Investigation into the Functional Role of the Stem Cell Marker CD133

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ABSTRACT

CD133 is a pentaspan membrane protein found on pseudopodia, microvilli and other plasma protrusions irrespective of cell type in both humans and mice. CD133 has been classified as a marker of primitive haemopoietic and neural stem cells. At the molecular level it interacts with cholesterol and is located within lipid micro domains known as lipid rafts. Using cellular and molecular techniques we investigated the functional role CD133 plays in stem cell biology. Prior to commencing the functional experiments, we determined that MUTZ-2, Caco-2 and primary $CD34^+$ cells provided the best characteristics to investigate CD133 function. We established growth conditions, patterns and CD133 expression for all 3 cell types and concluded that Caco-2 was the preferential cell line based on CD133 expression and cell stability.

A number of approaches were used to knock down CD133 using a variety of RNAi oligonucleotides. Plasmid vectors were used in an attempt to produce a permanent CD133 knockout Caco-2 cell line. However, after successfully inserting the plasmid, the cell line failed to proliferate. Ultimately, a 73% CD133 phenotypic knockdown was achieved using RNAi technology from Santa Cruz, with 50% CD133 re-expression within 5 days, confirmation by both PCR and flow cytometry. Knockdown of CD133 in Caco-2 cells, resulted in no change in proliferation or adhesive properties to plastic, however a slight increase in cell cycle activity was observed.

Gene profiling of CD133 knocked down Caco-2 and control cells was carried out using microarray technology. This was also applied to cells incubated with monoclonal antibodies against epitopes of CD133, as these are often used as a means of cell isolation for CD133 functional studies. A variety of genes were up and down regulated in both groups when compared to the control cells. CD133 knockdown caused an up-regulation of genes associated with cell migration, motility, cell cycle, Wnt and tyrosine kinase pathway inhibitors and lipid transport across the membrane and down-regulated cell adhesion genes and apoptotic related genes.

Caco-2 cells incubated with monoclonal antibodies against CD133 showed up regulation in genes associated with cell cycle, migration and DNA replication and down regulation of

genes associated with regulation of cell proliferation and apoptosis. This result is significant due to the extensive use of CD133 antibodies in functional CD133 and $CD133^+$ cell population studies.

Confocal studies showed partial co-localisation between the lipid rafts and CD133, removal of the lipid rafts using the drug Beta Methyl Cyclodextrine caused loss of CD133. However, lipid raft expression remained relatively constant on CD133 knockdown cells. Examining the distribution of CD133 on Caco-2 cells adhered to fibronectin compared to glass via confocal analysis, showed there is no direct involvement of CD133 with anchorage type cell adhesion. However, considering the confocal analysis showing the association of CD133 within lipid rafts and the results of microarray, this would imply a more indirect role of CD133 within the processes of cell adhesion.

This study has revealed that that CD133 plays a suppressive role in stem cell biology and play a regulatory role in maintaining quiescence, keeping the early stem and progenitor cells in a non proliferating, non motile state. A number of studies in recent years have attempted to determine the function of CD133 which still remains relatively elusive. However, this study has contributed to a greater understanding of CD133 function and identified key areas for further investigation.

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Abbreviation list

AA	Amino acids
AB	Abcam
ACDA	Acid citrate dextrose
AF488	Alexa fluor 488
AF555	Alexa fluor 555
ALCL	Anaplastic large-cell lymphoma
ALDH	Aldehyde dehydrogenase
AML	Acute myloid lymphoma
APAF-1	Apoptotic protease activating factor 1
APC	Allophycocyanin
APC^2	Adenomatous polyposis coli
ASODN	antisense oligodeoxynucleotide
ATP	Adenosine triphosphate
BMCD	Methyl-beta -cyclodextrin
BMME	Bone marrow microenvironment
CAM	Cell adhesion molecule
CaR	Calcium sensing receptor
CB	Cord blood
CFU-E	Colony forming unit- erythrocytes
CFU-G	Colony forming unit- granulocutes
CK1A	Casein kinase
CLL	chronic lymphatic leukemia
CLSM	Confocal laser scanning microscopy
CM	Conditioning medium
CMV	Cytomegalovirus
CoR	Co-repressor
CSC	Cancer stem cell
CSL	CMP-binding
CT-B	Cholera toxin subunit B
CXCR	Chemokine receptor
CY3/CY5	Cyanine3/Cyanine 5

DNA	Deoxyribonucleotide acid
DR	Death receptors
DRM	Detergent resistant membranes
DSH	Dishevelled
dsRNA	Double stranded RNA
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EPC	Endothelial progenitor cells
EPO	Erythropietin
ESC	Embryonic stem cell
FACScan	Fluorescence-activated cell scanner
FADD	Fas (TNFRSF6)-associated via death domain
FBS	Fetal bovine serum
FC	Fold change
FcR	Fc receptor
FDR	False discovery rate
FITC	Fluorescein isothiocuanate
FLIP	Flice like inhibitory protein
FLT-3	Fms-like tyrosine kinase receptor-3
FRET	Fluorescent resonance energy transfer
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GLI	Glioma-associated oncogene homolog
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GO	Gene ontology
GSI	Gamma- secretase inhibitors
GSK-3	Glycogen Synthase Kinase-3
HA	Hyaluronate
HAG	Human gamma globulin
HAT	Histone acetyltransferases
HDAc	Histone deacetylase
HGF	Haemopoietic growth factor
HPC	Haemopoietic progenitor cells

HSC	Haemopoietic stem cells
IAP	Inhibitor of apoptosis protein
IL	Interleukins
LTC-IC	Long tern culture initiating cells
LR	Lipid Rafts
Mab	Monoclonal antibody
MALDI	Matrix-assisted laser desorption/ionisation
MB	Miltenyi biotec
MCSF	Macrophage colony stimulating factor
M-CSF	Macrophage-colony stimulating factor
MDCK	Madin Darby canine kidney
MEM	Minimum essential medium
miRNA	microRNA
MMP-9	Matrix metalloproteinase-9
MNC	Mono nuclear cells
MS	Mass Spectrometry
NICD	Notch intracellular domain
NOD	Non obese diabetes
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PE	Phycoerythrin
PI	Propidium iodide
PMT	Photomultiplier tube
PTCH1	Patched-1
RISC	RNA-Induced Silencing Complex
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
SCF	Stem cell factor
SCID	Severe combined immunodeficiency disease
SHH	Sonic hedgehog
ShRNA	Small hairpin RNA
siRNA	Small interfering RNA
SMAC	second mitochondria-derived activator of caspases

SMO	Smoothened
SPR	Surface lasmon resonance
TEM	Transmission electron microscopy
TNF	Tumour necrosis factor
TRADD	Tumor necrosis factor receptor type 1-associated DEATH domain
TRAIL	TNF-related apoptosis-inducing ligand
WNT	Wingless-type MMTV integration site family

Chapter 1: General Introduction

1.1 CD133 Structure

CD133 was identified in 1997 via contra-lateral immunisation of New Zealand Black mice with foetal liver $CD34^+$ cells. This resulted in lymphocyte formation and antibody production against CD133 (Yin *et al.*, 1997) identified as the human correlate of the murine prominin 1 (Miraglia *et al.*, 1997; Yin *et al.*, 1997). Today there are 2 antibodies commercially available, AC141 and AC133 that both recognise distinct epitopes on the receptor. CD133 is a transmembrane glycoprotein specifically associated with plasma membrane protrusions. It displays a unique membrane topology with five membrane-spanning domains and two large N-glycosylated extracellular loops (over 250 residues each) as illustrated in figure 1(Corbeil *et al.*, 2001).

Figure 1: Schematic representation of CD133 antigen structure. N-linked glycans are indicated in red. The CD133 molecule features 5 transmembrane, 3 extracellular and 3 intracellular domains. (Adapted from Miraglia *et al.*, 1997).

The CD133 gene is located on chromosome 4p15.32 in humans and 5B3 in mice, it is composed of at least 37 exons that span more than 150kb and is under the control of five alternative promoters (Jaszai *et al.*, 2007). The cDNA of CD133 antigen encodes a single-

chain transmembrane glycoprotein of 866 amino acids (AA) (molecular weight 120 kDa) with a unique structure consisting of an N-terminus (105 AA), two additional extracellular domains (258, and 279 AA), five transmembrane domains (23 AA each), two intracellular domains of 29 and 21 AA, and a 59 AA carboxyterminal tail. On the basis of the sequence, eight potential N-glycosylation sites have been identified. This topology closely resembles that of mouse prominin, an 858 AA glycoprotein recognized by the murine monoclonal antibody (Mab) 13A4 (Wiegmann *et al.*1997) and expressed in microvilli of murine neuro epithelium and kidney. Despite the differences, human CD133 and mouse Prominin 1 share roughly 60% homology. Prominin 2, a recently discovered second member of the prominin family, shares about 26% and 29% homology with CD133 and mouse prominin 1 respectively (Corbeil *et al.*, 2001). Since the original work carried out by Yin *et al.*, (1997) and Miraglia *et al.*, (1997), CD133 has been shown to be expressed by a wide variety of different tissues of both healthy and oncogenic nature. Perhaps one of the most significant areas of interest for CD133 expression has been within haemopoietic stem cells.

1.2 Haemopoietic/progenitor stem cells

1.2.1 Haemopoietic Stem cells

HSC are the precursor to all different cell types from the myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells), and lymphoid lineages (T-cells, B-cells, NK-cells) and can be defined by certain functional properties: Stem cells are pluripotent, meaning they have the ability to differentiate into all the haemopoietic lineages. This was demonstrated by Till *et al.*, (1961) who demonstrated that a single stem cell could repopulate the spleen of myeloablated mice with cells of all haemopoietic lineages (Till and McCulloch, 1961).

Stem cells also have the ability to self-renew; they can maintain their own numbers throughout an individual's life, even though they are responsible for the continual production of approximately 10^{11} cells per day (McKenzie *et al.*, 1996). Self renewal may occur symmetrically or asymmetrically. This highly dynamic process of self renewal and differentiation to generate and maintain all blood lineages is known as haemopoiesis (Clark *et al.*, 2003).

1.2.2 Haemopoietic environment

The site of haemopoiesis changes throughout development until adult life. At the start of life the yolk sac is the only site of haemopoiesis and is described as the mesoblastic phase. The foetal liver starts to produce haemopoietic cells after six weeks of development, but does not become the main site of haemopoiesis until week twelve (Rodak, 1995). The liver continues to produce haemopoietic cells up until birth and is assisted by the spleen, kidney and lymph nodes. As birth approaches the bone marrow becomes increasingly important as a haemopoietic organ and a few weeks after birth is the major site for haemopoiesis (Rodak, 1995). For the first few years of life the vast majority of bones have active (red marrow) bone marrow. Subsequently, there is a gradual (10-15 yrs) replacement of active bone marrow by fatty tissue (yellow marrow) and in early adulthood, active bone marrow is restricted to the top of long bones, the sternum, ribs cranium, vertebrae and pelvis.

Within adult humans, the bone marrow is the major site of haemopoiesis containing the entire range of blood cells types. The bone marrow microenvironment supports the different developmental stages of all maturing cell lineages and contains stromal stem cells (mesenchymal stem cells), fibroblasts, endothelial cells, macrophages, osteoblasts and fat cells, together with the extracellular matrix consisting of collagen, fibronectin, laminin and proteoglycan constituents forming so called "niches" where stem cells reside. Both a healthy stem cell pool and bone marrow microenvironment (BMME) are necessary for haemopoietic cell production (see figure 2).

Figure 2: The bone marrow microenvironment. Haemopoiesis occurs in the BMME provided by a stromal matrix on which stem cells grow and divide. Recognition and adhesion sites, extracellular glycoproteins and other compounds are involved in their binding (adapted from Hoffbrand and Pettit, fifth edition, 2006).

1.2.3 The regulation of haemopoiesis

The proliferation, maturation and apoptosis of haemopoietic cells are governed by extrinsic signals, chemokines and cytokines in a complex system of soluble glycoproteins, named haemopoietic growth factors (HGF's) which direct the division and maturation of the progenitor cells along different lineages of differentiation as illustrated in figure 3.

Over 30 haemopoietic growth factors have been characterized and cloned. They control all growth stages within the different lineages of blood cells with more than one factor controlling cells in a single lineage, and most single factors influencing more than one lineage (Earle *et al.*, 2007).

Cytokines are not directly able to enter the cells so they exert their effect on receptors located on the cell membrane resulting in intracellular messengers being produced (Hart *et al.*, 2004). For example red cell production and release into the circulation is stimulated by erythropoietin (EPO) which is also a stem cell regulator. The ability of cells to respond to different growth factors is dependant on the expression of cell surface receptors, often linked to stages of differentiation e.g.: EPO and Macrophage colony stimulating factor (MCSF).

General characteristics of myeloid and lymphoid growth factors are listed below:

- Glycoproteins that act at very low concentrations.
- Multiple actions: proliferation, differentiation, maturation etc.
- Show synergistic or additive interactions with other growth factors.
- Usually affect more than one lineage.

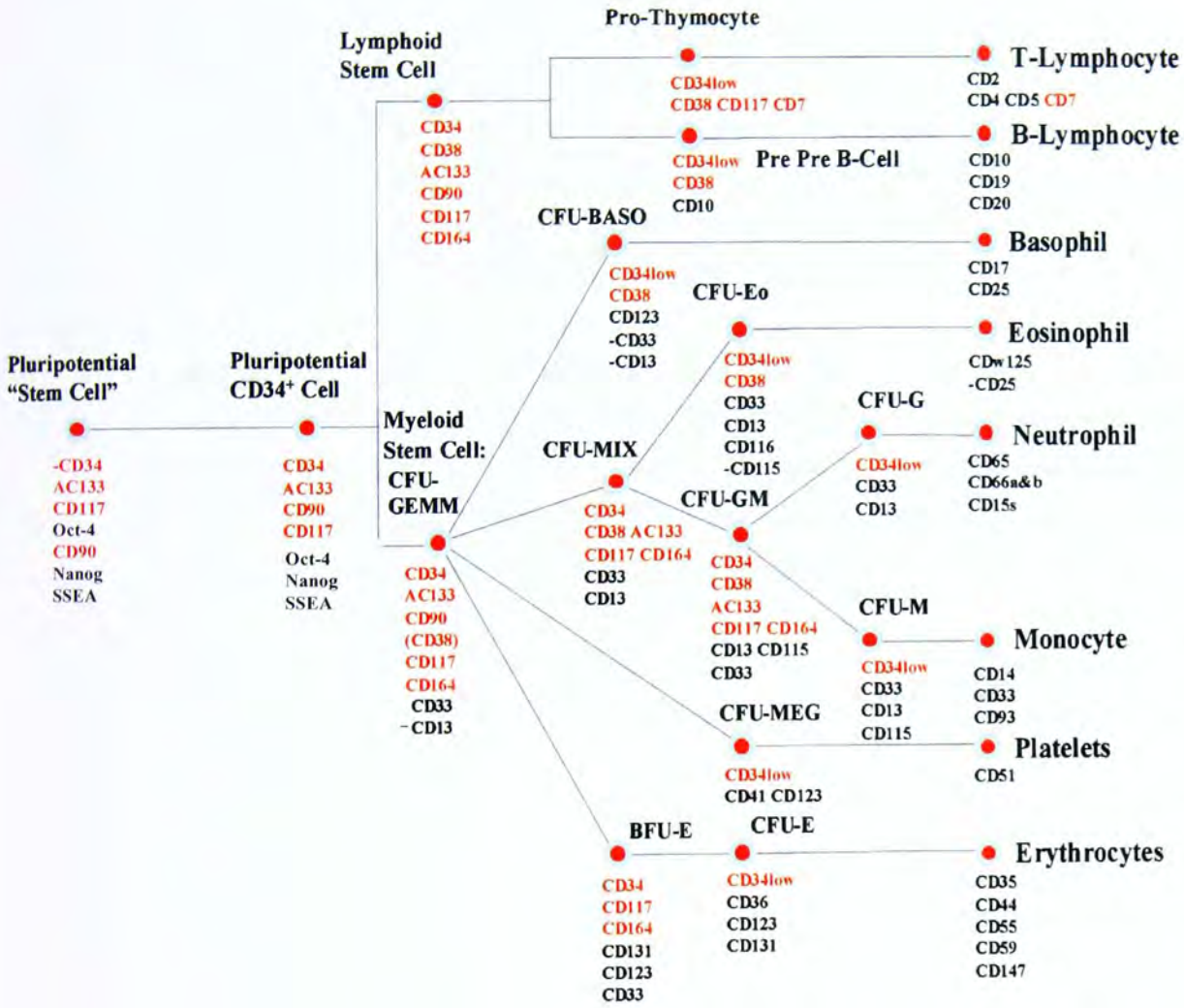

Figure 3: Schematic representation of the hierarchical model of cell markers in haemopoiesis. CFC = colony forming cell, BFU = burst forming unit, GEMM = capable of forming myeloid and erythroid lineages, BASO = Basophil, Eo = Eosinophil, G= Neutrophil, M = Macrophage, MEG = Megakaryocyte and E == Erythroid. Included are cell subset clusters of differentiation (CD) identifying markers. Not all of each cell's markers are displayed, rather markers that allow cell identification. Antigens in red were mentioned in this thesis. Where CD is prefixed by a '' –'' symbol, the cell subset is identified by lack of expression of the particular CD marker (Adapted from Hoffbrand *et al.*, 5th edition, 2007, Kishimoto T *et al.*, 1997).

To sustain haemopoiesis without exhausting the stem cell pool, two conflicting theories exist as to how stem cells make the choice between differentiating or self renewing.
The Stochastic theory suggests that the decision to self renew is a random one which is dependent on cytokine receptor expression of the receptor. HSC are exposed to a variety of cytokines in the bone marrow and failure to interact with the correct stimulus may lead to apoptosis (Ogawa, 1993). The deterministic theory suggests that exogenous stimuli such as specific cytokines are responsible for this decision. In the absence of suitable cytokines, the haemopoietic stem cell will undergo apoptosis (Ogawa, 1993).

As stem cells differentiate towards progenitors, precursors and eventually mature cells, they lose their ability to self renew. This is partially due to the stem cell being subjected to various external conditions which ultimately determine the lineage the cells will take. Chemokeines, growth factors and other cytokines determine the cells fate and are vital for haemopoietic stem cell regulation suggesting that both theories may be occurring.

1.3 CD133 expression on haemopoietic cells

CD133 is known to identify a very primitive population of HSC and was originally found on HSCs and HSCs derived from human fetal liver, bone marrow and peripheral blood (Pasino *et al.*, 2000). Yin *et al.*, (1997) has shown that the receptor is down regulated to undetectable levels as the haemopoietic stem cell matures (Yin *et al.*, 1997).

Up to 0.52% of bone marrow and 0.16% of cord blood mononuclear cells express CD133 (Bhatia *et al.*, 2001). CD133 is expressed on CD34+ cells known to contain up to 70% of Bone Marrow and 83% Cord Blood $CD34^+$ cells with the HSC being positive for CD133 (Yin *et al.*, 1997). Analysis showed that CD133 is dimly or not expressed on late progenitors, such as pre-B cells, CFU-E (colony forming unit-erythrocytes), and CFU-G (colony forming unit-granulocytes), and populations of $CD133^+$ cells are highly enriched for long term culture initiating cells (LTC-IC), the most primitive human haematopoietic cells which can be assayed *in vitro* (Matsumoto *et al.*, 2000). $CD133^+$ cells appear to be precursors to $CD34^+$ cells as Gallacher *et al.*, showed that CD133 cells were the only subset of amongst a $CD34^-CD38^-$ lineage population from human cord blood that could form $CD34^+$ cells in culture with an engraftment capability in NOD/SCID mice 400 fold greater than that observed in the $CD133^-$ subset (Gallacher *et al.*, 2000).This was also supported in part by Summers *et al.*, (2004) who generated $CD34^+$ cells from $CD133^+/CD34^-$ cells *in vitro* (Summers *et al.*, 2004). As the cells mature and become progenitor cells they begin to express markers such as CD38. Finally they begin to lose their haemopoietic/progenitor markers and adopt maturation markers such as CD13, CD33. A role for CD133 as an identifier of stem cells with the capacity to engraft and differentiate to form non haemopoietic adult lineages and contribute to disease amelioration through tissue regeneration has already been shown to be beneficial *in vivo*. Cord blood or bone marrow- derived $CD133^+$ cells have been used to restore myocardial tissue viability after infarction. $CD133^+$ cells were able to migrate, colonize and survive in the infarcted myocardium and support functional recovery by preventing scar thinning and diastolic dilation (Leor *et al.*, 2006) and also induce angiogenesis within the infarcted myocardium (Stamm *et al.*, 2003). In previous experiments this was suggested to be as a result of fibrosis rather than true myocyte regeneration, but functional myocytes have now been proven (Shmelkov *et al.*, 2005).

1.4 CD133 expression in normal tissues

$CD133^+$ expressing stem and progenitor cells can be found in many different tissues such as the liver, muscle, kidney, prostate and neural tissue and *in vitro* have been shown to have similar characteristics to endothelial cells, neural cells, hepatocytes, myocytes and osteoblasts. The general distribution of CD133 is described below:

1.5 Murine models:

1.5.1 Epithelial cells

In the prostate, CD133 expression was shown to be found on α2β1high basal cells, which were defined as the prostate epithelial stem cell population. When transplanted into athymic nude mice, $CD133^+$ cells from separated prostatic tissue regenerated a fully differentiated prostatic epithelium, including acini that secreted prostate-specific products (Richardson *et al.*, 2004). $CD133^+$ cells isolated from the human kidney could be differentiated *in vitro* into renal epithelial or endothelial cells. Upon transplantation into Severe Combined Immunodeficiency (SCID) mice with acute tubulonecrosis, CD133 cells migrated to the damaged kidneys forming tubular structures that expressed renal epithelial markers in damaged tubules (Bussolati *et al.*, 2005).

1.5.2 Endothelial cells

Endothelial progenitor cells (EPCs) play an essential role in postnatal neoangiogenesis and neovascularisation, and also as a potential treatment of ischemic or injured tissue and myocardial infarction (Yang *et al.*, 2004). After transplantation into nude mice with ischemic hind limb injury, enriched $CD133^+$ cord blood cells were able to incorporate into capillary networks, augment neovascularisation and improve ischemic limb salvage (Yang *et al.*, 2004). $CD133^+$ cells isolated from the bone marrow (Yang *et al.*, 2004), cord blood, mobilised and non-mobilised peripheral blood are capable of giving rise to endothelial cells *in vitro* in addition to the reconstitution of the haematopoietic system *in vivo* (Logues *et al.*, 2004) thus indicating that $CD133^+$ cells potentially contain hemangioblasts, the common precursor of HSCs, HPCs and EPCs (Logues *et al.*, 2004).

1.5.3 Neural cells

Rodent models of stroke damaged brains and spinal cord injuries in mice have also demonstrated the ability of injected $CD133^+$ neural stem cells to migrate to sites of lesions and to differentiate into functional neuronal phenotypes. In the case of spinal cord injuries, re-myelination, locomotive recovery and synapse formation were all observed (Cummings *et al.*, 2005). Lee *et al.*, (2005) isolated neural stem $CD133^+$ /lineage $^-$ cells (lacking markers of neuronal and glial lineages), which constituted approximately 0.1-0.3% of cells in the mouse developing cerebellum, and showed that these cells were capable of clonal expansion to form neurospheres *in vitro* (Lee *et al.*, 2005). CD133 has also been found within the mouse embryonic forebrain where it has been used to enrich for neural progenitors (Barraud *et al.*, 2007) and restricted to the ductal epithelial tree of the embryonic and adult mouse pancreas. These cells were capable of clonal expansion and multi lineage differentiation.

1.6 Human CD133 models

Within human tissue, CD133 expression is widespread and not solely restricted to haemopoietic stem or progenitor cells. Outlined below is the origin of these CD133 expressing cells and their possible functions.

Table 1: Represents cells expressing CD133 that are widely expressed by multiple tissues and function as stem cells.

Origin	Stem cell action	Reference
Adult human kidney	Endothelial and epithelial differentiation	(Bussolati *et al.*, 2005)
Human Bone marrow	Human liver regeneration	(Bitan *et al.*, 2005)
Human brain	Neural differentiation	(Corti *et al.*, 2008)
Human neonatal foreskin	Keratinocyte differentiation	(Yu *et al.*, 2002)
Human prostate basal cells	prostatic acinar differentiation in mice	(Richardson *et al.*, 2004)
Human Pancreas	Islet differentiation	(Koblas *et al.*, 2007)

$CD133^+$ cells isolated from fetal liver, umbilical cord blood, bone marrow and mobilized blood were capable of *in vitro* differentiation to neuronal cells as well as astrocytes, oligodendrocytes and glial cells (Jang *et al.*, 2004). Similarly, CD133 cells isolated from the human fetal brain or skin tissues were able to form self –renewing neurospheres *in vitro* and to differentiate into neurons and glia (Uchida *et al.*, 2000). Moreover when human $CD133^+$ neurosphere cells were transplanted into neonatal immunodeficient Non obese diabetes (NOD)-SCID mice, they proliferated, migrated and differentiated into fully integrated neurons and glial cells (Tamaki *et al.*, 2002). Kuci *et al.*, (2006) found that culture of highly purified $CD133^+$ cells from mobilized peripheral blood for 3–5 weeks in the presence of the appropriate micro environmental cues, differentiate into neural progenitor-like cells (NPLCs) and hepatocyte-like cells. (Kuci *et al.*, 2006).

$CD133^+$ cells from the stroma of human cornea have the capacity to proliferate *in vitro*; colonies derived from $CD133^+$ cells could be differentiated into fibroblastic cells, indicating that $CD133^+$ cells represent stem cell of the corneal stroma (Thill *et al.*, 2007). Human circulating $CD133^+$ cells were also induced to undergo endothelial or cardiomyocytic differentiation *in vitro* (Bonanno *et al.*, 2007). However, Lu *et al* (2007) compared colony formation and endothelial cell formation in 2 groups of cells expressing $CD133^+$ or $CD34^+$ from umbilical cord blood. The results showed that even though both groups formed similar numbers of granulocyte-macrophage colony-forming units, $CD133^+$ cells showed reduced formation of burst forming units erythroid colonies and were unable to differentiate to endothelial cells indicating that there may be no advantage of using $CD133^+$ cells in this instance compared to the proposed theory mentioned above or that of $CD133^+$ cord blood cells were able to incorporate into capillary networks, augment neovascularisation and improve ischemic limb salvage in the murine model (Lu *et al.*, 2007).

However, as previously stated, CD133 and Prominin only share approximately 60% homology. If CD133 is directly involved in these regenerative processes, then this disparity may be reflected in the varying results observed between human and murine models. Due also to the rarity of $CD133^+$ cells from within CB and BM and the potential unreliability of murine models, perhaps a more reliable model from which to study CD133 within human tissue is the use of $CD133^+$ cancer cell lines.

1.7 Introduction to cancer stem cells

According to the traditional model of carcinogenesis, a tumor can originate from any dividing cell of the body as a result of multiple mutations allowing an unlimited proliferation potential resulting in a tumour mass. In the last several years, evidence has suggested that the capacity of initiating a self renewable clonal population could be a rather unique characteristic of cells with stemness properties. Expansion of mammary stem cells in mouse breast cancer models prior to cancer development is also indicative of a potential connection between normal tissue stem cells and cancer stem cells (CSCs) (Shackleton *et al.*, 2006).

These so-called CSC's have been isolated from a variety of tumours and tend to make up a very minor population of cells that have the ability to self renew, differentiate and proliferate to various cell types observed in tumours. Factors that make cancer stem cells difficult to treat are their resistance to therapeutic drugs, partially due to the fact the cells are in the quiescent G0 phase and these drugs normally target homogeneous populations of rapidly dividing and differentiating tumour cells. Also the fact they have greater migratory capabilities than normal cancer cells which relates to a higher metastatic potential making it difficult to home in and target the cells in a localised area. It is therefore imperative to try and understand the mechanisms contributing to this resistance, but this can only be achieved by identifying the cancer stem cells, their regulatory mechanisms and possible signalling pathways involved in CSC maintenance. Many of the factors known to govern HSC migration are also critical mediators of cancer metastasis and may contribute to the problem. The laminin receptor which is known to play a key role in HSC migration from the bone marrow, has also been found to play an important role in cancer metastasis (Selleri *et al.*, 2006). Similarly, stromal cell derived factor and its receptor chemokine receptor 4 (CXCR4), critically involved in HSC migration and homing, also function in breast (Kang *et al.*, 2003), prostate (Bernards *et al.*, 2003) and other types of cancers.
Matrix metalloproteinase-9 (MMP-9) belongs to a family of MMPs that play a critical role during cancer cell invasion (Chinni *et al.*, 2006) and is involved in HSC homing and migration (Heissig *et al.*, 2002). Finally it has been shown that HSC lacking a particular calcium sensing receptor (CaR) are unable to localise to there endosteal niches in the bone. However elevated CaR expression in breast cancer samples positively correlate with

increased bone metastasis (Mihai *et al.*, 2006) perhaps due to lack of homing to its own tissue.

1.8 CD133 expression in malignant cancer stem cells and solid tumours

Cancer stem cells with stem cell like features were first observed in acute myeloid leukaemia and later found in other tumour types. In most cases such cells have been identified through the expression of specific cell surface markers.
The most frequent markers shared by CSC of different origins are CD20, CD24, CD34, CD44, CD90, CD117, CD133, aldehyde dehydrogenase (ALDH), nestin, the β1 integrin chain and EpCAM. Across tumour types the most common cancer stem cell marker used to identify CSC has been CD133.

Singh *et al.*, (2003) took a $CD133^+$ cell population from human brain tumours and was able to show that they exhibit stem cell like features, by proving they were capable of self renewal and replication of the original tumour when transfected into an immunodeficient mouse brain. He was able to show that injection of just 100 $CD133^+$ cells were able to cause a tumourgenic effect whereas injection of the same number of $CD133^-$ cells did not elicit a response (Singh *et al.*, 2003). Similarly, Chiou *et al.*, (2008) discovered that in atypical teratoid rhabdoid tumour, a malignant neoplasm in the central nervous system, the migration, invasion, malignancy and radio resistant capabilities of $CD133^-$ were significantly augmented when compared to $CD133^+$ cells (Chiou *et al.*, 2008). There have been many studies proving that cells expressing CD133 represent cancer stem cells, including those listed in table 2.

Interestingly Rappa *et al.*, (2008) investigated CD133 as a potential molecular therapeutic target for metastatic melanoma. They used FEMX-I melanoma cells and made retroviral plasmids to create a permanent CD133 down-regulated cell line. Functional analysis of the knocked out cell lines showed the down regulation of CD133 resulted in slower growth (growth decreased by 25% in FEMX-1A), and reduced capacity to form spheroids, suggesting that the cells have lost some CSC- associated properties. The down regulation of the receptor severely reduced the capacity of the cells to metastasize, indicating that

Table 2: CD133 expression in solid tumour tissues on human cells.

Antigenic Phenotype	Tumour Origin	Reference
CD133/CD44/α1β2	Human prostate tumour	Collins *et al.*, 2005
CD133/ABCG2	Human pancreatic adenocarcinoma	Olempka *et al.*, 2007
CD133	Human colon carcinoma	O'B rien *et al.*, 2007
CD133	Human hepatocellular carcinoma	Yin *et al.*, 2007
CD133/Nestin	Human neural tumours	Liu[1] *et al.*, 2006
CD133	Human renal tumours	Bruno *et al.*, 2006
CD133	Human lung tumours	Song *et al.*, 2008
CD133	Melanoma carcinoma	Monzani *et al.*, 2007
CD133	CNS carcinoma	Blazek *et al.*, 2007

CD133 may play a role in the tumour cells capacity to seed to distant sites (Rappa *et al.*, 2008). This was partially demonstrated by Horst *et al.*, (2009) showing that high CD133 expression correlates strongly with synchronous liver metastasis, but knockdown of the receptor showed no change in proliferation or colony formation (Horst *et al.*, 2009).
In contrast to this, various groups have expressed their concerns regarding CD133 as CSC marker. In Shmelkov's (2008), study, both $CD133^+$ and $CD133^-$ metastatic colon cancer cells were capable of long term tumourigenesis in SCID mice and the later formed larger tumours (Shmelkov *et al.*, 2008). Other studies have backed this work claiming that expression of CD133 in colonic cancer is not restricted to CSC, but it is also expressed on differentiated tumour cells (Kemper *et al.*, 2010) suggesting that $CD133^-$ human glioblastoma cells were also found to be tumour initiating. Ogden *et al.*, (2008) and Yoshikawa *et al.*, (2009) revealed that CD133 could be a biliary and progenitor cell marker *in vivo*, concluding that CD133 alone is not sufficient to detect tumor-initiating cells in human hepatocellular carcinoma cell lines (Yoshikawa *et al.*, 2009; Ogden *et al.*, 2008). Therefore, the validity of CD133 being a CSC marker is still very much under debate.

Another major therapeutic problem CSC's present is their resistance to treatment such as radiation and various chemodrugs which normally target homogeneous populations of rapidly growing, differentiating tumour cells. Alternative targets are being looked at. These

include the drug Salinomycin, a potassium ionophore that is capable of reducing the proportion of $CD133^+$ subpopulations in human CRC HT29 AND SW480 cells. Furthermore, salinomycin treatment decreases colony-forming ability and cell motility in HT29 cells and down regulates the expression of vimentin (mesenchymal stem cell marker) and induces E-cadherin expression in HT29 cells (Dong *et al.*, 2011).

It is therefore imperative to try and understand the mechanisms contributing to this resistance, but this can only be achieved by identifying the cancer stem cells, their regulatory mechanisms and possible signalling pathways involved in CSC maintenance. As discussed previously, resistance to radiation and chemo drugs has been a major therapeutic problem for combating CD133 associated CSC's.

1.9 Cellular distribution

The majority of work on CD133 within both CSC and normal stem cell populations, has predominantly used CD133 as a cell population identifier, although some studies have focused on the distribution of CD133 on different cell types and the possible significance of its distribution. The main characteristics of CD133 can be described as follows:

1. A profound preference for membrane extensions such as microvilli, which is illustrated by its concentration in various types of plasma membrane protrusions.
2. Associated with a cholesterol- based membrane microdomain in which CD133 interacts directly and specifically with membrane cholesterol.
3. Association with membrane particles (prominosomes) that are found in various body fluids including saliva and lacrimal fluid (Corbeil *et al.*, 2001).

In epithelial cells, CD133 is concentrated in microvilli and similar protrusions of the apical plasma membrane, and is lacking from the planar subdomain of the cell surface (Weigmann *et al.*, 1997). In non-epithelial cells, such as haemopoietic stem cells, CD133 is again enriched in plasma-membrane protrusions (Corbeil *et al.*, 2000). In rod photoreceptor cells, CD133 is concentrated in the plasma-membrane envaginations present at the base of the outer segment, which are essential precursor structures in the biogenesis of photoreceptive disks (Maw *et al.*, 2000). Figure 4 shows the distribution of CD133 for plasma membrane protrusions in various cell types (Corbeil *et al.*, 1999).

The molecule also displays a remarkable sub cellular localization. At the plasma membrane, it is confined to specific subdomains that although distinct in various cell types have one feature in common, namely that they all protrude from the planar region of the plasmalemma.

Figure 4: CD133's preference for plasma membrane protrusions in various cell types. **A)** In epithelial cells, newly synthesized prominin is directly targeted (arrow) from the trans-Golgi network (TGN) to the apical plasma membrane domain. Within this domain, prominin is selectively associated with microvilli (red) rather than the planar subdomain (blue) of the plasma membrane **B)** In hematopoietic progenitor cells, prominin is enriched in the sparse, small plasma membrane protrusions of these cells (red) **C)** In rod photoreceptor cells, prominin is concentrated in the plasma membrane envaginations (red) at the base of the outer segment **D)** In oligodendrocytes, prominin appears to be enriched in the plasma membrane protrusions forming the myelin sheath (red) **E)** In transfected fibroblasts, prominin is preferentially localized in microspikes, filopodia and at the leading edge of lamellipodia (red) (adapted from Corbeil *et al.*, 2001).

1.10 Cell membrane and lipid rafts

The cell membrane is a biological membrane that separates the interior of all cells from their external environments. Charles Ernest Overtone was the first to find that non-polar substances have the ability to pass surprisingly quickly across membranes of plant and animal cells. The cell membrane surrounds the protoplasm of a cell and consists of the phospholipid bilayer with embedded proteins. It is selectively-permeable to ions and organic molecules and controls the movement of substances in and out of cells. Cell membranes are involved in a variety of cellular processes such as cell adhesion, ion conductivity and cell signalling and serve as the attachment surface for the extracellular glycocalyx and cell membrane and intracellular cytoskeleton (Alberts *et al.*, 5th edition, 2007). The cell membrane also plays a role in anchoring the cytoskeleton to provide shape to the cell, and in attaching to the extracellular matrix and other cells to help group cells which together form tissues. The barrier is differentially permeable and able to regulate what enters and exits the cell, thus facilitating the transport of materials needed for survival. The movement of substances across the membrane can be passive, occurring without the input of cellular energy, or active, requiring the cell to expend energy (Mayor and Rao, 2004).

The apical membrane of a polarized cell is the surface of the plasma membrane that faces the lumen whilst the basolateral membrane is the surface of the plasma membrane that forms its basal and lateral surfaces. Tight junctions join epithelial cells near their apical surface preventing the migration of proteins from the basolateral membrane to the apical membrane. The cell membrane consists primarily of three main classes of amphipathic lipids: phospholipids, glycolipids, and cholesterols which make up about 30% of the cell membrane. Phospholoipids are spontaneously arranged so that the hydrophobic "tail" regions are shielded from the surrounding polar fluid, causing the more hydrophilic "head" regions to associate with the cytosolic and extracellular faces of the resulting bilayer. This forms a continuous spherical lipid bilayer maintained by hydrophobic interactions. Lipid bilayers have very low permeability for ions and most polar molecules. The arrangement of hydrophilic heads and hydrophobic tails of the lipid bilayer prevent polar solutes (e.g. amino acids, nucleic acids, carbohydrates, proteins, and ions) from diffusing across the membrane, but generally allows for the passive diffusion of hydrophobic molecules (Tristram-Nagle *et al.*, 2004).

Originally defined biochemically as detergent resistant membrane (DRM) fractions, lipid rafts are proposed to be highly dynamic, submicroscopic assemblies that are found on the cell membrane surface and consists of cholesterol and sphingolipid. Lipid rafts are more ordered and tightly packed than the surrounding bilayer, but float freely in the membrane bilayer with (Fantini *et al.,* 2002) the cholesterol acting as a "dynamic" glue holding the raft together. The tighter packing is due to the saturated hydrocarbon chains in raft sphingolipids and phospholipids compared with the unsaturated fatty acids of phospholipids in the non-raft phase (Simons and Vaz, 2004). These specialized membrane microdomains compartmentalize cellular processes by serving as organizing centers for the assembly of signalling molecules influencing membrane fluidity, membrane protein trafficking (e.g. apical delivery, membrane budding and fission), regulating neurotransmission and receptor trafficking (Korade *et al.,* 2008) (Figure 5). Two types of lipid rafts have been proposed: planar lipid rafts and caveolae. Planar rafts are defined as being continuous with the plane of the plasma membrane (not invaginated) (Janich *et al.,* 2007). Caveolae, on the other hand, are flask shaped invaginations of the plasma membrane that contain caveolin proteins and are the most readily-observed structures in lipid rafts (Hnasko *et al.,* 2003). Caveolins are widely expressed in the brain micro-vessels of the nervous system, endothelial cells, astrocytes, oligodendrocytes, schwann cells, dorsal root ganglia and hippocampal neurons (Sowa *et al.,* 2008). Planar rafts contain flotillin proteins and are found in neurons where caveolae is absent. Lipid rafts also have been defined largely according to their insolubility in nonionic detergents such as Triton X-100 (Calder *et al.,* 2007; Roper *et al.,* 2000) which probably relates to their structural characteristics.

Figure 5: The schematic structure of a lipid bilayer. Figure shows a cell plasma membrane showing amphipathic lipids in a bilayer. Polar phospholipid head group exposed to the aqueous cell interior and exterior but hydrophobic acyl chains associated with each other and make a barrier between cell interior and exterior. Integral or peripheral proteins of a lipid bilayer are free to move in the plane of the bilayer. (Adapted from Alberts *et al.*, 5th edition 2007).

1.11 CD133 association with cell membrane lipid rafts

Little is known about the precise delivery of CD133 to the cell surface, but we know that the receptor shows a polar distribution towards the apical membrane location. This was demonstrated by Karbonova *et al.*, (2008) using a novel monoclonal antibody (80B258) generated against the human prominin-1 polypeptide. Using glandular epithelial cells they observed 80B258 immunoreactivity at the apical or apicolateral membranes of polarized cells (Karbonova *et al.*, 2008) and its association with microvilli involved a cholesterol based lipid raft (Corbeil *et al.*, 2000). The relationship between CD133 and the cholesterol based lipid rafts was strengthened when it was discovered that a mild cholesterol depletion of the cell surface lead to a redistribution of prominin from microvilli over the entire apical plasma membrane (Roper *et al.*, 2000).

Surprisingly, the retention of CD133 in microvilli is not due to a direct interaction with the actin skeleton and it seems that the receptor is not a constituent of the classical Triton cholesterol-sphingolipid rafts but Lubrol rafts (terminology comes from the fact that they are soluble in the detergent Triton and insoluble in Lubrol). Interestingly the rafts are

assembled in the trans golgi network into relatively small lubrol rafts and then are delivered to the apical membrane directly. The small rafts cluster forming larger ones hence creating the microdomains. This was shown by Oshima *et al.*, (2007) using Madin-Darby Canine Kidney Cells (MDCK cells) to investigate the intracellular transport of Prominin (Oshima *et al.*, 2007).

There are 3 mechanisms that could possibly explain the retention of CD133 in the microvillar plasma membrane. Lubrol rafts may be linked to each other by numerous weak interactions involving the glycan moieties of their glycoproteins and glycolipids forming a stable structure (Figure 6A). Lubrol rafts may be linked due to a vertical interaction with the actin-based cytoskeleton of a Lubrol insoluble transmembrane protein distinct from prominin (Figure 6B). The membrane curvature at the base of the microvilli may act like a fence preventing lateral diffusion of the CD133 containing rafts and preventing escape from the microvilli.(Figure 6C) (Corbeil *et al.*, 2001).

Figure 6: Illustration of three hypothetical mechanisms underlying the retention of Prominin-containing Lubrol rafts in the microvillar plasma membrane. A) Retention due to lateral interactions of multiple rafts involving the glycan moieties of their glycoproteins and glycolipids WGA, wheat germ agglutinin. B) Retention due to vertical interaction with the actin-based cytoskeleton of a Lubrol-insoluble transmembrane protein distinct from CD133. C) Retention due to the negative membrane curvature which exists in the external membrane leaflet. (Adapted from Corbeil *et al.*, 2001).

It has also been reported that Prominin 1 (murine CD133) is associated with small membrane particles known as prominosomes. Prominin 2 (a structurally related protein) is confined to microvilli, cilia and other acetylated tubulin-positive protruding structures. Similar to Prominin 1, Prominin 2 is partly associated with detergent-resistant membranes in a cholesterol-dependent manner, suggesting its incorporation into membrane microdomains, and binds directly to plasma membrane cholesterol. It was also noted by the same group, that Prominin 2 is not restricted to the apical domain like Prominin 1 but is distributed in a non-polarized fashion between the apical and basolateral plasma membranes. (Florek *et al.*, 2007).

Prominosome production and release is unknown, however they may arise from microvilli or primary cilia where Prominin 1 appears concentrated at the tips. Prominosomes are released into the culture media and found in a variety of physiological fluids (Florek *et al.*, 2007). Marezesco *et al.*, (2005) discovered that prominosomes particles were found in various body fluids of adult humans, including saliva, seminal fluid and urine, and were released by the epithelial model cell line Caco-2 upon differentiation (Marzesco *et al.*, 2005). The fact they are released on differentiation supports the theory that their role in haemopoiesis may be a means of disposal of a "stem cell- specific membrane microdomain" allowing these cells to modify stem and progenitor cell properties (Bauer *et al.*, 2008). Alternatively the Prominin 1 containing membrane particles might play a role in intercellular communication by carrying specific signalling.

1.12 Postulated functions of CD133

1.12.1 Cell membrane organiser

The function of CD133 still remains to be defined. The distribution of the molecule within areas such as microvilli and pseudopodia suggest that of CD133 may be involved with the regulation of the plasma membrane or be an "organiser" of the plasma membrane topololgy. Marzesco *et al.*, (2005) showed that small CD133 containing vesicles known as prominosomes, were discovered in the ventricular fluid within the developing mouse neural tube, human saliva, seminal fluid and urine. The appearance of the prominosomes coincided with the regression of microvilli and the formation of large pleomorphic

protuberances on embryonic neuroepithelium suggesting CD133 mediated regulation of the plasma membrane structure (Marzesco *et al.,* 2005). CD133's interactions with cholesterol forming micro domains on the cell surface suggests that CD133 may be a cell surface lipid regulator (Fargeas at al, 2004). This was supported by Roper *et al.,* (2000) who showed that prominin, becomes associated in the trans-Golgi network of lipid rafts (Roper *et al.,* 2000). The role CD133 plays in association with lipid rafts is not clearly defined. It may have an indirect effect determining the lipid concentration within the membrane, which would not only affect signalling processes but ultimately affect cell motility and migration. Motility is related to cell membrane lipid composition, thus the correct cocktail and concentrations of lipids would ultimately increase the fluidity of the cell membrane facilitating movement. CD133 may have a direct effect on lipid raft formation, structural integrity and maintenance thus affecting all the processes associated with lipid rafts in the bilayer. No studies have yet confirmed this hypothesis.

1.12.2 Pseudopodia formation

It has been suggested that CD133 plays a role in formation of plasma membrane protrusions. CD133 is expressed in retinoblastoma cell lines and adult retina, however a single nucleotide deletion causing a frame shift mutation in the CD133 gene results in the loss of approximately half of the second extracellular loop, the final membrane spanning element and the cytoplasmic C-terminal domain. The resulting truncated protein cannot be transported to the cell surface resulting in impaired generation of the envaginations or impaired conversion of the envagination to disks by autosomal recessive degeneration of the photoreceptor disk (Maw *et al.,* 2000). This results in blindness and loss of peripheral vision from childhood which can progress to profound visual impairment. This data is also consistent with the postulated function of Prominin 1 as an organiser of plasma-membrane protrusions. Corbeil *et al.,* (2001) showed by using immunohistological and electron microscopic analyses that prominin-1 is:

(1) Confined to the apical surface of the epithelium all along the epididymal duct, with the exception of the initial segment.
(2) Concentrated in stereocilia of the epididymal duct epithelium.
(3) Found on the tail of developing spermatozoa in seminiferous tubules.
(4) Involved in the formation of epididymal stereocilia and the tail of spermatozoa.

This suggests that Prominin 1 is involved in the formation and/or stabilization of epididymal stereocilia as well as the tails of spermatozoa. However, Prominin 1 cannot be viewed as the underlying force behind the production of plasma membrane protrusions as over expression does not appear to result in an increase in plasmalemma protrusions. (Jaszai *et al.*, 2007).

1.12.3 Cell communication

CD133 may play an important role in specific cell to cell communication and within the BMME. Hayward-Costa showed via transmission electron microscopy (TEM) and immuno-gold analysis that the distribution of CD133 on $CD34^+$ selected umbilical cord blood was distributed along the length of long thin pseudopodia and that $CD133^+$ pseudopodia selectively contact distant cells. This further enhances the hypothesis that specific cell to cell contact is occurring. In addition it also raises the possibility that the cells may be communicating via the receptor, but this hypothesis still warrants further investigation (Hayward-Costa, Kingston University, unpublished work, 2000).

1.13 Adhesion and migration

Various studies indicate that $CD133^+$ cells have greater migratory potentials than $CD133^-$ cells. Moriyarna *et al.,* (2010) showed using pancreatic cancer cells that the cells expressing $CD133^+$ expressed a much more aggressive behaviour, such as increased cell proliferation, migration, and invasion, especially in the presence of pancreatic stromal cells compared to CD133- cells (Moriyarna *et al.,* 2010). Consistent with this theory Elsaba *et al.,* (2010) knocked down the CD133 gene in the HT29 cell line (which has a $CD133^+$ population of >95%) and a time course assay showed that CD133 inhibition produced a reduction in cell motility ($p<0.04$) (Elsaba *et al.,* 2010).

In support of the migration story, Rappa *et al.,* (2008) showed a significant decrease in motility of melanoma cells using a transwell assay where CD133 had been down-regulated. A 78% and 84% decrease in migration was observed for both clones of the cell line used (Rappa *et al.,* 2008) whilst Elsaba *et al.,* (2010) also knocked down CD133 in HT29 cells and demonstrated a reduction in motility (Elsaba *et al.,* 2010). The work of our group (Hayward-Costa, Kingston University, unpublished work, 2000) and Giebel *et al.,* (2004) showed that the stem cell marker CD133 is selectively concentrated in the uropod of polarized HSCs/HPCs and therefore is likely to be involved in migration of haemopoietic stem cells (Corbeil *et al.,* 2004). However Bauer *et al.,* (2008) looked at the polarisation of the receptor from another perspective and deduced that the polarised location of CD133 indicates its possible role in regulating proliferation. CD133 is concentrated in cell surface domains that correspond to the spindle pole region during metaphase. In telophase and cytokinesis, it is either equally or unequally distributed between the 2 nascent daughter cells (Bauer *et al.,* 2008). This contradicts the theory that the CD133 receptor acts as an adhesion molecule in the BMME and its loss leads to motility of the cells. However this effect on migration may be cell specific as the knock down of CD133 in cultured Caco-2 cells did not effect cell proliferation, invasion or migration of the cells (Horst *et al.,* 2009[2]).

Adhesion and migration are very much linked because in order for a cell to move or migrate, the up regulation of adhesion molecules are essential, allowing cells to bind and move within their environment. CD133 may be one of many other receptors that are up regulated contributing to cell adhesion or motility, however from a haemopoietic

perspective CD133 may act as an adhesion molecule preventing movement of cells within the bone marrow through adhesion to the basement membrane or stroma in the bone marrow. This theory was partially demonstrated by Suuronen *et al.,* (2006) who isolated adult human $CD133^+$ and $CD133^-$ cells from peripheral blood mononuclear cells and showed that the $CD133^+$ cells demonstrated improved adhesion to extracellular matrix and endothelial monolayer substrates, compared to freshly isolated $CD133^-$ cells (Suuronen *et al.,* 2006).

As mentioned there are other factors which contribute to adhesion in haemopoiesis. Cell adhesion molecules (CAMS) are expressed on haemopoietic cells and cells of the BMME. These allow for the retention of early cells within the environment whilst assisting at various stages in the homing and mobilization of stem cells. The expression of these cell surface receptors, both CAMS and growth factor receptors enables identification of cells not only within a specific lineage, but also can provide some means of identifying morphologically very similar cells at different stages of differentiation.

CD34 is the best known marker of human stem and progenitor cells within the haemopoietic system. It is a member of the sialomucin family of glycoproteins, which are heavily glycosylated molecules with potential adhesion and signalling capabilities. (Simmons *et al.,* 1997; Sutherland *et al.,* 1988). It is expressed on early haemopoietic stem and progenitor cells, small vessel endothelial cells, embryonic fibroblasts a well as a variety of cell lines (Steen *et al.,* 1998). $CD34^+$ cells account for ~0.8% of cord blood mononuclear cell preparations, 1-3% of bone marrow mononuclear cell preparations and 0.04-0.05% peripheral blood (Pasino *et al.,* 2000; Huang *et al.,* 1998). CD34 has 3 main binding epitopes, and 17 distinct anti-CD34 antibodies have been identified.

Despite the extensive use of CD34 as a primitive haemopoietic cell marker, its function still remains unknown. CD34 knockout mice feature a significantly reduced haemopoietic progenitor cell population. The effect is somewhat corrected by the expression of the full length CD34 molecule or the cytoplasmic domain truncated version (Cheng *et al.,* 1996), suggesting CD34 may be a direct regulator of haemopoiesis and the extra cellular portion of the molecule may play a passive (non signalling) role in this development.

Healy *et al.*, (1995) suggested that functionally, CD34 may play a direct role in cellular adhesion to the bone marrow microenvironment by showing that the ectopic expression of

human CD34 antigen on the surface of murine T cells caused the adhesion of murine T cells to human stroma and not to murine stroma. Anti Human CD34 blocking antibodies decreased the binding of human $CD34^+$ murine T cells to human bone marrow stroma (Healy *et al* 1995). The two proposed functions for CD34; firstly as an adhesion molecule and secondly a specific signalling molecule which may combine with other haemopoietic molecules to exert its function, suggests that it is an intrinsic molecule involved in the development and migration of HSC and HPC.

CD164, another haemopoietic stem cell marker is a heavily glycosylated type 1 membrane sialomucin protein, physiologically expressed as 368 amino acid long (160-180kDa) homodimer (Watt *et al.*, 1998). It has a similar structure to the CD34 with an extracellular bearing multiple O- and N- glycosylation sites. On average 63-82% of BM and 55-93% of UCB $CD34^+$ cells are positive for CD164 (Watt *et al.*, 1998). Studies have shown that CD164 may be a negative regulator of haemopoietic cell adhesion and proliferation (Zannettino *et al.*, 1998). There are various other cell markers that can be expressed such as Thy-1 (Negrin *et al.*, 2000), and CD117 (Ashman *et al.*, 1999), which are all expressed on early haemopoietic stem cells.

1.14 CD133 and cell cycle

Cell proliferation and cell death form a finely tuned equilibrium controlling the organisms homeostasis. The series of events elapsing between two cell divisions form the cell cycle, which is conventionally split into 4 phases: G1 phase (cellular growth, high metabolism and protein synthesis), the S phase (DNA repair and replication), the G2 phase (preparing cellular ultra structure for mitosis) and the M phase (cell division). An additional phase known as the G0 or quiescent phase has been associated with stem cells whereby they cease to proliferate but retain their capacity to re-enter the cell cycle via the G1 phase at a later date (see figure 7) (Harper *et al.*, 2005).

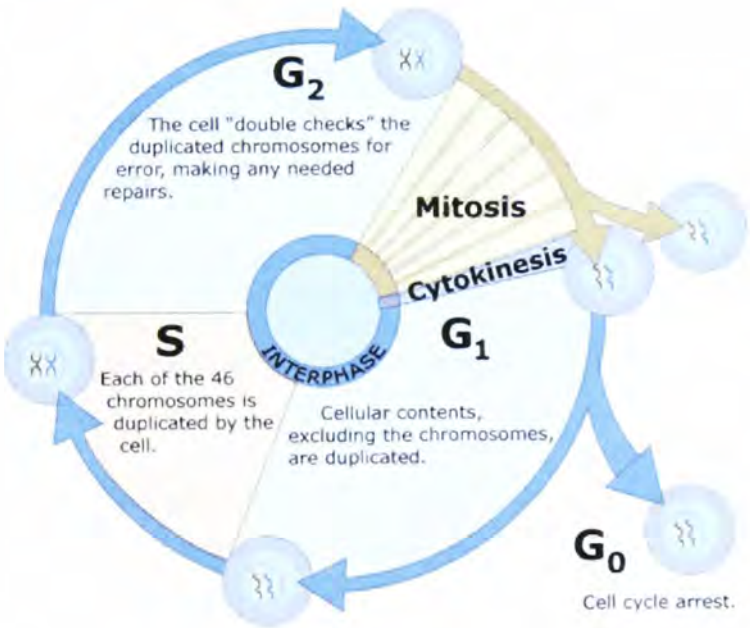

Figure 7: Overview of the cycle: Progression through the cycle is regulated by specific combinations of cyclin dependent protein kinases (Cdk) and cyclin proteins. The cycle is divided into the mitotic phase **(M phase)** during which the cell physically divides. **Interphase** represents the rest of the cycle and this phase is divided into three main stages. **G1 phase** in which the cells begin to commit to replication, **S phase** during which the DNA content doubles and the chromosomes replicate and the **G2 phase** in which cell organel les are copied and cytoplasmic volume is increased. Cells that are resting prior to division enter the G0 phase where they can remain for long period.

The quantitative measurement of the cells' DNA content was one of the earliest applications in flow cytometry. Propidium iodide (PI) is the most common DNA-fluorochrome used for flow cytometric analysis. PI binds stochiometrically to the double helix of nuclear DNA, hence allowing quantification of DNA status (figure 8) (Longobardi Givan., 2001).

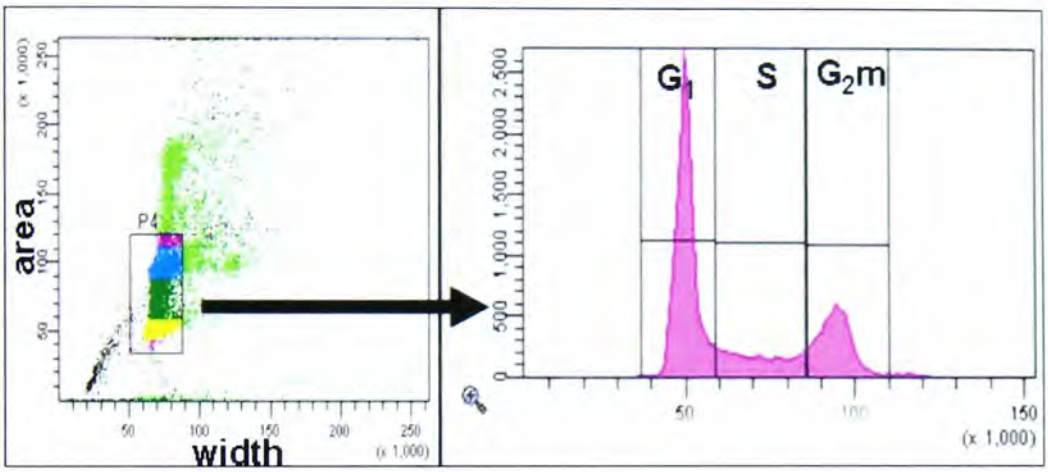

Figure 8: Flow cytometric analysis of cell cycle status. After Propidium Iodide DNA staining, the panel on the left incorporates a gating strategy on a dot plot representing FL2-A against FL-2W which allows discrimination between single cells and aggregates or debris. The second panel shows a DNA histogram representing the various stages of cell cycle.

The quiescent non proliferative phase (G0) of the cell cycle would require the cells to remain in the non proliferating phase of the cycle. According to the literature, most $CD133^+$ cells reside in the G0/G1 phase (Summers *et al.*, 2004). Grskovic, (2004) demonstrated that immediately after umbilical cord blood separation, 96.7^+/-0.5% of $CD133^+$ cells were in G0/G1-phase, while 2.0^+/-0.3% were in the S-phase, respectively (Grskovic *et al.*, 2004). The cell cycle of $CD133^+$ cells and $CD133^-$ cells were compared in U251, a primary glioblastoma multiforme culture cell line. The $CD133^+$ cells contained a higher percentage of G1/G0 phase cells 80.2%, with approximately 4.3% and 15.3% of U251 cells in S and G2 phase, respectively, compared to the $CD133^-$ fraction where 55.3% of the $CD133^+$ cells were in G1/G0 phase and approximately 25.4% and 20.3% cells in the S and G2 phase respectively (Qiang *et al.*, 2009).

