case the increased concentration resulted in non-recoverable loss of viability. Stable growth at 5nM doxorubicin was achieved within 4-5 weeks / 8 passages and cells were subcultured at least twice more prior to assays.

Western blot analysis and Hoechst 33342 / Rhodamine123 efflux analysis were performed as previously described (Materials and Methods / Chapter 3).

RESULTS

Sensitivity of Canine Cancer Cell Lines to Chemotherapy Drugs

The *in vitro* sensitivities of 3132, SB, REM134 and D17 canine cells, and human MCF-7 cells, to commonly used chemotherapy drugs were established by cell viability assay. There is little available information detailing expected serum concentrations of these drugs when used in dogs – drug effects were assessed over a range of concentrations based initially on levels achieved at therapeutic doses in humans (TPC=Therapeutic Plasma Concentration) (Materials and Methods chapter, Table 6).

Preliminary studies were conducted with $5x10^2$ cells per well of a 96-well plate, but these very low cell numbers gave IC_{50} values well below expected therapeutic serum concentrations. The existence of an "inoculum effect", whereby cytotoxicity is affected by cell density due to factors such as binding site saturation and acidification of the medium, is well documented for *in vitro* responses of cells to certain drugs (including doxorubicin and vincristine, though not carboplatin) (Ohnuma *et al.*, 1986). It is also likely that seeding at very low cell densities results in more variation in cell number seeded per well (proportional to the target density). Cell viability assays such as MTT and ATP-luminescence show good linearity up to $1x10^6$ cells per well (Twentyman *et al.*, 1989; Andreotti *et al.*, 1995; Cree *et al.*, 1995 / manufacturers' data). At $5x10^3$ cells per well, errors were found to be smaller and IC_{50} values more representative of plasma concentrations achieved *in vivo*, and so seeding at this density was used for further investigations. Results are summarised in Table 1 and Figures 3 - 4.

Drug	Cells	IC_{50}
Doxorubicin	3132	5.49nM
	D17	116.6nM
	REM134	32.18nM
	MCF7	91.87nM
	D17*	8.65nM
Mitoxantrone	3132	0.63nM
	D17	202.2nM
	REM134	18.02nM
	MCF7	19.42nM
Vincristine	3132	~0.65nM
	D17	3.26nM
	REM134	5.77nM
	MCF7	8.33nM
Carboplatin	3132*	3.55µM
	SB*	4.33µM
	REM134	168.1µM

Table 1 – Calculated IC_{50} values of chemotherapy drugs for canine (and human MCF-7) cancer cell lines. All cell viability assays were performed at a density of $5x10^3$ cells per well except where indicated by * ($5x10^2$ cells / well).

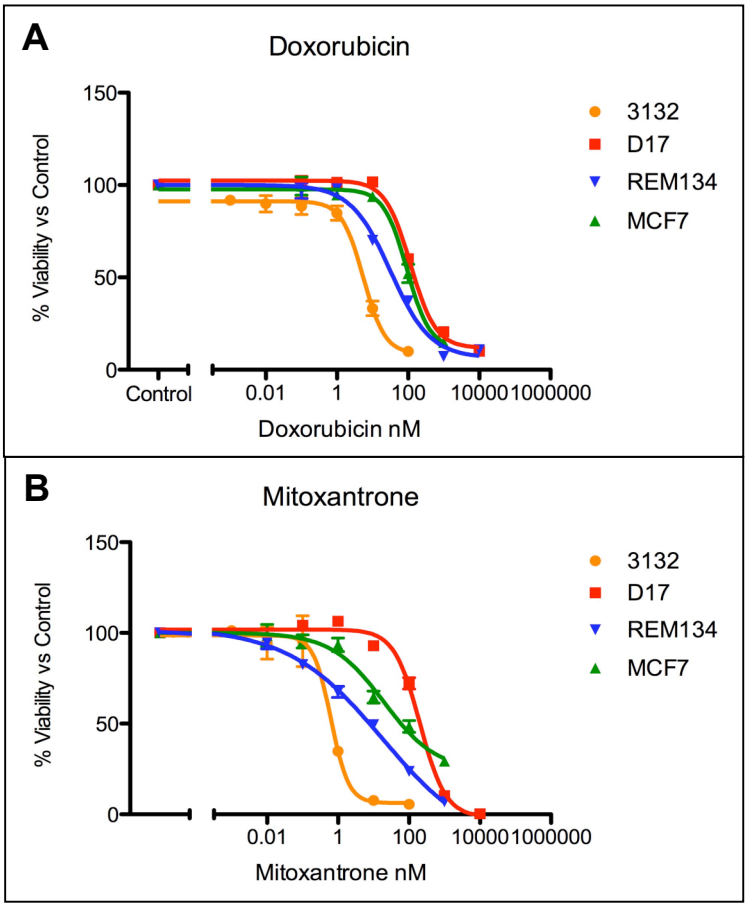


Figure 3 – Cell viability of canine (and human MCF7) cancer cell lines in response to increasing concentrations of (A) doxorubicin and (B) mitoxantrone.

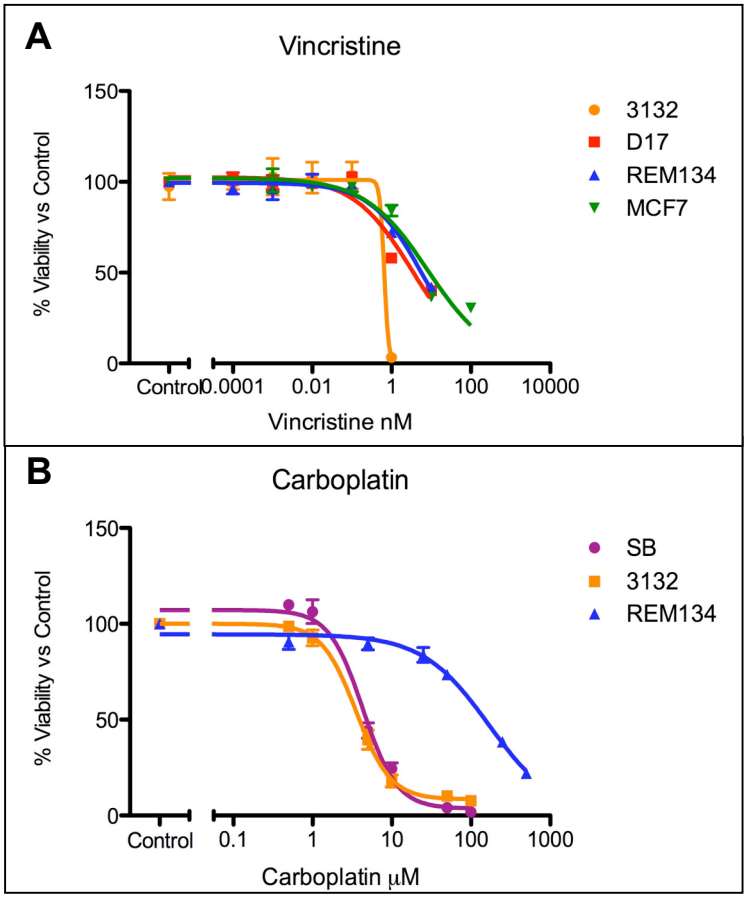


Figure 4 – Cell viability of canine (and human MCF7) cancer cell lines in response to increasing concentrations of (A) vincristine and (B) carboplatin.

D17 osteosarcoma cells showed the highest IC_{50} values for doxorubicin and mitoxantrone. For doxorubicin, D17 had an IC_{50} of 116.3nM when seeded at $5x10^3$ cells per well, exceeding that shown by MCF-7 (91.9nM) and more than three times that of REM134 (32.17nM) and twenty times that of 3132 (5.5nM) (Figure 3A). Even at low inoculums ($5x10^2$ cells per well), the IC_{50} of doxorubicin for D17 was 8.65nM.

For mitoxantrone, D17 showed an IC_{50} of 200.1nM, much higher than that of MCF-7 (19.43nM), REM134 (6.47nM) and 3132 (0.63nM). For this drug, even at the lower inoculum of 500 cells per well D17 was considerably more resistant than the other cell lines (IC_{50} = 43.54nM) (Figure 3B). Notably, all tested cells (including MCF-7) showed IC_{50} values for mitoxantrone well below initial maximum serum concentrations achieved at clinical doses in humans ($15mg/m^2 \Rightarrow 0.63\text{-}0.95\mu g/ml \equiv 1.42\text{-}2.14\mu M$) (Canal *et al.*, 1993). This may reflect differences between the pharmacokinetics of the drug *in vitro* (where drug is applied directly to the cells and concentration is sustained) and *in vivo,* where the drug shows high levels of plasma protein binding, accumulation within and slow release from lymphocytes, and decreasing plasma concentrations as the drug is redistributed and excreted in bile and urine.

3132 lymphoma cells showed greatest drug sensitivity overall, with the lowest IC_{50} values for doxorubicin, mitoxantrone and vincristine. They appeared particularly sensitive to vincristine (Figure 4A) – viability reduced dramatically with small increases in concentration and response to equivalent levels of drug varied between experiments [the latter was noted with other cell lines including D17 and MCF-7 but was most marked for 3132]. REM134 was the least sensitive of the canine cell lines to vincristine (IC_{50} 5.7nM).

Canine Cancer Cell Lines Show NFκB Transcription Factor Activity

NFκB activation in the canine cancer cell lines D17, J3T, REM134 and SB, and also human MCF-7 and feline Cat-MT cells, was assessed by electrophoretic mobility

shift assay (EMSA). This assay uses digoxigenin-labelled (DIG-labelled) NFκB DNA consensus sequence as a probe, to assess whether the protein is present within nuclear extract (as a result of induced or, for unstimulated cells, constitutive activation). A specific band (DIG-labelled probe bound to nuclear NFκB protein) was present for all cell lines (Figure 5). In addition, specificity of the reaction was confirmed - reduction in the DIG-labelled band was seen in the presence of unlabelled probe (which binds in competition with labelled oligonucleotide), and a supershift observed upon addition of a specific NFκB p65 (RelA) antibody (which binds the protein-DNA complex, retarding its movement through the gel). Thus, canine cancer cell lines demonstrate constitutive activation of the nuclear transcription factor NFκB.

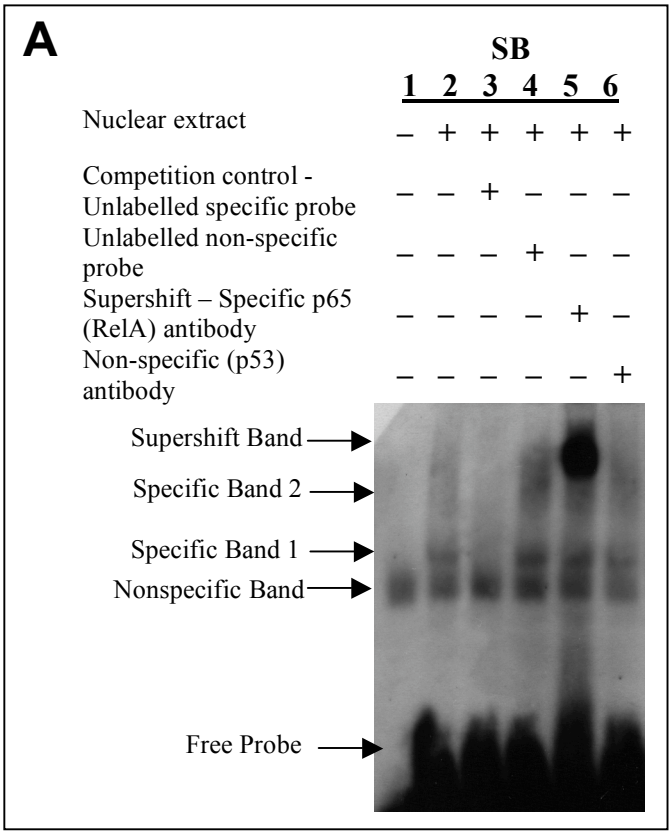
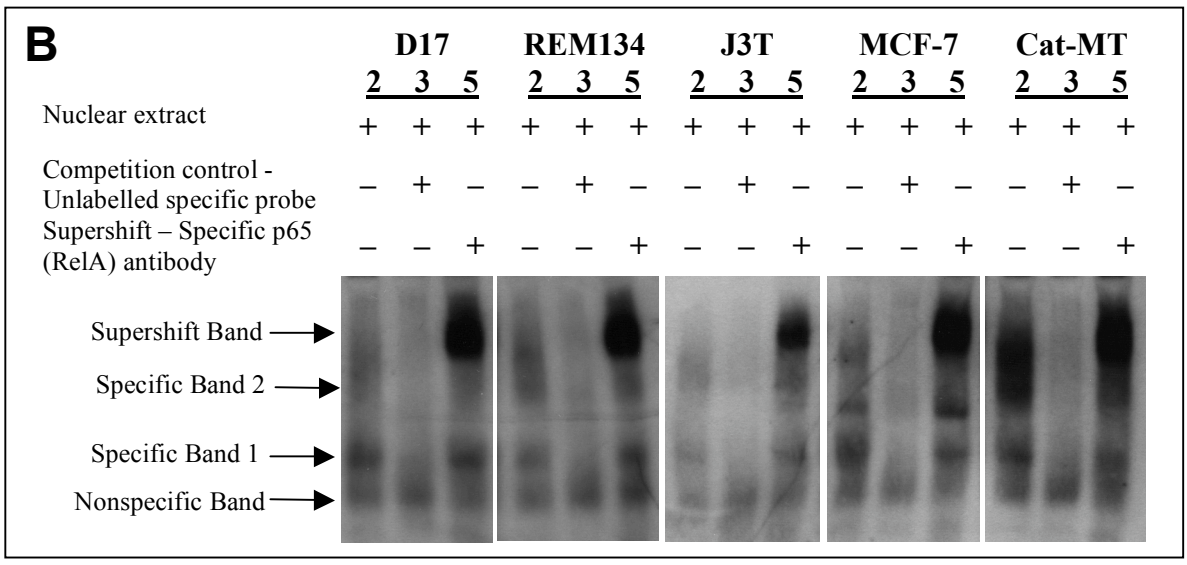


Figure 5 – Representative EMSA blots demonstrating nuclear NFκB activity in canine (and human and feline mammary) cancer cell lines.

A – SB haemangiosarcoma showing test and control gel shift binding reactions. "+" and "–" refer to components added to or omitted from standard reaction. DIG-labelled probe is included in all lanes.
1 – Negative Control
2 – Test – Specific band
3 – Competition control probe
4 – Non-specific probe
5 – Supershift – specific antibody
6 – Non-specific antibody

B – Test and specificity reactions for other cell lines.



Reactions:
1 – Negative control – no nuclear extract. No specific band.
2 – Test – DIG-labelled NFκB consensus oligonucleotide binds nuclear NFκB protein. Two specific bands representing probe-transcription factor complexes are seen due to the heterodimeric nature of NFκB. The larger of these ("Specific band 2") is more diffuse in canine/feline than in human cells.
3 – Unlabelled NFκB oligonucleotide competes for binding with labelled probe – loss of specific band.
4 – Non-specific (Oct2A) oligonucleotide does not compete for binding with labelled probe.
5 – Supershift – specific p65 antibody binds probe- NFκB complex and retards progress in the gel.
6 – Nonspecific antibody – does not bind probe-transcription factor complex – no supershift band.
A non-specific band is seen in all samples, including negative control.

Inhibition of NFκB in Canine Cancer Cell Lines

The effects of inhibition of the NFκB pathway in D17, REM134 and SB canine cells were assessed by cell viability assay. InSolution™ NFκB Activation Inhibitor (ISNI) is a quinazoline compound that directly inhibits the transcriptional activation NFκB (Tobe *et al.*, 2003). The effects of two indirect inhibitors acting more proximally in the pathway were also tested – these agents inhibit the activity of IKK, hence preventing the phosphorylation and degradation of IκB. IKK Inhibitor II Wedelolactone (WDL) is derived from the herbal medicine *Eclipta Alba*, and inhibits both IKKα and IKKβ (Li *et al.*, 2003; Kobori *et al.*, 2004). Similarly, IKK Inhibitor VII is a benzamido-pyrimidine compound that inhibits activity of IKKα, IKKβ, and the IKK complex, with slight selectivity for IKKβ / IKK complex, and as such may block NFκB activation by both canonical and alternative pathways (Waelchli *et al.*, 2006). Results are summarised in Table 2 and Figures 6-7.

Drug	Cells	IC_{50}
IKK Inhibitor VII	D17	2.82μM
	SB	2.50μM
IKK Inhibitor II Wedelolactone (WDL)	D17	97.46μM
	REM134	19.71μM
	SB	3.84μM
InSolution NFκB activation inhibitor (ISNI)	D17	2.85nM
	REM134	2.69nM
	SB	0.34nM

Table 2 – Calculated IC_{50} values of NFκB pathway inhibitors for canine cancer cell lines. All cell viability assays were performed at a density of $5x10^3$ cells/well

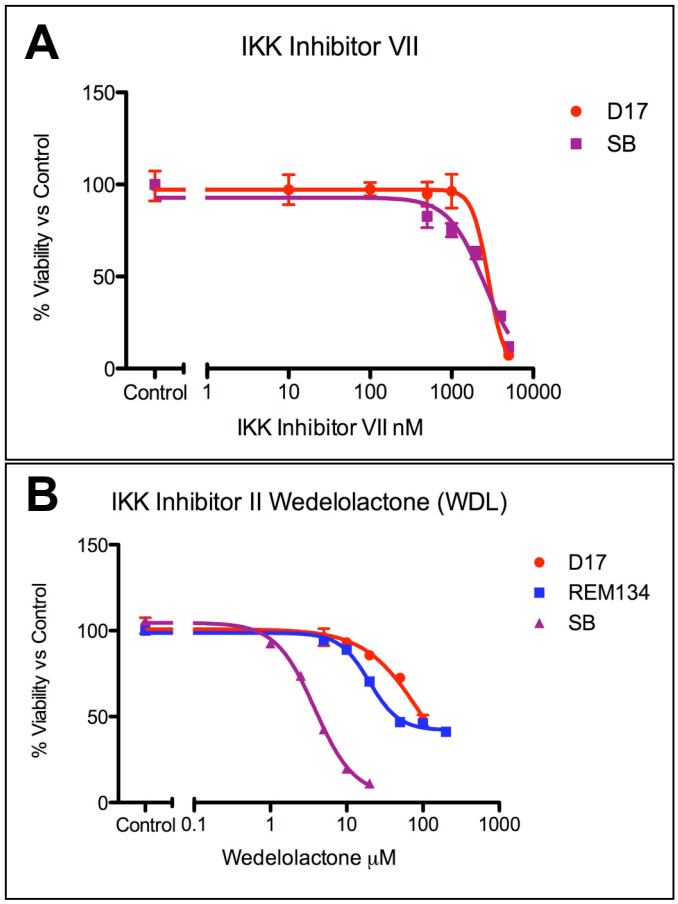


Figure 6 – Cell viability of canine cancer cell lines in response to indirect inhibition of NFκB activation - (A) IKK inhibitor VII and (B) WDL.

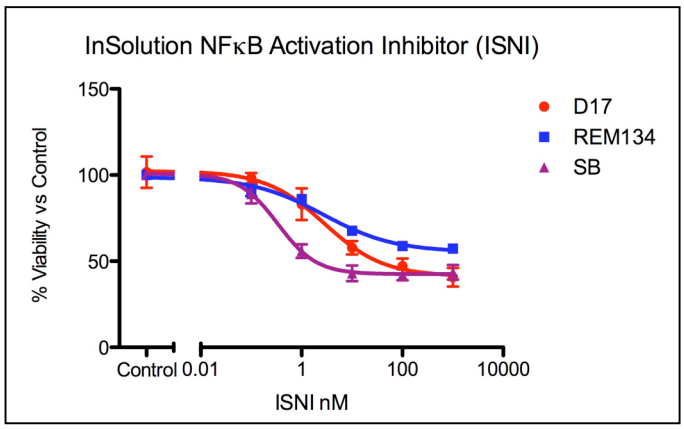


Figure 7 – Cell viability of canine cancer cell lines in response to direct inhibition of NFκB activation – ISNI.

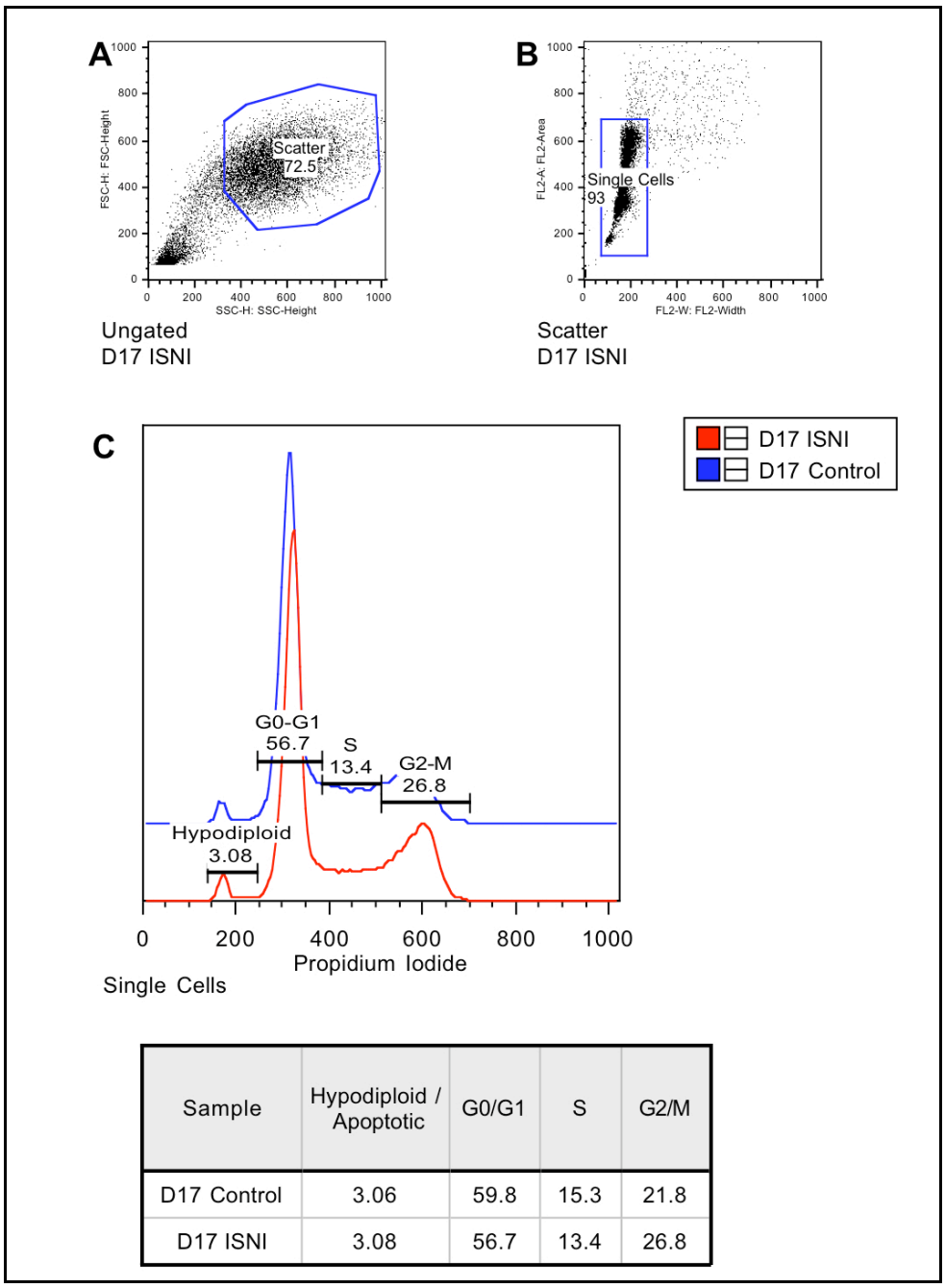
SB was the most sensitive of the three cell lines to inhibition of the pathway, with D17 showing least sensitivity, although both of these cell lines were relatively resistant to the effects of IKK Inhibitor VII. For this drug, viability was maintained up to high concentrations before a sudden drop-off in the micromolar range (SB IC_{50} 2.2μM, D17 IC_{50} 2.4μM) (Figure 6A). Published IC_{50} values for the (ATP-dependent) activity of this drug, based on responses by human HeLa cervical carcinoma cells, are in the nanomolar range (IKKβ 40nM, IKK complex 70nM, IKKα 200nM) (Waelchli *et al.*, 2006). Vehicle control samples indicated that the activity against the canine cancer cell lines was a drug-related toxicity rather than a DMSO carrier effect. Nonetheless, IC_{50} values for canine cells were orders of magnitude greater than those for human and rodent cells. It is possible that the drug is more specific for IKK in these species, or that effects on canine cell viability at these high concentrations were not mediated through inhibition of NFκB signalling.

