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Role of CD133 in colorectal cancer

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Abstract

CD133 is a pentaspan transmembrane glycoprotein of ~ 120 kDa, which was initially used to identify haematopoietic stem cells and, later on, used for the isolation and study of cancer stem cells in many different types of solid tumour including colorectal cancer. Although CD133 expressing cells are thought to represent cancer stem cells, little is known about the exact role of CD133 and the molecular mechanisms underlying control of CD133 expression. This project sought to investigate these questions in colorectal cancer.

Initially the expression of CD133 was tested by immunohistochemistry in a two tissue microarray (TMA) sets consisting of (a) 449 cases of primary colorectal cancer, and (b) 45 cases of primary and matched liver metastases. High CD133 expression was marginally associated with shorter overall survival (OS) (*p=0.05, Log-rank test*) but no difference in expression was found between primary tumours and corresponding metastases.

Next, the functional activity of CD133 was evaluated in colorectal cancer (CRC) cell lines by knockdown in cell lines with high CD133 expression. In order to identify appropriate cell lines, the expression of CD133 was tested by quantitative RT-PCR in a series of 29 CRC cell lines and 10 samples of normal mucosa and, in selected cell lines, validated by testing for protein expression by flow cytometry. CD133 mRNA was expressed in 24/29 colorectal cancer cell lines with a heterogenous level of expression. 10 cell lines were chosen on the basis of CD133 mRNA expression level to assess the protein level. CD133 mRNA and protein expression were generally correlated (r_s = *0.831, p= 0.003, spearman rank correlation coefficient test*) although, interestingly, CD133 mRNA level was higher in normal samples compared with that in cancer cell lines and was significantly higher in cell lines derived from metastatic sites than those derived from site of primary tumour (*p=0.009; Mann-whitney test*). In addition, it was noted that many cell lines had a stable biphasic phenotype containing CD133+ and CD133- cell populations. This allowed functional analysis of CD133 by sorting the two populations.

HT29 was identified as a high expresser of CD133 (95%) and was used for gene-knockdown studies, SW480 had a biphasic population consisting of

42% CD133+ cells and 58% CD133- cells and each population was isolated by cell sorting before functional analysis. Functional assays included proliferation, migration, colony formation and staurosporine induced apoptosis assays. These showed that CD133 expressing cells had greater cell motility (*p= 0.04, and p = 0.03, unpaired t-test, for knocked down cells and sorted populations respectively)* , enhanced colony forming abilities (*p=0.0001, and p=0.003, unpaired t-test for 2D and 3D colony formation respectively using sorted populations only)*, and increased resistance to staurosporine induced apoptosis (*p=0.01, and p=0.008, unpaired t-test, for knocked down and sorted populations respectively)* than CD133 negative counterparts. In addition, sorted monophasic populations reverted to a biphasic state in both CD133+/- populations from SW480. Further studies demonstrated that CD133-induced cell motility was independent of E-cadherin, β-catenin, and suggestive of not being regulated by Cten or Wnt, but further work is warranted to verify these results. In addition, regulation of CD133 was partly dependent on STAT3 signallingand on CD133 promoter methylation. Levels of mRNA of some stem cell related genes such as *KLF-4, Musashi-1, OCT4, Nanog, and Lgr5 were higher in CD133 + compared to CD133 negative cells (p=0.008, p=0.004, p=0.006, p=0.001, and p=0.11; unpaired t-test, respectively)*

In conclusion, in CRC, CD133 was found to be a significant prognostic factor which enhances cell motility and is associated with features of "stemness". It is a target of STAT3 signalling and partly regulated by promoter methylation. More in depth studies are warranted to discover the downstream and upstream targets of CD133 before translating these preclinical and laboratory investigations into clinical management of colorectal cancer.

Publications resulting from the thesis

Elsaba, T. M., L. Martinez-Pomares, et al. (2010). "The stem cell marker CD133 associates with enhanced colony formation and cell motility in colorectal cancer." PLoS One **5**(5): e10714.

Publications in preparation from the thesis

1- Elsaba TM, D Jackson, and M Ilyas
"CD133+ cells were associated with stem cell related genes expression"

2- Elsaba TM, D Jackson, and M Ilyas
"STAT3 and DNA methylation but not Wnt signalling regulates CD133 expression in colorectal cancer"

3- Elsaba TM, D Jackson, J. H. Scholefield, L. G. Durrant and M. Ilyas

"Clinical significance of CD133 in colorectal cancer: an immunohistochemistry study"

Declaration

This thesis, entitled **role of CD133 in colorectal cancer,** is entirely the result of my own investigations, except where otherwise stated. Other sources are acknowledged in the text. No part of this report has been submitted for a degree, diploma or other qualification at any other institutions.

Tarek Ahmed

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Abbreviations

5'UTR	5 prime untranslated region
5-Aza	5-aza-2'-deoxycytidine
ALDH1	Aldehyde dehydrogenase 1
AML	Acute myeloid leukemia
APC	Adenomatous polyposis coli
APES	3-aminopropyltriethoxysilane
ASCs	Adult stem cells
BMP	Bone morphogenetic protein
Brdu	Bromodeoxyuridine
cDNA	Complementary deoxyribonucleic acid
CHRDL1	Chordin-like 1
CRC	Colorectal cancer
CSCs	Cancer stem cells
Cten	C-terminal tensin-like
DKK1	Dickkopf-1(Wnt inhibitor)
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EpCAM	Epithelial cell adhesion molecule
ESA	Epithelial specific antigen
ESCs	Embryonic stem cells
FACS	Fluorescence activated cell sorting
Fzd	Frizzeld (Wnt receptor)
GLI	Glioma associated oncogene homolog
GM-CFC	Granulocyte macrophage colony forming cells
GREM1	Gremlin 1
GREM2	Gremlin 2
HEIR	Heat induced epitope retrieval
HER-2	human epidermal growth factor receptor 2

HPRT1	Hypoxanthine phosphoribosyl transferase 1
IL-6	Interleukin-6
iPS	Induced pluripotent stem cells
KLF-4	Krüppel-like factor 4
Lgr5	Leucine-rich repeat containing G protein coupled receptor 5
LRCs	Long term label retaining cells
M-MLV RT	Moloney Murine Leukemia Virus Reverse T ranscriptase enzyme
mRNA	Messenger ribonucleic acid
Msi-1	Musashi-1
NOD/SCID mice	Non-obese diabetic mice with severe combined immunodeficiency disease
NSCs	Normal stem cells
NSS	Normal swine serum
NTC	Non template control
Oct4	Octamer binding transcription factor 4
ORF	Open reading frame
PBS	Phosphate buffered saline
QRT-PCR	Quantitative reverse transcriptase- polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
RT-	Reverse transriptase minus
RT	Room temperature
SCs	Stem cells
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Shh	Sonic hedgehog
STAT3	Signal transducer and activator of transcription 3
Sv	Splice variant

TCF4	T-cell factor 4
TM	Transmembrane
Wnt	Wingless- type mouse mammary tumour virus integration site

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1 Chapter 1: General introduction

1.1 Introduction

CRC is one of the most common causes of deaths worldwide. In the UK, colorectal cancer is the third leading cause of cancer mortality with around 38, 610 new cases diagnosed every year, which accounts for more than 100 cases every day (Bowel cancer statistics 2010). Surgical treatment is the mainstay of CRC treatment; however, adjuvant chemotherapy could be applied dependent on the pathologic tumour features (Morris, Maughan et al. 2007).

CRC development is a multistep process that requires years and is accompanied by accumulation of a number of genetic changes (Vogelstein, Fearon et al. 1988). These genetic changes are mirrored as pathologic transformation of normal colonic epithelium to dysplastic epithelium (i.e. adenomas) and eventually development of invasive CRC (Todaro, Francipane et al. 2010).

During the last decade research in the field of CRC has been directed towards the exploration of dysregulated pathways involved in the development of CRC. It was found that activation of the WNT signalling pathway, due to mutations that lead to either inactivation of the adenomatous polyposis coli (APC) gene or stabilization of β- catenin (Korinek, Barker et al. 1997; Morin, Sparks et al. 1997), lead to the development of adenomas (Shibata, Toyama et al. 1997). Wnt signalling

activity leads to nuclear accumulation of *β-catenin* followed by over expression of the *c-myc* and *cyclin D1* oncogenes which contribute to the malignant features of CRC (He, Sparks et al. 1998; Tetsu and McCormick 1999). Several other pathways and genes were also found to be disrupted in development of CRC such as activation of *KRAS* and inactivation of tumour suppressor *TP53* gene (Vogelstein, Fearon et al. 1988), bone morphogenetic protein pathway *(BMP)* (Howe, Bair et al. 2001), and dysregulation of the Sonic hedgehog *(Shh)* pathway (Douard, Moutereau et al. 2006).

A well described feature of tumours is differentiation towards the tissue of origin. This feature is used in diagnostic practice (to identify the origin of tumours) and shows that, despite the presence of a full set of mutations, some cells will still show some evidence of terminal differentiation (Saaf, Halbleib et al. 2007) Thus although all the mutations are necessary for CRC formation, not every cell is likely to be able to form tumours. This has led to the development of the "stem cell hypothesis" of cancer in which it is thought that just small populations of cancer stem cells are responsible for the development of most of the tumour. Nowadays, these two subjects - dysregulated pathways and cancer stem cells, are the main focus of research in the field of cancer.

In CRC, a small population of cells capable of initiating tumour growth when they were inoculated in mice (Dalerba, Dylla et al. 2007; O'Brien,

Pollett et al. 2007; Ricci-Vitiani, Lombardi et al. 2007), was recently identified. These cells were characterized by the expression of CD133 and were thought to represent the CRC stem cells.

1.2 What are stem cells?

Stem cells (SCs) are undifferentiated cells that have the ability to give rise to one or more type of cells, through *asymmetric* cell division. In this type of division, SCs give rise to two daughter cells, one cell resembles the mother cell and will have the same SC features, whilst the other daughter cell is a more specialized cell (often called transient amplifying or progenitor cells) which later on differentiates to form tissue specific cells (Lin and Schagat 1997; Dingli, Traulsen et al. 2007). Stem cells undergo another type of division called *symmetric* division in which stem cells divide and develop into two identical daughter cells endowed with the same SC characteristics of the mother cells. This leads to replenishment of the stem cell store and prevents its exhaustion (Dingli, Traulsen et al. 2007) (figure 1-1).

Stem cells are of two types, embryonic stem cells (ESCs) and adult stem cells (ASCs). ESCs are pluripotent cells, meaning they are able to differentiate and give rise to all the three germ layers (ectoderm, endoderm and mesoderm) (Reubinoff, Pera et al. 2000), while ASCs are multipotent and can give rise to multiple cell types of the same organ but not other cell

types of different organs. However, recently, researchers have induced pluripotency in ASCs (Takahashi, Tanabe et al. 2007). ASCs remain in an undifferentiated state and retain their features by asymmetric division, and can give rise to the specific cells of the organ where they locate. Recently, ASCs were identified to participate in tissue repair after injury demonstrating their role in tissue homeostasis (Weissman 2000; Zhang, Zhang et al. 2004).

In different organs, ASCs reside in a specific microenvironment called "niches". These stem cell niches are composed of cells from the surrounding microenvironment which act as a protector that prevents differentiation stimuli and other stimuli that could affect stem cell reserves (Moore and Lemischka 2006).

Recently, stem cell biology researchers have been directed from an exploration of the mechanisms of development and maintenance of normal tissue to the implication of stem cells in tumourigenesis.

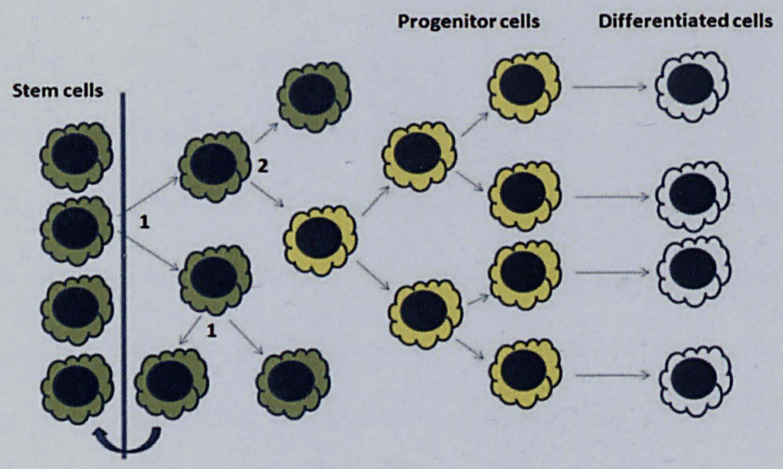


Figure 1-1: Schematic presentation of stem cell divisions.
Normal ASCs undergo two types of division, a symmetric division (number 1), and give rise to two identical daughter cells (green cells) with the same SC features which maintain the stem cell reserve. The other type of division named an asymmetric division (number 2) and leading to development of two daughter cells, one resemble the mother cells in having the SC features (green cell), and the other one is called progenitor/ transient amplifying cells (yellow cells). These progenitor cells undergo a number of replicative proliferation with terminal differentiation (White cells).

1.3 Colon stem cells

The normal human colon consists of millions of crypts. Each crypt contains about 2000 cells (Cheng, Bjerknes et al. 1984; Potten and Loeffler 1990; Potten, Kellett et al. 1992; Booth and Potten 2000) which are maintained by stem cells. Little is known about the number and the exact location of stem cells and they have been topics of debate which can be attributed to lack of specific stem cell markers (Brittan and Wright 2002). Unfortunately, the vast majority of data about colonic stem cells comes from mouse small intestine studies which differ from human colon (Bevins 2006). This difference arises from the fact that small intestine had finger-like projections called intestinal villi that are lacking in the colon. In addition,

there is a fourth cell type, Paneth cells, which reside at the bottom of the small intestinal crypt but not in the colon. This could mean that small intestine SCs endow molecular information to their progeny that is different from that endowed by colonic SCs (Boman and Huang 2008). Furthermore, the mutant mice used as models of human colon cancer develop tumours predominantly in the small intestine, not the colon (Corpet and Pierre 2003). Cheng and Leblond *et al.* presumed the unitarian hypothesis to describe the origin of epithelial cells in the gastrointestinal tract which is the slow proliferating SC (Cheng and Leblond 1974) . This theory was supported by the study of Paulus *et al.* which showed that only a single cell that escaped irradiation damage of the crypt was capable of regeneration of the damaged crypt (Paulus, Potten et al. 1992).

In the normal human colon, stem cells are present at the base of the crypt. They divide and migrate upward forming the transient amplifying/progenitor cells which occupy the lower two thirds of the crypt. Differentiated cells (which are produced from progenitor cells) occupy the upper third and migrate towards the surface where they undergo apoptosis or are extruded into the lumen (figure 1-2). The life span of epithelial cells is rapid and replaced within a week with the exception of stem cells (Potten 1998; Reya and Clevers 2005).

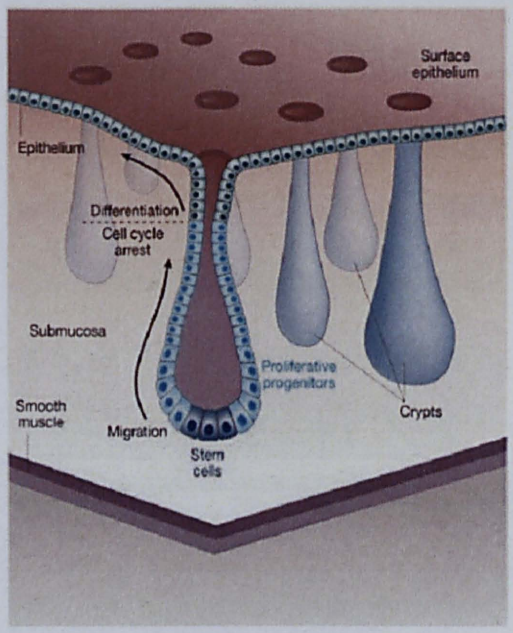


Figure 1-2: Normal colon crypt.
Normal colon crypt showing stem cells and proliferating cells occupying the lower two thirds (Reya and Clevers 2005)

1.4 Colon stem cell niches

In the colon, stem cells reside at the crypt base in a microenvironment that maintains their stemness features and which is named the "stem cell niche". The colonic stem cell niche is located at the base of the crypt and is formed by intestinal subepithelial myofibroblasts (also known as pericryptal myofibroblasts) that surrounds the stem cells (Powell, Mifflin et al. 1999). Pericryptal myofibroblasts are involved in various functions such as tissue repair, organogenesis and extracellular matrix metabolism (McKaig, Makh et al. 1999; Powell, Mifflin et al. 1999; Okuno, Andoh et al. 2002). Within the niche, several signalling pathways influence stem cell self-renewal and differentiation such as Wnt signalling pathway, Notch, and BMP pathways (He, Zhang et al. 2004; Andoh, Bamba et al. 2005).