Toren *et al.*, (2005) also showed that mutations arising in genes found solely in CD133 positive cells known as "stemness" genes lead to abnormal proliferation and leukaemia emphasizing that cells which express CD133 are inhibited from proliferating (regulated) and clearly loss of the receptor causes loss of regulation leading to uncontrolled proliferation (Toren *et al.*, 2005). It also seems that CD133 can exert its effect long after differentiation has occurred. This was shown by Bao *et al.*, (2010) who were able to take human L929 cells and using a recombinant CD133 plasmid (generated by PCR from a cDNA library of a foetus) transfect the CD133 gene into the human cell line L929. CD133-2/L929 cells caused inhibition of T cell proliferation and down regulation of the activation markers CD4/CD25 and CD8/CD25 on T cells, showing a more downstream inhibitory effect on proliferation and activation (Bao *et al.*, 2010). Furthermore, the expression of genes encoding cell adhesion molecules related to functionally important processes in HSC migration and homing was examined by Jaatinen *et al.*, (2006). Using $CD133^+$ cord blood, global expression analysis was carried out and among the 690 differentially expressed genes, 11 that encode adhesion molecules were up-regulated in $CD133^+$ cells. The overexpression of these genes (*CD34*, *IL-18*, *JUP*, *DST*, *COL5A1*, *TRO*, *DSG2*, *ITGA9*, *SEPP1*, *PKD2*, and *VAV3*) are also associated with cell cycle arrest (Jaatinen *et al.*, 2006).

An increase in adhesion molecules within the bone marrow as a result of CD133 expression may also have an effect on preventing motility of these cells. This process would allow the cell to mature and develop within the bone marrow to its desired level. Once this threshold has been reached, the cell would loose CD133 expression thus allowing motility, differentiation and proliferation, releasing the cell from the bone marrow to migrate to areas requiring regeneration. However this expression of genes encoding cell adhesion molecules may also contribute to migration and motility. Repression of differentiation can also be seen in cancer stem cells as was demonstrated by Takenobu *et al.*, (2011) who showed that CD133 repressed neuroblastoma cell differentiation, for example neurite extension and the expression of differentiation marker proteins, and CD133 expression was decreased by several differentiation stimuli (Takenobu *et al.*, 2011). However Feng *et al.*, (2010) was more cautious with the notion that CD133 represses differentiation. Their studies conclude that CD133 negatively correlates with cell differentiation, it is not a regulator of differentiation (Feng *et al.*, 2010). Marzesco *et al.*, (2005) showed that small CD133 containing vesicles found in human saliva, urine etc were released upon differentiation of the intestine-derived epithelial cell line Caco 2 cells. This

observation highlights the point that the expression of CD133 may sustain a stem cell phenotype and upon its release, differentiation might occur (Marzesco *et al.*, 2005) supported by similar studies showing that CD133 downregulation is observed upon differentiation of Caco-2 (Corbeil *et al.*, 2000).

1.15 CD133 function in malignant cells

In haemopoiesis, CD133 may stimulate and maintain haemopoietic stem cells, however on downregulation of CD133 in the presence of specific growth factors, this leads to cell differentiation and proliferation. The proposed function of CD133 in CSC's seems to differ although they may possess similar characteristics. A lot of the CD133 knock down work involving cancer stem cells or tumour lines have shown that CD133 stimulates proliferation and multi lineage differentiation *in vitro* to recapitulate the original tumour phenotype, consistent with CSC properties (Collins *et al.*, 2005) and loss of the receptor causes inhibition of proliferation. Yao *et al.*, (2009) took U251 human glioma cells and used CD133 antisense oligodeoxynucleotides to knock down CD133 expression and demonstrated that the knock down of these cells inhibited proliferation and decreased colony forming ability, showing that CD133 may play an important functional role in the growth of these tumour cells (Yao *et al.*, 2009).

Barsky *et al.*, (2009) studied MARY-X as well as other breast carcinoma cell lines (HCC70, HTB132, HCC1143) that expressed CD133. They knocked down CD133 using siRNA's and obtained a 10 fold knockdown of mRNA as well as protein in all of the lines studied. They showed that CD133 knockdown significantly suppressed growth in all of the breast carcinoma cell lines (Barsky *et al.*, 2009). Rappa *et al.*, (2008) recently reported that CD133 knock-down in human melanoma cells slowed their growth and motility, decreased the formation of spheroids and the capacity to metastasize. Therefore it seems quite obvious that the role of CD133 in CSC's may be that of a positive regulator of proliferation whereby in HSC, cells expressing CD133 reside within the G0/ G1 resting phase Rappa *et al.*, 2008).

Another important cellular process that seems to be affected by CD133 up-regulation in CSC is apoptosis. Apoptosis, or programmed cell death, plays an important role in many physiologic and pathologic processes and is genetically controlled by complex molecular

signalling systems. During apoptosis cells undergo an orderly, energy-dependent enzymatic breakdown into characteristic molecular fragments, DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage and formation of membrane enclosed apoptotic bodies which will degrade and become phagocytized (Alberts *et al.*, 5th edition, 2007) There are four main apoptotic stimuli groups; the first group includes ionizing radiation and alkylating agents. An important function of apoptosis lies in the elimination of damaged cells. For example, cells with genetic damage caused by exposure to carcinogens may be deleted by undergoing apoptosis, thereby preventing their replication and the accumulation of clones with abnormal cells.

The second group induces apoptosis via receptor mechanisms described later. The third group consists of biochemical agents that stimulate the downstream components of the apoptotic pathway such as phosphates and kinase inhibitors like calphostin C. Finally agents that cause direct cell membrane damage such as UV light and oxidizing agents.

1.16 Mechanism of apoptosis

Following an appropriate stimulus the first stage or "decision phase" of apoptosis is the genetic control point of cell death. This is followed by the second stage or execution phase which is responsible for the morphological changes associated with apoptosis. The main apoptotic processes are known as intrinsic or extrinsic pathways.

1.16.1 Extrinsic pathway

Activated cell surface death receptor induced apoptosis:

This pathway involves a variety of death receptors such as Fas receptor and tumor necrosis factor (TNF) receptor system. The Fas receptor (also known as Apo-1 or CD95) binds the Fas ligand (FasL), a transmembrane protein, part of the TNF family (Wajant *et al.*, 2002). The interaction between Fas and FasL results in the formation of the death-inducing signalling complex (DISC), which contains the Fas (TNFRSF6)-associated via death domain (FADD), caspase-8 and caspase-10. In some types of cells (type I), processed caspase-8 directly activates other members of the caspase family, and triggers the execution of apoptosis of the cell.

TNF is a cytokine produced mainly by activated macrophages. Most cells in the human body have two receptors for TNF: TNF-R1 and TNF-R2. The binding of TNF to TNF-R1 has been shown to initiate the pathway that leads to caspase activation via the intermediate membrane proteins, TNF receptor-associated death domain (TRADD) and FADD (Chan *et al.*, 2002).

1.16.2 Intrinsic Pathway

Mitochondrial proteins known as second mitochondria-derived activator of caspases (SMACs) are released into the cytosol following an increase in permeability. SMAC binds to the inhibitor of apoptosis proteins (IAPs) and deactivates them, preventing the IAPs from arresting the apoptotic process and therefore allowing apoptosis to proceed. IAP also normally suppresses the activity of a group of cysteine proteases called caspases (Fesik *et al.*, 2001) which carry out the degradation of the cell, therefore the actual degradation enzymes can be seen to be indirectly regulated by mitochondrial permeability. Cytochrome c is also released from mitochondria due to formation of a channel, MAC, in the outer mitochondrial membrane, and serves a regulatory function as it precedes morphological change associated with apoptosis (Dejean *et al.*, 2006). Once cytochrome c is released it binds with Apoptotic protease activating factor - 1 (Apaf-1) and Adenosine triphosphate (ATP), which then bind to pro-caspase-9 to create a protein complex known as an apoptosome. The apoptosome cleaves the pro-caspase to its active form of caspase-9, which in turn activates the effector caspase-3 (Kaufmann, 2007).

1.16.3 Decision phase:

Apoptosis is a genetically regulated process and controlled ultimately by two genes; Bcl-2 and P53. Bcl-2 is a family of genes found on mitochondrial membranes and endoplasmic reticulum that regulate apoptosis. It seems that there are a set of similar proteins that have the ability to stimulate or inhibit apoptosis. Proteins such as Bcl-2 and Bcl-xl prevent apoptosis (Yang *et al.*, 1997) whereas proteins such as Bax, Bak, Bad and Bcl_{xs} stimulate apoptosis (Savitz *et al.*, 1998).

The p53 gene is a 53 kDa nuclear phosphoprotein that binds to DNA to act as a transcription factor and controls cell proliferation and DNA repair. The tumour suppressing protein p53 accumulates when DNA is damaged due to a chain of biochemical factors. Part

of this pathway includes alpha-interferon and beta-interferon, which induce transcription of the p53 gene and result in the increase of p53 protein levels and enhancement of cancer cell-apoptosis (Takaoka *et al.*, 2004). p53 prevents the cell from replicating by stopping the cell cycle at G1, or interphase, to give the cell time to repair, however it will induce apoptosis if damage is extensive and repair efforts fail. Any disruption to the regulation of the p53 or interferon genes will result in impaired apoptosis and the possible formation of tumors, explaining why mutations in the p53 gene have been found in 50% of cancers and correlate with resistance to treatment.

Figure 9: Diagrammatic representation of apoptosis. Apoptosis is initiated via 2 main stimuli (1) signalling through cell membrane receptors such as FAS or TGF receptor or (2) release of cytochrome c from mitochondria. Membrane receptors signal apoptosis through an intracellular death domain leading to activation of caspases which digest DNA. Cytochrome c binds to the cytoplasmic protein Apaf-1 leading to the activation of caspases. The intracellular ratio of pro- (e.g. BAX) or anti apoptotic (e.g. BCL-2) members of the BCL-2 family may influence mitochondrial cytochrome c release. (adapted from Hoffbrand and Pettit, fifth edition, 2006).

1.16.4 Execution phase:

This phase involves proteolysis and mitochondrial inactivation. Cellular disruption results from activation of a family of cysteine proteases known as caspases. Almost 10 human caspases (1-10) have been discovered. Since all 10 proteins are cysteine proteases and are specific for cleavage after aspartic acid residues, they are mutually termed as "caspase". They can be divided into three sub groups. Group I includes caspases 1, 4 and 5 involved in processing of pro-inflammatory cytokines. Group II includes human caspases 2, 3 and 7 associated with the cleavage of apoptotic substrates. Group III caspases includes caspases 6, 8 and 9 whose function is to activate group II caspases (Arends *et al.*, 1991). Some intermediate genes like oncogene C-myc transcription for the E_2 F-1 (a positive regulator of myc expression) and R as oncoprotein are involved in the internal regulation of apoptosis.

It is known that the failure of apoptosis is an important factor in the evolution of certain cancers and their poor response to chemotherapy and radiation (Watson *et al.*, 1996). Inhibition of apoptosis causes an imbalance in normal tissue homeostasis promoting cell growth and allowing the survival of genetically damaged cells, both contributing to tumour development and progression (Koornstra at al, 2003). One of the characteristics that make cancer stem cells unique is their ability to resist apoptosis. Several mechanisms have been proposed to govern CSC resistance, including impaired apoptotic machinery such as the pro apoptotic effectors p53 and the p53 pathway, increased DNA damage repair following radio- and chemotherapy. Recent studies have demonstrated that CSCs that have been isolated from malignant gliomas have enhanced DNA repair mechanisms that can resolve the alkylation damage to DNA induced by temozolomide and carmustine which are both used for glioma chemotherapy (Johannessen *et al.*, 2008) and up regulation of multi drug resistance proteins. Angelastro *et al.*, (2010) showed that over expression of CD133 in C6 glioma cells showed a significant reluctance to undergo apoptosis from camptothecin and doxorubicin. Interestingly there was an up-regulation of ABC transporter proteins by 62% which may have contributed to the resistance, suggesting that CD133 may contribute to the observed resistance to apoptosis of $CD133^+$ cancer stem cells (Angelastro *et al.*, 2010). CSCs are also believed to be relatively quiescent residing in the G0 phase so as a consequence, following treatment, the decrease in tumour size might be due to the elimination of the fast-cycling tumour cells (Rich *et al.*, 2007) leaving the CSC behind to self renew and prolong the effects of the cancer.

It is difficult to conclude whether CD133 has a direct effect in apoptosis on CSCs or whether it just happens to be a bystander protein with no association with the apoptotic pathways. However the data tends to suggest that CD133 plays a role in the apoptotic process relating to CSC.

CD133 expression studies have shown loss of the receptor causes an up-regulation in apoptotic genes and programmed cell death as demonstrated by Barsky *et al.*, (2009), who showed that in all breast cancer lines tested, CD133 knockdown induced 100% apoptosis within 5 days (Barsky *et al.*, 2009). Although death receptors (DR) are expressed on normal and tumour cells, TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis preferentially in tumor cells (Ashkenazi *et al.*, 1999). However, many human cancers such as chronic lymphatic leukemia (CLL), astrocytoma, meningioma and medulloblastoma are resistant to TRAIL despite the expression of the death TRAIL receptors on the tumor surfaces (Dyer *et al.*, 2007).

Determination of the apoptotic pathways potentially blocked by CD133 again is unclear. Zobalova *et al.*, (2008) demonstrated that the CD133 population within the Jurkat T-cell acute leukaemia cell line and the breast cancer cell line MCF7 were reported to expressed higher levels of the FLICE-like inhibitory protein (FLIP) (Zobalova *et al.*, 2008). This evidence was supported by studies showing that cancer cells with high expression of CD133 are resistant to TRAIL-induced apoptosis, compared to their CD133-low counterparts. This resistance was related to the high expression of FLIP. Downregulation of FLIP by siRNA in CD133-high cells sensitised them to TRAIL killing. Thus, $CD133^{+}$ high cells may be resistant to TRAIL due to high expression of FLIP (Zobalova[2] *et al* 2008). The effects of FLIP are exerted on TNF which plays a major role in the extrinsic pathway of apoptosis indicating that CD133 exerts its effect as an inhibitor of the extrinsic pathway in CSCs. Moreover, in contrast to $CD133^{-}$ stem cells, $CD133^{+}$ glioma cells are resistant to TRAIL/APO2L due to downregulation of caspase-8 by promoter methylation (Capper *et al.*, 2009).

1.17 Signalling pathway

The molecular mechanism leading cells to self renew, differentiate or proliferate is unknown as well as their regulatory steps. The role of CD133 in normal cells and its role in malignancy means it is important to try and understand the signalling pathways utilised by $CD133^+$ cells. The fact that these mechanisms and regulatory processes will also vary depending on the surrounding niche environments or the tumours expressed makes understanding these processes more difficult. Cells use a large number of clearly defined signalling pathways to regulate their activity. These signalling pathways fall into two main groups depending on how they are activated. Most of them are activated by external stimuli and function to transfer information from the cell surface to internal effector systems. However, some of the signalling systems respond to information generated from within the cell, usually in the form of metabolic messengers.

For all of these signalling pathways, information is conveyed either through protein–protein interactions or it is transmitted via secondary messengers.

The signalling pathways involved in the typical stem cell processes mentioned are Notch, wingless type MMTV integration site family (Wnt), Bone morphogenetic protein and Hedgehog intracellular pathway. The Wnt signalling pathway functions primarily to maintain a balance between cell proliferation and differentiation, and dysregulation of this pathway underlies tumourigenesis in several tissues (Taipale *et al.*, 2001).

1.17.1 Wnt Pathway

The conical Wnt pathway describes a series of events that occur when Wnt proteins bind to cell surface receptors of the Frizzled family, causing the receptors to activate dishevelled family proteins, ultimately resulting in a change in the amount of β-catenin that reaches the nucleus. Dishevelled (DSH) is a key component of the membrane-associated Wnt receptor complex which when activated by Wnt binding inhibits a second complex of proteins that include axin, glycogen-synthase kinase 3β (GSK-3) and the protein adenomatous polyposis coli (APC^2). The axin/GSK-3/APC^2 complex normally promotes the proteolytic degradation of the β -catenin intracellular signalling molecule. This step is enhanced by the

phosphorylation of the β -catenin by the GSK-3 protein and also by casein kinase (CK1A). After this "β-catenin destruction complex" is inhibited, the concentration of β –catenin builds up in the cytoplasm and thus is able to also accumulate in the nucleus and activate the Wnt transcriptional regulation genes such as cyclin D1 and c-Myc (Sparks *et al.*, 1998).

A study by Nikolova *et al.*, (2007) assessed the changes in CD133 cells following culture in medium containing various Wnt signalling molecules. Wnt3a preserved an undifferentiated phenotype in CD133 cells by increasing the ratio of blast like cells to macrophages and increased the expression of the neural progenitor nestin. Wnt5a, Wnt11 andWnt4 induced expression of the endothelial marker CD31 and von Willebrand factor. Moreover Wnt5a led to increase mRNA β-catenin levels and elevated levels in the cytoplasm and nucleus consistent with the activation of the Wnt pathway (Nikolova *et al.*, 2007).

This was supported by Reya *et al.*, (2003) who showed β catenin as well as purified Wnt3a protein can promote self renewal of murine HSCs *in vitro* and enhance their ability to reconstitute the haemopoietic system of lethally irradiated mice *in vivo* (Reya *et al.*, 2003)
It was also shown that Wnt 5a treatment of human haemopoietic progenitor cells expressing CD133 in the presence of stromal cell contact promotes the expansion of undifferentiated progenitors *in vitro* (Van Den Berg *et al.*, 1998). Further evidence suggests that there is an interaction between CD133 and Wnt pathway. Whilst knocking down the CD133 receptor on FEMX cells, it was found that 10 of the 76 genes up-regulated on knockdown were established or putative inhibitors of the canonical Wnt pathway (Rappa *et al.*, 2008). Regulation of the differentiation and proliferation of $CD133^+$ cells by Wnt signalling fits with the role of CD133 as a CSC marker, as dysregulated Wnt signalling due to loss of function mutations in its downstream components such as APC^2 or β-catenin underlies the pathogenesis of several cancers (Fodde *et al.*, 2007). Studies showed that higher levels of the Wnt transcriptional gene C-myc were expressed in CD133 glioma stem cells compared with their normal counterparts (Wang *et al.*, 2008).

1.17.2 Notch pathway

The Notch signalling pathway is an evolutionarily conserved, intercellular signalling mechanism essential for proper embryonic development in all metazoan organisms. The Notch protein is a single-pass transmembrane receptor protein. It is a hetero-oligomer composed of a large extracellular portion, which associates in a calcium-dependent, non-covalent interaction with a smaller piece of the Notch protein composed of a short extracellular region, a single transmembrane-pass, and a small intracellular region. In vertebrates 2 notch proteins exist Notch1-Notch4 that are activated by the Delta (or Delta-like) and Jagged/Serrate families of membrane-bound ligands (Lindsell *et al.*, 1996). They are transported to the plasma membrane as cleaved, but otherwise intact polypeptides. Interaction with ligand leads to two additional proteolytic cleavages that liberate the Notch intracellular domain (NICD) from the plasma membrane. The NICD translocates to the nucleus, where it forms a complex with the DNA binding protein CSL and the transcriptional regulator CMP-binding factor 1, displacing a histone deacetylase (HDAc)-co-repressor (CoR) complex from CSL. Components of an activation complex, such as MAML1 and histone acetyltransferases (HATs), are recruited to the NICD-CSL complex, leading to the transcriptional activation of Notch target genes (Hansson *et al.*, 2010). The final effect of this cascade of events is the promotion of survival and growth of normal stem cells.

Notch pathway inhibition was investigated using a glioblastoma neurosphere cell line. Gamma- secretase inhibitors (GSI) appeared to down-regulate CD133, reduce neurosphere growth and clonogenicity and depleting stem-like cancer cells through reduced proliferation and increased apoptosis, whereas transfection of a gene up-regulating Notch 2 in the glioblastoma neurospheres increased their growth *in vitro*. (Fan *et al.*, 2010). This could be of therapeutic relevance, improving the survival of patients with glioblasoma and other malignant tumours.

1.17.3 Hedgehog pathway

Sonic hedgehog (SHH) is the best studied ligand of the vertebrate hedgehog pathway. When SHH reaches its target cell, it binds to the Patched-1 (PTCH1) receptor. In the absence of ligand, PTCH1 inhibits Smoothened (SMO), a downstream protein in the pathway. It has been suggested that SMO is regulated by a small molecule, the cellular localisation of which is controlled by PTCH (Taipale *et al.*, 2002).

A current theory of how PTCH regulates SMO is by removing oxysterols from SMO. PTCH acts like a sterol pump and removes oxysterols that have been created by 7-dehydrocholesterol reductase. Upon binding of a Hedgehog protein or a mutation in the sterol sensing domain (SSD) (which has shown to be essential for the suppression of SMO) of PTCH, the pump is turned off allowing oxysterols to accumulate around SMO. This accumulation of sterols allows SMO to become active or stay on the membrane for a longer period of time. The binding of SHH relieves SMO inhibition, leading to activation of the GLI transcription factors: the activators Gli1, Gli2 and the repressor Gli3. Activated GLI accumulates in the nucleus and controls the transcription of hedgehog target genes (Rahnama *et al.*, 2006) which have been shown to promote the proliferation of adult stem cells from various tissues, including primitive hematopoietic cells (Bhardwaj *et al.*, 2001) and mammary cells (Liu *et al.*, 2006). Neural glioma stem cells have also shown evidence that the SHH-GLI pathway is required to sustain the survival of $CD133^+$ cells in which it regulates the expression of stem cell regulated genes (Wong *et al.*, 2008).

Similar to Wnt and Notch, the hedgehog pathway has also been implicated in the development of cancer in various organs including brain, lung, mammary glands, prostate and skin. Basal cell carcinoma, the most common form of cancerous malignancy, has the closest association with hedgehog signalling. Loss-of-function mutations in Patched and activating mutations in Smoothened have been identified in patients with this disease (Xie *et al.*, 1998). Abnormal activation of the pathway probably leads to development of disease through possible transformation of adult stem cells into cancer stem cells that give rise to the tumour.

Figure 10: Molecular pathways regulating survival and growth of CD133+ stem cell like cells
APC: Adenomatous polyposis coli; BAD: B cell leukaemia/ lymphoma-associated factor 2-antagonist of cell death; CoA: Coenzyme A; CSL: CMP-binding factor 1, suppressor of hairless, and lymphocyte activation gene-1; DIFs: Differentiation-inducing factors; Dsh: Dishevelled; ERK: Extracellular signal-regulated kinase; FOXO: Forkhead box O; GLI: Glioma-associated oncogene homolog; GSK: Glycogen-synthase kinase; HES: Hairy and enhancer of split; HhAntag; Hedgehog antagonists; ICN: Intracellular Notch domain; LEF: Lymphoid enhancer-binding factor; MAML: Mastermind-like; mTOR: mammalian target of rapamycin; PDK: Orange boxes: Pharmacological inhibitors of specific molecular pathways; Phosphatidyl inositol-dependent kinase; PLC: Phopholipase C; PTEN: Phosphatase and tensin homolog; SHH: Sonic hedgehog; SUFU: Suppresor of fused; TCF: T cell factor; WNT: Wingless-type MMTV integration site family (Ferrandina *et al.*, 2009).

In summary, CD133 is a novel pentaspan membrane protein highly conserved across species. The receptor is found on haemopoietic stem cells and has been classified as a marker of primitive haemopoietic and neural stem cells. The highly restricted expression of CD133 family molecules on protrusion such as microvilli and pseudopodia of epithelial and other cell types, and its close association with cholesterol and lipid rafts suggests a possible role in plasma membrane organisation, cell communication and even motility.
CD133 represents a marker of tumour initiating cells in a number of human cancers relating to a variety of tissues. To further help with the understanding the functional properties of the receptor, studies have investigated the molecular mechanisms and signalling pathways that regulate the behaviour of CD133 expressing cells. Data has revealed that Wnt, Notch and BMP may play a role in $CD133^{+}$ cancer stem cell regulation and potentially within haemopoiesis.

In this thesis, we will investigate some of the hypothesis put forward relating to CD133 function by knocking down the receptor and conducting molecular and cellular experiments. Based on the literature various processes have been associated with CD133 function however for the purpose of our study we have limited the processes we will address to apoptosis, cell cycle, proliferation and cell adhesion. We will also examine any functional similarities between CD133's function within haemopoiesis and cancer stem cell biology.

Chapter 2: Methods and Materials

This chapter describes the biological methods and materials used throughout this thesis.

2.1 Cell line cultures

Cell lines that readily express the CD133 receptor are the tetratocarcinoma cell line NT-2 (Yin *et al.*, 1997), retina and retinoblastoma cell lines (Yin *et al.*, 1997) and the embryonic stem (ES) cell lines H1, H7 and H9 (Carpenter *et al.*, 2003). For the purpose of this experiment a baseline for cell proliferation and doubling times were established for MUTZ-2 and CACO-2 cell lines. We chose these specific cell lines because they are representatives of a haemopoietic and epithelial cell line respectively.

2.1.1 Preparation of 5637 feeder cell line

5637 cell line was purchased from the cell bank of DSMZ (DSMZ, Braunschweig, Germany). 5637 is derived from a human bladder carcinoma cell line of a 68 year old male patient. The cell line conditioned medium (5637 CM) stimulates proliferation of human growth factor-dependent leukemia cell lines such as MUTZ-2 (DSMZ) in a dose-dependent fashion. High levels of G-CSF and GM-CSF and lower levels of IL-1beta, M-CSF and SCF have been detected in 5637 CM through quantitative ELISA. 5637 cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. The basal growth media was RPMI 1640 (Gibco BRL life technologies LTD, Paisley, Scotland), supplemented with 10% heat inactivated Foetal Bovine Serum (FBS, Sigma- Aldrich Company Limited, Poole, Dorset, UK), 0.5% gentamycin (Gibco BRL life technologies LTD, Paisley, Scotland).

After 2 days of culture the supernatant was collected, centrifuged and stored at -20°C. Cells were re-cultured after trypsin detachment followed by washing with RPMI/FBS/Gentamycin solution at 1300RPM (Jouan, BR4i) for 10 minutes (RTP), counted using trypan blue (Gibco BRL life technologies LTD, Paisley, Scotland) exclusion and seeded at $1x10^6$cells/ml.

2.1.2 Preparation of MUTZ-2 cell line

The MUTZ-2 cell line was established from the peripheral blood of a 62-year-old man with acute myeloid leukaemia (AML) of the Fab classification M2 whose diagnosis was made in 1993 (Hu *et al.*, 1996) it was the first $CD133^+$ cell line and the only known cell line of haemopoietic origin (Kratz *et al.*, 1998). MUTZ-2 were purchased from DSMZ and grown at 37°C in a humidified atmosphere containing 5% CO_2. The basal growth media was alpha MEM, (Gibco BRL life technologies LTD, Paisley, Scotland) and was supplemented with 20% heat inactivated FBS, 0.5% gentamycin and 20% conditioning medium from the 5637 cell line. The cells were seeded at $5x10^5$ cells/ml in 24 well plates, (Nunclon, Denmark) and incubated for 48hrs, at which point the cells were washed at 1300RPM for 10 minutes at room temperature and counted using trypan blue (Gibco BRL life technologies LTD, Paisley, Scotland) exclusion and then re-seeded at $5x10^5$ cells/ml under the same conditions.

In order to establish a consistent and efficient way of culturing the MUTZ cells prior to functional analysis, a series of assays were conducted using defined growth factors and cytokines. Meyer *et al.*, (1999) reported that in MUTZ-2 cells, FLT-3 was able to extend survival by 64-135% compared to control cells. SCF also prolonged survival of MUTZ-2, and in combination with FLT-3 was active in promoting survival and proliferation of human AML cells (Meyer *et al.*, 1999).

We varied the concentration of 5637 Supernatant, SCF (R^+D systems, Germany) and FLT-3 (Sigma- Aldrich Company Limited, Poole, Dorset, UK). All cultures were carried out in duplicate. The total volume in the wells was made up to 1ml. The cells were then incubated for 48 hours, counted using trypan blue exclusion, topped up to 2ml using the supplemented Alpha MEM medium and/or cytokines then incubated for a further 48 hours. This process was repeated for 12 days in total. (Note supernatant was removed and replaced with fresh medium and cytokines every 48 hours maintaining the seeding density at approx $5x10^5$ cells /ml).

2.1.3 Preparation of Caco -2 cell lines

Caco 2 cells were also obtained from the cell bank of DMSZ. The Caco-2 cell line is a continuous line of heterogeneous human epithelial colorectal adenocarcinoma cells that grows as a confluent monolayer. This line was chosen as it is very manageable compared to the MUTZ-2 cell line, requiring a relatively simple culture and maintenance system and very high expression of CD133. The cells were seeded at 2 $x10^4$ cell/ml and maintained in RPMI supplemented with 10% FBS and 0.5% gentamicin. Cells were passaged every 2-3 days when confluent and the cells were removed from the monolayer by treatment with trypsin and EDTA (0.7mM).

2.2 Isolation and validation of HPSC population

2.2.1 Collection of Cord Blood

Ethical approval for the study was obtained and following informed consent, cord blood was collected following elective caesarean sections on full term neonates. CB samples, 5-90ml were collected within approximately 15 minutes of child birth and placed in a 50ml tube containing Acid citrate dextrose formula-A (ACDA, Baxter, Maurepas, France) 6%, 5mls per 35ml of cord blood. This was then diluted a 1:4 with PBS in preparation for density separation of mononuclear cells (MNC).

2.2.2 Isolation of Mononuclear cells

Cord Blood mononuclear cells were separated by gently layering the blood on a cushion of Ficoll- Plaque (d:1.077g/cm^3 Pharmacia Biotech, Sweden). The MNC were then centrifuged at 1300RPM for 30 minutes at room temperature. Once complete the mononuclear cell layer was removed and the cells washed twice in 10ml phosphate buffer solution (PBS, Sigma- Aldrich Company Limited, Poole, Dorset UK) (1300RPM, 10 minutes), supplemented with bovine serum albumin (0.5% fraction V, Sigma-Aldrich Company LTD, Poole, Dorset, UK) and ACDA, 0.6% . The solution was known as ACDA buffer. The MNC's were then resuspended in 2ml ACDA buffer at 4°C. Cell viability was

determined using haemocytometer and Trypan Blue exclusion. Cell number was determined using 3% Acetic acid (Sigma- Aldrich Company Limited, Poole, Dorset, UK).

2.2.3 Cell Counting and viability assessment

Cell number and viability was microscopically assessed using a haemocytometer. This uses a specialized glass with a grid of a known area 1.0mm^2 and depth of 0.1mm encompassing a defined volume of 1x10^4 ml. A 10µl cell suspension would be diluted to give a final dilution factor of 100 in 3% acetic acid, thus cell number = Mean cell number x 10^4 x dilution Factor x Volume of cell suspension. Cell viability for all cells was determined by using trypan blue, 0.25% in a 1:10 dilution.

2.2.4 Positive selection

Due to the low number of cells expressing CD34 and CD133, it was very important after MNC isolation to undertake positive immunomagnetic selection to ensure a more homologous population thus enriching for a population of cells expressing higher levels of the particular antigen. The CD34 antigen is expressed on approx 0.25% cord blood mononuclear Cells (CB MNC) and approximately 0.3-2.4% of CB MNC co-express the CD34 and CD133 antigens (de Wynter *et al.*, 1998).

2.2.5 CD34$^+$ Positive Selection

CD34$^+$ cells were isolated using a MACS Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotech, Bergish, Germany). Cells were incubated for 30 minutes (agitated every 10 minutes) at 4°C with 100µl Fc receptor (FcR) blocking reagent per 10^8 cells to prevent non specific or FcR mediated binding of CD34 beads. Cells were then labelled with 100µl CD34 microbeads per 10^8 total cells for 30 minutes at 4°C agitating every 10 minutes. Cells were washed with ACDA buffer (10ml) and centrifuged at 1300RPM for 10 minutes at 4°C. The supernatant was discarded and the cells kept on ice.

2.2.6 Selection column processing for positive cell isolation

This was carried out in accordance with manufacturer's instruction. In brief a medium MACS separation column previously kept at 4°C was placed into the Midi Macs magnet (Miltenyi Biotech. Bergish, Germany). 500ml of ACDA buffer was added to the column initially to allow the removal of surplus bead coating and collected as waste, this was repeated 3 times. The MNC were resuspended in 500ml ACDA buffer solution and added to the column and washed through with 500ml ACDA buffer four times. This was collected as negative fraction 1.1ml ACDA buffer was then added to the column directly, the magnet removed from the column and the buffer solution was plunged through the column and collected as positive fraction 1, the plunger was removed and another 1ml of buffer was added to the column and plunged thorough in a similar manner into the same positive fraction 1. The process was repeated using a fresh column kept at 4°C and positive fraction 1 cells used as the starting cells. This was done to enhance the purity of the sample. Cells were then washed once in ACDA buffer, centrifuged at 1300RPM for 10 minutes. The cell number and viability were assessed using trypan blue.

2.2.7 $CD34^+$ cell purity

To determine the purity of CD34 selected cells, phenotypic expression of CD34 was determined by direct immunofluorescent monoclonal antibody labelling and flow cytometric analysis. Cord blood $CD34^+$ immunomagnetically selected cells, $5x10^4$ were incubated with Human Gamma Globulin (HAG, 2% in PBS, Sigma-Aldrich Company Limited, Poole, Dorset, UK) for 30 minutes. The cells were then washed at 1300RPM for 10 minutes at 4°C and incubated with antibodies at a concentration of 5 µg/ml

Mouse anti-human CD34 (IgG,) Phycoerythrin (PE) BD- Pharmigen UK LTD, Cowley, Oxford, UK.
Mouse IgG1 PE BD- Pharmigen UK LTD, Cowley, Oxford, UK.

Cells were incubated for 30 minutes, washed with PBS (BSA/ACDA/NaAz) before centrifugation at 1300RPM, for 10 minutes at 4°C. The pellet was resuspended and fixed

in 200µl of 4% Paraformaldehyde (PFA) and analyzed on a Becton Dickinson FACScan flow cytometer.

2.3 Determination of steric hindrance

The definition of steric hindrance is the prevention or retardation of a chemical reaction, caused by the arrangement of atoms in a molecule. In this case it describes the process that occurs when a ligand specific to a particular epitope binds to the receptor. Due to the unknown proximity of the CD133 receptors within the cell membrane and the proximity of the Mab binding to the CD133 receptor it was important to establish whether binding of Mab CD133 to either epitope 1 or 2 would cause a conformational change altering the binding sites on the receptor or produce a physical blockage that could possibly prevent successful binding to the other epitope resulting in the inhibition of Mab specific to different epitopes on the receptor. As future experiments involved the binding/stimulation of the various epitopes on the CD133 receptor simultaneously it was important to determine weather steric hindrance could be a potential problem. This was investigated using direct immunofluorescent monoclonal antibody labelling. Fresh $CD34^+$ selected cells were labelled as follows giving a final antibody concentration of 5µg/ml.

Mouse anti-human CD133/1 PE; Miltenyi Biotech. Bergish, Germany.
Mouse anti-human CD133/2 APC; Miltenyi Biotech. Bergish, Germany.
Mouse IgG1 PE; BD- Pharmigen UK LTD, Cowley, Oxford, UK.
Mouse IgG1 APC; BD- Pharmigen UK LTD, Cowley, Oxford, UK.

Tube 1 cells were labelled with 5µl mouse anti human CD133/1 PE, incubated for 30 minutes at 4°C, washed, then incubated again with mouse anti human 5µl CD133/2 APC for another 30 minutes, washed and resuspended in PFA.

Tube 2 cells were labelled with mouse anti human 5µl CD133/2 APC incubated for 30 minutes at 4°C, washed, then incubated again with mouse anti human 5µl CD133/1 PE for another 30 minutes, washed and resuspended in PFA.

Tube 3 cells were labelled with matched isotype control; 5µl mouse IgG1 PE and 5µl mouse IgG1 APC. Analysis was carried out on a BD FACScan flow cytometer.

2.4 Effect on freezing $CD34^+$ cells

Cord blood was collected as described above and stored at -70°C. The cells were stored for periods up to 6 months so it was vital to establish weather freezing the cells for such a prolonged period would effect CD133 expression and the general health of the cells. This was done by comparing the phenotype of frozen $CD34^+$ selected cells for 3 months to that of fresh $CD34^+$ selected cells.

The analysis was carried out using direct immunofluorescent monoclonal antibody labelling. Labelling was carried out as described above for mouse anti human CD133/1 PE and CD133/2 APC for both frozen and fresh cells at a concentration of 5µg/ml.

Tube 1 cells were labelled with 5µl mouse anti human CD133/1 PE, incubated for 30 minutes at 4°C, washed, resuspended in PFA.

Tube 2 cells were labelled with 5µl mouse anti human CD133/2 APC incubated for 30 minutes at 4°C, washed, and then resuspended in PFA.

Tube 3 matched isotype control cells were labelled with matched isotype control; 5µl mouse IgG1 PE or 5µl mouse IgG1 APC.

This was carried out on both fresh and frozen $CD34^+$ cells and analysed on a BD FACScan flow cytometer.

2.5 Immunolabelling for flow cytometric analysis of Primary Cells ($CD34^+$), MUTZ-2, and Caco 2 cell surface markers

$1x10^5$ cells were incubated with 45µl 2% HAG for 30 minutes to block Fc receptors. Then direct immunofluorescent labelling was carried out with cells incubated at 4°C for 30 minutes, using the following antibodies at similar concentrations and amounts as stated before.

Mouse anti-human CD34 (IgG,) Phycoerythrin (PE) BD- Pharmigen, Oxford, UK.
Mouse anti-human CD133 (AC133/2, IgG1) FITC BD- Pharmigen, Oxford, UK Mouse anti-human CD13 (IgG1) (PE) BD- Pharmigen, Oxford, UK.
Mouse anti-human CD117 (IgG1) (PE) BD- Pharmigen, Oxford, UK.
Mouse anti-human CD33 (IgG1) (PE) BD- Pharmigen, Oxford, UK.

The matched isotopes were as follows:

Mouse IgG1 PE BD- Pharmigen UK LTD, Cowley, Oxford, UK.
Mouse IgG1 FITC BD- Pharmigen UK LTD, Cowley, Oxford, UK.

The cells were then washed with 3ml PBS supplemented with BSA (0.5% fraction V), ACDA, 0.6% and Sodium Azide 0.1% (Sigma-Aldrich Company Limited, Poole, Dorset, UK), and centrifuged at 1300RPM, for 10 minutes at 4°C. The cells were resuspended in approx 200µl of PFA (1%. PH7.2). Cells were then analyzed on a Becton Dickinson flow cytometer. A minimum of 10,000 events were acquired for each stain.

2.6 Flow Cytometry

Flow cytometry is the measurement of cells based on cell size, granularity, phenotype or other physical characteristics such as DNA and the excitation and emission of fluorescent light. A laser light source of single wavelength is directed onto a hydrodynamically focused stream of cells and fluid within a surrounding sheath fluid resulting in laminar flow (Rodak 1995). A number of detectors are aimed at the point where the stream passes through the light beam, one in line with the beam used to measure forward scatter, several perpendicular to those measuring side scatter. There are also a number of detectors which measure fluorescence. The laser light beam is scattered by the interference of each particle or cells passing through the beam with excitation of fluorescent conjugated antibodies and emission of light at a longer wavelength. The level of fluorescence can be correlated to the degree of expression of the specific receptor antibody labelled.

Typically, flow-cytometry can be used to measure or identify five to seven parameters such as different cell surface markers defining different cell types or the functional status of cells. The large amount of data generated can then be processed by a computer with programmed software. Flow-cytometry can be contrasted to conventional microscopy. In microscopy, the eye makes qualitative contrasts to conventional microscopy, making qualitative estimates of a large number of parameters on a few cells and record detail within each cell. In contrast, the flow-cytometer quantifies an average parameter for each cell but measures thousands of cells without selective bias. Flow-cytometry makes measurement on each cell individually, so that if there is a small subset of cells with a particular feature within a large group this will be recorded and analysed. Such subsets might not be detected by conventional microscopy (Darzynkiewicz at al 1994).

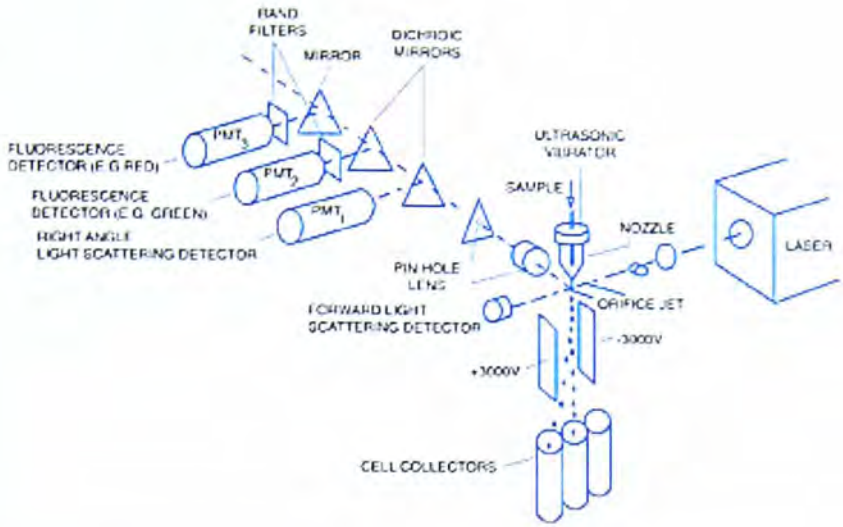

Figure 11: Diagram to show basic principles of flow cytometry
A Flow-cytometer consists of a light source, a flow cell, a lens system to focus the light into the flow cell, optical components to focus light of different emission wavelengths onto the detectors, an electronic component to amplify and process the resulting signals and a computer.

2.6.1 The Flow Cytometer

The flow cell is purposely designed to deliver the cells singly to a specific point at which the source of light is focused. This is achieved by injection of the sample into the centre of a stream of liquid called the sheath fluid. The cell is designed so that the sheath fluid hydrodnamically focuses the sample stream delivering the cells to the point of detection.
A standard flow-cytometer machine is usually fitted with an argon-ion laser which has blue fluorescence light with a wavelength of 488nm. The roles of the optical lenses are to focus the beam onto the sample stream. A simple lens is used to provide a beam with a 50µm cross-section. Secondly, to collect light reflected from the sample stream. These collection lenses will then deliver the collected light to the detectors (figure 11). The forward and side scattered light is detected by a sold state detector. For measuring fluorescence and orthogonal scatter, photomultipliers (PMTs) are used.

The process of collecting data from samples using the flow cytometer is termed "Acquisition". This process is mediated by a computer physically connected to the flow cytometer. The software is capable of adjusting parameters (i.e. voltage, compensation etc)

for the sample being tested. The data can be analysed through a series of dot plots or histograms in a logarithmic fashion.

2.6.2 Gating of flow cytometry

Modern flow cytometers are capable of analysing at least five parameters and to make full use of the information collected, "gating" is employed. Data from one or two parameters are displayed and regions of interest are defined to select certain populations of cells for display of further parameters. These regions are then called "gates". The cells of interest are often initially selected by gating on a cytogram of side versus forward scatter as shown in figure 12. In a study of a particular group of cells, the gates can exclude clumps, debris and possible dead cells. In multi-cellular systems, a particular type of cell may be delineated (Nirmala *et al* 2002).

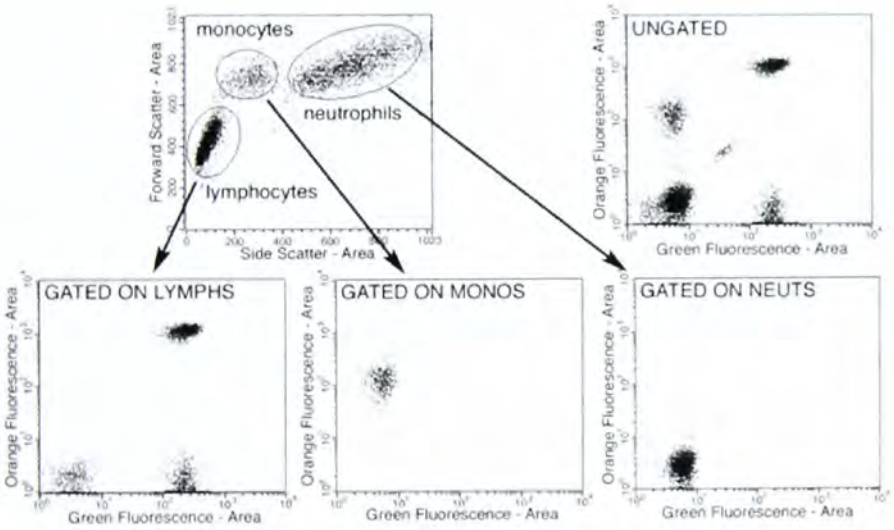

Figure 12: Diagrammatic representation of gating a population of peripheral blood mononuclear cells. Here one can select different populations of interest such as monocytes or neutrophils and exclude or include gated populations thus allowing further analysis on cell populations of interest.

2.6.3 Fluorescence and compensation

Fluorescence is the phenomenon in which absorption of light of a given wavelength by a fluorescent molecule is followed by the emission of light at a longer wavelength. The fluorescence excitation spectrum is the distribution of wavelength-dependent intensity of emitted energy that results in fluorescence. The main fluorochromes used within this work are described in table 3.

Table 3: summarises the maximum emission wavelength of the fluorochromes employed for general flow cytometry when stimulated by an argon-ion laser.

Fluorochrome	Maximal emission	Channel	Application
Fluorescein Isothiocynate (FITC)	525	FL1	Phenotypic
Alexa Fluor 488	519	FL1	Phenotypic
Phycoerythrin (PE)	576	FL2	Phenotypic
Alexa fluor 610	628	FL3	Phenotypic

When performing multi colour flow cytometric analysis, the emission spectra of the fluorochromes can overlap, leading to emission of a given fluorochrome into an "inappropriate detector" as shown below in figure 13. An FL1 (fluorescence 1) signal can spill and overlap into an FL2 (fluorescence 2) channel falsely enhancing the signal detected in FL2. To avoid data misinterpretation from false positive subsets, compensation was thus performed regularly. Compensation is the process by which the fluorescence "spillover" originating from a fluorochrome other than the one specified for a particular PMT detector is subtracted as a percentage of the signal from other PMT's. For example, spectral overlap between FITC and PE produces light which is detected by both the FL1 and FL2 detectors. The amount of FITC fluorescence being detected by the FL2 detector (i.e. PE detector) can be regarded as excess fluorescence and should therefore be compensated out as shown in figure 13.

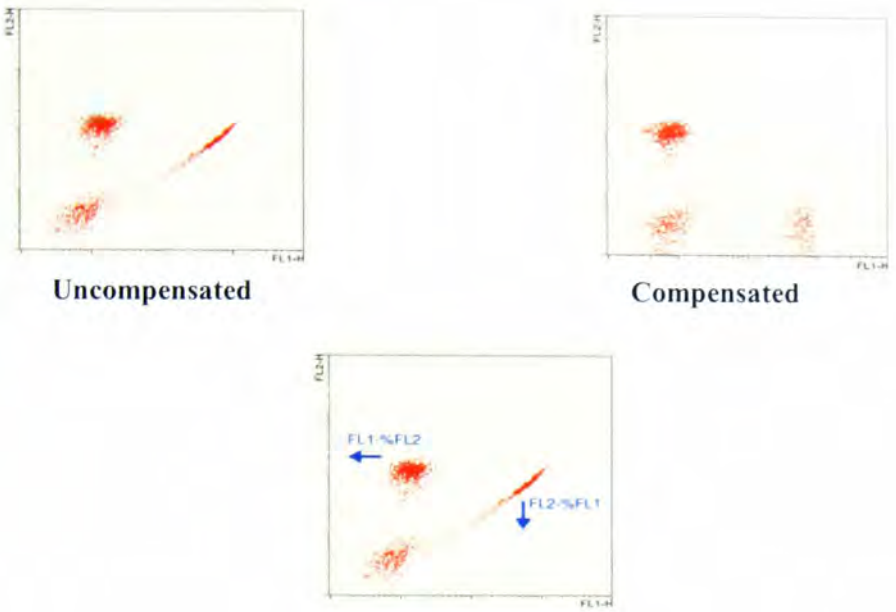

Figure 13: Diagrammatic representation to show the principles of compensation. In the left hand panel, uncompensated data are shown for a mixture of unstained, FITC-anti-CD8 positive and PE-anti-CD4 positive cells. Notice that the CD8-bright cells which should ideally only show a signal in the FL1 detector also show a large signal in the FL2 detector. Conversely the CD4-bright cells should show a signal in the FL2 detector also show a small signal in the FL1 detector. Compensated data are shown in the right hand panel. The compensation procedure involved setting FL2-%FL1 to approximately 19% and FL1-%FL2 to approximately 0.6%, and is illustrated in the lower

2.7 Methodology for knockdown experiments

2.7.1 Fast Forward transfection of Adherent cells with siRNA

Caco2 cells were seeded at $1.0x10^5$ cells per well of a 24 well plate in 0.5ml RPMI supplemented with 10% FBS and gentamicin. The cells were incubated under normal growth conditions of 37°C and 5% CO_2 overnight prior to transfection. The siRNA (Qiagen LTD, Crawley, West Sussex, UK) was diluted in varying amounts of culture medium without serum until the required concentration was obtained. For example 37.5ng siRNA in 100µl culture medium would give a siRNA concentration of 5nM. At this stage, 3µl of the Transfection Reagent (Qiagen LTD, Crawley, West Sussex, UK) was added to the siRNA and mixed by vortexing.

The samples were left for 10 minutes to allow the formation of the transfection complexes and then added drop wise onto the cells. 400µl RPMI supplemented with 10% FBS and gentamicin was then added to the cells containing the transfection complex and incubated for 48 hours. Cells were then trypsinised and washed with PBS and the RNA extracted. Real time and RT PCR was carried out as well as flow cytometry using conjugated anti CD133 Mab.

2.7.2 Isolation and quantification of RNA

Extraction of RNA from Caco2 cell lines was performed using RNeasy kit (Qiagen LTD, Crawley, West Sussex, UK. This was carried out after the cells were trypsinised to remove the adherent layer, washed in medium containing FBS to stop the reaction and then finally washed in PBS. A buffer containing guanidinium thiocyanate was added to lyse the cells prior to passing them through a shredder to obtain a homogenous lysate. Seventy percent ethanol (VWR international, Briare, France) was then added to the tube and mixed well. The sample including any precipitate formed was transferred into an RNeasy spin column. The lysates were mixed and transferred onto a spin column. The column was washed and DNA digestion carried out using the RNase-free DNase set. The column was then repeatedly washed using 2 different buffers to remove contaminants. The RNA was eluted from the column using RNase-free water in 50µl and immediately reverse transcribed, if reverse transcription could not be undertaken immediately the RNA was stored at -80°C.

The DNA was quantified using a Nanodrop 2000c (Thermo Scientific). 1µl of sample was required to provide the required concentration in ng/µl. Qualitative information was supplied via the wavelength ratios and the graphs produced.

2.7.3 Reverse Transcription (RT) for cDNA synthesis and Polymerase chain Reaction

First strand synthesis was performed using a superscript II RT kit (Invitrogen LTD, Paisley, Scotland) on approx 2-3µg of total RNA using 1µl oligo (dT) primer (500µg/ml, Invitrogen LTD, Paisley, Scotland) and 1µl of dNTP mix (10mM, Invitrogen LTD, Paisley, Scotland) made up to 12µl with water and heated to 65°C for 5 minutes followed by rapidly chilling the reaction on ice. Subsequently 4µl of first strand buffer, 2µl of 0.1M dithiothreitol (DTT) and 0.5µl RNAse out (40 units/µl) was added and further incubated for 2 minutes at 42°C. A volume of 1µl SuperScript II was added to the mix and the resultant reaction was incubated for a further 50 minutes at 42°C. The reaction was inactivated at 70°C for 15 minutes and the resultant cDNA was cooled on ice.

Amplification by PCR was performed using a PCR kit (Invitrogen LTD, Paisley, Scotland). The reaction mix (50µl) typically consisted of 0.5µl Taq DNA polymerase, 5µl 10X PCR Buffer, 1.5µl of 50mM MgCl, 1µl of 10mM DNTP mix, 2µl of forward and reverse primers 10µM), 0.4µl Taq DNA polymerase (5U/µl), 2µl cDNA from the first strand reaction and the reaction was then made up with distilled water to 50µl. The amplification was carried out using temperature cycling on a Thermo hybrid and the PCR amplification programme was carried out using the following parameters:

Denaturation: 94°C for 5 minutes; 30 cycles; 94°c for 30 seconds, 55°C for 30s, 72°C for 90s; extension: 72°C for 5 minutes.

The PCR products were purified using a QIAquick PCR purification Kit (Qiagen LTD, Crawley, West Sussex, UK) according to manufacturer's instructions, and samples stored at -20°C.

2.7.4 Agarose gel electrophoresis

Horizontal agarose gel electrophoresis was used to separate and detect PCR- amplified DNA fragments. Agarose was dissolved in Tris acetate (TAE, Fisher Scientific UK LTD, Loughborough, Leicestershire, UK) buffer by heating to a concentration of 2% (w/v). The

gel solution was allowed to cool slightly and 0.5μg/ml Ethidium Bromide (Sigma- Aldrich Company Limited, Poole, Dorset, UK) was added. The gel was poured into a mould, to which well-forming combs were added, and allowed to set. At this point the combs were removed and the gel placed in an electrophoresis tank, with TAE added to submerge the gel. DNA samples mixed with gel loading solution (5:1) were loaded into the wells. Electrophoresis was carried out at 90V for 30-60 minutes depending upon product size. DNA was visualised via a UV Trans illuminator and the image recorded using a Kodak DC290 digital camera.

2.7.5 Real Time PCR: Reverse Transcription

Total RNA was reverse transcribed with Superscript II oligo (dT) primer according to the protocol described earlier and resulting in a 20μl solution/sample.

2.7.6 Sybr Green real time PCR

The RT-PCR was performed using the Quantitect Sybr Green PCR kit (Qiagen LTD, Crawley, West Sussex, UK) on a Stratagene Mx3000 instrument for CD133 and GAPDH primers (Sigma- Aldrich Company Limited, Poole, Dorset, UK). 5μl (0.3μM) of primers (total for forward and reverse), 25μl Quantitect Sybr Green Master mix, 5μl DNA (400ng) and RNase free water were used for a 50μl of final volume reaction.

Table 4: Real time cycling conditions PCR technology consists of a series of 40 repeated temperature changes, called cycles, with each cycle commonly consisting of 3 discrete temperature steps known as Denaturation, Annealing and Extension.

STEP	TIME	TEMPERATURE
PCR Initial activate step	2 min	50°C
Denaturation	15min	95°
Annealing	30sec	60°C
Extension	30sec	72°C

Detection of fluorescence was carried out at 72°C. After amplification, the melting curve was performed by denaturation at 95°C for 60 seconds, annealing at 55°C for 30 seconds and increasing the temperature to 95°C with a ramp, the fluorescence was collected during the ramp increase temperature. The CD133 and GAPDH primers were purchased from Qiagen and there efficiency was optimal with the Quantitect Sybr PCR kit.

2.7.7 Knockdown analysis via immunofluorescent staining

To determine the phenotypic knockdown expression of CD133 on the Caco2 cell line, immunofluorescent monoclonal antibody labelling and flow cytometry single or dual stain analysis was used. After the cells had been exposed to their varying knockdown conditions, the cells were transferred to FACs tubes, washed with PBS, incubated with (2%) human gamma globulin and immuno-fluorescently labelled as described in the previous chapter. Mouse anti –human CD133 (AC133/2, IgG1) PE conjugated.

2.7.8 RNA Interference using a plasmid construct.

RNA interference as shown above is a useful tool for gene targeting. In this instance a plasmid construct is created that exogenously expresses short-hairpin RNAs (shRNAs). Using a plasmid vector to knock down a gene allows for long tem and permanent knockdown, without the need to generate knockout phenotypes.

2.7.9 P Silencer siRNA Expression Vector

The expression vector used was the pSilencer 3.1 H1 neo kit (Ambion LTD, Huntingdon, Cambridgeshire, UK), the vector map is shown in figure 14. The hairpin CD133 siRNA template oligonucleotides were designed using the target sequence web-based insert design tool from Ambion and constructed by Sigma- Aldrich Company Limited, Poole, Dorset,UK. The sequence used was an RNAi sequence previously designed and tested by Qiagen and the restriction enzymes used were BamH1 and Hind III (Sigma) (Table 5).

Table 5: showing the forward and reverse sequences of the RNAi oligonucleotide including the restriction site sequences.

Oligonucleotide Name	Sequence 5' to 3:	Restriction Site
CD133 Forward	GATCCGGTAAGAACCCGGATCAAATTCAAGAGATTTGATCCG GGTTCTTACCTGTTTTTTGGAAA	BamH I
CD133 Reverse	AGCTTTTCCAAAAAACAGGTAAGAACCCGGATCAAATCTCTT GAATTTGATCCGGGTTCTTACCG	Hind III

Figure 14: Schematic diagram representing the vector map from the 3.1-H1 plasmid purchased from Ambion. The vector uses the H1 RNA pol 111 promoter and has been linearized with both BamH1 and Hind111 to facilitate directional siRNA cloning. The vectors contain a Neomycin and Ampicillin resistant gene allowing antibiotic selection using G418 an analog of Neomycin (Qiagen, 2010).

The hairpin siRNA oligonucleotide was dissolved in 100µl of nuclease-free water, 1ul of each oligonucleotide was diluted to 1:100 in TE (10nm Tris, imM EDTA) and the concentration calculated in µg/ml using a nano drop. The oligonucleotides were then diluted to 1ng/ul. The annealing mixture was assembled as follows: 2µl of the sense and antisense template oligonucleotide and 46µl of DNA annealing solution were mixed. The mixture was heated to 90°C for 3 minutes then placed in a 37°C incubator and incubated for 1 hour. 5µl of the annealed siRNA template insert was diluted with 45µl nuclease-free water for a final concentration of 8ng/µl. Two 10µl ligation reactions; a plus-insert ligation and a minus-insert negative control were set up as shown in table 6, prior to incubation for 1-3 hours at room temperature.

Table 6: Showing the composition of the 10µl ligation reactions for both experimental (+ insert) and control (- insert).

Plus-insert	Minus-insert	Component
1µl	-	Dil. Annealed siRNA insert
-	1µl	1x DNA annealing solution
6µl	6µl	Nuclease free water
1µl	1µl	10x T4 DNA ligase buffer
1µl	1µl	pSilencer neo vector
1µl	1µl	T4 DNA ligase (5U/µl)

JM109 *E-Coli* (Promega UK, Southampton, UK) competent cells, 45µl, initially thawed on ice were mixed with 5µl of the ligation reaction and incubated on ice for 45minutes. The cells were heat shocked at 42°C for 1 minute and incubated on ice for a further 2 minutes. The cells from each plus and minus insert were plated on separate 100µg/ml Luria broth Ampicilin plates, inverted and incubated overnight at 37°C. Isolated colonies were picked from the plus insert plate and each clone was inoculated into 5ml aliquots of Luria broth with Ampicillin (Sigma- Aldrich Company Limited, Poole, Dorset, UK). They were then incubated in a 37°C shaker overnight. A glycerol stock of the individual bacterial clones was made and stored for future use. The cells were then pelleted by centrifugation at 1800RPM for 10 minutes at 4°C, the supernatant aspirated and the pellets frozen at -20°C.

2.7.10 Purification of Plasmid DNA

The DNA was prepared from the bacterial pellet using the Qiagen miniprep kit (Qiagen LTD, Crawley, West Sussex, UK) according to manufacturers instructions. The procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt. The procedure consists of 3 basic steps:

1. Preparation and clearing of bacterial lysate
2. Adsorption of DNA onto the QIAprep membrane
3. Washing and elution of plasmid DNA

The purified plasmid DNA was digested with the restriction endonucleases BamH1, Hind111 or Ecor1. The restriction digest reaction consisted of 8µg DNA, 2µl 10x reaction buffer and 2µl of enzyme in a final volume of 20µl made up with ddH_2O. The reaction was incubated at 37°C for a minimum of 4 hours.