D17 cells were also resistant to WDL – cell viability was almost 50% at 100μM - compared to REM134 (IC_{50} 19.71μM) and SB (IC_{50} 3.8μM) (Figure 6B). Maximal inhibition of REM134 cells by WDL was approximately 60% - at tested concentrations ≥100μM, vehicle control samples demonstrated that the DMSO carrier was having an inhibitory effect on the cells, and comparison of total responses with vehicle control responses indicated that the sigmoid drug inhibition curve had reached its lower plateau by this point. Inhibition of IKK by WDL in SB cells resulted in greater loss of viability, which was <5% (4.81%) at 20μM.

ISNI was active against all of the tested canine cell lines at nanomolar concentrations. The IC_{50} values for D17, REM134 and SB cells were 2.9nM, 3.5nM and 0.34nM, respectively. Inhibition reached a plateau in all cases, with at least 40% viability remaining as drug levels were increased (Figure 7). These results suggest that viability for each of the cell lines is at least partially dependent on the anti-apoptotic and pro-survival influence of NFκB activity. SB showed greater susceptibility to inhibition of IKK than to direct inhibition of NFκB activity. This discrepancy suggests that IKK activity may influence other cell survival processes in this cell line. For D17, the failure of IKK inhibition to produce the same cell-

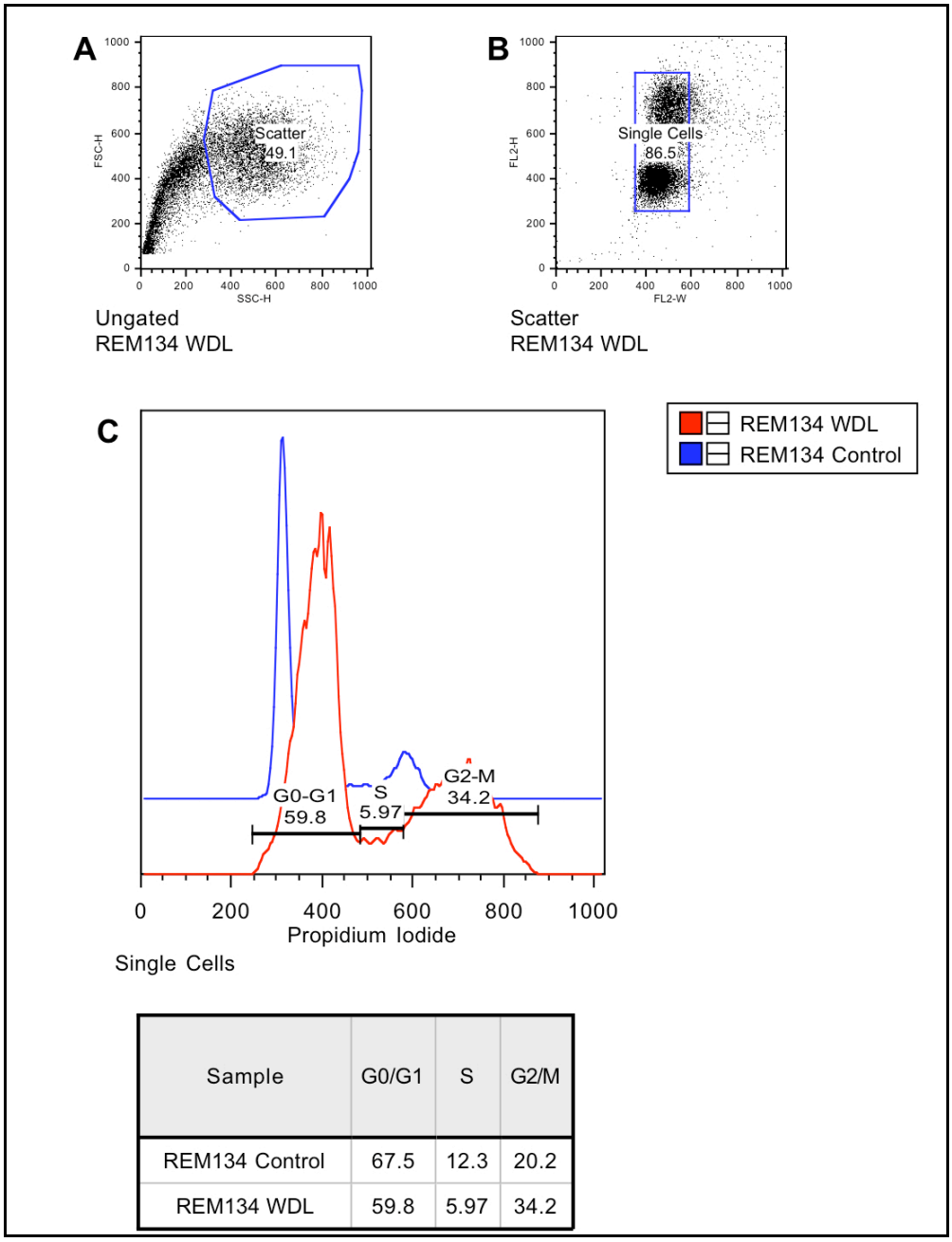
inhibitory effects as direct inhibition of NFκB transcriptional activation suggests that constitutive NFκB activity in this cell line is independent of IKK, and that alterations in the pathway may occur more distally (for example through defective IκB activity).

NFκB may transactivate mediators of cell cycle progression, such as Cyclins D1 and D2 (Ghosh and Karin, 2002; Greten and Karin, 2004; Ravi and Bedi, 2004). The cell cycle profiles of ISNI- and WDL-treated D17 and REM134 cells were analysed. Results were similar for both cell lines. Direct inhibition of NFκB transcriptional activation (ISNI) did not produce alterations in cell cycle distribution (Figure 8). Inhibition of IKK (WDL) resulted in an increased proportion of cells in G_2/M phase, which suggests that this drug may exert NFκB-independent effects on cellular proliferation. The drug also appeared to affect the integration of PI into DNA, leading to reduced definition of the cell cycle distribution profile (Figure 9). As well as reduction in the density of the monolayer, cells treated with both drugs (particularly WDL) demonstrated altered morphology (Figure 10).



Sample	Hypodiploid / Apoptotic	G0/G1	S	G2/M
D17 Control	3.06	59.8	15.3	21.8
D17 ISNI	3.08	56.7	13.4	26.8

Figure 8 – D17 cells treated with 3.5nM ISNI.
Gating strategy to select intact (A), single (B) cells for cell cycle analysis (C). Proportion of cells in each phase of the cell cycle, as well as hypodiploid peak (indicating apoptosis), is similar for control and treated cells



Sample	G0/G1	S	G2/M
REM134 Control	67.5	12.3	20.2
REM134 WDL	59.8	5.97	34.2

Figure 9 – REM134 cells treated with 40 μM WDL.
Gating strategy to select intact (A), single (B) cells for cell cycle analysis (C). Increased proportion of treated cells are in G_2/M, with fewer in G_0/G_1 or S phase compared to control. D17 cells showed a similar cell cycle distribution in response to WDL treatment.

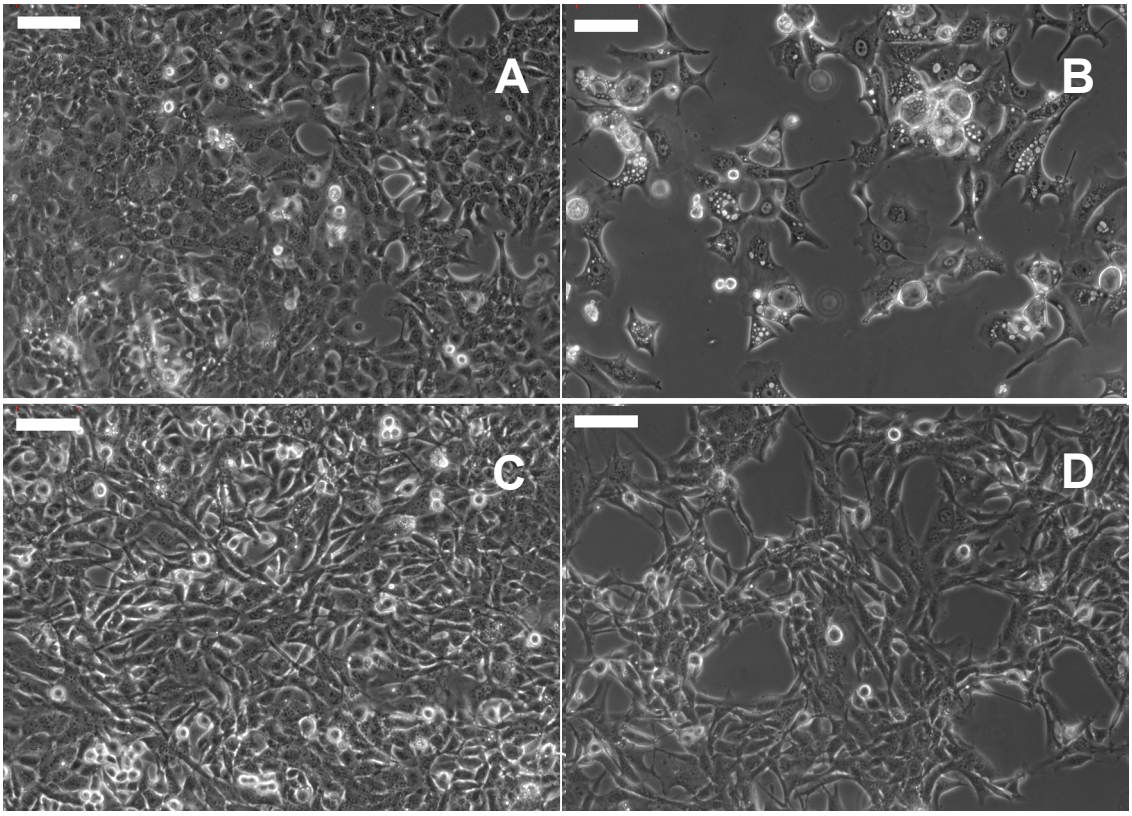


Figure 10 – Photomicrographs (100x objective) showing canine cancer cells after 72h treatment with NFκB pathway inhibitors. Bar = 100μm.
Upper panels – REM134 cells, untreated (A) and treated with 40μM WDL (B). Treated cells lose their normal regular epithelioid morphology and organisation, and are enlarged with prominent processes. Many show two adjacent nuclei, consistent with the increased proportion of cells in G_2M seen on cell cycle profile.
Lower panels – D17 cells, untreated (C) and treated with 3.5nM ISNI (D). Overall monolayer density is reduced. Morphologically, treated cells are more spindle-shaped, again with processes, but changes are less marked than with WDL.

Can Suppression of NFκB Signalling Potentiate the Effects of Conventional Chemotherapy on Canine Cells?

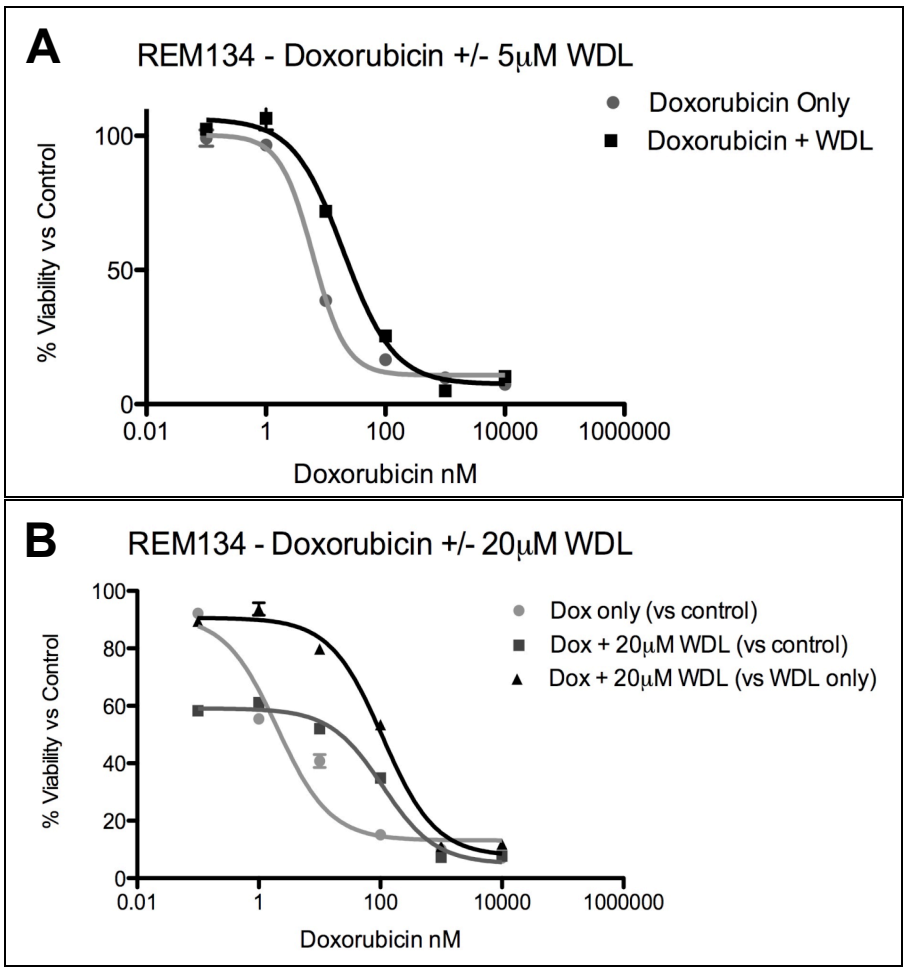
Synergy between the antiproliferative effects of NFκB pathway inhibitors and more conventional cytotoxic drugs has been demonstrated in some cancers and cancer cell lines (Cusack *et al.*, 2001; Mabuchi *et al.*, 2004; Avellino *et al.*, 2005; Nakanishi and Toi, 2005). Upregulation of anti-apoptotic signalling in response to cytotoxic challenge may be offset by concurrent suppression of NFκB activity. Alternatively, complementary activity may represent inhibition of both bulk progeny and CSC

components. REM134 and D17 cells were treated with combinations of an NFκB pathway inhibitor and doxorubicin, to assess whether this potentiated the inhibitory effects of either drug given individually.

REM134 cells were tested against combinations of doxorubicin and WDL – results are presented in Figure 11. Concentrations of doxorubicin were varied against a background of 5μM WDL (a relatively non-toxic dose). Not only was there no apparent synergy between the two drugs, but in combination with 5μM WDL, the IC_{50} of doxorubicin (19.9nM) was significantly greater than that of doxorubicin alone (6.1nM) ($p < 0.0001$) (Figure 11A).

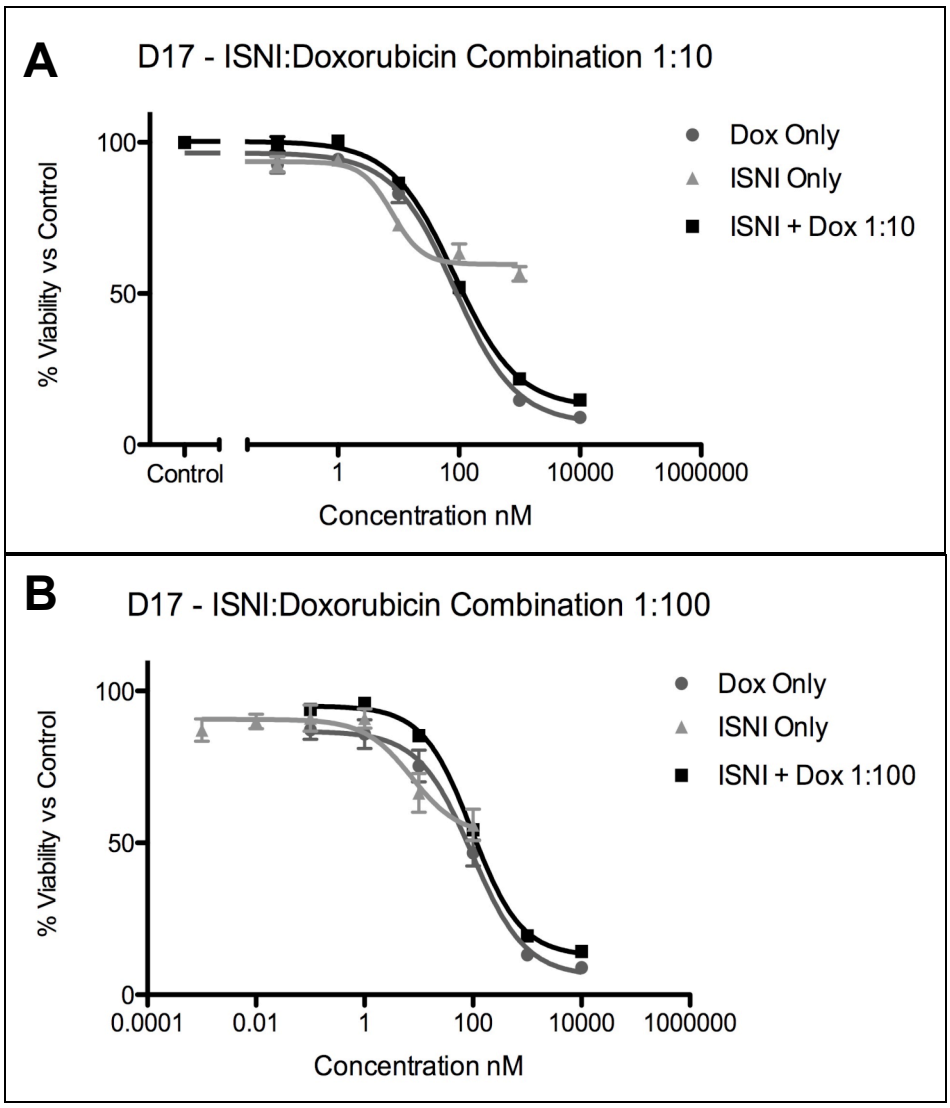
At an increased background concentration of 20μM WDL, there was a reduction in overall cell viability due to its inhibitory effect. However, the IC_{50} for doxorubicin against this background of NFκB inhibition increased to 108.8nM ($p < 0.0001$), representing a fifty-fold increase – the sigmoid curve for doxorubicin + WDL shifts considerably to the right. Thus, in the presence of WDL, much greater concentrations of doxorubicin were required for the same proportional effect on viability. For example, 1nM doxorubicin alone reduced viability by 44.6 ± 1.6%; in the presence of 20μM WDL, 1nM doxorubicin resulted in only 6.3 ± 2.1% loss of viability in addition to the background inhibition by WDL. This suggests that the effect of combining these drugs is in fact antagonistic (Figure 11B).

D17 were tested against combinations of doxorubicin and ISNI. Predetermined IC_{50} values fell within the nanomolar range for both drugs, with those for doxorubicin approximately 40-fold greater than those for ISNI. The drugs were tested in combination at concentration ratios of 1:10 and 1:100 ISNI:doxorubicin. Neither combination resulted in significantly greater cytotoxic effects than those of doxorubicin alone (Figure 12). At a 1:10 ISNI: doxorubicin ratio, the calculated IC_{50} value (75.4nM) was slightly lower than that for doxorubicin-only controls (91.8nM) but this difference was not significant ($p = 0.11$), and the fitted curves are very similar (Figure 12A).



Combination	Doxorubicin only IC_{50} / nM	Doxorubicin + WDL IC_{50} / nM	Significant Difference?	*p*-value
Doxorubicin + WDL 5μM	6.14	19.92	**Yes**	**<0.0001**
Doxorubicin + WDL 20μM	2.01	108.8	**Yes**	**<0.0001**

Figure 11 – WDL antagonises the cytotoxic effect of doxorubicin on REM134 cells. A – Increasing concentrations of doxorubicin on a background of 5μM WDL (a relatively non-toxic dose). Viability of cells exposed to the drug combination is greater than that of cells exposed to doxorubicin only.
B – Background concentration of 20μM WDL. When normalised to account for the inhibitory effect of WDL ("vs WDL only"), the sigmoid curve for the combination lies considerably to the right of the doxorubicin-only curve.



Combination	Doxorubicin only IC_{50} / nM	Doxorubicin + ISNI IC_{50} / nM	Significant Difference?	*p*-value
ISNI : Dox 1:10	91.77	75.35	No	0.112
ISNI : Dox 1:100	93.03	98.46	No	0.699

Figure 12 – ISNI does not enhance the cytotoxic effect of doxorubicin on D17 cells
A – Concentration ratio of 1:10 ISNI:doxorubicin
B – Concentration ratio of 1:100 ISNI:doxorubicin

There is no significant reduction in IC_{50} of doxorubicin when combined with ISNI at either ratio.

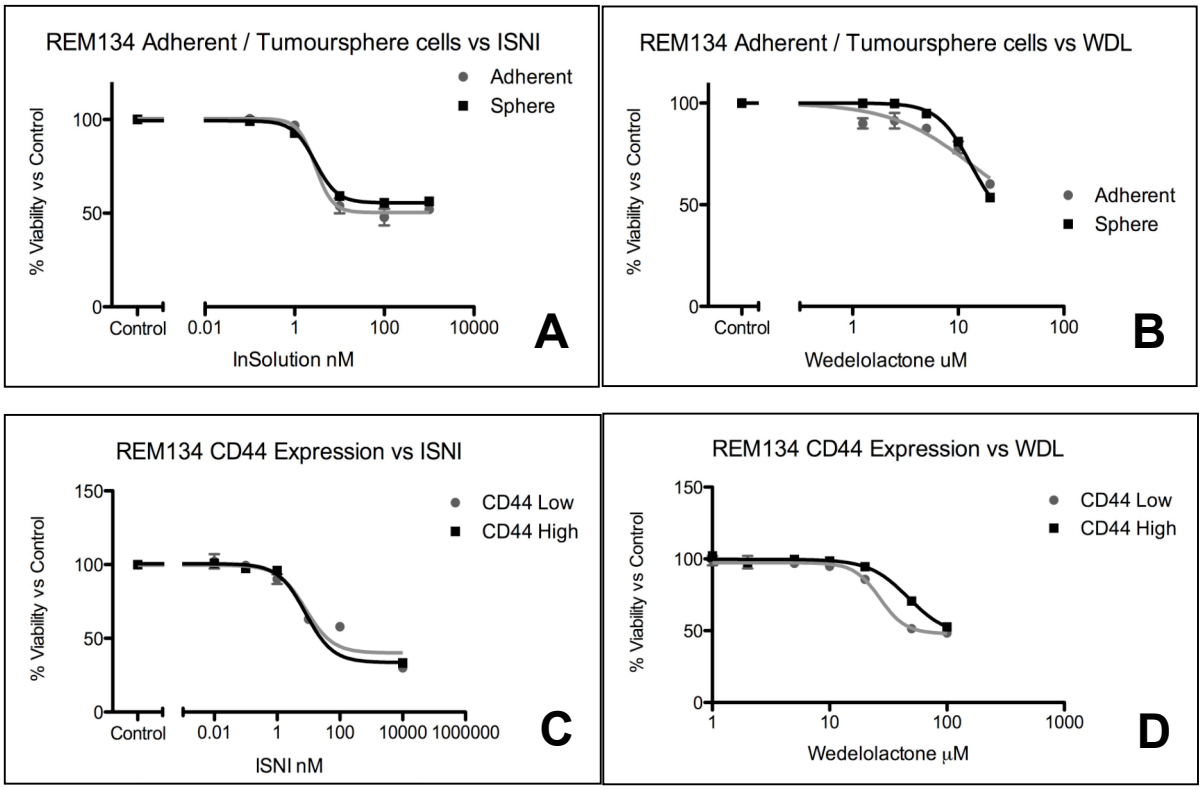
Again, rather than apparent synergy or even an additive effect, results suggested that the two agents may antagonise each other's effects. For example, mean inhibition of viability by ISNI only at 10nM was 27.3±1.5%, and by doxorubicin only at 100nM was 49.2±0.6%, yet the combination resulted in only 48.0±1.5% inhibition. Similarly, although 100nM ISNI alone produced 36.6±3.1% inhibition, and 1000nM doxorubicin 85.3±0.5%, the two drugs together led to only 78.3±0.7% loss of viability. At a combination ratio of 1:100 ISNI:doxorubicin, the IC_{50} (98.8nM) was in fact slightly greater than for doxorubicin-only controls (92.9nM), again suggesting antagonism, although the difference between the curves was not statistically significant ($p = 0.70$) (Figure 12B).

These results suggest that for D17 and REM134 cell lines, inhibition of NFκB activity does not augment the cytotoxic effect of doxorubicin.

Does NFκB Inhibition Preferentially Target Putative CSC Subsets in Canine Cancer?

NFκB pathway inhibitors have shown greater selectivity towards CSC than the bulk population of some tumours (Guzman *et al.*, 2005; Guzman *et al.*, 2007; Zhou *et al.*, 2008; Zhou *et al.*, 2009b). Two putative canine CSC subpopulations – REM134 tumoursphere cells and REM134 $CD44^{High}$ cells - were assessed for their responses to inhibition of IKK (WDL) and direct suppression of NFκB (ISNI).