Growing evidence has indicated that Wnt signalling has a discrete function within the intestinal crypts, with particular emphasis on progenitor and stem cells. In mouse models, deletion of T-cell factor 4 (TCF4) in the intestine leads to absence of the progenitor cells and the stem cells compartment of the crypt (Korinek, Barker et al. 1998). Similarly, ectopic expression of Dickkopf-1 (DKK1); a Wnt signalling inhibitor, leads to architectural disruption of the villi of the intestine and colon with complete loss of the crypt. (Pinto, Gregorieff et al. 2003; Kuhnert, Davis et al. 2004). Wnt signalling via nuclear expression of β-catenin stimulates the expression of ephrin type B receptor tyrosine kinases and their ligands which are involved in the epithelial cell proliferation and migration along the crypt villus axis (Batlle, Henderson et al. 2002). Although there is growing evidence suggesting the role of Wnt signalling in the maintenance of the stem cell niche, until recently the source of Wnt signalling was unclear. Recently, it was found that Wnt mRNA was expressed mainly in the pericryptal myofibroblasts supporting the role of intestinal myofibroblasts in the maintenance of stem cells. Moreover, Frizzled (Fzd) mRNA was expressed in both intestinal myofibroblasts and in the crypt epithelium suggesting autocrine and paracrine function of the Wnt ligand (Andoh, Bamba et al. 2005). Previous reports showed that *C-MYC* (myelocytomatosis oncogene) is one of the target genes of Wnt signalling (He, Sparks et al. 1998). It was found that c-myc expression inhibits $p21^{WAFI/CIP1}$ (Mitchell and El-Deiry 1999), which has been previously shown to be expressed by differentiated epithelial cells (El-Deiry, Tokino et al.

1995). A study has shown that Wnt activation stimulates c-myc expression which in turn represses $p21^{WAFI/CIP1}$ allowing cellular proliferation and suppression of differentiation (van de Wetering, Sancho et al. 2002). All these data support the enrolment of Wnt signalling pathway in the maintenance of stem cell niche and inhibition of differentiation at the crypt base.

BMP is another pathway involved in the intestinal stem cell self-renewal and differentiation (Crosnier, Stamataki et al. 2006). BMPs are members of transforming growth factor β superfamily. Kosiniski *et al.* noted that BMP members are differentially expressed along the crypt axis of the colon. BMP1, BMP2, BMP5, BMP7, SMAD7, and BMPR2 were highly expressed in the colon top; whilst, BMP inhibitors such as gremlin1 (GREM1), gremlin 2 (GREM2) and chordin-like 1 (CHRDL1) were enhanced at the crypt base (Kosinski, Li et al. 2007). It was found that GREM1, GREM2 and CHRDL1 originate from the intestinal myofibroblasts and smooth muscle that are located at the crypt base. Furthermore, GREM1 activates Wnt signalling confirming the speculation that these BMP antagonists are involved in the stem cell niche control through activation of Wnt signalling and inhibition of differentiation of the epithelial cells at the crypt base (Kosinski, Li et al. 2007). In addition, He *et al.* demonstrated that BMP inhibited the intestinal stem cells self-renewal through inhibiting activation of β-catenin, thus balancing the role of Wnt- β catenin in stem cell self-renewal (He, Zhang et al. 2004).

Apart from the Wnt and BMP pathways, the notch pathway is also involved in the regulation of stem cell niche (Crosnier, Stamataki et al. 2006). Several molecules of the Notch pathway are expressed in intestinal crypt suggesting a role of Notch in determining cell fate decision and differentiation (Sander and Powell 2004). Kosiniski *et al.* found that NOTCH1, NOTCH2 and NOTCH3 are highly expressed at the crypt base (Kosinski, Li et al. 2007). Deletion of Hes-1 (hairy and enhancer split 1), whose transcription is regulated by Notch activity (Jarriault, Brou et al. 1995), resulted in production of excessive numbers of secretory cells such as goblet, enteroendocrine and paneth cells (Jensen, Pedersen et al. 2000), supporting the role of Notch signalling in selecting cell fate and differentiation of the crypt cells.

Though these data demonstrated the importance of stem cell niche in maintaining a balance between stem cell self-renewal and differentiation through epithelial-mesenchymal interactions, the crypt villus structure could be generated from a single SC in the absence of mesenchymal niche. Sato *et al.* demonstrated that a single SC, when maintained in a long-term culture conditions, initiates organoids that contains all the cellular architecture present in the crypt villus unit of the adult mammals (Sato, Vries et al. 2009). Figure (1-3) demonstrates different signalling pathway in stem cell niche.

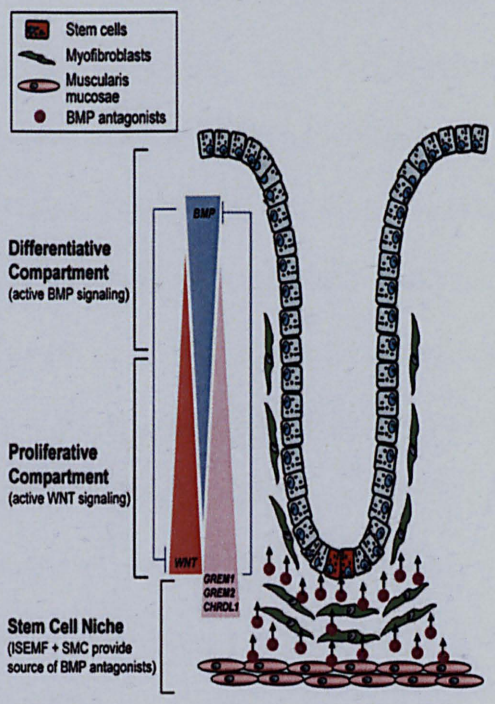


Figure 1-3: signalling pathways in stem cell niche

A diagram showing different signalling pathways that control stem cell self-renewal in the stem cell niche a model proposed by Kosiniski *et al.* (Kosinski, Li et al. 2007). ISEMFs surround the base of the crypt and exert its effect on stem cells through paracrine secretion of certain molecules such as BMP inhibitors (GREM1, GREM2, CHRDL1) which stimulates Wnt activity. These inhibitors are high at the bottom of the crypt and lower at the upper part. Wnt activity was higher at the base of the crypt and lowered with direction up toward differentiated cells. At the meanwhile, BMP activity is higher in the upper region to affect the cellular differentiation of the intestinal cells.

1.5 Molecular identification of normal colon SCs

Historically, DNA labelling using bromodeoxyuridine (Brdu) was used to identify the stem cells, based on the assumption that long term label retaining cells (LRCs) could be identified as either cells constituting or enriched with stem cells in many tissues including colon. These LRCs have the ability to retain DNA labeling as they have a slower cell cycle than the more rapidly dividing transit cells (Kim, Cheung et al. 2004). Nowadays, this method has been replaced by the identification of cell surface markers on the stem cells and these were considered as putative stem cell markers.

Musashi-1 (Msi-1), an RNA-binding protein was the first molecule identified as a putative marker for normal human colon SCs. The early information regarding its function demonstrated that it was essential for the preservation of neural stem cells (Nakamura, Okano et al. 1994; Okano, Kawahara et al. 2005). Then, Msi-1 expression was detected in the human colon crypt and mouse intestinal crypt (Nishimura, Wakabayashi et al. 2003; Potten, Booth et al. 2003). In human colon crypts, Nishimura *et al.*, by immunohistochemical analysis of Msi-1, showed that positivity of Msi-1 located at the lower part of the crypt at cell positions 1-10 that could correspond to the area of stem cells as well as early transient amplifying cells (Nishimura, Wakabayashi et al. 2003).

In 2002, Fujimoto *et al.* demonstrated that the integrin β1 subunit is a surface marker for the proliferative zone of the human colon crypt which include SCs and progenitor cells (Fujimoto, Beauchamp et al. 2002). Immunofluorescence study revealed that β1 integrin expression was higher in the lower part of the crypt than the remainder of the crypt. Employing clonogenic assays after separating crypt cells based on their expression of β1 integrin, revealed a cell population with enhanced ability to colony formation (Fujimoto, Beauchamp et al. 2002). Nonetheless, further experiments were required to confirm the specificity of β1 integrin as a putative marker for colon stem / progenitor cells.

Most recently, leucine-rich repeat containing G protein coupled receptor 5 (lgr5); a Wnt target, was identified to be a marker of normal colon SCs (Barker, van Es et al. 2007). Lgr5 was identified in the crypt base, marking active cycling cells and contradicting the concept that SCs are slow cycling cells. Moreover, a more recent study revealed that a single Lgr5+ cell can produce a structure similar to crypts and contain all the cellular architecture of the crypt when grown *in vitro* in cultured conditions for long term (Sato, Vries et al. 2009). In brief, Lgr5 could be considered as a promising marker for colon SCs.

1.6 Cancer stem cell hypothesis

For decades, the stochastic model for tumour development was held, which presumed that all tumour cells are equally able to form tumour growth. But, recently, the cancer stem cell theory has emerged and suggested that the tumourigenicity was limited to a small population of cells within the tumour, called cancer stem cells (CSCs). These CSCs are responsible for tumour initiation and maintain tumour growth (Wang and Dick 2005; Dalerba, Cho et al. 2007). Due to the heterogeneity of the tumour, CSCs were proposed to have stem-cell like features such as self-renewal and differentiation abilities. The efficacy of CSCs to initiate tumors was demonstrated by using xenografting models which is good *in vivo* evidence for existence of these cells (Bonnet and Dick 1997). Many cell surface markers were identified to isolate CSCs.

1.7 Molecular identification of cancer stem cells (CSCs)

CSCs were first identified in acute myeloid leukemia (AML) (Bonnet and Dick 1997). Bonnet and Dick revealed that a small population of AML cells, when injected into non-obese diabetic mice with severe combined immunodeficiency disease (NOD/SCID) mice, produced leukemic blasts. Moreover, these cells had a cell surface phenotype of being $CD34^+$ $CD38^-$ similar to that of normal haematopoietic stem cells (Bonnet and Dick 1997). Later on, research has surged towards identification of CSCs in other tumour types based on expression of specific cell surface markers, and the ability to self-renewal and differentiation.

In breast cancer, as few as 200 cells of a cell population with the cell surface phenotype of $CD44^+CD24^{-/Low}$ were capable of inducing tumours when implanted into NOD/SCID mice (Al-Hajj, Wicha et al. 2003). Moreover, the same cells showed the ability to grow as non adherent mammospheres *in vitro* (Ponti, Costa et al. 2005), in concordance with findings obtained with normal stem and progenitor cells of the breast (Dontu, Abdallah et al. 2003).

In pancreatic cancer, Li *et al.* initially identified that $CD44^+CD24^+ESA^+$ were tumourigenic as 100 cells with this cell surface phenotype were capable of initiating tumours that recapitulate the primary tumour from which they originate, indicating its characterization as CSCs in pancreatic

tumours (Li, Heidt et al. 2007). However, Herman *et al.* indicated that pancreatic CSCs were identified by the use of CD133 marker (Hermann, Huber et al. 2007).

Besides breast and pancreas, prostate CSCs were identified by the expression of CD44, CD133, and α2β1 integrins. It was found that cells of $CD44^{+}$ $\alpha2\beta1^{high}CD133^{+}$ have the ability to self renew and to regenerate tumours phenotypically similar to the original tumour (Collins, Berry et al. 2005). Furthermore, in brain tumours, Singh *et al.* demonstrated that a small number (100 cells) of CD133+ cells generated tumors in NOD/SCID mice that recapitulate the original tumour, while 10^5 of CD133- populations failed to generate tumours (Singh, Hawkins et al. 2004). In another study, CD133+ and Nestin+ cells isolated from brain tumors were able to proliferate and produce tumour spheres *in vitro* (Yi, Zhou et al. 2007)

In colon, several cell surface markers were proposed as putative markers for colon CSCs such as CD133, CD24, CD44, CD166, ESA, and aldehyde dehydrogenase 1 (ALDH1) (Dalerba, Dylla et al. 2007; O'Brien, Pollett et al. 2007; Ricci-Vitiani, Lombardi et al. 2007; Huang, Hynes et al. 2009). The first existence of colon CSCs was identified by O'Brien *et al.* where they demonstrated that only a small population of human colon cancers, isolated based on the expression of CD133, were able to induce tumour when implanted in NOD/SCID mice (O'Brien, Pollett et al. 2007). Moreover, CD133+ cells were able to grow *in vitro* as undifferentiated spheres in long

term culture (Ricci-Vitiani, Lombardi et al. 2007). Epithelial cell adhesion molecule (EpCAM), also known as ESA, and CD44 were used for identification of colon CSCs. A study reported that injection of 200 to 500 cells of EpCAM ^{high}CD44^{+} in NOD/SCID mice resulted in tumour formation, whilst injection of 10^4 of EpCAM ^{low}CD44^{-} cells did not produce any tumour; moreover, in the same study it was found that CD166 (a mesenchymal stem cell marker) could be used as a CSC marker in conjunction with EpCAM and CD44 (Dalerba, Dylla et al. 2007). Cells that express CD133 were more clonogenic than negative counterparts, and when the clonogenicity was tested based on co-expression of CD44, CD166, CD29 with CD133, no increase in the clonogenicity was reported. However, coexpression of CD133 with CD24 increased the clonogenicity of CD133 expressing cells. Moreover, CD133 and CD24 expression was lost upon differentiation followed by CD44, indicating that CD133 and CD24 could be used in conjunction for isolation of colon CSCs (Vermeulen, Todaro et al. 2008).

Recently, ALDH1 was proposed to be a marker for normal stem cells and colon CSCs. A study showed that ALDH1 was expressed at the bottom of the colon crypt where cells are also positive for CD133 and CD44. Isolation of human colon cancer cells based on the expression of ALDH1 and injection into NOD/SCID initiate tumours while the negative counterparts failed to do so. Moreover, isolation of cells using ALDH1 with another

marker such as CD44 or CD133 leads to a minor increase in the tumour initiating ability (Huang, Hynes et al. 2009).

So far it is thought that no single molecular signature can be used to identify CSCs, but likely, a combination of cell surface markers that are expressed or repressed is preferable. Within the same context, evidence suggesting the use of CD133, the most widely used marker, either alone or in combination with other markers to isolate CSCs in many solid tumours has grown. Some of the markers used for identification of cancer stem cells are summarised in (Table 1-1)

Table 1-1: list of some cancer stem cell markers.

Tumour type	Marker	Reference
Breast	$CD44^{+}CD24^{-/Low}$	(Al-Hajj, Wicha et al. 2003)
Pancreas	$CD44^{+}CD24^{+}ESA^{+}$	(Li, Heidt et al. 2007)
Pancreas	CD133	(Hermann, Huber et al. 2007)
Brain	CD133	(Singh, Hawkins et al. 2004)
Brain	$CD133^{+}Nestin^{+}$	(Yi, Zhou et al. 2007)
Colon	CD133	(O'Brien, Pollett et al. 2007) (Ricci-Vitiani, Lombardi et al. 2007)
Colon	$EpCAM^{high}CD44^{+}$	(Dalerba, Dylla et al. 2007)
Colon	$CD133^{+}CD24^{+}$	(Vermeulen, Todaro et al. 2008)
Colon	ALDH1	(Huang, Hynes et al. 2009)
AML	$CD34^{+}$ $CD38^{-}$	(Bonnet and Dick 1997)
Prostate	$CD44^{+}$ $\alpha2\beta1^{high}CD133^{+}$	(Collins, Berry et al. 2005)

1.8 CD133 (prominin-1)

1.8.1 Identification of CD133

CD133, also known as prominin-1, is a pentaspan transmembrane glycoprotein of the prominin family (Miraglia, Godfrey et al. 1997). In mouse, prominin-1 was located in the microvilli and plasma membrane protrusions of the apical surface of the epithelium (Weigmann, Corbeil et al. 1997). AC133 antibody was originally used to identify the cell surface CD133 antigen (Yin, Miraglia et al. 1997), which recognizes a glycosylation dependent epitope. Yin *et al.* reported that AC133 antigen was restricted to $CD34^+$ cells of fetal liver, bone marrow, adult bone marrow, and cord blood suggesting that it could function as a marker of haematopoietic progenitor cells (Yin, Miraglia et al. 1997). In the same study, another antibody named AC141 has been generated against CD133 antigen and also recognizes a glycosylated epitope that is different from the previous epitope recognized by AC133 antibody (Yin, Miraglia et al. 1997).