To confirm the successful cloning of the combined oligomers into the plasmid the restriction endonuclease reaction was subjected to electrophoresis using 1% agarose gels made by dissolving 1g of agarose (Sigma- Aldrich Company Limited, Poole, Dorset, UK) in 100ml of 1X TBE buffer (Tris-borate pH8.0, 1mM EDTA) and 1µl of 10mg/ml Ethidium Bromide. The running buffer used was 1X TBE buffer. DNA samples were mixed with 10X loading buffer and diluted to a final concentration of 1X loading buffer. A 100bp KB plus DNA ladder (Invitrogen LTD, Paisley, Scotland) was used as a standard for sizing DNA sample fragments. Electrophoresis was carried out at 100V. DNA was visualized under a UV light source and gel images were obtained using a Polaroid camera with Polaroid 667film or a Biorad gel doc imaging system.

2.7.11 Transfecting the pSilencer plasmid into mammalian cells

24 hours before transfection, Caco2 cells were plated in 24 well plates (Nunclon, Denmark) at $4x10^4$ in 500µl RPMI medium supplemented with 10% FBS and Gentamicin and incubated overnight at 37°C with 5% CO_2 under humid incubation.
In sterile polystyrene tubes, 1µl siPORT XP-1(Ambion LTD, Huntingdon, Cambridgeshire, UK) was diluted into Opti-MEM I medium (Gibco BRL life technologies LTD, Paisley, Scotland) producing a final volume of 50µl. The tubes were vortexed thoroughly and incubated at room temperature for 15 minutes. 200ng of plasmid DNA was added to the siPORT solution and mixed gently. The solution was incubated at room temperature for a further 15 minutes. The siPORT/DNA complex was added drop wise onto the cells and the dish rocked back and forth to evenly distribute the complexes. The cells were then incubated for 24 hours.

At this point the selection process was undertaken using G418 (Gibco BRL life technologies LTD, Paisley, Scotland) at a concentration of 600µg/ml. Two non transfected cell cultures were also set up. One is subjected to G418 selection to control for the fraction of cells that survive selection. The second control is grown without G418 selection as a positive control for cell viability. The gentamicin was added to the culture medium and cultured with the cells until all the cells in the non transfected control culture were killed. This assumed, the selection was complete and the remaining cells in the culture could be grown without antibiotics until confluent. The cells were analysed for knockdown of the target gene using PCR or flow cytometry.

2.8 Methodology for functional experiments on knocked down Caco-2 cells

2.8.1 Determining CD133 receptor re-expression after knockdown

Caco-2^{CD133-} (knocked down with the Santa Cruz siRNA) were purified using anti CD133 MACs beads producing a population of pure $CD133^-$ cells. A proportion of the $CD133^-$ cells were then stained with CD59 to show cell viability and to confirm that these were Caco-2 cells. The control sample expressing a scrambled control siRNA (Santa Cruz Inc, Heidelberg, Germany) Caco-2^{SSC} was also stained with CD59 (Pharmigen UK LTD, Cowley, Oxford, UK). At this point the cells were then cultured in 6 well plates under normal growth conditions at a concentration of $5x10^6$ cells per well. Samples were taken on a daily basis to assess the rate at which CD133 receptor re-expression occurred on Caco-2 cells using flow cytometry.

2.8.2 Proliferation Assays

A Caco-2 proliferation time course assay was performed after gene knockdown and cell sorting to isolate the CD133 population. Wells were seeded with 10^4 cells of Caco-2 and cultured in 2ng CD133 specific (Caco-2^{CD133-}) or cultured in 2ng of a scrambled control siRNA (Caco-2^{SSC}). Cell numbers were assessed on days 1, 3 and 5 and a methylene blue assay was used to quantify the number of viable cells.

2.8.3 Cytoxicity experiments

The cytotoxicity of anti- CD133 monoclonal antibodies was measured as follows; 300,000 cells were cultured in each well of a 6 well plate (Nunclon, Denmark) in a volume of 2ml of media. The cells used were Caco-2^{ssc}, Caco-2^{CD133-} and HL-60 (DMSZ, Germany), an acute myloid leukaemia cell line which lacks CD133 expression and was used as a negative control. Twenty four hours after incubation unconjugated Mab's directed against epitope 1 AC133 (Miltenyi Biotech, Bergish, Germany), epitope 1 AC133 (Abcam PLC, Cambridge, UK) and AC141 epitope 2 (Miltenyi Biotech, Bergish, Germany) were added at various concentrations to all 3 cell line groups. Seventy two hours later, the antibody

containing medium was removed, and the cytotoxic affect of anti- CD133 antibody was determined using trypan blue exclusion. The experiment was carried out in triplicates and repeated three times.

2.8.4 Binding capacity of Caco-2 cells to plastic

Each well of a 6 well plate was seeded with either Caco-2 $^{CD133-}$ or Caco-2^{SSC} cells 24 hours after transfection. The seeding density was 300,000 cells per well. At various time points 1 well from each experimental group was swirled gently and all the contents pipetted out into a testube. The cell number was then calculated and noted. This was repeated at regular intervals for a total period of four hours.

2.8.5 Cell cycle analysis of Caco-2 $^{CD133-}$ and Caco-2 ^{SSC} cells

The DNA content was measured in samples of $5x10^5$ cells/ well. Caco-2 $^{CD133-}$ and Caco-2^{SSC} were both seeded independently in 6 well plates and left for 24 hours under normal growing conditions before cell cycle activity began to improve the health of the cells. Synchronisation of the cells was then carried out to stabilize the cells.

Aphidicolin (Sigma- Aldrich Company Limited, Poole, Dorset, UK) which is a tetracyclic diterpene antibiotic with antiviral and antimitotical properties was used to synchronise the cells in G0/G1 resting phase. Aphidicolin is a reversible inhibitor of eukaryotic nuclear DNA replication, blocking the cell cycle at G1 phase. It acts by inhibiting DNA polymerase A and D in eukaryotic cells enabling us to track and record the rate at which the cells cycle from the G1 resting phase through to the mitotic phase of the cycle. The concentration of Aphidicolin was carefully determined by Cinatl *et al.*, (1992) who showed that high concentrations of the drug kills Caco-2 cells whilst having no affect on other cell lines. After establishing the correct incubation period for the drug to align the cells within the G1 phase, Aphidicolin was added to the cells at a concentration of 5ug/ml for a period 20 hours. 24 hours realistically being the longest time the cells would be subjected to the drug as studies showed that prolonged exposure may damage the cell and after going through the knockdown procedure the cells were potentially under stress (Cinatl *et al.*, 1992). The cells were thoroughly washed twice with PBS, seeded under normal culture conditions and left to recover for 24 hours as preliminary studies showed no cycle activity

occurred during this period. From this point, regular time intervals were set at every 12 hours. At each time period, cells were permeabilised in 70% ethanol and placed in the -20 freezer. On completion of the 36 hour time course the cells were then washed and stained.

For PI (Sigma- Aldrich Company Limited, Poole, Dorset, UK) DNA labelling, cells were initially trypsinized and centrifuged at 1300RPM for 10 minutes at 20°C. Cells were fixed by gradual addition of 2ml of ice cold 70% ethanol to enable cell permeabilization. The cells were then centrifuged at 1450RPM for 10 minutes at 20°C. After the removal of supernatant, the cells were resuspended in 5ml of PBS and further centrifuged at 1400RPM for 10 minutes at 20°C. The cells were then resuspended in 500ul of PBS/ ribonuclease-A (RNase) at a final concentration of 0.2mg/ml to lyse residual RNAs and then incubated for 30 minutes at 37°C. Finally the DNA was stained with PI at a concentration of 2mg/ml and analysed by Flow Cytometry with a FACScan and the attached CELLQuest software.

2.9 Micro array methodology

2.9.1 Microarray setup

The experiment was aimed at studying the gene expression levels on Caco-2 cells under various conditions affecting the CD133 receptor. We compared the expression levels of 3 experimental groups against a control group.

Group 1: Cells seeded at $2x10^5$ per well were subjected to knockdown of the CD133 receptor using the optimum concentration and conditions as described earlier for siRNA (Santa Cruz). The cells were incubated for 48 hours post siRNA transfection however we only achieved a 65% CD133 knockdown of Caco-2 cells so the remaining sample was purified using a CD133 cell isolation kit (see earlier chapter).

Group 2: $1x10^6$ cells were seeded in 3 wells of a 6 well plates and labelled with an IgG (Miltenyi Biotech, Bergish, Germany) control for 1 hour on ice at a concentration of 50µg/ml to eliminate non specific binding, and act as a further control to group 3.

Group 3: The Caco-2 cells were labelled with 3 CD133 monoclonal antibodies; CD133/1, CD133/2 (Miltenyi Biotec) and CD133/1* (Abcam) collectively for 1 hour on ice to try and mimic ligand stimulation and thus trigger any down stream reactions that may occur on binding to CD133. All three antibodies were added at 50µg/ml. The cells were seeded at $1x10^6$ cells/ well and the experiment was carried out in triplicate in an identical fashion to group 2.

Initially for groups 2 and 3, each experiment was carried out in triplicates with incubation periods of 1 hour and 12 hours respectively, however due to the cost of the gene array process the final process was carried out using the samples incubated for an hour and reducing the replicates to 2.

Group 4: The Caco-3 cells were seeded at $1x10^6$ cells in 6 well plates and cultured with growing medium. This group comprised of the control group which the other 3 groups were compared against.

The RNA was extracted using the Qiagen RNAeasy protocol described earlier and then quantified using a nanodrop. The samples from each group were then diluted to a concentration of 200ng. The RNA extracts from the samples were hybridized to Agilent chips (Agilent Technologies UK Ltd, Edinburgh, UK) and the expression level of 45015 probe sets were obtained for each sample and the experiments in duplicate.

2.9.2 One Colour Spike Mix

To prepare the one colour spike mix dilution appropriate for 200ng of total RNA, we heated the mix at 37°C for 5 minutes and then added 2µl of the one colour spike mix stock to 38µl of dilution buffer (supplied with agilent kit). 2ul of this mixture was then added to 48µl of dilution buffer and finally 4ul of this second dilution was added to 36µl of dilution buffer for the third dilution. The solution was mixed thoroughly and centrifuged briefly to collect all the liquid at the bottom of the tube.

2.9.3 Cy3 labelling

200ng of total RNA was diluted in 8.3ul RNase free water and placed in a micro centrifuge tube. 1.2ul of T7 promoter primer was added followed by 2ul of the spike mix. The primer and the template were denatured by incubating the reaction at 65°C for 10min and then cooled on ice for a further 5 minutes.

A cDNA master mix was made up consisting of; 4ul 5X First Strand Buffer, 2ul 0.1DTT, 1ul 10mM dNTP mix, 1ul MMLV-RT and 0.5ul RNaseOut giving a final volume of 8.5ul which was added to each sample tube.

The samples were then incubated at 40°C for 2 hours, then moved to 65°C for 15 minutes and finally kept on ice for 5 minutes. Samples were then micro centrifuged to collect the contents.

Next the transcription process occurs where a cRNA template is created from the cDNA produced in the previous step. 60ul of the transcription mix was added to each sample. The master mix contains; 15.3ul Nuclease-free water, 20ul 4X Transcription Buffer, 6ul 0.1 M DTT, 8ul NTP mix, 6.4ul 50% PEG, 0.5ul RNase OT, 0.6µl inorganic pyrophosphatase, 0.8µl T7 RNA polymerase, 2.4µl and Cyanine 3-CTP. The solution was gently mixed by pipetting and then incubated for 2 hours at 40°C.

2.9.4 Purifying the labelled/amplified RNA

The purification of the samples was done using an RNA easy Kit from Qiagen, the procedures were as follows, 20µl of nuclease free water was added to the samples producing a total volume of 100µl per sample. 350µl of RLT buffer was added followed by 250µl of ethanol (100%) and thoroughly mixed. 700µl of the sample was then transferred to an RNeasy mini column, centrifuged at 4°C for 30 seconds at 1300RPM. The flow through was discarded. 500µl of RPE buffer was then added to the column and centrifuged at 4°C for 60 seconds at 1300RPM discarding the flow through as before. To remove any buffer that may be remaining the column was placed in an empty 1.5ml tube and spun for 30 seconds at 1300RPM.The cleaned cRNA was then eluted by transferring the column to a 1.5ml collection tube, adding 30µl of RNase free water directly onto the column's membrane and centrifuging at 4°C for 30 seconds at 1300RPM.

2.9.5 Quantifying the cRNA

Quantification was performed on a Nanodrop ND-1000 UV-VIS spectrophotometer, making sure the Microarray measurement tab was clicked and the RNA-40 tab was also selected. The machine was initially blanked with nuclease free water and then 1µl of sample was loaded onto the instrument sample loading area.

2.9.6 Calculation to determine the yield and specific activity of the reaction

(Concentration of cRNA) X 30µl (elution volume) / 1000 = µg of cRNA

(Concentration of cCy3) / (Concentration of cRNA) X 1000 = pmol Cy3 per µgRN

2.9.7 Hybridization

500ul of nuclease free water was added to the vial containing lyophilized 10X blocking agent and mixed gently by vortexing. For the whole human genome 4 X 44K Microarray format, a fragmentation master mix was made up consisting of 1.65µg of Cyanine 3-labelled, amplified cRNA, 11µl 10X blocking agent, 52.8µl of nuclease-free water, 2.2µl 25X fragmentation buffer producing a final volume of 55µl. The samples were gently mixed and then incubated for 60°C for exactly 30 minutes to fragment RNA. 55µl of 2X GEx Hybridixation buffer HI-RPM was added to the 4 X 44k array to stop fragmentation.

The solution was mixed well then centrifuged for 1 minute at room temperature at 1300RPM, then placed on ice.

A clean gasket slide was loaded into the Agilent SureHyb chamber base with the label facing up and aligned with the rectangular section of the chamber base. 100µl of the hybridization sample was dispensed onto the gasket well in a "drag and dispense" manner. An array chip was placed "active side" down onto the SureHyb gasket slide and aligned correctly. The chamber cover was then placed onto the sandwiched slides, clamped and rotated to wet the gasket. The slide chamber was then placed in the hybridization oven and set to 65°C where hybridization occurred for 17 hours.

2.9.8 Washing of Microarray slides

Following removal of the hybridisation chamber from the incubator, the Caco-2 cells on the slides were washed three times in 3 separate dishes using two different buffers supplied with the kit GE wash buffer 1 and 2. The first wash known as the disassembly step entailed removing the array chip from the chamber in the wash and was done with GE buffer 1 at room temperature. The Microarray slide was then placed on a rack and washed in a new dish with Buffer 1 for 1 minute with a stirrer at room temperature. The final wash was carried out in the same manner but at an elevated temperature for 1 minute (Note 8 slides can be washed at any particular time). Slides were then immediately scanned and analysed.

2.10 Confocal microscopy methodology

2.10.1 Immunofluorescent staining for CD133 expression on adhered Caco-2 cells for CLSM analysis

13mm, 1.0 thickness round glass cover slips (VWR international, Briare, France) were deposited into each well of a 6 well plate, a maximum of 3 cover slips per well. Caco-2 cells were plated into the 6 well plates at 200,000 cells per well in 2ml of medium under normal growing conditions, and adhered to the coverslips overnight at 37°C, CO_2 5%. The cover slips were washed in PBS to remove un-adhered cells, before separating the coverslips into individual wells of a 6 well plate and incubated with HAG, 2%, 1ml for 30 minutes on ice.

Adhered cells were then labelled with the pure primary anti-CD133/1 antibody (Miltenyi Biotech, Bergish, Germany) at 2mg/ml. An irrelevant matched isotype antibody control was also used at the same concentration (IgG1) (Miltenyi Biotech, Bergish, Germany). Cells were incubated with 1ml of diluted antibody at 4°C for 30 minutes, then washed in excess PBS prior to secondary fluorescent conjugated antibody labelling using rabbit anti mouse Alexa Fluor 468 conjugated (Invitrogen LTD, Paisley, Scotland) or rabbit anti mouse Alexa Fluor 555 (Invitrogen LTD, Paisley, Scotland) was diluted 1:1000 with PBS and added at 1ml per well. Antibody incubations were carried out at 4°C for 30 minutes with the wells then washed in excess PBS (0.1% sodium azide). Cells were fixed with PFA (pH7.2, 4%), for 15 minutes then washed again in PBS wash.

2.10.2 Mounting of Caco-2 cells.

Vectashield DAPI anti fade reagent (Vector Laboratories, Peterborough, UK), 50µl was applied to the centre of the slide. The round cover slip was then inverted and placed over a 24x50mm, 1.5 thickness cover glass slide (VWR international, Briare, France) allowing the Vectashield to circulate around the coverslip. Clear acrylic nail varnish, was used around the coverslip to create a sealed chamber. The slides were CLSM analysed within two days of staining and were stored at 4°C in a blacked out slide box.

2.10.3 LR staining for CLSM analysis

The LRs were labelled using a Vybrant LR labelling kit (Invitrogen LTD, Paisley, Scotland) which contained a Cholera toxin Subunit B antibody conjugated to either AF468 or AF555 fluorochrome. The Cholera toxin subunit B is part of an oligomeric complex made up of 6 proteins: a single copy of the A subunit (enzyme) and five copies of the B subunit (Receptor binding) secreted by the bacterium Vibrio cholera. Once secreted, The B subunit ring of the bacterium will bind to the pentasaccharide chain of plasma membrane GM1 gangliosides on the surface of the host cells, which selectively partitions into LRs. An antibody that specifically recognises the Cholera toxin subunit B is then used to cross link the CT-B – labelled LRs into distinct patches on the plasma membrane, which are easily visualized by fluorescence microscopy.

The labelling protocol was the same for both adherent and confluent cells; a stock solution of the fluorescent CT-B conjugate was prepared by adding 100µl PBS to the vial creating a 1mg/ml stock solution. A working solution was then prepared by taking 2µl of the stock solution and adding it to 2ml of PBS producing a 1µg/ml solution. A 200 fold dilution of the anti-CT-B antibody was prepared by taking 10µl of the stock solution and adding it to 2ml of PBS.

After incubation with HAG 2%, 1ml for 30 minutes on ice and washing of cells, they were resuspended in 2ml of the fluorescent CT-B conjugate working solution for 10 minutes at 4°C, prior to washing the cells gently with PBS 3 times. 2ml of the anti-CT-B antibody working solution was added and incubated for 15 minutes at 4°C. Cells were washed, then fixed with paraformaldehyde and mounted as described above and according to the type of cells.

2.10.4 Dual fluorescent staining of lipid rafts and CD133 for CLSM analysis

Dual colour analysis was an important technique carried out enabling us to understand the relationship between CD133 and LR expression. The LRs were labelled initially as above. At the point were the anti CT-B antibody was added and the cells washed, the murine CD133 antibody was added followed by the RAM Alexa Fluror fluorochrome of choice. The cells were then fixed and mounted on to slides or coverslips. When staining using dual indirect immunolabelling, there is a possibility to create the potential for inappropriate staining by Bridge formation, where the murine antibody specific for the second antigen binds to free anti murine sites on the fluorochrome conjugated antibody labelling the first antigen or Cross reactivity between fluorochromes aimed at the second antigen and free sites on the murine antibody against the first antigen, This inappropriate fluorescent staining would result in potentially false positive fluorescent signals for the second antigen. These problems were overcome by using a directly conjugated antibody against LRs with LR immunolabelling prior to CD133 labelling thereby limiting the potential for bridge formation and cross reactivity.

2.10.5 Removal of Lipid Rafts using Beta Methyl Cyclodextrine

This was carried out using the drug Beta Methyl Cyclodextrine (BMCD) (Sigma- Aldrich Company Limited, Poole, Dorset, UK). BMCD comes from a family of compounds made up of sugar molecules bound together in a ring. The water-soluble BMCD is known to form soluble inclusion complexes with cholesterol, thereby enhancing its solubility in aqueous solution as the bulky and hydrophobic cholesterol molecule is easily lodged inside cyclodextrin rings that are then removed hence its role in disrupting LR. 1ml of a 2nM concentration of the drug was added to adherent cells grown on a coverslip in a 6 well plate. Time periods of 40 and 60 mins were initially set, however the BMCD drug has been known to be harsh on cells (Ulloth *et al.*, 2007) and viability assays (trypan blue) performed on both time points showed clearly that there was a high percentage of cell death after 60 minutes incubation. Therefore we proceeded with BMCD cell incubation for 40 minutes, at which point the cells were washed 3 times and the staining procedure carried out.

2.10.6 Immunofluorescent staining of Caco-2 cells for adhesion onto fibronectin

The immunofluorescent staining was carried out in 5ml polystyrene round bottom tubes (BD Biosciences, Erembodegem, Belgium) as opposed to slides or coverslips. The cells were washed with PBS and incubated with 2% HAG, 250µl for 30 minutes on ice. At this point they were washed twice with PBS and labelled with the pure primary CD133/1 antibody at a concentration of 10µg/ml. An irrelevant matched isotype antibody control was also used at the same concentration (IgG1). Antibody incubations were carried out at 4°C for 30 minutes. Cells were washed twice in 2ml of PBS prior to secondary fluorescent conjugated antibody labelling using: Rabbit anti mouse Alexa Fluor 468 (AF468) conjugated or Rabbit anti mouse Alexa Fluor 555 (AF555), diluted 1:1000 with PBS, 250µl per tube. Antibody incubations were carried out at 4°C for 30 minutes prior to washing three times in 2ml PBS (0.1% sodium azide). Cells were allowed to adhere onto glass coverslips or fibronectin coated coverslips prior to fixing with PFA (pH7.2, 4%), for 15 minutes, then washed twice with PBS.

Chapter 3: Devlopmental biology

3.1 Aims and objectives

The primary aims of this chapter were:

- To establish optimal growth conditions for the MUTZ-2 cell line and the CACO-2 cell line *in vitro*.
- Measuring growth patterns for MUTZ-2, CACO-2 and $CD34^+$ Primary cells taken from cord blood.
- Quantifying CD133 phenotypic expression on both MUTZ-2 and CACO-2 cell lines.
- Determining long term expression of CD133 on $CD34^+$ primary cells.
- Evaluating the effect of steric hindrance and freezing on $CD34^+$ primary cells.

3.2 Introduction

Due to the rare nature of primary $CD133^+$ cells, cell lines such as MUTZ-2 and CACO-2 have been used to establish the correct experimental conditions. However, as indicated in Chapter 1, there is a great deal of evidence to support the theory that CD133 may not carry out the same functional role within normal haemopoietic and CSC populations. Therefore, where possible, cells expressing CD133 from Umbilical Cord Blood (UCB) have been used. UCB was obtained via caesarean section with fully informed parental consent following local research and ethical approval.

3.3 Results

Three main sources of cells were used to investigate the functional properties of CD133; Caco-2, MUTZ-2 and $CD34^+$ primary cells selected from cord blood. Both cell lines MUTZ-2 and Caco-2 readily expressed CD133 at varying percentages, however, MUTZ-2 was the cell line that had the closest resemblance to the haemopoietic system expressing both CD34 and CD133 and was therefore the line of interest.

3.3.1 Immunophenotyping of MUTZ-2 cell line:

Phenotyping of MUTZ-2 cell line was accomplished using multicolor FACS analysis with a panel of conjugated antibodies directed to leukocyte surface structures known to be expressed on human haemopoietic stem or progenitor cells. The results show expression on cells that had been cultured in 5637 medium and SCF. Figure 15 shows the mean surface expression of CD133 on MUTZ-2 cells with a percentage expression of 44%. Typically figure 16 shows CD13, CD33, CD34 were all expressed at levels above 90%, whereas CXCR4 and CD117 were expressed at much lower levels in particularly (G) CXCR4 did not stain above the (B) isotype control level.

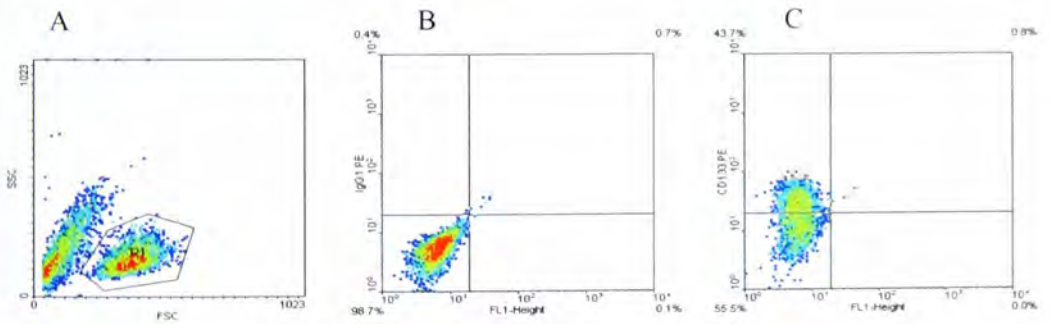

Figure 15: Mean expression of CD133 on MUTZ-2 cells. A) gating strategy employed during flow cytometric analysis of MUTZ-2 cells labelled with CD133. The gated region R1 was applied to **B)** irrelevant matched isotype control, and **C)** single positive CD133/1 PE immunofluorescent staining. Positive cell count indicated for each quadrant at the corresponding corner of plots. CD133 expression gave a mean percentage of 39% ± 6.7 Standard Error Mean (SEM), (n=4).

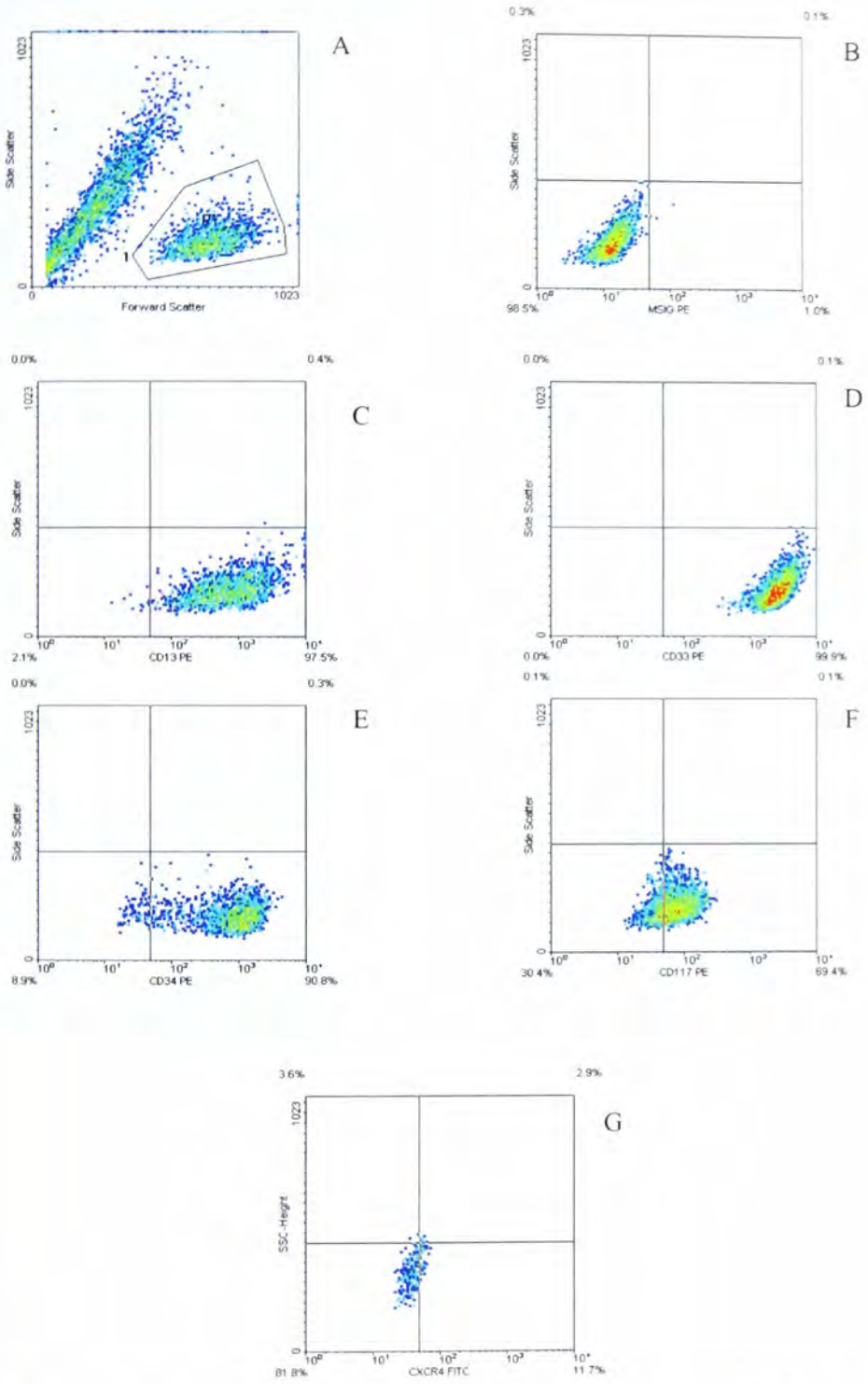

Figure 16: Phenotypic flow cytometry characterization of MUTZ-2 cell line. Representative analysis of: **A)** Profile of selected cell with R1 representing the gated cells analysed. **B)** IgG1 PE control. **C)** CD13 (97.5%± 2.2) **D)** CD33 (99.9%± 1.8). **E)** CD34 (90.8%± 4.6). **F)** CD117 (70%± 6.1). **G)** CXCR4 (12%± 8.5). All data was analyzed from 10,000 events (n=3).

3.3.2 Optimising growth conditions for MUTZ-2 cells line

It was important to try and create growing conditions that were not only optimum with respect to cell proliferation and cost, but also easily reproducible as any minor changes in the growing conditions could hugely affect downstream events. A major issue was the 5637 CM, we found that different batches stored would contain various levels of cytokines as cells may be at different cycle points before the experiment commenced. This would provide inconsistent growth patterns in experiments where all other parameters were identical making it difficult to achieve consistent results. The MUTZ-2 cell line grew in alpha MEM and FBS with conditioning medium from 5637 cell line grown independently as described earlier and figure 17 shows the growth pattern of MUTZ-2 cells under these conditions. It can be observed that by day 6 the cell number had increased by 2.5 fold and also the rate of proliferation decreased from days 8 to 12.

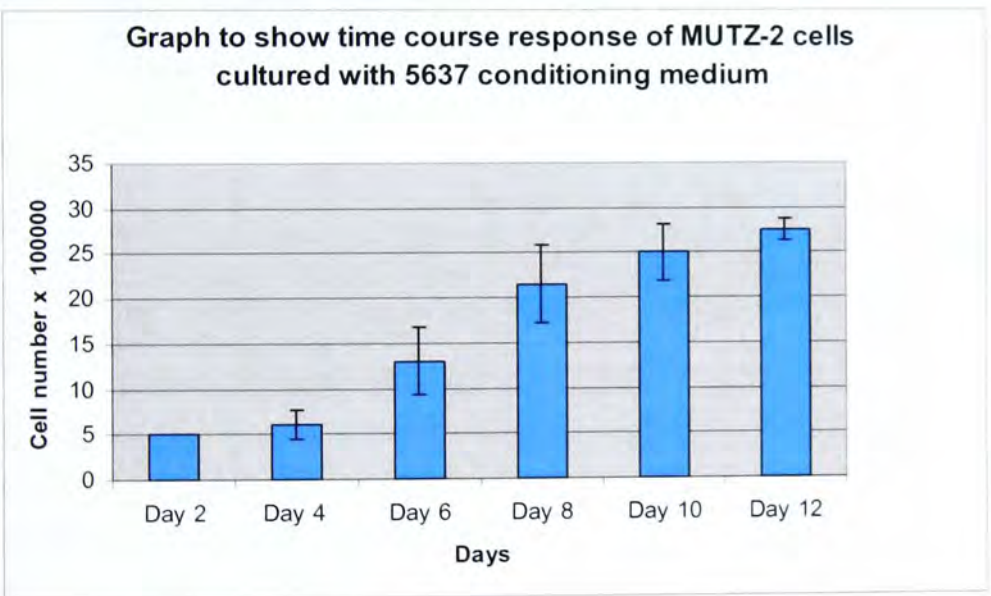

Figure 17: Proliferation of MUTZ-2 cells in response to 5637. Cells were plated at $5x10^5$ cells per well in 12 well plates under normal growing conditions with 20% 5637 conditioning medium. Every second day cell numbers were determined using trypan blue. (n=5).

Modifications were made to the culture protocol to enhance cell growth by the addition of stem cell factor (SCF) to 5637 cell culture supernatant. Figure 18 shows MUTZ-2 cells cultured with varying concentrations of SCF over a 12 day culture period, ranging from 5- 70ng/ml. Comparable cell numbers were achieved when using 5637 supernatant and

50ng/ml, 60ng/ml or 70ng/ml of SCF. 50ng/ml was functionally the best concentration to use as by day 12 total cell number in 50ng/ml, 60ng/ml and 70ng/ml were comparable. The rates of proliferation were also very similar, as higher concentrations did not further enhance the growth rate.

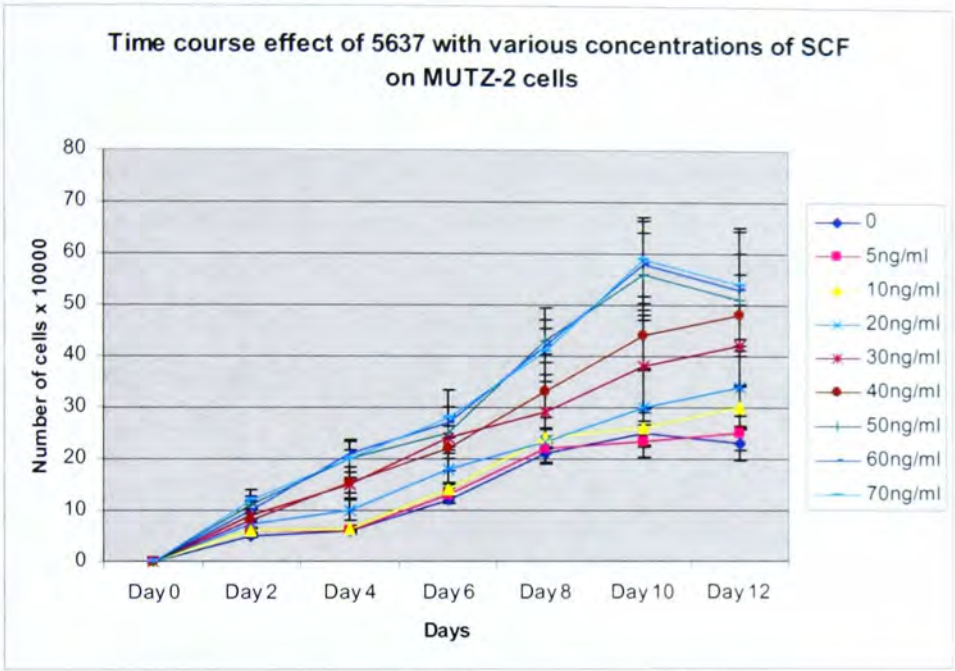

Figure 18: SCF dose response curves on MUTZ-2. Cells were plated at $5x10^5$ cells/ well as before. Varying concentrations of SCF were added to the cells on day 3, 6 and 9. Various SCF concentrations are represented by the different colored lines. 3 experiments were carried out in triplicates (n=3).

Even though higher levels of proliferation were observed with SCF, MUTZ-2 was still found to be unpredictable and difficult to maintain a steady level of growth, as proliferation levels could vary greatly. This can be demonstrated by the large error bars shown in figure 18. One of the factors responsible for this was the 5637 conditioning medium. It was very difficult to quantify cytokine levels in the conditioning medium as 5637 growth would vary and therefore collection points of the supernatant ultimately would vary also. It was decided to grow large batches of 5637, collect the supernatants over a period of time and then pool the supernatants together and freeze thus having a main stock. Although it was not possible to determine the concentration of cytokines, this would enable culture with consistent 5637 supplemented media. However, there was still variation in growth patterns for MUTZ-2. Cell culture would follow a similar growth pattern as illustrated in figure 18, with intermittent periods of no growth. No definable

reasoning could be determined for this. A number of different batches or MUTZ-2 were purchased from the supplier, and all were subject to similar inconsistent growth patterns.

An experiment was then set up to try and combine 5637 medium, SCF and FLT-3 (figure 19). The combination of SCF 5637 gave the highest proliferation rate and level of proliferation compared to the other groups. There was an increase of 4.5 fold compared to using 5637 alone whilst the addition of FLT-3 to 5637 enhanced the proliferation by 2.5 fold. Surprisingly, FLT-3 alone at the concentrations of 10ng/ml and 50ng/ml both produced higher proliferation levels on day 8 than SCF alone however the addition of FLT-3 to 5637 gave reasonable proliferation properties and was further investigated in the next experiment in combination with SCF. The concentrations of FLT-3 in combination samples was 10ng/ml (n=3).

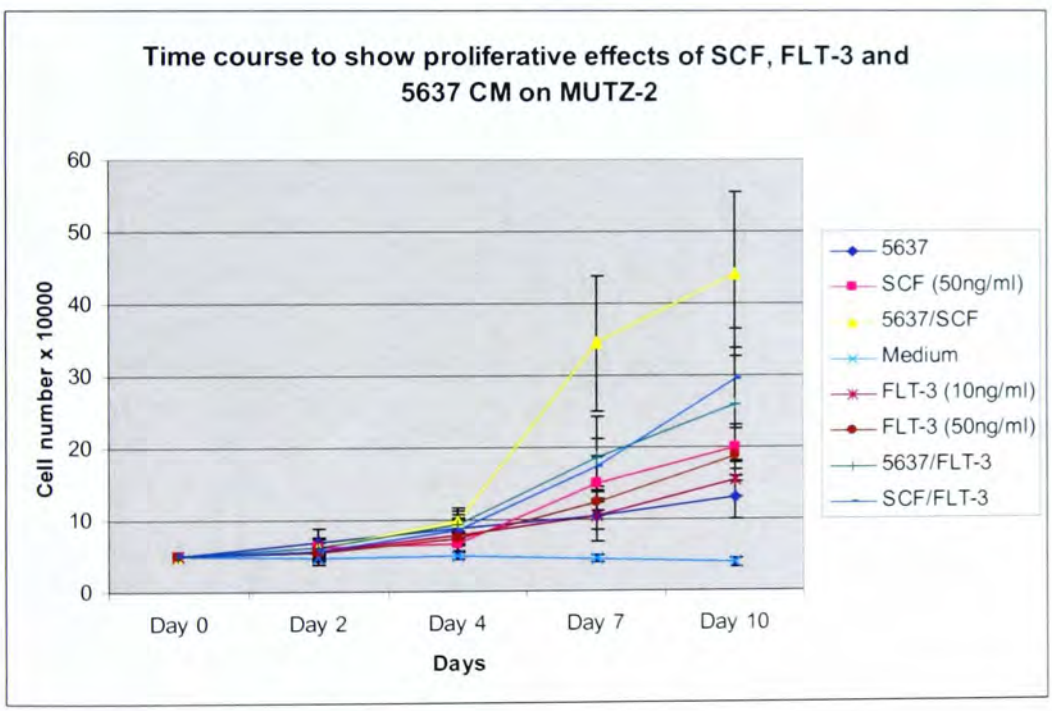

Figure 19: Proliferative effects of SCF, FLT-3 and CM on MUTZ-2 over a 10 day time course. Cells were seeded at $5x10^5$ cells per well and counted every 48hrs. 3 experiments were carried out in triplicates (n=3).

At this point we then further investigated the possible effects FLT-3 may have on proliferation by combining varying concentrations of FLT-3 with or without SCF and observing the effects on cell proliferation (figure 20). The combination of SCF and FLT-3 increased the proliferation compared to the other culture conditions. Note: concentrations of SCF remained constant at 50ng/ml throughout the experiment. As expected FLT-3 and 5637 gave the lowest cell yield just under $3x10^6$, however the combination of FLT-3 and SCF generated 1 million more cells by day 12. From the experiment it would be difficult to conclude that the addition of FLT-3 in combination with SCF enhances proliferation due to the large error bars produced.

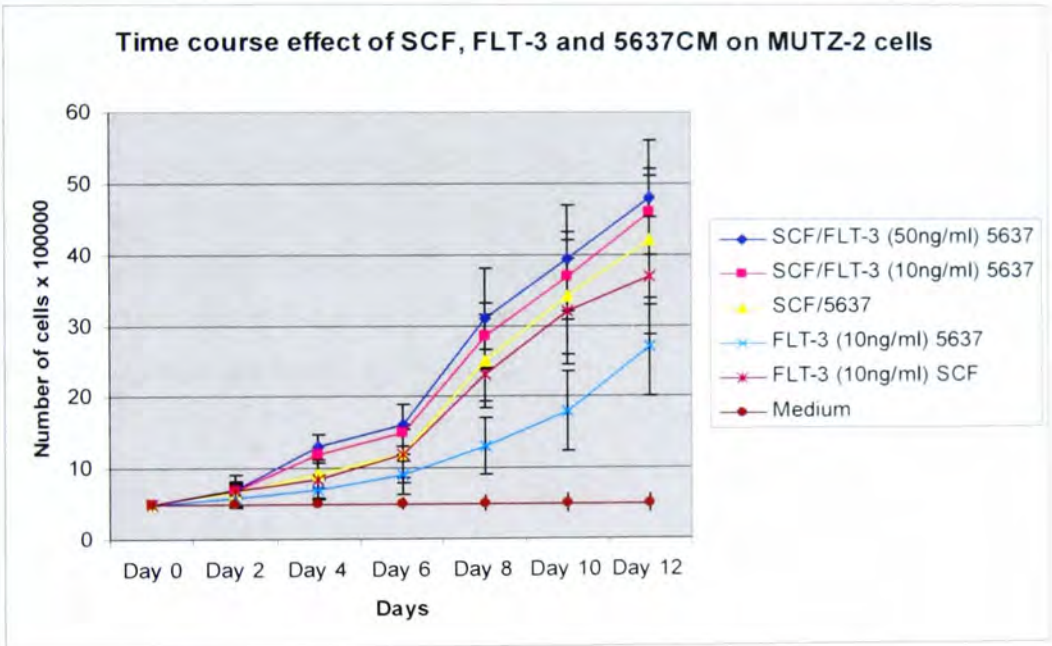

Figure 20: Graphical representation studying the effects of SCF, FLT-3 and 5637 CM on MUTZ-2. Cells were seeded at $5x10^5$ cells per well Note concentrations of SCF remained constant at 50ng/ml throughout the experiment. FLT-3 and 5637 produced the lowest cell yield just under $3x10^6$, however the combination of FLT-3 and SCF produced an extra 1 million cells by day 12. The experiment was carried out in triplicates (n=3).

These experiments suggest that the highest cell numbers were achieved with the 50ng/ml SCF, 10ng/ml FLT-3 and the conditioning medium 5637. Unfortunately it is not possible to quantify the contents of this conditioning medium and cytokines even though we pooled the supernatants creating one main batch. SCF alone produced poor proliferation results e.g.; in figure 20 on day 10 SCF alone produced 1 million cells whereas with 5637 and SCF there was a 4 fold increase. On day 12 SCF/FLT-3 results are comparable to using SCF/5637 producing a cell count of approx 4 million and was the fourth optimum condition in that experiment after SCF/5637 as shown in figure 20. Varying the concentration of FLT-3 used was also investigated but this did not seem to have a major effect on the cell number hence 10ug/ml was decided upon for further culture

From the results it became apparent that an alternative combination to the recommended cocktail of 5637CM and SCF was FLT-3 and SCF. The benefit of using these cytokines was that a known concentration of cytokine was being added to the cells as opposed to using the conditioning medium which contained unknown concentration of cytokines and growth factor. In summary, due to the large error bars produced it would be impossible to extract any meaningful conclusions from these results other than MUTZ-2 cells are capable of proliferation under various conditions but exhibit extremely unpredictable growth patterns making them unstable as a cell line.

3.3.3 CD133 expression on CACO-2 cell line

Caco-2, a colon cancer cell line was also investigated. In contrast to MUTZ-2 cell line it is an adherent cell line. The cell line readily expressed CD133 at values of about 98% for both CD133 epitopes (figure 21).

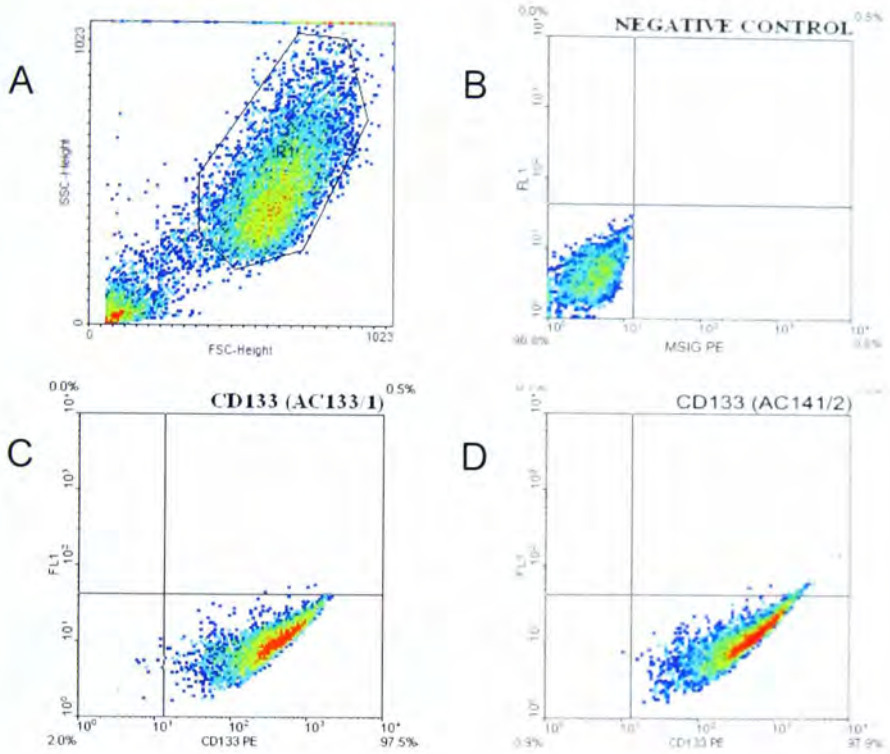

Figure 21: Phenotypic expression of CD133 (AC133 and AC141) on Caco-2 cells. A) gating strategy employed during flow cytometric analysis of MUTZ-2 cells labelled with CD133, The gated region R1 was applied to **B)** irrelevant matched isotype control, **C)** CD133/1 (AC133) PE immunofluorescent staining gave a positive result of 97.5%± 3.6 SEM expression (n=6) and **D)** single positive CD133/2 (AC141) PE immunofluorescent staining. Positive cell count indicated for each quadrant at the corresponding corner of plots. CD133 expression gave a mean percentage of 97.9%± 2.7 SEM.

3.3.4 Positive collection of $CD34^+$ cells from cord blood.

Primary cell were collected and cultured as the ultimate aim was to repeat the cell line work using these cells. Expression of CD133 was analysed using flow cytometry and expression was found to be 70% post selection.

The rarity of CD133 positive cells makes it necessary to enrich them before performing functional studies. We enriched for CD133 positive cells using CD34 which is co expressed on CD133 cells thus avoiding stimulation of the CD133 receptor and initiating downstream effects. After magnetic bead separation the purity of cells were measured. There was a 95% positive $CD34^+$ population as shown using FACs analysis (figure 22).

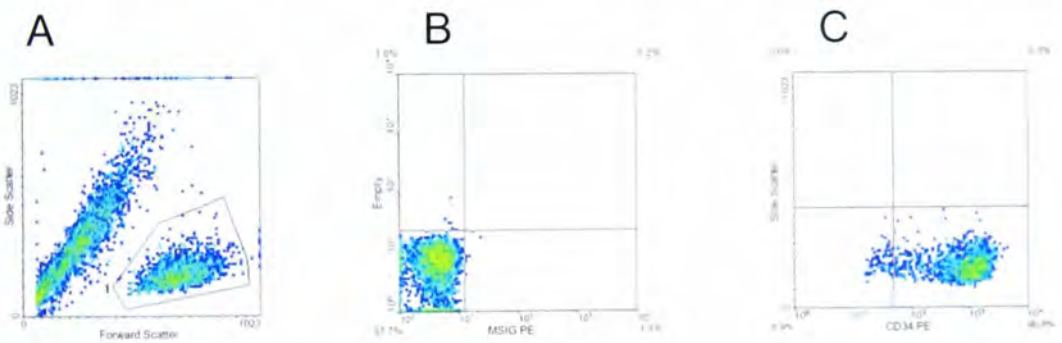

Figure 22: Flow cytometry analysis after Mini MACS purification of umbilical cord blood (UCB) cells. The eluted cells were double-stained with (B) non specific isotype controls and with (C) CD34 PE antibodies. The percentage of cells in each quadrant is indicated. Overall, the purity of CD34 cells was consistently more than 90%± 5.7 SEM (n=5).

3.3.5 Effects of freezing and thawing primary cells

It was also important to establish that freezing the primary $CD34^+$ cells for a prolonged period was not affecting the CD133 receptor sites in any way. The results show the differences between the frozen and fresh samples (figures 23 to 26).

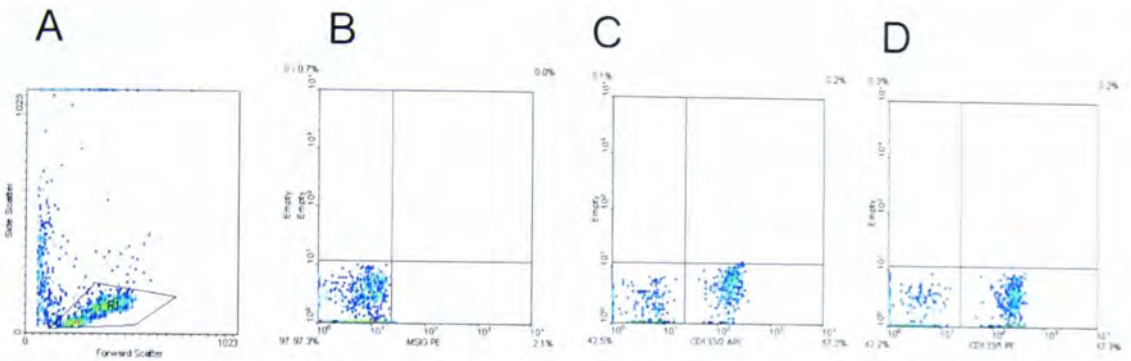

Figure 23: Flow cytometric analysis of CD133 expression on Patient X with Mab CD133/2 on fresh $CD34^+$ cells. Two experiments were conducted, and represented by dot-plot profile as shown. A: R1 indicates the gate for $CD34^+$ cells. B: indicates the MSIG control PE (C) CD133/2 APC Mab (57%± 2.6 SEM) (D) CD133/1 PE (57%± 3.8 SEM) (n=2).

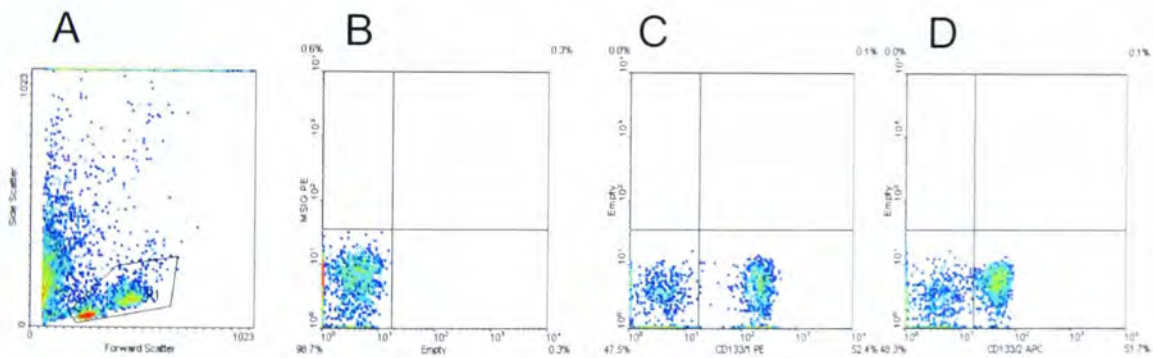

Figure 24: Flow cytometric analysis of CD133 expression on Patient X with Mab CD133/1 on frozen $CD34^+$ cells. Two experiments were conducted, and represented by dot-plot profile as shown. A: R1 indicates the gate for $CD34^+$ cells. B: indicates the MSIG control PE (C) CD133/1 PE Mab (52%± 2.0 SEM) (D) CD133/2 APC (52%± 3.5 SEM) Note cells were frozen for 2 weeks prior to staining (n=2).

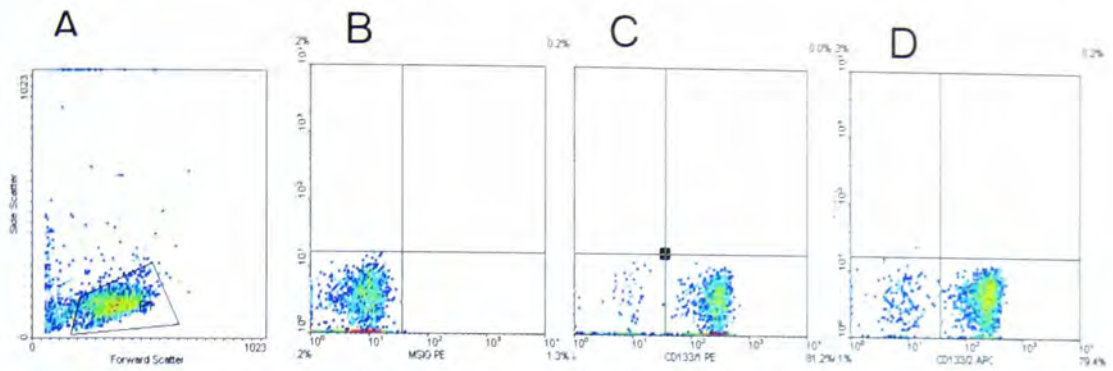

Figure 25: Flow cytometric analysis of CD133 expression on Patient Y with Mab CD133/2 on fresh CD34$^+$ cells. Two experiments were conducted, and represented by dot-plot profile as shown. A: R1 indicates the gate for CD34$^+$ cells. B: indicates the MSIG control PE (C) CD133/1 PE Mab (81%± 1.4 SEM) (D) CD133/2 APC (80%± 2.4 SEM) (n=2).

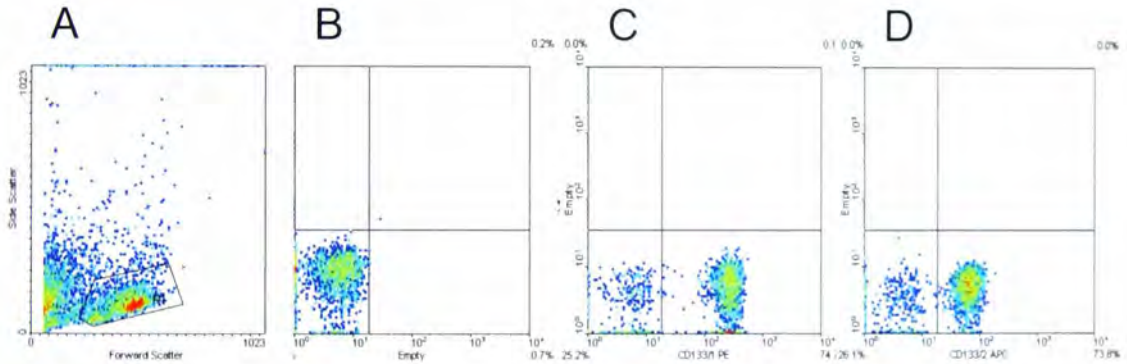

Figure 26: Flow cytometric analysis of CD133 expression on Patient Y with Mab CD133/1 on frozen CD34$^+$ cells. Two experiments were conducted, and represented by dot-plot profile as shown. A: R1 indicates the gate for CD34$^+$ cells. B: indicates the MSIG control PE (C) CD133/1 PE Mab 75%± 3.1SEM) (D) CD133/2 APC (74%± 4.3SEM) Note cells were frozen for 2 weeks prior to staining (n=2).

In patient A there was a 5% difference between the fresh and frozen sample for CD133/1 and a 5.3% difference for CD133/2. In both cases the frozen sample produced the lower percentage. In patient Y 6% difference was observed with CD133/1 and a 5.5% difference observed with CD133/2, again the lower percentage being the frozen sample. However there is no significant difference between the two results when comparing frozen and fresh CD34$^+$ cells in both patients.

3.3.6 Effects of steric hindrance on $CD34^+$ cells

Steric hindrance was determined using direct immunofluorescent monoclonal antibody labelling. The results showed that steric hindrance has no effect on the binding ability of C133 Mabs on either epitopes 1 or 2. It was important to note that the experiment was carried out using a directly conjugated antibody. Figure 27A shows the profile of the cells gated, figure B is the negative control. In figure C CD133 /1 was added initially to the $CD34^+$ cells, after which CD133/2 was added whereas in D CD133/2 was added first followed by CD133/1. Figures E-H are repeated using CD34 positive cells from a different patient. CD133 expression varied across patients but for each individual patient the results were comparable. Patient X showed in fig C a double positive percentage stain of 50.5% whilst in fig D the double positive percentage was 54.3%.

Patient Y also had similar percentages, fig G 29.9% and fig H 27.6% suggesting no steric hindrance.

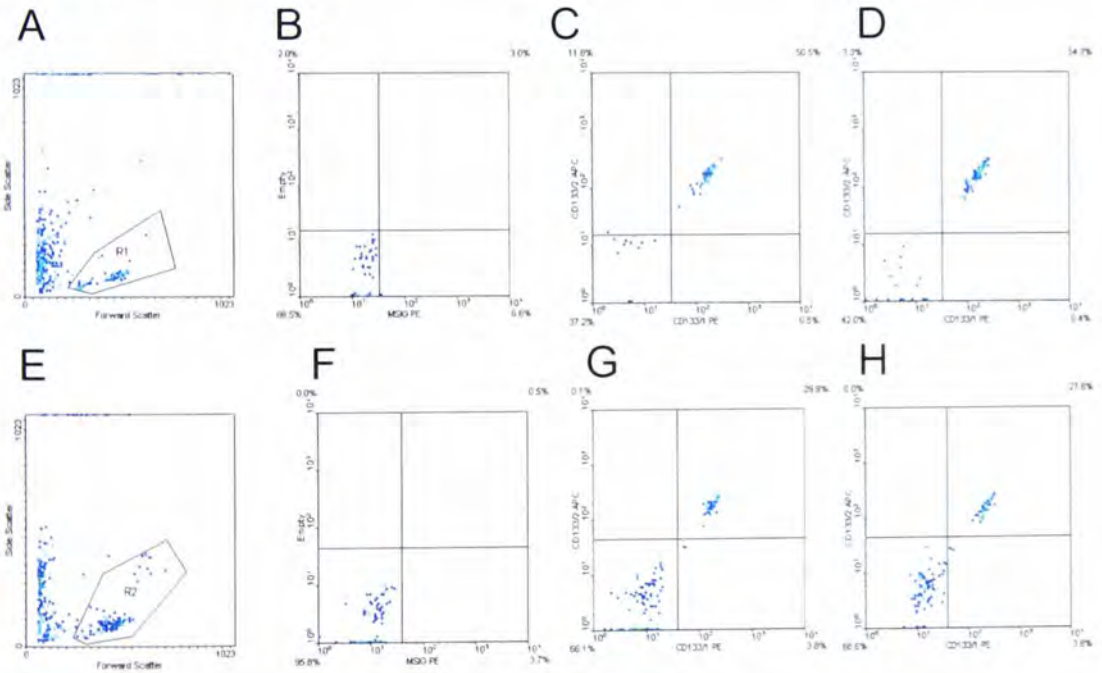

Figure 27: The CD133 Mab showed no steric hindrance of the CD133 receptor. A-D Flow cytometric analysis of CD133 expression on Patient X using fresh $CD34^+$ cells A: R1 indicates the gate for $CD34^+$ cells. B: shows the MSIG control PE (C) Mab CD133/1 PE was added initially followed by the addition of Mab CD133/2 APC. (51%± 3.2 SEM) (D) Mab CD133/2 APC was added firstly in this case followed by the addition of Mab CD133/1 PE (54%± 1.1 SEM). E-G Patient Y E: R1 indicates the gate for $CD34^+$ cells. F: shows the MSIG control PE (G) Mab CD133/1 PE was added initially followed by the addition of Mab CD133/2 APC (30%± 4.6 SEM). (H) Mab CD133/2 APC was added firstly in this case followed by the addition of Mab CD133/1 PE (28%± 3.1SEM) (n=2).