There were no significant differences between the responses of REM134 tumoursphere and adherent cells to ISNI (adherent =3.3nM, tumoursphere = 2.5nM) (Figure 13A). The IC_{50} values for WDL were also very similar (IC_{50} adherent = 13.53μM, tumoursphere = 12.99μM) (Figure 13B). Thus, NFκB inhibitors WDL and ISNI show similar activity against REM134 tumoursphere and adherent cells, with a considerable proportion of tumoursphere cells unaffected by both drugs at maximal activity.



Drug	REM134 Adherent	REM134 Tumoursphere	Significant Difference?	*p* - value
ISNI IC_{50} / nM	3.30	2.52	No	0.189
WDL IC_{50} / μM	13.53	12.99	No	0.603

Drug	REM134 $CD44^{Low}$	REM134 $CD44^{High}$	Significant Difference?	*p* - value
ISNI IC_{50} / nM	6.96	16.25	No	0.35
WDL IC_{50} / μM	26.76	46.89	**Yes**	**<0.0001**

Figure 13 – NFκB pathway inhibition does not selectively eliminate putative CSC populations derived from REM134
Upper panels –
Response of REM134 adherent and tumoursphere cells to ISNI (A) or WDL (B).
Lower panels –
(C) No significant difference between the responses of REM134 $CD44^{High}$ and $CD44^{Low}$ cells to ISNI.
(D) $CD44^{High}$ cells are less sensitive to WDL than $CD44^{Low}$ cells. Viability of both populations is almost 50% at maximal inhibitory concentrations.

FACS-sorted REM134 $CD44^{High}$ and $CD44^{Low}$ cells did not show significantly different responses to direct NFκB inhibition by ISNI (Figure 13C). Moreover, $CD44^{High}$ cells were significantly more resistant to WDL than $CD44^{Low}$ cells, with IC_{50} values of 46.9μM and 26.8μM, respectively (p = <0.0001) (Figure 13D). Thus, this putative CSC marker identifies cells with reduced, not greater, sensitivity to NFκB inhibition. However, whilst the $CD44^{Low}$ population responded to lower concentrations of drug, viabilities of both populations at maximal inhibition were similar, at almost 50%. Treatment with WDL produces alterations in the cell cycle profile with an increase in G_2M phase cells, and the $CD44^{High}$ phenotype is associated with active proliferation in REM134 cells (Chapter 5). It is therefore possible that this result represents a cell-cycle-specific effect rather than inhibition of a CSC subpopulation (particularly as direct NFκB inhibition did not produce differential effects).

Can Exposure to Cytotoxic Drugs Modulate the CSC Phenotype?

3132 cells were cultured in the presence of increasing concentrations of doxorubicin. Over a period of several weeks, stable growth at a concentration of 5nM was achieved. This is close to the measured IC_{50} for 3132, (5.5nM), and the drug-adapted population ("3132Drug") could be repeatedly subcultured, proliferating at a rate comparable to that of the drug-free parental cells ("3132Par"). Assays were performed at this level of adaptation, as further increases in drug concentration resulted in growth retardation, cell clumping and cell death. Equivalent populations of MCF-7 cells (MCF-7Par and MCF-7Drug) were also cultured in parallel to the 3132 cells.

Chemoresistance of cells chronically exposed to cytotoxic drugs

3132Drug cells showed increased resistance to doxorubicin, mitoxantrone and vincristine as compared to 3132Par cells. Although the overall shifts in the sigmoid curve for doxorubicin and mitoxantrone were small, IC_{50} values for the drug-adapted

population were at least double those of the drug-free cells (Figure 14). For doxorubicin, 3132Drug showed an IC_{50} of 22.4nM (95% confidence interval 14.3 – 35.1nM) where that of 3132Par was 10.6nM (95% confidence interval 6.88 – 16.27nM, $p = 0.0158$). For mitoxantrone, the IC_{50} of 3132Drug was 0.824nM (95% confidence interval 0.502 – 1.353nM); that of 3132Par was 0.298nM (95% confidence interval 0.133 - 0.672nM, $p = 0.0286$). When treated with vincristine both 3132Par and 3132Drug cells showed poor viability even at picomolar concentrations such that IC_{50} values were not obtained, but survival of 3132Drug cells was significantly greater at each level of drug ($p = 0.0313$, Wilcoxon signed rank test).

Under equivalent experimental conditions, there were no significant differences between the responses of MCF-7Par and MCF-7Drug cells (data not shown). Interestingly, when tested at low inoculums (500 cells/well) the IC_{50} values of MCF-7Drug were greater for doxorubicin (20.75nM *vs* 5.878nM for 3132Par, $p = 0.003$) and mitoxantrone (12.59nM *vs* 0.4155nM for 3132Par, $p = 0.0049$) (Figure 15 A, B). Although viability in the presence of vincristine was poor under these conditions, MCF-7Drug cells showed greater survival than MCF-7Par ($p = 0.0078$, Wilcoxon signed rank test) (Figure 15C). At this lower cell density, the IC_{50} of doxorubicin for parental cells (5.9nM) is close to the level of exposure in culture; at 5000 cells/well, the IC_{50} of doxorubicin is considerably greater (>90nM). This further emphasises the influence of cell density on these assays and during the process of drug adaptation. It is likely that chronic exposure of MCF-7 cells to greater concentrations of drug would be required to produce appreciable resistance at higher cell densities.

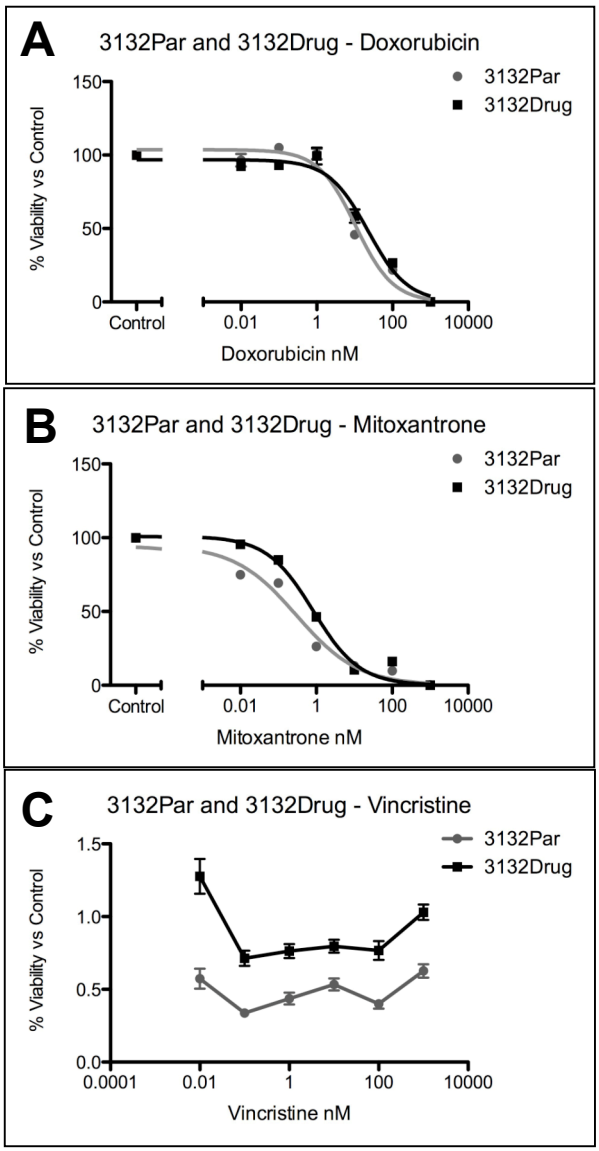


Figure 14 – Increased resistance of 3132 cells chronically exposed to doxorubicin (3132Drug) as compared to untreated (3132Par) cells.

A - Doxorubicin
B - Mitoxantrone
C - Vincristine

Although the level of resistance is modest, IC_{50} values of 3132Drug for both doxorubicin and mitoxantrone are at least twofold greater than for 3132Par cells.

Viability of both populations was poor upon exposure to vincristine. However, the survival of 3132Drug cells was again greater than that of 3132Par cells.

Drug	3132Par	3132Drug	Significant Difference?	*p* - value
Doxorubicin IC_{50} / nM	10.58	22.44	**Yes**	**0.0158**
Mitoxantrone IC_{50} / nM	0.298	0.824	**Yes**	**0.0268**
Vincristine IC_{50} / nM	-	-	**Yes***	**0.0313***

* Populations compared using Wilcoxon signed ranks test; 2-tailed *p* - value

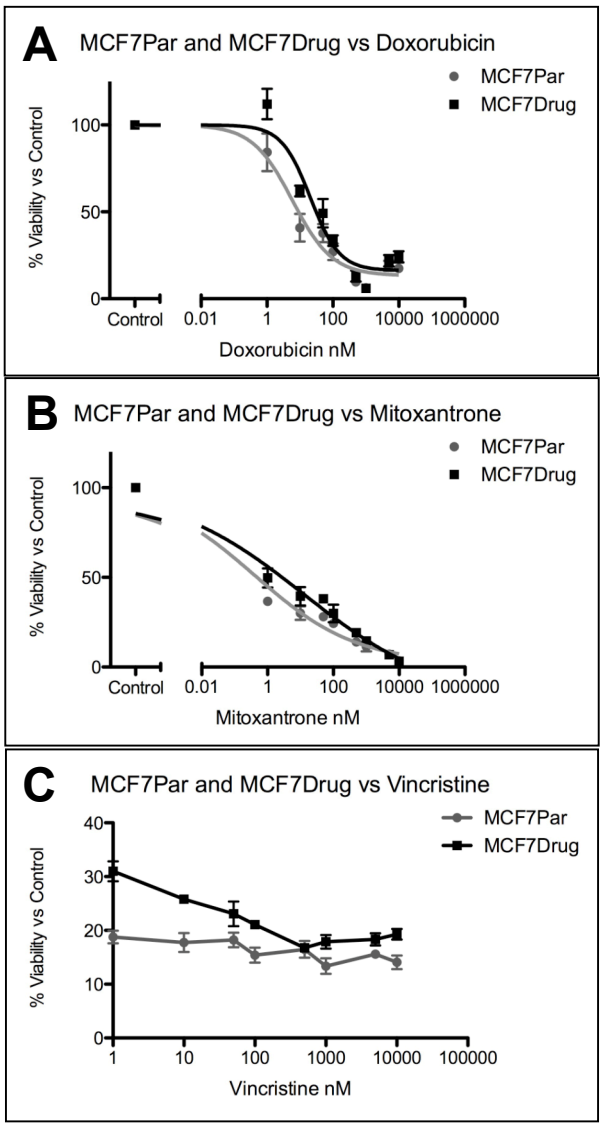


Figure 15 - Increased resistance of MCF7 cells chronically exposed to doxorubicin (MCF7Drug) as compared to untreated (MCF7Par) cells, assessed at 500 cells/well.

A - Doxorubicin
B - Mitoxantrone
C - Vincristine

Drug	MCF7Par	MCF7Drug	Significant Difference?	*p* - value
Doxorubicin IC_{50} / nM	5.87	20.75	**Yes**	**0.003**
Mitoxantrone IC_{50} / nM	0.416	12.59	**Yes**	**0.0049**
Vincristine IC_{50} / nM	-	-	**Yes***	**0.0078***

* Populations compared using Wilcoxon signed ranks test; 2-tailed *p* - value

Side population and rhodamine efflux analysis of drug-adapted cells

Hoechst 33342 efflux assays were performed to compare drug-free and drug-adapted cells. MCF-7Par cells showed an SP-like population of 0.7-0.8%, which was reduced in the presence of verapamil. The SP frequency within MCF-7Drug cells was greater, representing 1.0-1.6% of cells, and again reduced in the presence of verapamil (Figure 16). Whilst 3132Par cells did not demonstrate any SP profile, 3132Drug cells showed a candidate population of 0.3%, which was reduced in the presence of verapamil (Figure 16). As was frequently found when analysing Hoechst efflux in canine cells, the appearance of this profile was not absolutely consistent, and on one occasion an SP profile was seen in the 3132Drug verapamil control sample. Nonetheless, these results suggest that for both MCF-7 and 3132 cells, chronic adaptation to cytotoxic drugs may induce an SP-like profile.

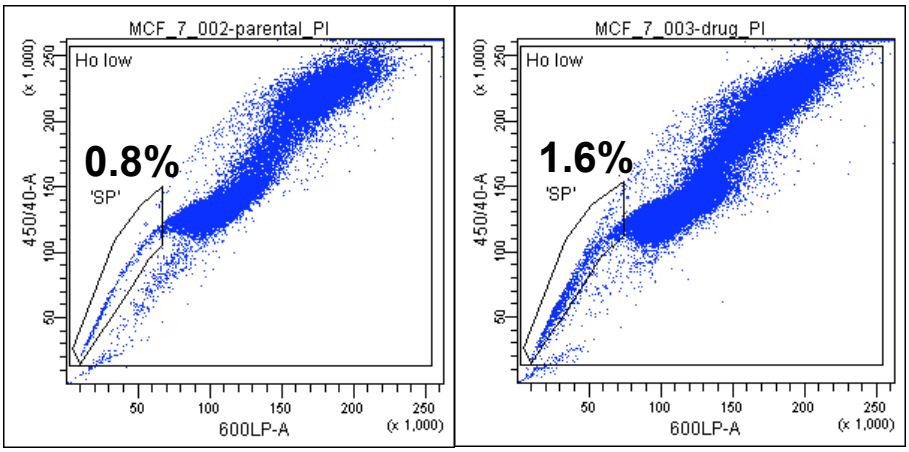


Figure 16 – Increased SP within cells chronically exposed to doxorubicin.
Above – MCF7Par (*left)* and MCF7Drug (*right*) cells.
Below – 3132Par (*left)* and 3132Drug (*right)* cells.

Rhodamine123 efflux may be mediated by either P-gP or ABCG2^{482T} (Honjo *et al.*, 2001; Robey *et al.*, 2003). 3132Par and 3132Drug cells were tested for Rhodamine 123 efflux. As with previous Rho efflux analyses of these cells, a discrete Rho^{low} subpopulation was seen, but this was lost when dead cells were excluded based on light scatter profile or propidium iodide fluorescence. When the analysis included only live 3132Par and 3132Drug cells, Rho123 fluorescence was equivalent for both test and control (no efflux period) samples (Figure 17).

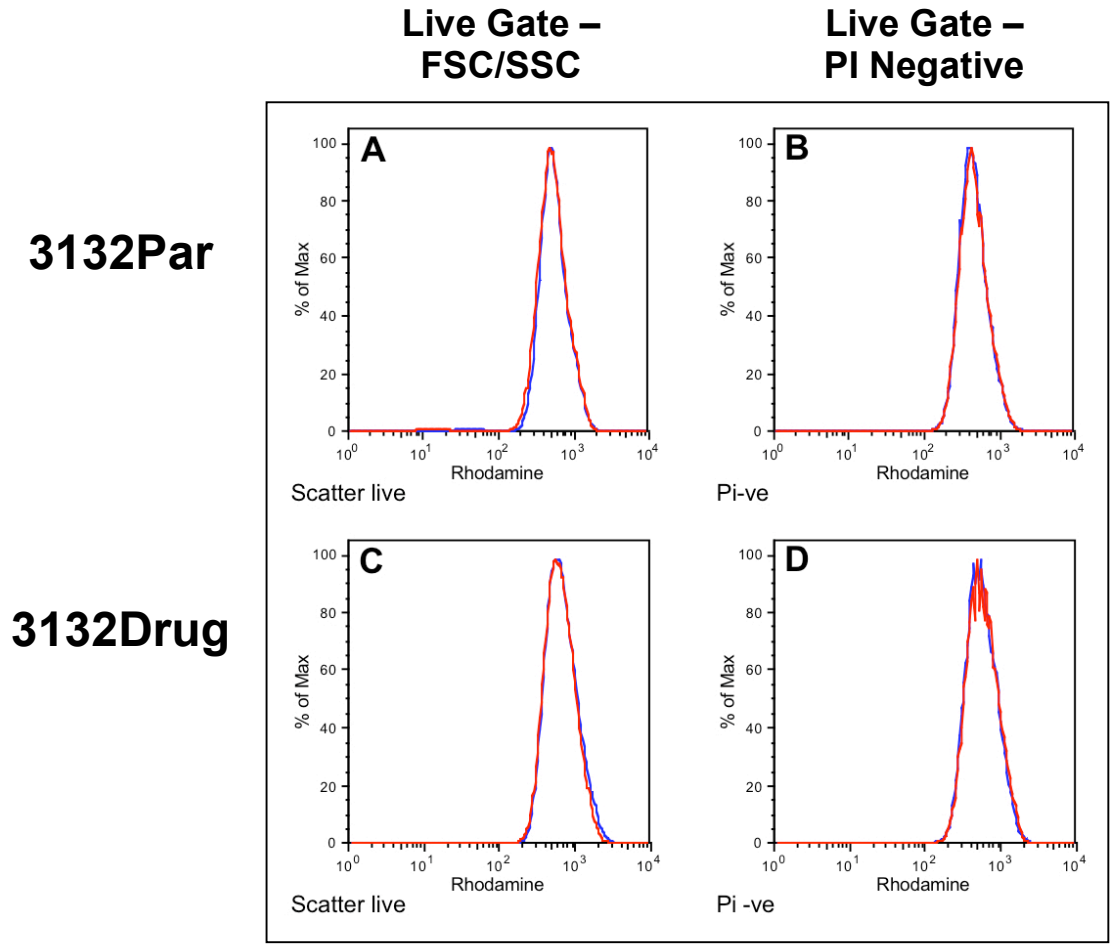


Figure 17 – Chronic exposure of 3132 cells to 5nM doxorubicin does not induce Rhodamine efflux capacity.
Overlaid histograms showing Rho123 fluorescence of test (red) and control (blue) cells.
A, B – 3132Par ; C, D – 3132Drug. Analysis including only intact / live PI-ve cells as indicated.

Thus, chronic treatment of 3132 or MCF-7 cells with 5nM doxorubicin increased the frequency of SP cells, but did not induce appreciable Rho123 efflux capacity.

Expression of ABCG2 by drug-adapted cells

ABCG2 expression by 3132Par, 3132Drug, MCF-7Par and MCF-7Drug cells was analysed using Western blot analysis. A protein band of the predicted size (73kDa) was seen for each population. Neither 3132Drug or MCF-7-Drug cells demonstrated any appreciable upregulation of protein expression - signal strength was equivalent to that of the β-actin loading control for all samples (Figure 18). Thus, despite the induction/enhancement of an SP-like profile in 3132 and MCF-7 cells by chronic treatment with 5nM doxorubicin, no clear changes in ABCG2 expression are apparent at this level of drug adaptation.

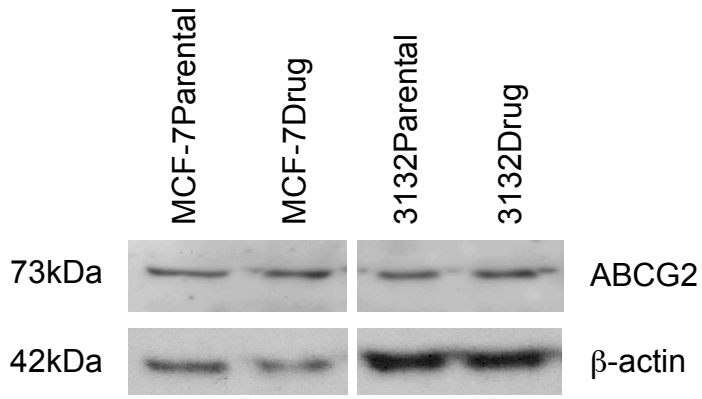
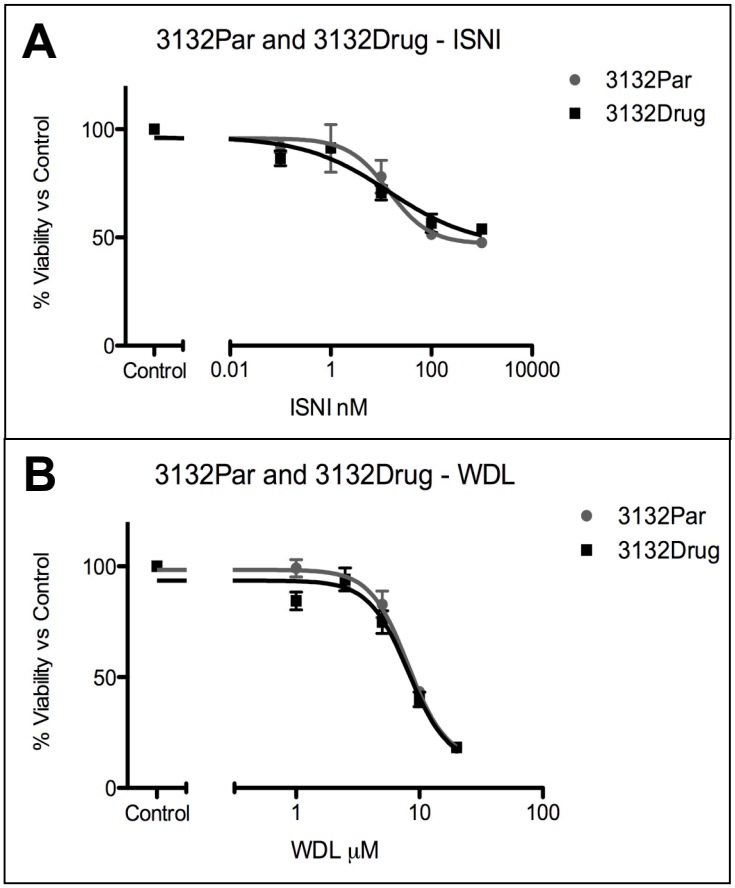


Figure 18 – ABCG2 detection by Western blot analysis of 3132 and MCF-7 cells chronically exposed to doxorubicin.

Inhibition of NFκB in drug-adapted cell lines

Resistance to chemotherapy drugs may be mediated by upregulation of antiapoptotic regulators such as NFκB (Das and White, 1997; Tergaonkar *et al.*, 2002; Bottero *et al.*, 2003). 3132Par and 3132Drug cells were assessed for sensitivity to ISNI and WDL to assess whether or not chronic exposure to doxorubicin led to increased

dependence on NFκB signalling. Inhibitory activity of both drugs was similar for 3132Par and 3132Drug, with no significant difference between IC_{50} values (Figure 19). Similarly, there were no significant differences between responses of MCF-7Par and MCF-7Drug to ISNI (to which both populations were resistant) or WDL.



Drug	3132Par	3132Drug	Significant Difference?	*p* - value
ISNI IC_{50} / nM	16.30	7.53	No	0.486
WDL IC_{50} / μM	8.42	7.75	No	0.181

Figure 19 – 3132Drug cells do not show increased sensitivity to inhibition of NFκB signalling.
A – ISNI NFκB activation inhibitor; B – WDL IKK inhibitor.
IC_{50} values of both drugs are slightly lower for 3132Drug cells, but these differences are not significant.

DISCUSSION

NFκB as a Therapeutic Target in Canine Cancer

All of the canine cancer cell lines examined in this study demonstrated nuclear NFκB transcriptional activity. In most cases inhibition of signalling resulted in only partial reduction in cell viability – this is consistent with the findings of other investigators (Hideshima *et al.*, 2002; Zhou *et al.*, 2008; Zhou *et al.*, 2009b). The supershift seen at EMSA with specific antibody to RelA/p65 demonstrates activation of the canonical pathway; compensatory non-canonical (alternative) signalling cannot be ruled out. The IKK inhibitors used in this study suppress both IKKα and IKKβ activation, so should inhibit both arms of the pathway. Whilst it is possible that genetic alterations of these proteins or their target (IκB) could reduce susceptibility, direct inhibition of NFκB transcriptional activation also produced a subtotal reduction in cell viability for all of the cell lines. This suggests that, as a population, the cells are not entirely dependent on the anti-apoptotic / pro-survival activity of NFκB for viability and proliferation, but that survival is compromised where the pathway is inhibited.

An alternative possibility for the subtotal response of the cell lines to NFκB inhibition is that only a subset of cells is reliant on signalling, with blockade leaving others relatively unaffected. Suppression of NFκB activity is reported to inhibit putative CSC, with more limited efficacy against the bulk cancer cell population, in some malignancies including canine leukaemia (Guzman *et al.*, 2007; Zhou *et al.*, 2008).

Two cellular subpopulations of REM134 mammary carcinoma cells – tumoursphere and $CD44^{High}$ cells – were examined for increased sensitivity to inhibition of IKK and NFκB. NFκB pathway inhibitors did not selectively inhibit REM134 tumoursphere cells, by contrast with the findings of Zhou *et al* (who reported that putative CSC, derived as tumourspheres from the human mammary MCF-7 cell line, were preferentially inhibited by NFκB blockade) (Zhou *et al.*, 2008). The REM134

$CD44^{High}$ cells were in fact more resistant to WDL than $CD44^{Low}$ cells. It seems likely that this effect is to some extent cell cycle-dependent, and may occur via NFκB-independent mechanisms, as there was no differential response to direct NFκB inhibition with ISNI. Moreover, although there was a shift in the response curve, almost 50% viability remained for both populations. The author acknowledges that neither tumoursphere formation or CD44 expression have been demonstrated to be reliable indicators of CSC identity for this cell line. Nonetheless, it is interesting that cytotoxicity of WDL was evident at lower concentrations for $CD44^{Low}$ cells, which are predominantly in G_0/G_1, than for actively proliferating cells, by contrast with the *in vivo* activity of many conventional chemotherapy agents. This may indicate potential for selective eradication of slow-cycling subpopulations of canine cancer cells.