CD133 antigen was detected in the stem and progenitor cells of different organs (Yin, Miraglia et al. 1997; Bhatia 2001), and in neoplastic cells (Corbeil, Roper et al. 2000; Florek, Haase et al. 2005), In Caco2, a human colon cancer cell line, CD133 antigen was downregulated upon cellular differentiation whilst the CD133 mRNA (messenger ribonucleic acid) was slightly increased (Corbeil, Roper et al. 2000). In the same study, CD133 mRNA was detected in many tissues including kidney, brain, liver, heart, lung, placenta, colon and small intestine which contradict the absence of

AC133 antigen (Miraglia, Godfrey et al. 1997). A plausible explanation for this contradiction is that CD133 antigen may be inhibited from translation or the fact that antibodies used, AC133 and AC144, detect a glycosylated epitope and the glycosylation process varies during cellular differentiation and malignant transformation (Corbeil, Roper et al. 2000). In support for the latter speculation, Florek *et al.* generated an antibody named αhE2 which could recognize glycosylated and non-glycosylated CD133 antigen. This led to detection of CD133 antigen in Caco2 even after differentiation. Moreover, CD133 immunoreactivity was detected in adult kidney and mammary glands (which showed higher CD133 mRNA) using αhE2, but no AC133 immunoreactivity (Florek, Haase et al. 2005). A more recent finding which is in line with these data reported that the AC133 glycosylated epitope could not be detected with differentiation of CSCs but CD133 could be detected in the differentiated cells (Kemper, Sprick et al. 2010). Therefore, it could be concluded that the glycosylation epitopes recognized by AC133 and AC141 antibodies were restricted to stem and progenitor cells, and lost in adult tissue and upon differentiation of cells.

1.8.2 The molecular profile of *CD133* gene

CD133 gene maps to the short arm of chromosome 4 (4p15.32) . The *CD133* gene consists initially of 27 exons. A 5 prime untranslated region (5'UTR) and the start codon are contained within exon 1, while the stop codon is in exon 26. Exons 1-26 range in size from 27 to 217 base pairs

(bp), while exon 27 is large containing at least 1143 bp (Yu, Flint et al. 2002). The complementary Deoxy ribonucleic acid (cDNA) that encodes CD133 protein consist of 3794 nucleotide, with a 37 nucleotides 5 'UTR, and an 1159 nucleotide 3' UTR, and contains a long open reading frame of 2596 nucleotides that forms a protein of 865 amino acids (AA) (Miraglia, Godfrey et al. 1997). Later on, Shmelkov *et al.* reported that CD133 consisted of 28 exons and the transcription of CD133 gene is controlled by 5 alternative promoters and 9 distinct 5' UTR exons results in the formation of seven alternatively spliced isoforms (Shmelkov, Jun et al. 2004).

1.8.3 Structure of CD133 protein

The CD133 protein is a 5 transmembrane (TM) domain glycoprotein. The protein consists of 865 AA with a molecular weight of ~ 97 kd (unglycosylated), and of ~ 120 kd (when glycosylated). There is an extracellular N-terminus (104 AA) to which is attached a signal peptide that is cleaved when the protein reaches the plasma membrane, two small intracellular loops (30 AA each), two extracellular domains(258 and 279 AA) at which 8 N-linked glycosylation site are present, and ends with a 59 AA carboxy-terminal tail (Figure1-4) (Miraglia, Godfrey et al. 1997).

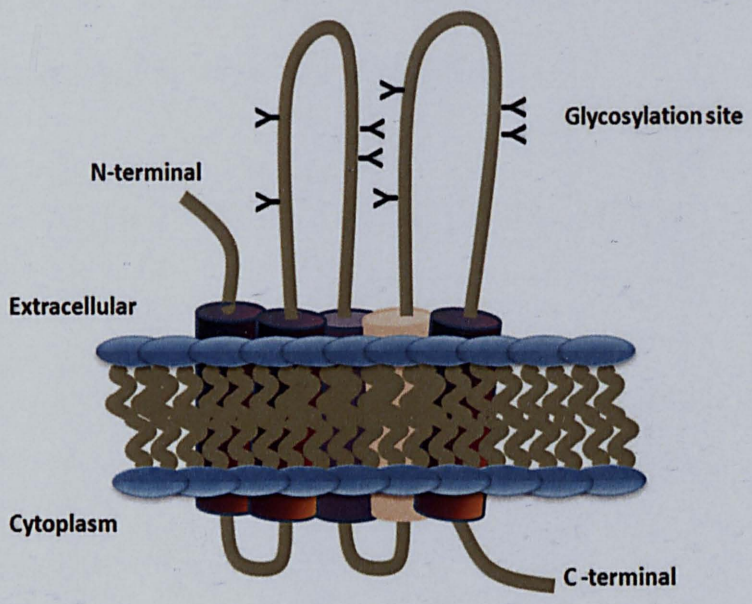


Figure 1-4: A schematic presentation for the expected structure of CD133.
A structural model of CD133 as proposed by Miraglia *et al.* (Miraglia, Godfrey et al. 1997), showing that CD133 composed of 5 TM domains, extracellular N-terminus end, two intracellular loops, two extracellular loops containing 8 N-linked glycosylation sites, and a C-terminus end.

1.8.4 CD133 splice variants

Initially, Miraglia *et al.* reported that CD133 gene is composed of 27 exons (Miraglia, Godfrey et al. 1997) and was referred as AC133-1. Later on, another study showed the presence of a splice variant of CD133 which lacks exon 3 (consisting of 27 nucleotides, 9 AA) and this splicing out of this exon does not affect the degree of glycosylation (Yu, Flint et al. 2002). In 2004, Shmelkov *et al.* discovered an extra exon in the 5'UTR area of the *CD133* gene upstream to the previous exon 1 (which become exon 2), and contains 5 alternative promoter areas which can result in 7 alternatively spliced variants of CD133 transcripts which are expressed in a tissue

dependent manner and methylation plays a role in their regulation (Figure1-5)(Shmelkov, Jun et al. 2004).

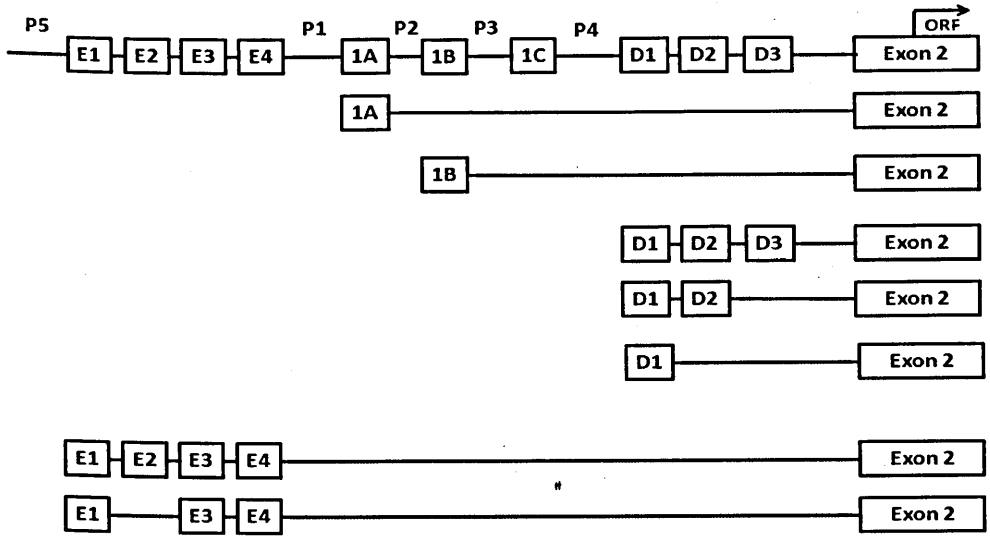


Figure 1-5: 5'UTR region and different alternative promoter sites of CD133.
A schematic presentation of 5'UTR region of CD133 showing different promoter areas based on Shemelkov *et al.* study (Shmelkov, Jun et al. 2004). 5' RACE (rapid amplification of cDNA ends) analysis revealed the presence of several exons in the 5'UTR region of CD133 such as exon 1A, 1B, which clustered as D1, D2 and D3, and exon 1E which clustered as E1, E2, E3 and E4. These exons are alternative spliced to exon 2. P1, P2, P3, P4, and P5 represent possible alternative promoters of CD133 gene that their activity gives rise to alternative spliced variants of CD133 mRNA.

Although this study showed the differential expression of alternative splice variants by alternative promoter activity in the 5'UTR, it does not show any splice variants affecting the ORF (open reading frame). Fargeas et al reported alternative splice variants resulting in differential transcript variants (Figure 1-6, and Table 1-2) (Fargeas, Huttner et al. 2007). Currently, the biological value of these variants is unclear.

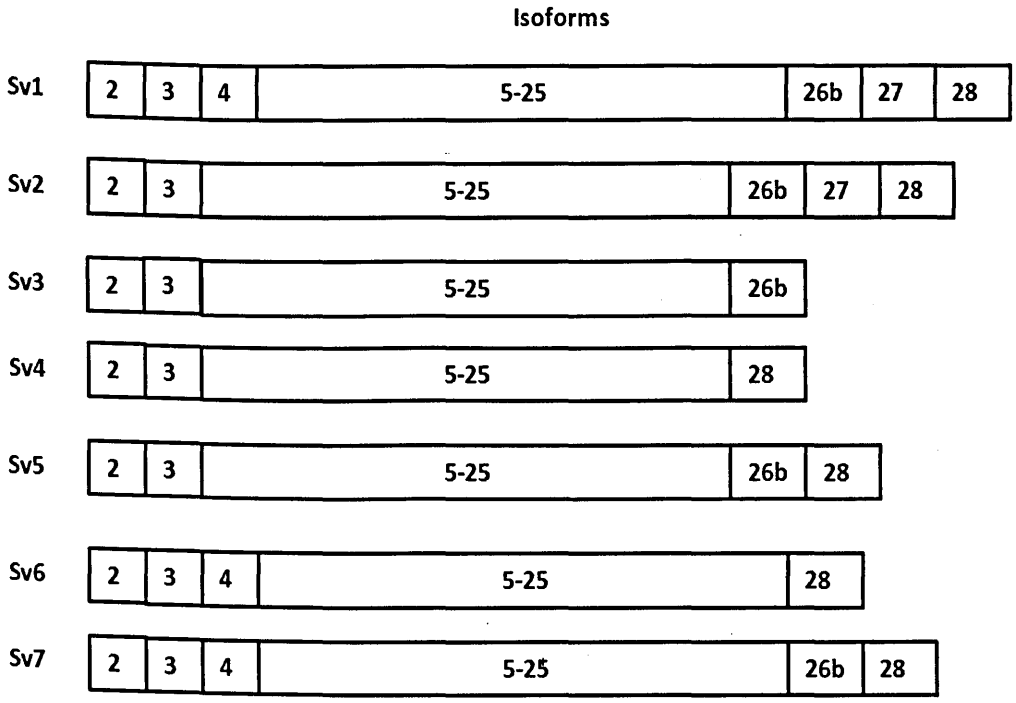


Figure 1-6: CD133 Splice variants
A schematic presentation illustrates seven different splice variants of CD133. These Svs are variable in their expression of exon 4, 26b, and/or 27.

Table 1-2: CD133 splice variants (Svs)
This table showed the CD133 Svs, their CBI nomenclature, AA, and accession number in NCBI Database (Fargeas, Huttner et al. 2007)

Splice variants	NCBI nomenclature	AA	Accession Number
Sv1	Isoform 1	865	NM_006017
Sv2	Isoform 2	856	NM_001145847
Sv3	Isoform 3	830	AY449689
Sv4	Isoform 4	825	AY449690
Sv5	Isoform 5	833	AY449691
Sv6	Isoform 6	834	AY449692
Sv7	Isoform 7	842	AY449693

1.8.5 Function of CD133

The exact function of CD133 is still elusive. Growing evidence suggests a role in the regulation of plasma membrane topography due to its localization in the plasma membrane protrusions (Corbeil, Roper et al. 2001). Possibly, CD133 could be involved in signal transduction, as it is known that CD133 has a cholesterol binding lipid raft microdomain (Roper, Corbeil et al. 2000), and lipid rafts often represent areas in the plasma membrane that are involved in signal transduction (Simons and Toomre 2000). It has been reported that membrane particles containing prominin-1 are released into a number of body fluids such as neural tube fluid, seminal fluid, urine and lacrimal fluid (Marzesco, Janich et al. 2005). Given that prominin-1 is a stem cell marker (Weigmann, Corbeil et al. 1997; Yin, Miraglia et al. 1997), it was postulated that its release in membrane particles could be involved in the down-regulation of stem cell properties or their differentiation. Supporting this postulation, the prominin-1 containing membrane particles were released upon differentiation of Caco2 cells (Marzesco, Janich et al. 2005).

Interestingly, the migrating $CD34^+$ haematopoietic stem cells acquire a polarized morphology, and during this process some molecules are redistributed including CD133, which is redistributed into the uropod, thus suggesting a role for CD133 in cell migration (Giebel, Corbeil et al. 2004). Uropod is a plasma membrane protrusion in which cytoskeletal proteins, signalling and adhesions receptors are present, and these might be

involved in many functions such as cell migration, intercellular adhesion (Sanchez-Madrid and Serrador 2009)

Genetic analysis of family members with retinal degeneration revealed that deletion of a single nucleotide from CD133 gene leads to a frame shift mutation. This mutation resulted in formation of a truncated protein that lost half of the second extracellular loop, the last transmembrane domain, and the cytoplasmic C-terminal domain. In the same study, mouse mutant PROM that mimics human PROM does not reach the cell surface and immunohistochemical staining of mouse retina showed that CD133 can be found on the membranes of rod photoreceptors implying a role in photoreceptor disk morphogenesis. (Maw, Corbeil et al. 2000) . Although many studies have proposed a biological role for CD133, till now its exact function is unclear.

1.8.6 CD133 and normal stem cells (NSCs)

Since its original description as a marker for hematopoietic stem/progenitor cells (Yin, Miraglia et al. 1997), much attention has been paid to evaluating the validity of CD133 as a putative stem cell marker in many tissue types such as hematopoietic system, renal, brain, endothelial cells and even in the field of regenerative medicine. CD133 was identified as a marker for hematopoietic stem and progenitor cells due to its selective expression in a subset of $CD34^+$ fetal, cord and bone marrow cells (Yin, Miraglia et al. 1997). Similarly, it has been stated that CD34+/AC133+ cells have 10-100

fold higher capability to produce granulocyte macrophage colony forming cells (GM-CFC) compared to CD34+/AC133- cells. In addition, human CD34+/AC133+ cells have higher engraftment rate with reconstitution of bone marrow of sub-lethaly irradiated NOD/SCID mice compared to CD34+/AC133- counterparts (de Wynter, Buck et al. 1998) . Moreover, Gallacher *et al.* demonstrated that hematopoietic AC133+/CD34-/Lin- cells were 400 fold more clonogenic than AC133-/CD34-/Lin- counterparts, and they were able to produce CD34+ cells (Gallacher, Murdoch et al. 2000). Lang *et al.* showed that injection of either CD133+ or CD34+ cells in leukemic patients produce immune and platelet cells recovery (Lang, Bader et al. 2004). Within the same context, Bitan *et al.* reported that successful mismatched transplantation of CD133+ cells in five high risk leukemic patients recovered the neutrophils and platelet cells with avoidance of lethal acute graft-vs-host disease (Bitan, Shapira et al. 2005).