3.3.7 Short term expression of CD133 on primary cells

Maintenance of the selected $CD34^+$ cell was achieved using 50ng/ml SCF and 10ng/ml FLT-3 (Dr. Abuakwa, St Georges, London, Personal Communication) as mentioned earlier. The rate at which the $CD34^+$ cells proliferated was encouraging, but issues arose when measuring expression of CD133. Flow cytometry indicated that as $CD34^+$ cells were being maintained *in vitro,* they were differentiating and loosing CD133 expression.

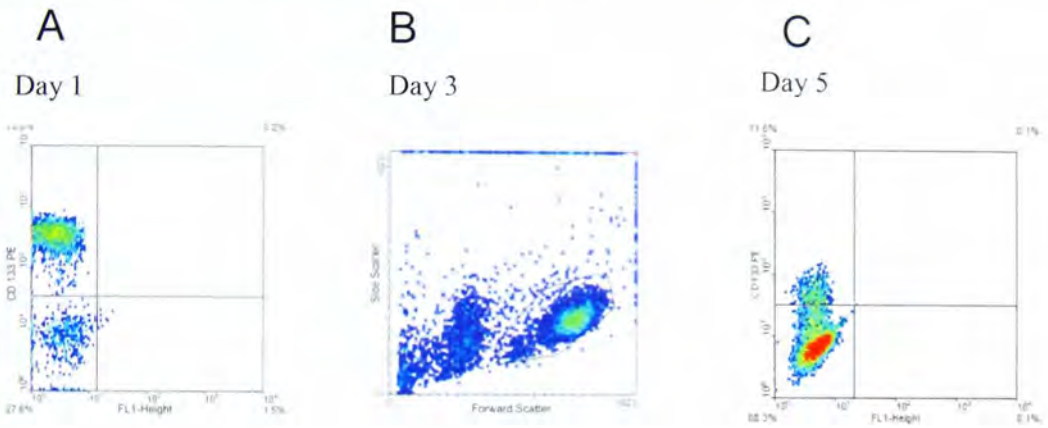

Figure 28: Graphical representation depicting the change in CD133 expression over 5 days on $CD34^+$ cells The variation in expression on $CD34^+$ cells cultured with SCF and FLT-3. **A)** Shows day 1 with 71%± 4.3 SEM expression of CD133 receptor, **B)** shows forward/side scatter profile on day 5, R1 gate depicts the live cells in the sample and **C)** shows expression has now dropped to 12%± 7.8 SEM

The profile of the cells as shown in figure 28 indicated that the cells were not dying and the proliferation data clearly indicate the cells were dividing but the CD133 expression was decreasing. The cytokines and growth factors were varied to try and overcome this problem but could not maintain $CD133^+$ cells for sufficient time to conduct useful long term experimentation.

3.4 Discussion

The work carried out in this chapter was aimed at determining the correct culture conditions of the cells used and any further confounding factors which may influence further experimentation. CD133 expression is found on a variety of cell lines for example colonic, gastric, lung, pancreatic, haemopoietic and melanoma. The basic findings suggest that MUTZ-2 would have been the ideal cell line to functionally study CD133 due to its haemopoietic background. The cell lines express CD133 at levels of 40% which is a sufficient enough level to visualize expression. Importantly low enough to prevent any problems we may encounter due to the down regulating and knocking down expression such as partial receptor.

Standardising culture conditions for MUTZ-2 was difficult and still required undefined media ie 5637. Even under optimised condition the proliferation of the MUTZ-2 cell line was unpredictable. Initially we thought cell stasis may be due to cell senescence owing to high cell passage numbers or CMV infection. New cells were purchased and the experiment carried out but still growth problems occurred. We then investigated the likelihood that the bulk 5637 conditioning media, was contaminated or again for some reason inactive, however the cells failed to grow with SCF and FLT-3 alone whereas previously growth was possible. We investigated whether this problem was unique and found that the cell line had only ever been were used by Drexler's group (1996) to investigate the effect FLT-3 had on proliferation and survival growing the cells for a short time period. No other citations were found in association with MUTZ-2.

Looking for alternative CD133 positive cells we investigated endothelial cell lines as endothelial stem cells express CD133. Human Umbilical Vein Endothelial Cells are generated from endothelium of the cord. It is known that CD133 is found on endothelial stem cells and also endothelial progenitor cells (Hilbe *et al.*, 2004) and thus one would assume the presence of CD133 on their surface. Phenotypic analysis of HUVEC cells from 2 individuals obtained from umbilical cords, taken with consent from St Georges Hospital, and cultured showed no CD133 expression (results not shown). A literature review confirmed lack of CD133 expression on the available endothelial cell lines.

Embryonic stem (ES) cells are derived from totipotent cells of the early mammalian embryo and are capable of unlimited, undifferentiated proliferation *in vitro*. H1, H7 and H9 embryonic cell lines all express CD133 (Carpenter *et al.*, 2003) and would be a close lineage to a haemopoietic cell line. However due to ethical reasons a handling license is required to work with these cell lines making them impossible for us to study.

CD133 expression is found in a number of colonic cell lines such as colo 201, HT29, Lovo, WiDr, CaR-1 and Caco-2 (Ieta *et al.*, 2008). Caco-2 cells although of epithelial origin were investigated as an alternate cell line and expressed CD133 at high levels of 98%, which potentially could be a problem due to the high CD133 density on the cells. Caco-2 became the preferred cell line of choice due to the fact they readily expressed CD133 and growth conditions/growth curves were reproducible compared to the MUTZ-2 line. Many of the other colonic cell lines minimally expressed CD133 and were difficult to culture therefore Caco-2 was an obvious choice. The only drawback was they are not of haemopoietic lineage, so drawing conclusions on function from a haemopoietic perspective may prove to be difficult.

We investigated using primary cells and were able to select positively, producing 90% $CD34^+$ cell purity after Immuno magnetic separation. The sample was then CD133 enriched to 70%. We investigated the effect of steric hindrance and freezing on primary cells using flow cytometry. Results showed that neither factors influenced the expression of CD133 or the phenotypic cell profile shown by the forward side scatter plot.
However, the rate at which CD133 expression was lost from primary cells in culture posed a problem for experiments. Cells lost 60% CD133 expression from day 0 to 5. This caused a potential problem when considering these cells for the knockdown experiments which required 4 days for loss of CD133 phenotype through knockdown. It would be difficult to conclude whether CD133 was being down-regulated by the knockdown procedure or merely loosing expression due to differentiation, limiting the extent to which primary cord blood cells can be utilised in this study.

Chapter 4: Knockdown of CD133 cell surface expression and functional analysis of CD133

4.1 Aims and objectives

This chapter aims to knock down the expression of CD133 on the surface of MUTZ-2 and Caco-2 cell lines and carry out functional experiments to determine a potential role for CD133 in cell biology.

The primary aims of this chapter were:

- To create a permanent CD133 knock out cell line using a plasmid vector on Caco-2 cells and MUTZ-2 cells.
- To knock down CD133 expression using RNAi technology in both cell lines.
- To determine the rate of recycling of the CD133 receptor on the cell lines after knockdown.
- To investigate knockdown affects of CD133 on proliferation, adhesion, Mab cytoxicity and cell cycle.

4.2 Introduction

4.2.1 CD133 gene knockdown using RNAi interference

RNA interference (RNAi) is a natural cellular process that regulates gene expression and provides an innate defense mechanism against invading viruses and transposable elements (Kim *et al.,* 2007).Within living cells it also helps to control which genes are active and how active they are. Two types of small RNA molecules microRNA (miRNA) and small interfering RNA (siRNA) are central to RNA interference (Munker *at al,* 2011). RNAs are the direct products of genes, and these small RNAs can bind to specific other RNAs and either increase or decrease their activity, for example by preventing a messenger RNA from producing a protein. RNA interference has an important role in defending cells against parasitic genes, viruses and transposons but also in directing development as well as gene expression in general (Davidson *et al.,* 2011).

4.2.2 The Mechanism of RNA Interference

Long double-stranded RNAs (dsRNAs; typically >200 nt) can be used to silence the expression of target genes in a variety of organisms and cell types (e.g., worms, fruit flies, and plants). Upon introduction, the long dsRNAs enter a cellular pathway that is commonly referred to as the RNA interference (RNAi) pathway (Sidahmed *et al.,* 2010). First, the dsRNAs are processed into 20-25 nucleotide (nt) small interfering RNAs (siRNAs) by an RNase III-like enzyme called Dicer (initiation step). Subsequently, the siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs), unwinding in the process. The siRNA strands guide the RISCs to complementary RNA molecules, where they cleave and destroy the cognate RNA (affecter step). Cleavage of cognate RNA takes place near the middle of the region bound by the siRNA strand.

4.3 Knockdown Results

4.3.1 CD133 knockdown using the psilencer neo siRNA vector

When initially attempting the knock down work, siRNA's from Invitrogen and Ambion were used. Unfortunately after varying both concentrations of RNA, incubation times and transfection reagent volume no knockdown could be detected using RT PCR and cellular techniques (data not shown).

The next step was to create a plasmid or a permanent knockout cell line lacking the CD133 gene. Again the product was purchased from Ambion, a psilencer neo siRNA vector. It also contained a neomycin resistant gene enabling antibiotic selection for enrichment of the successfully transfected cells. Colonies from plasmids 1, 2 and 3 were analysed to confirm the existence of the inserted anti CD133-RNAi DNA using the restriction enzymes Hind III and BamH I and the DNA subjected to electrophoresis. An oligonucleotide of 40bp was also used as a control to confirm the 60bp band. The primer was a 40mer MPa1.31, and was used because the 100bp ladder had no bands below 100bp. RNAseA enzyme was also added to the samples to remove any RNA that may contaminate and blur the gel. The presence of a band size approx 65bp just above the primer band and slightly below the 100bp ladder confirms that the insert was the anti CD133 RNAi insert. Lanes were run containing plasmid alone with no restriction enzymes to act as controls (figure 29).
For the purpose of confirming the presence of the 65 base pair band encoding the relevant sequence and to also show that the orientation of the insert was correct the purified plasma DNA was then digested with the enzymes HindIII and EcoRI. The results show quite clearly that the majority of colonies analysed including plasmids 1, 2 and 3 from figure 29 excised a 170 base pair product, as would be expected from the correctly constructed plasmid (Figure 30). Orientation was also confirmed by the size of the band produced. If the oligonucleotide had been inserted backwards the size of the vector produced would be less than 170bp producing a visual band of 101bp.

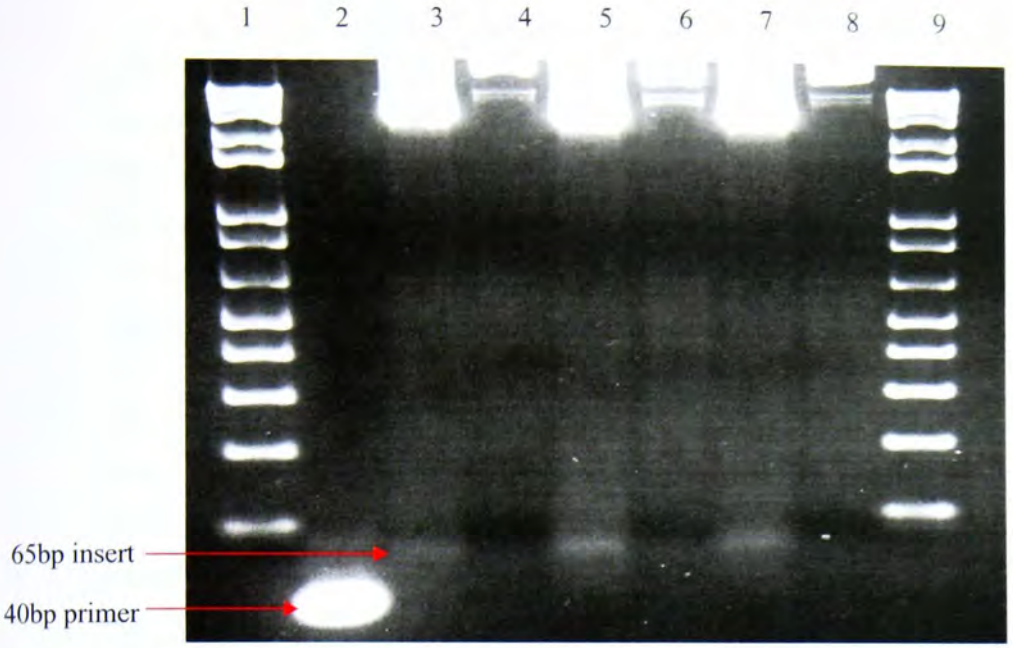

Figure 29: Gel electrophesis showing the presence of the CD133 insert band with control primer. Figure **A** Lane 1 and 9 contain Kb plus DNA ladder, lane 2 contains a 40bp primer (40mer MPa1.31) and was used as a control, lanes 3- 8 contain different cloned plasmids containing the insert. The plasmids were cut with the restriction enzymes BamH I and Hind III producing a band width of about 65bp shown in figure 6. Lanes 3, 5 and 7 contained the plasmid and restriction enzymes and clearly show a band below the 100 bp ladder and above the 40 bp primer showing a 60bp product. Lanes 4, 6 and 8 show the uncut plasmid with no restriction enzymes added with no DNA fragments produced. Note RNAse A was added to the samples to eliminate the possibility of RNA contamination.

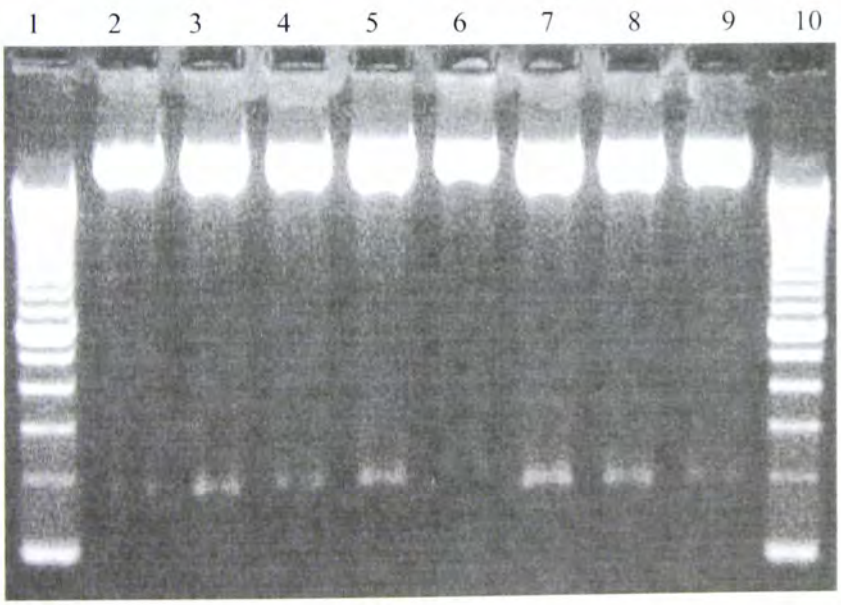

Figure 30: Gel electrophesis confirming the presence and correct orientation of the CD133 insert band. Figure **A** Lane 1 and 10 contain Kb plus DNA ladder, lanes 2- 9 contain different cloned plasmids containing the insert. The plasmids were cut with the restriction enzymes HindIII and EcoRI producing a band length of 170bp.

A G418 curve (Kill curve) was created; this identifies the lowest level of G418 that kills non transfected cells within 10 days by testing antibiotic concentrations from 200-1000µg/ml whilst keeping all other culture conditions equal (Figure 31).
We decided to use the Gentamicin at a concentration of 600µg/ml to select for transfected cells. The cells were cultured for 14 days with the Gentamicin and other cell culture reagents and tested for knockdown every couple of days using flow cytometry.

After culturing the cells for 10 days the cells in the control culture containing G418 were all dead. Unfortunately 98% of the cells in the plasmid culture were also dead showing that the cells did not successfully take up the plasmid vector or there was a problem with the selection process, but this was unlikely, as our control cell culture showed the cells were killed after 14 days. The few cells that remained were washed and analysed using flow cytometry and showed no level of knockdown (results not shown). This was repeated numerous times varying seeding densities, plasmid concentrations and gentamicin concentrations however we were unable to successfully transfect and isolate the cells.

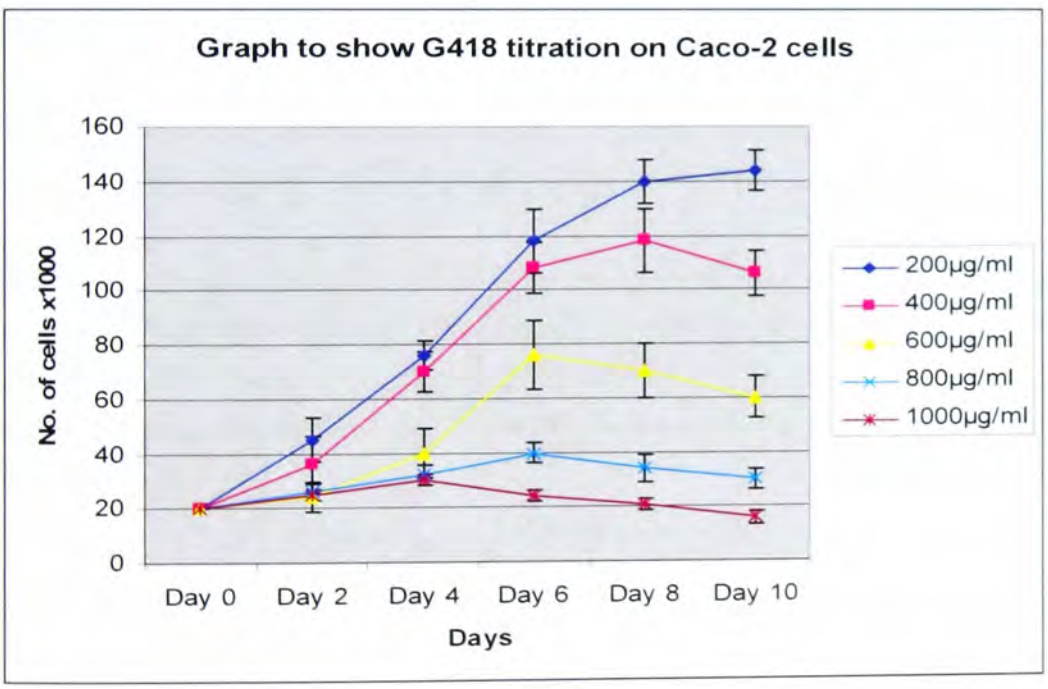

Figure 31: Gentamicin kill curve was plotted by measuring the proliferation of Caco-2 cells subjected to various concentrations of gentamicin ranging from 200µg/ml to 1000µg/ml. The concentration suitable for selecting cells containing the plasmid was 600-800µg/ml so cells were set up using 600, 700 and 800µg/ml of gentamicin. At day 15 all cells subjected to concentrations above 600µg/ml were all dead (n=2).

4.3.2 CD133 knockdown on Caco-2 and MUTZ-2 cell lines using Qiagen siRNA oligonucleotides

We then returned back to RNAi technology purchasing oligonucleotides from Qiagen LTD, Crawley, West Sussex, UK. In total, five different CD133 RNAi oligonucleotides (CD133 RNAi_1 to 5) were tested to knock down CD133 expression. Initially the concentration recommended by Qiagen was 50nM. However the experiments quickly showed that at this concentration CD133 knockdown was minimal.

As well as varying the concentrations of RNAi we also looked at various time points to determine CD133 knockdown, 24 hours, 48 hours and then 72 hours. We established that the ideal time point was 48 hours as 24 hours was too early to appreciate the full extent of the knockdown and there was not much difference between 48 hours and 72 hours other than the cell profile was beginning to change, showing possible stress and in some cases the level of knockdown was also lower. At such low levels of interference it would be very difficult to conclusively state 48 hours was the best time period relying on RNA alone, but this was also validated later on when high levels of surface antigen knock down were achieved. The level of knockdown in Caco-2 cells was investigated phenotypically and transcriptionally using flow cytometry and real time PCR respectively.

Phenotypic expression of CD133 was examined after using a variety of different concentrations of RNAi oligonucleotides ranging from 10nM to 200nM and were analysed after 48 hours incubation. The phenotypic data showed that knockdown occurred at the higher concentrations of RNAi, such as 200nM where a 6.5%±3.8 SEM knockdown was observed (Figure 32).

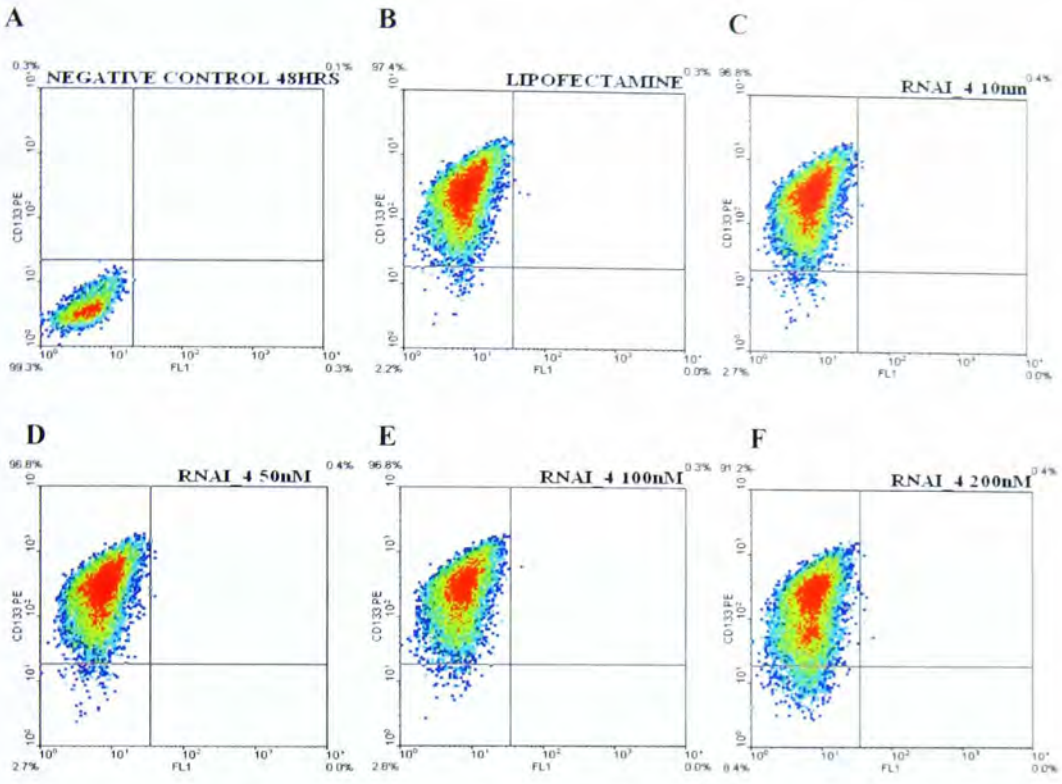

Figure 32: Caco-2 cells stained with CD133 PE to determine CD133 expression. The gated region R1not shown was drawn to exclude cell debris and dead cells and was applied to all samples **A)** Cells transfected with transfection reagent alone (irrelevant matched isotype control IgG1 PE). **B)** Caco-2 cells transfected with the transfection reagent alone thus showing normal expression of CD133.**C)** Cells transfected with the 10nM RNAi_4 oligonucleotide producing a knockdown in CD133 expression of <1% **D)** 50nm RNAi_4 oligonucleotide producing <1% knockdown, E) 100nM RNAi_4 oligonucleotide producing a <1% knockdown, **F)** 200nM RNAi_4 oligonucleotide producing a knockdown of 6.5%±3.8 SEM (n=3).

The results obtained did not give any indication that the percentages expressed were relative to the dose of oligonucleotides. The 200nM may have been the threshold dose needed to elicit a response. On attaining this low level of knockdown it was important to analyse the molecular affects of the RNAi molecules and determine if there were any changes in CD133 mRNA levels. The RNAi may be working at a genotypic level but not phenotypically. PCR analysis was carried out on two concentrations 100nmol and 200nmol using a (GAPDH) primer as a control. Samples containing cells alone and cells incubated with lipofectamine were also analysed as controls, Figure 33 shows the amplification plots for the control primer, GAPDH. This primer acts as a statutory control eliminating sample

variation error factors. All the samples measured were normalized against GAPDH expression. The experiment was carried out in duplicates with the blue and yellow circles representing control cells, red square and turquoise circle represent cells that had been incubated with transfection reagent alone, grey diamond and orange triangle represent cells incubated with 100nmol CD133_4 RNAi and green triangle and purple square represent a 200nmol concentration of RNAi. Cells were incubated for 48 hours before PCR analysis. The threshold value (vertical line), gives the threshold cycle for each control and sample. Average Ct values for samples containing cells alone was 19.8, Cells (lipofectamine control) 19.8, RNAi 100nmol 20.21 and for RNAi 200nmol 20.22. The variation in GAPDH mRNA between samples was minimal with a Ct range of 0.4.

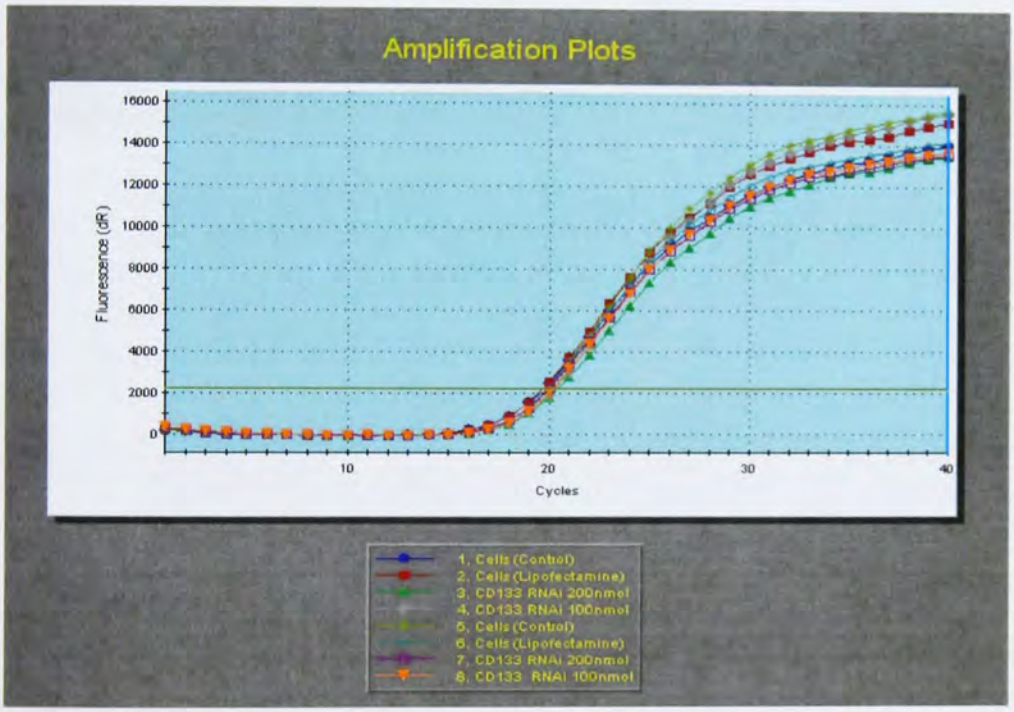

Figure 33: Amplification plots with GAPDH primer.
RNAi knockdown was performed on Caco-2 cells using various RNAi concentrations for 48hours at which point real time PCR was performed. Average Ct values for samples containing cells alone was 19.8, Cells (lipofectamine control) 19.8, RNAi 100nmol 20.21 and for RNAi 200nmol 20.22. The variation in GAPDH mRNA between samples was minimal with a Ct range of 0.4.

Figure 34 shows the amplification plots for the CD133 primer. Again the experiment was carried out in duplicates with the blue and yellow circles representing control cells, red square and turquoise circle represent cells that had been incubated with transfection reagent alone, grey diamond and orange triangle represent cells incubated with 100nmol CD133_4 RNAi and green triangle and purple square represent a 200nmol concentration of RNAi. Cells were incubated for 48 hours before PCR analysis. The threshold value (vertical line), gives the threshold cycle for each control and sample.

Average Ct values for samples containing cells alone was 16.1, Cells (lipofectamine control) 16.3, RNAi 100nmol 18.6 and for RNAi 200nmol 19.2. Fold decreases were established later using the GAPDH values and the Pear Formula.

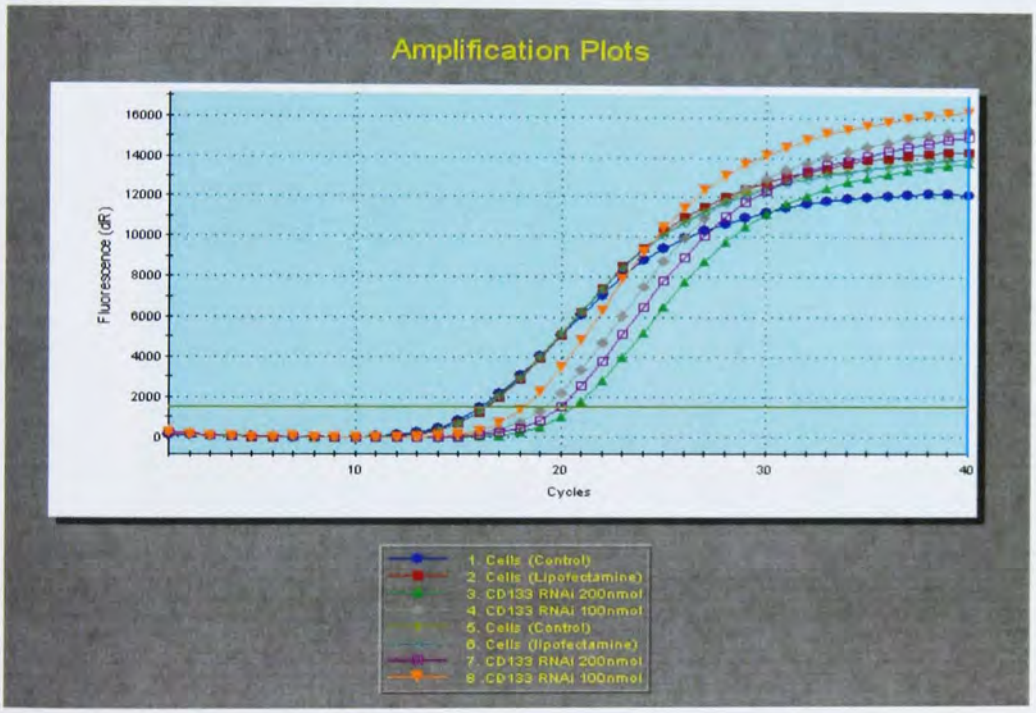

Figure 34: Amplification plots with CD133 primer.
RNAi knockdown was performed on Caco-2 cells using various concentrations for 48hours at which point Sybr Green real time PCR was performed. Average Ct values for samples containing cells alone was 16.1, Cells (lipofectamine control) 16.3, RNAi 100nmol 18.6 and for RNAi 200nmol 19.2. Fold decreases were established later using the GAPDH values and the Pear Formula.

It was important to run a dissociation curve following the real time PCR. This is due to the fact that SYBR Green will detect any double stranded DNA including primer dimers, contaminating DNA, and PCR product from misannealed primer. A dissociation curve ensures that the desired amplicon was detected.It is apparent from figure 35 that the Tm (melting point) of the amplicon) occurs at 78°C for the CD133 gene product and 84°C for GAPDH. Also we can see that no contaminating products were present in this reaction. Contaminating DNA, nonspecific products or primer dimers would show up as an additional peak separate from the desired amplicon peaks.

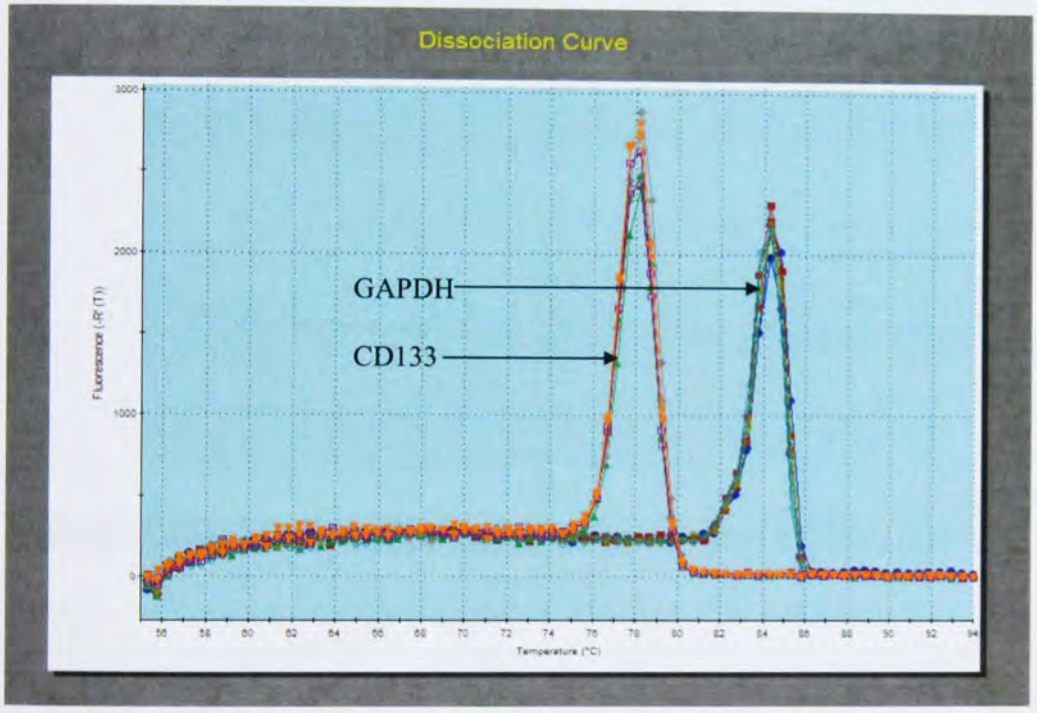

Figure 35: SYBR Green dissociation curve for the CD133 gene product at an Amplicon Tm of 78°C and GAPDH at 84°C.

To quantitatively determine the level of knockdown the efficiency of each primer had to be determined. This was done by creating a dilution curve for each primer (figure 36 shows dilution curve shown for CD133 only). Here the cDNA is diluted 1:10 creating a concentration gradient. The primer is added and the threashold cycle increases as the samples becomes more dilute. From this graph a standard curve is created as shown in figures 37 and 38 for CD133 and GAPDH respectively. From the slope of the curve the

efficiency of the primer (E) can be obtained. The efficiencies for GAPDH and CD133 were 86.4% and 98.4% respectively. E is important as it takes into consideration the fact that amplifications that occur during the PCR process are not perfect (that is, the template is not exactly doubled in each amplification cycle) thus this value indicates how efficient the primer is at amplifying the product providing a more accurate fold change.

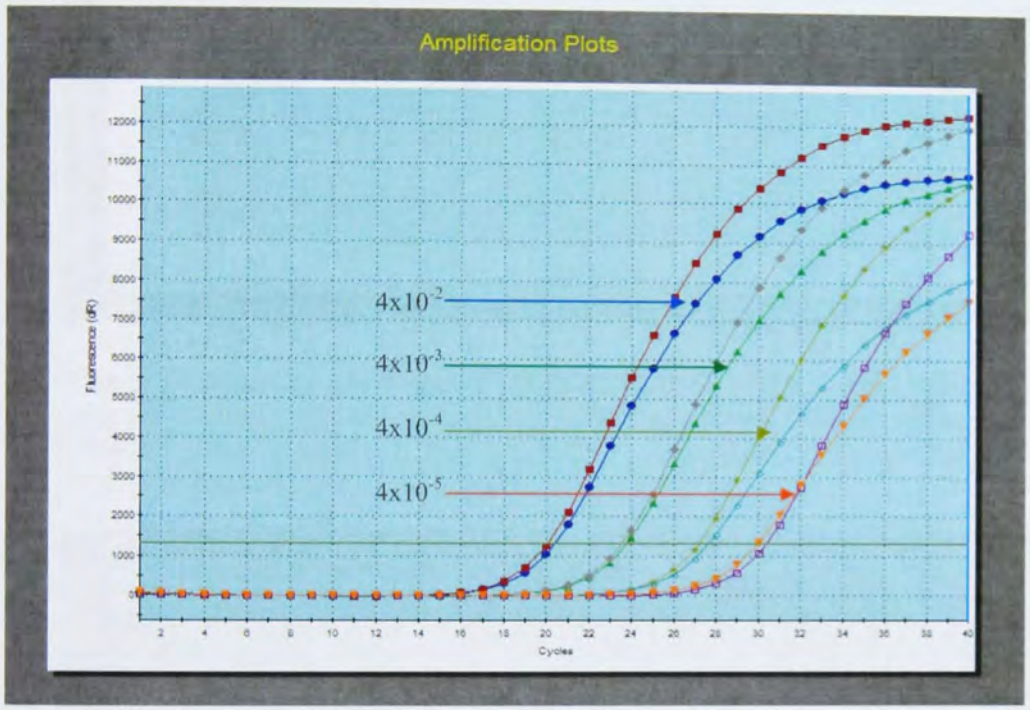

Figure 36: Amplification plots of CD133 mRNA levels carried out in duplicates were measured by real time quantitative PCR. Standard samples of $4x10^{-2}$ µg (red and blue lines), $4x10^{-3}$ µg (green and grey lines), $4x10^{-4}$ µg (light green and blue lines) and $4x10^{-5}$ µg (orange and purple lines)µg were amplified, and the ΔRn (fluorescent emission) was plotted against the cycle number.

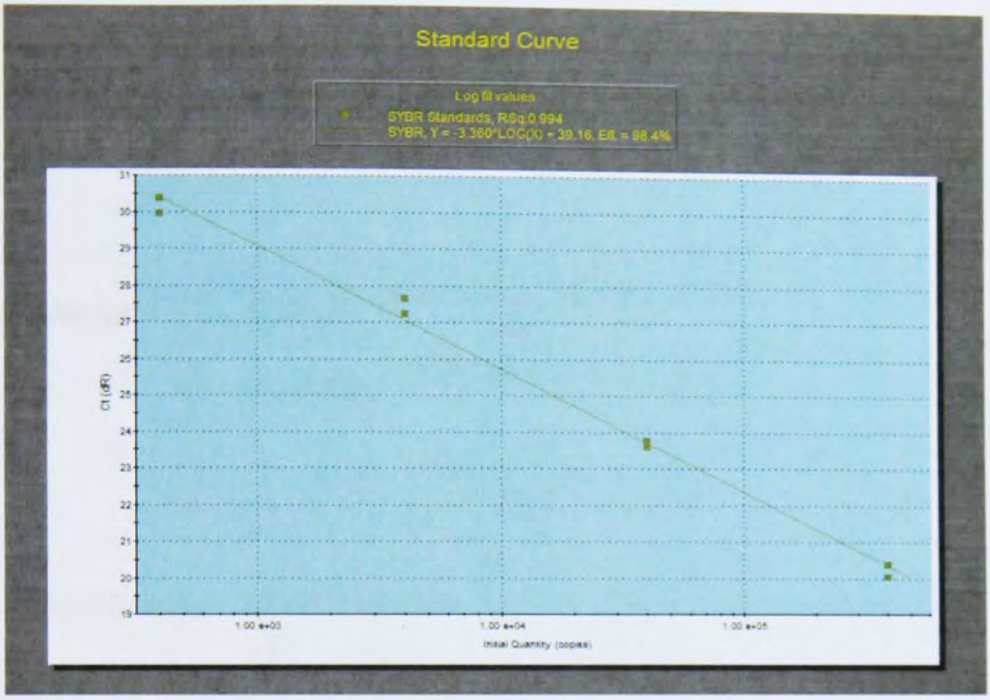

Figure 37: Standard curve for CD133 primer. mRNA levels measured by real time quantitative PCR. The amounts of standard samples were plotted against the threshold cycles in duplicates shown in figure 40. The gradient of the slope is used to work out the efficiency of the primer which was 98.4%.

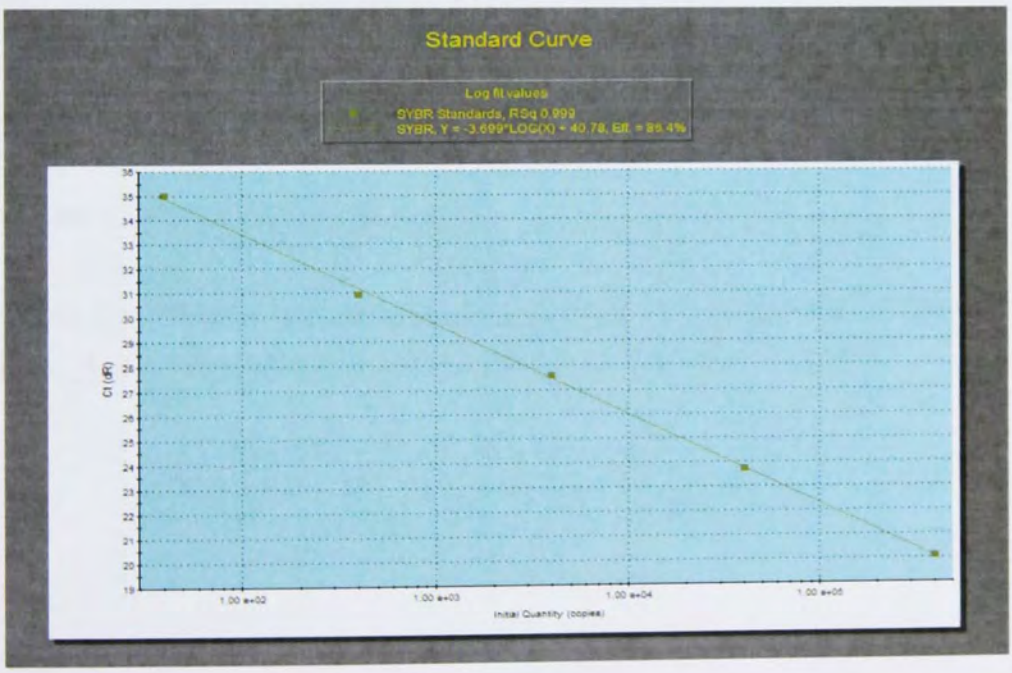

Figure 38: Standard curve for GAPDH primer. mRNA levels measured by real time quantitative PCR. The amounts of standard samples were plotted against the threshold cycles in duplicates (amplification plots for GAPDH primer not shown). The gradient of the slope is used to work out the efficiency of the primer which was 86.4%.

Now that the Ct values and the primer efficiencies have been calculated. It is possible to deduce the affect the RNAi oligonucleotides had on fold change. The formula used to calculate fold change is called the Pearsons formula.

$$\textbf{Fold Change} = \frac{(\text{E. Target})^{\text{Ct CD133 (CONTROL - EXPT)}}}{(\text{E. Refer})^{\text{Ct GAPDH (CONTROL - EXPT)}}}$$

For RNAi 100nmol = $1.984^{2.3}$ / 1.860.43

= 4.84 / 1.30

= 3.7 fold change decrease

For RNAi 200nmol = $1.984^{2.9}$ / $1.86^{0.42}$

= 7.29 / 1.30

= 5.6 fold change decrease

This can be expressed as a percentage value using the formula

% decrease = 100-1/ (Fold change X 100)

For RNAi_4 at 100nmol concentration the percentage fold change was 73% and for the RNAi_4 at 200nmol concentration the percentage fold change was 82%

Looking at the fold change in CD133 mRNA a knockdown of 82% was achieved in the 200nmol sample. Unfortunately knockdown at a phenotypic level was only 6.5% and definitely not adequate enough to perform the functional cell experiments, as the receptor would still be heavily present on the surface. Therefore other means had to be investigated Using real time PCR we investigated the mRNA levels of another RNAi oligonucleotide; RNAi_5 to measure its knockdown capabilities genotypically (Figure 39). The levels of knock down on average were similar to results obtained with the CD133_4 RNAi (n=3).

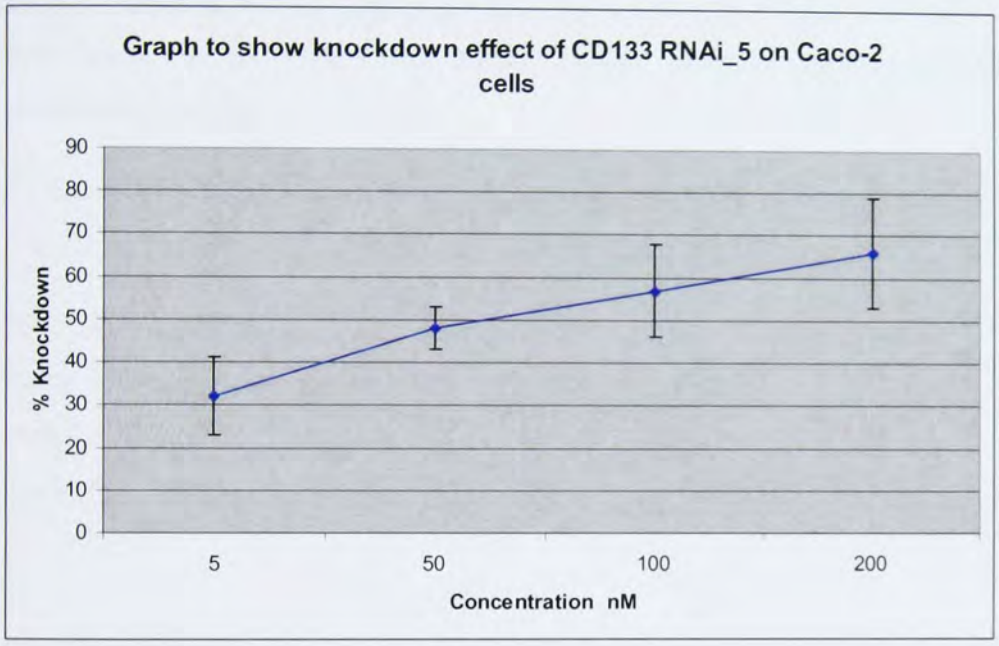

Figure 39: CD133 mRNA levels were measured by real time quantitative PCR after 48hr incubation with CD133_5 RNAi at concentrations of 0, 5, 50, 100 and 200nM. The experiment was carried out in duplicates. The highest level of knockdown was attained with a 200nmol concentration producing a 66% knockdown value. CD133 mRNA level was compared after correcting it using GAPDH mRNA level as an internal standard as described earlier (n=4).

We measured the percentage knockdown in all four CD133 RNAi oligonucleotides using flow cytometry and showed that the highest percentage knockdown was achieved by the Prom1_4 oligonucleotide knocking down CD133 by 7.2%. This was followed by the Prom1_5 oligonucleotide which had a knock down value of 6.4% (table 7). Unfortunately knock down values of 7.2% would not be adequate enough to perform functional experiments.

Table 7: showing the average percentage knock down on Caco-2 cells analysed by flow cytometry for the various four Qiagen RNAi's against the different concentration (n=3).

	5nM	10nM	50nM	100nM	200nM
Prom1_2	0	0	3.5	4	5.2
Prom1_3	0	2	2.3	4.1	6.3
Prom1_4	0	1.2	3	6.1	7.2
Prom1_5	0	0	3.4	5.3	6.4

Flow cytometry analysis was repeated on the MUTZ-2 cell line to investigate whether the RNAi oligonucleotides were cell line specific using all 4 RNAi oligonucleotides but unfortunately no level of knockdown was observed. Time periods of 48hrs and 72hrs were analysed with MUTZ-2 cells however no differences were observed. CD133 mRNA levels were also measured but no changes were observed (Figure 40).

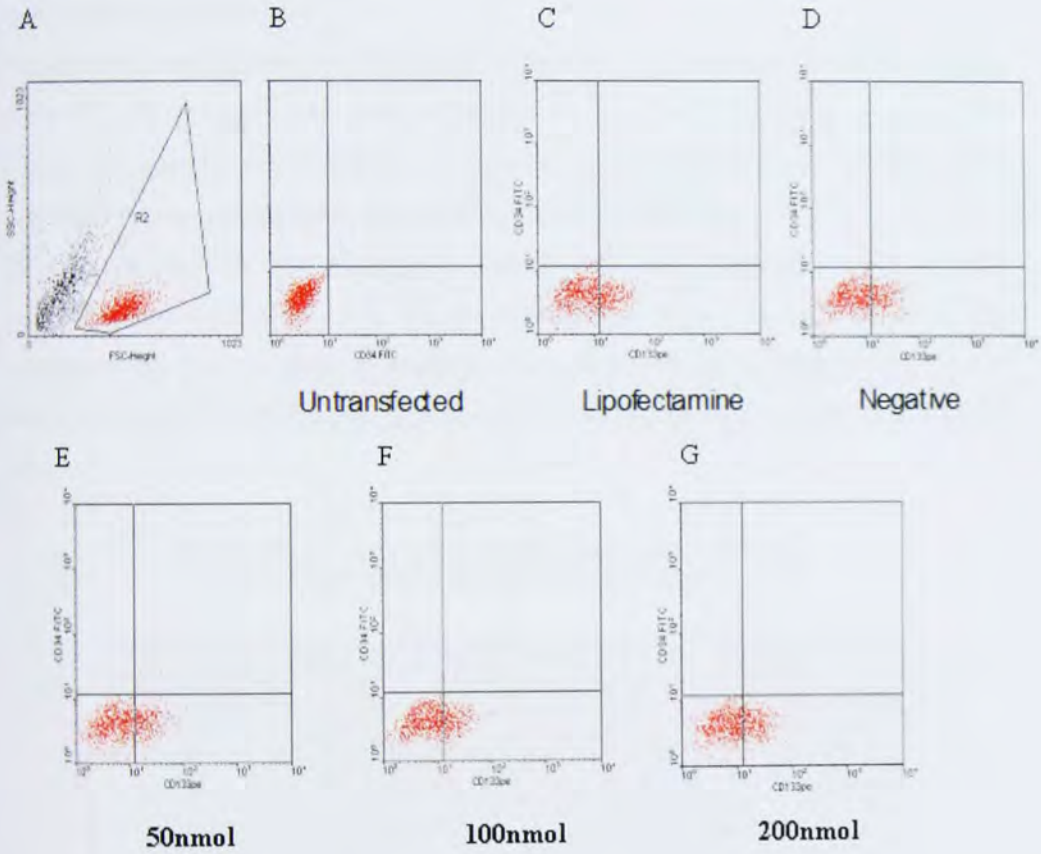

Figure 40: MUTZ-2 cells stained with CD133 FITC to determine CD133 expression **A)** gating strategy employed during flow cytometric analysis of MUTZ-2 cells after 48 hours. The gated region R1not shown was drawn to exclude cell debris and dead cells. The gated region R1 was applied to **B)** untransfected Caco-2 cells showing normal expression of CD133 (irrelevant matched isotype control IgG1 PE).**C)** Cells transfected with lipofectamine alone **D)** 100nmol scrambled sequence acting as a negative control **E)** 50nmol RNAi_4 oligonucleotide **F)** 100nmol RNAi_4 oligonucleotide, G 200nm RNAi_4 oligonucleotide. No significant change in expression was detected with all 3 RNAi concentrations ($p>0.05$ for all 3 concentrations). (n=5).

4.3.3 CD133 knockdown on Caco-2 and MUTZ-2 cell lines using Santa Cruz siRNA oligonucleotides

Due to the poor phenotypic knockdown achieved by the previous RNAi purchased from Qiagen, we attempted a repeat of the experiments using an siRNA oligonucleotide from Santa Cruz Inc, Heidelberg, Germany. Their product consisted of one siRNA which we tested initially using flow cytometry at various concentrations of siRNA.

Experiments were carried out in 6 well plates with concentrations of siRNA ranging from 0.5 – 6µg. Again 48 hours was used as a time period at which to analyse the extent of knockdown and initially the level of transfection reagent was used at the optimum volume suggested in the protocol which was 8µl/well.

The results showed that phenotypic knockdown was achieved over a different concentration range (figure 41). We concluded the highest level of knockdown was attained using 2µg of siRNA producing a knockdown of approximately 70% after 48 hours and the FACs profiles for this concentration and also 4µg can be seen in figure 42.

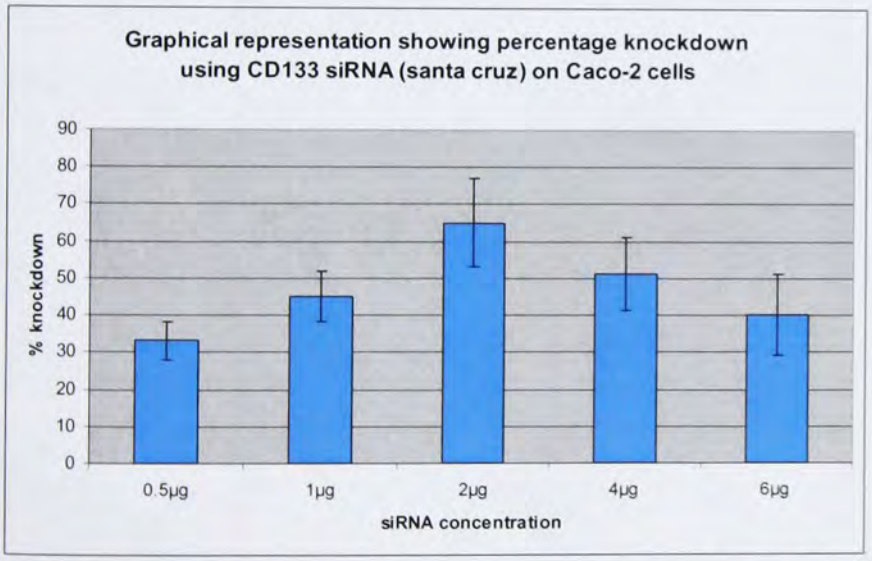

Figure 41: Quantitative comparisons using flow cytometry of CD133 knockdown on Caco-2 cells attained whilst incubating with RNAi (Santa Cruz) at concentrations of 0.5, 1.0, 2.0, 4.0 and 6.0µg.
Cells were incubated for 48hrs before analysis. The highest average percentage knockdown of 66% was achieved using 2ug RNAi and the lowest knockdown expression was 33% using 0.5µg. (n=4 in total but for 2.0ug concentration (n=7).

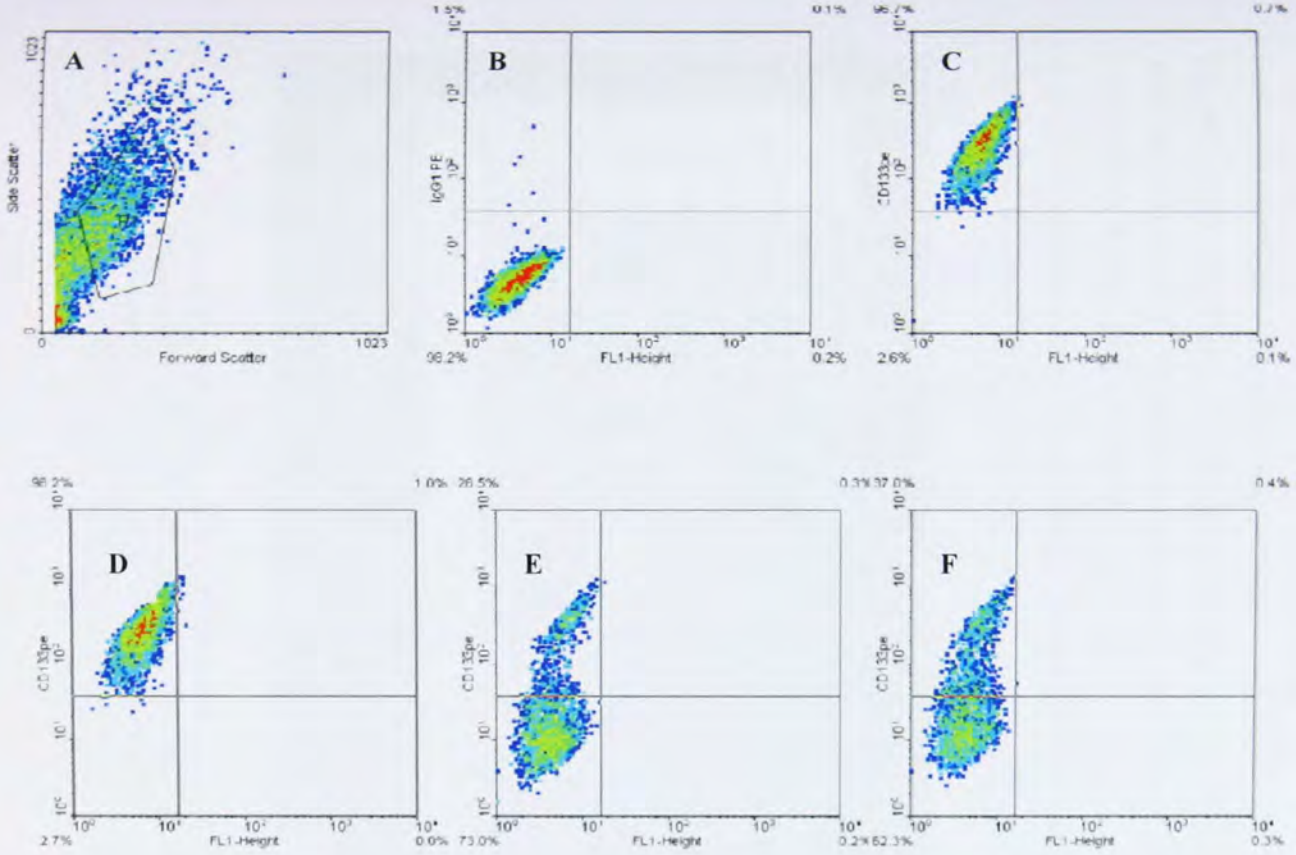

Figure 42: Caco-2 cells stained with CD133 PE to quantitate CD133 knockdown
A) Gating strategy employed during flow cytometric analysis of Caco-2 cells. The gated region R1 was drawn to exclude cell debris and dead cells. The gated region R1 was applied to **B)** cells transfected with lipofectamine alone (irrelevant matched isotype control IgG1 PE), **C)** Positive control; cells transfected with lipofectamine alone **D)** Negative control; 2µg scrambled sequence **E)** Cells transfected with 2µg RNAi (Santa Cruz) producing a knockdown in CD133 expression of 73% **F)** Cells transfected with 4µg RNAi (Santa Cruz) producing a knockdown of 62%.Cells harvested 48 hours before analysis.

Having successful achieved phenotypic knockdown of CD133 with the siRNA (Santa Cruz) we then investigated knock down at the mRNA level using real time PCR as demonstrated earlier. Real time PCR was carried out using the 2μg sample in triplicates. Figure 43 shows the amplification plots for the control GAPDH primer. The experiment was carried out in triplicates red square, turquoise circle and silver circle representing cells that had been incubated with transfection reagent alone and the grey circle, the orange square and the red triangle representing cells incubated with 2μg CD133 RNAi (Santa Cruz). Cells were incubated for 48 hours before PCR analysis. The threshold value (horizontal axis) was set at a fluorescence of 656.619. Average Ct values for samples containing cells with lipofectamine alone were 16.12 and for cells incubated with 2μg RNAi 16.31.