Although according to the hierarchical CSC paradigm it should be possible to treat a tumour by eradicating only the stem cell component – the "dandelion root" – it is more likely that therapy must eliminate both the parent and the progeny cells for successful outcome (Kern and Shibata, 2007; Jordan, 2009; Zhou *et al.*, 2009a). Mathematical modelling suggests that elimination of only CSC would produce only slow clinical responses, allowing the opportunity for development of further mutations (Dingli and Michor, 2006) and progression of disease may occur *in vivo* even where the CSC compartment is selectively and markedly reduced (Guzman *et al.*, 2007).

The effect of combining NFκB inhibition and conventional cytotoxic therapy was investigated, to assess whether actions on different cellular subpopulations produced a complementary effect. Synergy between drugs is a topic of considerable debate, as the term is often rather overused – definitively, it refers to the situation where the effect of a drug combination is greater than the *sum* of either alone, i.e. *more than* additive (Chou and Talalay, 1983; Chou, 1998). Complex pharmacological methods such as the isobologram or Chou-Talalay combination index may be used to demonstrate true synergy (Chou and Talalay, 1983). Nonetheless, in the context of this study, even an additive effect resulting from mutually exclusive drug effects

could be relevant (i.e. if NFκB inhibition preferentially eliminated a specific CSC subset, or enabled reduction of doxorubicin concentrations for a given cytocidal effect).

However, for both REM134 and D17 cells, inhibition of NFκB signalling not only failed to increase the efficacy of doxorubicin, but appeared to antagonise its cytotoxicity. WDL at 40μM was shown to modulate the cell cycle profile, increasing the proportion of cells in G_2/M. Where drugs affect cell cycle distribution, marked sequence-dependent effects are seen for some combinations – for example, marked cell cycle-associated resistance is seen when flavopiridol (which arrests cell cycle progression) is given prior to paclitaxel (an M-phase specific taxane) (Shah and Schwartz, 2001). However, the effects of doxorubicin are not cell cycle phase-specific – moreover, even at a WDL concentration with minimal effects on REM134 viability (5μM), the IC_{50} of doxorubicin was increased threefold.

Treatment with ISNI did not alter the cell cycle profile, yet antagonism was also seen when this drug was combined with doxorubicin – the inhibitory effect was less than the sum of that caused by either drug alone. Further work would be required to ascertain why inhibition of NFκB resulted in reduced sensitivity to doxorubicin. It remains possible that combinations using alternative inhibitors of the pathway, or different cytotoxic agents, could show synergistic activity.

Mechanisms of Acquired Drug Resistance and Effects on the CSC Phenotype

Chronic exposure of 3132 cells to 5nM doxorubicin resulted in increased resistance to doxorubicin, mitoxantrone and vincristine. The more consistent appearance of an SP profile for 3132Drug cells implicates upregulation of membrane pump transporter function. The ABCG2 transporter permits doxorubicin and mitoxantrone efflux, and commonly (although not exclusively) mediates the SP phenotype (Bunting *et al.*, 2000; Zhou *et al.*, 2001; Scharenberg *et al.*, 2002; Jonker *et al.*, 2005). Although there was no marked upregulation of ABCG2 protein expression, it is acknowledged

that the resistance levels achieved in this study were modest - continued adaptation to higher concentrations of doxorubicin could result in a greater differential.

Expression of ABCG2 protein by MCF-7Drug cells was also similar to that of parental cells. ABCG2 was originally characterised in MCF-7 through chronic adaptation to doxorubicin, but drug levels achieved were orders of magnitude greater (1μg/ml≡1.8μM) than those used in this study (5nM) (Doyle *et al.*, 1998). There are, however, no reports in the literature of whether this adaptation results in changes to the SP phenotype – we have shown that even comparatively low levels of drug exposure result in a consistent increase in the proportion of cells in the SP, and that this is reduced by verapamil.

P-gP expression may also confer anthracycline resistance, and in some cells is responsible for an SP phenotype (Bunting *et al.*, 2000). Moreover, no single ABC protein has the capacity to efflux all three drugs to which the cells showed enhanced resistance (neither P-gP nor ABCG2 efflux vincristine, unlike the multidrug resistance protein MRP (ABCC1)) (Dean *et al.*, 2005). It is therefore possible that additional resistance mechanisms were induced during the course of drug induction. Although 3132Drug cells showed no increase in sensitivity to inhibition of NFκB, the contribution of other survival pathways (such as Ras/Akt or mTOR) could be investigated further using protein expression studies and specific inhibitors of signalling.

Nonetheless, alteration of SP profile for both 3132 and MCF-7 cells suggests that for canine cancer cell lines, appearance of a cell in the SP is dependent on more than just the presence or absence of ABCG2 expression. If only the cells expressing the relevant pump appear in the SP, then for the 3132Drug cells this would represent a very small (0.3 – 0.6%) proportion of the population as a whole. It seems unlikely that survival of such a minority subset could produce the differential chemosensitivity seen when 3132Drug cells were treated with cytotoxic agents. Additionally, the increase in SP frequency correlated with an increase in overall resistance despite no apparent upregulation of transporter expression.

The results of the drug induction study are more suggestive that SP in cell lines may be an indicator of the functional activity of membrane transporters, rather than expression by a distinct population of cells. This is supported by the findings of other investigators. Although ABCG2 expression may distinguish the SP cells of normal bone marrow (Zhou *et al.*, 2001), in some human cancer cell lines, cells isolated according to presence in the SP show different properties from those isolated according to ABCG2 expression (Patrawala *et al.*, 2005). Moreover, Hu *et al* found that in hepatocellular carcinoma cell lines, ABCG2 was diffusely expressed amongst the population, and that SP cells did not show greater clonogenicity than non-SP cells. However, the SP cells *did* exhibit decreased drug retention and increased pump activity (Hu *et al.*, 2008). The implications of SP in cell lines may therefore be different from those in primary tissues.

If all cells express low levels of ABCG2 yet only certain of these appear in the SP, it questions the concept that the SP itself marks out a specific, resistant CSC population within a cell line. The results presented here suggest that SP frequency may be more representative of the drug efflux capacity within the population as a whole. This could be further investigated using specific surface staining for ABCG2, to determine directly the frequency of cells expressing the protein. Moreover, investigations should be extended to primary neoplastic cells. The implications of SP in cancer remain incompletely understood and may be context dependent. However, if the Hoechst efflux assay can identify altered drug sensitivity occurring due to altered membrane pump function, prior to demonstrably increased protein expression, it could represent a potentially valuable means of assessing the existence or development of drug resistance within tumours.

It is accepted that there are limitations to the interpretation of the results obtained through these studies of chronic doxorubicin exposure, because the degree of drug adaptation was relatively modest, with levels close to the IC_{50} of the parental cell line. Development of drug-resistant cell lines is a protracted process; many of the lines described in the literature have been adapted to exposure over a period of many months or years (Taylor *et al.*, 1991; Doyle *et al.*, 1998; Coley, 2004; Uozurmi *et al.*,

2005; Yang *et al.*, 2005). In practice, increasing the concentration of doxorubicin for 3132Drug cells beyond low nanomolar concentrations proved challenging, and resulted frequently in cessation of proliferation followed by cell death. The adaptation of canine lymphoma cell lines to drug exposure has been reported (Uozurmi *et al.*, 2005); however, 3132 cells were generally very sensitive to all tested cytotoxic drugs, and it may be more rewarding to attempt drug adaptation of one of the more robust lines (e.g. REM134) to further investigate the effects of chronic exposure.

Implications of CSC-Associated Resistance Mechanisms

The CSC paradigm opens up the possibility that the cells responsible for tumour progression might be selectively destroyed, improving clinical outcomes and also patient welfare during therapy. In this context, the ability to prospectively identify such cells is paramount. Although initial reports of LSC isolation showed great promise in this regard, it is now clear that there is unlikely to be any single reliable marker of "cancer stemness", particularly for solid tumours.

Elimination of LSC as defined by surface markers such as CD44 and CD123 has shown promise in some models of haematological malignancy (Jin *et al.*, 2006; Jin *et al.*, 2009). The use of surface markers to define tumourigenic cells in the context of targetted treatment is complicated by their lack of specificity or sensitivity, particularly for solid tumours. Some are overly permissive, with expression seen within tumourigenic and non-tumourigenic populations. Other markers or combinations may be too restrictive, such that a significant proportion of tumourigenic cells exists outwith the selected population. Moreover, if continued clonal evolution of tumourigenic cells is accompanied by changes in expression profile (Shipitsin and Polyak, 2008), this could allow escape from treatments which target CSC on the basis of surface phenotype.

As CSC are functionally defined, it may be more rational to target specific functional properties such as unlimited self renewal or enhanced antiapoptotic and survival

mechanisms. Multiple cellular signalling pathways have been implicated as contributing to the self renewal potential of CSC, including Notch, Wnt/β-catenin, Bmi-1 and Hedgehog, and much work has focussed on developing means to selectively inhibit these pathways (Lessard and Sauvageau, 2003; Glinsky *et al.*, 2005; Gal *et al.*, 2006; Liu *et al.*, 2006; Peacock *et al.*, 2007; Malanchi *et al.*, 2008; Zeilstra *et al.*, 2008; Majeti *et al.*, 2009; Misaghian *et al.*, 2009; Tanaka *et al.*, 2009; Zhao *et al.*, 2009). Self renewal is an essential capability for normal stem cells, and it is essential that for these cells it is *not* inhibited if they are to repopulate normal tissues after insults incurred during cancer therapy. Finding agents with this kind of selectivity is likely to prove challenging.

Anti-apoptotic and survival mechanisms such as PI3K / Akt / mTOR and NFκB are aberrantly activated in many cancers, and inhibition of these pathways has been demonstrated as a potential means of selectively inhibiting CSC (Guzman *et al.*, 2005; Dancey, 2006; Zhou *et al.*, 2007; Hambardzumyan *et al.*, 2008; Zhou *et al.*, 2008; Bleau *et al.*, 2009; Dubrovska *et al.*, 2009). Hypothetically, as targets they may pose less risk to normal stem cells than the inhibition of self-renewal mechanisms. Cancer cells which have accrued genetic mutations, DNA damage and other abnormalities which would cause self destruction, i.e. apoptosis, in an otherwise normal cell, may be considered as existing under an increased level of cellular stress (Evan and Vousden, 2001). "Unmasking" of these stresses by inhibiting the compensatory survival mechanisms could tip these abnormal cells into apoptosis, but should be less inherently problematic for (normal stem) cells which do not carry additional mutations.

Thus, NFκB inhibition may be a means of targetting CSC without unacceptable side effects on repopulation capacity in normal tissues. Nonetheless, NFκB signalling plays an important role in mediating immune and inflammatory responses (Ghosh *et al.*, 1998), and any strategy for inhibiting the pathway would have to address the potential for immunocompromising the patient.

In this study, constitutive NFκB nuclear activity was demonstrated in all of the tested canine cancer cell lines. Moreover, inhibition of this activity resulted in an overall reduction in cell viability. Although the specific combinations of NFκB inhibitors and doxorubicin did not show complementary effects for the REM134 and D17 cell lines, further investigation may reveal alternative combinations of NFκB inhibitor / cytotoxic drug which produce more promising results, through suppression of anti-apoptotic survival mechanisms.

Marked selectivity of NFκB inhibitors for putative CSC, shown in some studies, was not demonstrated. However, there are inherent difficulties in isolating stable specific CSC populations from cell lines, as previously addressed. Evaluation of primary tumour cells may prove more valuable in this regard - as these are more likely to demonstrate true heterogeneity, evaluation *in vitro* of the responses of distinct subpopulations might more closely reflect differential sensitivity to NFκB blockade. This may help to identify tumours where this could be included as part of a combined chemotherapy regime.

The appearance of an SP for 3132Drug cells and its increase on MCF-7 is significant, as it suggests that properties used to identify CSC may be acquired or modulated during the life history of a tumour. Again, the inherent lack of heterogeneity within continuous cancer cell lines may not adequately represent the natural situation in spontaneous tumours *in vivo*. It is likely that SP analysis of primary cells, both before and during chemotherapy, might give more insight into how drug resistance is acquired, and by which cells. There is increasing recognition that CSC are unlikely to be a static entity, and that continued clonal evolution is likely, particularly in the face of cytotoxic challenge (Clarke *et al.*, 2006; Adams and Strasser, 2008; Visvader and Lindeman, 2008; Shackleton *et al.*, 2009). If therapies are to be directed specifically to CSC populations, any potential for these to change over time must be addressed.

If a specific subpopulation of cancer cells is identifiable by the expression of drug efflux ABC proteins, this could be exploited to target these cells. Many ABC

inhibitors have been characterised, showing varying levels of selectivity and potency for different members of the superfamily (Doyle and Ross, 2003; Dean *et al.*, 2005). Although showing promise *in vitro*, clinical trials with ABC inhibitors such as verapamil and cyclosporine have been largely disappointing (Fojo and Bates, 2003; Dean *et al.*, 2005). However, work has concentrated on expression in the context of the entire cellular population, rather than expression by specific CSC. Also, most trialled strategies have been aimed at inhibition of P-gP, whereas resistance of many putative CSC subsets is mediated by other transporters such as ABCG2.

It has been proposed that devising ways to target specific subpopulations of cells with specific inhibitors could prove more rewarding, although the means of achieving this remain undetermined (Dean *et al.*, 2005; Jordan, 2009). It may be possible to exploit phenomena such as "collateral sensitivity", whereby cells expressing drug resistance pumps are paradoxically sensitive to certain agents, as part of combination strategies targetting CSC (Turk *et al.*, 2009). Of course, when considering inhibition of membrane transporters such as ABCG2, it is vital to address the effects on normal stem cells, particularly those where tissue turnover is rapid. Their ability to efflux cytotoxic agents helps them to survive chemotherapy and repopulate vulnerable tissues like intestinal epithelium and haematopoietic cells, so blanket inhibition of this capability might render patients unacceptably sensitive to the effects of anticancer agents.

Signalling mechanisms which may be aberrantly upregulated in CSC, such as those controlling differentiation, self-renewal or survival, may also directly modulate the activity of membrane transporter pumps. NFκB may upregulate P-gP, and suppression of signalling may inhibit their expression and enhance uptake of chemotherapy drugs (Zhou and Kuo, 1997; Bentires-Alj *et al.*, 2003). Hedgehog signalling is reported to enhance expression of ABCG2 and P-gP in carcinoma cell lines; inhibition of the pathway with cyclopamine, combined with low dose chemotherapy, inhibits transporter expression and activity to increase overall cell kill (Sims-Mourtada *et al.*, 2007). The serine-threonine kinase Akt, involved in multiple signalling mechanisms including the PI3K / Akt / mTOR pathway, may modulate the

SP of hepatocellular carcinoma cell lines: Akt inhibition was reported to reduce the SP, inhibiting drug efflux capacity of these cells (Hu *et al.*, 2008). Thus, targetting of the proliferation or survival mechanisms of CSC as part of combination chemotherapy regimes may provide additional benefits through inhibiting their drug resistance mechanisms.

Interestingly, it appears that novel tyrosine kinase inhibitors (TKI) such as imatinib and the EGFR inhibitors gefitinib (Iressa, AstraZeneca) and erlotinib (Tarceva, Genentech) may also act to inhibit the function of ABCG2 and P-glycoprotein, increasing the sensitivity of drug-resistant cell lines to conventional drugs (Nakamura *et al.*, 2005; Yang *et al.*, 2005; Shi *et al.*, 2007; Chu *et al.*, 2008). These effects are reported in combination with diverse chemotherapeutics, such as the intercalating agents doxorubicin (Chu *et al.*, 2008) and mitoxantrone (Nakamura *et al.*, 2005; Shi *et al.*, 2007), the mitotic spindle poison paclitaxel (Shi *et al.*, 2007), the topoisomerase I inhibitor etoposide (Yang *et al.*, 2005) and the topoisomerase II inhibitors irinotecan and topotecan (Nakamura *et al.*, 2005). Moreover, efficacy is reported even where cells do not overexpress the relevant tyrosine kinase (Nakamura *et al.*, 2005), suggesting a direct inhibitory effect on the transporter.

Two TKIs have been recently licensed for use in veterinary medicine. Toceranib (Palladia, Pfizer) and masitinib (Masivet, ABScience) have been released for treatment of canine mast cell tumours, based on their activity against c-Kit (Pryer *et al.*, 2003; Hahn *et al.*, 2008). Efficacy is reported towards other tumour types, and may also relate to inhibition of other tyrosine kinases – for example, toceranib may suppress PDGFRβ and VEGFR2 (London *et al.*, 2003; London *et al.*, 2009). Drug resistant canine cancer cell models should provide a platform to assess whether these TKIs modulate the activity of membrane transporter pumps, and might therefore show potential in drug combinations aimed at targetting resistant or CSC populations.

In summary, despite continuing controversy over what identifies a CSC, the goal of treatment must be to eliminate all cells which may drive progression or persist after

therapy. Therefore, it is perhaps more important to focus not on specific phenotypic identifiers, but what makes these cells resistant. Whatever the mechanism underlying heterogeneity within a given tumour, any cell with inherently increased resilience to treatment is more likely to persist, and thus have the opportunity to garner further mechanisms of resistance. Moreover, a reduction in tumour size may result in an increased growth fraction and decreased doubling time, increasing the proportion of cycling cells and the risk of further resistance developing (Dingli and Michor, 2006).

Thus, rapid and simultaneous eradicaton of all tumourigenic subpopulations will reduce the potential for further mutation of surviving cells – conversely, therapies that show only an increased selectivity towards putative CSC, but fail to completely eradicate them, may not ultimately produce better clinical outcomes. The clinical relevance of the CSC hypothesis lies largely in the fact that, whatever their phenotype, certain cells survive treatment. It therefore makes sense that identifying and exploiting their resistance mechanisms may be the most successful means to eliminate them.

CHAPTER 7

DISCUSSION

According to the cancer stem cell hypothesis, eradication of specific CSC subpopulations is critical to the successful treatment of neoplasia, without risk of recurrence. Notwithstanding the debate surrounding the hypothesis, it is rational to try and identify and characterise CSC, or clonally evolved subpopulations, which might possess enhanced malignant capabilities or persist after therapy. In dogs, as in humans, cancer therapy is confounded by the two major problems of relapse and metastatic spread, and so spontaneous cancer in the dog represents a valuable comparative model of human disease.

In this study, evaluation of the surface expression patterns of cancer cell lines did not reveal discrete candidate CSC populations. In the canine (and human and feline) cell lines tested, the flow cytometry histogram for a given marker appeared as a bell-shaped curve, from the lowest to the highest level of expression, rather than as a positively-stained population separate from the bulk. In a population of clonal cells, this distribution for a given antigen may arise through fluctuation of expression levels around the overall population mean (Brock *et al.*, 2009). A foundation of the CSC hypothesis is that the CSC and non-CSC populations are biologically distinct, with different inherent properties. For the cell lines examined here, expression of stem cell-associated surface markers (CD24, CD34, CD44, CD117, CD133) demonstrated no such distinctions. Cells were either negative for the tested antigen, or where positive (i.e. expression of CD44) showed transient and fluctuating surface expression levels in association with proliferation status.

Similarly, studies of drug efflux or detoxification capacity failed to demonstrate distinct and consistent subpopulations of cells with enhanced resistance mechanisms. Although Hoechst efflux analysis identified a side population within the tested cancer cell lines, the inconsistency of the assay precluded further isolation and evaluation of these cells. Moreover, the relationship between SP and expression of drug efflux pumps remains ambiguous. Evidence presented here from the study of drug resistant cells, as well as by other investigators, suggests that in cell lines appearance of a cell in the SP is multifactorial. Whilst the magnitude of the SP may indicate the overall functional activity of membrane transporters, cells within a

population of inherently similar ABCG2$^+$ cells may fall within the SP according to transient factors such as proliferation or developmental status. Thus, although presence within the SP may indicate the status of individual cells at a particular timepoint, it may not reflect a biologically distinct cellular subpopulation.

Although heterogeneity may exist within cell lines, the existence of biologically discrete subpopulations within clonal populations, which have been passaged over many generations, is less likely than in primary cells. Whilst the resurgence of the paradigm has been accompanied by numerous reports of putative CSC in well-established tumour cell lines, the concept that cell lines are maintained by a specific subpopulation of self-renewing cells remains contentious (Locke *et al.*, 2005; Zheng *et al.*, 2007; Yoo and Hatfield, 2008). For example, the formation of colonies in soft agar by only a limited proportion of cells has been suggested as evidence of this biological distinction (Lou and Dean, 2007). However, the evidence obtained through studies of CD44 expression by canine cancer cell lines strongly suggests that transient factors such as proliferation status can have a profound effect on colony formation. Thus, apparent heterogeneity in cellular behaviour within cell lines is not necessarily the result of a predetermined hierarchy. Modelling of the population dynamics within continuous cell lines suggests that a situation in which the bulk of cells are unable to replicate extensively, but are propagated by a limited subpopulation, may not be sustainable (Figure 1).

Moreover, within continuous cell lines, novel phenotypes may arise as a result of extrinsic influences, which will include culture conditions and technique. This may be one reason behind the conflicting data obtained through investigation by different groups (even for well characterised cell lines such as human MCF-7 mammary carcinoma (Kondo *et al.*, 2004; Patrawala *et al.*, 2005; Zhou *et al.*, 2007; Engelmann *et al.*, 2008; Steiniger *et al.*, 2008; Tanaka *et al.*, 2009) or rat C6 glioma (Kondo *et al.*, 2004; Zheng *et al.*, 2007)), and further confounds interpretation of the literature. Undoubtedly, cell culture techniques became more refined during the course of conducting the experiments presented here, due to greater operator experience.

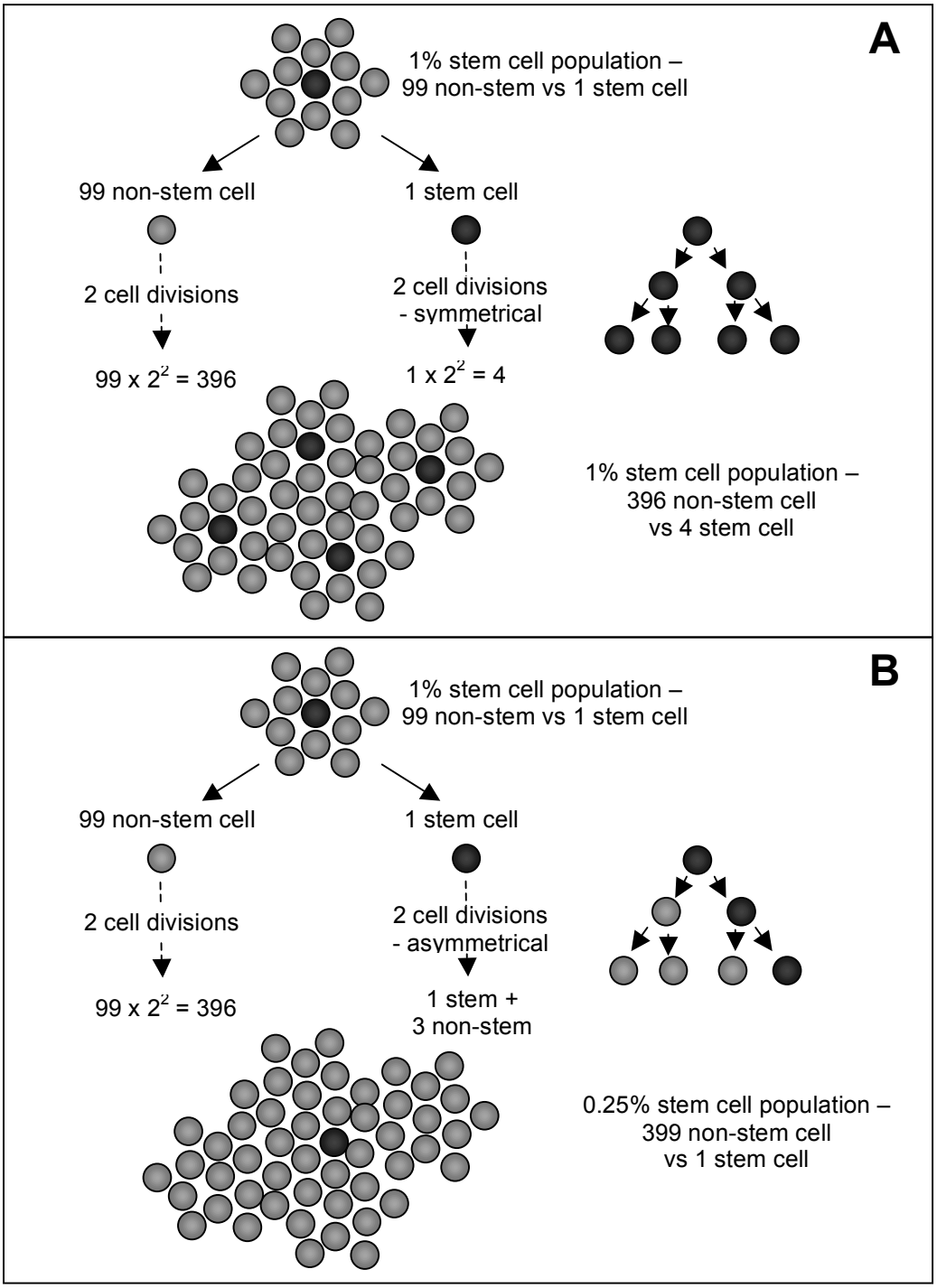


Figure 1 – Modelling the maintenance of a rare and specific CSC population within a cell line. Model assumes that non-stem cells may participate in population expansion, as must be the case in logarithmic growth. Symmetrical division of stem cells maintains overall proportion through exponential expansion (A). If stem cells must divide asymmetrically to produce non-stem cells, then the overall propoportion of stem cells will be reduced even over a limited number of divisions (B), unless proliferation rate of stem cell fraction is significantly greater, or non-stem cells and their descendents die soon after proliferation. (Adapted from Zheng *et al*, 2007 *Cancer Research*, **67** (8), 3691-7).