Apart from the hematopoietic system, CD133 can be used as a putative marker for stem cells in several other tissues. A long term culture model has been developed allowing the propagation of neural stem/progenitor cells, and these neural cells can grow as either monolayers or as clusters known as neurospheres (Ray, Peterson et al. 1993). Using the neurosphere assay, it was revealed that sorted CD133+ cells from human fetal brain tissue can grow as neurospheres; in addition, a single cell from these neurospheres could differentiate into neurone and astrocytes when grown in conditioned medium. Moreover, injection of human CD133+ cells

into the brain of NOD/SCID mice showed engraftment, proliferation and migration of these cells in the whole brain tissue (Uchida, Buck et al. 2000). Likewise, neural stem cells from murine cerebellum expressing CD133 and negative for other neural markers, were capable of forming neurospheres; even if they were cultured as single cells, in contrast to CD133- cells. Also, they were able to differentiate into neurone, astrocytes, and oligodendrocytes both *in vitro* and *in vivo* (Lee, Kessler et al. 2005).

CD133 expressing cells isolated from normal adult human kidney have been shown to be capable of self renewal and differentiation *in vitro* and *in vivo* into renal epithelium (Bussolati, Bruno et al. 2005). The renal origin of differentiated cells was confirmed by the expression of cytokeratin, E-cadherin, and markers of fully differentiated renal epithelium such as alkaline phosphatase and amine peptidase which are normally expressed by the epithelium of the proximal tubules (Mentzel, Dijkman et al. 1996).

Interestingly enough, the ability of hematopoietic CD133+ cells to engraft and differentiate into non-hematopoietic cells was directed to the field of tissue regeneration and disease improvement. Torrent *et al.* reported the ability of the isolated human AC133+ blood cells to self renew and differentiate into a myogenic lineage under certain culture conditions *in vitro*, and to be recruited into muscle fibers when transplanted into SCID/mdx mice and ameliorate the muscular dystrophy disease (Torrente, Belicchi et al. 2004). In a like manner, injection of autologous, CD133+

bone marrow stem cells into the liver was associated with enhanced physiologic regeneration of liver cells (am Esch, Knoefel et al. 2005). In brief, these studies stated that CD133 expression marks more primitive cells.

1.8.7 CD133 expression in human tumours

The CSC hypothesis states that tumourigenic potential is restricted to a small population of cells within the tumour which have the ability to self-renew, differentiate into multiple lineages, and regenerate tumour recapitulating the original one. These cells were named as CSCs or tumour initiating cells and postulated to originate from normal stem cells, as they are the most likely cells to initiate tumours. CD133 was identified as a putative CSC marker in various human tumours.

By using Flow cytometry analysis of cell surface expression of CD133 protein in different types of brain tumours such as medulloblastoma, oligodendroglioma, and astrocytoma it was shown that CD133+ cells were able to form neurospheres, differentiate, and form tumours *in vivo* that recapitulate the original tumours (Blazek, Foutch et al. 2007; Yi, Zhou et al. 2007). In addition these CD133+ cells were chemo- and radioresistant (Liu, Yuan et al. 2006; Blazek, Foutch et al. 2007). Similarly, in prostate cancer, the CSCs have a CD44+/α2β1high/CD133+ phenotype, were shown to be able to self-renew and differentiate (Collins, Berry et al. 2005). CD133+

cells have more proliferative and colony forming abilities, higher tumourigenic capacity *in vivo*, and are more chemoresistant than CD133- counterpart (Suetsugu, Nagaki et al. 2006; Ma, Chan et al. 2007; Yin, Li et al. 2007; Ma, Lee et al. 2008).

In pancreatic cancer, CD133+ cells were more tumourigenic *in vivo* and highly resistant to chemotherapy than CD133- cells; what is more, CD133 expression together with CXCR antigen showed higher invasive and metastatic potential (Hermann, Huber et al. 2007). Also, in anaplastic thyroid carcinoma, CD133+ cells showed higher proliferation rate, self-renewal ability, higher resistance to chemotherapy, and expression of stem cell marker such as OCT4 (Zito, Richiusa et al. 2008). Moreover, CD133+ ovarian cancer cells were characterized by higher proliferative capability and exhibited enhanced clonogenic efficiency compared to CD133- cells. Furthermore, CD133 expression was higher in ovarian cancer than normal or benign tumours, and interestingly was lower in metastatic ovarian tumours compared to the primary ovarian cancer (Ferrandina, Bonanno et al. 2008).

Recently, O'Brien *et al.* and Ricci-Vitiani et al isolated CD133+ colon cancer cells and showed that these are more tumourigenic *in vivo* than their negative counterparts. It has been shown that CD133+ cells can grow for a long time as tumour spheres and have the ability to xengraft and

induce tumours recapitulating the original one (O'Brien, Pollett et al. 2007; Ricci-Vitiani, Lombardi et al. 2007).

Using immunohistochemistry, the CD133 expression pattern has been found to be variable. Expression of CD133 can be detected at the apical surface and the luminal border of the tumour glands as reported in many tumours such as colon cancer (Horst, Kriegl et al. 2008), pancreatic cancer (Immervoll, Hoem et al. 2008), whilst cytoplasmic staining alone or in combination with the previous pattern is described in ovarian cancer (Ferrandina, Martinelli et al. 2009), and hepatocellular carcinoma (Song, Li et al. 2008). Differences of cellular localization have been seen in other proteins. For example, human epidermal growth factor receptor 2 (HER-2) by immunohistochemistry has been reported to be both membranous and cytoplasmic in colorectal cancer (Pavlakis, Kountourakis et al. 2007). In this case cytoplasmic expression of Her-2 was proposed to be either a cross reacting protein or a precursor form of the mature protein. The differences in cellular localization of CD133 expression could also be explained by this or may reflect a difference in cellular function at these different sites. However, these different patterns warrant further investigations.

1.8.8 Signalling pathways in CD133+ cells

Recently, several signalling pathways have been implicated in the regulation of CD133+ cancer stem cells. For example, the

Hedgehog/Glioma-associated oncogen homolog 1 (*GLI1*) pathway was reported to be involved in the survival and tumourigenicity of glioma cell culture. In addition, blockage of Hedgehog signalling pathway in CD133+ glioma stem cells reduces the clonogenicity, and expression of some stemness related genes such as Oct4, Nanog, and Sox2 (Clement, Sanchez et al. 2007). Similar to the hedgehog pathway, blockage of the Notch pathway decreased the tumour forming ability due to depletion of the CD133+ and nestin + medulloblastoma stem cells (Fan, Matsui et al. 2006). This depletion of stem cells was found to be due to decreased cellular proliferation and increased apoptosis through reduction of phosphorylation of Signal transducer and activator of transcription 3 (STAT3) and AKT (Fan, Khaki et al. 2010).

Wnt signalling is another pathway that was reported to be involved in the control of function of CD133+ cells. It has been stated that CD133 + cord blood hematopoietic stem cells could be differentiated into non-hematopoietic lineage such as neuronal cells, astrocytes and oligodendrocytes when grown in conditioned culture media (Jang, Park et al. 2004). Furthermore, CD34+AC133+ hematopoietic stem cells have an endothelial potential with a role in neo-angiogenesis (Peichev, Naiyer et al. 2000). The exact link between the commitment of these stem/ progenitor cells and lineage differentiation is not clear. Nikolova *et al.* evaluated the effect of culturing CD133+ cells in conditioned media containing Wnt signalling molecules. It was found that Wnt5a and Wnt11 increased the

expression of CD31+ cells, whereas, Wnt3a maintain the undifferentiated blast phenotype of CD133+ cells. Moreover, Wnt5a increased the expression and nuclear localization of β- catenin in CD133+ cells indicating activation of Wnt signalling. (Nikolova, Wu et al. 2007). Nuclear localization of β- catenin regulates the expression of several genes such as c-myc (He, Sparks et al. 1998). The latter was shown to be highly expressed in CD133+ glioma stem cells and involved in the regulation of proliferation and survival of these cells (Wang, Wang et al. 2008).

The BMP pathway is also involved in the control of brain stem cells. Treatment of glioblastoma tumour with BMP4, a ligand of the transforming growth factor β family, decreases the proliferation, reduces the tumour formation ability *in vivo*, increases the marker of neuronal differentiation and decreases the size of CD133 expressing cells (Piccirillo, Reynolds et al. 2006). Furthermore, in CD133+ cells of hepatocellular carcinoma the chemotherapy resistance was associated with activation of AKT/PKB pathway and survival molecule such as BCL-2 (Ma, Lee et al. 2008). Figure 1-7, simply represent the possible pathways regulating CD133 expressing cells. These studies demonstrated the involvement of these pathways in CD133+ cancer cells but the direct involvement of CD133 in these pathway remains elusive.

Uncovering the role CD133 plays in cancer biology, and the mechanisms regulating its expression and function are pivotal in considering CD133 as a potential target therapy.

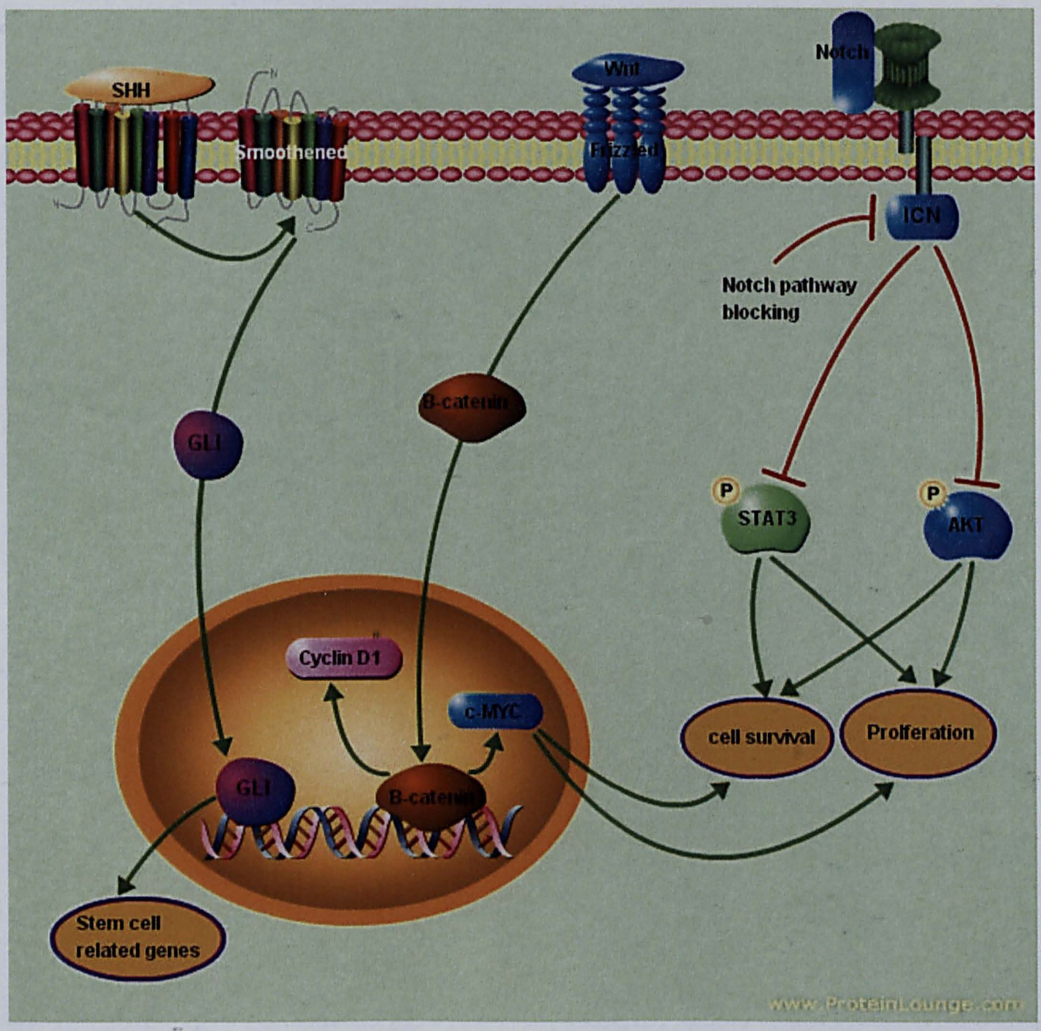


Figure 1-7: Signalling pathway regulating the function of CD133+ cells.
A simple schematic presentation of signalling pathway involved in the regulation of CD133+ cancer stem cells such as the Notch, Wnt, and Hedgehog pathways. Fan *et al.* found that blockage of Notch pathway decreases cellular proliferation and increased apoptosis through decrease phosphorylation of STAT3 and AKT not the total protein (Fan, Khaki et al. 2010). Hedgehog pathway increased survival of glioma cells through activation of GLI (a transcription factor) and its inhibition lead to reduced clonogenicity and decreased stem cell related genes such Oct4, Nanog and Sox2 (Clement, Sanchez et al. 2007). Wnt signalling stimulated nuclear localization of β-catenin which increased level of c-MYC that was increased in CD133+ glioma cells and was found to be involved in proliferation and increased survival

1.8.9 Hypothesis and aims

Considerable knowledge about characteristics of CD133 expressing cells in many tumours such as brain tumours, and hepatocellular carcinoma has been acquired. However, at the beginning of this project, two earlier studies by O'brien *et al.* and Ricci-Vitiani *et al.* revealed that CD133 could be used as a marker for tumor initiating cells in colon (O'Brien, Pollett et al. 2007; Ricci-Vitiani, Lombardi et al. 2007). In this case we hypothesized that, if CD133 marks CSC populations that are responsible for tumour growth and resistance to treatment, then CD133 expressing cells will have increased tumourigenicity and resistance to apoptosis and association with patient survival.

To test this hypothesis, the aims of the current study, which will form the groundwork of subsequent chapters as indicated, were to:

(1) Evaluate the expression of CD133 in colorectal cancer clinical samples and find its correlation with the different clinicopathological variables and patients clinical outcome (Chapter 3).

(2) Screening of different cell lines for CD133 expression as a preliminary step for gene functional studies (Chapter 4)

(3) Study the biological characteristics of CD133 expressing cells through study of functions such as proliferation, migration, colony forming, and staurosporine induced apoptosis (Chapter 5).

(4) Discover any of the upstream or downstream target molecules that could reflect the mechanism of action of CD133 (Chapter 6).

(5) Assess the expression of stemness related genes in CD133+ colorectal cancer cells (Chapter 7).

2 Chapter 2: Material and methods

2.1 Immunohistochemistry

2.1.1 Patients and specimens

This study encompassed use of two tissue Micro Array (TMA) sets which were studied after ethical approval by the Nottingham Local Research Ethics Committee under REC reference numbers Q1020402, and 05/Q2402/72. The first TMA set, kindly supplied by Prof. Lindy Durrant (Division of Oncology, University of Nottingham, UK), consisted of 449 cases of primary operable colorectal cancer patients who underwent elective resection at the Nottingham University Hospital between Januray 1994 and December 2000. All cases are represented as cores on a tissue micro array (TMA) construct prepared as previously described (Kononen, Bubendorf et al. 1998). Clinical and pathological data including histological type, histological grade, tumor site, TNM stage, and the presence or absence of vascular invasion were prospectively collected. The follow-up was considered from the date of primary tumor resection, and all surviving cases were censored for data analysis at December 2003. Clinical characteristics of patients are summarized in table 2-1.

The second TMA set used in this study was independent of the first TMA series; it comprised 45 samples from primary colorectal adenocarcinoma and their corresponding liver metastases, diagnosed between 1993 and 2009 and entered into the Nottingham CRC Series .The cases were arrayed onto TMA set as previously described (Kononen, Bubendorf et al.

1998) by Dr. Wakkas Fadhil (PhD student, Division of Pathology, School of Molecular Medical Sciences, University of Nottingham). Clinical characteristics of patients are summarized in table 2-2.