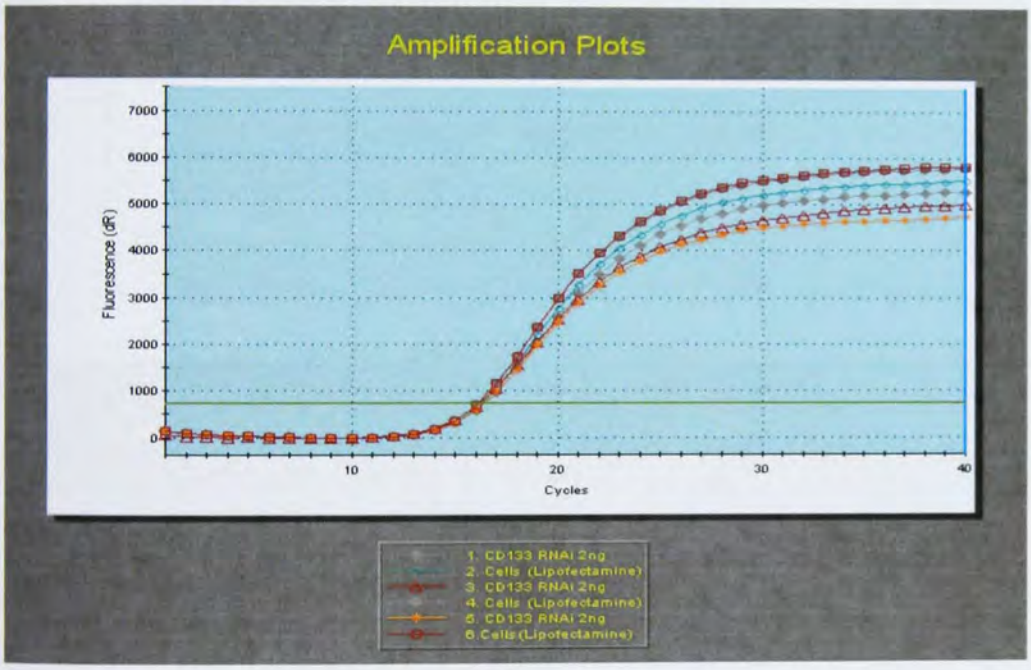

Figure 43: Amplification plots with GAPDH primer.
RNAi knockdown was performed using RNAi (Santa Cruz) at a concentration of 2ng for 48hours at which point real time PCR was performed. Average Ct values for samples containing cells with lipofectamine alone were 16.12 and for cells incubated with 2ng RNAi 16.31.

Figure 44 shows the amplification plots for the CD133 primer. Again the experiment was carried out in triplicates red square, turquoise circle and silver circle representing cells that had been incubated with transfection reagent alone and the blue circle, yellow circle and the green triangle representing cells incubated with 2µg CD133 RNAi (Santa Cruz). Cells were incubated for 48 hours before PCR analysis. The threshold value (vertical line) was set at a fluorescence of 656.619. Average Ct values for samples containing cells with lipofectamine alone were 18.86 and for cells incubated with 2µg RNAi 22.02.

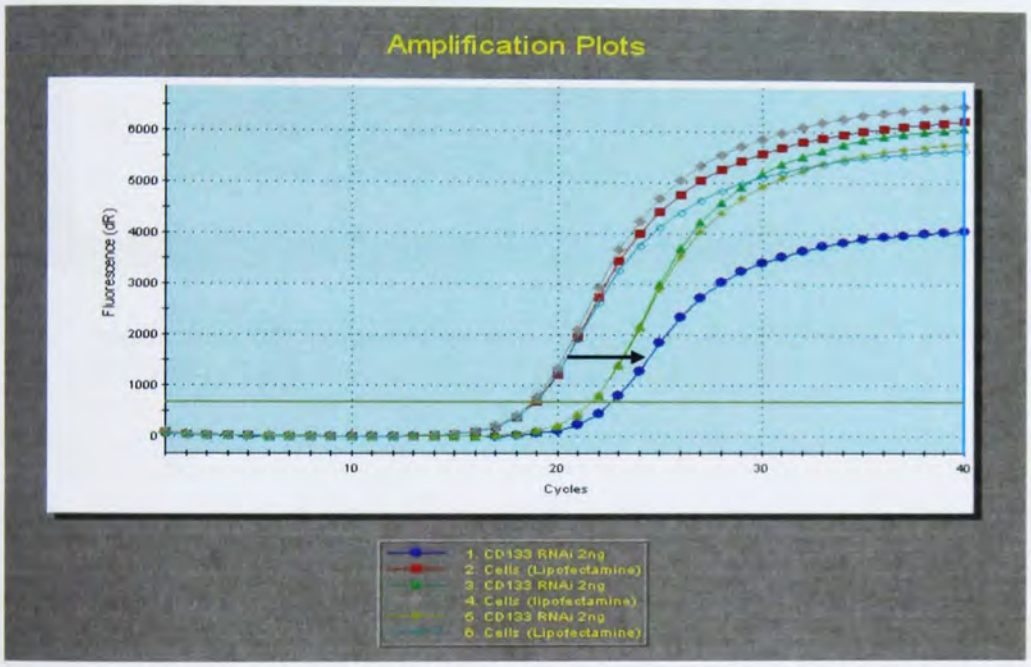

Figure 44: Amplification plots with CD133 primer.
RNAi knockdown was performed using RNAi (Santa Cruz) at a concentration of 2µg for 48 hours at which point real time PCR was performed. The black arrow represents an increase in cycle count which correlates to a reduction in CD133 mRNA. Average Ct values for samples containing cells with lipofectamine alone were 18.86 and for cells incubated with 2µg RNAi 22.02.

A dissociation curve was generated. However, as the primers used were exactly the same primers used in the previous experiments the Tm's were identical; 78°C for the CD133 gene product and 84°C for GAPDH (Figure 45).

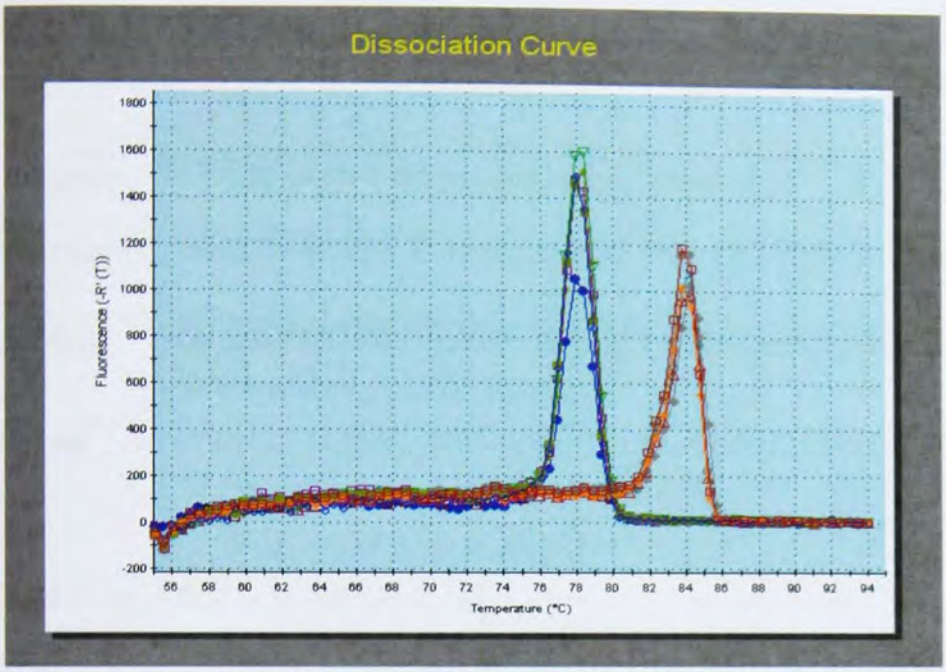

Figure 45: SYBR green dissociation curves for both CD133 and GAPDH primers Note that the Tm 78°C of the CD133 gene product is and for GAPDH gene product is Tm 84°C.

The fold changes were then measured using the Pearson formula. Primer efficiencies had already been established earlier; GAPDH efficiency: 86.4% and CD133 efficiency: 98.4%. The Ct values were taken as an average value of the three triplicate samples in each group.

$$\textbf{Fold Change} = \frac{(\text{E. Target})^{\text{Ct CD133 (CONTROL - EXPT)}}}{(\text{E. Refer})^{\text{Ct GAPDH (CONTROL - EXPT)}}}$$

For RNAi (Santa Cruz) at 2μg $= 1.984^{3.16} / 1.86^{0.19}$

$= 8.71 / 1.30$

$=$ **6.7 fold change decrease**

This can be expressed as a percentage value using the formula described earlier giving a knock down percentage of 85.1%.

Table 8: Showing the oligonucleotide sequences of the 10 siRNA's peptides purchased from the companies listed.

Company	CD133 siRNA Oligonucleotide Sequence (5' – 3')
Invitrogen	GGACAAGGCGTTCACAGAT, GCATTGGCATCTTCTTATGG
Ambion	GCAUUCUCUGUUUAUGUUA, CCAGCGACAGAAGGAAAAU GGUAAGAACCCGGAUCAAA
Qiagen	CGUUAUAGUCCAUGGUCCA GGCUAAGUACUAUCGUCGA GGUAAGAACCCGGAUCAAA, CUUUGAGUUUGGUCCCUAA
Santa Cruz	(CD133 siRNA (h): sc-42820) Undisclosed sequences comprising of 3 pooled target specific siRNA's

4.4 Functional results

Once a sufficient level of knockdown was established, we investigated functional affects loss of the receptor would have on Caco-2 cells, enabling us to further understand the possible functions CD133 may have in cell biology.

4.4.1 The affect knocking down the CD133 receptor has on re-expression

We investigated the re-expression of the knocked down CD133 receptor. All samples were also stained with CD59 to show cell viability and confirm that these were Caco-2 cells. The results show we attained 100% pure Caco-2 cells lacking CD133 expression figure 46F. Expression of CD133 remains low for the first two days reaching 12% by day 3 (figure 47). On day 4, the expression rose to 45% and by day 6 returned to its baseline level.

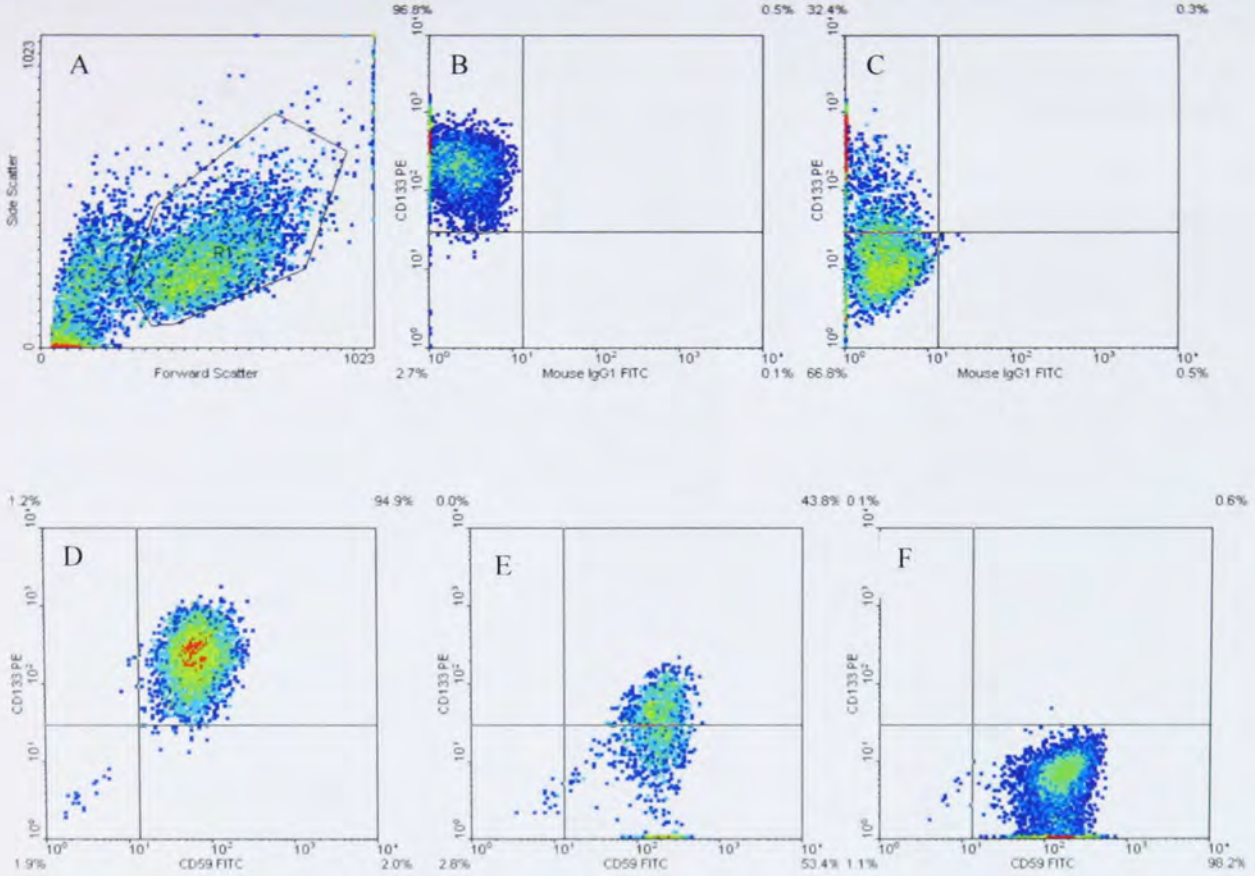

Figure 46: FACS analysis of Caco-2 cells stained with CD133 PE to determine purity of the CD133 knocked down sample. A) Shows the profile of Caco-2 cells, the gated region R1 was drawn to exclude cell debris and dead cells. This region R1 was applied to **B)** Caco 2 cells transfected with the transfection reagent alone thus showing normal expression of CD133. **C)** Cells transfected with the RNAi at 2µg producing a knockdown in CD133 expression of 67% **D)** shows the ($Caco\text{-}2^{SSC}$) control transfected cells expressing both CD133 and CD59 (CD59 was used as a marker to indicate the cells selected were alive and Caco-2 cells) **E)** Cells transfected with the RNAi were positively selected for CD133. **F)** The negative selection showing no CD133 cells remaining within the fraction. This was the sample used for the recycling studies.

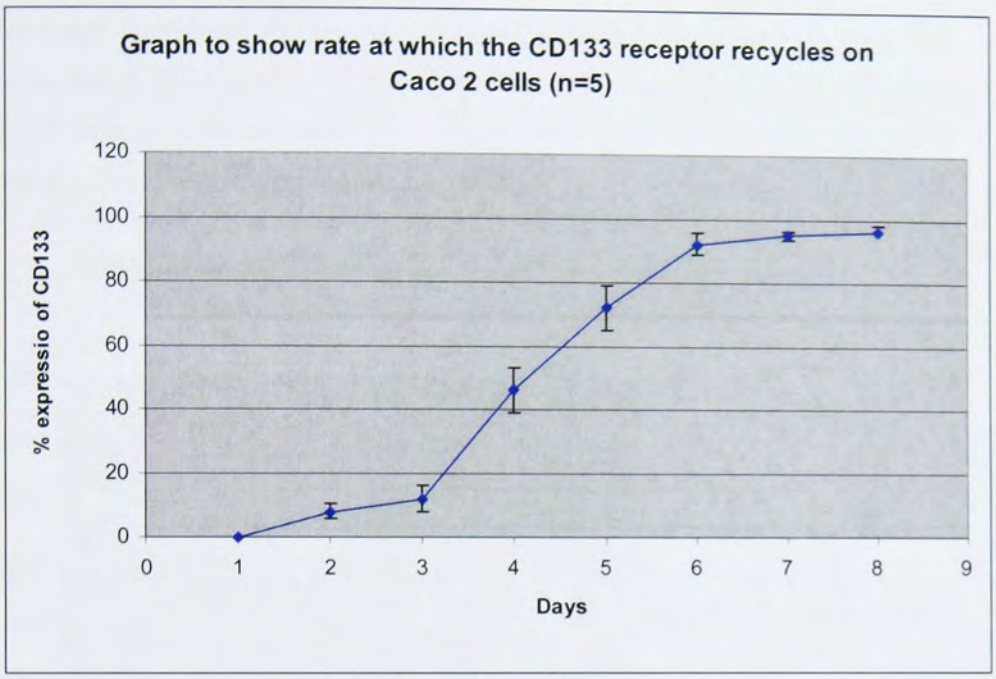

Figure 47: Phenotypic re expression of CD133 receptor on Caco-2 cells determined by flow cytometry. Purification of knocked down CD133 cells was carried out on Caco-2 cells, the cells were plated at 2 million per 6 well plates and the rate of recycling of the receptor was measured daily. Partial re expression is achieved by day 4 reaching values of 45% surface expression whilst full expression of the receptor was reached by day 6. (n=5).

4.4.2 Binding to plastic

No significant differences in binding capacity to plastic were observed when both control and knock down groups were compared (P=0.82) (figure 48). Cells were plated at 300,000 cells per well and after 240 minutes, 98% of the cells in both knockdown and control groups had attached to the plastic wells.

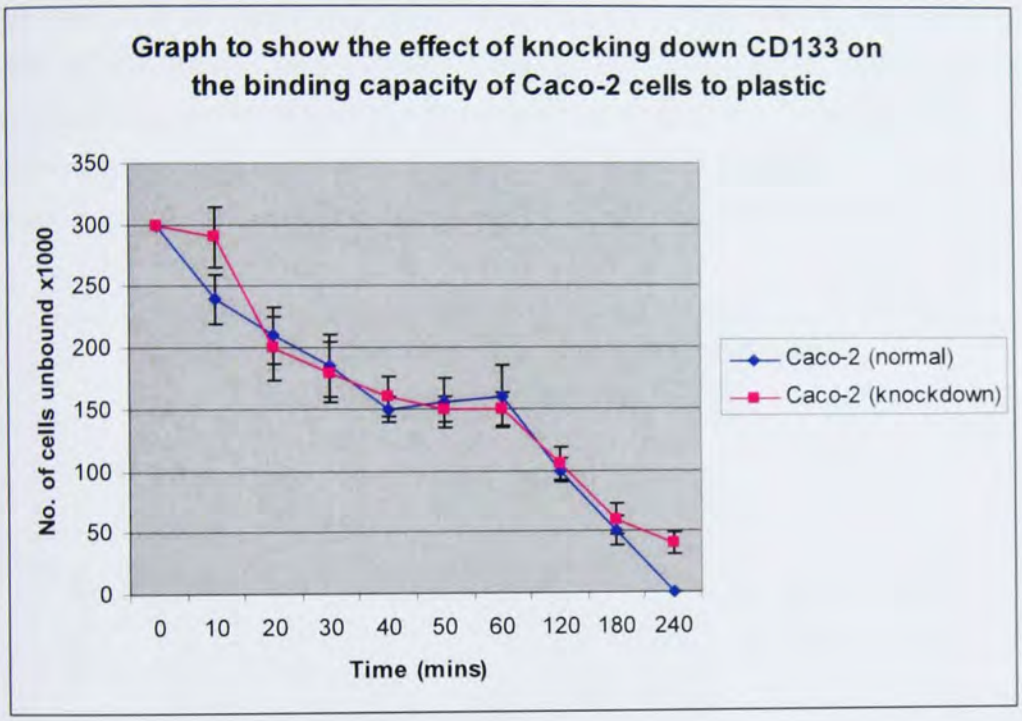

Figure 48; Caco-2 $^{CD133-}$ and Caco-2 ^{SSC} were plated on 6 well plates and at various time points, the number of cells unbound were counted. As time progressed more cells became attached to the plastic plate. After approx 4 hours all the cells were bound to the well. Knock down of CD133 does not seem to appear to affect the binding capacity of the cells to plastic, as both lines seem to follow similar trends. Experiment was carried out in triplicates and mean values from 3 experiments were plotted.

4.4.3 Association of CD133 expression with cell proliferation

The proliferation rate between cells expressing CD133 was compared. In Caco-2 the CD133 protein was knocked down as previously described and purified producing a pure CD133^{-}ve population and the cell numbers monitored for 5 days. The experiment showed that there was a steady increase in proliferation with time. At day 3 the proliferation in the control cells reached 4 million cells compared to the knocked down cells of 3.9 million. Betweens day 4 and 5 there was a slight increase in the proliferation rates for the knocked down cells compared to the controls however the differences were minimal. Proliferation was measured using trypan blue exclusion. The experiment show below was performed in triplicates and repeated four times

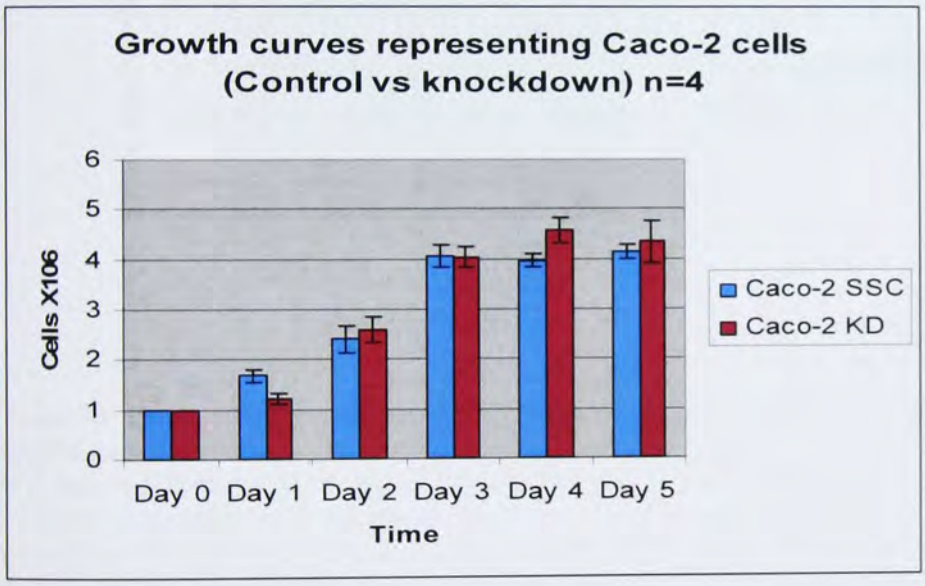

Figure 49: Affect of CD133 on proliferation. Cell proliferation was evaluated after knockdown of CD133in Caco-2 and comparing the proliferation rates against Caco-2 cells expressing CD133. Cells were seeded ay $1x10^{6}$ cells per well. A time course assay was performed over several days with no real association between CD133 and cell number (n=4).

4.4.4 Cytotoxic affect of anti-CD133 Mab

A dose-dependent cytotoxic affect of the anti-CD133 Mabs AC133, AC141 and CD133 on Caco-2 cells are shown in figure 50. All three antibodies had similar cytotoxic affects on the Caco-2 cells. 1µg of antibody produced a 50% reduction in cell viability for mAb CD133 2 (MB) and CD133 1(AB) and a 40% reduction for Mab CD133 1(MB). By 2µg of antibody concentration cell death had risen to 80% in all 3 Mab.

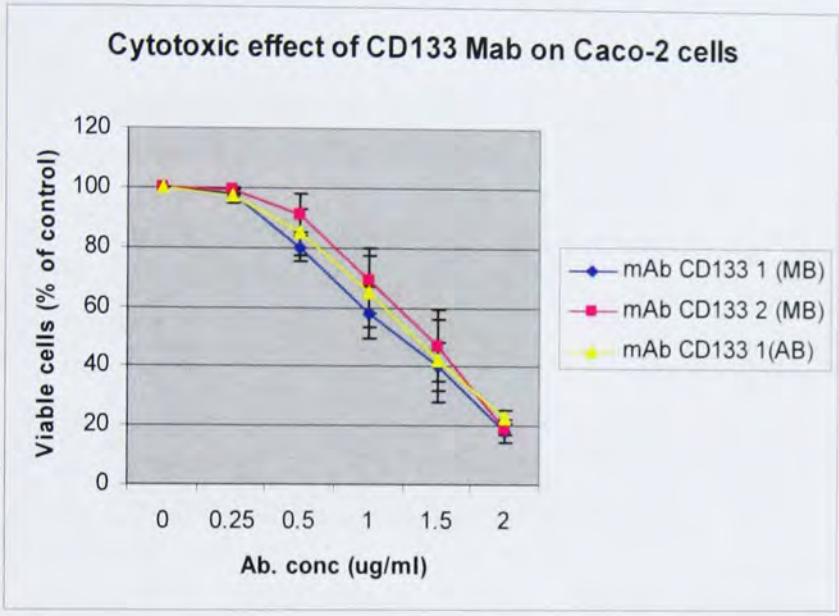

Figure 50: Cytotoxic affects of anti-CD133 Mabs. Cells were plated in complete medium and exposed 24 hours later to various concentrations of CD133 monoclonal antibodies for 72 hours. Cells were plated in 6 well plates at 400,000 cells per well. Cytotoxity was measured using trypan blue exclusion and comparing viable cells against a positive control consisting of Caco-2 cells with no Mab additions. The affect of three monoclonal antibodies on Caco-2 cells expressing CD133 was shown. The blue line represents AC133 epitope 1 antibody. The pink line represents AC141 epitope 2 antibody (both from Miltenyi Biotec) and the yellow line represents CD133 epitope 1 from Abcam. Data shows that all three Mabs have a toxic affect on the cell as the antibody concentration increases with the viability dropping to 20% at 2ug/ml of antibody. Note; when no antibody was added cell viability was 100% Experiment was carried out in triplicates and mean values from 3 experiments were plotted in the figures above.

The affect of knocking down the CD133 receptor on cell cytoxicity is shown in figure 51. We used an acute myeloid lymphoma cell line HL60, which did not express CD133 as a control. The viable cell count was calculated by measuring against a positive control consisting of Caco-2 cells with no additional CD133 mAb present. In the

CD133 knocked down Caco-2 line, no cytoxicity was observed and the cell viability remained constant at approximately 100% throughout the various ranges of antibody concentrations tested. Control HL60 cell line which lacks CD133 expression manipulated in the same way also showed 100% viability (figure 51). The viability levels of the Caco-2 control samples are similar to the results attained in figure 50. Similarly, 50% viability was observed at a concentration of approximately 1.2µg/ml in mAb CD133 (1) and a concentration of 1.4µg/ml in Mab CD133 (2).

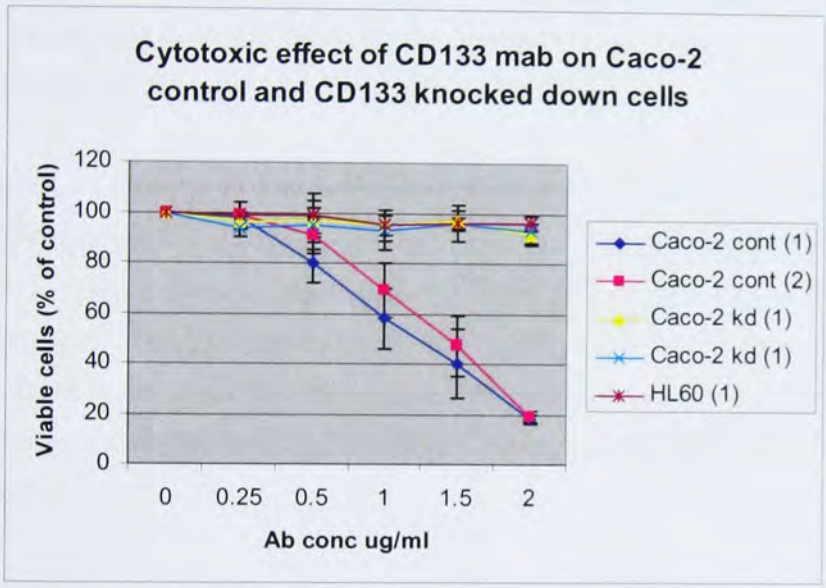

Figure 51: Cytotoxic affects of anti-CD133 Mabs. Cells were plated in complete medium and exposed 24 hours later to concentrations of CD133 monoclonal antibodies ranging from 0-2ug/ml for 72 hours. Cells were plated in 6 well plates at 400,000 cells per well. Cytotoxicity was measured using trypan blue exclusion and comparing viable cells against a positive control consisting of Caco-2 cells with no Mab additions. Mabs for epitope 1 and 2 were used against CD133 expressing cells (blue and pink), CD133 knocked down cells (yellow and turquoise) and a negative control cell line HL60 that does not express CD133 (red). It can clearly be seen that the cell line lacking CD133 expression show no viable cell change with the addition of the two Mabs.

4.4.5 Cell Cycle

The affect of knocking down CD133 in Caco-2 cells was examined on the cell cycle. Before analysis the cells were synchronised using Aphidicolin at 5μg/ml for 20 hours. Figures 52A and 53A show the cells synchronised in the G0/G1 phase with between 44% and 47% of the cells falling into the G1 portion. Preliminary studies showed that on removal of the drug from the medium the cells required 24 hours to recover and commence cycling. So we sampled from 24 hours at 12 hour intervals to measure cell cycle in Caco-2 cells. Figure 52 shows cell cycling through a 72 hour period with the highest percentage of cells (29%) in S phase (figure 52D) at the 48 hour point and dropping down to 10% at the 72 hour time point (figure 52F).

In figure 53 we show the cell cycle of the CD133 knocked down Caco-2 cells up to 36 hours. The experiment was terminated at 36 hours because CD133 re expression occurred by day 3 and so prolonging the experiment would not reflect a true measurement of CD133 knockdown on cell cycle. At 36 hours the comparisons between the knocked down cell and controls were analysed. We noticed a 7% increase in percentage of cells within the S phase in the knocked down cells compared to normal Caco-2 cells.

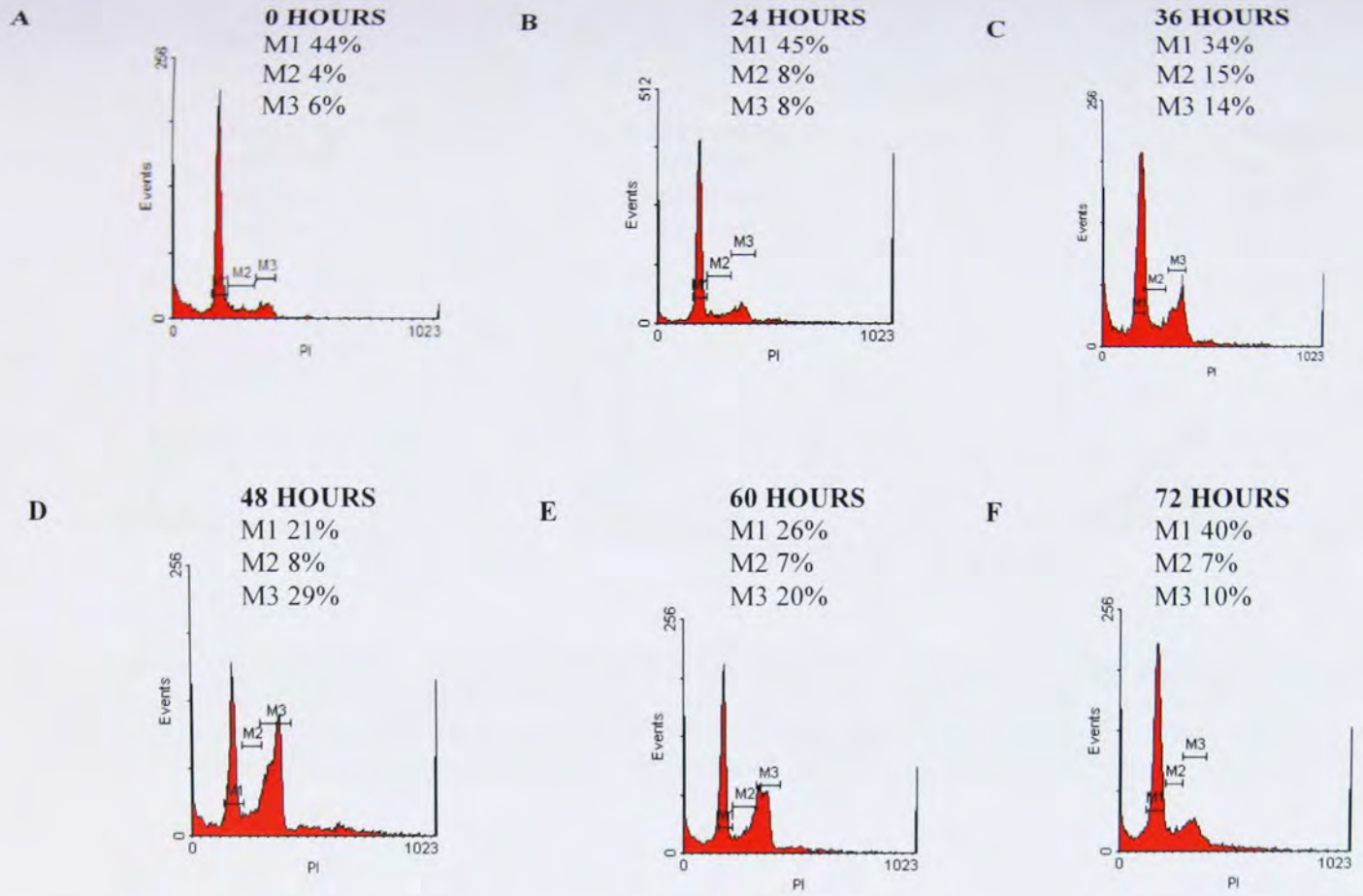

Figure 52: Flow cytometric analysis of synchronised Caco-2 cell cycling profiles. Following synchronisation with Aphidicholin **A)** Time 0 at the point the Aphidicholin was removed. **B, C, D, E** and **F** show samples taken at time points 24, 36 48 60 and 72hrs. M1 represents cells in G1 phase, M2 represents cells in S phase and M3 represents cells in G2/M phase of cycle. At 0 hours 44% of the cells were in G0 phase with only 6% in S phase. Over a period of 48 hours the cells have cycled with an increase to 29% of cells in S phase and a decrease to 21% of cells in G0 phase. At this point the cells begin to cycle back to there original synchronised state with 40% of cells in G0 phase and 10% of cells in S phase at 72 hours (n=1).

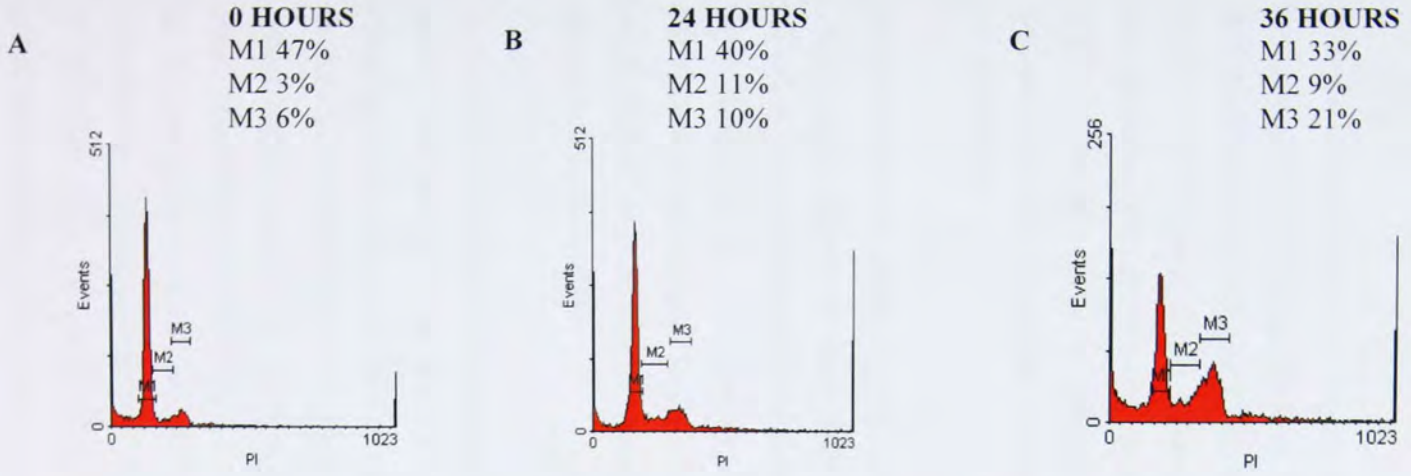

Figure 53: Flow cytometric analysis showing of CD133 Knock down Caco-2 cell cycle profiles. Following synchronisation with Aphidicholin. **A)** Time 0 at the point the Aphidicholin was removed. **B** and **C** show samples taken at time points 24 and 36hrs. M1 represents cells in G1 phase, M2 represents cells in S phase and M3 represents cells in G2/M phase of cycle. At 0 hours 47% of the cells were in G0 phase with only 6% in S phase. Over a period of 36 hours the cells have cycled with an increase to 21% of cells in S phase (n=1).

4.5 Discussion

Functionally experiments on CD133 knockdown cells were limited by rapid re-expression of CD133. These results showed that CD133 was partially re expressed to levels of 40% on the cell surface after knockdown by day 3 and full expression occurred within 6 days.

Different siRNA's may have various long term or short term affects on the cell. It has been shown in studies using CD133 RNAi that the Caco-2 cells can be knocked down for up to ten days (Horst *et al.*, 2009). Different cell lines or tissues may also exhibit different characteristics that may vary the efficacy of the siRNA's. An interesting question would be what determines the period of knockdown? Is it related to the cell line or dependent on the siRNA purchased? From our results it appears that it is more cell related as we varied concentrations of RNAi and did not see any increase in cell surface knockdown with the Qiagen RNAi's.

Our results showed no affect of knocking down CD133 on proliferation and binding of Caco-2 cells to plastic. Similarly Horst *et al.*, 2009 demonstrated on a colon cancer cell line that CD133 knocked down cells had no significant affect on proliferation or migration which could indirectly be related to binding (Horst *et al.*, 2009). However Yao *et al.*, (2009) showed that the knock down of CD133 on U251 human glioma cells inhibited proliferation (Yao *et al.*, 2009). This again poses the question: does CD133's function depend on cellular location?

The anti-CD133 Mab binding experiments showed that CD133 may contribute to binding passively but were by no means solely responsible. The cytoxicity data suggests that the interaction of the Abs with CD133 on the control Caco-2 cells is sufficient to trigger the Ab-induced cytotoxic events compared to CD133 knocked down cells. This was also demonstrated by Rappa *et al.*, (2008) who in a similar experiment observed that both of the CD133 knocked down FEMX cell lines were less sensitive to cytotoxic activity suggesting that the interaction of the Abs with the residual CD133 surface molecules is not sufficient to produce any cytotoxic events (Rappa *et al.*, 2008). The results we produced indicate that CD133 Mab's have the ability to successfully stimulate the receptor leading to the initiation of signalling events during functional experiments. This is verified in the next chapter.

Comparisons between the 2 groups indicate a slight increase in cells within the S phase of the cycle for Caco-2^{CD133-}. This would indicate a slight increase in cell cycle rate which has been observed by other groups. However our experiment would have to be repeated for statistical purposes. Yao *et al.,* (2009) showed using antisense oligodeoxynucleotides (ASODNs) of CD133 to knock-down CD133 expression in Huh-7 cells that the $CD133^{-}$ knocked down cells after 36 hours showed a 5% increase in cells within the S phase portion compared to the control cells and a significant increase ($P<0.005$) in S phase ratio and a non significant decrease in G0-G1 ratio between the two groups (Yao *et al.,* 2009). The cell cycle rate in the knocked down cell line were only measured to 36 hours and must be measured through the whole 72 hours as carried out with the control cell line in figure 56. The affects of the knockdown may not be immediate and may become apparent later on as the cycle progresses. However for this to be determined, a permanent knockdown of the CD133 receptor must be established so that the likelihood of re-expression is eliminated. Another concern was the health of the cells. The profile of the cells showed that the cells were not under duress or physically damaged. However the knockdown procedure is an exhaustive process for the cells and a further 20 hours in Aphidicolin may have affected viability and recovery may have been only partial thus affecting the rate at which they may cycle. Our result could indicate (assuming the changes in S phase for both groups are real) that, by controlling the expression level of CD133, we may move the cells out from their quiescent phase into their proliferating phase.

In summary the irrational growth patterns of the MUTZ-2 cell lines made it very difficult to knock down CD133. Caco-2 cells on the other hand were knocked down successfully and this was verified using flow cytometry and RT-PCR but rapidly re-expressed the receptor. However difficulty arose when trying to create a stable CD133 knockout cell line. We successfully created plasmids containing the anti CD133 RNAi insert but unfortunately were unable to transfect the plasmid into the Caco-2 cells.

Functional experiments in the knocked down Caco-2 cell showed that knocking down the receptor had no affect on the cells binding capacity to plastics or on cell proliferation. The viability of the cells within the knockdown group was not affected when subjected to high concentrations of CD133 Mab compared to the control cell line where the viability dropped to 20%. Cell cycle was also measured between the 2 groups. Analysing the cells

in the S phase we noticed that at 36 hours there was a 7% increase in cells within this portion in the knock down group suggesting an increase in cell cycle activity in this group. However this experiment was carried out only once therefore it would be impossible statistically to propose that this result was a true indication of CD133's role in cell cycle.

Chapter 5: Gene expression profiling of CD133 knocked down Caco-2 cells

5.1 Aims and objectives

This chapter aims to investigate the effect CD133 has on gene expression on Caco-2 cells under various conditions affecting the CD133 receptor. The conditions involved knocking down the receptor, stimulating the receptor with CD133 Mab's and an IgG control group. Micro array technology will be used to measure gene activity.

The primary aims of this chapter were:

- To investigate the effect the various conditions had on gene expression using Micro array.
- To analyse up and down regulated genes within each group and classify them into various gene ontology's, molecular processes and signalling pathways.
- To compare the groups with each other to further understand the effect Mab binding is having on the receptor.
- To extrapolate this to potential functions of CD133 in cell biology.

5.2 Introduction

5.2.1 Origin of DNA microarrays

Microarray technology has emerged in the last decade as a key method for analysing large numbers of nucleic acid sequences in parallel. Microarrays can be considered as a continuation of the development of molecular biology hybridisation methods, and as an extension of the use of fluorescence microscopy in biology, as well as using a solid surface as a means of reducing the amount of required analytes in assays (Southern, 2001).

By reversing the Northern blotting principle so that the labelled nucleic acids are derived from the mRNA sample and the immobilized fractions are the known sequences traditionally known as probes, filter –based gene expression analysis has enabled simultaneous determination of expression levels of thousands of genes in one experiment (Lennom and Lehrach, 1991). Therefore by combining fluorescence analysis of multiplexed probes with microscopy, fluorescent in situ hybridisation (FISH) has enabled detection of nucleic acid within cells and chromosomes, and has been found useful in gene expression and genomic analysis.

These methods of analysis were brought together by advancements made in attaching nucleic acid sequences to a glass support. Photolithography was used to synthesize oligonucleotides directly onto a glass support to construct in situ synthesised oligonucleotide arrays (Fodor *et al.*, 1991). Separately a procedure called contact printing was used to deposit purified cDNA clones onto a slide surface (Schena *et al.*, 1995).

5.2.2 DNA Microarray technology

A DNA microarray is a solid support upon which specific nucleic acids have been placed at defined locations (spots) at high density either by printing or in situ synthesis. The elements on the array consist either of synthetic oligonucleotides (referred to as oligonucleotide arrays) or polymerase chain reaction (PCR) products known as cDNA arrays.

5.2.3 Array Probes

Production of arrays begins with the selection of the probes to be immobilised on the array. The choice of the probes is largely dependent on the biological question to be answered. Probes may be of different types: purified cDNA clones (Duggan *et al.*, 1999) exon specific PCR products derived from genomic DNA (Attia *et al.*, 2003) or oligonucleotide probes (Lipshutz *et al.*, 1999).

5.2.4 Substrates

The probes are spotted or synthesised on different kinds of suitable non porous solid surfaces such as glass, with fluorescent labelling and detection to which the DNA is then fixed (Schena *et al.*, 1995). The probe can be bound to the glass support in two main ways. The glass surface can be modified using a positively charged layer (eg poly-L-lysine) to bind the negatively charged DNA fragments (Dufva *et al.*, 2005). Alternatively, DNA molecules that carry a modified 5' end can be covalently bound to a glass surface that carries reactive groups (Rogers *et al.*, 1999).

5.2.5 Target and probe reaction

The biological samples to be tested (mRNA or genomic DNA) are labelled with suitable labels and allowed to hybridise with the array probes under the appropriate conditions. Traditionally radioactive isotopes were used in filter based assays but now fluorescence is the labelling system of choice (Lockhart and Winzeler, 2000). Currently, the fluorophores most commonly used are Cy3 and Cy5 which are cyanine dyes. Multicolour fluorescence in microarray experiments allows the multiplexing of biological samples. Once the labelled

targets are reacted with the array probes, the unbound material is washed away and the remaining bound targets are detected by using a suitable imager system.

5.2.6 Data analysis

The image produced by scanning is converted to a digital format to reveal an informative subset of the total array elements which are quantitated and interpreted. Quantitation is usually made by superimposing a grid over the microarray image and computing an intensity value for each element with automated software. Intensity values can be further manipulated and converted into biologically relevant outputs using data analysis software. For simple experiments comparing only two conditions, it is logical to look at the extremes, genes with significant differential expression in individual samples. Although this simple approach can be effective for focussing on groups with differentially expressed transcripts, it does not take advantage of full genome scale experiments to improve our understanding of cellular biology provided by analysis of the entire repertoire of transcripts in a cell (Eisen *et al.,* 1998). A more holistic approach which investigates patterns from the whole data is required. Statistical algorithms can be applied to detect and extract patterns within expression profiling data. A common approach is to group genes on the basis of similarities in their expression profiles (Bassett *et al.,* 1999). However, similarity of gene expression profile does not mandate similarity of function and regulation, and it may also be coincidental. Nevertheless, the idea of clustering genes on the basis of their expression patterns is well established and cluster analysis has become the most widely used approach applied to large-scale gene expression data (Sherlock *et al.,* 2000).

5.2.7 DNA microarray applications and gene expression

Microarrays have a variety of different applications such as gene expression monitoring, mutational analysis and re-sequencing.

A gene to be expressed is transcribed into mRNA, which is then translated into a protein. The collection of genes, which are expressed from genomic DNA in a given cell, is the main controlling factor of cell function and phenotype. The differences in expression profile are responsible for morphological and phenotypic differences and determine the response of the cell to environmental stimuli and perturbations. To understand the

biological role of a gene, it is essential to know when, where and to what extent it is expressed.

It is known that the relative abundance of mRNA transcripts represents the cellular response to a particular state or stimulus. DNA microarrays have been used to study gene expression in a variety of organisms including human (Schena *et al.*, 1995). Microarray analysis has a key advantage over other methods available for detecting or quantitating gene expression in that it produces expression profiles that reflect the transcriptional response of thousands of genes in response to a pharmacological agent at the same time in a single experiment. The aim of such experiments is the identification of new genes involved in a pathway, or diagnostic and prognostic markers that characterise a disease state. In a trial to identify new diagnostic markers for different lymphomas, filtered based array were used to profile 31 different lymphoma cell lines and 36 cases of primary anaplastic large-cell lymphoma (ALCL). The study revealed clustering to be specifically expressed in all anaplastic large cell lymphoma and in all the primary cases examined, providing a new marker for diagnosis of ALCL (Wellmann *et al.*, 2000).

Global gene expression analysis can reveal patterns of gene expression that can be utilised to predict gene function. This is achieved by grouping genes into clusters with similar expression profiles produced by multiple experiments as mentioned earlier. Derisi *et al.*, 1999 used differential gene expression to examine the transcriptional response of yeast undergoing the shift from anaerobic to aerobic metabolism, known as the diauxic shift. Using cluster algorithms, distinct patterns of gene expression were identified and genes were grouped on the basis of their expression profiles. For example, cytochrome c related genes and genes involved in carbohydrate storage were co-ordinately induced during the diauxic shift (Derisi *et al.*, 1999).

5.3 Results

The aim was to obtain differentially expressed genes from each group, relate this to gene ontology and from there determine the possible function of CD133.
The data analysis procedure involved sample processing which comprised of probe level background correction and normalization, quality check, differential expression analysis and bio analysis.

5.3.1 Sample Processing

In the results that followed, the different groups were referred to as samples thus;

Sample 1 = Group 1 (CD133 knocked down group)
Sample 2 = Group 2 (IgG control labelled group)
Sample 3 = Group 3 (CD133 Ab labelled group)
Sample 4 = Group 4 (Control cells)

Initially the samples had to be normalized which adjusts the microarray data, accounting for effects which arise from variation in the technology rather than from biological differences between the RNA samples or between the printed probes. The intensity distributions of samples before and after normalization are shown below. In order to stabilize the variation effect across samples, the intensities were log transformed and quantile normalization was used to generate normalized data (figures 54 and 55). The intensity distribution of samples was similar, facilitating the sample comparison.

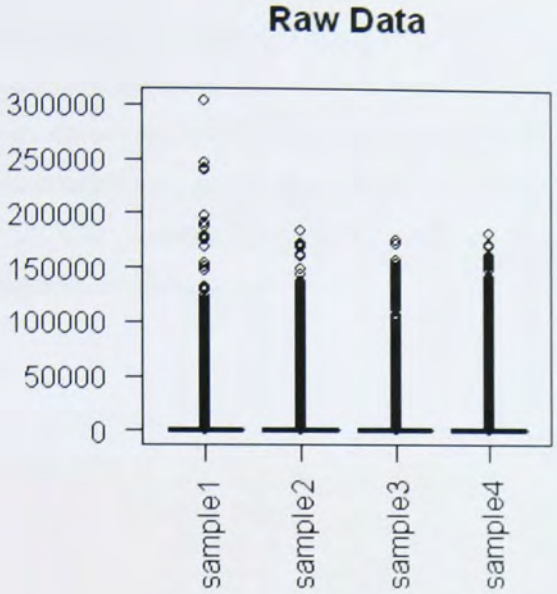

Figure 54: Box plot before normalisation

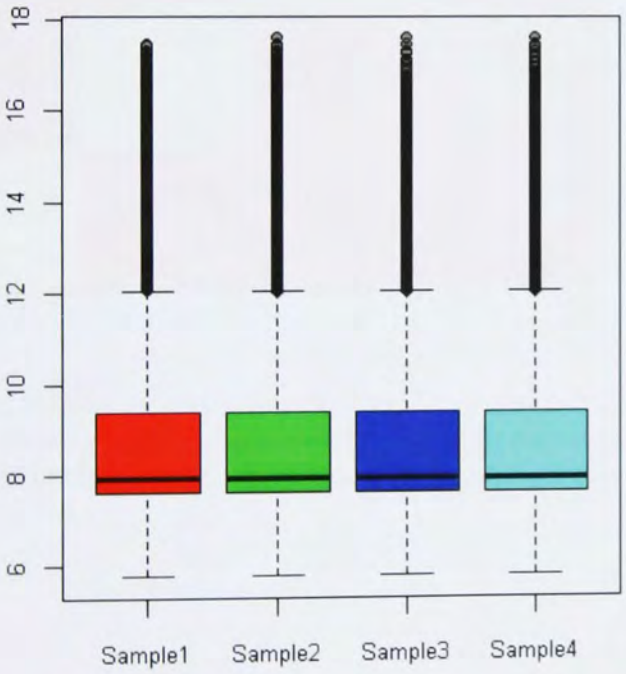

Figure 55: Box plot after Log Transformation and Quantile Normalisation

5.3.2 Sample Correction Analysis

The pair wise correlation between the expression values for samples was studied through Pearsons correlation coefficient. The analysis resulted in a correlation matrix indicating the extent of linear relationship amongst samples (figure 56). A coefficient value close to 1.0 indicates linear relation between the two arrays. The image plot depicting the correlations is shown below. The minimum correlation between the samples was 0.94, which was above the acceptable criterion of 0.8.

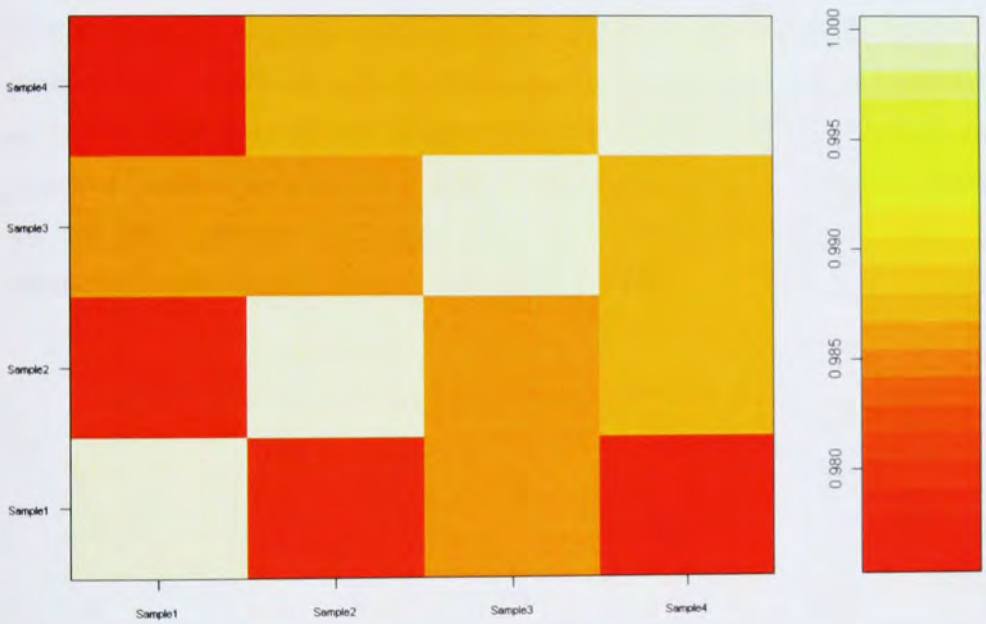

Figure 56: Correlation plot of 4 samples. Sample 1 (CD133 knocked down group), Sample 2 (IgG control labeled group), Sample 3 (CD133 Ab labelled group), Sample 4 (Control cells).

5.3.3 Differential Expression Analysis

The primary objective was to assess the up regulation or down regulation of mRNA transcript levels in the various groups, hence identifying the genes that show significant difference. This was generally measured by looking at the fold increase or decrease of the particular gene. Because a low number of samples were analysed (triplicates), it is important that the results give a true representation of the gene profile within the particular group. This was established by working out the FDR False Discovery Rate.

This in theory is the expected proportion of false positives among all significant hypotheses. For example if 100 genes were experimentally predicted to be different and the FDR for these observations was 0.10, then 10 of these genes would be expected to be false positives. This is different to calculating the p value which states that (in a similar scenario as the one mentioned above) a p value of 0.1 would indicate that the difference initially observed would be larger in 10 of these genes. In this analysis the FDR was fixed to 0.05 for the three groups. The scatter plot with differential expressed genes for both comparisons (Control Vs Group) is shown in figure 57.

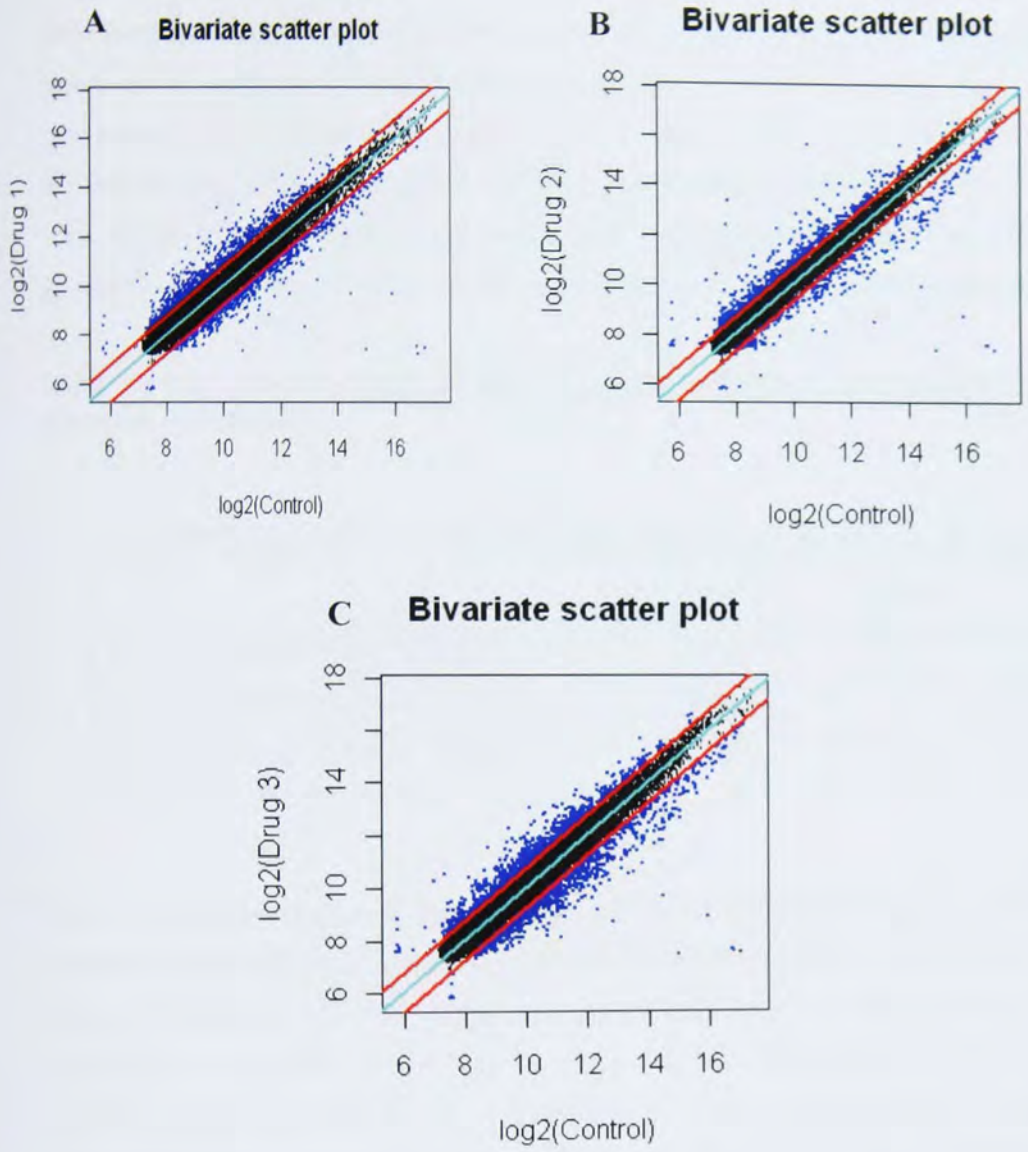

Figure 57: Scatter plots to show differentially expressed genes for the 3 different groups (drugs) against the control

The genes obtained through differential expression analysis were studied for their overabundance in different Gene Ontology (GO) terms as well as pathways. The GO terms were categorized into biological processes, molecular function and cellular component.The overabundance of a particular term was decided based on the number of significant genes in the analysis, the number of significant genes relevant to the term. Fisher's exact test was used to determine the significance of each GO term. If a term is significant with for example $p < 0.05$, then it was considered to be enriched with genes.

Table 9: The number of up and down regulated genes with FDR and fold change values in all 3 groups

Group	Comparison	FDR	Fold Change	Up-regulated Genes	Down-regulated Genes
1	Group 1 Vs Control	0.05	1.7	930	754
2	Group 2 Vs Control	0.05	1.58	642	404
3	Group 3 Vs Control	0.05	1.7	995	1065

Table 9 shows the number of genes up and down regulated. Fold change values represent the threshold values at which a gene change can be classed as significant and thus classed as up or down regulated. This number may vary depending on the experiment. The initial observation is the low gene profile for group 2 compared to group 3. This result is encouraging as it suggests that the CD133 antibodies had a specific effect on the receptor, 350 genes were up regulated and 660 genes down regulated in this group compared to the IgG control group in 2. If the antibodies were having no specific effect we would expect to see a similar gene profile in groups 2 and 3. An example of a list of genes up regulated in group 1 and their top gene ontologys are shown below, the remaining data is in the appendix.