Investigation of the effects of culture protocol (including cellular confluence at harvest, culture medium and split ratio) may help to elucidate how this may affect the results of assays for CSC, such as Hoechst-effluxing SP or expression of cell surface markers. Nevertheless, it appears that continuous cell lines have limitations when investigating concepts of cellular heterogeneity and hierarchies in neoplasia, as stable and intrinsic biological distinctions between cells are less likely to be evident than in spontaneous tumours.

By contrast, within the kt-osa populations derived from a primary canine osteosarcoma, discrete subpopulations expressing CSC-associated antigens such as CD117 and CD133 could be identified. The comparatively rapid reduction in these subpopulations over time in adherent culture suggests that heterogeneous expression patterns will be best represented in primary cells derived directly from tumours *in vivo*. This is particularly germane when considering rare-event and / or putatively slow-cycling populations. However, this also presents practical challenges, not only in terms of access to samples, but also when considering the cell numbers that will need to be isolated in order to carry out comparative assays.

Tumoursphere culture represents a potential means of expanding putative CSC *ex vivo* for further characterisation. Many studies have reported the use of the assay as successfully isolating candidate CSC populations from cancer cell lines, and all of the canine cell lines examined showed some degree of tumoursphere formation. However, clonal and population analyses for the cell lines tested in this study suggested that this did not represent proliferation of a biologically distinct population of canine tumour cells. Proliferation as tumourspheres by a fixed proportion of CSC should result in a linear relationship between cell density and sphere formation - this was not seen for any of the tested cell lines. The formation of tumourspheres by $CD44^{High}$, but not $CD44^{Low/-}$, REM134 cells also indicated the influence of other factors, such as cell cycle/proliferation status at plating.

It is accepted that these ambiguities may be specific to the cell lines examined in the study, but the findings certainly emphasise how results obtained through

tumoursphere culture must be carefully interpreted (Visvader and Lindeman, 2008). For example, claims that sphere formation equates to CSC proliferation should at least be qualified by clonal analysis, to demonstrate self-renewal capacity (as this is one of the principal cellular characteristics for which low-density, serum-free culture hypothetically selects).

In comparison to the continuous cell lines, for which most cells appeared to participate in tumoursphere formation in bulk cultures, observations of tumoursphere formation by kt-osa populations were more suggestive of selective proliferation by certain cells. Morphological distinctions between spheres derived from early and later-passage cells again suggest that tumoursphere culture of cells derived directly from tumours may more accurately reflect their heterogeneous composition. Notably, however, proliferation of kt-osa cells in low-density culture was limited, and did not permit propagation beyond 2-3 passages, despite the fact that the cells were derived from an aggressive metastatic tumour which led rapidly to the death of the patient.

Thus, whilst the fitness of cells to proliferate as tumourspheres in low-density serum-free culture is frequently cited as evidence of a CSC population, it is clear that the tumour-propagating cells of some malignancies may not be isolated using this assay. Additionally, whilst tumoursphere culture may be a useful means of expanding putative CSC populations for characterisation, multiple factors (such as differences in growth phase, drug penetration and oxygenation, and the persistence of contact-related resistance in 3-D cultures) would suggest that direct comparison of tumoursphere and adherent cultures may not give reliable results when inferring differential sensitivities to anticancer agents.

The demonstration of an $ALDH^{Bright}$ population within the BMMNC and PBMNC of a dog with leukaemia (and also weakly positive cells within the kt-osa populations at early passage) also supports the concept that subpopulations may be more readily identified within primary cells than cell lines – none of the tested canine cell lines tested positive using the Aldefluor assay. It is acknowledged that the role of ALDH

in normal canine stem and progenitor cells is not well established - whilst it appears to identify stem-like cells of multiple human tissues, its potential as a marker of undifferentiated cells in other species remains to be determined. Nonetheless, these findings suggest heterogeneity within these primary cell populations for drug detoxification capacity, and the $ALDH^{Bright}$ population increased during the course of treatment with the alkylating agent cyclophosphamide.

Significantly, the Aldefluor assay did not demonstrate a strong candidate for normal HSC within normal canine BMMNC. Similarly, $CD34^{+}$ leukaemic BMMNC showed a scatter profile distribution distinct from that expected for normal HSC. This is consistent with the now widely accepted concept that the CSC is not necessarily equivalent to a transformed tissue stem cell, and as such, the tumour-propagating cell will not necessarily display the same phenotypic characteristics as the stem cell which maintains the corresponding normal tissue. However, the functional capacity of ALDH to detoxify xenobiotic substances suggests that the Aldefluor assay may represent a potential means of identifying drug resistant cells, which may exert a critical influence on the biological behaviour of a tumour.

Thus, the results obtained through the course of these studies suggest that attempts to prospectively isolate subpopulations of putative CSC should concentrate on primary tissues. Of course, whilst the studies examined several canine cancer cell lines of diverse origin, this does not rule out the existence within other lines of stable, biologically distinct CSC fractions - but the lack of demonstrably discrete subpopulations within cell lines is by no means exclusive to this study. However, cell lines remain invaluable for developing techniques and assays, especially when optimising sensitive assays for characterisation of "precious" primary cells, which may be available on a limited basis.

One of the most enticing prospects raised by the CSC hypothesis was the possibility that there might be a consistent and universal marker (for example, CD133 or presence of a side population) enabling identification of the tumour-propagating fraction. It has now become clear that there exists considerable variation both

between and within tumours and tumour types. This will complicate interpretation of results obtained from primary samples – this is particularly relevant to studies of canine neoplasia, where detailed immunophenotyping data from large numbers of similar tumours is less readily available than for comparable human disease. Thus, cell lines also have an important place when assessing the implications of certain phenotypes. For example, in not just one but six canine (and one feline) cancer cell lines, an association between CD44 and cellular proliferation status was demonstrated.

The data gathered through studies of CD44 expression in canine cancer cell lines suggest that, as a transiently-expressed marker of proliferating cells, it may have limited utility as a CSC identifier. Interestingly, other studies where data suggests that the putative CSC marker may be proliferation-associated include the seminal brain tumour stem cell work published by Singh *et al* in 2003. In that study, $CD133^+$ cells showed increased proliferative capacity and tumoursphere formation compared with $CD133^-$ cells; moreover, when sorted cells were tested for purity, that of the $CD133^-$ population was much higher than the $CD133^+$ subset ($CD133^-$ 92.6 – 97.3%; $CD133^+$ 46.9 – 79.8%), which may indicate transient expression. Notably, these were cells derived from primary tumours, rather than cell lines (Singh *et al.*, 2003). The association of CD44 with proliferation does not preclude its potential as a prognostic indicator in cancer. Published observations have been equivocal, but have involved limited characterisation of variant isoforms, and on the basis of the findings presented here, the molecule warrants further investigation in canine tumours.

The demonstration of NFkB activation in multiple canine cancer cell lines similarly merits further investigation of the impact that this pro-survival pathway may have on the progression of canine neoplasia. Although it was unexpected to see antagonistic effects between the tested NFkB inhibitors and doxorubicin, assessment of other compounds may reveal drugs with more promise in this regard (published drug screens generally involve selection of a lead compound for further testing, which may suggest that of those assayed, not all produce the anticipated results!). The tested inhibitors showed little selectivity for populations derived from the REM134

mammary carcinoma cell line on the basis of putative CSC markers (tumoursphere formation / CD44 expression), but it appears that these models may not isolate biologically distinct cellular subsets. It remains possible that *ex vivo* sensitivity testing of specific cellular fractions directly derived from spontaneous tumours will demonstrate a greater differential effect. However, these findings emphasise the complexity of the pathway, and its multiple context-dependent interactions with other signalling mechanisms, such that it may be difficult to predict the outcomes associated with its suppression in diverse cell types (Hideshima *et al.*, 2009).

The key implication of the CSC hypothesis for cancer therapy lies in the concept that the behaviour of cancer cells will be predictable, based on their status as CSC (tumourigenic) or non-CSC (non-tumourigenic). This implies that therapy could effectively "ignore" certain cells within a tumour – as long as treatment eliminates the tumour-propagating CSC population, the rest of the cells should be unable to sustain the malignancy and the tumour will regress. There is now sufficient evidence to support the existence of cellular hierarchies within multiple tumour types, particularly haematopoietic malignancies. However, it is now widely accepted that CSC themselves may mutate, and their phenotypic profile change over time. It could be argued that, as a consequence, it will never be possible definitively to predict which cells represent the CSC fraction such that all other cells may be considered non-tumourigenic. Moreover, an individual tumour may comprise more than one tumourigenic population. This has led some commentators, such as the eminent cancer biologist and clinician Isaiah Fidler, to conclude that even where the hierarchical CSC model applies, specific therapeutic targetting of CSC is unlikely to improve clinical outcomes (Rowan, 2009).

Even staunch proponents of the CSC hypothesis concede that the published literature has become confused, leading not only to difficulty when evaluating its implications, but also increased scepticism. This has arisen not least as a result of overinterpretation of the model. Indeed, in a recent interview John Dick, leader of the group which performed the seminal work on stem cell hierarchies in AML, welcomed some of this controversy:

> "Controversy sparks better and better science. What it does is it actually eliminates sloppy thinking. There's been a real rush onto the cancer stem cell bandwagon in the last couple of years. People are talking about cancer stem cells here, there and everywhere, and in any old cell line. There was a huge slippage in the kind of criteria and rigor. People were using this terminology without any thought or any rigor based on some cell-surface marker or something like that." (Baker, 2009)

Fundamentally, characterisation of CSC must be based on their functional properties – that is, self-renewal and the capacity to differentiate into all relevant cell lineages. This may be supported by evidence of other CSC-associated properties, such as expression patterns associated with normal tissue or embryonic stem cells, growth in low-density tumoursphere conditions or the appearance of a side population – however, in order to substantiate a claim that a cancer cell is a CSC, these alone are not sufficient. Equally, where some cells show self-renewal capacity and multipotentiality but are no more resistant to standard therapies, or indeed if *all* cells within the population may show these properties (such as with some cancer cell lines), the CSC hypothesis will not be clinically relevant.

Nonetheless, against a background of consistently high rates of recurrence after therapy, the CSC hypothesis draws attention to the concept that eradication of specific cellular subpopulations, with different potencies and vulnerabilities, may be required to eliminate some cancers. This ties in with an increasing recognition that the most successful treatment outcomes in cancer are likely to be obtained through tailoring therapeutic regimes to individual tumours. The acknowledgement of clonal evolution amongst CSC populations complicates the issue of direct targetting – although the "dandelion root" concept of CSC-based therapy suggests that elimination of only these cells should be sufficient to destroy a tumour, this will only be a safe approach where it can be proven definitively that the remaining cells have no tumourigenic potential. It can be argued that, owing to the caveats associated even with "gold standard" methodologies such as serial transplantation in laboratory

animals, there are currently no assays sufficient to provide this level of evidence. Thus, CSC-based therapies are likely to be most successful as part of a combination regime which includes destruction of the bulk population. Moreover, even where the hypothesis is applicable, it is unlikely to replace the need for thorough characterisation and vigilant monitoring of cancer.

If CSC show clonal variation within and between tumours, the assessment of spontaneous, naturally heterogeneous tumours such as those occurring in the pet dog population may provide more relevant information than more homogeneous, artificially-induced neoplasms in laboratory animals. Although human tumour xenografts may be propagated in immunosuppressed murine hosts, the cellular population is likely to have undergone a degree of selection for growth in a foreign environment; moreover, interactions with host factors such as the tumour niche and immune system will be dissimilar to those experienced by the parental tumour.

The assays developed during the course of these studies should enable more detailed assessment of clinical cases, to characterise cellular heterogeneity within spontaneous canine tumours. Using a panel of flow cytometric markers, cells may be characterised according to surface phenotype – not only will this demonstrate the presence of subpopulations expressing CSC-associated markers, but testing over the course of treatment (for example with haematological malignancies) may indicate whether certain cellular subpopulations appear to be more resilient to a given treatment protocol. Isolation by FACS and/or expansion in tumoursphere culture, with *ex vivo* sensitivity testing of specific subsets, may build up a picture of how putative CSC respond to specific interventions, helping to inform therapeutic choices.

Moreover, assessment of tumours throughout the course of treatment using assays for CSC properties may help to determine the progression of disease, or monitor the development of chemoresistance. For example, analysis of Hoechst efflux by drug-exposed cells showed that the frequency of SP cells may increase as resistance develops. SP, rhodamine efflux and Aldefluor analysis of spontaneous tumours may

suggest a likely drug sensitivity profile or identify resistant subsets, allowing selection of agents which will be more likely to eliminate neoplastic cells and avoidance of those which may permit persistence of putative CSC.

The cancer stem cell hypothesis remains a topic of great interest and debate, with considerable scope for further investigation. Through following the literature, and in the course of these investigations, it has become clear that the CSC model is not the straightforward, universal paradigm that many had perhaps hoped it would be – in the words of John Dick,

> "There was a lot of hype of CSC being the answer to everything. Now we are in the phase of asking, "How valid is it? How universal is it?"" (Baker, 2009)

However, where CSC hierarchies exist in tumours, they may present novel ways to confront major clinical challenges in cancer, such as metastasis and disease relapse. Not only will naturally heterogeneous spontaneous cancer in the dog provide a suitable model for human disease, advances in understanding will also serve to improve the welfare of canine patients. It is hoped that the work presented here will provide a basis upon which can be built further insight into the CSC hypothesis, and the role of tumour cell hierarchies in cancer.

CHAPTER 8

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Review

From viruses to cancer stem cells: Dissecting the pathways to malignancy

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Abstract

Cancer is a disease of all vertebrate species and has been well documented throughout history with fossil records indicating that dinosaurs of the Jurassic period suffered from the disease. The Greek physician, Galen is accredited with describing human tumours as having the shape of a crab, with leg like tendrils invading deep into surrounding tissues – hence the term cancer. Today cancer can be defined as any malignant growth or tumour caused by abnormal and uncontrolled cell division that is able to invade tissues locally and spread to other parts of the body through the lymphatic system or the blood stream. This is obviously a simplistic attempt at describing a complex disease that can utilize a myriad of biological pathways to sustain growth and proliferation. Dissecting these pathways has been the challenge of cancer researchers for decades in the search for new treatment strategies. This review attempts to condense our understanding of cancer and to offer insights into an alternative theory regarding the existence of true cancer stem cells and how this will inform the development of new therapeutics.

Keywords: Cancer; Viral oncogenesis; Stem cell; Dog; Therapies

Introduction

Fundamental to our basic understanding of mammalian physiology is the concept of homeostasis. If we consider the body as a multi-cellular unit, then cells within this unit form part of a specialized society that cooperates to promote survival of the organism. In terms of homeostasis, cell division, proliferation and differentiation are strictly controlled and a balance exists between normal cell birth and natural cell death (Argyle and Khanna, 2006). In simple terms, cancer can be considered as a breakdown in cellular homeostasis leading to uncontrolled cell division and proliferation, which ultimately leads to a disease state.

The mechanisms of this breakdown are the subject of intense research, especially considering the high incidence of cancer in both humans and domestic animals. However, despite the fact that cancer is a common disease, and considering the number of cells making up an organism, the change from normal cell to cancer cell is actually a very rare event (Evan and Littlewood, 1998). This is because evolution has allowed the development of many fail-safe mechanisms within the cell that react to DNA damage by arresting the cell cycle (to allow repair) or allow the cell to die naturally. In this short synopsis of cancer biology we will consider the current understanding of cancer biology and discuss some of the ways that this is leading to informed drug development. Further, we will consider the role of stem cells in cancer and how they are challenging conventional wisdom.

The pathways to cancer: A stochastic model

For many years, cancer researchers have considered a stochastic model of cancer development (McCance and Roberts, 1999). In this model, cancer formation is the

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phenotypic end result of a whole series of changes that may have taken a long period of time to develop. Following an initiation step produced by a cancer forming agent on a cell, there follows a period of tumour promotion (Fig. 1). The initiating step is rapid and affects the genetic material of the cell. If the cell does not repair this damage, then promoting factors may progress the cell toward a malignant phenotype. In contrast to initiation, progression may be a very slow process, and may not even manifest in the lifetime of the animal.

Over the past four decades, cancer research has generated a rich and complex body of information revealing that cancer is a disease involving dynamic changes in the genome. Each stage of multi-step carcinogenesis reflects genetic changes in the cell with a selection advantage that drives the progression towards a highly malignant cell. The age-dependent incidence of cancer suggests a requirement for between four and seven rate limiting stochastic events to produce the malignant phenotype.

Oncogenes

Seminal to our understanding of cancer biology has been the discovery of the so called "cancer genes", or oncogenes, and tumour suppressor genes. Mutations that produce oncogenes with dominant gain of function, and tumour suppressor genes with recessive loss of function have been identified through their alteration in human and animal cancer cells and by their elicitation of cancer phenotypes in experimental models. The initial observation came in 1910 when Rous demonstrated that a filterable agent (later classified as a retrovirus termed avian leukosis virus) was capable of producing lymphoid tumours in chickens. Retroviral sequences that are responsible for transforming properties are called viral oncogenes (*v-onc*). Viral oncogenes were subsequently shown to have cellular homologues called cellular oncogenes (*c-onc*). Later the term proto-oncogene was used to describe cellular oncogenes that do not have transforming potential to form tumours in their native state but can be altered to lead to malignancy.

Most proto-oncogenes are key genes involved in the control of cell growth and proliferation and their roles are complex. For simplicity, their sites and modes of action in the normal cell can be divided as follows: growth factors, growth factor receptor, protein kinases, signal transducers, nuclear proteins and transcription factors (Hanahan and Weinberg, 2000) (Table 1 and Fig. 2). The conversion of a proto-oncogene to an oncogene is a result of somatic events in the genetic material of the target tissue. The activated allele of the oncogene dominates the wild-type allele and results in a dominant gain of function. This means that only one allele has to be affected to obtain phenotypic change and is in contrast to tumour suppressor genes where both alleles have to be lost for phenotypic change. The mechanisms of oncogene activation include the following.

Chromosomal translocation

Where proto-oncogenes are translocated within the genome (i.e. from one chromosome to another), their function can be altered. In human chronic myeloid leukaemia (CML), a chromosomal breakpoint produces a translocation of the c-abl oncogene on chromosome 9 to a gene on chromosome 22 (bcr). The bcr/abl hybrid gene produces a novel transcript whose protein product has tyrosine

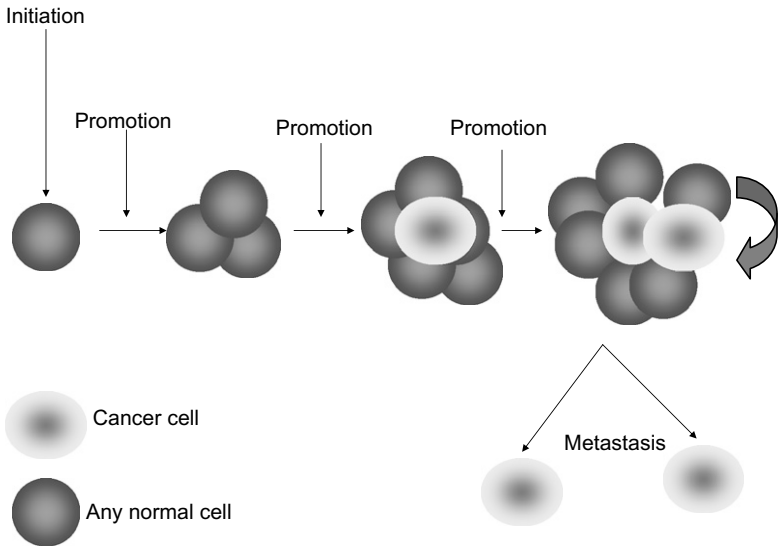

Fig. 1. *The stochastic model of carcinogenesis*: Cancer formation is the phenotypic end result of a whole series of changes that may have taken a long period of time to develop. They can occur in any cell type in the body. Following an initiation step produced by a cancer forming agent on a cell, there follows a period of tumour promotion. Each stage of multi-step carcinogenesis reflects genetic changes in the cell with a selection advantage that drives the progression towards a highly malignant cell. The age-dependent incidence of cancer suggests a requirement for between four and seven rate limiting, stochastic events to produce the malignant phenotype.

Table 1
Oncogenes can be growth factors, growth factor receptors, protein kinases, signal transducers, nuclear proteins and transcription factors

Oncogene class	Examples
Growth factors	Platelet derived growth factor (PDGF) Epidermal growth factor (EGF) Insulin like growth factor-1 (ILGF-1) Vascular endothelial growth factor (VEGF) Transforming growth factor-β (TGF-β) Interleukin-2 (IL-2)
Growth factor receptors	PDGF-receptor (PDGF-R) EGFR-receptor (erbB-1) ILGF-1 receptor (ILGF-R) VEGF-receptor (VEGFR) IL-2 receptor (IL-2R) Hepatocyte growth factor receptor (met) Heregulin receptor (neu/erbB-2) Stem cell factor receptor (kit)
Protein kinases	Tyrosine kinase e.g.: bcr-abl, src Serine-threonine kinase e.g.: raf/mil, mos
G-Protein signal transducers	GTPase e.g.: H-*ras* , K-*ras* , N-*ras*
Nuclear proteins	Transcription factors, e.g., ets, jun, fos, myb, myc, rel

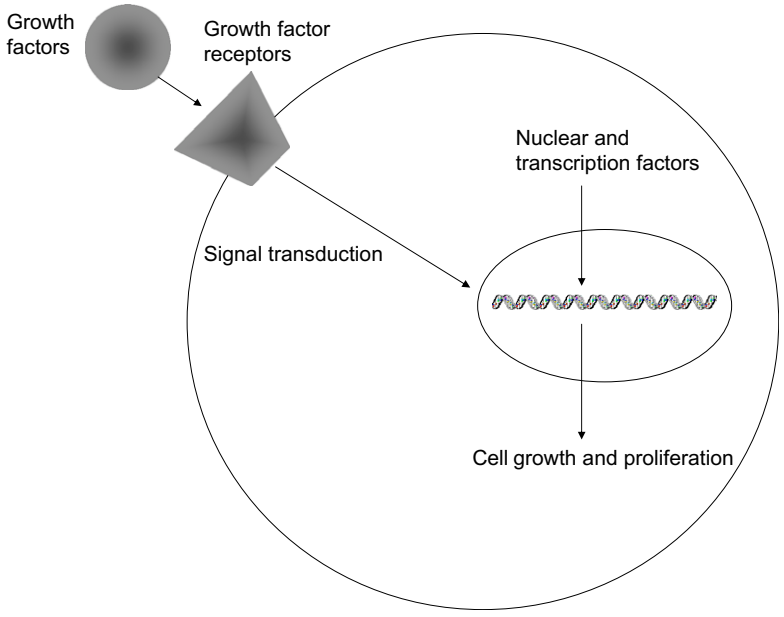

Fig. 2. *Oncogenes are normal cellular genes involved in cell growth and proliferation*: Most proto-oncogenes are key genes involved in the control of cell growth and proliferation and include growth factors, growth factor receptors, protein kinases, signal transducers, nuclear proteins and transcription factors. The conversion of a proto-oncogene to an oncogene is a result of somatic events in the genetic material of the target tissue. The activated allele of the oncogene dominates the wild-type allele and results in a dominant gain of function. The mechanisms of oncogene activation include chromosomal translocation, gene amplification, point mutations, and viral insertions.

kinase activity and can contribute to uncontrolled cellular proliferation (Heaney and Holyoake, 2007). This tyrosine kinase activity has become a major target for therapeutic intervention, with many drugs such as imatinib (a tyrosine kinase inhibitor) in human clinical trials.