Table 2-1:Clinicopathological features of patients cohort *n=449*

Age (years)	
Median	72
Range	57-89
Sex	
Male	257(57%)
Female	192 (43%)
Status	
Alive	167 (37%)
Dead (colorectal cancer related deaths)	220 (49%)
Dead (unrelated causes)	60 (13%)
Unknown*	2
Histological type	
Adenocarcinoma	382 (85%)
Mucinous adenocarcinoma	49 (11%)
Columnar adenocarcinoma	4 (1%)
Signet ring mucinous adenocarcinoma	6 (1%)
Unknown	8 (2%)
Histological grade	
Well differentiated	28(6%)
Moderately differentiated	345 (77%)
Poorly differentiated	67 (15%)
Unknown	9 (2%)
Tumour site	
Colon	230 (52%)
Rectal	177 (39%)
Unknown	42 (9%)
TNM stage	
0 (T_{is})	3 (1%)
1	67 (15%)
2	172 (38%)
3	149 (33%)
4	51 (11%)
Unknown	7 (2%)
Extramural vascular invasion	
Negative	219 (49%)
Positive	121 (27%)
Unknown	109(24%)

*Unknown: means either the data is not registered or no information found in the database

Table 2-2: Clinicopathological features of patients cohort n= 45

Clinicopathological variables	Frequency n (%)
Age (years)	
Median	70
Range	43-88
Sex	
Male	16 (35.6)
Female	29 (64.4)
T stage	
T1	1 (2.3)
T2	2 (4.6)
T3	23 (53.4)
T4	17 (39.7)
Vascular invasion	
Negative	14 (32.6)
Positive	29 (67.4)

2.1.2 Immunohistochemical staining with CD133

For immunohistochemistry, 4µm thick formalin-fixed paraffin - embedded tissue sections were cut and mounted onto glass slides pre-coated with 3-aminopropyltriethoxysilane (APES). Sections were dewaxed by immersing in xylene (twice; 5 minutes each), and rehydrated through graded alcohol (100, 90, and 70%; 10 seconds each). For antigen epitope retrieval, heat induced epitope retrieval (HEIR) method was employed, where sections were boiled in citrate buffer (0.1M concentration at PH 6.0) using a microwave for 20 minutes. Endogenous hydrogen peroxidase activity was blocked by applying 0.3% hydrogen peroxidase in methanol for 5 minutes. For blocking of nonspecific binding, sections were treated with 200 µl of normal swine serum (NSS) for 5 minutes at room temperature (RT). Sections were incubated for 30 minutes at RT with primary rabbit anti-

CD133 antibody (rabbit momoclonal antibody, C24B9, Cell Signalling Technology, product number # 3663, UK), diluted 1:100 (optimum dilution). After washing unbound primary antibody, immunostaining was performed using Dako REAL™ kit following the manufacturer's instructions (Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse, Code K5007). Bound antibody was developed by using 3, 3'-Diaminobenzidine tetrahydrochloride (Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse, Code K5007) for 6 minutes at RT. Sections were then counterstained with Mayer's haematoxylin, dehydrated with ascending graded alcohol, cleared in xylene, and mounted with DPX. Negative control was stained following the same protocol with the omission of the primary antibody to confirm the specificity of the stain. Positive controls using retinal tissue should be performed in order to judge the staining of CD133, but unfortunately we do not have reach to this type of tissue.

2.1.3 Evaluation of CD133 immunostaining

CD133 positivity was identified as expression of CD133 at the apical luminal surface of the colon cancer cells and staining of the shed intraluminal cellular debris that mirrors CD133 expression of the surrounding tumour cells (Horst, Kriegl et al. 2008). CD133 expression was scored on the basis of positive tumour glands (glands with either apical luminal staining or staining of intraglandular cellular debris) and expressed as a percentage of CD133 positive tumour glands compared to the total

tumour glands within each core. Assessment of CD133 positivity was carried out by two observers. First observer, Tarek Elsaba, assessed the whole TMA series blinded to the patients' clinicopathological and survival data. A second observer, Ahmed Benhasouna, also blinded to the patients' clinicopathological and survival data, assessed 25% of the TMA sections. A cutoff value of 50% was used. Tumours containing less than 50% positivity were considered CD133 negative/low, and those with ≥ 50% were considered CD133 high as previously described (Horst, Kriegl et al. 2008; Horst, Kriegl et al. 2009; Takahashi, Kamiyama et al. 2010). Cytoplasmic staining was evaluated as positive or negative according to the presence or absence of cytoplasmic staining.

2.1.4 Statistical analysis

For assessment of association between CD133 and other clinicopathological categories, specimens were categorized using the cut-off value previously described. To assess clinical significance between different categories, chi-square test and cross tabulation were used. Multiple testing corrections were applied if necessary. Kaplan-Meier curve was used to plot the difference in disease specific survival between groups, and the significance of difference between groups estimated by the log-rank test. Multivariate analysis using Cox regression hazard method to identify the relative risk and independent variables significance was performed. *p-values* < 0.05 were considered as statistically significant. Kappa (*K*) agreement was used to evaluate the agreement between two

observers. All statistical analysis was done by using SPSS package (version 15.0 for windows, SPSS Inc., Chicago, IL).

2.2 Cell culture condition

Colorectal cancer cell lines were maintained in Dulbecco Modified Eagle Medium (DMEM) (Invitrogen, UK) supplemented with 10% fetal bovine serum (FBS) (Sigma, UK), streptomycin 100 µg/ml (Sigma, UK) and penicillin100 units/ml (Sigma, UK) and were grown in T75 flasks (Corning Incorporation, Coaster, UK). Flasks were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The tissue culture medium was changed every 2 days and cells were passaged before they become confluent. Briefly, when the cells reach 90% confluence, the cells were rinsed with sterile phosphate buffered saline (PBS) (Sigma, UK), then 3ml of diluted Trypsin/EDTA (Ethylenediaminetetraacetic acid) were added [(10X) from Sigma, Cat# T4174]. After cell detachment, a new 5 ml medium was added to cells. Then 1 ml of these cells was transferred into a new T75 flask containing 10 ml fresh medium and incubated in the incubator.

2.3 Analysis of protein expression using flow cytometry

For analysis of cell surface protein expression in colorectal cancer cell lines using flow cytometry, the following protocol was used. In brief, the cells were rinsed with PBS, and then were detached using cell dissociation solution (1X non enzymatic Cell dissociation solution, Sigma, UK).

Carefully, cells were collected and centrifuged at 1000 rpm for 5 minutes at 4°C. Cells were resuspended in cold blocking buffer (PBS 1X; 5% heat-inactivated rabbit serum; 0.5% BSA; 2mM NaN_3; 5mM EDTA). Cells were counted and the number was calculated per ml by making a suitable dilution in trypan blue and using a haemocytometer. Cells were diluted to $5X10^5$ per 50µl then 50µl of mouse IgG1anti-human CD133/1 antibody (AC133) conjugated to R- phycoerythrin (PE) (Miltenybiotec, UK, Cat. 130-080-801) optimally diluted 1:50 (final working dilution is 1:100) in wash buffer (PBS 1X, 0.5% BSA; 2mM NaN_3; 5mM EDTA) were added to the cells, and incubated in dark at 4°C for 15 minutes. Unbound antibody was washed with wash buffer and centrifuged at 1000 rpm (twice, 5 minutes each). Labeled cells were resuspended with 250µl of wash buffer, and then fixed with 250µl of 2% formaldehyde. Negative control (with the omission of antibody and replace it with wash buffer), and isotype matched control [using monoclonal Mouse IgG1antibody conjugated to R-phycoerythrin (PE)] were performed following the previous staining procedures. At least fifty thousand events were detected using Epics Altra flow cytometry machine (Beckman Coulter), and the results were analyzed by WinMDi 2.9 or Weasel computer software. Background fluorescence was measured using negative populations, and gating parameters between positive and negative populations was implemented on the basis of cells labeled with IgG isotype control.

2.4 *Fluorescence activated cell sorting (FACS)*

Cells were stained using the previous staining protocol but without the final fixation step. To insure the purity of the sorted cells, the gating was stringently conducted to include the highly expressing cells and the lowest negative ones. To check the purity of the sorted populations, the sorted cells were re-analysed again by flow cytometry. Sorting was done using Epics Altra flow cytometry machine (Beckman Coulter).

2.5 *Magnetic cell sorting (MACS)*

For MACS sorting of colorectal cancer cell lines, we used anti-PE MicroBeads. First, the cells were stained with a R-Phycoerythrin (PE) conjugated primary antibody. Second, the cells are magnetically labeled with Anti-PE MicroBeads. Last, cells were filtered through a column placed in a magnetic field to retain the positively labeled cells within the column, and the negative cells (cells passed through the column) were filtered again through another column to increase the purity of the sorted cells. Briefly, the cells were first stained with mouse IgG1anti-human CD133/1 antibody (AC133) conjugated to R- phycoerythrin (PE) (Miltenybiotec, UK, Cat. 130-080-801) following the same staining protocol used with flow cytometry analysis without the final fixation step, and cells were resuspended in 80µl of wash buffer per 10^7 total cells. Then 20µl of anti-PE MicroBeads were added per 10^7 total cells, mixed well and the cells were incubated for 15 minutes in the refrigerator. Cells were washed with buffer

and centrifuged for 10 minutes. Then, supernatant was aspirated and cells were resuspended in 500µl buffer. Finally, cells were passed through MS column placed in a magnetic field of the MACS separator, where the unlabelled cells moved across the column while the labeled ones were retained in the column. The retained cells were eluted as positively selected portion.

2.6 Ribonucleic acid (RNA) extraction from cell lines

Total RNA was extracted from the CRC cell lines using RNeasy Mini Kit , including DNase treatment (QIAGEN house, West Susex, UK) following the manufacturer's instructions. Briefly, $1X10^7$ cells were washed with PBS and detached using Trypsin/EDTA. After detachment of the cells, medium was added and the cells were centrifuged at 1000 RPM for 5 minutes. After discarding the supernatant, 600 µl of lysis buffer were added to the cells and mixed by pipetting. Subsequently, the lysate was placed into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuged for 2 min at full speed. Then, 1 volume of 70% ethanol was added to the lysate to homogenize it and mixed well by pipetting. The lysate was transferred to an RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15 s at 13, 000 rpm. The RNeasy spin column membrane was washed with 700 µl of washing buffer (buffer RW1 supplied with the Kit) and centrifuged for 15 seconds at 13000 rpm. After discarding the flow-through, the column membrane was washed with 350 µl of wash buffer

(buffer RW1), and centrifuged for 15 seconds at 13000 rpm. At the same time, RNase-Free DNase enzyme was prepared by adding 10µl of DNase buffer with 70µl of DNase enzyme (supplied with Kit) and the mix was placed directly to the spin column and left on the benchplace for 15 minutes at (20-30^{0}C). Then, the column was washed with washing buffer and centrifuged at 13000 rpm for 15 seconds. After discarding the flow-through, the column membrane was washed twice with another wash buffer (buffer RPE, supplied with the kit) and centrifuged at 13000 rpm for 15 seconds (the first wash), and for 2 minutes (the second wash). Lastly, RNA was eluted by applying 30-50 µl of RNase-free water and centrifuged at 13000 rpm for 1 minute. The quantity and purity of eluted RNA were checked using a NanoDrop ND-1000 UV-Vis Spectrophotometer (LabTech International Ltd, Ringmer, UK). Samples had an A260/A280 ratio between (1.8-2.00) indicating a relative purity of the RNA. The eluted RNA was stored at - 80^{0}C. RNA samples from normal colonic mucosa were obtained following a study on inflammatory bowel disease (BD) by Professor Ilyas (Division of Pathology, University of Nottingham, UK).

2.7 Complementary Deoxyribonucleic acid (cDNA) synthesis

RNA was reverse transcriped into complementary deoxyribonucleic acid (DNA) using reverse transcription reaction. In short, a total of 1µg RNA was made up in a total of 18µl water and incubated with 1µl of random hexamer (pDN6) at 70°C for 10 minutes as initial denaturation step. Then a

mixture of 1 µl (200 units) of Moloney Murine Leukemia Virus Reverse Transcriptase enzyme [M-MLV RT (Invitrogen, UK)], 10mM of Dithiothreitol [DTT (Invitrogen, UK)] and 0.5mM each of Deoxyribonucleotide triphosphate (dNTP) was prepared, added to the denatured RNA making up a final volume of 50µl. Subsequently, the mix was incubated at 37°C for 60 minutes, then for 95°C for 10 minutes using thermocycler (Gene Amp PCR System 9700). Reverse transcriptase minus (RT-) negative control was performed with RNA and all the reagents with the exception of M-MLV RT enzyme.

2.8 Quantitative reverse transcriptase- polymerase chain reaction (QRT-PCR)

All the experiments of QRT-PCR were carried out in triplicates and values were normalized to the reference gene *Homo sapiens Hypoxanthine phosphoribosyltransferase 1 (HPRT1)* that was found to be the most accurate and economic single normalization gene that could be used as an alternative for use of multiple housekeeping genes when compared to another 12 endogenous housekeeping genes (de Kok, Roelofs et al. 2004) Primers were designed with the help of Primer3 web 0.4.0 which is a web based primer design software tool. PCR amplification was conducted using the SYBR green II dye as a reporter. For each quantitative reaction, a final volume of 25 µl was used; containing 12.5µl of 1X SYBR Green Master Mix (Stratagen, UK), 1 µl of each primer (250 nM, a final concentration),

and 5µl of cDNA templates (10 ng/5µl). Cycling conditions for the reactions were 10 minutes denaturation at 95°C followed by 40 cycles of 30 seconds denaturation at 95°C; 30 seconds annealing at a temperature according to the primer used (see table 2-3); 30 seconds extension at 72°C and a final melt for 60 seconds. The reaction was conducted using thermal cycler (MX3005P Stratagene, UK). The data for Q-PCR were analyzed by the MxPro-QPCR software version 3.20 using the standard curve method. For each QRT-PCR experiment, a standard curve was generated for each target gene. Standard curves were generated using 10 folds serial dilution of either image clone in case of CD133 and HPRT1 or 2 folds dilution of neat cDNA of highly expressing cell line for the rest of the genes. No template control (NTC) reactions which contain all the reagents except for DNA template, and (RT-) negative control were performed for each primer.

Table 2-3: List of primers used for qRT-PCR.
A table showed list of primers use and the annealing temperature and the product size

Gene	Primers	Amplicon size in bp	AN. T
CD133	Forward primer: 5' ACAGGGAATGGATTGTTGGA 3' Reverse primer: 5' CTCCCATACTTCTTAGTTTCCTCAA 3'	119	59°C
CD133 (Sv)	Forward primer : 5'-ACCCATTGGCATTCTCTTTG 3' Reverse primer: 5`CCCCAGGACACAGCATAGAA 3`	199 172	56°C
HPRT	Forward primer: 5' AAATTCTTTGCTGACCTGCTG 3' Reverse primer: 5' TCCCCTGTTGACTGGTCATT 3'	122	61°C
KLF-4	Forward primer: 5' **CCCACACAGGTGAGAAACCT 3'** Reverse primer: 5' ATGTGTAAGGCGAGGTGGTC 3'	169	56°C
Msi-1	Forward primer: 5' **ACAGCCCAAGATGGTGACTC** 3' Reverse primer: 5' CCACGATGTCCTCACTCTCA 3'	191	56°C
Oct4	Forward primer: 5' **GAAGGATGTGGTCCGAGTGT 3'** Reverse primer: 5' **GTGAAGTGAGGGCTCCCATA 3'**	183	58°C
Nanog	Forward primer: 5' **TTCCTTCCTCCATGGATCTG 3'**	213	57°C

	Reverse primer: 5' TCTGCTGGAGGCTGAGGTAT 3'		
Lgr5	Forward primer: 5'CTCTTCCTCAAACCGTCTGC 3'	181	59°C
	Reverse primer: 5' GATCGGAGGCTAAGCAACTG 3'		
c-MYC	Forward primer: 5' TTCGGGTAGTGGAAAACCAG 3'	203	59°C
	Reverse primer: 5' **CAGCAGCTCGAATTTCTTCC** 3'		

2.9 Construction of CD133 expression plasmid (pcDNA3.1-CD133)

The pcDNA™3.1 Directional TOPO® Expression Kit (Invitrogen, UK) was used to clone a blunt end PCR product (CD133 coding sequence) into pcDNA™3.1D/V5-His-TOPO®. (Vector map see appendix 9.1)

2.9.1 PCR amplification of CD133 coding sequence

In order to allow the directional cloning of CD133 coding sequence, the forward PCR primer must contain the sequence, CACC, at the 5' end of the primer and directly before the ATG start codon. This sequence allows direct ligation with overhang sequence GTGG in pcDNA™3.1D/V5-His-TOPO® vector (a polylinker map see appendix 9.2). The amplification primers were designed manually and their sequence were as follow;

forward primer 5'- CACCATGGCCCTCGTACTCGG-3; ***reverse primer*** 5'-TCAATGTTGTGATGGGCTTGTCAT-3' and were purchased from MWG-BiotecH AG).