Table 10: Group 1 (CD133 Knockdown) Up-regulated gene list (top 60)

S100P	S100 calcium binding protein P	10.41073484
N6AMT2	N-6 adenine-specific DNA methyltransferase 2 (putative)	8.938297105
PTPN12	protein tyrosine phosphatase, non-receptor type 12	8.75434961
THRAP1	thyroid hormone receptor associated protein 1	7.835362381
NUPR1	nuclear protein 1	6.91629785
CHAC1	ChaC, cation transport regulator homolog 1 (E. coli)	6.868523492
DDIT4	DNA-damage-inducible transcript 4	6.498019171
SEC13	SEC13 homolog (S. cerevisiae)	6.498019171
SAE1	SUMO1 activating enzyme subunit 1	6.233316637
KIAA1109	KIAA1109	6.105036836
CNOT2	CCR4-NOT transcription complex, subunit 2	5.540437872
TMEM92	transmembrane protein 92	5.314743256
LOC643471	similar to Keratin, type I cytoskeletal 18 (Cytokeratin-18) (CK-18) (Keratin-18) (K18)	5.278031643
PCK2	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	5.241573615
LYCAT	lysocardiolipin acyltransferase	5.205367422
PPIAL4	peptidylprolyl isomerase A (cyclophilin A)-like 4	5.169411323
TGFBI	transforming growth factor, beta-induced, 68kDa	5.028053498
CD44	CD44 molecule (Indian blood group)	4.675105754
LOC392878	similar to 60S ribosomal protein L35	4.658934346
CFLP1	cofilin pseudogene 1	4.626752736
IL21R	interleukin 21 receptor	4.469148552
PTPN2	protein tyrosine phosphatase, non-receptor type 2	4.377174805
LOC391271	hypothetical LOC391271	4.316912946
LOC402562	similar to Heterogeneous nuclear ribonucleoprotein A1 (Helix-destabilizing protein) (Single-strand binding protein) (hnRNP core protein A1) (HDP-1) (Topoisomerase-inhibitor suppressed)	4.198866734
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	4.141059695
LGI4	leucine-rich repeat LGI family, member 4	4.141059695
NOX1	NADPH oxidase 1	4.112455307
ANKRD38	ankyrin repeat domain 38	4.055837919
RABEPK	Rab9 effector protein with kelch motifs	4.0278222
ASNS	asparagine synthetase	4
PSAT1	phosphoserine aminotransferase 1	4
KLK8	kallikrein-related peptidase 8	4
LIN7A	lin-7 homolog A (C. elegans)	3.972369982
RPL23AP7	ribosomal protein L23a pseudogene 7	3.944930818
PSMD10	proteasome (prosome, macropain) 26S subunit, non-ATPase, 10	3.944930818
CEACAM1	carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	3.91768119
LOC647094	similar to acidic ribosomal phosphoprotein P0	3.863745316

Table 11: Group 1 (CD133 Knockdown) Up-regulated gene ontology list (top 30)

Gene Ontology term	P-value
DNA replication	5.90E-010
lipoprotein metabolic process	1.22E-009
cholesterol efflux	2.52E-009
cell cycle	5.38E-009
cellular amino acid biosynthetic process	9.07E-009
response to stress	1.21E-008
Transport	4.41E-008
lipid transport	4.48E-008
high-density lipoprotein particle remodelling	6.06E-008
oxidation reduction	1.16E-007
metabolic process	1.17E-007
acute-phase response	1.69E-007
L-serine metabolic process	3.13E-007
very-low-density lipoprotein particle assembly	1.11E-006
extracellular matrix organization	1.24E-006
negative regulation of fatty acid biosynthetic process	1.87E-006
positive regulation of cholesterol esterification	1.87E-006
phospholipid biosynthetic process	2.52E-006
Translation	2.56E-006
interspecies interaction between organisms	3.08E-006
protein folding	4.39E-006
neutral amino acid transport	4.59E-006
Mitosis	5.78E-006
cholesterol metabolic process	6.34E-006
cholesterol homeostasis	6.62E-006
phospholipid efflux	6.77E-006
regulation of transcription	8.60E-006
lipid metabolic process	1.02E-005
negative regulation of cell proliferation	1.07E-005
Apoptosis	1.14E-005

Table 12: Group 1(CD133 Knockdown) Down-regulated gene list (top 60)

GeneName	Fold Change
tudor domain containing 10	0.586417475
similar to Tetratricopeptide repeat protein 3 (TPR repeat protein 3) (TPR repeat protein D)	0.586417475
reticulon 3	0.586417475
similar to heterogeneous nuclear ribonucleoprotein K	0.586417475
chromosome 10 open reading frame 114	0.586417475
component of oligomeric golgi complex 8	0.586417475
schwannomin interacting protein 1	0.586417475
protein disulfide isomerase family A, member 3	0.586417475
inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	0.586417475
coiled-coil domain containing 96	0.586417475
glycosyltransferase 8 domain containing 3	0.583174281
hypothetical gene supported by BC008048	0.582366793
transmembrane 4 L six family member 1	0.582366793
solute carrier family 12 (sodium/potassium/chloride transporters), member 2	0.582366793
tropomyosin 4	0.582366793
high-mobility group nucleosomal binding domain 2	0.582366793
ferritin, heavy polypeptide 1	0.582366793
deformed epidermal autoregulatory factor 1 (Drosophila)	0.582366793
PRP8 pre-mRNA processing factor 8 homolog (S. cerevisiae)	0.582366793
OTU domain containing 1	0.582366793
protein tyrosine phosphatase, non-receptor type 11 (Noonan syndrome 1)	0.582366793
similar to Williams Beuren syndrome chromosome region 19	0.582366793
SHC (Src homology 2 domain containing) transforming protein 2	0.582366793
chromosome 1 open reading frame 53	0.582366793
hypothetical LOC401317	0.582366793
ferritin, light polypeptide	0.578344092
IQ motif and Sec7 domain 2	0.578344092
hypothetical protein DKFZP434I0714	0.578344092
chromosome 2 open reading frame 13	0.578344092
ST7 overlapping transcript 1 (antisense non-coding RNA)	0.578344092
CDC28 protein kinase regulatory subunit 1B	0.578344092
hypothetical protein LOC153546	0.578344092
nudix (nucleoside diphosphate linked moiety X)-type motif 4	0.578344092
hypothetical protein LOC253012	0.578344092
bicaudal C homolog 1 (Drosophila)	0.578344092
hypothetical protein LOC731997	0.578344092
copper metabolism (Murr1) domain containing 1	0.578344092
similar to 14-3-3 protein epsilon (14-3-3E) (Mitochondrial import stimulation factor L subunit) (MSF L)	0.578344092

Table 13: Group 1 (CD133 Knockdown) Down-regulated gene ontology list (top 30)

Gene Ontology term	P-value
Apoptosis	3.70E-014
Angiogenesis	1.44E-009
cell adhesion	9.94E-009
cytokine-mediated signalling pathway	1.79E-008
signal transduction	3.80E-008
response to drug	5.74E-008
negative regulation of transcription from RNA polymerase II promoter	1.80E-007
negative regulation of cell proliferation	2.18E-007
multicellular organismal development	3.09E-007
heart development	4.48E-007
oxidation reduction	4.89E-007
response to protein stimulus	7.91E-007
Vasculogenesis	9.23E-007
extracellular matrix organization	1.05E-006
response to peptide hormone stimulus	1.05E-006
small GTPase mediated signal transduction	1.05E-006
positive regulation of transcription from RNA polymerase II promoter	1.49E-006
induction of apoptosis by extracellular signals	2.91E-006
glucose transport	3.62E-006
muscle contraction	4.89E-006
response to stress	4.99E-006
negative regulation of transcription	5.17E-006
Transport	5.57E-006
cellular component movement	5.76E-006
nitric oxide mediated signal transduction	6.79E-006
nucleosome assembly	7.05E-006
response to oxidative stress	7.94E-006
anti-apoptosis	8.11E-006
focal adhesion assembly	8.95E-006
positive regulation of smooth muscle cell proliferation	1.15E-005
positive regulation of protein kinase activity	1.16E-005
nervous system development	1.17E-005
cyclic-nucleotide-mediated signalling	1.27E-005
intussusceptive angiogenesis	1.27E-005

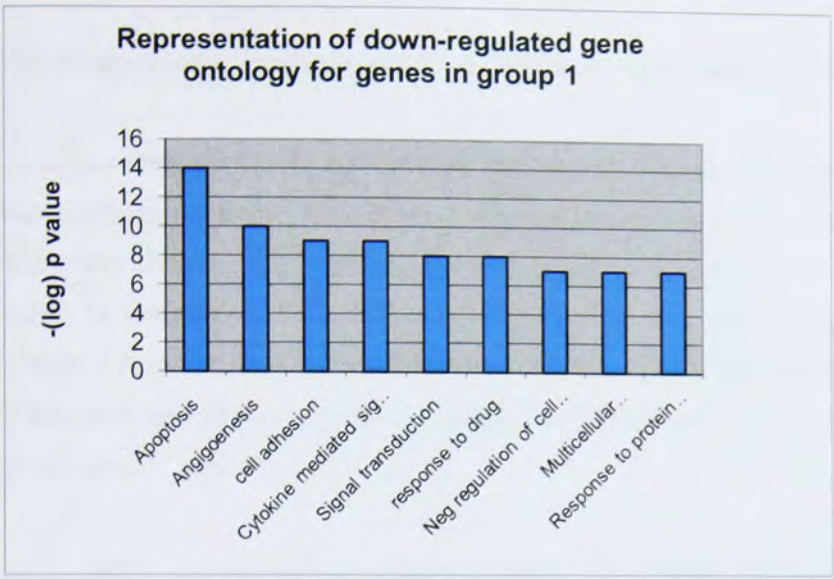

Figure 58: Group 1 down-regulated gene list

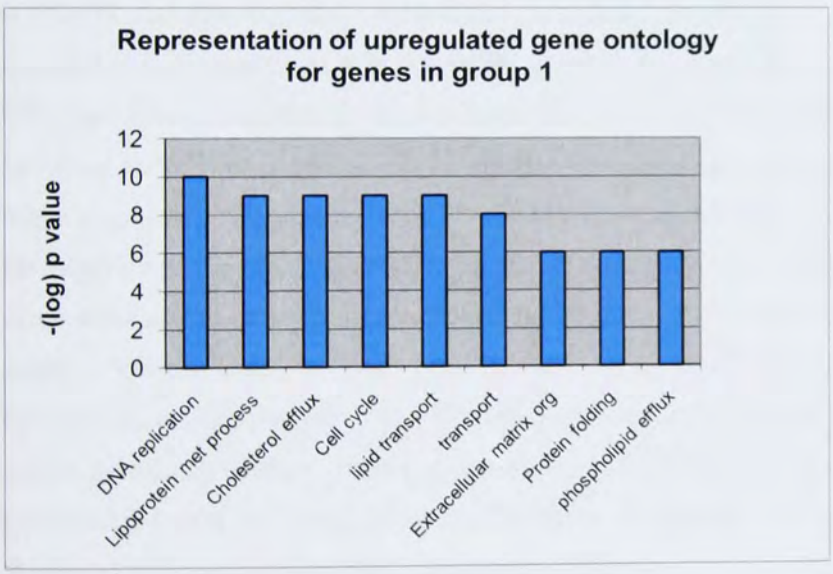

Figure 59: Group 1 up-regulated gene list

5.3.4 Comparisons between Group 1 (Knockdown) and Group 3 (Ab labelled)

It was now important to try and establish whether our findings in Group 1 would mirror the results obtained in Group 3 comprising of Mab CD133 Ab labelled cells.
Before any analysis of group 3 could occur, group 2 consisting of cells labelled with IgG had to be considered. Thus we analysed both gene lists and removed genes that were present in both lists from Group 3. This was done to try and eliminate unspecific binding of the antibody that may contribute to background noise causing the up or down regulation of non specific genes.

- **Group 3 (Up regulated genes) - Group 2 (Up regulated genes)**
- **Group 3 (Down regulated genes) - Group 2 (Down regulated genes)**

This then gave us a true reflection of specific gene up and down regulation with respects to CD133 and specific Mab interaction. On completion the gene profile in Group 3 consisted of 625 genes up regulated and 784 genes down regulated, a decrease of 30% and 36% respectively compared to the total including the IgG genes in group 2. Unfortunately the ligand for CD133 is still unknown. However, we used the anti-CD133 antibodies as a means of possibly interacting with CD133 in a ligand like manner. So for the purpose of this experiment we have to assume that the Mab are not just binding to a site on the receptor but actually mimicking the ligand and triggering the receptor causing downstream activity. We can safely say that group 1 knock down of the receptor is repressing any down stream activity and obviously preventing binding of the ligand as the receptor is not present on the cell surface. Both groups were investigated collectively to determine what genes CD133 may be involved in simulating or inhibiting. This was carried out by comparing different parameters in both groups 1 and 3.

Figure 60: Genes that have been down regulated in Group 1 (knockdown) were compared against the genes that have been up regulated in Group 3 (Ab labelled)
Figure 61: Genes that have been up regulated in Group 1 (knockdown) were compared against genes that have been down regulated on Group 3 (Ab labelled).

The genes that overlap both groups are the genes of importance in this experiment and are genes that may truly reflect the inhibitory/stimulatory function of CD133.
The results are shown on the Venn diagrams below (figures 60 and 61)

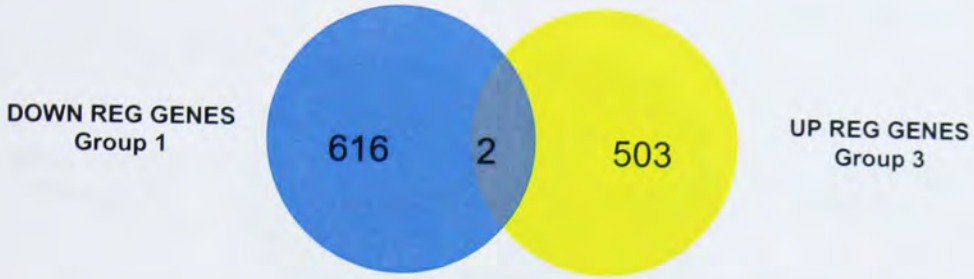

Figure 60: Venn diagram to show group 1 down regulated (knockdown) genes Vs up regulated genes in group 3(Ab labelled).

Figure 61: Venn diagram to show group 1 up regulated (knockdown) genes Vs up regulated genes in group 3 (Ab labelled).

Since a low number of overlapping genes occurred in both instances, it may be likely that the Mab's may not be providing a stimulatory effect on the receptor so we repeated the cross examination of both groups but changed the parameters slightly by analysing up regulation of both groups simultaneously and vice versa.

Figure 62: Genes that have been up regulated in Group 1 (knockdown) were compared against the genes that have regulated in Group 3 (Ab labelled)

Figure 63: Genes that have been up regulated in Group 1 (knockdown) were compared against genes that have been up regulated on Group 3 (Ab labelled).

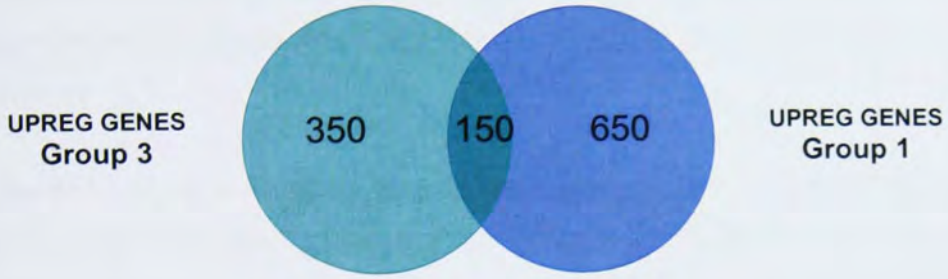

Figure 62 Venn diagram to show group 3up regulated genes (stimulation) Vs up regulated genes in group 1 (knockdown).

Figure 63: Venn diagram to show group 3 down regulated (Ab labelled) genes Vs down regulated genes in group 1 (knockdown).

The results clearly indicate that there is significant number of overlapping genes expressed in both figure 62 and 63, strongly implying that Mab may be inhibitory. The fact that the percentage of genes co-expressed in both groups 3 and 1 (figure 62) ranges from about 20% - 30% and in figure 63 was 50% - 60% between groups 3 and 1 suggests that binding to CD133 using Mab's may have a similar effect to knocking down the receptor, suggesting an inhibitory role for the antibodies. Comparing the genes up and down regulated in these groups should also give us a clearer indication of the receptor's possible function.

The next step was to determine the possible functional roles of the co expressed genes - in both up and down regulated genes. This was established by firstly categorising the genes under their relevant Gene Ontologys (see table 14 and 15), then individually looking at the genes that contributed to the specific ontologies.

Table 14: Top 5 Gene Ontology's for genes that have been up regulated in Group 1 and Group 3

Gene Ontology	P-value
Membrane enclosed lumen	1.0E-6
DNA replication	7.2E-6
Cell Cycle	4.1E-3
Acetylation	2.7E-3
Phosphoprotein	1.8E-3

Table 15: Top 5 Gene Ontology's for genes that have been down regulated in Group 1 and Group 3

Gene Ontology	P-value
Cell Death	3.1E-6
Death	3.4E-6
Nucleosome	3.5E-6
Reg of Cell Proliferation	1.4E-5
Reg of Apoptosis	2.0E-5

5.4 Discussion/Analysis

Our results share many similarities to work previously carried out. The group we concerned ourselves with mainly was the knock out group 3. We know that CD133 functions as a protein present within the lipid rafts of the cell membrane and is likely to function in cell to cell communication as we have seen evidence of CD133 rich pseudopodia from one cell extending to another $CD133^+$ cell in what appears to be a targeted manner.

Cell motility may also be an important function as CD133 congregates towards the leading edge of a cell, its expression within the lipid rafts could relate to a signalling function that may relate to lipid movement to and from the membrane making CD133 a "cell membrane organiser". The presence of CD133 may increase motility and migration within the bone marrow and to other locations. The presence of CD133 on early progenitor cells may also function to keep the cells in a quiescent, undifferentiated anti apoptotic state within the bone marrow. Loss of the receptor would then allow the cells to differentiate, proliferate and ultimately die.

When CD133 was knocked down we observed a variety of changes relating to GO profiles, the main changes involved: 1) down-regulation of adhesion genes (P value 3rd highest), 2) down-regulation of apoptosis related genes (P value 1st highest), 3) down-regulation of cell signalling receptor (P value 5th highest), 4) up regulation of genes relating to cell cycle (P value 4th highest), 5) up-regulation of cell motility genes, 6) Up-regulation of proliferative genes, 7) Up-regulation of lipid interaction genes (P value 3rd highest), 8) Up-regulation of apoptosis related genes. These changes have strong correlations to previously published work. Published data can be split into 2 segments; primary cell and cell line experiments.

The primary cell data suggests CD133 may have a role in the majority of processes listed but direct comparisons are difficult as the studies tend to look at a positive and negative fraction of cells expressing CD133 and compare the effects of CD133 on gene profile or GO. This is somewhat different to our experiments where we knocked down the receptor and then looked at the gene profiles and GO of the knocked down portion of cells.

To try and compensate for this, a comparison was made between up-regulation of GO's in CD133+ cells within the published data against GO's down-regulated in knocked down cells in our studies.

5.4.1 Lipid interactions

The localization of CD133 in a variety of plasma-membrane protrusions and its interaction with cholesterol suggest that it may act as an organizer of these structures.
CD133 could be a regulator of lipid transport namely cholesterol and loss of the receptor may lead to loss of regulation of the process. CD133 is present within the lipid rafts found on membrane protrusions and the lipid content within these rafts may be determined by the receptor. CD133 knock out leads to the up regulation of lipid transport across the plasma membrane. *NPC1* was up regulated by 2 fold and is involved in cholesterol homeostasis. It is critical for the uptake of cholesterol across the plasma membrane and may function in the transport of multiple lipids and homeostasis. A lack of activity leads to the abnormal accumulation of cholesterol and other lipids within cells and also multiple transport defects (Fernandez-Valero *et al.*, 2005). This increase in lipid activity could also relate to motility as an increase in genes that would enhance lipid membrane activity would also promote motility of the cell.

5.4.2 Apoptosis

Apoptosis linked genes had the highest gene ontology with respects to down regulation ($3.7x10^{-14}$) however the role CD133 knockdown plays in apoptosis is not clear.
Liu *et al.*, (2006)[2] investigated the chemo resistance of $CD133^+$ cancer stem cells in glioblastoma and suggested that gene profiling proved that the resistance is probably contributed to by the high expression levels of BCRP1 (important in drug resistance in stem cells) and MGMT (the DNA repair protein), as well as the anti apoptosis protein and inhibitors of apoptosis families. Genes such as Bcl-2, FLIP, BCL-XL and Class 1-3 IAPs expressed higher mRNA levels in $CD133^+$ cells than in $CD133^-$ cells (Liu *et al.*, 2006)[2].
On knock down a down regulation of genes relating to programmed cell death occurred which made it difficult to define the role of CD133 in conjunction with apoptosis. Many of the genes down regulated positively regulate apoptosis such as; *BCL-2 assoc X protein* accelerates programmed cell death by binding to, and antagonizing the apoptosis repressor

BCL2 or its adenovirus homolog E1B 19k protein. It induces the release of cytochrome c, activation of CASP3, and thereby apoptosis. *BCL-2* (3) in an essential mediator of p53-dependent and p53-independent apoptosis. PRKC is a pro-apoptotic protein capable of selectively inducing apoptosis in cancer cells, sensitizing the cells to diverse apoptotic stimuli and causing regression of tumours in animal models. *Caspase 7* is involved in the activation cascade of caspases responsible for apoptosis execution, cleaves and activates sterol regulatory element binding proteins (SREBPs). *Death associated protein kinase 3* is a Serine/threonine kinase which acts as a positive regulator of apoptosis. *Serine/threonine kinase 17b* acts as a positive regulator of apoptosis and *Kruppel like factor 10* a transcriptional repressor involved in the regulation of cell growth by inhibiting cell growth.

However, there were also genes down regulated that were anti apoptotic and negative regulators of apoptosis such as *NUAK family,presillin1, TNF member 2, ubiquitin, cyclin dependent kinase inhibitor 2D.* Within the same group there was also pro-apoptotic up regulated genes such as *BCL2- like 14, unc-5 homolog B, tribbles homolog 3 and DNA damage inducible transcript 4.*

5.4.3 The Wingless Signalling Pathway

The WNT signalling pathway plays an important role in colon cancer carcinogenesis and metastasis, but has also been linked to CD133 expression. Various studies have shown the relationship between WNT and CD133 namely Rappa *et al.*, who showed that after knocking down CD133 in FEMX melanoma cell line, ten of the 76 up regulated genes coded for WNT inhibitors, suggesting an interaction between CD133 and the canonical Wnt pathway. The conical WNT pathway describes a series of events that occur when WNT proteins bind to cell surface receptors of the Frizzled family, causing the receptors to activate dishevelled family proteins and ultimately resulting in a change in the amount of β -Cantenin that reaches the nucleus. This in turn regulates diverse processes during development such as cell fate determination, structural remodelling, cell polarity and morphology, cell adhesion, and growth.

Deng *et al.*, (2010) showed by using colon cancer cells that the WNT activity is higher in CD133 positive DLD1 cells than in $CD133^-$ DLD1 cells (Deng *et al.*, 2010). Barcelos *et al.*, (2009) showed that foetal $CD133^+$ cells expressed high levels of wingless (WNT)

genes, which were down regulated following differentiation into $CD133^-$ cells, and were ultimately able to show that $CD133^+$ cells stimulate wound healing by paracrine mechanisms that activate WNT signalling pathway in recipients. Here we observed down regulation of *FZD5* by 2 fold, a receptor for WNT proteins, thus also indicating a relationship between the pathway and CD133. There was an up- regulation of the dickkopf homolog 1 (DKK1) gene by 3.5 fold. *DKK1* is an inhibitor of the WNT pathway (β Cantenin antagonist), which acts by interacting with the lipoprotein receptor-related protein (LRP), resulting in endocytosis to prevent formation of the complex with Wnt and Frizzled (Fz) for the canonical pathway.

DKK1 up-regulation was also observed by Rappa *et al.*, (2008) producing the highest fold change in their experiments and also observed by Kim *et al.*, (2006) who detected an up regulation of DKK1 in human mesenchymal cells and Ieta *et al.*, (2008) who studied the biological and genetic effects of $CD133^+$ tumor- initiating cells in 12 colon cancer cell lines. Comparing $CD133^+$ Vs $CD133^-$ cells they found several genes associated with stem/progenitor cells up regulated including the WNT pathway inhibitor DKK1 (Ieta *et al.*, 2008).

In our studies we also found up-regulation of the WNT inhibitor gene DKK1. *Beta Cantenin* was down regulated by knocking down CD133 this also would have an inhibitory effect on the WNT pathway. *FZD3 and FZD4* were up regulated by approximately 2 fold. Ligands such as *Wnt10A, 10B*, 9B, 6 and 3* were also down regulated but the fold change was very minimal. The data suggests that loss of CD133 seems to inhibit the WNT pathway via a variety of methods from up regulating inhibitory molecules and down regulating WNT ligands and receptors. The results show quite clearly that the WNT pathway and CD133 are connected and more than likely signalling occurs via this pathway. The MAPK signalling pathway may also have some connection with CD133 as we know that Src and Fyn were both shown to phosphorylate CD133 on its cytoplasmic tail (Boivin *et al.*, 2009). The third highest up regulated gene with a fold increase of 8.75 was Protein tyrosine phosphatase (PTPN12) a known inhibitor of MAPK signal transducers (Sun *et al.*, 2011).

5.4.4 Gene list comparison between groups 1 and 3

We carried out analysis comparing groups 1 and 3 to determine if we could further understand the mechanisms behind CD133 stimulation. It is important to understand that in theory the genes down regulated when CD133 is stimulated by the Mab's (Group 3) (based on the assumption mentioned above) should have similarities to the genes up regulated in the knock out group (Group 1). However genes that overlap between both groups would be extremely important from a functional analysis perspective as we are investigating 2 independent factors that should if the hypothesis is correct, contribute to the same outcome. Strangely we only saw one overlapping gene in this collective study which was a Zinc finger protein 206, an embryonic transcription factor that plays a role in regulating pluripotency of embryonic stem cells (Wang *et al.*, 2007). Yu *et al.*, (2009) showed that Zfp206 is a key component of the core transcriptional regulatory network and together with Oct4 and Sox2 regulates differentiation of ESC (Yu *et al.*, 2009).

For the second group the 2 overlapping genes that were found had ascension numbers A_24_P41250 and A_24_P840868 but no relevant gene ID's. This led us to believe that since a low number of overlapping genes occurred in both instances, the Mab's we initially thought were stimulating the receptor may possibly be exerting its influence by inhibiting or blocking function so we repeated the cross examination of both groups and analysed up regulation of both groups simultaneously and vice versa. If our new hypothesis was correct we would expect to see a good proportion of the genes to be overlapping both groups. We then deduced from the results in figures 62 and 63 that these processes are inhibited, suppressed or negatively regulated by the presence of CD133, as knockdown or "inhibition" seems to also lead to an up-regulation of these processes. This data backs up some of the work done previously when we looked at the knockdown group independently and commented that cell cycle was definitely up-regulated when CD133 was knocked down.

5.4.5 Gene ontology comparisons

Gene ontology of up-regulated genes

Within the set of genes that fall between the overlaps, we investigated their gene ontology's (table 14 in results). A thorough look into the specific genes up-regulated in the cell cycle gene ontology shows many common genes e.g.: Cdk5 and Abl enzyme substrate 1, cell division cycle 25 required for the progression of cells from G1 to S phase. E2F3 and E2F2 both transcription activators that binds DNA and functions in the control of cell cycle progression from G1 to S phase and also the cell proliferation gene S100P was found to be up-regulated in both groups. The low P values for the phosphorylation of proteins and the acetylation of protein molecules was difficult to explain as we did not see these observations earlier. DNA replication and cell cycle are closely related as processes thus one would expect an up-regulation of DNA replication to compensate the increased cell cycle activity.

Gene ontology of down-regulated genes

Gene ontology's were analysed in this set of overlapped genes too (table 15). Apoptosis or cell death seemed to dominate the ontology list. As observed earlier apoptosis was up regulated and down regulated in Group 1 and posed to cause some confusion as to CD133's role within the ontology's listed. Looking specifically at the genes down regulated between the two groups 1 and 3, it seems a good proportion of the genes correlate to stimulation or mediation of apoptosis such as BBC3, DAK, TNF and TP53INP1 whilst the rest act in an anti apoptotic manner such as CDKN2D which inhibits CDK4 and CDK6, TNFAIP3, a protein that interacts with NAF1 and inhibits TNF-induced NF-kappa-B-dependent gene expression and inhibits programmed cell death and CCL2, another inhibitor of apoptosis. The fact that apoptotic and anti-apoptotic genes are both present with CD133 expression indicates that CD133 may have more of a regulatory role in relation to apoptosis allowing the switch to be "turned on or off ". During haemopoiesis CD133 is present on early haemopoietic stem cells. On maturation the receptor is lost and the cells then differentiate and proliferate. CD133 may act as a positive regulator of stem cells. Cells that fail to remain quiescent and begin differentiating and proliferating prematurely may lack certain receptors and characteristics causing the stem cells to

become non functional or over active leading to possible cancers. The potential role of CD133 could be to activate the apoptotic genes in these cases eliminating potentially harmful anergic stem cells. Conversely if the cell matures and develops steadily the anti-apoptotic genes are activated and the cell lives.

In summary this work has shown that the addition of CD133 Ab to the Caco-2 cells seems to be inhibitory. This was shown by comparing the gene profiles in both group1 (Knockdown) and group 3 (CD133 Ab labelled). The results showed a 26% overlap in co expression of genes up-regulated and a 56% overlap in co expression of genes down-regulated when both groups were compared. The inhibition may be caused competitively preventing the natural ligand to bind to the receptor. Binding of the Mab may be causing a conformational change to the receptor preventing ligand binding or moreover a partial signal is being produced by the Mab which may cause anergy/shutdown of the CD133 receptor.

Knocking down CD133 receptor lead to an increase in proliferative and cell cycle genes, the highest up regulated gene was S100P by 10.4 fold which stimulates cell proliferation and survival. The up-regulation of theses processes indicates that CD133 may contribute to the quiescent, non-proliferating and non differentiating phenotype associated with stem cells. Motility was also enhanced when the receptor was knocked down. This was achieved through a variety of processes such as a general decrease in adhesion molecules and chemokines causing disruption from the basement membrane. An up-regulation of CD44 and cofflin also contributed to cell migration and directional motility respectively suggesting that the role of CD133 maybe to hold the stem cells within the protected stem cell niche. Lipid interactions were also increased, the uptake of cholesterol and lipids were increased which would suggest that CD133 may be acting as a cell membrane organiser regulating the fluidity of the cell membrane and possibly enhancing other processes such as motility. Surprisingly inhibitors for the WNT pathway and the MAPK pathways were also up-regulated leading to an inhibition of both pathways which would stimulate or inhibit proliferation and differentiation and both genes promoting and inhibiting apoptosis were up-regulated.

Chapter 6: Functional analysis of CD133 using Confocal Laser Scanning Microscopy

6.1 Aims and objectives

The primary aims of this chapter were:

- To investigate the association of CD133 within Lipid Raft.
- To determine whether Beta methyl cyclodextrine can remove Lipid Rafts from the cell surface.
- To establish whether CD133 is necessary for Lipid Raft formation and conversely if Lipid Rafts are necessary for CD133 expression.
- To determine whether Caco-2 cell adhesion to fibronectin affects the expression or movement of CD133 on the cell surface.

6.2 Introduction

6.2.1 The Principles of Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) provides a technique for optically sectioning fluorescent labelled whole cells or tissues. Optical resolution and contrast are enhanced by using point illumination and a spatial pinhole to eliminate out of focus light in specimens that are thicker than the focal plane (White and Fordham 1987). Full field conventional microscopes tend to flood the specimen with light thus all parts of the specimen are excited and the resultant image contains both in focus and out of focus background imaging. With confocal technology the back ground is eliminated creating a sharper image. An additional advantage with confocal microscopy in comparison to conventional techniques is the ability to reconstruct three-dimensional structures from the obtained image stacks.

Laser light of various wavelengths are used to excite the fluorescent probes or conjugated antibodies (Dunn *et al.*, 1994) thus making it possible to undertake multi –fluorescent analysis. The excitation light is refracted by a dichroic mirror and focussed onto the specimen by the objective lens. The fluorescent and refracted light is then collected by the same objective lens. The dichroic mirror splits the fluorescent and refracted light, only allowing the fluorescent light to pass and removing the refracted light from the final image. Filters are also present to ensure that reflected laser light is not allowed to reach the confocal aperture and contribute to the final image. The emitted fluorescent light is then focussed onto the confocal imaging aperture. The focal point of the aperture and specimen are on conjugated planes restricting light from above and below the focal plane and resulting in high axial resolution free from stray light. The dept of focus is dependent on the size of the confocal aperture, with a smaller aperture producing a thinner focal plane, forming an image of an individual slice through a region of the cell.

Figure 64: Diagrammatic representation of the CLSM beam path
The laser light is filtered by the dichroic mirror and scanned across the specimen by the scanning mirror, the objective lens focuses the light on the specimen and then collects the emitted fluorescent light. Within the inverted microscope the light passes through filters which allow the required wavelength of light to be emitted. The refracted and fluorescent lights are split through a dichroic mirror which focuses the fluorescent light through a pinhole (confocal aperture) onto a photo detector tube (www.ifr.ac.uk).

By recording only in focus light, it creates the possibility for the microscope to act as if there were an infinite depth of field. By using an automated microscope stage the depth of the focal plane can also be adjusted whilst undertaking simultaneous x, y scanning to create an axial z scan. A complete in focus representation of the specimen throughout the z-axis as illustrated in figure 65A, Z scan slices can be achieved (Sheppard and Shotton, 1997). CLSM optics enables the observation of cells that are adherent whilst minimising the interference from non attached cells floating above the cell layer within the mounting medium and free fluorochromes or non specific fluorescent debris above the cell layer (Zemanova *et al.*, 2003).

Figure 65: Diagrammatic representation of Z scan slices through a 3-D image
A) Diagram to show slices thickness, dependent on the size of the confocal aperture pinhole (Pawley *et al.*, 2006). **B)** Representation of 3D mapping of Lipid Rafts (red) on Caco-2 cells using Imaris (Bitplane Scientific Software).

As confocal analysis permits removal of signal which sits out of the focal plane, this allows for a more accurate analysis of antigen distribution within a 3-dimensional view (representation provided in figure 65B) of cells when compared to conventional fluorescent microscopy. This was applied to investigate the distribution of CD133 and lipid rafts (LR) on Caco-2 cells to determine a possible relationship between CD133 and LR distribution. Five key points were evaluated using CLSM analysis; 1) are LR and CD133 co-localised, 2) can LR be removed successfully from the cell (using Beta Methyl Cyclodextrine), 3) does removal of LR result in removal of CD133, 4) does removal of CD133 result in removal or reduction in LR, and 5) does CD133 associate with the apical or basolateral regions of the cell relative to cell adhesion +/- fibronectin?

Visual analysis alone is often subject to operator or viewer bias. The use of analytical software to standardise data, generating numerical values suitable for quantitative and statistical analysis allows for a more unbiased approach. MATLAB® (MathWorks) is a numerical computing environment and fourth-generation programming language used in our study to determine statistical values. MATLAB allows matrix manipulations, plotting of functions and data and implementation of algorithms. MATLAB® algorithms were developed by Dr Andreas Hoppe of the Digital Imaging Resource Centre at Kingston University, and applied to the CLSM data for the quantification of signal to background

ratio in the determination of LR and CD133 distribution on Caco-2 cells under a number of different experimental conditions.

Signal to background ratio was determined by the masking of regions of positive signal. This was using a relatively crude technique, but one which allowed for removed of debris stained signal, and accommodation of relatively high background noise where encountered. This also permitted analysis of single cells where more than one cell was evident on the CLSM image. By using signal to background ratio, this also allowed for differences in digital zoon between different CLSM images making the data sets comparable.

6.3 Results

6.3.1 Fluorescence Specificity Confirmed using Isotype Controls

Irrelevant matched isotype control Immunolabelling was carried out using mouse pure IgG1 alongside each positive stain. The matched isotype control slides (Figure 66) showed no non-specific fluorescent labelling. The green and red haze within the cells represents internalisation of the fluorochromes and potential signal bleed through by DAPI.

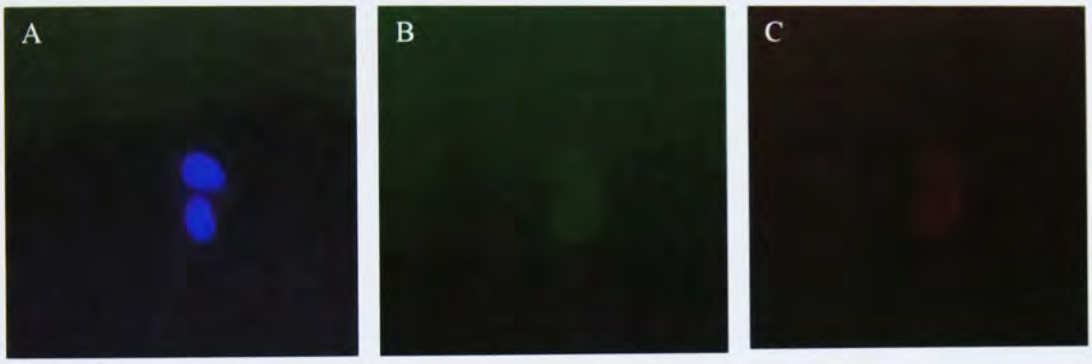

Figure 66: CLSM fluorescent staining showing irrelevant matched isotype antibody controls. A) Shows staining with DAPI alone. **B)** Mouse pure – IgG1 and Alexa Fluor 488 (green). **C)** Mouse pure IgG1 and Alexa Fluor 555 (red). Obj x63, N.A 1.4.

6.3.2 The association of CD133 within Lipid Rafts

Immunofluorescent labelling of CD133 using Alexa Fluror 468, figure 67A and LRs using Alexa Fluror 555 figure 67B on the Caco-2 cell line shows CD133 distributed in dense pockets around the cell membrane whilst LR distribution demonstrates a brighter peripheral halo around the cell. Regions where CD133 and LR are co-expressed are denoted by yellow as shown in figure 67C. Not all LR (red) are associated with CD133 (green) and not all CD133 is associated within LR.

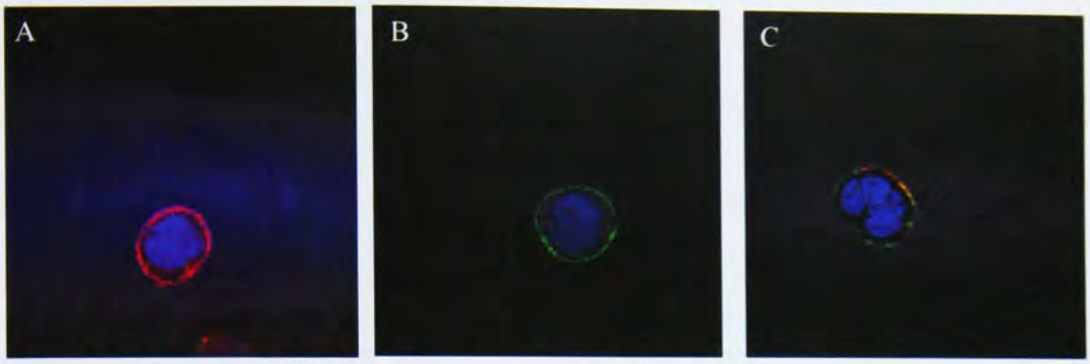

Figure 67: Caco2 cell line showing CD133 and Lipid Raft fluorescent distribution. A) Image showing Lipid raft fluorescent staining red (Alexa Fluror 555). Lipid Rafts distributed within a dense peripheral halo. **B)** Image showing CD133 staining green (Alexa Fluor 488). CD133 distributed within bright pockets surrounded by a slight peripheral halo. **C)** Dual staining of both Lipid rafts and CD133 showing individual staining, red and green respectively and co-localisation as yellow. Obj x63, N.A 1.4.

6.3.3 Removal of Lipid Rafts by Beta methyl cyclodextrine

Removal of the Lipid rafts was carried out using the drug BMCD which causes a physical disruption of the LR proteins. Removal of the LRs is shown in figures 68A and B on Caco-2 cell LRs stained with Alexa Fluror 555, and CLSM analysis. Minimal levels of LR remained after BMCD disruption. Cell signal to background ratio was also determined by Matlab analysis of CLSM images for BMCD and non BMCD treated Caco-2 cells and shows in figure 68B there was significantly less ($p=0.008$) LR expression on cells having undergone BMCD treatment when compared to none treated cells.

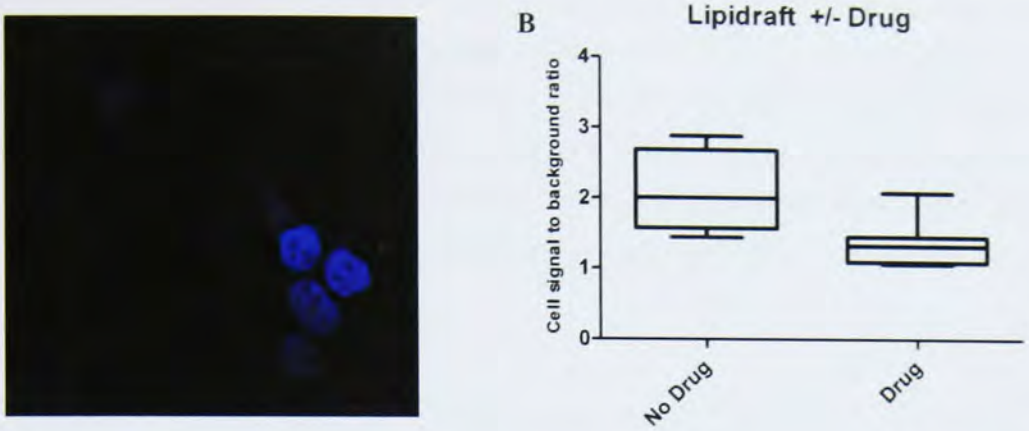

Figure 68: Effect of Beta methyl cyclodextrine on Lipid Raft expression on Caco-2 cells. A) Caco-2 cells treated with 2mM Beta methyl cyclodextrine were immunofluorescent labelled against Lipid rafts with AF555 (red) and shows minimal fluorescent signal (n=11) Obj x63, N.A 1.4. **B)** Comparison between cell signal and background signal ratio for CLSM Z section slices of treated (n=14) and un-treated (n=10) cells show a significant reduction in signal level for LR (Mann Whitney, paired

6.3.4 Are Lipid Rafts necessary for CD133 expression?

To determine the importance of LRs in CD133 expression removal of the LRs was carried out using the drug BMCD which causes a physical disruption of the LR proteins. Confirmation of CD133 loss in the presence of BMCD was carried out at 20mM BMCD for incubation periods of 40min and 1 hour, and direct immunofluorescence of CD133 was analysed by flow cytometry. Both time points show considerable loss of CD133 expression as shown in figures 69D and E. However, at 1 hour incubation, there was also considerable loss of cell viability as shown by the distribution of cells against the Y axis in figure 69E and suggested previously during cell viability monitoring using trypan blue. For all subsequent work, a concentration of 20mM of BMCD with an incubation periods of 40min was used.

Removal of the LR could be detected by CLSM, by staining the rafts with Alexa Fluror 555 with CD133 expression also lost after removal of the LRs Figure 70A. Small amounts of the LR remained after BMCD disruption however CD133 was no longer expressed. Loss of CD133 expression could be a consequence of lipid membrane damage, or the action of BMCD in removing cell surface receptors on the cell membrane indiscriminately.

An alternative approach to test this would be to knock down caveolin1, which is a protein that contributes quite heavily to the structural formation of the LRs and thus having no direct physical effect on the CD133 protein. Cell signal to background ratio for CD133 staining was also determined by Matlab analysis of CLSM images for BMCD and non BMCD treated Caco-2 cells and shows in figure 70B there was significantly less ($p=0.0002$) CD133 expression on cells having undergone BMCD treatment when compared to none treated cells.

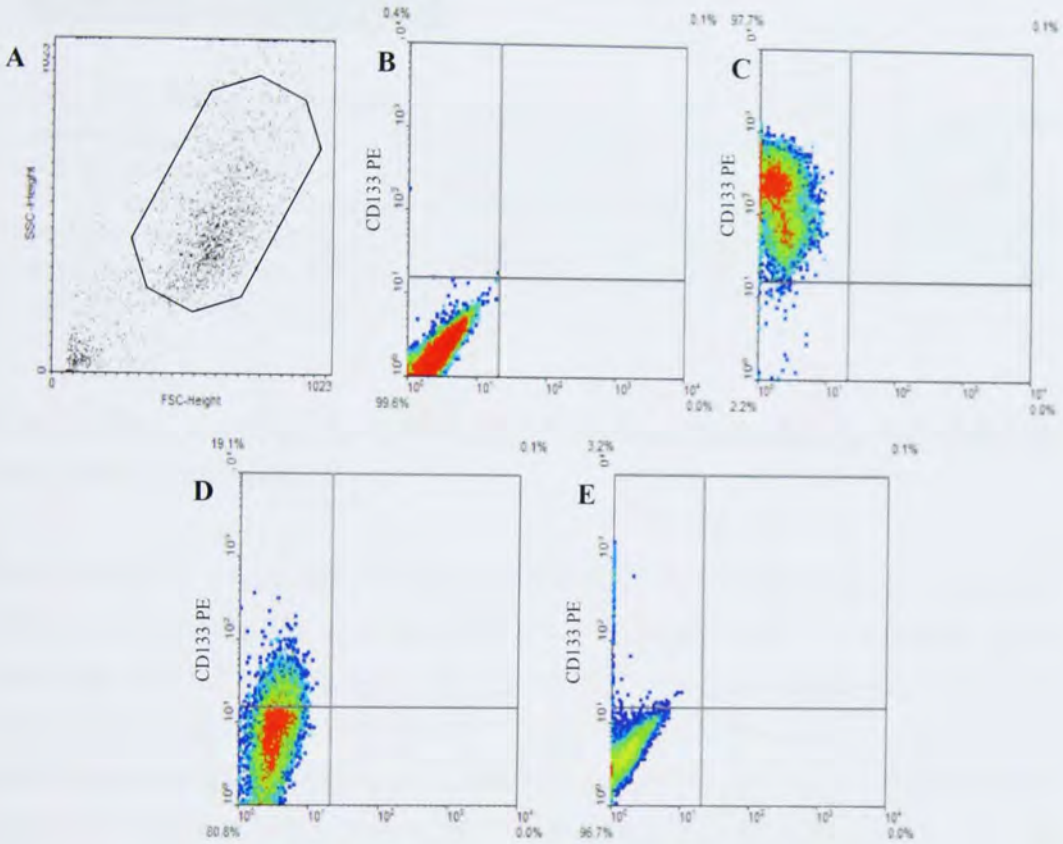

Figure 69: Effect of Beta methyl cyclodextrine on CD133 expression on Caco-2 cells analysed using flow cytometry. **A)** gated population B) PE isotype controls **C)** CD133 PE expression on membrane surface. **(D and E)** CD133 expression with Beta methyl cyclodextrine at 20mM for 40min and 20mM for 1 hour, producing knock downs of 80.8%±7 SEM and 96.7%±9.2 SEM respectively (n=3).

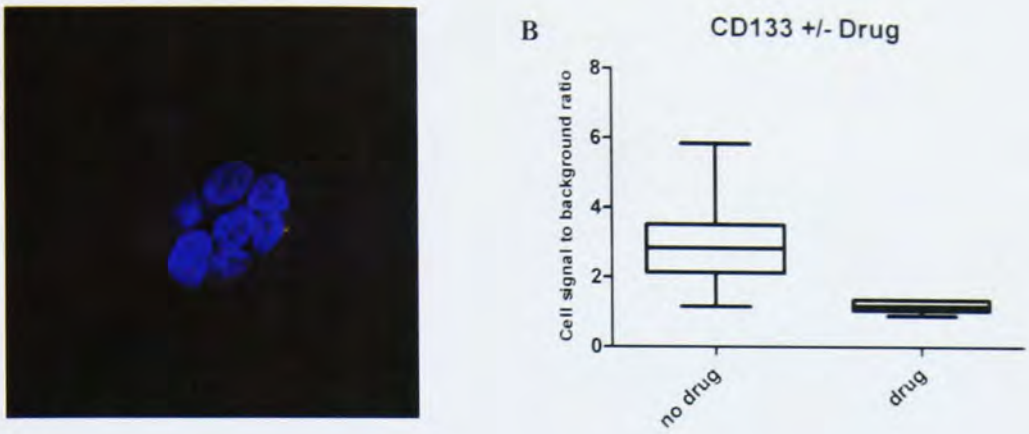

Figure 70: Effect of Beta methyl cyclodextrine on CD133 expression on Caco-2 cells analysed by CLSM. A) Cells were immunofluorescent labelled against CD133 with AF488 (green) and against Lipid raft with AF555 (red), showing negative staining for CD133 and minimal LR staining (n=10) Obj x63, N.A 1.4. **B)** Comparison between cell signal and background signal ratio for CLSM Z section slices of CD133 expression on treated (n=13) and non treated (n=11) cells shows a significant reduction in signal level for CD133 (Mann Whitney, paired test).

6.3.5 Is CD133 necessary for Lipid Raft formation and thus indirectly responsible for cell to cell communication?

We investigated whether CD133 expression was necessary for LR formation by knocking down CD133 expression using the CD133 RNAi (Santa Cruz) and depletion of the remaining $CD133^{+}$ cells through immunomagnetic selection. Caco-2 cells were then immunofluroescent stained with AF555. Figure 71 shows the population of Caco-2 cells before knockdown expressing high levels of CD133 (97%) and after purification of the CD133-ve cells by CD133 depletion. CD59 was used to confirm that firstly the cells were Caco-2 and secondly they were still viable as shown in figure 71D. Dual staining of CD133 and LRs on Caco-2 cells as shown in figure 72A clearly indicates the presence of both receptors on the cell membrane. Figure 72B shows dual staining with CD133 AF488 and LR AF555 depicting that the level of LR expression has not been affected by the loss of CD133.

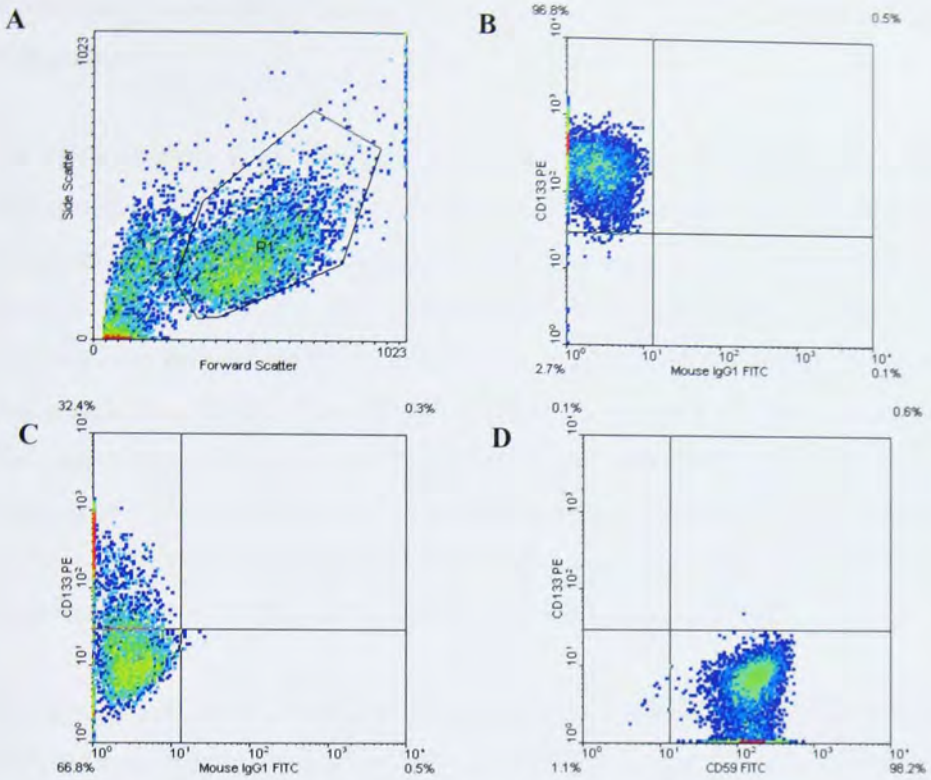

Figure 71: Confirmation of CD133 knockdown by flow cytometry A) un-gated Caco-2 cells **B)** R1 gated Caco-2 cells expressing CD133 receptor showing 96.8%%±3.6 positivity. **C)** Cells knocked down by RNAi CD133 (Santa Cruz) showing 67.8%%±11 $CD133^{+}$, and **D)** Purified population of $CD133^{-}$ cells, with 98.8%±2.9 SEM expressing CD59 as a Caco-2 marker and 0.7%±2.2 SEM $CD133^{+}$ (n=6).

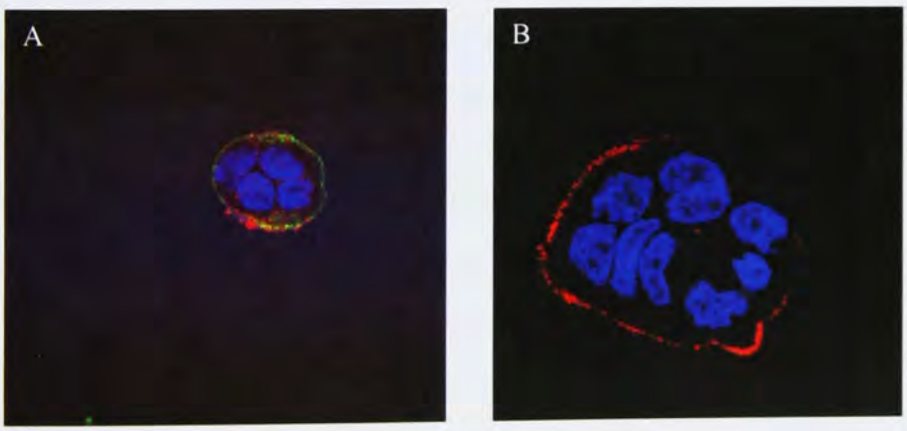

Figure 72: CLSM analysis of CD133 and Lipid raft staining on normal and knockdown CD133 Caco-2 cells. A) Shows dual fluorescent staining of CD133 (green) and Lipid rafts (Red), co-localisation (yellow) on normal cells. **B)** Showing lipid rafts (red) and no CD133 (green) on knockdown CD133 Caco-2 cells. (n=5) Obi x63. N.A 1.4.

6.3.6 Does Caco-2 cell adhesion to fibronectin affect the Distribution of CD133 on the cell surface?

This was determined by comparing Z scan data of Caco-2 cells adhered to uncoated and fibronectin coated slides. CD133 fluorescence was measured using Alexa Fluror 555 for both groups. The Z section galleries in figure 73 (A and B) shows the CD133 distribution through the z-axis of the cell for Caco-2 cells adhered to glass slides or fibronectin. Although the fluorochrome used (AF 555) to stain CD133 emits red fluorescent light and the nuclear stain (DAPI) blue, the imaging software (Zeiss Zen 2009 Light Edition) used in the construction of the gallery images, auto-converts for display purposes to green and red respectively. Both galleries show that there is no great difference in distribution between the two adhesion conditions and there appears to be a moderate increase in CD133 distributed within the Z section slices towards the bottom of the cell.

For the quantification of signal to background ratio and distribution of CD133, the cell Z sections were simply divided into top and bottom slices based on the number of Z sections and central Z section slice. Signal to background ratios were determined for each slice of the Z sections and comparison made between top and bottom regions within cells adhered to glass and top and bottom regions for cells adhered to fibronectin. Comparison was also made between the top distribution and bottom distribution of signal to background ratio between the glass and fibronectin adhered cells. One Way ANOVAs and bonferroni post hoc test analysis was carried out and showed there to be significantly more signal to background ratio, representing CD133 distribution, towards the top of the cells for both the glass and fibronectin adhered cells when compared to the bottom of the cells ($p<0.005$) as shown in figure 74. There was no significant difference ($p=0.05$) when comparing the overall CD133 distribution between glass and fibronectin adhered cells.

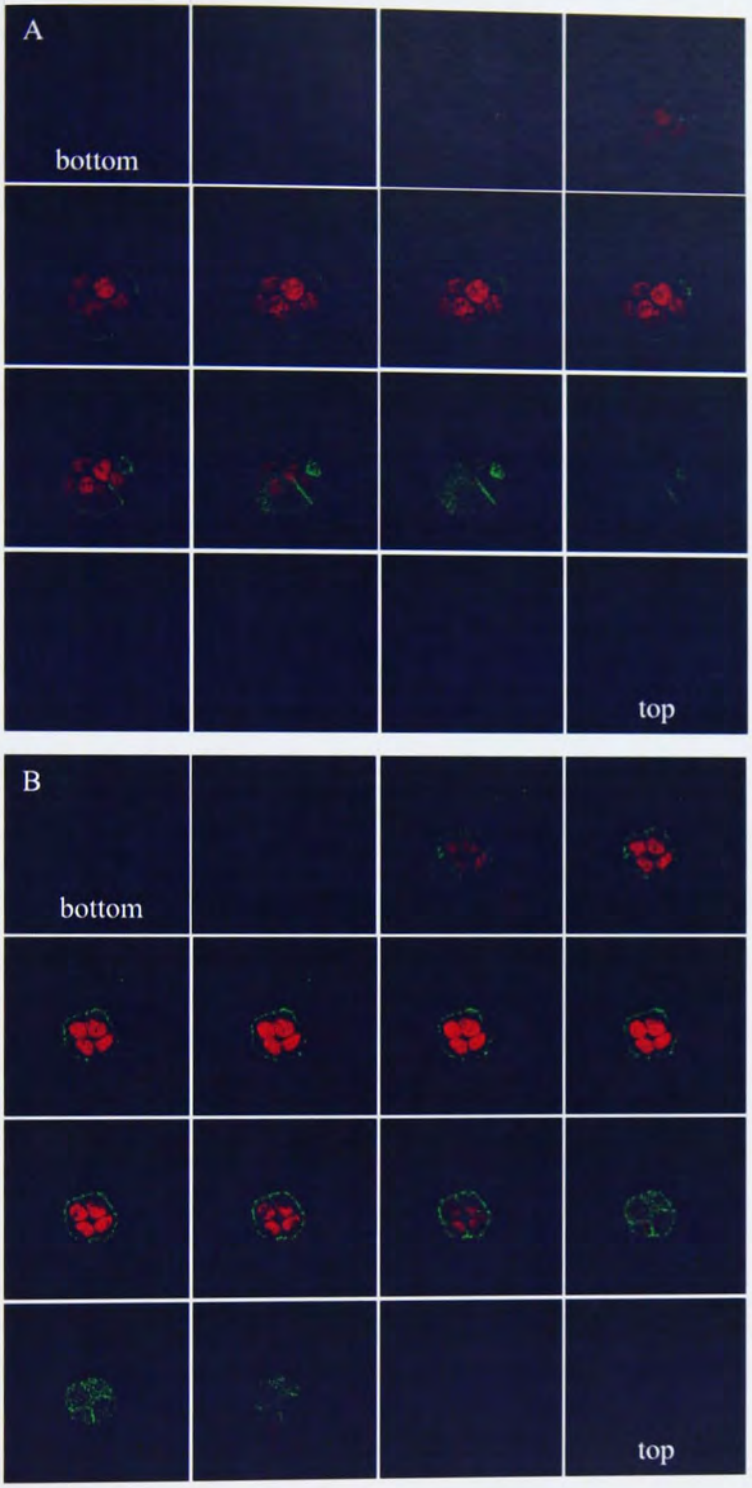

Figure 73: CLSM Z-section gallery of CD133 distribution on Caco-2 cells adhered to glass slides or fibronectin. A) Caco-2 cell z-section gallery of CD133 (green) distribution and nucleus (red) on cells adhered to glass slides. **B)** Caco-2 cell z-section gallery of CD133 (green) distribution and nucleus (red) on cells adhered to fibronectin. (n=6). Galleries constructed using Zeiss CLSM reconstruction software. Obj x63, N.A 1.4.