Gene amplification

Amplification of oncogenes (i.e. multiple gene copies) can occur in a number of tumour types and has been demonstrated in domestic animal cancers. As an example, the MDM2 proto-oncogene has been identified in dogs and horses and has been shown to be amplified in a proportion of canine soft-tissue sarcomas (Nasir et al., 2001).

Point mutations

These are single base changes in the DNA sequence of proto-oncogenes leading to the production of abnormal proteins. For example, point mutations in the Ras

proto-oncogene are a consistent finding in a number of human tumours (Konstantinopoulos et al., 2007).

Viral insertions

Studies of the tumour causing viruses allowed for the discovery of oncogenes. In some circumstances proto-oncogene function can be damaged by the insertion of viral elements. A more detailed account of viral oncogenesis is given below.

Tumour suppressor genes

Changes in genes can lead to either a stimulatory or inhibitory effect on cell growth and proliferation. The stimulatory effects are provided by the proto-oncogenes described above. Mutations or translocations of these genes produce positive signals leading to uncontrolled growth. In contrast, tumour formation can result from a loss of inhibitory functions associated with another class of cellular genes called the tumour suppressor genes. The Retinoblastoma gene (Rb) was the first gene to inform mechanisms of tumour suppressor genes (Weinberg, 1995).

The retinoblastoma tumour suppressor Rb is the principal member of a family of proteins that also encompass pRb2/p130 and p107. Rb plays a central role in regulating cell cycle progression in G1 and disruption of Rb function has been found to be a common feature of many human cancers as well as the classical retinoblastoma tumour. Rb function can be abrogated by point mutations, deletions, or by complex formation with viral oncoproteins such as SV40 large T antigen or adenoviral E1a protein (Knudsen et al., 2006). In a cell with only one normal allele of a tumour suppressor gene such as Rb, that allele usually produces enough tumour suppressor product to remain normal. Mutations in tumour suppressor genes behave very differently from oncogene mutations. Whereas activating oncogene mutations are dominant to wild-type (they emit their proliferating signals regardless of the wild-type gene product), suppressor mutations are recessive. Mutation in one gene copy usually has no effect, as long as a reasonable amount of wild-type protein remains (Fig. 3).

The discovery of the p53 gene, another tumour suppressor, revolutionised our understanding of molecular oncology (Harris, 1996). p53 is a gene whose product is intimately involved in cell cycle control; it has been described as the guardian of the genome, by virtue of its ability to promote cell cycle arrest or apoptosis depending on the degree of DNA damage (Fig. 4). Consequently, the p53 tumour suppressor gene plays an important role in cell cycle progression, regulation of gene expression and in the cellular response mechanisms to DNA damage.

Under normal physiological conditions, wild type p53 can bind specific DNA sequences and regulate transcription of a number of genes involved in cell cycle progression and apoptotic pathways including $p21^{waf1/cip1}$ and bax. The p53-mediated mechanisms are responsible for tumour suppression and prevent accumulation of potentially oncogenic mutations and genomic instability. Failure by p53 to activate such cellular functions may ultimately result in abnormal uncontrolled cell growth leading to tumorigenic transformation (Fuster et al., 2007). p53 is the most frequently inactivated gene in human neoplasia with functional loss commonly occurring through gene mutational events including non-sense, mis-sense and splice site mutations, allelic loss, rearrangements and deletions (Lane, 1992; Levine, 1997). However, p53 function can also be abrogated by several non-mutational mechanisms including nuclear exclusion, complex formation with a number of viral proteins and through over expression of the cellular oncogene MDM2 (mouse double minute-2) (Haupt et al., 1997).

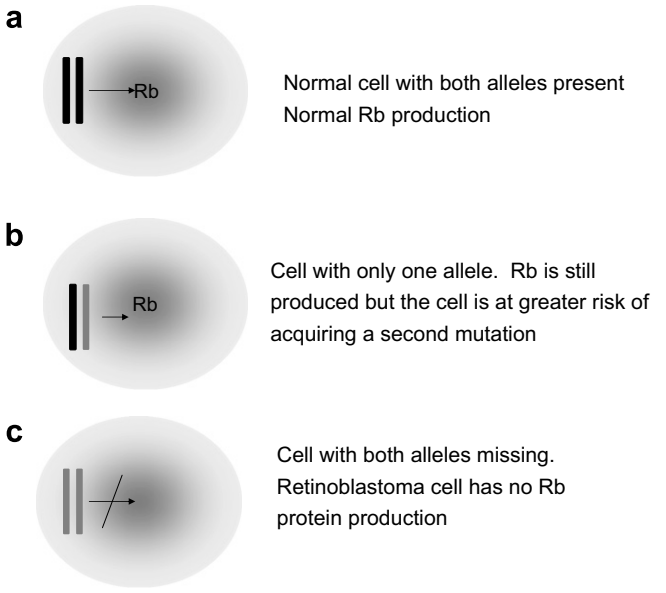

Fig. 3. *In contrast to oncogene mutations, suppressor effects are recessive.* Normal cell (a). Mutation in one copy (b) usually has no effect but the cell is at risk. Cells with both alleles affected produce no tumour suppressor effects (c).

Oncogenenic viruses provided the first evidence that genetic factors play a role in the development of cancer (Jarrett and Onions, 1992). These viruses are a diverse group of pathogens that include all the major families of the DNA viruses and a class of RNA viruses known as Retroviruses. Although diverse, one almost universal feature is the importance of a DNA stage in the replication of the viral genome.

Retroviruses and cancer

Retroviruses are important oncogenic viruses of cats, cattle and chickens, the studies of which have been seminal to our understanding of viral and non-viral oncogenesis. The structure and basic replication cycle of a typical retrovirus is shown in Fig. 5. Retroviruses can promote carcinogenesis through the activation of cellular oncogenes by integrating adjacent to them. A good example of this is

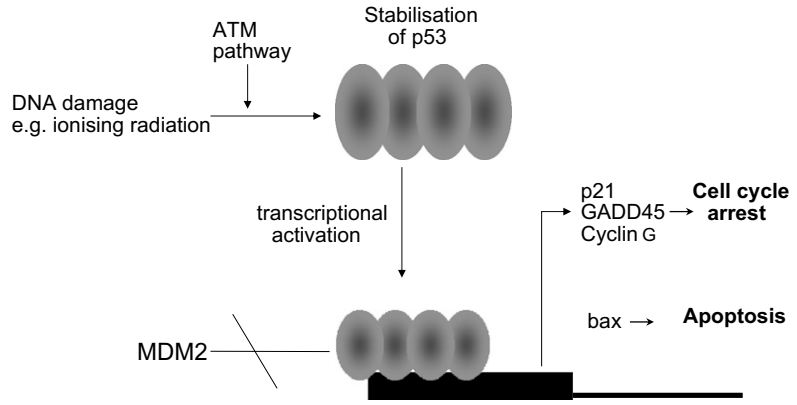

Fig. 4. *p53 is considered a genomic guardian*: The p53 tumour suppressor gene plays an important role in cell cycle progression, regulation of gene expression and in the cellular response mechanisms to DNA damage. Under normal physiological conditions, wild type p53 can bind specific DNA sequences and regulate transcription of a number of genes involved in cell cycle progression and apoptotic pathways including $p21^{waf1/cip1}$ and bax. The p53-mediated mechanisms are responsible for tumour suppression and prevent accumulation of potentially oncogenic mutations and genomic instability. Failure by p53 to activate such cellular functions may ultimately result in abnormal uncontrolled cell growth leading to tumorigenic transformation. p53 is the most frequently inactivated gene in human neoplasia with functional loss commonly occurring through gene mutational events including non-sense, mis-sense and splice site mutations, allelic loss, rearrangements and deletions.

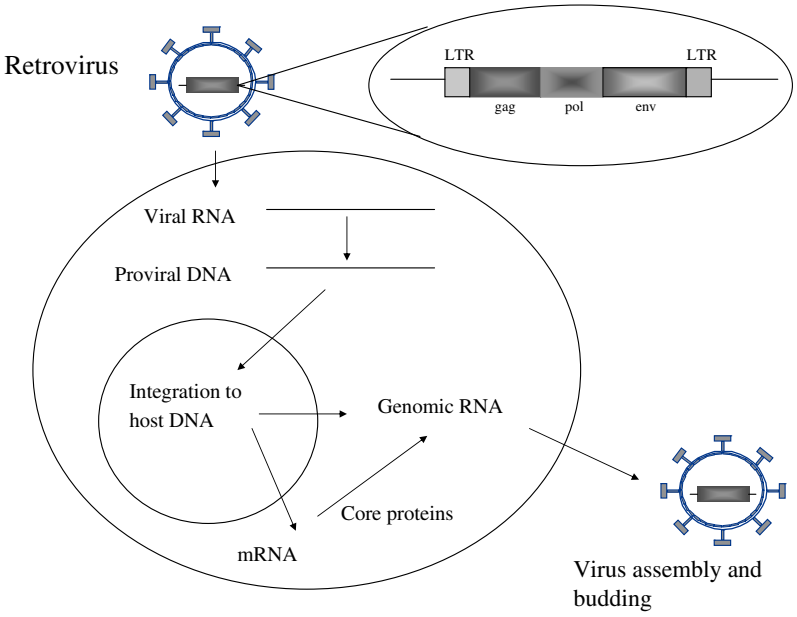

Fig. 5. *The structure and replication lifecycle of a typical retrovirus*. The retrovirus is a double stranded RNA virus, which, on entry to the cell, reverse transcribes into proviral DNA. This DNA can integrate into the host genome.

the *myc* gene, which is frequently activated in B-cell tumours of chickens caused by avian leukosis virus (ALV) and feline T cell lymphomas caused by feline leukaemia virus (FeLV). (Neil et al., 1984). *Myc* is an oncogene intimately associated with cell cycle progression and proliferation. When there is viral insertion close to the *myc* locus, the gene becomes controlled by the powerful viral promoters leading to up-regulated *myc* expression and prevention of cells entering Go of the cell cycle (Fig. 6).

In cattle, a leukaemia/lymphoma complex occurs both as a sporadic and an enzootic form, the latter being associated with infection with bovine leukaemia virus (BLV) (Gillet et al., 2007). In contrast to FeLV and ALV, BLV has a remarkable cell association and is only found in the latent form in B cells. Although all three viruses (ALV, FeLV, BLV) are considered type C retroviruses (based on electron microscopy), BLV cases have no free virus in the blood. In further contrast to the feline virus, BLV also contains an additional tax gene to gag, pol and env. This gene regulates the transcription of the pro-virus, which is often transcriptionally silent. When it is activated, the first mRNA produced encodes the tax protein, which activates other cellular proteins that bind the LTR and up-regulate transcription. Consequently, the expression of the tax

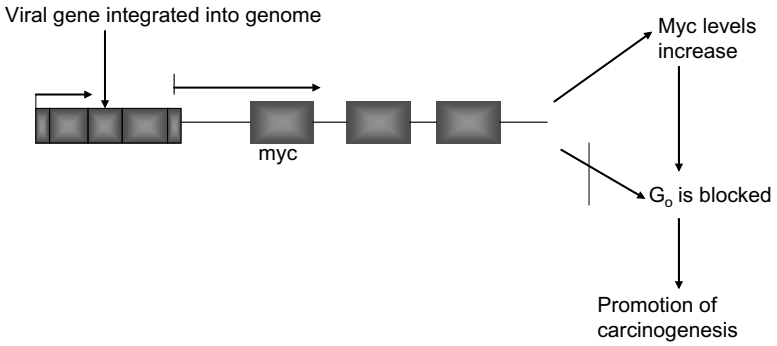

Fig. 6. *Oncogenesis through insertional mutagenesis*. In this scenario, the *myc* gene comes under control of the integrated retroviral promoters. There is a failure of cells to enter Go of the cell cycle, leading to uncontrolled proliferation.

protein acts as a positive feedback loop in the replication cycle BLV. The tax protein can also trans-activate certain cellular genes that may be involved in tumour production.

The DNA viruses

Many DNA viruses have been associated with the development of cancer in animals and humans. In particular, the papilloma viruses (which are small DNA viruses) have long been known to cause wart lesions, which can become malignant depending on a number of several other predisposing factors (Carrillo-Infante et al., 2007). Most often, wart lesions are overcome by the immune system and disappear from the animal over a 6-month period. The life cycle of the virus is tightly coupled with the differentiation process of the epithelial cell and, in certain circumstances, the benign wart can persist and ultimately become transformed to become a squamous cell carcinoma.

The most extensively studied of the papilloma viruses are the bovine papilloma viruses (BPV) and these have also been used as model systems to study the role of co-carcinogens in the development of cancer (Gaukroger et al., 1993). BPV fall into two groups: Subgroup A, comprising the fibropapillomaviruses BPV-1, 2 and 5, and Subgroup B, comprising the epitheliotropic papillomaviruses BPV 3, 4 and 6. BPV-2 is the common virus of common cutaneous warts in cattle. The high risk viruses have early gene products known as E6 and E7, which can immortalise cells and thus contribute to the development of malignancy. However, as we have discussed, the progression to produce a malignant cell requires a number of genetic insults in addition to the effects of E6 and E7 proteins. These proteins can bind to both p53 and Rb proteins thereby conferring a further growth advantage of the infected cell.

In healthy cattle the papillomas normally regress, but in cattle exposed to co-carcinogens, there is a positive correlation between warts and the development of cancer. BPV-2 has been associated with bladder cancer in cattle and BPV-4 is associated with a syndrome of upper alimentary tract cancer in bracken fed cattle. Bracken fern is a co-carcinogen where cattle are infected with papilloma virus.

Field cases of alimentary tract cancer were found to occur at high frequency in areas such as the Nasampolai Valley in Kenya and the Western Highlands of Scotland where the cattle were grazing on bracken (*Pteridium aquilinum*) infected land. Bracken-fed cattle become chronically immunosuppressed, develop chronic enzootic haematuria and bladder tumours and show a high incidence of alimentary tract cancers. The development of papillomas at this site and the concurrent transformation to cancer is through the immunosuppressive and carcinogenic effects of the sesquiterpene pterosins and pterosides present in bracken (Campo et al., 1992, 1994). Additional agents such as ptaquiloside and a-ecdysone, are also found in bracken and are associated with producing changes in cells such as chromosomal aberrations. More recently, it has been demonstrated experimentally that the components of bracken fern may activate viral oncogenes such as E7 to drive the cells towards malignancy. Further, activation of the ras proto-oncogene and inactivation of the p53 tumour suppressor gene may also have a role to play in the pathogenesis of this disease.

In contrast to the papilloma viruses, herpes viruses are large DNA viruses and are known to cause Marek's disease in chickens. The herpes viruses are the subject of extensive studies in man through their involvement in epstein barr virus (EBV) associated lymphomas and Kaposi's sarcoma.

Cancer arises through multiple molecular mechanisms

From the preceding section we can conclude that (1) cancer is a genetic disease, involving fundamental changes in the cell at the genetic level; (2) changes in oncogenes or tumour suppressor genes may contribute to carcinogenesis, and (3) one mechanism by which tumours can arise is through infection with oncogenic viruses.

However, the last two decades of cancer research has demonstrated that, despite the many potential causes of cancer and carcinogenic pathways, transformation of a normal cell into a malignant cell actually requires very few molecular, biochemical and cellular changes. These changes can be considered as the acquired capabilities of a cancer cell that allow it to be regarded as displaying a

malignant phenotype. Further, despite the wide diversity of cancer types, these acquired capabilities appear to be common to all types of cancer. An optimistic view of increasing simplicity in cancer biology is further endorsed by the fact that all normal cells, irrespective of origin and phenotype, carry similar molecular machineries that regulate cell proliferation, differentiation, aging and cell death.

Consequently, we can consider that the vast array of cancer genotypes is a manifestation of only seven alterations in cellular physiology that collectively dictate malignant growth (Hanahan and Weinberg, 2000). These characteristics are acquired during the process of carcinogenesis and can be considered as:

(1) A self sufficiency in growth
(2) An insensitivity to anti-growth signals
(3) An ability to evade programmed cell death (apoptosis)
(4) Limitless replicative potential (mainly through reactivation of telomerase)
(5) An ability to sustain angiogenesis
(6) An ability to invade and metastasise
(7) An ability to evade host immunity (Fig. 7)

It is important to stress that the pathways for cells becoming malignant are highly variable. Mutations in certain oncogenes can occur early in the progression of some tumours, and late in others. As a consequence, the acquisition of the essential cancer characteristics may appear at different times in the progression of different cancers. Furthermore, in certain tumours, a specific genetic event may, on its own, contribute only partially to the acquisition of a single capability, whilst in others it may contribute to the simultaneous acquisition of multiple capabilities. Irrespective of the path taken, the hallmark capabilities of cancer will remain common for multiple cancer types and will help clarify mechanisms, prognosis and the development of new treatments.

A challenge to the stochastic model of carcinogenesis: The cancer stem cell theory

For decades, the accepted model of carcinogenesis has been a stochastic model, whereby any cell in the body has the potential for malignant transformation. However, this model is sometimes difficult to reconcile with what happens in the animal body. The majority of cells making up the various organ systems have a finite life-span, dictated largely through progressive telomeric attrition at each cell division. The question then arises as to how a cell would live long enough to acquire the number of mutations required to become a cancer cell? A challenge to the stochastic model is the cancer stem cell (CSC) theory, which suggests that cancer is, in fact, a true stem cell disease (Reya et al., 2001).

Stem cells are cells that have the ability to self-renew and are capable of asymmetric cell division, giving rise to another stem cell and a cell that gives rise to the phenotypically diverse range of cell types in the body (Moore and Lemischka, 2006). In the normal body we identify two broad classes of stem cells, namely embryonic stem cells (ESC) that are present in the inner cell mass of the early embryo and give rise to all cell types in the body, and adult stem sells (ASC) that are tissue specific and are responsible for cell replenishment in that organ system. The CSC theory states that malignant transformation occurs in the adult stem cell and gives rise to a cancer stem cell (Al-Hajj and Clarke, 2004) (Fig. 8). This would reconcile how a cell would survive long enough to acquire the appropriate number of genetic changes, as stem cells are long-lived.

Many parallels can be drawn between normal adult stem cells and cancer stem cells in terms of clonality and

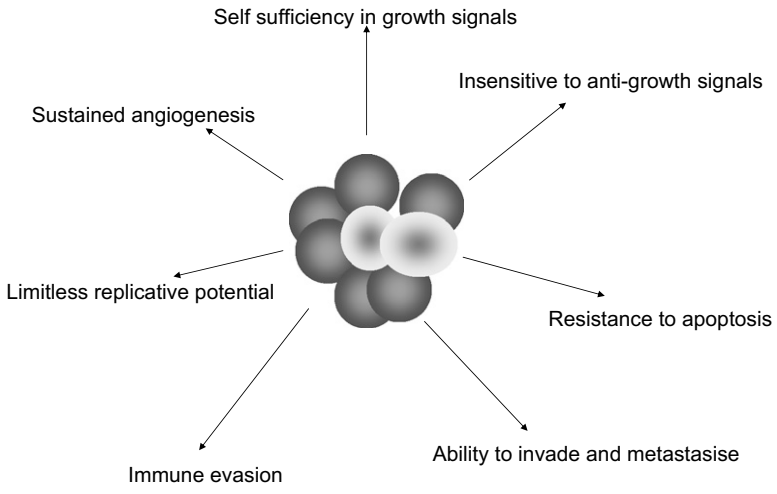

Fig. 7. *The pathways to cancer*. Despite the complexity of cancer as a disease, it can be defined on the basis of the acquisition of seven fundamental characteristics: self sufficiency in growth, an insensitivity to anti-growth signals, an ability to evade programmed cell death (apoptosis), limitless replicative potential (mainly through reactivation of telomerase), an ability to sustain angiogenesis, an ability to invade and metastasise, and an ability to evade host immunity.

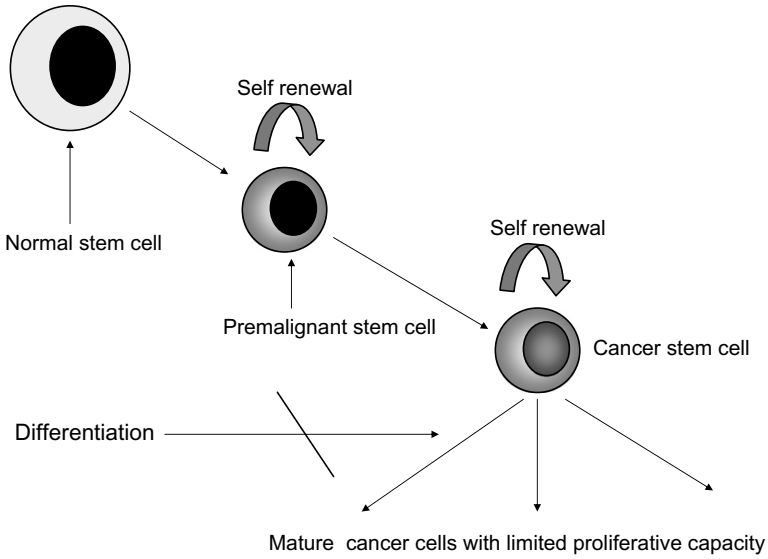

Fig. 8. *The cancer stem cell theory*: This theory challenges the stochastic model presented in Fig. 1 and suggests that malignant transformation is restricted to adult stem cells. Progression to a full malignant cell then leads to the formation of an asymmetrically dividing cancer cell capable of self-renewal and the production of daughter cells. In a similar way to the production of committed cells from normal stem cells, daughter cancer cells have a limited proliferative capacity.

asymmetric division, the implication being that this very small population of CSCs gives rise to daughter cancer cells that represent the bulk of a tumour mass. In support of this, it has been demonstrated that when cancer cells of different types are subjected to both in vitro and in vivo assays, that only a small minority of cells are able to proliferate extensively (Reya et al., 2001). This has given rise to the concept that tumours are composed of both CSCs, which have a large proliferative capacity, and a daughter population of cells, with a limited proliferative potential.

Do cancer stem cells exist?

The evidence for true CSCs was first documented for haematopoietic malignancies such as acute myeloid leukaemia (AML) and multiple myeloma. Using a NOD-SCID (severe combined immunodeficient) mouse model of AML, isolation of cells with a $CD34^{++}CD38^{-}$ phenotype (representing only 0.2% of the tumour population) could reproduce the phenotype of the original tumour in a recipient animal. The heterogeneous AML population required far more cell numbers to achieve this result (Park et al., 1971; Huntly and Gilliland, 2005; Kamel-Reid et al., 1989; Lapidot et al., 1994; Sirard et al., 1996; Bonnet and Dick, 1997).

Similar studies have also been performed to show that cells of solid tumours are phenotypically heterogeneous and only a small proportion of cells are clonogenic in culture and in vivo (Fidler and Kripke, 1977; Fidler and Hart, 1982; Heppner, 1984; Nowell, 1986; Southam and Brunschwig, 1961). For example, only 1 in 1,000 to 1 in 5,000 lung cancer, ovarian cancer and neuroblastoma cells have been found to form colonies in soft agar. A number of reports have now emerged that suggest that cancers of the brain, colon, breast, pancreas and prostate arise from transformed stem cells (Reynolds et al., 1992; Ignatova et al., 2002; Hemmati et al., 2003; Singh et al., 2003, 2004; Balic et al., 2006; O'Brien et al., 2007). Taking either tumour tissues or cell lines, researchers have subjected cells to serum-free, low density culture conditions and demonstrated selection of colonies of cells (spheres) that exhibit stem cell properties, defined by their capacity for self-renewal, gene expression profiles, and their ability to recapitulate the tumour in model systems.

As an example, Dontu et al. (2003) reported a low-adherence, clonal density serum-free culture system which could be used to propagate "mammospheres", enriched in mammary stem/progenitor cells, in vitro from normal human mammary tissue. In this assay system, most primary mammary epithelial cells died under these conditions, but a few generated colonies of cells capable of self-renewal (passage) and differentiation into the three cellular lineages seen in adult mammary tissue.

Al-Hajj et al. (2003) used flow cytometry to separate cells from human primary and metastatic breast carcinomas according to the expression of cell surface markers. It was shown that $CD44^{+}CD24^{-/low}Lineage^{-}$ cells (i.e. those expressing primitive cellular markers) required initial cell inoculums of 2–10% of those required for unsorted cells to form tumours in immunosuppressed mice.

These studies have highlighted that using either a 'sphere' assay or sorting by flow cytometry (FACS), it is possible to identify a small, sub-population of cells that have stem cell properties. However, despite the evidence supporting the theory that cancer is initiated and propagated by cells with stem-like characteristics, it remains unclear whether the CSC is a normal tissue stem cell which has undergone malignant transformation, or a more differ-

entiated cell which has acquired more primitive, stem-like characteristics as a result of mutation or dedifferentiation. Many of the attributes of normal stem cells make them attractive candidates for malignant transformation into CSCs – they are programmed for self-renewal and multilineage differentiation; they persist and continue to divide for the lifetime of the host, allowing them more opportunity to accrue transforming mutations; isolated tumour-initiating cells show many phenotypic similarities to the corresponding normal tissue stem cell (e.g. cell-surface markers, protein expression, telomerase activity).