To amplify a blunt end CD133 coding sequence with CACC sequence at the 5' end of the PCR product, CD133 image clone (accession No. BC012089 , IMAGE:4644690), and the aforementioned designed primers were used in a final volume of 50µl PCR reaction using the Pfu DNA polymerase enzyme (Promega, cat. No. M774A). In a sterile, nuclease-free

0.5 ml tube, the PCR reaction contain the following components; 5 µl of 10x Pfu DNA polymerase buffer with $MgSO_4$ (final concentration of 1X), 2µl of each forward and reverse primers (final concentration of 250nM), 1µl of Pfu DNA polymerase enzyme (2-3u/µl), 1µl of dNTPs mix (10mM)(making a final concentration of 200µM), and 29µl of nuclease-free water.

The reaction was performed in thermal cycler (Perkin Elmer GeneAmp PCR system 2400), and allowed to run for 40 cycles with the following cyclic conditions; 95°C for 2 minutes (initial denaturation), 95°C for 1 minute (denaturation), 60°C for 1 minute (annealing), 72°C for 2 minutes (elongation) and 72°C for 10 minutes (final extension). PCR product was checked for a single band suitable for cloning using 1 % agarose gel.

2.9.2 Agarose gel electrophoresis

1% agarose gel was prepared by adding 1 gm of agarose (Gibco-BR Life Technologies, USA) into 100 ml of 1% Tris Borate EDTA (TBE) buffer (Sigma, USA) and heated in a microwave for 60 seconds. The gel was allowed to cool down at room temperature. 10µl of Sybersafe dye (1000x) (Sigma, USA) was added and the gel was then poured into the gel apparatus and an appropriate comb was inserted. After solidification of the gel, 12 µl of previously prepared DNA samples (10µl of DNA samples mixed with 2µl loading dye) were loaded into the gel lanes, and 1KB DNA ladder (New England BioLabs, UK) was loaded for sizing of the samples. Gels were allowed to run at 90 volts for 45 minutes using 1X TBE solution

as a running buffer. Visualization of the PCR products on the gel was done using an ultraviolet transilluminator (UVP Inc., USA).

2.9.3 Purification of PCR product

The PCR product was purified using QIAquick PCR Purification Kit (Qiagen) following the manufacturer's protocol. Briefly, 5 volumes of buffer PBI were added to one volume of PCR sample and mixed.the mixture was added to the QIAquick column and centrifuged for 60 seconds. Then, column was washed by adding 750µl of buffer PE and centrifuged for 60 seconds. Lastly, DNA was eluted in a clean microcentrifuge tube using 50 µl of water. The purified DNA was then analyzed on a gel and the final amount of purified DNA was quantified using NanoDrop ND-1000 UV-Vis Spectrophotometer (LabTech International Ltd, Ringmer, UK). A ratio of 1.8 – 2.0 was considered an indication of relative purity.

2.9.4 Cloning of CD133 into in pcDNA™3.1D/V5-His-TOPO® vector and transformation

To clone CD133 PCR product into pcDNA™3.1D/V5-His-TOPO® vector, TOPO cloning reaction was performed. The reaction was done by making a mixture of 1µl of purified PCR product, 1µl of Salt Solution (1.2 M NaCl and 0.06 M MgCl2), 1 µl of TOPO vector, and sterile water to a final volume of 5µl. The mixture was incubated at room temperature (22°-23°C) for 15 minutes. Then, the reaction was placed on ice for 5 minutes, and preceded to one shot chemical transformation following the manufacturer's instructions.

One shot chemical transformation was conducted by mixing gently 4µl of the TOPO cloning reaction (as performed above) into a vial of One Shot®TOP10 Chemically Competent *E. coli* and incubated on ice for 30 minutes. Then, the cells were heat-shocked for 30 seconds at 42°C without shaking, and the tube immediately transferred to ice. A 250µ of SOC medium was added to the tube, tightly closed and was then placed in a shaker (New Brunswick Scientific Co. Ltd., USA) at 37°C for 1 hour. 60 µl from the transformation were spread on a LB agar plate containing 50µg/ml ampicillin as a selection antibiotic to allow growth of bacteria containing the plasmid and the plate was incubated overnight at 37°C.

2.9.5 Analyzing transformants

Eight colonies were picked and cultured overnight in LB broth medium (Sigma, UK) containing 50µl/ml ampicillin. Then, plasmid DNA was isolated using GenElute™Plasmid Miniprep Kit (Sigma Aldrich, UK) following the manufacturer's instructions. Briefly, 1-5 ml of the recombinant *E.coli* were pelleted by centrifugation at 13000rpm for 1 minute and then resuspended in 200 µl of resuspension solution (supplied with the kit). Cells were lysed by adding 200 µl of lysis solution and mixed gently followed by adding 350µl of neutralization/binding solution in order to precipitate the cell debris. This was then centrifuged at maximum speed for 10 minutes. Subsequently, the lysate was added to a GenElute Miniprep Binding Column and centrifuged at 13000 rpm for 1 minute. After discarding the flow-through, a 750µl of wash solution was added to the column and

centrifuged at 13000 rpm for 1 minute. The flow-through was discarded and samples were centrifuged at maximum speed for 2 minutes. Finally the plasmid DNA was eluted by adding 100µl of water to the column and cetntrifuged at 13000rpm for 1 minute. The quantity and purity of the eluted plasmid were checked by using using NanoDrop ND-1000 UV-Vis Spectrophotometer (LabTech International Ltd, Ringmer, UK) A ratio of 1.8 – 2.0 was considered an indication of relative purity.

The plasmid DNA was analyzed by restriction enzyme analysis method to confirm the presence of the insert in the construct. EcoRV enzyme was used for the restriction digestion analysis. In a sterile 0.5 ml tube, the reaction mix was prepared in a final volume of 20µl as follow: 2µl of 10x buffer 3 (Promega, UK), 0.2µl of 100X BSA (bovine serum Albumin) ((Promega, UK), 1µl of EcoRV enzyme (Biolabs, UK), 6µl of plasmid DNA, 10.8µl of distilled water. The reaction was incubated for two hours at 37°C. Gel electrophoresis was done by using 1% agarose gel and 1Kb ladder to visualize the results of restriction analysis. Furthermore, to confirm the correct orientation of the insert, the plasmid DNA was subjected to direct sequencing.

2.10 RNA silencing

The small interfering RNA (siRNA)(stealth type) was used to knockdown CD133 and STAT3 in CRC cell lines. The following siRNA was used:

CD133 siRNA, 5'AUU GCUAUC UGC CAG UUU CCG ACU C-3'., CD133 control siRNA 5' GAG GGA ACA GUC GGA UAG ACC UAA U 3', STAT3 siRNA 5' UGGCCCAAUGGAAUCAGCUACAGCA 3', AND STAT3 control siRNA 5' UGG ACU AGG AAC UGA UCC AAC CGC A 3'. The siRNA for CD133 was synthesized by the help of BLOCK-iT™ RNAi designer which is a web-based tool for designing and customizing synthetic siRNA from nucleotide target sequences (Supplied free by Invitrogen). After making a selection, duplexes was ordered from the same company (Invitrogen, UK). The STAT3 siRNA was kindly gifted by one of our colleagues in the Lab (Dr. Kanwal Baloch; a PhD student, department of Pathology), and was designed by the same way as CD133. For gene knockdown, lipofectamine™ 2000 transfection reagent (Invitrogen, UK) was used following the manufacturer's guidelines. One day before transfection, cells were seeded in 1 ml of antibiotic-free growth medium in 6 well plate (Corning Incorporation, Coaster, UK) in order to allow cells to be 30-40% confluent before transfection. On the day of transfection, medium was aspirated, cells were washed with PBS, and then 2 ml of Opti-MEM® I Reduced Serum Medium without serum was added. For each transfection reaction, oligomer –lipofectamine 2000 complexes were prepared as follow: A 100 pmol siRNA (equal to 5µl of siRNA) was diluted in 250 µl of Opti-MEM® I Reduced Serum Medium without serum (final concentration of siRNA when added to the cells is will be 33 nM), and mixed gently. Then, 5 µl of lipofectamine 2000 was diluted at 250µl of Opti-MEM® I Reduced Serum Medium without serum, mixed gently and incubated for 5 minutes.

After the incubation, the diluted oligomer and lipofectamine 2000 were combined, mixed gently, and incubated for 20 minutes at room temperature. Lastly, the complexes were added to the wells and mixed gently by rocking the well, and incubated at 37°C in 5% CO_2 atmosphere. 6 hours later the medium was replaced by the normal DMEM medium. Seventy two hours later, transfected cells were used in functional assays, flow cytometry analysis, protein extraction for western blot analysis, and RNA isolation for PCR reactions.

2.11 Plasmid (pcDNA3.1-CD133) DNA transfection

For plasmid DNA transfection, lipfectamine®2000 transfection reagent was used following the manufacturer's protocol. One day before transfection, the proper number of cells were seeded in 1 ml antibiotic-free growth medium in 6 well plate, so that they will be 60-70% confluent at the transfection day. At the day of transfection, medium was aspirated, cells were washed with PBS, and then 2 ml of Opti-MEM® I Reduced Serum Medium without serum was added. For each transfection reaction complexes were prepared as follow: A 4µg plasmid DNA was diluted in 250 µl of Opti-MEM® I Reduced Serum Medium without serum, and mixed gently. Then, 12 µl of lipofectamine 2000 was diluted at 250µl of Opti-MEM® I Reduced Serum Medium without serum, mixed gently and incubated for 5 minutes. After the incubation, the diluted plasmid DNA and lipofectamine 2000 were combined, mixed gently, and incubated for 20

minutes at room temperature. Lastly, the complexes were added to the wells and mixed gently by rocking the well, and incubated at 37°C in 5% CO_2 atmosphere. 6 hours later the medium was changed. Twenty four or 48 hours later, cells were used for flow cytometry analysis, protein extraction for western blot analysis, and RNA isolation for PCR experiments.

2.12 Western blot (WB) analysis

2.12.1 Protein preparation and quantitation

Either after gene knockdown or gene forced expression, protein preparation was performed by removing the culture medium, then rinsing the cells with ice-cold 1X PBS. Cells were homogenized with 495 µl of lysis buffer (RIPA buffer) made fresh [20 mM Tris, pH 7.5, 150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 1M EDTA, 0.1% SDS] enriched with 5µl of protease and phosphatase inhibitors cocktail (Sigma, UK). Cells were scraped off with a syringe plunger, and cell lysate was transferred into 1.5 ml eppendorf tube, and incubated on ice for 30 minutes. Afterwards, the cell lysate was centrifuged at 13000 rpm at 4°C for 20 minutes. The supernatant fluid (total cell lysate) was transferred into new small Eppendorf tube and stored at -20°C.

Protein quantification was done using BCA (bicinchoninic acid) Protein Assay Kit (Pierce, thermo-scientific, Cat. No.23225), following the manufacturer's instructions (Appendix 9.4).

2.12.2 Electrophoresis and blotting

30 µg of cell lysate; prepared previously; were then loaded with 4X loading dye [100mM Tris-HCl (pH 6.8), 200mM DTT, 4% SDS, 0.2% glycerol and 0.2% bromophenol blue] supplemented with 5% β-mercaptoethanol. Samples were boiled for 5 minutes in a water bath at 90°C, and then electrophoresed using 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) at 60mA. Subsequently, the protein samples on the gel were transferred to a PVDF membrane (Amersham Hybond-P PVDF membrane, GE Healthcare, UK) using a semi-dry transfer method at 60mA for 2 hours. After blocking the membranes for 1 hour at room temperature using 5% dried milk dissolved in Tris buffer saline (TBS) with tween, they were incubated with primary antibodies overnight with agitation at room temperature. Membranes were washed with wash buffer (TBS with 0.1% Tween-20) three times, 5 minutes each. Appropriate horse raddish peroxidase conjugated secondary antibody was added to the membrane and incubated with agitation for 1 hour at room temperature. Proteins were visualized using Enhanced Chemiluminescence detection kit (Supersignal West Pico Chemiluminescent Substrate, Thermoscientific, UK) and exposure to X-ray film (Kodak, UK). Antibodies applied in this study include anti-CD133 (1:1000, Cell signalling, C24B9; UK), anti-STAT3 (1:50, Abcam, ab7966), anti-β-catenin (1:1500; clone β-catenin-1, Dako), anti-E-Cadherin (1:1500; clone NCH-38; Dako) , anti-B-actin (1:2000, Sigma; UK)., anti-c- c-Myc (1:1000, clone 9E10, ab32, Abcam), anti-CTEN

(1:1000; Sigma, UK product number WH0084951M1). Detailed steps of western blot experiment see (see appendix 9.5)

2.13 Functional Assays

2.13.1 Proliferation assay

A time course assay was conducted to compare the number of proliferating cells between two different populations regarding their CD133 expression. A 24 well plate (Corning Incorporation , Coaster, UK) was used, seeded with $1X10^4$ cells and cell number was measured at specific time using methylene blue assay (Dvory-Sobol, Sagiv et al. 2007). First, cells were washed with PBS once then fixed in 500µl of absolute methanol for 30 minutes. Cells were allowed to air dry for 5 minutes after removal of methanol, followed by staining with 1% methylene blue [1gm methylene blue hydrate (Sigma, UK), and 100 ml distilled water] for 30 minutes. Then methylene blue was removed and wells are washed with distilled water (thrice). Lastly, 500µl of 0.1% HCl in ethanol was added , then 100µl of each well was transferred into 96 well plate, and absorbance was measured at a wave length of 570nm using a plate reader (Labsystems, UK). Each assay was performed in triplicate and repeated in at least two independent experiments

2.13.2 Cell Migration assay

In-vitro migration ability of the cells was assessed using Transwell cell migration assays and wound healing assay. Transwell cell migration assay was performed using a Boyden chamber containing a polycarbonate filter with an 8 µm pore size (Costar, UK). A 600 µl of culture medium(DMEM medium) supplemented with 20% FBS (Sigma, UK) was added to the lower chamber and $2.5x10^5$ cells were seeded in 100µl of culture medium supplemented with 10% FBS in the upper chamber. The number of cells migrating through the membrane was manually counted either after 24 or 48 hours. Assays were performed in triplicate and on at least two separate occasions. Cell migration was also measured using a cell wounding assay performed in 6 well plates (Costar, UK). Cells were grown to confluence and then starved for 24 hours in serum free medium. A sterile 200µl pipette tip was used to create three separate parallel wounds and migration of the cells across the wound line was assessed after 24 and 48 hours. Photographs were taken using a charge-coupled device (CCD) camera (Canon, Japan) attached to the inverted phase-contrast microscope at a power of X40. The distance between the edges was measured at 6 equally distributed points using ImageJ software.(Rasband WS, Image J. U. S. National Institutes of Health, Bethesda, Maryland, USA, 1997–2009. Available from URL: http://rsb.info.nih.gov/ij/) and then analysed using a two tailed t-test. Experiments were repeated on at least two separate occasions

2.13.3 2D / 3D colony formation assay

The ability of isolated single CD133+ and CD133- cells to form colonies was tested in both 2 dimensional (2D) culture and 3 dimensional (3D) culture. For 2D culture, 300 freshly sorted cells were seeded into individual wells of a 6 well plate and cultured for 14 days. The cells were then stained with methylene blue and colonies containing more than 20 cells were counted. The experiments were carried out in triplicate and on two separate occasions. 3D (soft agar assay) culture was performed to assess the clonogenic ability of the sorted populations in non adherent conditions. Cells (2500 from each population CD133+ and CD133-cells) were counted and resuspended as single cells in 0.7% DNA grade agarose (Sigma Aldrich).This was overlaid on a base of 1% DNA grade agar (Sigma Aldrich) and both top and base layers were mixed with 2X DMEM. Experiments were set up in triplicate and medium changed twice a week. After two weeks, the number of colonies that developed within each well was counted and visualized under a microscope after staining with 0.05% crystal violet for 1 hour and representative fields were photographed. For both 3D and 2D culture. Colony forming efficiency (CFE) was calculated as follow, % CFE = (Number of obtained colonies / Number of cultured cells) X 100 (Arango, Chamorro et al. 2005). The original values have skewed distributions; therefore log transformed values rather than the original values were used for statistical analysis.