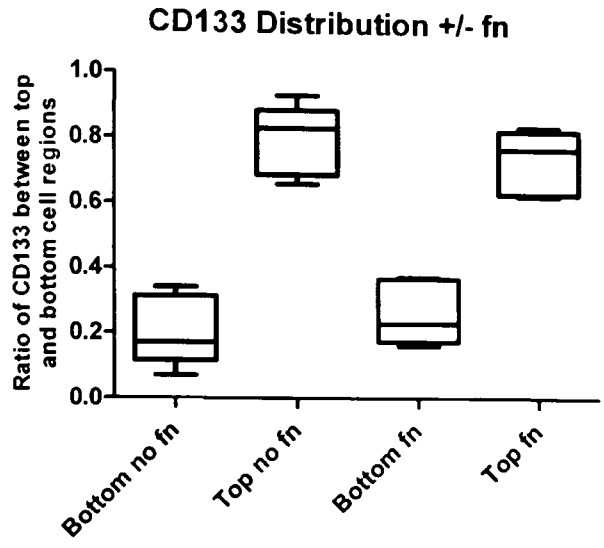

Figure 74: Comparison of CD133 signal to background ratio distribution on Caco-2 cells adhered to glass slides or fibronectin. Caco-2 cell z-sections divided into top and bottom segments for analysis of signal to background ratio and comparison of CD133 distribution within the top and bottom cell region. Showing significant difference ($p<0.005$) in CD133 distribution for cells adhered to either glass or fibronectin. Comparison of top region for glass and fibronectin adhered cells and bottom region for glass and fibronectin adhered cells shows no significant difference ($p=0.05$) ($n=6$). Analysis carried out by One Way ANOVA.

6.4 Discussion

Our studies showed that CD133 was partially co-localised within LRs on the membrane of Caco-2 cells. The drug BCMD significantly removed both LRs and CD133 from the cell surface compared to the control group with no drug added. The confocal images showed that removal of LRs resulted in decreased expression of CD133, but conversely, knocking down CD133 had no significant effect on LR expression. Finally CD133 distribution on the cell surface was not affected by cell adhesion to fibronectin.

Our first question would be to determine the role of CD133 within the LRs and why the receptor is found in this location? Is there a functional reason for its location or is it merely coincidental? LRs are involved in influencing membrane fluidity and membrane protein trafficking (Korade *et al.*, 2008). It is well established that, in the case of tyrosine kinase signalling, adaptors, scaffolds and enzymes are recruited to the cytoplasmic side of the plasma membrane as a result of ligand activation (Hunter *et al.*, 2000). CD133 contains a short C-terminal cytoplasmic domain with five tyrosine residues, including a consensus tyrosine phosphorylation site. Boivin *et al.*, (2009) showed that the cytoplasmic domain of the CD133 receptor is tyrosine phosphorylated in human medulloblastoma D283 and Daoy cells, in a Src family kinase-dependent manner (Boivin *et al.*, 2009) suggesting that CD133 signalling may be occurring via the tyrosine kinase pathway within the LRs hence their location.

Our studies also showed that BMCD was able to remove LRs but also had a significant effect on CD133 expression indicating the drug was physically responsible for the removal of CD133. Unfortunately, we were not able to fully determine the effect LRs had on CD133 expression as the harshness of the drug to the cell membrane, removed a significant amount of CD133 receptor from the membrane and this result maybe be irrelevant of LR expression. To determine whether LR expression is necessary for CD133 expression the rafts would have to be knocked down using RNAi technology. The expression levels of CD133 can then be measured using confocal analysis or flow cytometry. Conversely our results showed that knocking down CD133 had no effect on LR formation and expression. This supports studies by Roper *et al.*, (2000), who proposed that CD133 is incorporated into LRs and Giebel *et al.*, (2004) who suggest it is very likely that LRs organize the

delivery and/or retention of CD133. This concludes CD133 is found within the rafts and are not intercalated or a physical entity of the rafts themselves.

The distribution analysis of CD133 quite clearly shows that the CD133 congregated towards the top of the cell. This would correlate with the well documented evidence that CD133 is found on the apical membrane and not on the basolateral membrane of cells and normally associated with membrane protrusions not directly adhesion-anchorage related. Binding to fibronectin had no effect on the distribution of the receptor indicating that CD133 does not physically get involved with anchorage or adhesion as part of the migratory process in Caco-2 cells. However we know that cell polarity is important to migration. Polarisation is thought to be connected to the PI3K pathway and inhibition of this pathway causes primary human bone marrow $CD34^+$ cells to lose their polarised morphology and also lose their migrating potential (Wang *et al.*, 2000). CD133 is selectively concentrated in the uropod of polarized HSC/HPC suggesting a possible indirect role in motility (Roper *et al.*, 2000). $CD34^+$ cells have been known to acquire a polarized cell shape on adhesion to fibronectin which ultimately would lead to a change in CD133 distribution within the cell (Fruehauf *et al.*, 2002). However we failed to observe this suggesting this may only be apparent with non-adherant cells.

Based of these results alone it would be difficult to define the role CD133 plays within LRs. An important factor to consider initially is the type of cell being investigated. It seems the role of LRs may be dependent on whether the cells are adherent in nature or normally free in suspension. Cells in suspension enable local polarization contributing to cell motility (Ikonen *et al.*, 2001), which may also explain their connection with CD133. Generally CD133's role has often been associated with either lipid bilayer organization, whether it is formation of microvilli, pseudopodia or regulation of lipid membrane concentrations.

Chapter 7 General Discussion:

CD133 was discovered nearly 15 years ago and classified as a marker of primitive haemopoietic / early progenitor cells within the bone marrow (Yin *et al.*, 1997). It is also found on neural stem cells (Corti *et al.*, 2008), undifferentiated epithelium (Bussolati *et al.*, 2005) and myogenic cells (Negroni *et al.*, 2009). In tissues, CD133 is expressed in cells within the brain, kidney, prostate, colon, liver, pancreas and skin (Corbeil *et al.*, 2000; Weigmenn *et al.*, 1997). The functional role CD133 plays on the other hand is still an enigma. This study investigated the potential roles CD133 might play in normal and malignant cells and its overall contribution to haemopoiesis. Our results have led to a number of interesting questions that will now be considered.

Little is known regarding the roles of CD133 in normal and malignant cells. This work is consistent with studies suggesting that CD133 may play a role in plasma membrane organisation due to its location on microvilli on the plasma membrane (Mizrak *et al.*, 2008, Fargeas *et al.*, 2006; Zobalova *et al.*, 2011). The data using confocal analysis indicates that CD133 is significantly localised with cholesterol lipids rafts on the cell membrane. These serve as organisers of various plasma membrane activities such as maintaining lipid composition within the membrane (Roper *et al.*, 2000). CD133's role within the plasma membrane is also supported by the fact that a mutation in the CD133 gene causes retinal degeneration (Corbeil *et al.*, 2001) and that loss of Prominin-1 in CD133 knockout mice causes photoreceptor cell degeneration (Zacchigna *et al.*, 2009).

Previous studies in this laboratory demonstrated $CD133^{+}$ pseudopodia from primary human umbilical cord blood selectively contacting a distant cell, strongly suggesting a role within directional cell communication to another CD133 positive cell. On plastic or stromal adhesion CD133 relocates to polar regions of the cell, whereas prior to stimulation CD133 is distributed in pockets across the cell membrane (Whiting *et al.*, 2003; Hayward-Costa *et al.*, 2000). It may be that the distribution on the leading edge of motile cells and directional movement is unique to circulating cells. Certainly, adhesion, migration and cell cycle have all been linked to CD133 expression especially within haemopoiesis (Hemmoranta *et al.*, 2006; Jaatinen *et al.*, 2006). Studies have shown that cells expressing CD133 demonstrated improved adhesion to extracellular matrix and endothelial monolayer

substrates, compared to freshly isolated $CD133^-$ (Suuronen *et al.*, 2006). The majority of $CD133^+$ cells remain in the G0/G1 phase indicating that the expression of the receptor inhibits cell cycle (Jaksch *et al.*, 2008). Haemopoietic blood stem cells are mobile by definition and it may be that within the bone marrow or tissue sites, adhesion to specific cells within the microenvironment or other stem cells perhaps to maintain the cells in a quiescent, undifferentiated state rather than directional movement is the dominant function.

Kim *et al.*, (2006) studied the molecular signatures specific to various stem cells. It was not a direct observation of the effects of CD133 on the various cell types, but merely to detect if any patterns could be observed in the gene profile of embryonic, haemopoietic and mesenchymal stem cells. All 3 cell types were shown to regularly express CD133 and were compared against universal human RNA. More importantly the expression profiling was split into 2 categories for the HSC; CD34 and CD133 expressing cells. 653 genes were up regulated more than 2 fold and 842 genes down regulated more than 2 fold for the CD133 category. The most highly up regulated genes were involved in signalling pathways and transduction, transcription factors and regulation, DNA binding, cell cycle, receptor/surface markers/surface membrane and metabolism. The genes up regulated in all stem cells include those that encode a signalling protein RHOG, a ras homolog gene family member G, a transcription factor HIF1A and several proteins with known functions such as protein transport TLOC1 and catabolic activities (Kim *et al.*, 2006).

In a similar experiment Jaatinen *et al.*, (2006) took human cord blood derived $CD133^+$ cells and performed a global expression analysis on the cells. The $CD133^-$ fraction was used as a control. In this case 690 transcripts were over expressed between $CD133^+$ and $CD133^-$. 393 were increased and 297 were decreased in $CD133^+$ cells. The fractions were separated using immunomagnetic beads which would cause a similar response to labelling the receptor with CD133 Mab as was carried out in Group 3 of our experiment.
Biological processes over expressed included cell proliferation, transport, signal transduction, cell organization and biogenesis, cell adhesion, response to stimulus, development and metabolism. Biological processes under expressed were cell communication, chemotaxis, apoptosis, organogenesis and immune response. Several HSC associated genes were over expressed; CD133, CD34, KIT, TIE,SCA-1 MEISI. Genes supporting self renewal, such as GATA2, MPLV and STAT5A and TCF7L2 were also up

regulated. HOX genes (A5, A9, A10) also thought to be involved in HSC regulation were also up regulated (Jaatinen *et al.*, 2006).

These studies suggest that CD133 has a potential role in a variety of processes so it would be fair to assume that knocking down CD133 would inhibit or at least dampen these processes listed. As our results suggest, CD133 has a stimulatory role in cell adhesion, migration and cell signalling and this was supported by both studies quoted. However our results reflected that CD133 had an inhibitory effect on cell cycle and proliferative genes.

Most of these experiments revolved around purifying CD133 cells and comparing them against the negative fraction in primary cells or cell lines. The problem with this approach is that one cannot really understand the true functions of CD133 due to other undefined receptors being present on the $CD133^+$ cells and not on the $CD133^-$ fraction and which are potentially influencing the gene profiles produced. It was then important to try and really understand the direct effect of CD133 on the cells and ultimately on gene profile. This could be done in a variety of ways, the main approach would be to try and attempt to stimulate the receptor using an antibody raised against the receptor or even better using the actual ligand but because the ligand for CD133 was unknown, attempted stimulation using an antibody would be the only alternative.

An alternative approach would be knocking out the receptor and analyzing a change in gene profile between the control and knocked down cell line. This was achieved by Rappa *et al.*, (2008) using a melanoma cell line FEMX-1. They created 2 knock down cell lines using two different short hairpin RNA's and performed various functional assays to try and determine whether CD133 could be a molecular therapeutic target receptor. Down-regulation of CD133 resulted in slower cell growth, reduced cell motility and decreased capacity to form spheroids under stem cell like growth conditions. The rate of metastasis was also reduced which could be linked to the decrease in motility
In total only 76 and 67 genes were up and down regulated respectively. The up-regulated genes included 10 established or putative inhibitors of the canonical Wnt pathway, namely DKK1 (B catenin antagonist), DACT1 (Dapper), LRP6, three members of the insulin growth factor binding protein (IGFBP) family, N-cadherin, AXL, EFS and FerIL3. They propose that CD133, directly or through its interaction with signalling pathways such as WNT, and their functional experiments suggest influences on cell polarity, migration and

the interaction of CSC's with surrounding cells and the extracellular matrix contributing to the metastatic potential (Rappa *et al.*, 2008). Our results indicated that CD133 knockdown stimulated proliferation, cell cycle and motility activity in contrast to Rappa *et al.*, (2008) however we did observe inhibition of the Wnt pathway at various points.

CD133 has been shown to interact with the TGF-Beta signalling pathway. Like CD133, TGF-Beta receptors localize to cholesterol rich lipid rafts as well as endosomes and lysosomes (Meyer *et al.*, 2011) and TGF-Beta signalling has been shown to up-regulate CD133 transcription by demethylation of its promoters (You *et al.*, 2010). Rappa *et al.*, (2008) showed a 2.96 fold up-regulation of TGFBI transcripts after CD133 knockdown, and we found an even greater induction of 3.91 fold after CD133 knockdown. TGFBI is a protein induced by TGF-Beta signalling that is involved in collagen adhesion and motility. Under normal conditions, TGF-Beta induces cell cycle arrest and apoptosis, but in injured liver tissue it may induce epithelial to mesenchymal transition conferring motility and stem like properties on target cells through interaction with MAPK signalling pathways (Fischer *et al.*, 2005). Indeed putative liver cancer stem cells which may be isolated by CD133 expression have been shown to undergo a similar transition increasing invasiveness and tumour progression (Gotzmann *et al.*, 2006). Together this suggests that CD133 may interact with TGF-Beta signalling to regulate cell motility and proliferation in normal and cancer stem cells.

Gene profiling of cell lines expressing CD133 similar to the melanoma cell line mentioned above have produced varying results and hypothesis. The cell line use in the gene array experiments was a colon cancer line, Caco-2. Cancer cell lines have been used quite commonly to try and understand the function of CD133. Botchkina *et al.*, (2009) used the colon cancer HCT116 cell line to analyse gene expression between an enriched $CD133^{+}$ population versus the bulk tumor cells lacking CD133 expression. In this case there was an over expression of 826 genes and reduced activity in 162 genes in the $CD133^{+}$ population. Over expression was related to anti apoptosis, stemness, cell cycle and proliferation, transcription factors and DNA repair. The most significantly down regulated genes were HLI4 which is responsible for heterophilic cell adhesion (motility, migration, metastasis), apoptosis- related cytochrome c, COX6A1 and BCL2L1, which regulates the release of cytochrome c from mitochondria. CXCL14 involved in cell signalling also responsible for cell motility, migration etc. other down regulated genes were involved in apoptosis, cell

cycle/proliferation, cell motility, genes involved in negative regulation of proliferation, signal transduction and heat shock protein genes (Botchkina *et al.*, 2009).

Garcia *et al.*, (2010) showed that gene expression analysis of $CD133^+$ vs. $CD133^-$ cell populations from various fresh, primary tumours resulted in an up regulation of genes involved in angiogenesis, permeability and a down regulation of genes implicated in cell assembly, neural cell organization and neurological disorders (Garcia *et al.*, 2010). Beier *et al.*, 2007 looked at primary glioblastoma derived cancer stem cells expressing $CD133^+$ and compared the gene profiles against cells not expressing the receptor. The genes up regulated in $CD133^+$ CSC were related to Growth factors/intracellular signalling, migration, ECM associated genes, DNA interacting proteins and transcription factors. Genes up regulated in $CD133^-$ CSC involved MHC class II proteins (Beier *et al.*, 2007).

Various members of the Apolipoprotein family were also up regulated by CD133 knock down. *A2;* Stabilizes HDL by association with lipids and effects HDL metabolism, *A4*, *C1* Inhibits binding of beta VLDL to the LDL receptor protein,*C3;* inhibits Lipoprotein lipase and hepatic lipase and decreases the uptake of lymph chylomicrons, *H*; Binds to negatively charged substances- heparin, phosoholipid and dextran sulphate- may prevent activation of the intrinsic blood coagulation cascade by binding to phospholipids on the surface of damaged cells., *L6*; May effect the movement of lipids in the cytoplasm or allow the binding of lipids to organelles, and *M*; Involved in lipid transport. *PLLPNA* catalyses transfer of lipids between membranes. *MTTP* catalyses the transport of triglyceride, cholesteryl ester and phospholipids between phospholipid surfaces and *Choline Kinase Alpha* has a regulatory role in phosphatidylcholine synthesis. We can conclude that knocking down CD133 leads to an up regulation of transport across the plasma membrane. Therefore CD133 expression may act to inhibit genes that promote transport. This supports the results attained for motility, stating lipid movement across the membrane would facilitate motility. Loss of the receptor has caused an increase in genes relating to cell motility but also increased cell motility by decreasing genes responsible for cell to cell, cell to matrix interactions but also increasing adhesion molecules responsible for migration like CD44 and cofflin.

It is possible CD133 could also be acting as a cell membrane lipid regulator as various other processes occur via lipid membranes on the surface of the cells. The fact that they are

found within the Lipid Rafts could suggest a signalling role, as this area of the cell membrane is known for the occurrence of various signalling activities. Interestingly *Caveolin 2,* a protein that physically contributes to the make up of the Lipid Rafts on the cell surface was also down regulated on CD133 knock down. The protein is a major component of the inner surface of caveolae, small invaginations of the plasma membrane, and is involved in essential cellular functions, including signal transduction, lipid metabolism, cellular growth control and apoptosis. However our functional confocal studies showed that completely knocking down CD133 using RNAi technology had no visual effect on the expression of the lipid rafts on the cell surface. Lipid rafts may have a slow receycling rate therefore mRNA down-regulation of caveolin which was observed using micro array technology might fail to have an instantaneous effect on protein expression.

Also in differentiated 3T3-L1 adipocytes, cholesterol depletion leads to a 2.6-fold increase in caveolin 2 mRNA level assessed by real-time reverse transcription-polymerase chain reaction. While lovastatin treatment also causes caveolin 2 reduction to about 79% in 3T3-L1 adipocytes. This was mirrored in our experiments as an up regulation in cholesterol homeostasis and lipids caused the down regulation of caveolin 2 gene suggesting that caveolin may also have a role in lipid metabolism and storage.

As discussed previously, there were many similarities when comparing our work with other studies. In order to really appreciate CD133 biological effect one has to analyse the individual genes within the different processes. Quoting that "CD133 up-regulates cell cycle genes" for example is extremely vague and does not give us an appreciation as to what role CD133 plays in cell cycle biology. We looked at the various processes and the main genes up or down regulated in these processes to try and further understand and define their roles

The up regulation of cell proliferation genes suggests that the presence of CD133 allows the cell to remain in an undifferentiated quiescent state whilst loss of the receptor removes this suppression allowing genes such as *KIT ligand* and *S100P* to increase in expression. KIT ligand stimulates proliferation and survival in haemopoietic cells and also migration to the bone marrow. S100P similarly stimulates cell proliferation and survival; this was demonstrated by Jiang *et al.*, (2011) who knocked down the gene expression of S100P in

colon cancer cells using lentivirus-mediated RNA interference and found a significant inhibition of cancer cell growth, migration and invasion *in vitro*, as well as tumor growth and liver metastasis *in vivo* (Jiang *et al.*, 2011). Interestingly in our study S100P was the gene with the highest fold increase of 10.4. This would also explain the large increase in cell cycle positive regulators seen on knockdown of the receptor. Previous studies have shown that CD133 expression keeps cells in the GO/G1 phase whilst cells that have lost CD133 expression cycle quite readily with the majority of the cells being in cycle. An up regulation of various cell cycle genes support this claim as *CDC25A* is required for progression from G1 to the S phase of the cell cycle and was readily up regulated on knockdown of CD133. It is competent to activate the G1/S cyclin-dependent kinases CDK4 and CDK2 by removing inhibitory phosphate groups from adjacent tyrosine and threonine residues; it can also activate Cdc2 (Cdk1), the principal mitotic Cdk.

CLASP 2, a gene that plays an important role in spindle and kinetochore function especially during anaphase. *PLK1* a Serine/threonine-protein kinase that performs several important functions throughout M phase of the cell cycle, including the regulation of centrosome maturation and spindle assembly, the removal of cohesins from chromosome arms, the inactivation of APC/C inhibitors, and the regulation of mitotic exit and cytokinesis, NIMA a gene required for chromosome segregation at metaphase-anaphase transition and therefore for mitotic progression. TIPIN, important for normal progression of S-phase and required for cell survival after DNA damage or replication stress.

E2F3 and E2F2 both transcription activators that binds DNA and functions in the control of cell cycle progression from G1 to S phase. CDK3 inhibitor was also up regulated however this may play a role in cell cycle regulation as opposed to inhibition.
The up-regulation of genes that seem to be associated with cell cycle progression such as CDC25A, and DNA replication reinforce the theory held by Jaatineen *et al.*, (2006) who took human cord blood derived CD133+ cells and performed a global expression analysis on the cells. The CD133- fraction was used as a control. They observed within the cell cycle that that expression of GATA2 (FC 7) and N-MYC (FC 15) that keep the HSC's in undifferentiated state were significantly elevated in CD133 cells, down regulation of these genes would initiate the cell cycle. DST (FC 5.3) and PLAGl1 (FC 9.1) which support cell cycle arrest, were also up regulated. A cell cycle inhibitor and negative regulator of proliferation NME1, was over expressed in CD133 cells by 3.7 fold. Even though the

majority of the $CD133^+$ cells have shown to be in the G0 phase, genes such as SKB1, STAG1,ANAPC7 and MPHOSPH9 were over expressed by an average fold change of 2.3, suggesting that a portion of the CD133 cells are cycling. This hypothesis that $CD133^+$ cells may be cycling is supported by Jacksch *et al.*, (2008) who claim that the majority of $CD133^+$ cells isolated from cord blood are in G2/M phase (Jacksch *et al.*, 2008). It seems that the role of CD133 within cells form different sources may affect the cell cycle phase the cells may fall into.

Cancer stem cells are known to be resistant to anti-cancer drugs and survive much longer than cells that don't express the CD133 receptor, This in part would also explain why this occurs as CD133 is able to up-regulate and activate a set of anti apoptotic genes preventing apoptosis and cell death. Cell proliferation genes were also down regulated in both groups. A closer look at the gene profile showed that inhibition or knockdown of the receptor down regulated anti apoptotic genes such as BTG family member 2; an anti proliferative protein, Kruppel like factor 10; a transcriptional repressor involved in the regulation of cell growth, TAX1BP3 a gene that may act as a WNT inhibitor and control proliferation via the WNT pathway, interestingly, IL-11 was also down regulated in the 2 groups; IL-11 directly stimulates the proliferation of hematopoietic stem cells and megakaryocyte progenitor cells and induces megakaryocyte maturation resulting in increased platelet production. Generally our data is in line with the data obtained when looking at group 1 alone as we saw an up-regulation in proliferative genes when the cell receptor was knocked out, thus enhancing growth, which mirrors a down regulation in anti-proliferative genes on knock down. There was also a down regulation of closely related chemokines chemokine (C-C motif) ligand 2, chemokine (C-X-C motif) ligand 3, chemokine (C-X-C motif) ligand 3 and chemokine (C-X-C motif) receptor 7. The significance of this is still unclear.

CD133 expression has also been related to motility of haemopoietic cells as studies have shown that CD133 congregates at the leading edge of cancer stem cells. It would also explain why cancer cells expressing CD133 were much more potent and malignant compared to cancerous cells lacking CD133 expression. Movement of the cancer around the body would be enhanced by CD133 expression hence spreading the disease systemically making it much harder to combat and eradicate. This was demonstrated by Rappa *et al.*, (2008) who showed that down regulation of CD133 severely reduced the capacity of the cells to metastasis.

Motility and cell adhesion can be described in various ways. Cell adhesion does not just relate to motility and the direct movement of a cell, but may also relate to cell to cell adhesion and adhesion to basement membranes and surroundings that may ultimately contribute to direct movement from one site to another or fixed adhesion. Overlap between receptors, adhesion molecules and chemokines made it difficult to conclusively depict the role CD133 plays in cell adhesion. Interestingly, knocking out CD133 leads to a general decrease in adhesion molecules enabling cell motility and migration. Loss of CD133 leads to a down regulation of a variety of genes related to cell-cell interactions. Disruption of *Cadherin, Cantenin and Actinin 1* would lead to a loosening of cells within the tissues and cell to cell contact allowing the cells to be free and mobile. *Paxillin*, a cytoskeleton protein involved in actin-membrane attachment at sites of cell adhesion to the ECM was also down regulated. This would result in loosening the tight cell-matrix interaction ultimately promoting motility. Genes such as *IGFBP-7 and Laminin* were also down regulated and inhibit motility. However, *fibronectin leucine rich TM protein* was also down regulated but contributes to motility as a possible function due to the fact that the presence of CD133 would increase the level of this gene thereby increasing its function which is cell adhesion, growth and migration .

On the converse we also showed that there was an up regulation of CD44 by a 4.6 fold increase (15^{th}/931 genes) when CD133 was knocked out. CD44 is a cell surface glycoprotein and member of the hyaluronate receptor family of adhesion molecules. The human CD44 gene has been mapped to the chromosomal locus 11p13 and is composed of two groups of exons (Naor *et al.*, 2008). One group, comprising exons 1-5 and 16-20, are spliced together to form ubiquitously expressed standard isoform CD44s which contains 363 amino acids and has a molecular mass of 37kDa. The ten variable exons (6-15) (also known as v1-10) can be alternatively spliced and included within the standard exons (Tolg *et al.*, 1993). The principal ligand of CD44 is hyaluronate which is an abundant extracellular polysaccharide found in mammalian ECM. Other CD44 ligands include osteopontin, serglycin, collagens, fibronectin and laminin (Fujiwara *et al.*, 2008). The major physiological role of CD44 is to maintain organ and tissue structure via cell-cell and cell-matrix adhesion. Epithelial cells undergoing proliferation or cells under repair appear to up-regulate both CD44 and Hyaluronate (HA) production Hertweck *et al.*, 2011).

Studies have shown that CD44 is up-regulated in various cancers namely breast cancer (Fillmore *et al.,* 2007) and that its expression can influence tumour growth and development. The requirement for CD44-HA binding in tumour development has been shown by the suppression of tumour formation in the presence of soluble human CD44 soluble proteins (Zawadzki *et al.,* 1998) and it has become a marker of cancer stem cells. However angiogenesis was the 2nd highest down regulated gene ontology in this group, a lack of CD133 expression leads to a down regulation of these genes that would ultimately be responsible for blood vessel formation, angiogenesis and permeability which are essential functions in tumour progression thereby backing the claims that CD133 contributes to the survival of prolonged cancer stem calls

Subramaniam *et al.,* (2007) have shown that CD44 regulates cell migration in colon cancer cells and that knock down of CD44 leads to a decrease in cofflin which is involved in the directional motility of cells. Cofflin was also up regulated in our results by a fold change of 4.6 in this GO thereby suggesting loss of CD133 increases the directional motility of cells (Subramaniam *et al.,* 2007). CD44 involvement in cell to cell and cell to matrix adhesion interactions suggests any up regulation of this gene would clearly indicate that CD133 expression may be suppressing or inhibiting the cell to cell and matrix interactions that would ultimately allow motility.

Cells expressing CD133 are early progenitor stem cells within a bone marrow niche, and are within a protected and carefully regulated environment. There may still be developmental and maturation processes that the cells need to acquire before they become fully fledged stem cells able to be released out of the bone marrow to specific tissue sites to begin differentiation, proliferation etc. Prevention of movement i.e. cell motility and keeping the cells attached to the extra cellular matrix may be a necessary requisite allowing the cells to fully develop and preventing premature release. The loss of CD133 then allows the cell to break loose from its surroundings and migrate to its relevant site. Cancer stem cells on the other hand would benefit from this increased motility and thus a different mechanism may be in place because expression of CD133 in this case seems to inhibit movement.

The study of CD133 function within primary and malignant haemopoietic cells will remain a challenge both because of the low number of $CD133^+$ cells in primary tissue, the volume

of cord blood available and more importantly the loss of CD133 expression in cultured cells. Down regulation of CD133 in cultures was also observed in the malignant haemopoietic MUTZ-2 cell line prior to senescence. Whilst using MUTZ-2 as a haemopoietic primary cancer cell line and Caco-2 as an epithelial cell line was practical, the results may not be generalisable. Caution needs to be exercised in interpreting the results of experiments such as these where cells have undergone mutations that may alter their expression and regulation making them different from their original counterparts. Regulatory processes that may govern proliferation, differentiation, apoptosis and other functions in a normal cell may not govern the same processes in a cancerous cell.

Reproducibly growing MUTZ-2 proved difficult, perhaps due to dysregulated differentiation of a proportion of the cells in culture resulting in apoptosis. CD133 expression also decreased over time in cell cultures. Live cells were analysed therefore if cells differentiate and apoptosed they would not be detected as a $CD133^{+}$ cell by the detection methods, but it may also be that CD133 is being down-regulated in cell culture as observed in primary cells. This most likely represents the limitations of current culture media rather than a function of CD133 or differentiation of these cells.

Caco-2 cells could be grown reproducibly but were an adherent epithelial colon cancer cell line. It may well be that the function of CD133 in this cell line differs from cells of a haemopoietic lineage. It would be valuable to study the embryonic stem cell lines H1, H7 and H9 that express CD133 and are capable of capable of unlimited, undifferentiated proliferation *in vitro* (Carpenter *et al.*, 2003). The primitive nature of these cells is more appropriate to the early involvement of CD133 on stem cells (Kaufman *et al.*, 2001) as they more closely represent early haemopoietic stem / progenitor cells than the more differentiated and adherent Caco-2 cells. This was prohibited in this present study by the requirement for a special license and ethical applications.

Other studies would suggest further functions for CD133. Todaro *et al.*, (2010) suggests that the cancer stem cell is responsible for tumour initiation, metastasis and resistance to treatments (Todaro *et al.*, 2010). The function of CD133 within cancer stem cells seems to differ depending on the tissue of origin. Knock down studies of CD133 in cancer stem cells taken from a variety of tissues suggests CD133 may stimulate proliferation, self renewal, differentiation, migration and prevent apoptosis (Rappa *et al.*, 2008; Zhang *et al.*, 2010,

Takenobu *et al.*, 2011; Bissels *et al.*, 2011). These characteristics are not necessarily common in all cancer types. Cancer stem cells would represent a rare tumour population which may originate from the transformation of normal stem cells or progenitor cells differentiated through several generations. CD133 expression may be retained through several generations and be expressed on both sets of cells which may explain why functional overlaps occur. Interpretation of the function of CD133 may be affected by the use of monoclonal antibodies that directly bind CD133 to identify and isolate putative cancer stem cell populations from malignant tumours of brain, prostate, liver, pancreas, lung, and colon. Thus binding of ligands may affect their function.

There is some evidence that the role CD133 plays in haemopoiesis may not be identical to that of cancer stem cells. CD133 may play a lesser role in CSC biology compared to stem cell biology as mutations to the cells may effect downstream signals and dysregulate or alter CD133 function without effecting expression. CD133 antibody binding in Caco-2 cells led to inhibition cell cycle, proliferation, motility and apoptosis to an extent. These features are crucial to normal and malignant stem cell survival and function alike. The reduced proliferation lowers the toxic insult to cells allowing malignant cells to be chemotherapy and radiation resistant. The down regulation of apoptotic proteins contributes to this resistance.

An increase in motility and migration with cancer stem cells is expected as their metastatic potential is much greater than cells lacking CD133 expression, but the full functional capacity of CD133 may not be translated through different cell origins expressing the receptor. Taking cells that are $CD133^-$ and comparing them to $CD133^+$ has limited value as there is considerable redundancy in protein function in most cells and there may well be alternate mechanism or pathways that cells may utilise to compensate for the lack of CD133 function.

Creating either a primary CD133 knockout cell line or a CD133 knocked down primary cell line is a much more effective and cleaner way of measuring potential function in different tissues as well as primitive and more mature cells. Attempts to generate a permanent CD133 knockout cell line were unsuccessful. This may have been a technical issue as CD133 mice have been bred, but it is possible that CD133 plays a vital role in normal cell function and may be essential to functions such as structural integrity, adhesion

or motility in humans. Permanently knocking out the receptor may inhibit these normal functions rendering the cells non-viable. Suggestive of this is the fact that there are no CD133 malignant or normal knockout human cell lines. Studies where the receptor has been "knocked down" as oppose to "knocked out" in cell lines tend to leave a residual amount of receptor on the surface but more importantly re-expression is rapid, therefore the cell is only affected temporarily.

The identity of the ligand for CD133 receptor remains elusive. In addition their involvement in cell communication is still unclear. It is not known precisely where the CD133 Ab binds on the receptor. Binding on or close to the receptor would prevent binding of another ligand and may result in stimulation or an inhibition of cellular function. It is also possible that the Ab may bind to the receptor site without triggering a function but cause downstream activity that cause activation or inhibition. Alternatively the antibody may be partially triggering the receptor causing the cell to become anergic. However it is known that the Mab affects the cells as a specific dose-dependent cytotoxic effect in Caco-2 cells was observed whilst using CD133 Mab's against both epitopes. This dose dependent cytotoxic effect was not observed when the knocked down cells were utilised.
When the Mab was added to the cells there was a 26% overlap in genes up regulated and 56% in genes down-regulated when comparing gene profiles for both knockdown and Mab binding groups. It then became apparent that the antibodies may be having an inhibitory or suppressive role on the cell, as binding of the antibodies produced a similar profile to cells in which the receptor had been knocked down.

There are no other proposed ligands for CD133 however our results and those of others pose some interesting suggestions;
There is scope to suggest that the leucine zipper motif on the second extracellular loop of CD133 (Miraglia *et al.*, 1997) may enable CD133 to dimerise and be involved in direct HPSC to HPSC communication within the BMME. A leucine zipper is a common three-dimensional structural motif in proteins. These motifs are usually found as part of a DNA-binding domain in various transcription factors, and are therefore involved in regulating gene expression (Nijhawan *et al.*, 2008). Structurally the leucine zipper is made up of two alpha-helical segments of protein that have leucines facing each other along the length of the helices, allowing them to dimerise and form a symmetric interface that can bind to the DNA on both sides of the double helix (Tsutsumi *et al.*, 2009). The purpose of this could

be to initiate quiescence and anergy in the cell. Loss of this interaction may then inhibit the suppression of the cells initiating cell cycle, proliferation and motility. In this way CD133 may also contribute to the regulation and maintenance of the stem cell pool. Direct receptor-receptor interaction can be assessed by the use of fluorescent resonance energy transfer (FRET). FRET is a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule (Seegar *et al.,* 2010). In this case the acceptor molecule and donor molecule would both be CD133 on independent cells, labelled with different fluorochromes. When the two cells interact becoming in close proximity to each other (10-100 angstroms), the fluorescent peaks overlap and a large proportion of energy is transferred from the donor to the acceptor molecule Muñoz-Losa *et al.,* 2009) creating a much larger emission peak compared to the peak that would be generated independently from the cells.

The ligand may also be secreted by $CD133^+$ cells for example down regulation of the receptor or non binding may cause the cell to secrete a protein that will bind to $CD133^+$ cells causing either suppression or excitation. It is possible that this ligand maybe present in the culture medium. A method to test this would be "Ligand fishing". Antigen fishing is the use of surface plasmon resonance (SPR) coupled with mass spectrometry (MS) to identify and characterize proteins that bind to particular ligands. There are several techniques that may be employed for SPR or MS procedures, but in principal all antigen fishing work flows requires fixation of the receptor or "bait" molecule (CD133) to the surface of a chip, a flow system to cover the chip in medium containing the ligand known as the "target" and either direct detection of the target molecule by matrix-assisted laser desorption/ionisation (MALDI) MS or elution of the target for use in other MS procedures (Thillaivinayagalingam *et al.,* 2010; Buij and Franklin, 2005). When prey molecules bind to the bait ligand they disrupt the resonance field of the chip, which is then detected as a disruption in the chips electro-magnetic field. The target may then be eluted and identified by MS. More advanced techniques using on chip MALDI MS techniques, allow the arrangement of chips in an array format for screening of multiple ligands (Bellon *et al.,* 2009). This latter with purified CD133 solutions.

There is some evidence that CD133 has a functional role in the cell membrane and that it may have a role in cell signalling. The association of CD133 with pseudopodia and its interaction

with cholesterol within the lipid rafts (Roper *et al.*, 2000), suggests a possible functional role for CD133 as a lipid membrane regulator (Fargeas *et al.*, 2004). We have used spatial distribution and quantitative analysis of CD133 and the lipid raft proteins to verify that there was significant co-localisation between the two proteins and that removal of lipid rafts led to removal of CD133. We also showed that a CD133 knockdown led to a down-regulation in the lipid raft protein caveolin. This down-regulation was not observed phenotypically with confocal analysis as CD133 downregulation had no effect on lipid raft expression. A cause of this discrepancy may be due to the recycling properties of caveolin. In future experiments it would be important to confirm that the reagent used to remove the lipid rafts (BMCD) was not removing CD133 physically from the cell surface. One could validate this by molecularly knocking down the lipid rafts protein preventing cell surface expression of the rafts and then fluorescently staining to determine CD133 expression levels using confocal or flow cytometry.

Our studies and those of Rappa *et al.*, (2008) showed that CD133 knock down led to an up regulation in Wnt and tyrosine kinase pathway inhibitors. We also observed a down regulation in Wnt ligands. Both Wnt and tyrosine kinase pathways regulate proliferation and differentiation within cells so an up-regulation of pathway inhibitors, which we found when the receptor was knocked down, may result in dysregulation of the pathway leading to cancers. In normal tissues there are tight proliferation and differentiation regulatory mechanisms, so therefore CD133 expression promotes the Wnt and tyrosine kinase pathway causing the high level of regulation needed to keep the cells in a quiescent, pluripotent state as observed in the circulating blood stem cells or the bone marrow stem cell niche.

CD133 evidently has a role in adhesion, motility and migration. The results suggest that motility and migration are stimulated by loss of CD133 expression. Various migratory molecules such as CD44 and cofflin were up-regulated on CD133 knockdown. We discovered a down-regulation of proteins such as actinin 1, cantenin and cadherin which would stimulate detachment of cells from the basement membrane and extracellular matrix contributing to motility. CD133 is located within the apical membrane of cells so therefore its relationship with actinin, cadherin and cantenin up-regulation is not a direct one. We are suggesting from our results that CD133 does not bind to the basement membrane of cells physically preventing motility, but merely stimulates up or down regulation of basement

membrane adhesion proteins. In this study the confocal data clearly showed that there was no significant change in receptor expression or movement of CD133 receptor on the cell surface when CD133 was incubated with fibronectin, a protein found within the extracellular matrix showing that CD133 has no direct relationship with the basement membrane. This is consistent with the functional cell adhesion experiments where no differences in binding capacities between both control and knock down groups was observed indicating that CD133 has no direct effect on anchorage or binding to plastic. This fits with the role CD133 plays within haemopoiesis in that cells expressing the receptor are held within the stem cell niche in the bone marrow, due to increase adhesion to the matrix. Reduction of motility and migration allows the early progenitor cells to develop, mature and maintain primitive status. However, under extreme circumstances such as stress and trauma primitive cells expressing $CD133^{+}$ are able to move out of the bone marrow to specific areas for tissue repair or to escape damage (Jensen *et al.*, 2007), suggesting there are various receptors and signals other than CD133 responsible for initiating motility and migration. Loss of CD133 may act to modify regulation of other receptors that will ultimately lead to migration. It is possible that the CD133 pathway may be bypassed allowing migration as observed in these extreme cases.

The result attained suggests that CD133 down-regulation promotes motility and migration. It was also shown that a redistribution of the receptor when the cell is activated by plastic or stromal cell adhesion or activated led to the formation of pseudopodia (Whiting *et al.*, 2003, Hayward-Costa *et al.*, 2000) It was not demonstrated whether total cell CD133 was up or down-regulated. In accordance with our data Corbeil *et al.*, (2000) have shown that CD133 congregates at the leading edge of primary cells suggesting a possible role in migration. In support of this Rappa *et al.*, (2008) and Elsaba *et al.*, (2010) showed an increase in metastatic potential within CD133 expressing cells in FEMX (melanoma) and HT29 (colonic) cell lines respectively compared to the CD133 knocked down cells in both cell lines supporting CD133's importance in migration.

Our data would also indicate involvement of CD133 in cell quiescence. The role of cell turnover was positively affected by loss of CD133 expression. There was an increase in cell cycling protein genes and a 7% increase in S phase cells in Caco-2 cells after knock down of CD133 compared to the control cell line. This data is further evidence of a role for CD133 in maintaining cell quiescence with loss of the receptor allows the cell to cycle.

This information is pertinent to the debate concerning the role the cell cycle in haemopoiesis. The accepted position is that CD133 positive cells actually reside in the G0/G1 phase (Summers *et al.*, 2004; Qiang *et al.*, 2009; Grskovic *et al.*, 2004), however, Sun *et al.*, (2009) showed that in neural stem cells, cell cycle profiling revealed CD133 negative cells reside in that G1 phase suggesting the CD133's role in cell cycle may be tissue specific.

It is also possible to comment from the data on the homogeneity of function of CD133 across tissue types. In all cell types, CD133 is located on protrusions such as pseudopodia and microvilli on the cell membrane and found within the lipid rafts in association with cholesterol. However, studies have shown that function and location of CD133 are not necessarily related. If motility is examined the molecular studies indicated that CD133 knockdown led to an up-regulation in motility and migration, both directly by the up regulation of specific migratory molecules and also indirectly by the facilitation of other processes related to motility, indicating lipid membrane activity and the decrease in cell adhesion molecules. However, other studies in the melanoma line FEMX (Rappa *et al.*, 2008), Colon cancer line HT29 cells (Elasaba *et al.*, 2010), Pancreatic cell lines SUIT-2 and KP-2 (Moriyarna *et al.*, 2010) show that the lack of CD133 expression leads to a down regulation in motility and migration. In HSC expressing CD133, the receptor accumulates at the leading edge of the cell again indicating a possible role of CD133 in migration in primary cells. Interestingly, Horst *et al.*, (2009) found that knocking down CD133 in Caco-2 cells had no functional effect on migration. Our study supports this data by showing that CD133 knockdown had no effect on adhesion *in vitro*. The functional variation within the different tissues suggests that the methodology to assess CD133 needs to be standardised across tissue types and caution is needed with the interpretation of microarray data. The up-regulation of genes correlating to a particular process such as cell migration may not necessary lead to the translation of the proteins necessary for the process to occur as some of the genes up regulated may be dormant. Others may require co-stimulation from another receptor or a secondary messenger for translation to be initiated. The micro array data showed an up-regulation of genes relating to cell migration, whereas Horst *et al.*, (2009) showed within the same cell line, there were no functional effects on migration. This discrepancy can possibly be explained by the fact that more than one factor is likely to contribute to cell movement. Many of the processes that have been related to CD133 function may require additional stimuli indicating that CD133 is part of a complex network

of receptors and signals necessary for cell function. Transwell experiments would have been helpful to investigate downstream effects on CD133 knocked down cells to determine if the up regulation observed in genes associated with migration can be translated to functional migration experiments. Unfortunately due to the rapid rate at which Caco-2 cells re express CD133 after knock down, it would not be feasible to carry out these experiments on this particular cell line. However, if a permanent knock out Caco-2 cell line could be created then that would eradicate the problem of early re-expression.

With consideration it is possible to design experimental modifications to overcome methodological problems in future studies. The main techniques used to carry out functional analysis were microarray technology, confocal analysis and cell cycle/proliferation assays. In the micro array studies cells were knocked down in one group and CD133 Mab added to another. The results obtained should only be taken as a guide line as to potential CD133 functions as the correlation between induced mRNA and induced levels of protein were not always well aligned. Differential gene expression needs to be confirmed through direct examination of selected gene expressions. This analysis would entail real time PCR to examine if transcripts of a specific gene are up or down-regulated.

Intracellular, membrane and secreted proteins could be examined with CD133 antibody treatment using flow cytometry, mass spectrometry and western blotting, with specific proteonomics and metabolonomics to further investigate function. Translational and post-translational regulatory mechanisms that affect the activity of various cellular proteins are not examined by microarray technology. Other limitations of microarray analysis include the impact of alternative splicing during transcript processing and the limited detectability of unstable mRNAs. The requirement for large amounts of high-quality mRNA in microarray analysis may be preclusive to the analysis of primary cells.

Confocal laser scanning microscopy was also used to investigate the potential function of CD133. The combined use of fluorescent conjugated antibodies and CLSM Z-scan analysis for the identification of cellular components as reported here has enabled the direct visualisation of CD133 distribution and interaction with other cell surface proteins or stimuli. A major analytical drawback was photobleaching of fluorescent probes within a few minutes of illumination. A more suitable method to analyse extracellular components

and possible function would be immunofluorescent labelling of live cells. Further investigations examining the intracellular expressions of CD133 and its relationship with intracellular components (lipid rafts, golgi complexes, signal transduction) through immunofluorescent labelling would rely on cell fixation and permeabilisation prior to antibody labelling for microscopic analysis. Antibody labelling also raises the question of antibody/fluorescent charge, activation or inhibition of the receptor and antibody cross reactivity thereby limiting the extent to which this work could be extended by immunolabelling alone. The live cell processing of CD133 by transfecting cells with a CD133 DNA sequence including a green fluorescent protein (GFP) tag. Fluorescent expression would allow tracking of CD133 receptor distribution from cytoplasmic packaging and transport processing, to cell membrane incorporation. The combined use of the GFP and confocal techniques would enable visualisation of the CD133 receptor distribution within viable cells. This could be applied to investigate antigen distribution and migration over time on live cells in response to various cellular stimuli and conditions, contributing to our understanding of receptor interactions and its functional capacity.

Clearly CD133 influences crucial biological properties of haemopoietic stem cells and progenitor stem cells. It is conceivable that CD133, directly or through its interaction with signalling pathways such as Wnt and tyrosine kinase, may influence cell cycle, proliferation and the interaction of stem cells with surrounding cells and the extracellular matrix as well as contributing to motility and migration. Our data suggests that CD133 may play a suppressive role in stem cell biology and a regulatory role in maintaining quiescence, keeping the early stem and progenitor cells in a non proliferating, non motile state. Within haemopoiesis this would occur within the bone marrow, allowing the stem cells to self renew or with appropriate stimuli mature and proliferate, thereby maintaining haemopoiesis. With the loss or ligand dissociation of CD133 expression cells are allowed to proliferate cycle and migrate to areas were tissue regeneration is required.

Future studies

Studying cancer stem cells has proven useful in trying to comprehend CD133 function, however there is no format to mutations and studies have shown that cancers expressed in various tissues and cells exhibit different functions. This may be due to functional changes or differences in experimental conditions between various labs. A way to resolve this would be to analyse a range of cell lines expressing CD133 under the same conditions and determining whether any generic functions are associated with expression.

More specifically generation of a CD133 knockout cell line, ideally from a haemopoietic background would be crucial in determining the role CD133 plays in biology. This would prevent receptor recycling problems and allow research to be conducted on a range of functional experiments with no time constraints attached. Transwell experiments would also be very informative as we failed to carry out any experiments in this thesis. This would determine CD133's relationship to motility and migration and whether knockdown of CD133 or Mab binding would affect these processes as we suggested. An alternative to "knocking out" is "knocking in" CD133 into a cell line lacking expression and measuring changes in the function of the cell. This could be developed further by generating "knock in" cell lines that express a fusion of a fluorescent protein (such as red mCherry) with the C-terminus of CD133 allowing us to visualise and trace $CD133^+$ cells.

One would expect CD133's role within haemopoiesis to be structured and possibly easier to investigate in contrast to malignant cells. This could be examined using primary cells. In this thesis we attempted to culture cord blood cells, however CD133 is also expressed on mesenchymal and embryonic stem cells. Establishing a method to culture primary cells in a manner that would inhibit differentiation but allow proliferation is crucial to manipulating these cells and successfully conducting functional experiments.

Experimentally, various other aspects of functionality can be measured other than the methods outlined in this thesis. Micro array analysis could be carried out in a broader fashion with no pre conceived notions as to possible outcome with the aim of uncovering novel genes and pathway previously unrelated to CD133. Metabolonomics and proteonomics experiments may also be carried out to better define the down stream pathways and signalling molecules CD133 may be utilising.

Finally, to determine whether the suppressive effects observed from binding of the Mab CD133 on Caco-2 cells are epitope dependent or whether it requires the stimulation of both epitopes. If on binding to their specific epitopes, Mab AC141 fails to provide downstream events and Mab AC133 caused downstream signalling, this would lead to an alteration in the gene profiles produced by the different antibodies. This would suggest that AC141 would be an ideal candidate to use when purifying and enriching $CD133^{+}$ cells, whilst AC133 would be a useful ligand alternative.

References

Alberts,B., Johnson,A., Lewis,J., Raff,M., Roberts,K., Walter,P. Molecular biology of the cell, 5th edition, 2007. Garland Publishers.

am Esch,J.S., Knoefel,W.T., Klein,M., Ghodsizad,A., Fuerst,G., Poll,L.W., Piechaczek,C., Burchardt,E.R., Feifel,N., Stoldt,V., Stockschlader,M., Stoecklein,N., Tustas,R.Y., Eisenberger,C.F., Peiper,M., Haussinger,D., & Hosch,S.B. (2005) Portal application of autologous CD133+ bone marrow cells to the liver: a novel concept to support hepatic regeneration. *Stem Cells*, **23**, 463-470.

Anderson,L.H., Boulanger,C.A., Smith,G.H., Carmeliet,P., & Watson,C.J. (2011) Stem cell marker prominin-1 regulates branching morphogenesis, but not regenerative capacity, in the mammary gland. *Dev.Dyn.*

Angelastro,J.M. & Lame,M.W. (2010) Overexpression of CD133 promotes drug resistance in C6 glioma cells1. *Mol.Cancer Res.*, **8**, 1105-1115.

Arends,M.J. & Wyllie,A.H. (1991) Apoptosis: mechanisms and roles in pathology1. *Int.Rev.Exp.Pathol.*, **32**, 223-254.

Ashkenazi,A., Pai,R.C., Fong,S., Leung,S., Lawrence,D.A., Marsters,S.A., Blackie,C., Chang,L., McMurtrey,A.E., Hebert,A., DeForge,L., Koumenis,I.L., Lewis,D., Harris,L., Bussiere,J., Koeppen,H., Shahrokh,Z., & Schwall,R.H. (1999) Safety and antitumor activity of recombinant soluble Apo2 ligand 5. *J.Clin.Invest*, **104**, 155-162.

Ashman,L.K. (1999) The biology of stem cell factor and its receptor C-kit. *Int.J.Biochem.Cell Biol.*, **31**, 1037-1051.

Attia,M.A., Welsh,J.P., Laing,K., Butcher,P.D., Gibson,F.M., & Rutherford,T.R. (2003) Fidelity and reproducibility of antisense RNA amplification for the study of gene expression in human CD34+ haemopoietic stem and progenitor cells 29. *Br.J.Haematol.*, **122**, 498-505.

Bao,W., Bussel,J.B., Heck,S., He,W., Karpoff,M., Boulad,N., & Yazdanbakhsh,K. (2010) Improved regulatory T-cell activity in patients with chronic immune thrombocytopenia treated with thrombopoietic agents. *Blood*, **116**, 4639-4645.

Barcelos,L.S., Duplaa,C., Krankel,N., Graiani,G., Invernici,G., Katare,R., Siragusa,M., Meloni,M., Campesi,I., Monica,M., Simm,A., Campagnolo,P., Mangialardi,G., Stevanato,L., Alessandri,G., Emanueli,C., & Madeddu,P. (2009) Human CD133+ progenitor cells promote the healing of diabetic ischemic ulcers by paracrine stimulation of angiogenesis and activation of Wnt signalling. *Circ.Res.*, **104**, 1095-1102.

Barfield,R.C., Hale,G.A., Burnette,K., Behm,F.G., Knapp,K., Eldridge,P., & Handgretinger,R. (2007) Autologous transplantation of CD133 selected hematopoietic progenitor cells for treatment of relapsed acute lymphoblastic leukemia. *Pediatr.Blood Cancer*, **48**, 349-353.

Barraud,P., Stott,S., Mollgard,K., Parmar,M., & Bjorklund,A. (2007) *In vitro* characterization of a human neural progenitor cell coexpressing SSEA4 and CD133. *J.Neurosci.Res.*, **85**, 250-259.

Barsky,S. (2009) CD133, a stem cell surface marker in inflammatory breast cancer, functions in tumor cell survival . *FASEB J.*

Bassett,D.E., Jr., Eisen,M.B., & Boguski,M.S. (1999) Gene expression informatics--it's all in your mine 47. *Nat.Genet.*, **21**, 51-55.

Bauer,N., Fonseca,A.V., Florek,M., Freund,D., Jaszai,J., Bornhauser,M., Fargeas,C.A., & Corbeil,D. (2008) New insights into the cell biology of hematopoietic progenitors by studying prominin-1 (CD133). *Cells Tissues.Organs*, **188**, 127-138.

Beier,D., Hau,P., Proescholdt,M., Lohmeier,A., Wischhusen,J., Oefner,P.J., Aigner,L., Brawanski,A., Bogdahn,U., & Beier,C.P. (2007) CD133(+) and CD133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. *Cancer Res.*, **67**, 4010-4015.

Bellon,S., Buchmann,W., Gonnet,F., Jarroux,N., Anger-Leroy,M., Guillonneau,F., & Daniel,R. (2009) Hyphenation of surface plasmon resonance imaging to matrix-assisted laser desorption ionization mass spectrometry by on-chip mass spectrometry and tandem mass spectrometry analysis. *Anal.Chem.*, **81**, 7695-7702.

Bernards,R. (2003) Cancer: cues for migration. *Nature*, **425**, 247-248.

Bhardwaj,G., Murdoch,B., Wu,D., Baker,D.P., Williams,K.P., Chadwick,K., Ling,L.E., Karanu,F.N., & Bhatia,M. (2001) Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. *Nat.Immunol.*, **2**, 172-180.

Bhatia,M. (2001) AC133 expression in human stem cells. *Leukemia*, **15**, 1685-1688.

Bissels,U., Wild,S., Tomiuk,S., Hafner,M., Scheel,H., Mihailovic,A., Choi,Y.H., Tuschl,T., & Bosio,A. (2011) Combined characterization of microRNA and mRNA profiles delineates early differentiation pathways of CD133+ and CD34+ hematopoietic stem and progenitor cells. *Stem Cells*, **29**, 847-857.

Bitan,M., Shapira,M.Y., Resnick,I.B., Zilberman,I., Miron,S., Samuel,S., Ackerstein,A., Elad,S., Israel,S., Amar,A., Fibach,E., Or,R., & Slavin,S. (2005) Successful transplantation of haploidentically mismatched peripheral blood stem cells using CD133+-purified stem cells. *Exp.Hematol.*, **33**, 713-718.

Blazek,E.R., Foutch,J.L., & Maki,G. (2007) Daoy medulloblastoma cells that express CD133 are radioresistant relative to C. *Int.J.Radiat.Oncol.Biol.Phys.*, **67**, 1-5.

Bodo,M., Baroni,T., Bellucci,C., Lilli,C., De,I.M., Bonifacio,E., Moretti,L., Becchetti,E., Bellocchio,S., Delfini,C., Lumare,E., & Tabilio,A. (2006) Unique human CD133+ leukemia cell line and its modulation towards a mesenchymal phenotype by FGF2 and TGFbeta1. *J.Cell Physiol*, **206**, 682-692.

Boivin,D., Labbe,D., Fontaine,N., Lamy,S., Beaulieu,E., Gingras,D., & Beliveau,R. (2009) The stem cell marker CD133 (prominin-1) is phosphorylated on cytoplasmic tyrosine-828 and tyrosine-852 by Src and Fyn tyrosine kinases. *Biochemistry*, **48**, 3998-4007.

Bonanno,G., Mariotti,A., Procoli,A., Corallo,M., Rutella,S., Pessina,G., Scambia,G., Mancuso,S., & Pierelli,L. (2007) Human cord blood CD133+ cells immunoselected by a clinical-grade apparatus differentiate *in vitro* into endothelial- and cardiomyocyte-like cells. *Transfusion*, **47**, 280-289.

Botchkina,I.L., Rowehl,R.A., Rivadeneira,D.E., Karpeh,M.S., Jr., Crawford,H., Dufour,A., Ju,J., Wang,Y., Leyfman,Y., & Botchkina,G.I. (2009) Phenotypic subpopulations of metastatic colon cancer stem cells: genomic analysis. *Cancer Genomics Proteomics.*, **6**, 19-29.

Bruno,S., Bussolati,B., Scacciatella,P., Marra,S., Sanavio,F., Tarella,C., & Camussi,G. (2006) Combined administration of G-CSF and GM-CSF stimulates monocyte-derived pro-angiogenic cells in patients with acute myocardial infarction. *Cytokine*, **34**, 56-65.

Buhring,H.J., Seiffert,M., Bock,T.A., Scheding,S., Thiel,A., Scheffold,A., Kanz,L., & Brugger,W. (1999) Expression of novel surface antigens on early hematopoietic cells. *Ann.N.Y.Acad.Sci.*, **872**, 25-38.

Buijs,J. & Franklin,G.C. (2005) SPR-MS in functional proteomics. Brief.Funct.Genomic *Proteomic.*, **4**, 39-47

Bussolati,B., Bruno,S., Grange,C., Buttiglieri,S., Deregibus,M.C., Cantino,D., & Camussi,G. (2005) Isolation of renal progenitor cells from adult human kidney. *Am.J.Pathol.*, **166**, 545-555.

Hayward-Costa,C., Electron microscopy demonstration of CD34 antigen on progenitor pseudopodia. Exp.Hematol. 28[12], 1494-1495. 2000.

Calder,P.C. & Yaqoob,P. (2007) Lipid rafts--composition, characterization, and controversies. *J.Nutr.*, **137**, 545-547.

Capper,D., Gaiser,T., Hartmann,C., Habel,A., Mueller,W., Herold-Mende,C., von,D.A., & Siegelin,M.D. (2009) Stem-cell-like glioma cells are resistant to TRAIL/Apo2L and exhibit down-regulation of caspase-8 by promoter methylation. *Acta Neuropathol.*, **117**, 445-456.

Carpenter,M.K., Rosler,E., & Rao,M.S. (2003) Characterization and differentiation of human embryonic stem cells 23. *Cloning Stem Cells*, **5**, 79-88.

Chan,W.Y., Lorke,D.E., Tiu,S.C., & Yew,D.T. (2002) Proliferation and apoptosis in the developing human neocortex. *Anat.Rec.*, **267**, 261-276.

Cheng,J., Baumhueter,S., Cacalano,G., Carver-Moore,K., Thibodeaux,H., Thomas,R., Broxmeyer,H.E., Cooper,S., Hague,N., Moore,M., & Lasky,L.A. (1996) Hematopoietic defects in mice lacking the sialomucin CD34. *Blood*, **87**, 479-490.

Chinni,S.R., Sivalogan,S., Dong,Z., Filho,J.C., Deng,X., Bonfil,R.D., & Cher,M.L. (2006) CXCL12/CXCR4 signalling activates Akt-1 and MMP-9 expression in prostate cancer cells: the role of bone microenvironment-associated CXCL12. *Prostate*, **66**, 32-48.