Polyak and Hahn (2006) proposed three models for development of malignancy involving stem cells:

(1) A mutation causes dysregulation of asymmetric division in a tissue stem cell (TSC) and is passed on to all progeny; progression to full transformation occurs in this population as further mutations are acquired.
(2) The TSC itself acquires mutations sufficient for malignancy, and passes these on to all progeny.
(3) The transit-amplifying cells or more differentiated progeny accrue mutations leading to dedifferentiation and acquisition of stem-cell like properties; TSCs themselves are not involved.

The present evidence suggests that (1) and (2) are more likely, but more work is required to truly identify the target cell.

Evidence for cancer stem cells in the dog

Using similar culture techniques, the authors have isolated CSCs from canine osteosarcomas (Wilson et al., in press). In addition we have achieved similar results with canine lymphoma, melanoma and haemangiosarcoma (unpublished results). These cells were characterized by their ability to grow in harsh culture conditions, and through their expression of proteins such as Nanog and Oct 3/4 that are responsible for the maintenance of pluripotency. The identification of such cells in the dog opens up opportunities for the identification of therapeutic pathways to target these cells, and further supports developing the dog as a model to study human disease.

The niche environment

In vivo studies in mouse models have highlighted the importance of the niche environment during tumour initiation and development (Polyak and Hahn, 2006; Mueller and Fusing, 2004; Bjerkvig et al., 2005). The acquisition of the malignant phenotype by stem cells, may in part be a reflection of the cellular environment. For example, regions of severe oxygen deprivation (hypoxia) arise in tumours due to rapid cell division and aberrant blood vessel formation. The hypoxia-inducible factors (HIFs) mediate transcriptional responses to localised hypoxia in normal tissues and in cancers and can promote tumour progression by altering cellular metabolism and stimulating angiogenesis.

Recently, HIFs have been shown to activate specific signalling pathways such as Notch and the expression of transcription factors such as Oct4 that control stem cell self renewal and multipotency. As the CSC theory evolves, it may suggest that a hypoxic environment may support the initiation of cancer in adult stem cell populations (Keith and Simon, 2007).

Mobilisation and metastasis

Studies on haematopoietic stem cells have shown that normal bone marrow stem cells possess the capacity to mobilise and migrate in the circulation to distant sites in response to tissue damage and stress with complex, coordinated homing mechanisms being involved (Lapidot et al., 2005). Stem cells from different tissues share common genetic programmes (Terskikh et al., 2001) and bone marrow stem cells display plasticity allowing them to differentiate into a variety of cell types (Bjerkvig et al., 2005; Kucia et al., 2005). There are many similarities between the mechanisms governing the migration of normal stem cells and the metastatic dissemination of tumour cells, such as the interaction between the cell surface CXCR4 receptor and its ligand, stromal-derived factor (SDF)-1, secreted by the niche (Macpherson et al., 2005). Cancer cells may disseminate to distant sites but never develop into true metastases despite remaining detectable in remote tissues (Brabletz et al., 2005). It would seem plausible within the CSC theory that the formation of metastatic deposits within tissues may require migration of CSCs into an appropriate niche environment. There is also recent evidence to suggest that the formation of metastatic deposits is intimately linked with the bone marrow. Kaplan et al. (2005) suggests that the primary tumour may signal the bone marrow to mobilise cells to various sites in the body to create a niche environment for metastatic deposition.

Are non-haematopoietic cancers bone-marrow-derived?

Most of the work on CSCs has been based upon an assumption that cancers are derived from adult stem cells. An alternative hypothesis has been suggested by some workers that implicate the bone marrow as the site from where cancer cells are derived. In elegant work by Houghton et al. (2004) using a mouse model of gastric cancer, marker cell studies showed that carcinoma cells in forming gastric carcinomas had their origins as haematopoietic cells. The implications of this are immense but much more work is required to validate this theory.

Cancer stem cells and the implications for therapy

If a population of CSCs is responsible for the propagation of a tumour, then this has huge implications for therapy. The evidence suggests that daughter cells, which make

up the bulk population of tumours, may be sensitive to the effects of conventional treatments such as radiation and/or chemotherapy (Fig. 9). However, stem cell populations tend to harbour strong resistance mechanisms, entering periods of quiescence during which they are resistant to strategies aimed at eradicating cycling cells.

Many molecular mechanisms have been identified in association with stem-like behaviour in cancer cells. Often these mirror the differential expression patterns that mark out normal tissue stem cells. The side population (SP) phenotype was first identified in haematopoietic stem cells (HSCs), when a subset of cells with low uptake of the dye Hoechst 33342 was isolated by FACS and found to be enriched for HSCs (Goodell et al., 1997). The dye-efflux SP phenotype for haematopoietic and several other tissue progenitor cells has been shown both in vitro and in vivo to be conferred by the ATP binding cassette (ABC) transporter ABCG2 (Zhou et al., 2001; Scharenberg et al., 2002), which has also been identified for its role in multiple drug resistance (Bunting, 2002), and shown to confer to breast cancer cells the ability to efflux chemotherapeutic drugs (Doyle and Ross, 2003). This would tie in with the high frequency of cancer relapse following initial remission after chemotherapy.

If conventional therapies are not appropriate for killing CSCs, then it would follow that alternative pathways in these cells need to be identified. Mutations in many of the signalling pathways and genetic mechanisms regulating normal stem cells have been demonstrated in human cancers. Polycomb genes, particularly *Bmi-1*, *HOX* transcription factors, and the Wnt-βcatenin, Notch and Sonic Hedgehog (SHh) pathways, are important in self-renewal and other functional stem cell properties, and may offer an opportunity for therapeutic targeting (Huntly and Gilliland, 2005; Taipale and Beachy, 2001; Pardal et al., 2003; Behbod and Rosen, 2005).

Other treatment modalities might also preferentially target putative CSCs. Transiently inactivating the causative oncogene in transgenic mouse model of osteogenic sarcomas can cause the tumours to regress, with reactivation leading to apoptosis (Jain et al., 2002). It would seem logical that forcing CSCs down a symmetrical division pathway, whereby two more committed daughter cells are produced, would deprive a tumour of its self renewal potential and effect a cure. Differentiation therapy with retinoids is effective in a majority of cases of human acute promyelocytic leukaemia, although responses in other malignancies have been variable (Sell, 2004).

Treatments directed solely at CSCs, however, may not have an effect on the differentiated progenitor and daughter cells. Therefore, the bulk of the tumour may remain intact while the CSCs are being destroyed – the dandelion phenomenon (Huff et al., 2006). This theory states that cutting off a dandelion at the roots, or treating the bulk of the tumour, takes away the disease that one can see, however the weed will still re-grow because the root has not been destroyed. Conversely, destroying the root, or CSCs, of the weed leaves the flower above soil initially. However,

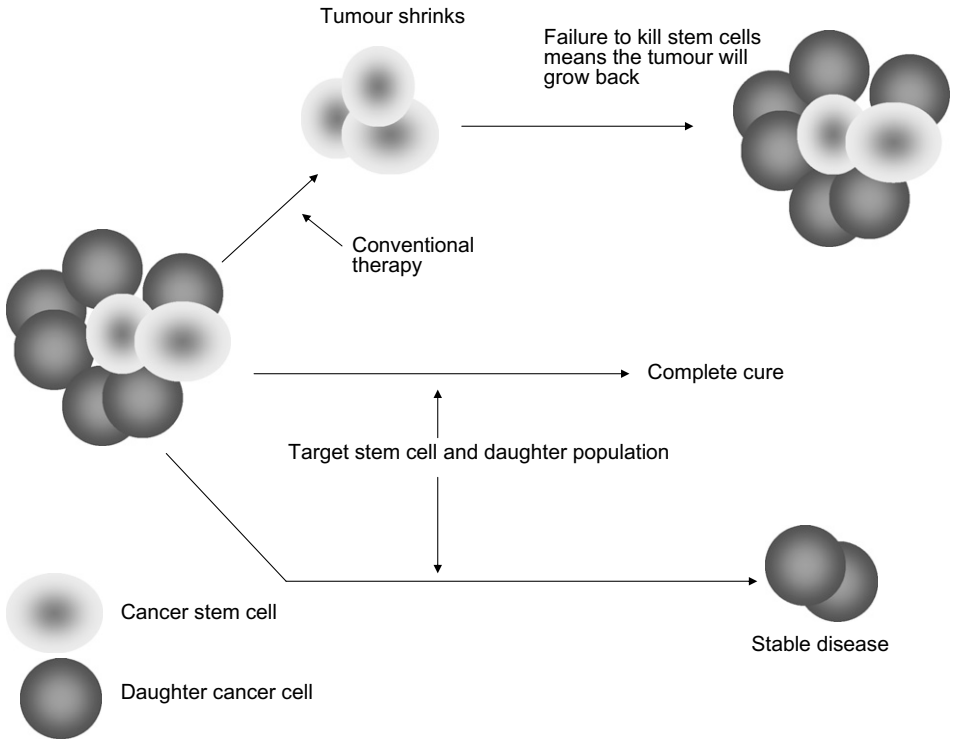

Fig. 9. *Cancer stem cells may inform new therapies*: Currently conventional cancer therapies are directed at non-stem cell populations. Consequently, any tumour has the capacity to re-grow the original cancer. Therapies targeted at the cancer stem cell and the daughter cells would have the capacity to cause either stable disease or complete tumour eradication.

as the root dies, the portion above ground will eventually wither and die without the root.

According to the dandelion phenomenon of CSC therapy, a treatment regimen may be abandoned prematurely if we only consider remission of the tumour bulk. This response is likely to lag behind the destruction of CSCs. Conversely, treatment of chronic phase CML with imatinib mesylate has achieved high rates of complete remission despite the fact that BCR/ABL-expressing progenitors are spared (Bhatia et al., 2003). This may indicate the significance of the niche provided to a stem cell by its surrounding daughter cell population – removal of paracrine factors in targeting the tumour bulk may sometimes be sufficient to arrest the progress of disease (Polyak and Hahn, 2006).

Given that clinical response may not be an ideal way to monitor for response to therapies that target CSCs, overall survival is left as a common monitoring criteria. This requires long study times that can be impractical. Animal models are an ideal way to continue to use survival as criteria for determining effectiveness of therapy. Animal models of spontaneously occurring tumours often progress much faster than the human form of the disease. Many canine tumour models are very similar or even identical to their human counterparts (Gorlick et al., 2003; Khanna et al., 2006) and the natural canine model may represent an ideal testing ground for novel compounds directed at the CSC.

Future perspectives

The identification of CSCs in both humans and dogs has been a defining moment in cancer research. If the theory is correct, then future efforts must be made to characterise these cells with a view to identifying therapeutic targets. In our laboratory we have already begun the laborious task of characterising canine stem cells using microarray technology and marker analysis. However, it is highly likely that elimination of these cell populations will require targeting of self-renewal pathways that may be common to normal stem cells. As with most cancer treatments, we will be left developing drugs whose efficacy will depend on the degree of 'off target' effects.

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Is cancer a stem cell disease? Theory, evidence and implications

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Introduction

Cells of muticellular organisms form part of a specialized society that cooperates to promote survival of the organism. In this, cell division, proliferation and differentiation are strictly controlled and a balance exists between normal cell birth and cell death. Inextricably linked to this fundamental concept is the role of the adult stem cell (ASC) whose progeny and microenvironment make up the anatomical structure that coordinates normal homeostatic production of functional mature cells. ASCs have been best characterized in the haematopoietic system but exist in all major organ systems. These cells are characterized by a capacity for self-renewal, being undifferentiated but capable of multilineage differentiation, slowly cycling cells but clonogenic, and capable of asymmetric division. Further, ASC reside in particular 'niche' environments that support an appropriate spatiotemporal dialogue between ASC and microenvironmental cells in order to fulfil the lifelong demands for normal differentiated cells.[1]

For decades, advances in molecular techniques have allowed us to dissect the mechanisms of carcinogenesis, most work focusing on the accepted model of multistage carcinogenesis underpinned by progressive genetic changes that drive malignant transformation. In this model, any cell in the body has the potential for malignant transformation. More recently, attention has focused on an alternative model where the tumour is maintained by a cancer stem cell (CSC) which gives rise to a tumour composed mostly of daughter cancer cells and a small number of CSCs that drive tumour growth and expansion.

Cancer stem cells

The CSC theory probably represents a modern day interpretation of a similar proposal made by Virchow and Cohnheim nearly 150 years ago, proposing that cancer resulted from activation of dormant embryonic tissue. This theory was reawakened in the 1960s and 1970s with suggested theories of maturation arrest in tissue-specific stem cells,[2,3] and then ultimately with the identification of the leukaemic stem cell in seminal experiments performed by Fialkow *et al.* in the late 1960s.[4] In the current context, the CSC can be considered a cell that has the ability to self renew and is capable of asymmetric cell division, giving rise to another malignant stem cell and a cell that gives rise to the phenotypically diverse tumour cell population (Fig. 1).

Proof that CSC exists as a phenotypically different population of cancer cells requires isolation of different populations of cancer cells and demonstration that one or more groups are efficient at producing tumours while other groups lack this ability.[5] However, these cells cannot be definitively called CSC until it is possible to show that a single transplanted cancer cell can give rise to a diverse population of cancer cells within a tumour.[5]

Evidence for CSC

Many parallels can be drawn between normal ASC and CSC in terms of clonality and asymmetric division (Fig. 2). Further, it has been shown that when

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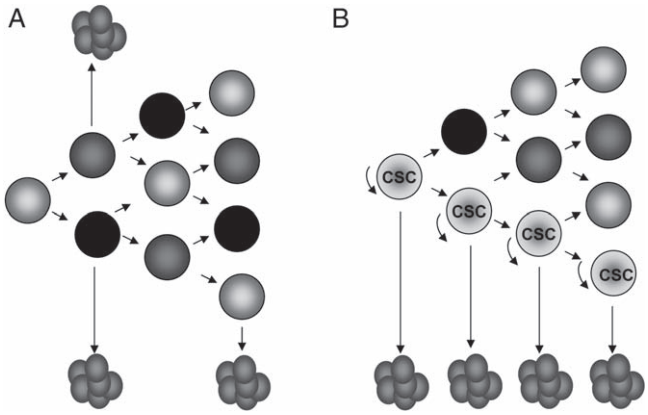

Figure 1. Two general models of cancer presented. In both (A) and (B) there is tumour cell heterogeneity. However, in (A) many different cancer cell phenotypes have the potential to proliferate extensively to cause a tumour. In (B) we predict that a small subset of cancer cells [the cancer stem cell (CSC)] are the only population that can form a new tumour upon transplantation.

cancer cells of different types are subjected to both *in vitro* and *in vivo* assays, that only a small minority of cells are able to proliferate extensively.[6] This has given rise to the concept that tumours are composed of both CSC, which have a large proliferative capacity, and a daughter population of cells, with a limited proliferative potential.

The evidence for true CSC was first documented for haematopoietic malignancies such as acute myeloid leukaemia (AML) and multiple myeloma. In seminal studies, it was found that when mouse myeloma cells were obtained from mouse ascites, and subjected to *in vitro* colony-forming assays, only 1 in 10 000 to 1 in 100 cancer cells were able to form colonies.[7] The normal haematopoietic stem cell (HSC), whose nature has still not been fully characterized, was first shown according to its ability to reconstitute the bone marrow of lethally irradiated

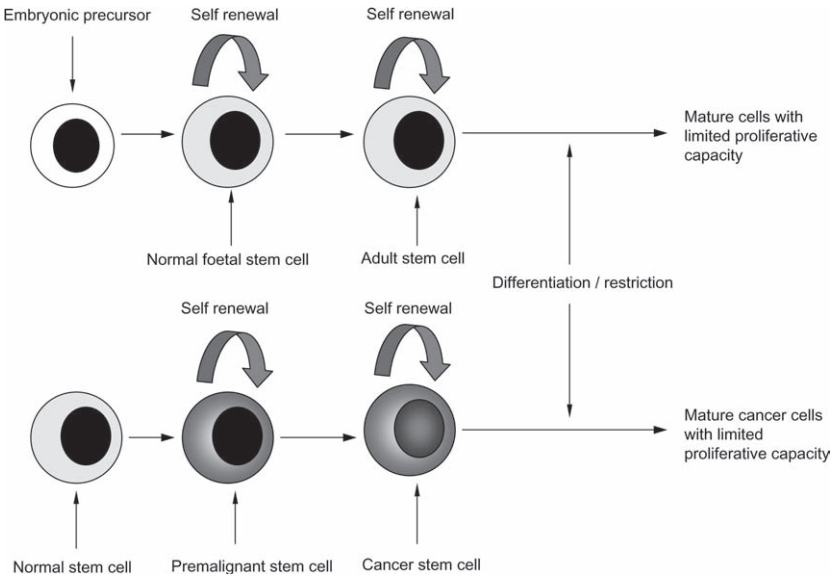

Figure 2. Parallels can be drawn between normal stem cell development and cancer stem cell development. Both pathways share properties of self-renewal and asymmetric division. Ultimately both pathways give rise to cells with limited proliferative potential.

mice. Mirroring this work, experiments using severe combined immunodeficient (SCID) mice indicated that normal haematopoietic stem cells become engrafted in the bone marrow of these immunodeficient animals (which lack B and T lymphocytes) such that all mature lineages, other than T-cells, were produced. Furthermore, transplantation of cells from human AML, chronic myeloid leukaemia (CML) and acute lymphoblastic leukaemia (ALL) could be performed in the same way and recapitulate the human cancer in the recipient mouse.[8–11]

However, the SCID mouse model had a number of limitations, which prevented definitive demonstration that the 'SCID leukaemia-initiating cell' (SL-IC) could recapitulate tumours; similarities between HSC and SL-IC cell-surface markers could only be shown for one leukaemia subtype.[10] It was only after the development of the non-obese diabetic (NOD)/SCID mouse, which has additional immune response deficits (e.g. natural killer cell activity, complement activation), that the engraftment experiments were refined to allow Dick and colleagues to show properties in the SL-ICs which met the definition of CSC. One problem with the SCID model was the need to transplant large numbers of host cells to ensure engraftment in the recipient. It was shown that 10–20 times fewer cells were required in the NOD–SCID mice to achieve the same level of engraftment.[12]

The resulting experiments showed that, for a range of AML subtypes, both unfractionated bone marrow samples and purified $CD34^+$ $CD38^-$ cells could reproduce the phenotype of the original human tumour in the recipient animal. This was seen even when the $CD34^+$ purified fraction represented a tiny (0.2%) proportion of blast cells. The heterogeneous human-derived blast population, with CD38 expressed in most, even when transplanted cells had been purified according to the absence of this cell-surface marker, seemed to evoke normal haematopoietic differentiation. Cells could be further transplanted into a secondary recipient, recapitulating the tumour; it was calculated that the SL-ICs population must have expanded 30-fold, providing further evidence for self-renewal properties. These findings were compelling evidence for AML being a stem cell disorder.[12]

CSCs and solid cancers

The CSC theory has only been applied to solid cancers in recent years, although similar studies had been performed to show that cells of solid tumours are phenotypically heterogeneous and only a small proportion of cells are clonogenic in culture and *in vivo*.[13–17] For example, only 1 in 1000 to 1 in 5000 lung cancer, ovarian cancer and neuroblastoma cells were found to form colonies in soft agar in studies performed in the 1970s. Despite this, the extension of the CSC hypothesis to solid tumours has been more challenging experimentally. Normal haematopoietic differentiation is better understood than the corresponding process in most solid tissues – importantly, cell-surface markers for normal haematopoietic stem cells and their progeny have been identified, allowing their isolation. For many solid tissue stem cells, assays have yet to be developed. A further problem is the physical nature of the tissues. Cells from solid tissues are often larger and more fragile than blood cells, cells are often less accessible for sampling, and creation of viable single-cell suspensions is challenging.

In 1992, Reynolds showed that a <1% subpopulation of embryonic striatal cells were viable after 5 days *in vitro* when plated in serum-free, low-density culture in the presence of the mitogen epidermal growth factor (EGF). These EGF-responsive striatal progenitors initially divided to form clusters of cells (neurospheres), with most of the constituent cells showing immunoreactivity for nestin (a cytoskeletal intermediate filament protein expressed in neuroepithelial stem cells). After 14 divisions, the clusters had continued to expand, and now two distinct populations of daughter cells stained positive for neurone-specific enolase and glial fibrillary acidic protein, markers for the neuronal and astrocyte lineages, respectively. Thus, the assay conditions and presence of EGF seemed to select for multipotent stem cells, defined by their capacity for self-renewal and multilineage differentiation.[18]

Experiments using modifications of this system, some exerting additional selection pressure by suspending cells in methylcellulose in low-adherence culture wells have been widely used to test the CSC hypothesis in solid tumours.[19,20] Experiments inducing over-expression of the proto-oncogene

Myc, Ras, Akt and platelet-derived growth factor (PDGF) showed that neural progenitors underwent transformation (increased proliferation[21] and tumour formation[22,23]) more readily than more differentiated cell types.

Subsequently, reports were published of the isolation of CNS CSC from a variety of human cancers including astrocytomas, glioblastomas, medulloblastomas and ependymomas, by virtue of the ability of the cells to form neurosphere clones in serum-free culture in the presence of pleiotropic growth factors, and to differentiate into cells phenotypically similar to the lineages seen in the initial tumour.[19,24–26]

The foetal neuronal stem cell marker CD133 was widely expressed on these multipotent tumour cells.[24,25] Karyotypic abnormalities and aberrant differentiation profiles indicated that they were not normal neural stem cells migrating within the tumour,[25] but part of the tumour cell population. The *in vivo* ability of putative tumour stem cells to recapitulate the primary tumour mass was tested, by implanting cells isolated by sorting for CD133[26] or growth in serum-starved, clonal density conditions[27] into immunosuppressed mice, subcutaneously and/or intracranially. The resulting xenografts showed striking similarities to the tumours from which the progenitors were derived[26,27] albeit with a 'peculiar histomorphology' in some cases.[27]

Dontu *et al.* in 2003 reported a low-adherence, clonal density serum-free culture system, which could be used to propagate 'mammospheres', enriched in mammary stem/progenitor cells, *in vitro* from normal human mammary tissue. As with the neurosphere assay, most primary mammary epithelial cells died under these conditions, but a few generated colonies of cells capable of self-renewal (passage) and differentiation into the three cellular lineages seen in adult mammary tissue.[28]

Mammosphere initiating cells were contained within the side population (SP) of cells capable of excluding Hoechst 33342 dye and showed upregulation of genes coding for membrane and cytoskeletal proteins, transcription factors, signalling and cell adhesion molecules, cell cycle regulators and metalloproteinases when compared with cells grown in differentiating conditions.[28] The mammosphere system was used to show that activation of the Notch signalling pathway (Notch has been implicated as a proto-oncogene) affected lineage specification, and promoted self-renewal and proliferation of mammosphere cells. Conversely, alterations in signalling did not have any significant effect on fully committed mammary epithelial cells.[29]

Al-Hajj *et al.* used flow cytometry to separate cells from human primary and metastatic breast carcinomas according to the expression of cell-surface markers. In some cases, tumour cells were derived directly from patients and in others they had undergone one or two passages in mice. In all but one tumour, it was shown that $CD44^+$ $CD24^{-/low}Lineage^-$ cells required initial cell inoculums of 2–10% of those required for unsorted cells to form tumours in immunosuppressed mice. $CD24^+Lineage$ cells were unable to form tumours except in one subject. The $CD44^+$ $CD24^{-/low}Lineage$ population showed similar cell cycle distribution to the nontumorigenic cells, excluding this as the cause of tumorigenicity. $CD44^+$ $CD24^{-/low}Lineage^-$ cells could be serially passaged in mice, forming tumours from which further $CD44^+$ $CD24^{-/low}Lineage^-$ cells could be isolated (i.e. self-renewal capacity) as well as the other nontumorigenic cell populations found in the original tumour (i.e. multilineage differentiation).[30]

It is interesting, given the use of metastatic breast cancer cells (pleural effusion) in eight out of the nine tumour types investigated, that in another study the prevalence of the putative $CD44^+$ $CD24^{-/low}$ tumour stem cell phenotype in breast tumours did not correlate with tumour progression or prognosis. A greater prevalence *was* associated, however, with a tendency for distant metastasis upon recurrence.[31] The presence of disseminated tumour cells in breast cancer can be detected with immunohistochemistry for cytokeratins (CK). Balic *et al.* assessed CK+ bone marrow samples from 50 patients with early breast cancer and found that all specimens had detectable $CD44^+$ $CD24^{-/low}$ cells, with prevalence (33–100%, median 65%) much greater than that seen in primary tumour masses, again associating the putative breast CSC population with a tendency towards metastasis.[32]