2.13.4 Staurosporine induced apoptosis assay

Staurosporine was used to evaluate the resistance conferred by CD133 to exogenous apoptotic stresses. The protocol was modified for individual cell lines. For HT29, each well was seeded with either $HT29^{CD133-}$ (cells transfected with CD133 siRNA) or $HT29^{ssc}$ (cells transfected with siRNA control)cells 72 hours after transfection, Approximately 5 X 10^4 cells were seeded per well and incubated with staurosporine (Staurosporine readymade solution, Sigma, UK) at a final concentration of 8μM for 24 hours. Control cells were treated with DMSO. After this period, viable cells were measured using methylene blue assay. For SW480, each well of a 96 well plate was seeded with 10^5 cells of the sorted cells and staurosporine was added 24 hours later at a concentration of 8μM. Control cells were also treated with DMSO. After another 24 hours, the numbers of viable cells was assessed using the previously described methylene blue assay. The assay was performed in triplicate and repeated in at least two independent experiments

2.13.5 Statistical analysis

All evaluations were done using unpaired two tailed Student's t-test For studies involving cell and colony counting, cell numbers were analysed. For studies with a numerical fluorescence output, the raw fluorescence values were used.

2.14 5-aza-2' deoxycytidine effect

To test the epigenetic control involved in colorectal cancer, a DNA methyltransferase (DNTM) inhibitor (5-aza-2'-deoxycytidine, DAC) was used. Cultured cells were treated with 5-aza-2'-deoxycytidine (5-Aza) (DAC; Sigma Adldrich, UK)) at a concentration of 1µmol for 72 hours, and media changed every 24 hour. Control cells were treated with DMSO. After 72 hours, treated cells with either vehicle or 5-Aza were evaluated for CD133 expression by flow cytometry.

Results

3 Chapter 3: The expression of CD133 in colorectal cancer and its correlation with clinico-pathological features

3.1 *Abstract*

Background and aims: CD133, a transmembrane glycoprotein of unknown function, was originally described as a stem cell marker in haemopoietic cells. Subsequently it has been reported as a marker of cancer stem cells or "tumour initiating cells" in many solid tumours including colon, liver, pancreas, prostate and brain tumours. The aim of this study is to (1) evaluate the expression of CD133 in colorectal cancer and is correlation with clinico-pathological features, and (2) compare CD133 expression in primary colorectal cancers and their corresponding liver metastases cases

Methods: Formalin fixed paraffin embedded blocks of previously prepared tissue microarrays (TMAs) of 449 colorectal cancer cases, and 45 matched primary colorectal cancer and their corresponding liver metastases were stained for CD133 using standard immunohistochemistry protocols. CD133 expression was scored as "low" (<50% tumour cells positive) or "high" (>50% tumour cells positive) and correlated with the clinico-pathological parameters and patients survival. The level of CD133 expression was compared between primary lesions and liver metastases cases.

Results: A total of 323/449 cores could be evaluated after immunostaining. Staining for CD133 was usually confined to the lumen of tumour glands. 28/323 (8.6%) showed high expression whilst 295/323 showed low expression. There was no association between CD133 expression and pathological features. A borderline significant association

was found between low CD133 expression and better overall survival *(p = 0.05; Log-rank test)*, and multivariate analysis showed that CD133 was an independent prognostic marker in colorectal cancer. No significant change in CD133 expression level in the primary and corresponding liver metastases. However, a significant correlation was found between CD133 expression in primary tumours and liver metastases (r = 0.46, p = 0.001; *Spearman rank correlation coefficient test*).

Conclusion: In these TMA series, CD133 protein was found to be an independent prognostic significance in colorectal cancer which may reflect its clinical importance as a predictor of survival, but with no difference in primary and metastatic cases.

3.2 Introduction

Recently, much effort has been focused towards the cancer stem cell (CSC) model of tumour growth. Growing evidence suggests that the tumour is hierarchically structured, and according to the CSC model, the tumour contains a minority of cells having stem cell-like features, termed "cancer stem cells (CSC)" or "tumour initiating cells (TIC)", and these are responsible for the maintenance of the tumour (Reya, Morrison et al. 2001; Shmelkov, Jun et al. 2004; Burkert, Wright et al. 2006; Dalerba, Cho et al. 2007). Two recent studies stated that the CSC model could apply in colorectal cancer. (Ricci-Vitiani, Lombardi et al. 2007; Todaro, Alea et al. 2007). and CD133 was proposed as a marker that can be used to characterize colon cancer stem cells (Co-CSCs) (O'Brien, Pollett et al. 2007; Ricci-Vitiani, Lombardi et al. 2007).

Although little is known about CD133 function it is reported to be an independent prognostic factor for overall survival (OS) in many tumours such as pancreas (Maeda, Shinchi et al. 2008), hepatocellular carcinoma (Song, Li et al. 2008), brain tumours (Pallini, Ricci-Vitiani et al. 2008). Furthermore, CD133 mRNA in peripheral blood mononuclear cells of patients with different cancer types was elevated in patient with bone metastases (Mehra, Penning et al. 2006), and can predict colon cancer recurrence (Lin, Hassan et al. 2007) Therefore, the aim of this study is 1) to assess the expression of CD133 in colorectal cancer; 2) correlate

CD133 expression with different clinicopathological variables and patients' outcome; 3) compare the expression of CD133 in primary tumours and their corresponding liver metastases.

Briefly, in this study we stained formalin fixed paraffin embedded TMA sections comprised of 449 cases of primary colon cancer. In order to further evaluate the role CD133 may play in metastasis, another TMA comprising 45 cases of primary tumours and their corresponding liver metastases cases were stained with rabbit monoclonal antibody (C24B9, Cell Signalling, UK) Western blotting was employed with whole cell lystaes from Caco2 and DLD1Cell lines to validate anti-CD133 antibody specificity. Details regarding the immunostaining protocol, evaluation criteria of CD133 expression, western blot experiment, and statistical methods are described in detail in the material and methods section.

3.3 Results

3.3.1 Validation of the antibody

The performed Western blotting analysis with lysates from the CD133+ colon cancer cell lines Caco2, and DLD1 which were reported as high and negative expressers of CD133, respectively (Corbeil, Roper et al. 2000; Horst, Kriegl et al. 2008) revealed a specific band at the predicted size (~120 kDa) of the CD133 protein confirming the specificity of the antibody used in IHC staining (Figure 3-1). The anti-CD133 antibody used (C24B9)

in western blot analysis and immunohistochemistry can detect both glycosylated and non-glycosylated epitopes of CD133 protein. Horst *et al.* revealed that immunohistochemistry detection of CD133 protein in colorectal cancer using three different clones of CD133 antibodies (two of them detected glycosylated epitopes) showed comparable levels of CD133 expression. Therefore, we used this clone to allow direct comparison with a previous study (Horst, Kriegl et al. 2008).

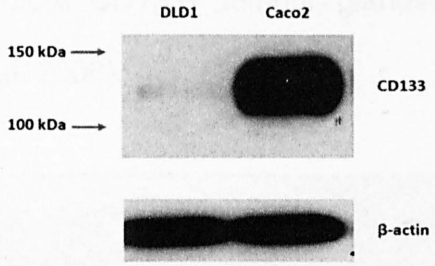


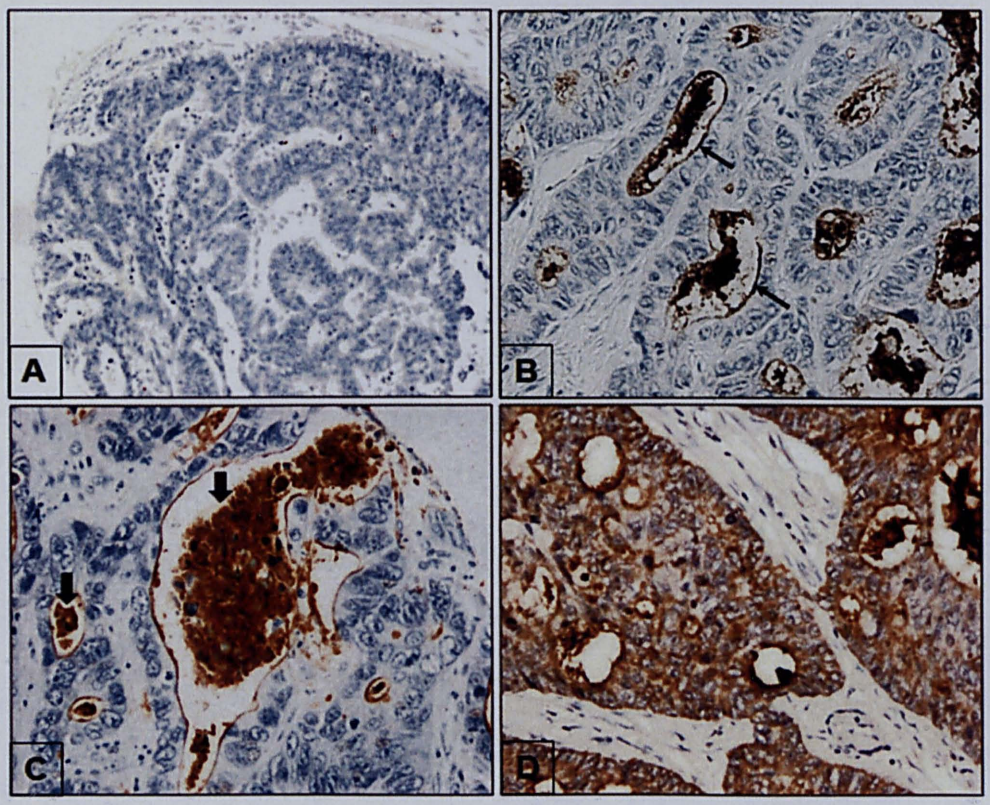
Figure 3-1: CD133 antibody specificity
Western blot analysis using anti-CD133 (C294B) rabbit monoclonal antibody used for immunohistochemistry showed specific band at the expected size (120 kDa). Protein lysates of Caco2 (CD133+) and DLD1 (CD133-) were used. B- actin was used to indicate equal loading of proteins.

3.3.2 Immunostaining for CD133

CD133 expression was investigated in TMA section of 449 cases, and 45 cases of primary CRC and matched live metastases. The CD133 staining pattern in colorectal cancer was detected mainly in the apical surface of the colon cancer glands (figure 3-2B) as well as the shed cellular debris into the glandular lumen of the CD133+ glands (figure 3-2C). In addition, mixed cytoplasmic staining with apical luminal and positive shed cellular

debris was observed in a few cases (9/323 and 4/45 cases showed cytoplasmic staining (Figure 3-2D).

CD133 expression was scored by determining the percentage of CD133+ tumour glands by two observers (see material and methods section), and a K > 0.86 was obtained, indicating a very good correlation between observers. And accordingly the cohort was dichotomized into two categories as CD133 low (0% to <50% CD133+ tumour glands), and CD133 high (≥ 50% CD133+ tumour glands) as previously described (Horst, Kriegl et al. 2008).



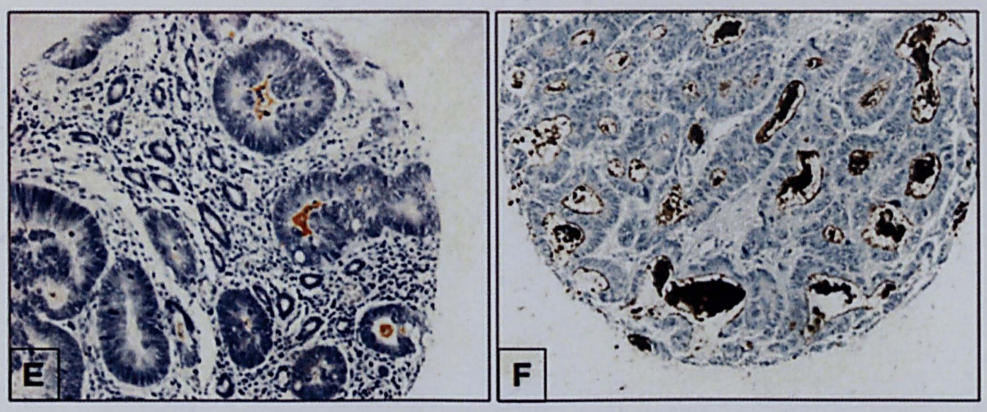


Figure 3-2. Pattern of CD133 expression in colorectal cancer.

(a) represents a negative control , **(b)** CD133 staining of the apical luminal surface of the gland (thin arrows) (200X magnification), **(c)** CD133 positive staining of shedded intraglandular cellular debris (thick arrows), positivity was considered when either the apical luminal surface or intraglandular cellular debris or both of them are stained (200x magnification), **(D)** mixed staining pattern of CD133 including cytoplasm, apical lumina, and intraglandular cellular debris, the stroma between tumour glands was negative indicating the specificity of CD133 staining (400X magnification), **(E)** represents a case with CD133 expression less than 50% (100X), and **(F)** represents a case of CD133 expression of more than 50% (100X magnification)

3.3.3 Correlation of CD133 expression with clinicopathological parameters

After excluding the uninformative TMA cores (due to loss of tissue, folding, and absence of invasive tumours in the cores), 323 cases were applicable for statistical analysis. Applying the aforementioned scoring criterion, it was found that 28/323 (8.6%) were CD133 high and 295/323 (91.4%) were CD133 negative/low expressors. Figure (3-3) shows a distribution of percentage score for CD133. A cross tabulation and χ^2 test was performed to assess the correlation of CD133 expression status with different clinico-pathological parameters. No association was observed between CD133

expression and histologic type, vascular invasion, TNM stage, and distant metastasis (Table 3-1).

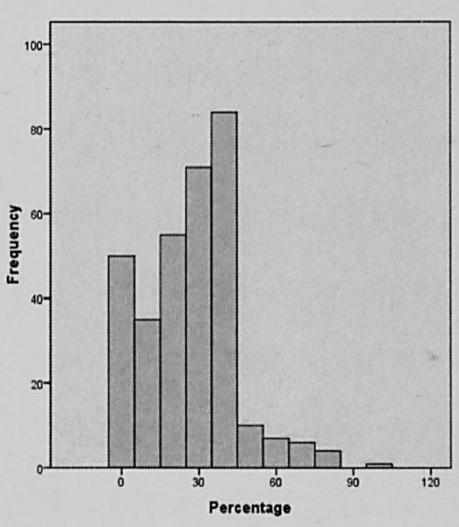


Figure 3-3: A histogram showing the distribution of percentage scores,

Table 3-1: Correlation of CD133 with the clinicopathological variables in 323 colorectal cancer cases.

Variables	Total	CD133 expression		χ^2	*p-value*
		Low	High		
Histologic types					
Adenocarcinoma	285	258	27	2.74	0.601
Mucinous	33	32	1		
Columnar	4	4	0		
Signet ring adeno.	1	1	0		
Vascular Invasion					
No	159	142	17	2.751	0.151
Yes	88	84	4		
TNM stage					
I	56	53	3	4.073	0.539
II	116	104	12		
III	108	101	7		
IV	40	34	6		
Distant Metastasis					
No	281	259	22	1.104	0.350
Positive	39	34	5		

3.3.4 Correlation of CD133 expression with patients' outcome

Applying Kaplan Meier curve analysis demonstrated that patients with high CD133 expression had significantly poor prognosis than those with low CD133 expression. Univariate analysis revealed that patients with CD133 high expression associated with short overall survival (OS) which was borderline significant (*Log Rank, LR=3.761, p = 0.05, Log-rank test*) (figure 3-4). Furthermore, multivariate Cox proportional hazard analysis including tumour stage, distant metastasis, and CD133 expression showed that CD133 expression was an independent predictor of low survival (*HR=0.478, p = 0.01, 95% CI = 0.261 - 0.875*; *Cox regression test*) (Table 3-2)

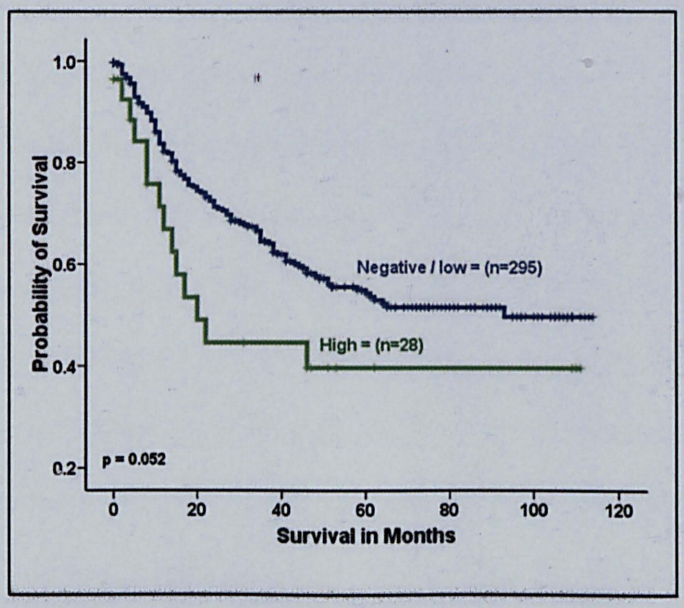


Figure 3-4. Association between CD133 expression and patients overall survival in CRC series.
Kaplan-Meier plot revealed that high expression of C133 is associated with short overall survival (*Log Rank, LR = 3.761, p = 0.05*; *Log-rank test*).