Chiou,S.H., Kao,C.L., Chen,Y.W., Chien,C.S., Hung,S.C., Lo,J.F., Chen,Y.J., Ku,H.H., Hsu,M.T., & Wong,T.T. (2008) Identification of CD133-positive radioresistant cells in atypical teratoid/rhabdoid tumor. *PLoS.One.*, **3**, e2090.

Cinatl,J., Cinatl,J., Mainke,M., Weissflog,A., Steigmann,G., Rabenau,H., Doerr,H.W., & Kornhuber,B. (1992) Aphidicolin selectively kills neuroblastoma cells *in vitro*. *Cancer Lett.*, **67**, 199-206.

Clark,A.D., Jorgensen,H.G., Mountford,J., & Holyoake,T.L. (2003) Isolation and therapeutic potential of human haemopoietic stem cells. *Cytotechnology*, **41**, 111-131.

Collins,A.T., Berry,P.A., Hyde,C., Stower,M.J., & Maitland,N.J. (2005) Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res.*, **65**, 10946-10951.

Corbeil,D., Roper,K., Hannah,M.J., Hellwig,A., & Huttner,W.B. (1999) Selective localization of the polytopic membrane protein prominin in microvilli of epithelial cells - a combination of apical sorting and retention in plasma membrane protrusions. *J.Cell Sci.*, **112 (Pt 7)**, 1023-1033.

Corbeil,D., Roper,K., Hellwig,A., Tavian,M., Miraglia,S., Watt,S.M., Simmons,P.J., Peault,B., Buck,D.W., & Huttner,W.B. (2000) The human AC133 hematopoietic stem cell antigen is also expressed in epithelial cells and targeted to plasma membrane protrusions. *J.Biol.Chem.*, **275**, 5512-5520.

Corbeil,D., Roper,K., Fargeas,C.A., Joester,A., & Huttner,W.B. (2001) Prominin: a story of cholesterol, plasma membrane protrusions and human pathology. *Traffic.*, **2**, 82-91.

Corti,S., Nizzardo,M., Nardini,M., Donadoni,C., Salani,S., Ronchi,D., Saladino,F., Bordoni,A., Fortunato,F., Del,B.R., Papadimitriou,D., Locatelli,F., Menozzi,G., Strazzer,S., Bresolin,N., & Comi,G.P. (2008) Neural stem cell transplantation can ameliorate the phenotype of a mouse model of spinal muscular atrophy. *J.Clin.Invest*, **118**, 3316-3330.

Craig,C.E., Quaglia,A., Selden,C., Lowdell,M., Hodgson,H., & Dhillon,A.P. (2004) The histopathology of regeneration in massive hepatic necrosis. *Semin.Liver Dis.*, **24**, 49-64.

Cummings,B.J., Uchida,N., Tamaki,S.J., Salazar,D.L., Hooshmand,M., Summers,R., Gage,F.H., & Anderson,A.J. (2005) Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. *Proc.Natl.Acad.Sci.U.S.A*, **102**, 14069-14074.

Darzynkiewicz,Z., Traganos,F., Zhao,H., Halicka,H.D., & Li,J. (2011) Cytometry of DNA replication and RNA synthesis: Historical perspective and recent advances based on "click chemistry" 4. *Cytometry A*, **79**, 328-337.

Davidson,B.L. & McCray,P.B., Jr. (2011) Current prospects for RNA interference-based therapies 7. *Nat.Rev.Genet.*, **12**, 329-340.

de Wynter,E.A., Buck,D., Hart,C., Heywood,R., Coutinho,L.H., Clayton,A., Rafferty,J.A., Burt,D., Guenechea,G., Bueren,J.A., Gagen,D., Fairbairn,L.J., Lord,B.I., & Testa,N.G. (1998) CD34+AC133+ cells isolated from cord blood are highly enriched in long-term culture-initiating cells, NOD/SCID-repopulating cells and dendritic cell progenitors 15. *Stem Cells*, **16**, 387-396.

Dejean,L.M., Martinez-Caballero,S., & Kinnally,K.W. (2006) Is MAC the knife that cuts cytochrome c from mitochondria during apoptosis? *Cell Death.Differ.*, **13**, 1387-1395.

Deng,Y.H., Pu,X.X., Huang,M.J., Xiao,J., Zhou,J.M., Lin,T.Y., & Lin,E.H. (2010) 5-Fluorouracil upregulates the activity of Wnt signalling pathway in CD133-positive colon cancer stem-like cells. *Chin J.Cancer*, **29**, 810-815.

Derisi,J.L. & Iyer,V.R. (1999) Genomics and array technology. *Curr.Opin.Oncol.*, **11**, 76-79.

Domen,J. & Weissman,I.L. (2003) Hematopoietic stem cells and other hematopoietic cells show broad resistance to chemotherapeutic agents *in vivo* when overexpressing bcl-2. *Exp.Hematol.*, **31**, 631-639.

Dong,T.T., Zhou,H.M., Wang,L.L., Feng,B., Lv,B., & Zheng,M.H. (2011) Salinomycin Selectively Targets 'CD133+' Cell Subpopulations and Decreases Malignant Traits in Colorectal Cancer Lines. *Ann.Surg.Oncol.* , **18**, 1797-1804.

Dua,H.S., Joseph,A., Shanmuganathan,V.A., & Jones,R.E. (2003) Stem cell differentiation and the effects of deficiency. *Eye (Lond)*, **17**, 877-885.

Dufva,M. (2005) Fabrication of high quality microarrays. *Biomol.Eng*, **22**, 173-184.

Duggan,D.J., Bittner,M., Chen,Y., Meltzer,P., & Trent,J.M. (1999) Expression profiling using cDNA microarrays. *Nat.Genet.*, **21**, 10-14.

Dunn,K.W., Mayor,S., Myers,J.N., & Maxfield,F.R. (1994) Applications of ratio fluorescence microscopy in the study of cell physiology. *FASEB J.*, **8**, 573-582.

Dyer,M.J., MacFarlane,M., & Cohen,G.M. (2007) Barriers to effective TRAIL-targeted therapy of malignancy. *J.Clin.Oncol.*, **25**, 4505-4506.

Earle,V.L., Ross,F., Fisher,A., Strike,P., Berrington,S., Chiecchio,L., Cabanas,E.D., Washbourne,R., Watts,K., & Grand,F. (2007) Haemopoietic growth factors significantly improve the mitotic index and chromosome quality in cytogenetic cultures of myeloid neoplasia. *Genes Chromosomes.Cancer*, **46**, 670-674.

Eisen,M.B., Spellman,P.T., Brown,P.O., & Botstein,D. (1998) Cluster analysis and display of genome-wide expression patterns. *Proc.Natl.Acad.Sci.U.S.A.*, **95**, 14863-14868.

Elsaba,T.M., Martinez-Pomares,L., Robins,A.R., Crook,S., Seth,R., Jackson,D., McCart,A., Silver,A.R., Tomlinson,I.P., & Ilyas,M. (2010) The stem cell marker CD133 associates with enhanced colony formation and cell motility in colorectal cancer. *PLoS.One.*, **5**, e10714.

Fan,X., Khaki,L., Zhu,T.S., Soules,M.E., Talsma,C.E., Gul,N., Koh,C., Zhang,J., Li,Y.M., Maciaczyk,J., Nikkhah,G., Dimeco,F., Piccirillo,S., Vescovi,A.L., & Eberhart,C.G. (2010) NOTCH pathway blockade depletes CD133-positive glioblastoma cells and inhibits growth of tumor neurospheres and xenografts. *Stem Cells*, **28**, 5-16.

Fantini,J., Garmy,N., Mahfoud,R., & Yahi,N. (2002) Lipid rafts: structure, function and role in HIV, Alzheimer's and prion diseases. *Expert.Rev.Mol.Med.*, **4**, 1-22.

Fargeas,C.A., Joester,A., Missol-Kolka,E., Hellwig,A., Huttner,W.B., & Corbeil,D. (2004) Identification of novel Prominin-1/CD133 splice variants with alternative C-termini and their expression in epididymis and testis. *J.Cell Sci.*, **117**, 4301-4311.

Feng,H.L., Liu,Y.Q., Yang,L.J., Bian,X.C., Yang,Z.L., Gu,B., Zhang,H., Wang,C.J., Su,X.L., & Zhao,X.M. (2010) Expression of CD133 correlates with differentiation of human colon cancer cells. *Cancer Biol.Ther.*, **9**, 216-223.

Fernandez-Valero,E.M., Ballart,A., Iturriaga,C., Lluch,M., Macias,J., Vanier,M.T., Pineda,M., & Coll,M.J. (2005) Identification of 25 new mutations in 40 unrelated Spanish Niemann-Pick type C patients: genotype-phenotype correlations. *Clin.Genet.*, **68**, 245-254.

Ferrandina,G., Petrillo,M., Bonanno,G., & Scambia,G. (2009) Targeting CD133 antigen in cancer. 2. *Expert.Opin.Ther.Targets.*, **13**, 823-837.

Fesik,S.W. & Shi,Y. (2001) Structural biology. Controlling the caspases. *Science*, **294**, 1477-1478.

Fillmore,C. & Kuperwasser,C. (2007) Human breast cancer stem cell markers CD44 and CD24: enriching for cells with functional properties in mice or in man? *Breast Cancer Res.*, **9**, 303.

Finney,M.R., Greco,N.J., Haynesworth,S.E., Martin,J.M., Hedrick,D.P., Swan,J.Z., Winter,D.G., Kadereit,S., Joseph,M.E., Fu,P., Pompili,V.J., & Laughlin,M.J. (2006) Direct comparison of umbilical cord blood versus bone marrow-derived endothelial precursor cells in mediating neovascularization in response to vascular ischemia. *Biol.Blood Marrow Transplant.*, **12**, 585-593.

Fischer,A.N., Herrera,B., Mikula,M., Proell,V., Fuchs,E., Gotzmann,J., Schulte-Hermann,R., Beug,H., & Mikulits,W. (2005) Integration of Ras subeffector signalling in TGF-beta mediated late stage hepatocarcinogenesis. *Carcinogenesis*, **26**, 931-942.

Florek,M., Bauer,N., Janich,P., Wilsch-Braeuninger,M., Fargeas,C.A., Marzesco,A.M., Ehninger,G., Thiele,C., Huttner,W.B., & Corbeil,D. (2007) Prominin-2 is a cholesterol-binding protein associated with apical and basolateral plasmalemmal protrusions in polarized epithelial cells and released into urine. *Cell Tissue Res.*, **328**, 31-47.

Fodde,R. & Brabletz,T. (2007) Wnt/beta-catenin signalling in cancer stemness and malignant behavior. *Curr.Opin.Cell Biol.*, **19**, 150-158.

Fodor S.P.A. Light-directed, spatially addressable parallel chemical synthesis. Read J.L., Pirrung M. C. Stryer L. Lu A. T. Solas D. Science 251, 467-470. 1991. Ref Type: Generic

Fruehauf,S., Srbic,K., Seggewiss,R., Topaly,J., & Ho,A.D. (2002) Functional characterization of podia formation in normal and malignant hematopoietic cells. *J.Leukoc.Biol.*, **71**, 425-432.

Fujiwara,T., Kawakatsu,T., Tayama,S., Kobayashi,Y., Sugiura,N., Kimata,K., & Takai,Y. (2008) Hyaluronan-CD44 pathway regulates orientation of mitotic spindle in normal epithelial cells. *Genes Cells*, **13**, 759-770.

Gallacher,L., Murdoch,B., Wu,D.M., Karanu,F.N., Keeney,M., & Bhatia,M. (2000) Isolation and characterization of human CD34(-)Lin(-) and CD34(+)Lin(-) hematopoietic stem cells using cell surface markers AC133 and CD7. *Blood*, **95**, 2813-2820.

Garcia,J.L., Perez-Caro,M., Gomez-Moreta,J.A., Gonzalez,F., Ortiz,J., Blanco,O., Sancho,M., Hernandez-Rivas,J.M., Gonzalez-Sarmiento,R., & Sanchez-Martin,M. (2010) Molecular analysis of ex-vivo CD133+ GBM cells revealed a common invasive and angiogenic profile but different proliferative signatures among high grade gliomas. *BMC.Cancer*, **10**, 454.

Gehling,U.M., Willems,M., Dandri,M., Petersen,J., Berna,M., Thill,M., Wulf,T., Muller,L., Pollok,J.M., Schlagner,K., Faltz,C., Hossfeld,D.K., & Rogiers,X. (2005) Partial hepatectomy induces mobilization of a unique population of haematopoietic progenitor cells in human healthy liver donors. *J.Hepatol.*, **43**, 845-853.

Giebel,B., Corbeil,D., Beckmann,J., Hohn,J., Freund,D., Giesen,K., Fischer,J., Kogler,G., & Wernet,P. (2004) Segregation of lipid raft markers including CD133 in polarized human hematopoietic stem and progenitor cells. *Blood*, **104**, 2332-2338.

Giebel,B., Corbeil,D., Beckmann,J., Hohn,J., Freund,D., Giesen,K., Fischer,J., Kogler,G., & Wernet,P. (2004) Segregation of lipid raft markers including CD133 in polarized human hematopoietic stem and progenitor cells. *Blood*, **104**, 2332-2338.

Gotzmann,J., Fischer,A.N., Zojer,M., Mikula,M., Proell,V., Huber,H., Jechlinger,M., Waerner,T., Weith,A., Beug,H., & Mikulits,W. (2006) A crucial function of PDGF in TGF-beta-mediated cancer progression of hepatocytes. *Oncogene*, **25**, 3170-3185.

Grskovic,B., Ruzicka,K., Karimi,A., Qujeq,D., & Muller,M.M. (2004) Cell cycle analysis of the CD133+ and C. *Clin.Chim.Acta*, **343**, 173-178.

Hansson,E.M., Lanner,F., Das,D., Mutvei,A., Marklund,U., Ericson,J., Farnebo,F., Stumm,G., Stenmark,H., Andersson,E.R., & Lendahl,U. (2010) Control of Notch-ligand endocytosis by ligand-receptor interaction. *J.Cell Sci.*, **123**, 2931-2942.

Hao,H.N., Zhao,J., Thomas,R.L., Parker,G.C., & Lyman,W.D. (2003) Fetal human hematopoietic stem cells can differentiate sequentially into neural stem cells and then astrocytes *in vitro*. *J.Hematother.Stem Cell Res.*, **12**, 23-32.

Harper,J.V. & Brooks,G. (2005) The mammalian cell cycle: an overview. *Methods Mol.Biol.*, **296**, 113-153.

Hart,C., Drewel,D., Mueller,G., Grassinger,J., Zaiss,M., Kunz-Schughart,L.A., Andreesen,R., Reichle,A., Holler,E., & Hennemann,B. (2004) Expression and function of homing-essential molecules and enhanced *in vivo* homing ability of human peripheral blood-derived hematopoietic progenitor cells after stimulation with stem cell factor. *Stem Cells*, **22**, 580-589.

Hayward-Costa, Kingston University, unpublished work, 2000.

Healy,L., May,G., Gale,K., Grosveld,F., Greaves,M., & Enver,T. (1995) The stem cell antigen CD34 functions as a regulator of hemopoietic cell adhesion. *Proc.Natl.Acad.Sci.U.S.A*, **92**, 12240-12244.

Heissig,B., Hattori,K., Dias,S., Friedrich,M., Ferris,B., Hackett,N.R., Crystal,R.G., Besmer,P., Lyden,D., Moore,M.A., Werb,Z., & Rafii,S. (2002) Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell*, **109**, 625-637.

Hemmoranta,H., Hautaniemi,S., Niemi,J., Nicorici,D., Laine,J., Yli-Harja,O., Partanen,J., & Jaatinen,T. (2006) Transcriptional profiling reflects shared and unique characters for CD34+ and CD133+ cells. *Stem Cells Dev.*, **15**, 839-851.

Hertweck,M.K., Erdfelder,F., & Kreuzer,K.A. (2011) CD44 in hematological neoplasias. *Ann.Hematol.*, **90**, 493-508.

Hilbe,W., Dirnhofer,S., Oberwasserlechner,F., Schmid,T., Gunsilius,E., Hilbe,G., Woll,E., & Kahler,C.M. (2004) CD133 positive endothelial progenitor cells contribute to the tumour vasculature in non-small cell lung cancer. *J.Clin.Pathol.*, **57**, 965-969.

Hirst,J.D., Vieth,M., Skolnick,J., & Brooks,C.L., III (1996) Predicting leucine zipper structures from sequence. *Protein Eng* , **9**, 657-662.

Hnasko,R. & Lisanti,M.P. (2003) The biology of caveolae: lessons from caveolin knockout mice and implications for human disease. *Mol.Interv.*, **3**, 445-464.

Hoffbrand and Pettit, Essential Haematology, fifth edition, 2006, Blackwell Publishing.

Horst,D., Kriegl,L., Engel,J., Kirchner,T., & Jung,A. (2008) CD133 expression is an independent prognostic marker for low survival in colorectal cancer. *Br.J.Cancer*, **99**, 1285-1289.

Horst,D., Kriegl,L., Engel,J., Jung,A., & Kirchner,T. (2009) CD133 and nuclear beta-catenin: the marker combination to detect high risk cases of low stage colorectal cancer. *Eur.J.Cancer*, **45**, 2034-2040.

Horst,D., Scheel,S.K., Liebmann,S., Neumann,J., Maatz,S., Kirchner,T., & Jung,A. (2009) The cancer stem cell marker CD133 has high prognostic impact but unknown functional relevance for the metastasis of human colon cancer [2]. *J.Pathol.*, **219**, 427-434.

Hu,Z.B., Ma,W., Zaborski,M., MacLeod,R., Quentmeier,H., & Drexler,H.G. (1996) Establishment and characterization of two novel cytokine-responsive acute myeloid and monocytic leukemia cell lines, MUTZ-2 and MUTZ-3. *Leukemia*, **10**, 1025-1040.

Huang,W.H., Wu,J.Z., Hu,Z.X., Feng,S.Z., Huang,X.Y., Li,A., & Robinson,W.A. (1988) Bone marrow granulopoietic response to scalds and wound infection in mice. *Burns Incl.Therm.Inj.*, **14**, 292-296.

Hunter,A.J., Ottoson,N., Boerth,N., Koretzky,G.A., & Shimizu,Y. (2000) Cutting edge: a novel function for the SLAP-130/FYB adapter protein in beta 1 integrin signalling and T lymphocyte migration. *J.Immunol.*, **164**, 1143-1147.

Ieta,K., Tanaka,F., Haraguchi,N., Kita,Y., Sakashita,H., Mimori,K., Matsumoto,T., Inoue,H., Kuwano,H., & Mori,M. (2008) Biological and genetic characteristics of tumor-initiating cells in colon cancer. *Ann.Surg.Oncol.*, **15**, 638-648.

Ikonen,E. (2001) Roles of lipid rafts in membrane transport. *Curr.Opin.Cell Biol.*, **13**, 470-477.

Jaatinen,T., Hemmoranta,H., Hautaniemi,S., Niemi,J., Nicorici,D., Laine,J., Yli-Harja,O., & Partanen,J. (2006) Global gene expression profile of human cord blood-derived CD133+ cells. *Stem Cells*, **24**, 631-641.

Jaksch,M., Munera,J., Bajpai,R., Terskikh,A., & Oshima,R.G. (2008) Cell cycle-dependent variation of a CD133 epitope in human embryonic stem cell, colon cancer, and melanoma cell lines. *Cancer Res.* , **68**, 7882-7886.

James Pawley (2006) *Handbook of Biological Confocal Microscopy,* Springer.

Jang,Y.K., Park,J.J., Lee,M.C., Yoon,B.H., Yang,Y.S., Yang,S.E., & Kim,S.U. (2004) Retinoic acid-mediated induction of neurons and glial cells from human umbilical cord-derived hematopoietic stem cells. *J.Neurosci.Res.*, **75**, 573-584.

Janich,P. & Corbeil,D. (2007) GM1 and GM3 gangliosides highlight distinct lipid microdomains within the apical domain of epithelial cells. *FEBS Lett.*, **581**, 1783-1787.

Jaszai,J., Fargeas,C.A., Florek,M., Huttner,W.B., & Corbeil,D. (2007) Focus on molecules: prominin-1 (CD133). *Exp.Eye Res.*, **85**, 585-586.

Jensen,G.S., Hart,A.N., Zaske,L.A., Drapeau,C., Gupta,N., Schaeffer,D.J., & Cruickshank,J.A. (2007) Mobilization of human CD34+ CD133+ and CD34+ CD133(-) stem cells *in vivo* by consumption of an extract from Aphanizomenon flos-aquae--related to modulation of CXCR4 expression by an L-selectin ligand? *Cardiovasc.Revasc.Med.*, **8**, 189-202.

Jiang,L., Lai,Y.K., Zhang,J., Wang,H., Lin,M.C., He,M.L., & Kung,H.F. (2011) Targeting S100P inhibits colon cancer growth and metastasis by lentivirus-mediated RNA interference and proteomic analysis. *Mol.Med.*

Johannessen,T.C., Bjerkvig,R., & Tysnes,B.B. (2008) DNA repair and cancer stem-like cells--potential partners in glioma drug resistance? *Cancer Treat.Rev.*, **34**, 558-567.

Kang,Y., Siegel,P.M., Shu,W., Drobnjak,M., Kakonen,S.M., Cordon-Cardo,C., Guise,T.A., & Massague,J. (2003) A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell*, **3**, 537-549.

Karbanova,J., Missol-Kolka,E., Fonseca,A.V., Lorra,C., Janich,P., Hollerova,H., Jaszai,J., Ehrmann,J., Kolar,Z., Liebers,C., Arl,S., Subrtova,D., Freund,D., Mokry,J., Huttner,W.B., & Corbeil,D. (2008) The stem cell marker CD133 (Prominin-1) is expressed in various human glandular epithelia. *J.Histochem.Cytochem.*, **56**, 977-993.

Kaufman,D.S., Hanson,E.T., Lewis,R.L., Auerbach,R., & Thomson,J.A. (2001) Hematopoietic colony-forming cells derived from human embryonic stem cells. *Proc.Natl.Acad.Sci.U.S.A*, **98**, 10716-10721.

Kawano,Y., Kobune,M., Chiba,H., Nakamura,K., Takimoto,R., Takada,K., Ito,Y., Kato,J., Hamada,H., & Niitsu,Y. (2006) Ex vivo expansion of G-CSF-mobilized peripheral blood CD133+ progenitor cells on coculture with human stromal cells. *Exp.Hematol.*, **34**, 150-158.

Kemper,K., Grandela,C., & Medema,J.P. (2010) Molecular identification and targeting of colorectal cancer stem cells. *Oncotarget.*, **1**, 387-395.

Kim,C.G., Lee,J.J., Jung,D.Y., Jeon,J., Heo,H.S., Kang,H.C., Shin,J.H., Cho,Y.S., Cha,K.J., Kim,C.G., Do,B.R., Kim,K.S., & Kim,H.S. (2006) Profiling of differentially expressed genes in human stem cells by cDNA microarray. *Mol.Cells*, **21**, 343-355.

Kim,D.H. & Rossi,J.J. (2007) Strategies for silencing human disease using RNA interference. *Nat.Rev.Genet.*, **8**, 173-184.

Kim,K.J., Lee,K.H., Kim,H.S., Moon,K.S., Jung,T.Y., Jung,S., & Lee,M.C. (2011) The presence of stem cell marker-expressing cells is not prognostically significant in glioblastomas. *Neuropathology.*

Kishimoto,T., Goyert,S., Kikutani,H., Mason,D., Miyasaka,M., Moretta,L., Ohno,T., Okumura,K., Shaw,S., Springer,T.A., Sugamura,K., Sugawara,H., von dem Borne,A.E., & Zola,H. (1997) Update of CD antigens, 1996. *J.Immunol.*, **158**, 3035-3036.

Koblas,T., Zacharovova,K., Berkova,Z., Mindlova,M., Girman,P., Dovolilova,E., Karasova,L., & Saudek,F. (2007) Isolation and characterization of human CXCR4-positive pancreatic cells. *Folia Biol.(Praha)*, **53**, 13-22.

Koornstra,J.J., Kleibeuker,J.H., van Geelen,C.M., Rijcken,F.E., Hollema,H., de Vries,E.G., & de,J.S. (2003) Expression of TRAIL (TNF-related apoptosis-inducing ligand) and its receptors in normal colonic mucosa, adenomas, and carcinomas. *J.Pathol.*, **200**, 327-335.

Korade,Z. & Kenworthy,A.K. (2008) Lipid rafts, cholesterol, and the brain. *Neuropharmacology*, **55**, 1265-1273.

Kratz-Albers,K., Zuhlsdorp,M., Leo,R., Berdel,W.L., Buchner,T., & Serve,H. (1998) Expression of a AC133, a novel stem cell marker, on human leukemic blasts lacking CD34-antigen and on a human CD34+ leukemic line:MUTZ-2. *Blood*, **92**, 4485-4487.

Kuci,Z., Seitz,G., Kuci,S., Kreyenberg,H., Schumm,M., Lang,P., Niethammer,D., Handgretinger,R., & Bruchelt,G. (2006) Pitfalls in detection of contaminating neuroblastoma cells by tyrosine hydroxylase RT-PCR due to catecholamine-producing hematopoietic cells. *Anticancer Res.*, **26**, 2075-2080.

Leclercq,L., Bricout,H., Tilloy,S., & Monflier,E. (2007) Biphasic aqueous organometallic catalysis promoted by cyclodextrins: can surface tension measurements explain the efficiency of chemically modified cyclodextrins? *J.Colloid Interface Sci.*, **307**, 481-487.

Lee,A., Kessler,J.D., Read,T.A., Kaiser,C., Corbeil,D., Huttner,W.B., Johnson,J.E., & Wechsler-Reya,R.J. (2005) Isolation of neural stem cells from the postnatal cerebellum. *Nat.Neurosci.*, **8**, 723-729.

Lennon G.G. Hybridization analyses of arrayed cDNA libraries. Lehrach H. Trends Genet. 7, 314-317. 1-1-1991.
Ref Type: Generic

Leor,J., Guetta,E., Feinberg,M.S., Galski,H., Bar,I., Holbova,R., Miller,L., Zarin,P., Castel,D., Barbash,I.M., & Nagler,A. (2006) Human umbilical cord blood-derived CD133+ cells enhance function and repair of the infarcted myocardium. *Stem Cells*, **24**, 772-780.

Lindsell,C.E., Boulter,J., diSibio,G., Gossler,A., & Weinmaster,G. (1996) Expression patterns of Jagged, Delta1, Notch1, Notch2, and Notch3 genes identify ligand-receptor pairs that may function in neural development. *Mol.Cell Neurosci.*, **8**, 14-27.

Lipshutz,R.J., Fodor,S.P., Gingeras,T.R., & Lockhart,D.J. (1999) High density synthetic oligonucleotide arrays. *Nat.Genet.*, **21**, 20-24.

Liu,G., Yuan,X., Zeng,Z., Tunici,P., Ng,H., Abdulkadir,I.R., Lu,L., Irvin,D., Black,K.L., & Yu,J.S. (2006) Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma [2] . *Mol.Cancer*, **5**, 67.

Liu,S., Dontu,G., Mantle,I.D., Patel,S., Ahn,N.S., Jackson,K.W., Suri,P., & Wicha,M.S. (2006) Hedgehog signalling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. *Cancer Res.*, **66**, 6063-6071.

Lockhart,D.J. & Winzeler,E.A. (2000) Genomics, gene expression and DNA arrays. *Nature*, **405**, 827-836.

Loges,S., Fehse,B., Brockmann,M.A., Lamszus,K., Butzal,M., Guckenbiehl,M., Schuch,G., Ergun,S., Fischer,U., Zander,A.R., Hossfeld,D.K., Fiedler,W., & Gehling,U.M. (2004) Identification of the adult human hemangioblast. *Stem Cells Dev.*, **13**, 229-242.

Longobardi Givan. Flow cytometry first principles. 2001. Wiley-Liss.

Lu,F., Gao,J.H., Mizuro,H., Ogawa,R., & Hyakusoku,H. (2007) [Experimental study of adipose tissue differentiation using adipose-derived stem cells harvested from GFP transgenic mice]. *Zhonghua Zheng.Xing.Wai Ke.Za Zhi.*, **23**, 412-416.

Ma,N., Ladilov,Y., Moebius,J.M., Ong,L., Piechaczek,C., David,A., Kaminski,A., Choi,Y.H., Li,W., Egger,D., Stamm,C., & Steinhoff,G. (2006) Intramyocardial delivery of human CD133+ cells in a SCID mouse cryoinjury model: Bone marrow vs. cord blood-derived cells. *Cardiovasc.Res.*, **71**, 158-169.

Majka,M., Kucia,M., & Ratajczak,M.Z. (2005) Stem cell biology: a never ending quest for understanding. *Acta Biochim.Pol.*, **52**, 353-358.

Marzesco,A.M., Janich,P., Wilsch-Brauninger,M., Dubreuil,V., Langenfeld,K., Corbeil,D., & Huttner,W.B. (2005) Release of extracellular membrane particles carrying the stem cell marker prominin-1 (CD133) from neural progenitors and other epithelial cells. *J.Cell Sci.*, **118**, 2849-2858.

Matsumoto,K., Yasui,K., Yamashita,N., Horie,Y., Yamada,T., Tani,Y., Shibata,H., & Nakano,T. (2000) *In vitro* proliferation potential of AC133 positive cells in peripheral blood. *Stem Cells*, **18**, 196-203.

Maw,M.A., Corbeil,D., Koch,J., Hellwig,A., Wilson-Wheeler,J.C., Bridges,R.J., Kumaramanickavel,G., John,S., Nancarrow,D., Roper,K., Weigmann,A., Huttner,W.B., & Denton,M.J. (2000) A frameshift mutation in prominin (mouse)-like 1 causes human retinal degeneration. *Hum.Mol.Genet.*, **9**, 27-34.

Mayor,S. & Rao,M. (2004) Rafts: scale-dependent, active lipid organization at the cell surface. *Traffic.*, **5**, 231-240.

McGuckin,C.P., Forraz,N., Pettengell,R., & Thompson,A. (2004) Thrombopoietin, flt3-ligand and c-kit-ligand modulate HOX gene expression in expanding cord blood CD133 cells. *Cell Prolif.*, **37**, 295-306.

McKenzie,S.B. Textbook of Haematology. 1-1-1996. Williams and Wilkins.

Meyer,C. & Drexler,H.G. (1999) FLT3 ligand inhibits apoptosis and promotes survival of myeloid leukemia cell lines. *Leuk.Lymphoma*, **32**, 577-581.

Meyer,C., Godoy,P., Bachmann,A., Liu,Y., Barzan,D., Ilkavets,I., Maier,P., Herskind,C., Hengstler,J.G., & Dooley,S. (2011) Distinct role of endocytosis for Smad and non-Smad TGF-beta signalling regulation in hepatocytes. *J.Hepatol.*, **55**, 369-378.

Mihai,R., Stevens,J., McKinney,C., & Ibrahim,N.B. (2006) Expression of the calcium receptor in human breast cancer--a potential new marker predicting the risk of bone metastases. *Eur.J.Surg.Oncol.*, **32**, 511-515.

Miraglia,S., Godfrey,W., Yin,A.H., Atkins,K., Warnke,R., Holden,J.T., Bray,R.A., Waller,E.K., & Buck,D.W. (1997) A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. *Blood*, **90**, 5013-5021.

Miraglia,S., Godfrey,W., & Buck,D. (1998) A response to AC133 hematopoietic stem cell antigen: human homologue of mouse kidney prominin or distinct member of a novel protein family? *Blood*, **91**, 4390-4391.

Monzani,E., Facchetti,F., Galmozzi,E., Corsini,E., Benetti,A., Cavazzin,C., Gritti,A., Piccinini,A., Porro,D., Santinami,M., Invernici,G., Parati,E., Alessandri,G., & La Porta,C.A. (2007) Melanoma contains CD133 and ABCG2 positive cells with enhanced tumourigenic potential. *Eur.J.Cancer*, **43**, 935-946.

Moriyama,T., Ohuchida,K., Mizumoto,K., Cui,L., Ikenaga,N., Sato,N., & Tanaka,M. (2010) Enhanced cell migration and invasion of CD133+ pancreatic cancer cells cocultured with pancreatic stromal cells. *Cancer*, **116**, 3357-3368.

Munker,R. & Calin,G.A. (2011) MicroRNA profiling in cancer. *Clin.Sci.(Lond)*, **121**, 141-158.

Munoz-Losa,A., Curutchet,C., Krueger,B.P., Hartsell,L.R., & Mennucci,B. (2009) Fretting about FRET: failure of the ideal dipole approximation. *Biophys.J.*, **96**, 4779-4788.

Naor,D., Wallach-Dayan,S.B., Zahalka,M.A., & Sionov,R.V. (2008) Involvement of CD44, a molecule with a thousand faces, in cancer dissemination. *Semin.Cancer Biol.*, **18**, 260-267.

Negrin,R.S., Atkinson,K., Leemhuis,T., Hanania,E., Juttner,C., Tierney,K., Hu,W.W., Johnston,L.J., Shizurn,J.A., Stockerl-Goldstein,K.E., Blume,K.G., Weissman,I.L., Bower,S., Baynes,R., Dansey,R., Karanes,C., Peters,W., & Klein,J. (2000) Transplantation of highly purified CD34+Thy-1+ hematopoietic stem cells in patients with metastatic breast cancer. *Biol.Blood Marrow Transplant.*, **6**, 262-271.

Negroni,E., Riederer,I., Chaouch,S., Belicchi,M., Razini,P., Di,S.J., Torrente,Y., Butler-Browne,G.S., & Mouly,V. (2009) *In vivo* myogenic potential of human CD133+ muscle-derived stem cells: a quantitative study. *Mol.Ther.*, **17**, 1771-1778.

Neuzil,J., Stantic,M., Zobalova,R., Chladova,J., Wang,X., Prochazka,L., Dong,L., Andera,L., & Ralph,S.J. (2007) Tumour-initiating cells vs. cancer 'stem' cells and CD133: what's in the name? *Biochem.Biophys.Res.Commun.*, **355**, 855-859.

Nijhawan,A., Jain,M., Tyagi,A.K., & Khurana,J.P. (2008) Genomic survey and gene expression analysis of the basic leucine zipper transcription factor family in rice. *Plant Physiol*, **146**, 333-350.

Nikolova,T., Wu,M., Brumbarov,K., Alt,R., Opitz,H., Boheler,K.R., Cross,M., & Wobus,A.M. (2007) WNT-conditioned media differentially affect the proliferation and differentiation of cord blood-derived CD133+ cells *in vitro*. *Differentiation*, **75**, 100-111.

Nirmala,R. & Narayanan,P.R. (2002) Flow cytometry--a rapid tool to correlate functional activities of human peripheral blood lymphocytes with their corresponding phenotypes after *in vitro* stimulation. *BMC.Immunol.*, **3**, 9.

Nishino,T., Miyaji,K., Ishiwata,N., Arai,K., Yui,M., Asai,Y., Nakauchi,H., & Iwama,A. (2009) Ex vivo expansion of human hematopoietic stem cells by a small-molecule agonist of c-MPL. *Exp.Hematol.*, **37**, 1364-1377.

O'Brien,C.A., Pollett,A., Gallinger,S., & Dick,J.E. (2007) A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature*, **445**, 106-110.

Ogawa,M. (1993) Differentiation and proliferation of hematopoietic stem cells. *Blood*, **81**, 2844-2853.

Ogden,A.T., Waziri,A.E., Lochhead,R.A., Fusco,D., Lopez,K., Ellis,J.A., Kang,J., Assanah,M., McKhann,G.M., Sisti,M.B., McCormick,P.C., Canoll,P., & Bruce,J.N. (2008) Identification of A2B5+C. *Neurosurgery* , **62**, 505-514.

Olempska,M., Eisenach,P.A., Ammerpohl,O., Ungefroren,H., Fandrich,F., & Kalthoff,H. (2007) Detection of tumor stem cell markers in pancreatic carcinoma cell lines. *Hepatobiliary.Pancreat.Dis.Int.*, **6**, 92-97.

Oshima,Y., Suzuki,A., Kawashimo,K., Ishikawa,M., Ohkohchi,N., & Taniguchi,H. (2007) Isolation of mouse pancreatic ductal progenitor cells expressing CD133 and c-Met by flow cytometric cell sorting. *Gastroenterology*, **132**, 720-732.

Padovan,C.S., Jahn,K., Birnbaum,T., Reich,P., Sostak,P., Strupp,M., & Straube,A. (2003) Expression of neuronal markers in differentiated marrow stromal cells and CD133+ stem-like cells. *Cell Transplant.*, **12**, 839-848.

Pasino,M., Lanza,T., Marotta,F., Scarso,L., De,B.P., Amato,S., Corcione,A., Pistoia,V., & Mori,P.G. (2000) Flow cytometric and functional characterization of AC133+ cells from human umbilical cord blood. *Br.J.Haematol.*, **108**, 793-800.

Potgens,A.J., Bolte,M., Huppertz,B., Kaufmann,P., & Frank,H.G. (2001) Human trophoblast contains an intracellular protein reactive with an antibody against CD133--a novel marker for trophoblast. *Placenta*, **22**, 639-645.

Qiang,L., Yang,Y., Ma,Y.J., Chen,F.H., Zhang,L.B., Liu,W., Qi,Q., Lu,N., Tao,L., Wang,X.T., You,Q.D., & Guo,Q.L. (2009) Isolation and characterization of cancer stem like cells in human glioblastoma cell lines. *Cancer Lett.*, **279**, 13-21.

Quirici,N., Soligo,D., Caneva,L., Servida,F., Bossolasco,P., & Deliliers,G.L. (2001) Differentiation and expansion of endothelial cells from human bone marrow CD133(+) cells. *Br.J.Haematol.*, **115**, 186-194.

Rahnama,F., Shimokawa,T., Lauth,M., Finta,C., Kogerman,P., Teglund,S., Toftgard,R., & Zaphiropoulos,P.G. (2006) Inhibition of GLI1 gene activation by Patched. *Biochem.J.*, **394**, 19-26.

Rappa,G., Fodstad,O., & Lorico,A. (2008) The stem cell-associated antigen CD133 (Prominin-1) is a molecular therapeutic target for metastatic melanoma. *Stem Cells*, **26**, 3008-3017.

Reya,T., Duncan,A.W., Ailles,L., Domen,J., Scherer,D.C., Willert,K., Hintz,L., Nusse,R., & Weissman,I.L. (2003) A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature*, **423**, 409-414.

Rich,J.N. (2007) Cancer stem cells in radiation resistance. *Cancer Res.*, **67**, 8980-8984.

Richardson,G.D., Robson,C.N., Lang,S.H., Neal,D.E., Maitland,N.J., & Collins,A.T. (2004) CD133, a novel marker for human prostatic epithelial stem cells. *J.Cell Sci.*, **117**, 3539-3545.

Rizzo,S., Attard,G., & Hudson,D.L. (2005) Prostate epithelial stem cells. *Cell Prolif.*, **38**, 363-374.

Rodak,B.F. (1995) *Diagnostic Hematology23,* W.B Saunders Company.

Rogers,K.R., Apostol,A., Madsen,S.J., & Spencer,C.W. (1999) Detection of low dose radiation induced DNA damage using temperature differential fluorescence assay 142. *Anal.Chem.*, **71**, 4423-4426.

Roper,K., Corbeil,D., & Huttner,W.B. (2000) Retention of prominin in microvilli reveals distinct cholesterol-based lipid micro-domains in the apical plasma membrane. *Nat.Cell Biol.*, **2**, 582-592.

Savitz,S. (1998) Role of apoptosis in health and disease. *JAMA*, **279**, 1699-1700.

Schena M (1995) Quantitative monitoring of gene expression patterns with a omplementary DNA microarray. *Science*, **270**, 467-470.

Seegar,T. & Barton,W. (2010) Imaging protein-protein interactions *in vivo*. *J.Vis.Exp.*

Selleri,C., Ragno,P., Ricci,P., Visconte,V., Scarpato,N., Carriero,M.V., Rotoli,B., Rossi,G., & Montuori,N. (2006) The metastasis-associated 67-kDa laminin receptor is involved in G-CSF-induced hematopoietic stem cell mobilization. *Blood*, **108**, 2476-2484.

Shackleton,M., Vaillant,F., Simpson,K.J., Stingl,J., Smyth,G.K., Asselin-Labat,M.L., Wu,L., Lindeman,G.J., & Visvader,J.E. (2006) Generation of a functional mammary gland from a single stem cell. *Nature*, **439**, 84-88.

Sheppard,C.J.R. Confocal Laser Scanning Microscopy. Shotton, D. M. BIOS Scientific Publishers,Oxford, UK.
Ref Type: Generic

Sherlock,G. (2000) Analysis of large-scale gene expression data. *Curr.Opin.Immunol.*, **12**, 201-205.

Shmelkov,S.V., St,C.R., Lyden,D., & Rafii,S. (2005) AC133/CD133/Prominin-1. *Int.J.Biochem.Cell Biol.*, **37**, 715-719.

Shmelkov,S.V., Butler,J.M., Hooper,A.T., Hormigo,A., Kushner,J., Milde,T., St,C.R., Baljevic,M., White,I., Jin,D.K., Chadburn,A., Murphy,A.J., Valenzuela,D.M., Gale,N.W., Thurston,G., Yancopoulos,G.D., D'Angelica,M., Kemeny,N., Lyden,D., & Rafii,S. (2008) CD133 expression is not restricted to stem cells, and both CD133+ and C. *J.Clin.Invest*, **118**, 2111-2120.

Sidahmed,A.M. & Wilkie,B. (2010) Endogenous antiviral mechanisms of RNA interference: a comparative biology perspective. *Methods Mol.Biol.*, **623**, 3-19.

Simmons,P.J., Levesque,J.P., & Zannettino,A.C. (1997) Adhesion molecules in haemopoiesis. *Baillieres Clin.Haematol.*, **10**, 485-505.

Simons,K. & Vaz,W.L. (2004) Model systems, lipid rafts, and cell membranes. *Annu.Rev.Biophys.Biomol.Struct.*, **33**, 269-295.

Singh,S.K., Clarke,I.D., Terasaki,M., Bonn,V.E., Hawkins,C., Squire,J., & Dirks,P.B. (2003) Identification of a cancer stem cell in human brain tumors. *Cancer Res.*, **63**, 5821-5828.

Song,W., Li,H., Tao,K., Li,R., Song,Z., Zhao,Q., Zhang,F., & Dou,K. (2008) Expression and clinical significance of the stem cell marker CD133 in hepatocellular carcinoma. *Int.J.Clin.Pract.*, **62**, 1212-1218.

Southern,E.M., Maskos,U., & Elder,J.K. (1992) Analyzing and comparing nucleic acid sequences by hybridization to arrays of oligonucleotides: evaluation using experimental models. *Genomics*, **13**, 1008-1017.

Sowa,G., Xie,L., Xu,L., & Sessa,W.C. (2008) Serine 23 and 36 phosphorylation of caveolin-2 is differentially regulated by targeting to lipid raft/caveolae and in mitotic endothelial cells. *Biochemistry*, **47**, 101-111.

Sparks,A.B., Morin,P.J., Vogelstein,B., & Kinzler,K.W. (1998) Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. *Cancer Res.*, **58**, 1130-1134.

Stamm,C., Westphal,B., Kleine,H.D., Petzsch,M., Kittner,C., Klinge,H., Schumichen,C., Nienaber,C.A., Freund,M., & Steinhoff,G. (2003) Autologous bone-marrow stem-cell transplantation for myocardial regeneration. *Lancet*, **361**, 45-46.

Steen,R. & Egeland,T. (1998) CD34 molecule epitope distribution on cells of haematopoietic origin. *Leuk.Lymphoma*, **30**, 23-30.

Subramaniam,V., Vincent,I.R., Gardner,H., Chan,E., Dhamko,H., & Jothy,S. (2007) CD44 regulates cell migration in human colon cancer cells via Lyn kinase and AKT phosphorylation. *Exp.Mol.Pathol.*, **83**, 207-215.

Summers,Y.J., Heyworth,C.M., de Wynter,E.A., Hart,C.A., Chang,J., & Testa,N.G. (2004) AC133+ G0 cells from cord blood show a high incidence of long-term culture-initiating cells and a capacity for more than 100 million-fold amplification of colony-forming cells *in vitro*. *Stem Cells*, **22**, 704-715.

Sun,Y., Kong,W., Falk,A., Hu,J., Zhou,L., Pollard,S., & Smith,A. (2009) CD133 (Prominin) negative human neural stem cells are clonogenic and tripotent. *PLoS.One.*, **4**, e5498.

Sun,Y., Moretti,L., Giacalone,N.J., Schleicher,S., Speirs,C.K., Carbone,D.P., & Lu,B. (2011) Inhibition of JAK2 signalling by TG101209 enhances radiotherapy in lung cancer models. *J.Thorac.Oncol.*, **6**, 699-706.

Sutherland,D.R., Watt,S.M., Dowden,G., Karhi,K., Baker,M.A., Greaves,M.F., & Smart,J.E. (1988) Structural and partial amino acid sequence analysis of the human hemopoietic progenitor cell antigen CD34. *Leukemia*, **2**, 793-803.

Suuronen,E.J., Wong,S., Kapila,V., Waghray,G., Whitman,S.C., Mesana,T.G., & Ruel,M. (2006) Generation of CD133+ cells from C. *Cardiovasc.Res.*, **70**, 126-135.

Taipale,J. & Beachy,P.A. (2001) The Hedgehog and Wnt signalling pathways in cancer. *Nature*, **411**, 349-354.

Taipale,J., Cooper,M.K., Maiti,T., & Beachy,P.A. (2002) Patched acts catalytically to suppress the activity of Smoothened. *Nature*, **418**, 892-897.

Takaoka,M., Harada,H., Andl,C.D., Oyama,K., Naomoto,Y., Dempsey,K.L., Klein-Szanto,A.J., El-Deiry,W.S., Grimberg,A., & Nakagawa,H. (2004) Epidermal growth factor receptor regulates aberrant expression of insulin-like growth factor-binding protein 3. *Cancer Res.*, **64**, 7711-7723.

Takenobu,H., Shimozato,O., Nakamura,T., Ochiai,H., Yamaguchi,Y., Ohira,M., Nakagawara,A., & Kamijo,T. (2011) CD133 suppresses neuroblastoma cell differentiation via signal pathway modification. *Oncogene*, **30**, 97-105.

Tamaki,S., Eckert,K., He,D., Sutton,R., Doshe,M., Jain,G., Tushinski,R., Reitsma,M., Harris,B., Tsukamoto,A., Gage,F., Weissman,I., & Uchida,N. (2002) Engraftment of sorted/expanded human central nervous system stem cells from fetal brain. *J.Neurosci.Res.*, **69**, 976-986.

Thill,M., Schlagner,K., Altenahr,S., Ergun,S., Faragher,R.G., Kilic,N., Bednarz,J., Vohwinkel,G., Rogiers,X., Hossfeld,D.K., Richard,G., & Gehling,U.M. (2007) A novel population of repair cells identified in the stroma of the human cornea. *Stem Cells Dev.*, **16**, 733-745.

Thillaivinayagalingam,P., Gommeaux,J., McLoughlin,M., Collins,D., & Newcombe,A.R. (2010) Biopharmaceutical production: Applications of surface plasmon resonance biosensors. *J.Chromatogr.B Analyt.Technol.Biomed.Life Sci.*, **878**, 149-153.

Till,J.E. & McCulloch,E.A. (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat.Res.*, **14**, 213-222.

Todaro,M., Francipane,M.G., Medema,J.P., & Stassi,G. (2010) Colon cancer stem cells: promise of targeted therapy. *Gastroenterology*, **138**, 2151-2162.

Tolg,C., Hofmann,M., Herrlich,P., & Ponta,H. (1993) Splicing choice from ten variant exons establishes CD44 variability. *Nucleic Acids Res.*, **21**, 1225-1229.

Tondreau,T., Meuleman,N., Delforge,A., Dejeneffe,M., Leroy,R., Massy,M., Mortier,C., Bron,D., & Lagneaux,L. (2005) Mesenchymal stem cells derived from CD133-positive cells in mobilized peripheral blood and cord blood: proliferation, Oct4 expression, and plasticity. *Stem Cells*, **23**, 1105-1112.

Toren,A., Bielorai,B., Jacob-Hirsch,J., Fisher,T., Kreiser,D., Moran,O., Zeligson,S., Givol,D., Yitzhaky,A., Itskovitz-Eldor,J., Kventsel,I., Rosenthal,E., Amariglio,N., & Rechavi,G. (2005) CD133-positive hematopoietic stem cell "stemness" genes contain many genes mutated or abnormally expressed in leukemia. *Stem Cells*, **23**, 1142-1153.

Tristram-Nagle,S. & Nagle,J.F. (2004) Lipid bilayers: thermodynamics, structure, fluctuations, and interactions. *Chem.Phys.Lipids*, **127**, 3-14.

Tsutsumi,H., Nomura,W., Abe,S., Mino,T., Masuda,A., Ohashi,N., Tanaka,T., Ohba,K., Yamamoto,N., Akiyoshi,K., & Tamamura,H. (2009) Fluorogenically active leucine zipper peptides as tag-probe pairs for protein imaging in living cells. *Angew.Chem.Int.Ed Engl.*, **48**, 9164-9166.

Uchida,N., Buck,D.W., He,D., Reitsma,M.J., Masek,M., Phan,T.V., Tsukamoto,A.S., Gage,F.H., & Weissman,I.L. (2000) Direct isolation of human central nervous system stem cells. *Proc.Natl.Acad.Sci.U.S.A*, **97**, 14720-14725.

Ulloth,J.E., Almaguel,F.G., Padilla,A., Bu,L., Liu,J.W., & De,L.M. (2007) Characterization of methyl-beta-cyclodextrin toxicity in NGF-differentiated PC12 cell death. *Neurotoxicology*, **28**, 613-621.

Untergasser,G., Koeck,R., Wolf,D., Rumpold,H., Ott,H., Debbage,P., Koppelstaetter,C., & Gunsilius,E. (2006) CD34+/C. *Exp.Gerontol.*, **41**, 600-608.

van den Berg,D.J., Sharma,A.K., Bruno,E., & Hoffman,R. (1998) Role of members of the Wnt gene family in human hematopoiesis. *Blood*, **92**, 3189-3202.

Wajant,H., Pfizenmaier,K., & Scheurich,P. (2002) TNF-related apoptosis inducing ligand (TRAIL) and its receptors in tumor surveillance and cancer therapy. *Apoptosis.*, **7**, 449-459.

Wang,J., Wang,H., Li,Z., Wu,Q., Lathia,J.D., McLendon,R.E., Hjelmeland,A.B., & Rich,J.N. (2008) c-Myc is required for maintenance of glioma cancer stem cells. *PLoS.One.*, **3** , e3769.

Wang,J.F., Park,I.W., & Groopman,J.E. (2000) Stromal cell-derived factor-1alpha stimulates tyrosine phosphorylation of multiple focal adhesion proteins and induces migration of hematopoietic progenitor cells: roles of phosphoinositide-3 kinase and protein kinase C. *Blood*, **95**, 2505-2513.

Wang,Z.X., Kueh,J.L., Teh,C.H., Rossbach,M., Lim,L., Li,P., Wong,K.Y., Lufkin,T., Robson,P., & Stanton,L.W. (2007) Zfp206 is a transcription factor that controls pluripotency of embryonic stem cells. *Stem Cells*, **25**, 2173-2182.

Watson,A.J., Merritt,A.J., Jones,L.S., Askew,J.N., Anderson,E., Becciolini,A., Balzi,M., Potten,C.S., & Hickman,J.A. (1996) Evidence of reciprocity of bcl-2 and p53 expression in human colorectal adenomas and carcinomas. *Br.J.Cancer*, **73**, 889-895.

Watt,S.M., Buhring,H.J., Rappold,I., Chan,J.Y., Lee-Prudhoe,J., Jones,T., Zannettino,A.C., Simmons,P.J., Doyonnas,R., Sheer,D., & Butler,L.H. (1998) CD164, a novel sialomucin on CD34(+) and erythroid subsets, is located on human chromosome 6q21. *Blood*, **92**, 849-866.

Weigmann,A., Corbeil,D., Hellwig,A., & Huttner,W.B. (1997) Prominin, a novel microvilli-specific polytopic membrane protein of the apical surface of epithelial cells, is targeted to plasmalemmal protrusions of non-epithelial cells. *Proc.Natl.Acad.Sci.U.S.A*, **94**, 12425-12430.

Wellmann,A., Thieblemont,C., Pittaluga,S., Sakai,A., Jaffe,E.S., Siebert,P., & Raffeld,M. (2000) Detection of differentially expressed genes in lymphomas using cDNA arrays: identification of clusterin as a new diagnostic marker for anaplastic large-cell lymphomas. *Blood*, **96**, 398-404.

White,J.G., Amos,W.B., & Fordham,M. (1987) An evaluation of confocal versus conventional imaging of biological structures by fluorescence light microscopy. *J.Cell Biol.*, **105**, 41-48.

Whiting,K.A., McGuckin,C.P., Wertheim,D., Pearce,D., & Pettengell,R. (2003) Three-dimensional analysis of CD34 sialomucin distribution on cord blood and bone marrow. *Br.J.Haematol.*, **122**, 771-777.

Wong,S.Y. & Reiter,J.F. (2008) The primary cilium at the crossroads of mammalian hedgehog signalling. *Curr.Top.Dev.Biol.*, **85**, 225-260.

www.ifr.ac.uk/materials/fractures/Confocal_microscopy.

Xie,J., Murone,M., Luoh,S.M., Ryan,A., Gu,Q., Zhang,C., Bonifas,J.M., Lam,C.W., Hynes,M., Goddard,A., Rosenthal,A., Epstein,E.H., Jr., & de Sauvage,F.J. (1998) Activating Smoothened mutations in sporadic basal-cell carcinoma. *Nature*, **391**, 90-92.

Yang,C., Zhang,Z.H., Li,Z.J., Yang,R.C., Qian,G.Q., & Han,Z.C. (2004) Enhancement of neovascularization with cord blood CD133+ cell-derived endothelial progenitor cell transplantation. *Thromb.Haemost.*, **91**, 1202-1212.

Yang,J., Liu,X., Bhalla,K., Kim,C.N., Ibrado,A.M., Cai,J., Peng,T.I., Jones,D.P., & Wang,X. (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science*, **275**, 1129-1132.

Yao,J., Zhang,T., Ren,J., Yu,M., & Wu,G. (2009) Effect of CD133/prominin-1 antisense oligodeoxynucleotide on *in vitro* growth characteristics of Huh-7 human hepatocarcinoma cells and U251 human glioma cells 2. *Oncol.Rep.*, **22**, 781-787.

Yin,A.H., Miraglia,S., Zanjani,E.D., Almeida-Porada,G., Ogawa,M., Leary,A.G., Olweus,J., Kearney,J., & Buck,D.W. (1997) AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood*, **90**, 5002-5012.

Ying,Z., Gonzalez-Martinez,J., Tilelli,C., Bingaman,W., & Najm,I. (2005) Expression of neural stem cell surface marker CD133 in balloon cells of human focal cortical dysplasia. *Epilepsia*, **46**, 1716-1723.

Yoshikawa,S., Zen,Y., Fujii,T., Sato,Y., Ohta,T., Aoyagi,Y., & Nakanuma,Y. (2009) Characterization of CD133+ parenchymal cells in the liver: histology and culture. *World J.Gastroenterol.*, **15**, 4896-4906.

You,H., Ding,W., & Rountree,C.B. (2010) Epigenetic regulation of cancer stem cell marker CD133 by transforming growth factor-beta. *Hepatology*, **51**, 1635-1644.

Yu,H.B., Kunarso,G., Hong,F.H., & Stanton,L.W. (2009) Zfp206, Oct4, and Sox2 are integrated components of a transcriptional regulatory network in embryonic stem cells. *J.Biol.Chem.*, **284**, 31327-31335.

Yu,Y., Tang,L., Wang,J., Liu,S., Wang,W., An,H., Qi,R., Zhang,M., & Cao,X. (2002) Psoriatic lesional keratinocytes promote the maturation of human monocyte-derived Langerhans cells. *Dermatology*, **204**, 94-99.

Zacchigna,S., Oh,H., Wilsch-Brauninger,M., Missol-Kolka,E., Jaszai,J., Jansen,S., Tanimoto,N., Tonagel,F., Seeliger,M., Huttner,W.B., Corbeil,D., Dewerchin,M., Vinckier,S., Moons,L., & Carmeliet,P. (2009) Loss of the cholesterol-binding protein prominin-1/CD133 causes disk dysmorphogenesis and photoreceptor degeneration. *J.Neurosci.*, **29**, 2297-2308.

Zannettino,A.C., Buhring,H.J., Niutta,S., Watt,S.M., Benton,M.A., & Simmons,P.J. (1998) The sialomucin CD164 (MGC-24v) is an adhesive glycoprotein expressed by human hematopoietic progenitors and bone marrow stromal cells that serves as a potent negative regulator of hematopoiesis. *Blood*, **92**, 2613-2628.

Zawadzki,V., Perschl,A., Rosel,M., Hekele,A., & Zoller,M. (1998) Blockade of metastasis formation by CD44-receptor globulin. *Int.J.Cancer*, **75**, 919-924.

Zemanova,L., Schenk,A., Valler,M.J., Nienhaus,G.U., & Heilker,R. (2003) Confocal optics microscopy for biochemical and cellular high-throughput screening. *Drug Discov.Today*, **8**, 1085-1093.

Zobalova,R., McDermott,L., Stantic,M., Prokopova,K., Dong,L.F., & Neuzil,J. (2008) CD133-positive cells are resistant to TRAIL due to up-regulation of FLIP ^{2}. *Biochem.Biophys.Res.Commun.*, **373**, 567-571.

Zobalova,R., Stantic,M., Prokopova,K., Dong,L.F., & Neuzil,J. (2008) Cancer cells with high expression of CD133 exert FLIP upregulation and resistance to TRAIL-induced apoptosis. *Biofactors*, **34**, 231-235.

Zobalova,R., Prokopova,K., Stantic,M., Stapelberg,M., Dong,L.F., Ralph,S.J., Akporiaye,E., & Neuzil,J. (2011) The Potential Role of CD133 in Immune Surveillance and Apoptosis: A Mitochondrial Connection? *Antioxid.Redox.Signal*

Appendix 1

Micro Array analysis of Caco-2 cells

Compact Disk – Appendix/ Chapter 4 Micro Array Data

Group 1 **(CD133 knocked down group)**

Group 2 **(IgG control labelled group)**

Group 3 **(CD133 Ab labelled group)**

All groups were compared to a control group consisting of unstimulated cells

Folder A: Contains normalised and raw data for all three groups and control group.

Folder B: Gene lists for all three conditions compared to the control group for both up and down-regulated genes.

Folder C: Gene ontology's for all three groups both up and down regulated genes. The ontology's were split into 3 different areas. Biological processes, Molecular functions and Cellular components.

Folder D: Molecular pathways for all three groups both up and down regulated.

Appendix 2

CLSM and 2-Dimensional imaging

Compact Disk – Appendix/ Chapter 5 Confocal Data

The following table illustrates the folders found within appendix 2 on the enclosed CD. These folders contain examples of the original CLSM data and 2-D images

Folder A: BMCD removal of Lipid Rafts

Folder B: CD133 association with Lipid Rafts

Folder C: CD133 knockdown effect on lipid Rafts

Folder D: CD133 distribution in the presence of Fibronectin

Folder	Image	CD133	Lipid Raft	Magnification
Folder A	1	488 (Green)	555 (Red)	63x oil
	2	488	555	63x oil
Folder B	3	488	555	63x oil
	4	488	555	63x oil
	5	488	555	63x oil
Folder C	6	488	555	63x oil
	7	488	555	63x oil
Folder D	**8a** – Fibronectin	488	555	63x oil
	9a + Fibronectin	488	555	63x oil

Images 8b and 9b in folder D represent 2D Z scan images of Caco-2 cells showing the distribution of CD133 on the cell surface (apical, middle or basolateral).