Gibbs *et al.* describe 'sarcospheres' created in low-adherence, serum-free culture from a variety of osteosarcomas and chondrosarcomas[20] – these phenotypically distinct tumours occur primarily in

childhood and adulthood, respectively, yet the expression patterns and behaviour of the putative bone sarcoma stem cells were very similar. Self-renewal properties and multilineage gene expression were demonstrated, along with expression of the proteins Oct 3/4 and Nanog, associated with pluripotency and self-renewal in embryonic stem cells, and shared attributes with normal mesenchymal stem cells.[20]

Seaberg and van der Kooy showed the significance of serial passaging in the identification of stem cells.[33] Early progenitor cells have limited self-renewing capabilities; however, they are able to form secondary and even tertiary spheres in non-adherent serum-starved-media conditions typically reserved for isolation of stem cell populations. It is important to differentiate these early progenitor cells from true CSC by serially passaging them many times. CSCs should, theoretically, be capable of indefinite self-renewal under these conditions with very little change in their ability to self renew as well as form differentiated progeny.[34]

Two groups of investigators have found that the minority $CD133^+$-cell population from human colon carcinomas can reproduce the primary tumour in immunusuppressed mice, whereas $CD133^-$ cells do not have this capacity. Dick and colleagues showed that all human colon cancer-initiating cells (CC-IC) were $CD133^+$, and that while 1 in 57 000 unsorted tumour cells were CC-IC, this was enriched to one in less than 300 $CD133^+$ cells.[35] Unlimited self-renewal potential and capacity for differentiation into all cell types seen in the primary tumour was established *in vivo* with serial transplantation in mice and also *in vitro* with a serum-free sphere culture system.[35,36]

CSC regulators and markers

ABCG2

Many molecular mechanisms have been identified in association with stem-like behaviour in cancer cells. Often these mirror the differential expression patterns that mark out normal tissue stem cells. The SP phenotype was first identified in haematopoietic stem cells, when a subset of cells with low uptake of the dye Hoechst 33342 was isolated by fluorescence-activated cell sorting (FACS) and found to be enriched for HSCs.[37] The dye–efflux SP phenotype for haematopoietic and several other tissue progenitor cells has been shown both *in vitro* and *in vivo* to be conferred by the ATP-binding cassette (ABC) transporter ABCG2,[38,39] which has also been identified for its role in multiple drug resistance,[40] and shown to confer to breast cancer cells the ability to efflux chemotherapeutic drugs.[41]

This would tie in with the high frequency of cancer relapse following initial remission after chemotherapy, and a SP was detected in approximately 30% of cultured tumour cell lines from a variety of tissues including breast, colon, ovary and glioma. SP cells from breast, prostate and brain cancers were shown to be more tumorigenic than non-SP cells. However, when sorted according to ABCG2 expression, both $ABCG2^+$ and $ABCG2^-$ cells were tumorigenic to similar degrees. It was postulated that $ABCG2^+$ cells are tumour progenitors with more rapid turnover but that they themselves arise from more primitive, slow-cycling cells within the $ABCG2^-$ population with more long-term self-renewal capacity.[42]

Signalling pathways

Mutations in many of the signalling pathways and genetic mechanisms regulating normal stem cells have been shown in human cancers. Polycomb genes, particularly *Bmi-1*, *HOX* transcription factors, and the Wnt–β-catenin, Notch and Sonic Hedgehog pathways, are important in self-renewal and other functional stem cell properties; alterations in expression have been implicated in tumours of both blood and solid tissues.[8,43–45]

Bmi-1

Bmi-1 is a member of the Polycomb group of transcriptional repressor proteins, which acts through the *ink4a* locus to downregulate the tumour suppressors encoded there – $p16^{INK4a}$ and $p19^{ARF}$ (Fig. 3). Bmi-1 over-expression in mouse embryonic fibroblasts leads to their immortalization, and in co-operation with *ras* can cause neoplastic transformation.[46] Correspondingly, under-expression is associated with reduced proliferative capacity in both normal haematopoietic precursors derived from foetal liver cells and leukaemic stem cells in a

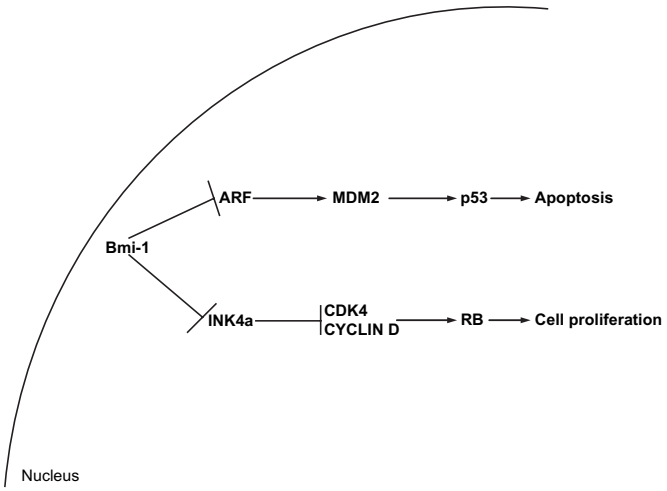

Figure 3. Bmi-1 signalling: Bmi-1 inhibits the transcription of two cyclin-dependent-kinase inhibitors, INK4A (p16) and ARF (p14). If INK4A is blocked, Retinoblastoma Protein (RP) becomes phosphorylated and inactivated by a complex of Cyclin-dependent kinase 4 (CDK4) and Cyclin D. This allows cells to enter the cell cycle. If ARF is blocked, MDM2 inhibits p53-dependent expression of genes that cause apoptosis.

mouse model of AML.[47] Bmi-$1^{-/-}$ leukaemias were not transplantable into secondary recipients, although this capacity could be rescued by introduction of a retroviral *Bmi-1* provirus. Interestingly, this Bmi-1-mediated rescue was also seen in Bmi-$1^{-/-}$ clones with defects in the expression of $p16^{INK4a}$ and $p19^{ARF}$, indicating additional pathways through which the molecule exerts its effects.[47] Glinsky *et al.* investigated the role of Bmi-1 in human prostate cancer using microarray analysis; elevation in expression was seen in all cancer cell lines, with greater increases seen in more metastatic tumour types. An 11-gene signature associated with Bmi-1 function in normal stem cells was found to be expressed in 11 different types of cancer, and consistently to predict metastasis and poor prognosis.[48]

Notch

The four Notch transmembrane receptors found in mammals are activated by their ligands Delta, Jagged and other members of the DSL (Delta, Serrate and Lag-3) family. Binding initiates a signalling pathway, which leads to the activation of the CSL transcription factor, along with mastermind-like (MAML) co-activators. This leads to transcription of genes associated with processes such as cell fate determination during development and self-renewal in adult tissues.[49]

Notch signalling has been shown to be oncogenic in mouse models of T-cell acute lymphoblastic leukaemia (T-ALL),[50] and can collaborate with the c-neu/erbB2 oncogene in the development of mammary tumours.[51] Dontu *et al.* showed that activation of Notch signalling promoted self-renewal and proliferation of normal mammary stem/progenitor cells cultured in mammospheres, but had no effect on fully committed mammary epithelial cells, suggesting that it exerts its oncogenic potential at the progenitor level.[29] Interestingly, loss-of-function mutations have been shown to contribute to neoplastic transformation,[52] showing that notch-activated gene expression has varied roles depending on the context.[49]

This may account for the differential expression of the Notch ligands, Delta and Jagged-1 and 2 between neurosphere clones derived from normal brain and glial tumour tissue. *Delta* expressed by normal tissue and neurosphere clones which had been allowed to attach to a substrate, but not suspended neurospheres; *Jagged-2* was expressed by normal clones but not those originating from tumours.[19] Conversely, recent microarray analysis of $CD34^{+}$ $CD38^{-}$ leukaemic stem cells (LSC) from AML has indicated over-expression of *Jagged-2*,

with inhibition of *Jagged* and *Notch* signalling reducing LSC growth in colony-forming assays.[50]

Wnt and β-catenin

Wnt signalling influences cell migration and developmental patterning, proliferation and survival, through the binding of β-catenin to the LEF/Tcf transcription factors and activation of downstream genes (Fig. 4). The binding of Wnt proteins to their Frizzled cell-surface receptors inhibits the cytoplasmic 'destruction complex' in which β-catenin is normally held, allowing it to accumulate in the cytoplasm and translocate to the nucleus. Over-expression of β-catenin in transgenic murine HSCs increased their self-renewal capacity; over time, while controls appeared to differentiate down myelo-monocytic lineage, an increased proportion of the β-catenin-transduced HSC population remained as lineage negative, proliferative cells. Inhibition of Wnt-signalling suppressed HSC growth between four- and sevenfold, suggesting that the pathway is required for normal HSC function.[51] It remains possible, however, that this is an *in vitro* phenomenon and that *in vivo*, alternative mechanisms are involved and Wnt is less significant.[44]

Wnt signalling is also involved in self-renewal of epithelial cells in other tissues [e.g. skin, intestine and central nervous system (CNS)]; over-activation of the pathway and increased nuclear β-catenin has been implicated in colon, prostate, ovarian, CNS and skin tumours as well as haematological malignancies.[44] The Adenomatous Polyposis Coli (APC) tumour suppressor gene mutated in familial adenomatous polyposis is part of the β-catenin destruction complex inhibited by Wnt signalling. It has been proposed that both germline and somatic mutations of APC confers a selective advantage to a cell, with increased proliferation in response to the Wnt-pathway dysregulation, but that APC function must not be so impaired so as to lead to apoptosis. Evidence exists for both the 'top down' model of colon cancer, whereby somatic APC mutation occurs first at the luminal surface and spreads down into the crypts, and the 'bottom up' model, where the mutation is propagated in, and spreads from the stem cell located in the crypt

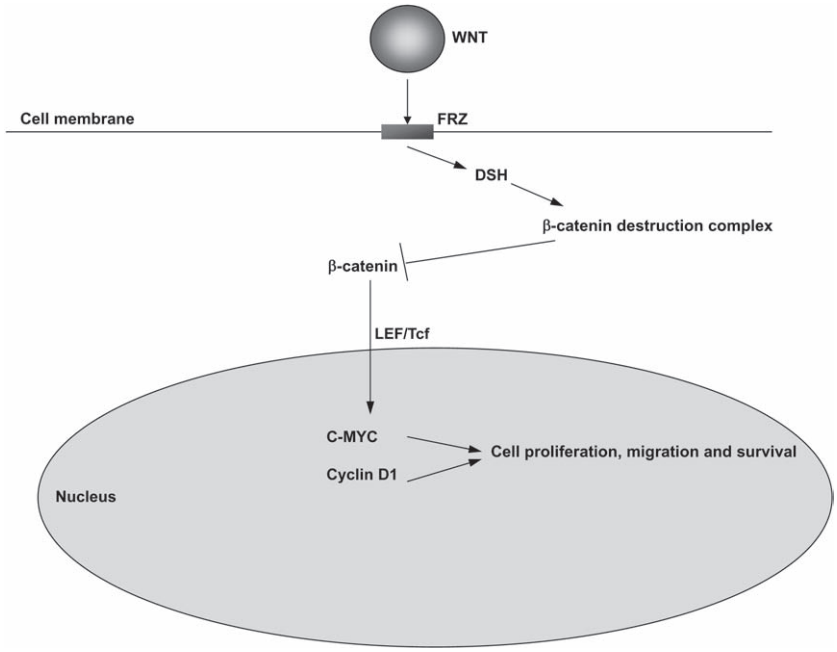

Figure 4. Wnt binds to Frizzled (FRZ) receptors and activates Dishevelled (DSH). This disrupts a β-catenin destruction complex and allows β-catenin to accumulate and translocate to the nucleus. In the nucleus β-catenin binds to LEF/Tcf transcription factors leading to expression of target genes (e.g. c-MYC) that promote survival, proliferation and cellular migration.

base – obviously the latter being more easily reconciled with the CSC hypothesis.[52]

Telomeres and telomerase

When grown in tissue culture, the phenomenon of *senescence* limits the replicative potential of cells, in that eventually the cells will stop dividing.[53] This is partly governed by the gradual loss of the protective telomeres, tandem repeats of a 6-bp sequence, which are present at the ends of chromosomes. DNA polymerises cannot fully replicate the 3' end of the DNA strand, such that upon each cell division there is a loss of 50–100 bp at each end of every chromosome. The telomeres buffer this loss but are of finite length, so they gradually become shorter through the life of a dividing cell – this protective mechanism means that the cell will be directed to senescence or apoptosis after a certain number of divisions.[54,55]

Stem cells circumvent this 'end replication problem' with the expression of low levels of *Telomerase*, an enzyme which catalyses the addition of telomeric repeat sequences on to the ends of chromosomes, such that they have lifelong replicative potential. Telomerase is also expressed in over 80% of human cancers, and has been presumed to confer unlimited cell cycling ability. Conversely, many tumours show foreshortened telomeres. A two-step process has been proposed, whereby early telomere shortening promotes chromosomal instability and mutation, and then telomerase activity stabilizes the telomeres and allows uncontrolled replication. In most haematological malignancies, short telomeres and telomerase activity are detected; telomerase activity levels of between 2 and 50 times that of normal haematopoietic precursors has been shown.[55]

Protein expression

The protein products of normal stem cells have been seen to be differentially expressed within tumours, with populations enriched for putative CSC showing higher levels of expression. Nestin is an intermediate filament protein and a marker for neuroepithelial precursors[56]; in undifferentiated clonal neurospheres from both normal brain[18] and brain tumour tissue[24,25] increased immunoreactivity is seen. Nanog and Oct 3/4 are homeoproteins involved in self-renewal and pluripotency of embryonic stem cells, with immunoreactivity demonstrable on tumour sections. Sarcospheres grown from osteosarcomas and chondrosarcomas show preferential expression of these proteins; the proportion of positively staining cells decreases as the spheres grow out and cells begin to differentiate.[20]

Which cell is the target for transformation?

Despite the evidence supporting the theory that cancer is initiated and propagated by cells with stem-like characteristics, it remains unclear whether the CSC is a normal tissue stem cell, which has undergone malignant transformation, or a more differentiated cell, which has acquired more primitive, stem-like characteristics as a result of mutation or dedifferentiation.

As discussed, many of the attributes of normal stem cells make them attractive candidates for malignant transformation into CSC – they are programmed for self-renewal and multilineage differentiation; they persist and continue to divide for the lifetime of the host, allowing them more opportunity to accrue transforming mutations; isolated tumour-initiating cells show many phenotypic similarities to the corresponding normal tissue stem cell (e.g. cell-surface markers, protein expression and telomerase activity).

At the same time, pluripotent stem cells represent a small minority of cells within a tissue. An individual stem cell would be a very small target population for the accumulation of sufficient mutations to confer a neoplastic phenotype.[57] Also, mutations of pathways which control normal stem cell function, such as the Wnt–β-catenin pathway, are seen in many cancers[57,58] – this would perhaps be unexpected if normal stem cell processes had simply been co-opted to lead to tumorigenesis, rather than independent acquisition of such functions through mutation. It has been suggested that normal stem cells may be protected from the

effects of mutations, but that these may manifest in their more downstream progeny.[8]

Certainly evidence exists that tumour-initiating cells may lie in a more committed cell population than the CSC. Both committed myeloid progenitors and haematopoietic stem cells produce a transplantable murine model of AML when transduced with a mixed lineage leukaemia fusion gene. Moreover, the phenotypic characteristics and stage of maturation arrest was identical in leukaemias derived from transplanted HSCs, common myeloid progenitors or granulocyte–macrophage progenitors (GMP).[59] Analysis of the different cellular compartments in CML showed increased nuclear β-catenin in myeloid progenitors as the disease advanced to blast crisis, while levels in HSCs remained stable. Also *in vitro* self-renewal capacity was shown both by GMPs from leukaemic patients and normal GMPs with forced β-catenin expression.[60] (It is of note that the cellular compartments in the leukaemic samples were defined according to the surface marker phenotypes of the normal haematopoietic hierarchy – this may be inappropriate, given that markers of normal differentiation would be expected to show derangement in leukaemia, particularly at blast crisis stage.)

Polyak and Hahn[57] propose three models for development of malignancy involving stem cells: (1) a mutation causes dysregulation of asymmetric division in a CSC and is passed on to all progeny; progression to full transformation occurs in this population as further mutations are acquired; (2) the CSC itself acquires mutations sufficient for malignancy, and passes these on to all progeny; and (3) the transit-amplifying cells or more differentiated progeny accrue mutations leading to dedifferentiation and acquisition of stem-cell-like properties; CSCs themselves are not involved.

Although the dedifferentiation of a committed cell into one with more primitive, stem-like properties seems an unlikely event, it has been shown in *Drosophila melanogaster* that cells differentiating in four- to eight-cell cysts during early development can be induced to revert to germinal stem cells.[61] Autocrine signalling of PDGF has been implicated for its role in the development of human gliomas. Over-expression in not only neural progenitors but also (albeit with less efficiency) differentiated astrocytes *in vitro* leads to increased proliferation, and *in vivo* can induce tumour formation in mice; this is enhanced by loss-of-function of the *Ink4a* locus.[23]

It has been proposed that perhaps, rather than unlimited self-renewal capacity being conferred on CSC by gain-of-function mutations, that it is in fact a 'default' pathway (seen, for example, in most single-celled organisms). If tissue specialization relies on a balance between self-renewal, differentiation and cell death, then could any cell in which apoptosis is prevented or differentiation is blocked (the effects of many of the mutations seen in cancer) act as a self-renewing CSC?[62]

Recently, interesting findings have suggested that there exists an even more primitive population of precursors within the bone marrow, the putative 'haemangioblast'. One hallmark feature of CML, present in 95% of patients, is the Philadelphia chromosome – this truncated chromosome 22 results from a reciprocal translocation with chromosome 9, and produces the BCR–ABL fusion protein. The protein is found in multiple haematopoietic lineages in CML, suggesting that the translocation event arises in an HSC; however, demonstration of BCR–ABL expression in endothelial cells from a patient with CML may point to the mutational event having occurred in a cell preceding the HSC in the haematological hierarchy, with greater differentiation potential.[63] Certainly, as more is understood about the stem cell hierarchies present in normal tissues, understanding of the role of stem or stem-like cells in cancer will be enhanced.

The importance of the niche

The potent effects imparted by the environment in which a cell exists, or 'niche', cannot be disregarded when considering the evolution of a tumour.[57,64,65] The tumour interacts with its surrounding stroma in a reciprocal manner similar to the communications between cells in normal tissues, and can influence the stroma such that it is more conducive to tumour growth. Indeed, epithelial tumour formation can be enhanced by inducing mutations in cleared (epithelial cell-free) stromal environments, and then introducing normal untreated epithelial cells.[64]

Normal bone marrow stem cells possess the capacity to mobilize and migrate in the circulation to distant sites in response to tissue damage and stress; complex, co-ordinated homing mechanisms are involved.[66] Stem cells from different tissues share common genetic programs,[67] and bone marrow stem cells display plasticity allowing them to differentiate into a variety of cell types.[65,68] There are many similarities between the mechanisms governing the migration of normal stem cells and the metastatic dissemination of tumour cells, such as the interaction between the cell-surface CXCR4 receptor and its ligand, stromal-derived factor 1, secreted by the niche.[69]

Cancer cells may disseminate to distant sites; however, while this may lead to the formation of metastases (whose cellular heterogeneity frequently resemble that of the primary tumour[70]), some of these migrating cells never develop further but remain detectable in remote tissues.[6] It has been proposed that it is the migration of the CSC population which allows the expansion of the tumour mass – manifesting as local invasion over short distances and metastatic spread for longer migrations.[70]

The cell fusion hypothesis

An alternative mechanism proposed for the apparent plasticity shown by stem cells is cell fusion. Fusion of bone marrow stem cells with a variety of different cell types has been shown *in vivo* and *in vitro*. If this hypothesis is extended to cancer, the fusion of a stem cell with a somatic cell carrying mutations could result in a cell with genetic and karyotypic abnormalities, which has the properties of a CSC.[65] The case for cell fusion remains equivocal. It has been best shown in models involving severe tissue injury; it remains to be established whether it occurs in diploid, adult cells *in vivo* in the absence of cell damage.[71]

Implications for cancer therapy

If a population of CSC is responsible for the propagation of a tumour, then these must be eliminated to effect a cure (Fig. 5). This may not be achieved by conventional strategies, which target rapidly dividing cells – stem cells may enter periods of quiescence during which they will be resistant to strategies aimed at eradicating cycling cells. This is evident in the treatment of acute leukaemias, in which total bone marrow ablation followed by reconstitution is required in a significant proportion of patients.[71] Guzman *et al.* report the use of MG-132, a proteasome inhibitor, and idarubicin, an anthracycline, in preferential targeting of leukaemic stem cells in *in vitro* and *in vivo* models of AML – the cells are driven to apoptosis, but normal HSCs are spared.[72]

Other treatment modalities might also preferentially target putative CSC. Transiently inactivating the causative oncogene in transgenic mouse model of osteogenic sarcomas can cause the tumours to regress, with reactivation leading to apoptosis.[73] It would seem logical that forcing CSC down a symmetrical division pathway, whereby two more committed daughter cells are produced, would deprive a tumour of its self-renewal potential and effect a cure. Differentiation therapy with retinoids is effective in a majority of cases of human acute promyelocytic leukaemia, although responses in other malignancies have been variable.[71]

Treatments directed solely at CSCs, however, may not have an effect on the differentiated progenitor and daughter cells. Therefore, the bulk of the tumour may remain intact while the CSCs are being destroyed. Jones *et al.* describes this theory as the dandelion phenomenon.[74] This theory states that cutting off a dandelion at the roots, or treating the bulk of the tumour, takes away the disease that one can see, however, the weed will still regrow because the root has not been destroyed. Conversely, destroying the root, or CSC, of the weed leaves the flower above soil initially. However, as the root dies, the portion above ground will eventually wither and die without the root.

According to this dandelion phenomenon of CSC therapy, a treatment regimen may be abandoned too quickly if the only judge of a response to therapy is a remission of the bulk of the tumour. This response is likely to lag behind the destruction of CSC. Conversely, treatment of chronic phase CML with imatinib mesylate, a tyrosine kinase inhibitor, has achieved high rates of complete remission despite the fact that BCR–ABL-expressing progenitors are spared.[75] This may indicate the significance of the

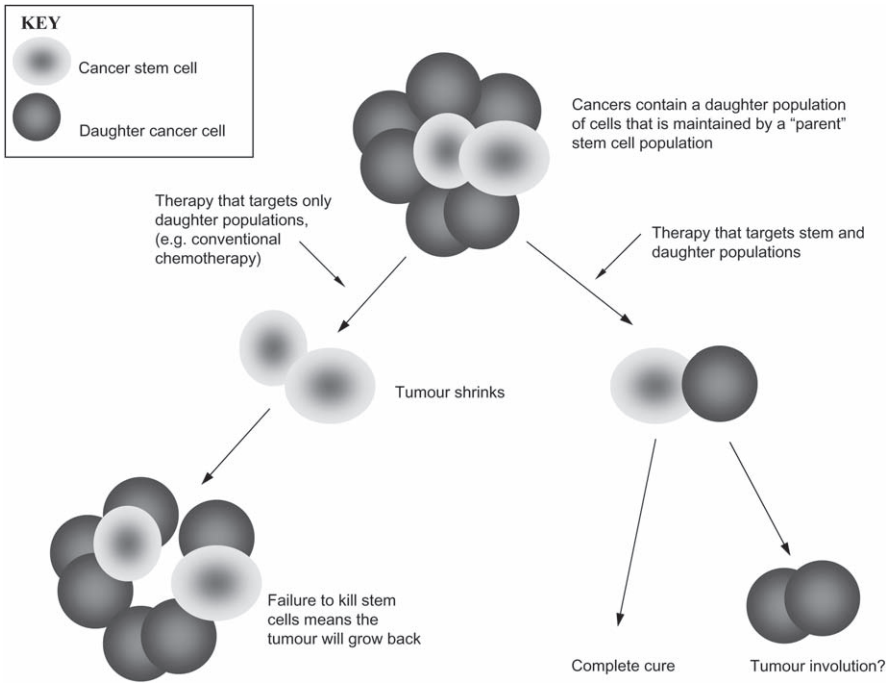

Figure 5. Currently conventional cancer therapies are directed at non-stem cell populations. Consequently, any tumour has the capacity to regrow and recapitulate the original cancer. Therapies targeted at the cancer stem cell and/or the daughter cells would have the capacity to cause either involution or complete tumour eradication.

niche provided to a stem cell by its surrounding daughter cell population – removal of paracrine factors, in targeting the tumour bulk, may sometimes be sufficient to arrest the progress of disease.[57]

Given that clinical response may not be an ideal way to monitor for response to therapies that target CSC, overall survival is left as common monitoring criteria. This requires long study times that can be impractical. Animal models are an ideal way to continue to use survival as criteria for determining effectiveness of therapy. Animal models of spontaneously occurring tumours often progress much faster than the human form of the disease. Many canine tumour models are very similar or even identical to their human counterparts,[76,77] and the natural canine model may represent an ideal testing ground for novel compounds directed at the CSC.

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