Table 3-2. Multivariate Cox proportional hazard analysis of CD133 expression in CRC.

Variables	Hazard ratio	95% CI		P-value
		Low	High	
TNM stage	0.167	0.096	0.291	<0.001
Vascular Invasion	0.480	0.318	0.723	<0.001
CD133 expression	0.478	0.261	0.875	0.01

CD133 is an independent prognostic factor for low survival in CRC.

3.3.5 CD133 expression in primary and corresponding liver metastasis cases

In the 45 cases, the percentage of CD133 positivity was evaluated for each case. Using CD133 expression as a continuous variable (percentage of CD133 positivity), it was found that there was no significant difference in the expression level between primary CRC and liver metastases cases. However, a significant correlation was found between CD133 expression in primary and metastatic lesions (*r = 0.46, p = 0.001*; *Spearman rank correlation coefficient test*) (Figure 3-5). The CD133 expression level (negative/low expression vs high expression) in liver metastatic cases was compared with that in primary lesions (Table 3-3). It was found that 26/32 (81.25%) cases of liver metastases derived from CD133 negative/low primary tumours were also CD133 negative/low, while, 6/32 cases (18.75%) were CD133 high liver metastasis in patients with CD133 negative/low expression primary tumours. Liver metastases originating

from CD133 high primary tumours were also heterogeneous for CD133 expression level. 4/13 cases (30.7%) of liver metastasis derived from CD133 high primary tumours were CD133 negative/low. Also, it could be noted that there was a slight tendency for liver metastatic lesion to exhibit more CD133 high expression (33.3%) than was found in primary tumours (28.9%) (*p=0.002, Fisher's exact test*). Figure 3-6 showed the expression of CD133 in primary tumours and liver metastases.

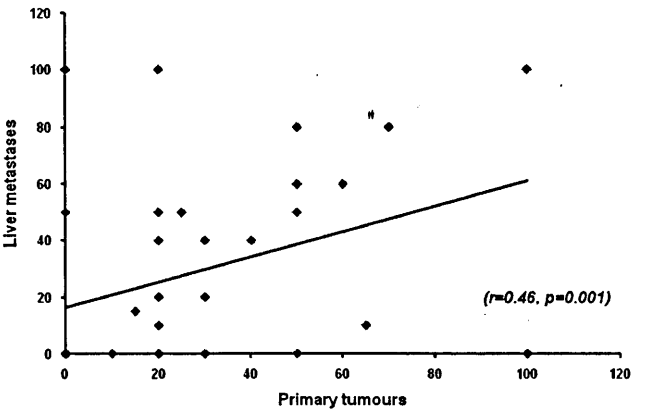


Figure 3-5: Correlation of CD133 in primary and liver metastases cases.
A scatter blot graph showed that the correlation between CD133 mRNA expression in primary tumours and their corresponding liver metastases was statistically significant *(r = 0.46; p = 0.001)*

Table 3-3: CD133 expression level in primary CRC and liver metastases

		Liver metastasis		
		CD133 negative/low	**CD133 high**	**Total**
Primary CRC	**CD133 negative/low**	26	6	32
	CD133 high	4	9	13
Total		30	15	45

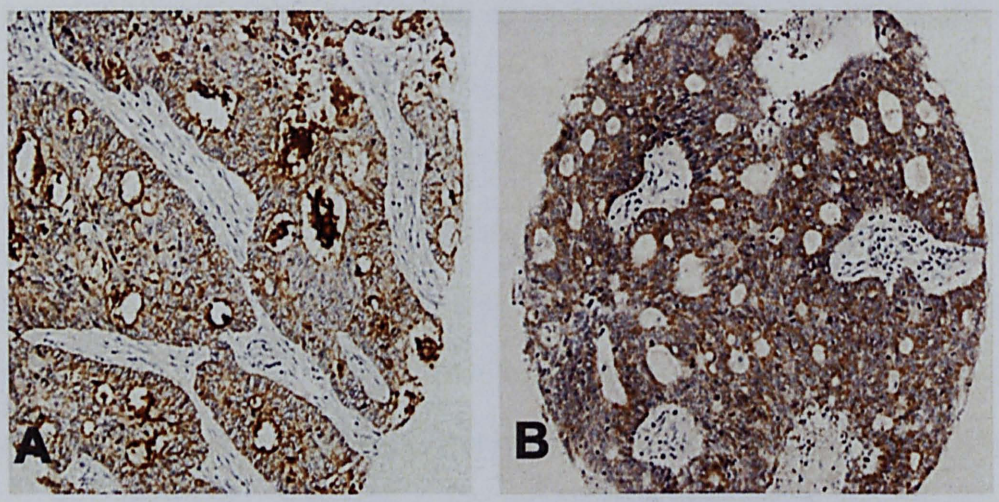


Figure 3-6: CD133 staining pattern in primary and liver metastases.
CD133 expression level was the same in primary colorectal cancer (200X magnification) **(a)**, and the corresponding liver metastasis **(b).**

3.4 Discussion

This study examined the expression of CD133 in colorectal cancer and correlated this expression with the clinicopathological findings and patients outcome.

CD133 was formerly identified on the apical surface of the colon cancer cell line Caco2 and many human embryonic tissues including gut, neural tube and kidney (Corbeil, Roper et al. 2000). In the studies of Horst *et al.* and Kojima *et al.* CD133 immunostaining was detected on the luminal surface of the tumour glands and intraglandular shed cellular debris (Horst, Kriegl et al. 2008; Kojima, Ishii et al. 2008). However, studies have also shown cytoplasmic staining of CD133 expression in pancreatic and CRC

as well as glioblastoma multiforme (Immervoll, Hoem et al. 2008; Kojima, Ishii et al. 2008; Maeda, Shinchi et al. 2008; Pallini, Ricci-Vitiani et al. 2008). In line with the previous studies, the positivity of CD133 was mainly identified on the luminal surface of tumour glands and/or shed intraglandular cellular debris although a few cases also showed cytoplasmic staining (9/323 and 4/45 cases). This low number of cases will lead to lowering the power of statistical test used when correlating this cytoplasmic pattern of staining with other clinicopathological variables and patients' survival. Therefore, a larger series with a larger number of cytoplasmic cases should be considered in the future to build up an accurate conclusion regarding the impact of cytoplasmic expression of CD133.

The results of this study demonstrated low incidence of CD133 high expression (≥ 50%) in a large series of colorectal cancer. This incidence was similar to the findings of Li *et al.* that CD133 high expression was found in 7/104 (6.5%) cases of colorectal cancer (Li, Li et al. 2009). However, in other studies in colorectal cancer, variable incidences of CD133 high expression were detected. For instance, Horst *et al.* found that CD133 high expression represented 26% of the cases (Horst, Kriegl et al. 2008). Whilst, another study showed that 36% of cases were correspond to CD133 high expression (Horst, Kriegl et al. 2009). Moreover, a more recent study revealed that the frequency of CD133 high expression was 27% (Takahashi, Kamiyama et al. 2010). Such a discrepancy between

these studies could be attributed to different selection criteria of colorectal cancer cases studied. In this study as well as Li *et al.* study (Li, Li et al. 2009), the selected cases included different histopathologic types of adenocarcinoma and different grades; whilst, one study of Horst *et al.* (Horst, Kriegl et al. 2008) the selected cases were only moderately differentiated adenocarcinoma, and the other study, contained only grade 2 and grade 3 and no other histopathologic types of adenocarcinoma (Horst, Kriegl et al. 2009). In addition, the Takahashi *et al.* study contained well and moderately differentiated adenocarcinoma only (Takahashi, Kamiyama et al. 2010). Accordingly, these selection criteria could affect the expression level of CD133 through a postulation that colorectal cancer has different histopathologic subtypes with different expression level of CD133.

The current study revealed that CD133 expression was not correlated with any of the clinico-pathological parameters including histologic types, TNM stage, vascular invasion and distant metastasis in the series of TMA examined. Similarly, Kojima *et al.* examined 189 cases of CRC, and showed that CD133 expression is not correlated with any of the clinicopathological features such as histology, LN metastasis, distant metastasis, liver metastasis, tumour stage and vascular invasion. However, when the series was stratified, a significant correlation was found between CD133 expression in well and moderately differentiated adenocarcinoma and distant metastasis (Kojima, Ishii et al. 2008). In line with our data, many studies in colorectal cancer showed that CD133

expression was not associated with any of the clinicopathological characteristics (Horst, Kriegl et al. 2008; Choi, Lee et al. 2009; Horst, Kriegl et al. 2009; Li, Li et al. 2009; Lugli, Iezzi et al. 2010). On the other hand, a previous study of CD133 expression in human colorectal cancer showed correlation between CD133 expression and lymph node metastasis and liver metastases (Horst, Scheel et al. 2009). In another study, CD133 was correlated with tumour differentiation, lymphovascular invasion and tumour stage (Wang, Chen et al. 2009). In addition, Takahashi *et al.* reported that cytoplasmic expression of CD133 was correlated with TNM stage, vascular invasion and lymphatic invasion, while membranous staining of CD133 was significantly correlated with histology of the tumour (Takahashi, Kamiyama et al. 2010). In our study the cytoplasmic expression of CD133 was noted in only a small numbers of cases, which will lead to lowering the power of the test when compared to any of the clinicopathological variables and lead to inaccurate interpretation of the results.

Interestingly enough, in this study, although the frequency of CD133 high expression in this large series of colorectal cancer was only 8.5%, CD133 high expression was found to be associated with shorter OS compared to CD133 negative/low expression cases, although it was marginally significant ($p=0.05$). Moreover, multivariate analysis revealed that CD133 is an independent predictor factor for survival. In agreement with these data, many studies in CRC have reported the same findings (Horst, Kriegl et al. 2008; Horst, Kriegl et al. 2009; Horst, Scheel et al. 2009; Li, Li et al. 2009;

Wang, Chen et al. 2009; Takahashi, Kamiyama et al. 2010). The association of CD133 high expression with shorter survival could be due to the following reasons. First, CD133 expression may confer resistant phenotype to the conventional treatment (chemo-radiotherapy). Supporting this notion, in colorectal cancer Ong *et al.* found that CD133 expressing tumours had no survival benefits from 5-fluorouracil chemotherapy (Ong, Kim et al. 2010). Furthermore, an *in vitro* study, revealed that the resistant clone of H29 cell line to 5-fluorouracil and oxaliplatin showed an increase in CD133 expression by 16 and 30 fold increase compared to parent cells, suggesting that CD133 expression could predict resistance to treatment (Dallas, Xia et al. 2009). Second, CD133 expression may be associated with metastasis which is a prognostic factor in colorectal cancer. In support of this speculation, association of CD133 expression with liver metastases, lymph node meatstases, and lymphovascular invasion was reported in many studies (Horst, Scheel et al. 2009; Wang, Chen et al. 2009; Takahashi, Kamiyama et al. 2010). On the contrary, two studies reported that there was no association between CD133 expression and survival in colorectal cancer (Choi, Lee et al. 2009; Lugli, Iezzi et al. 2010).

Such discrepancies between these studies as regarding the association between CD133 expression with the clinicopathological features and clinical patient outcome could be possibly explained by several factors such as differences in number of cases, selection criteria, antibody used, immunohistochmistry protocols, and cut-off values. Some studies were

small sized (less than 90 cases) (Horst, Scheel et al. 2009), the antibody used is different from that of this study, some may pick up only the glycosylated epitope, while ours pick up both glycosylated and non-glycosylated epitopes (Choi, Lee et al. 2009; Wang, Chen et al. 2009). In the Choi *et al.* study, CD133 expression was associated with advanced T stage but not with distant metastasis, lymphatic invasion and vascular invasion, and had no effect on overall survival (Choi, Lee et al. 2009). While Wang *et al.* study showed that CD133 correlated with lymphovascular invasion, tumour differentiation, TNM stage and tumour regression after preoperative radiotherapy and is of prognostic value (Wang, Chen et al. 2009). On the other hand, a study using the same antibody clone as ours showed that CD133 expression was not correlated with any of the clinicopathological variables and associated with poor disease free survival after chemoradiotherapy (Saigusa, Tanaka et al. 2009), while another study showed that CD133 only correlate with the degree of differentiation and stage but not with any other clinicopathological features and associated with poor survival (Takahashi, Kamiyama et al. 2010). The cut-off values determining the level of CD133 whether high or low were different from that used in this study (Choi, Lee et al. 2009; Wang, Chen et al. 2009; Lugli, Iezzi et al. 2010). Accordingly, it is worth considering optimization of cut-off point selection by using either receiver-operating characteristic (ROC) curve analysis (Zlobec, Steele et al. 2007), or an X-tile program (Camp, Dolled-Filhart et al. 2004). The latter is used to determine an outcome-based cut point and is widely used in

many studies (Aleskandarany, Rakha et al. 2010; Stratford, Bentrem et al. 2010). Therefore, a rationale should be applied in particular regarding the cut-off point and the antibody used; moreover, the prognostic and predictive value of CD133 should be confirmed in a large prospective study Comparing the association of CD133 expression with the clinicopathological parameters and patient clinical outcome in colorectal cancer and other human solid tumours, also, it was found that CD133 has no correlation with the clinicopathological features in ovarian cancer (Ferrandina, Martinelli et al. 2009), endometrial cancer (Nakamura, Kyo et al. 2010), breast cancer (Liu, Li et al. 2009), and lung cancer (Li, Zeng et al. 2010). In contrast, in pancreatic cancer, Maeda *et al.* reported an association between CD133 expression and histologic type, lymphatic invasion and lymph node metastases (Maeda, Shinchi et al. 2008). In addition, in hepatocellular carcinoma a significant association was reported between CD133 expression and tumour grade and stage (Song, Li et al. 2008). Similarly, an association between CD133 and some of the clinicopathological features was detected in gastric cancer (Ishigami, Ueno et al. 2010).

Likewise in many human solid tumours such as glioma (Zeppernick, Ahmadi et al. 2008), endometrial cancer (Nakamura, Kyo et al. 2010), hepatocellular carcinoma (Song, Li et al. 2008; Sasaki, Kamiyama et al. 2010), cholangiocarcinoma (Shimada, Sugimoto et al. 2010), and gastric cancer (Ishigami, Ueno et al. 2010; Zhao, Li et al. 2010), CD133 was an

independent prognostic factor for low patient survival. Contradicting these data, in ovarian cancer (Ferrandina, Martinelli et al. 2009), and lung cancer (Salnikov, Gladkich et al. 2010), CD133 was reported to be of no prognostic value for patient survival. Arising out of these findings, CD133 expression has a prognostic role in colorectal cancer and many other solid tumours that could have a role in the clinical setting.

Comparing CD133 expression level in 45 cases of matched primary CRC and corresponding liver metastases demonstrated that no significant difference in the expression level between primary and metastatic cases was found. This similar level of CD133 is in line with the cancer stem cell model (Brabletz, Jung et al. 2005). According to this model, within the tumour there are two types of cancer stem cells, stationary cancer stem cells present at the main bulk of the tumour and migrating cancer stem cells found at the invasive front where they undergo epithelial mesenchymal transition with the help of environmental signals. These migrating cancer stem cells when reaching the metastatic site they revert to the stationary state and differentiate due to loss of the microenvironment signals (Brabletz, Jung et al. 2005). Also, it was noted that the frequency of CD133 high expression was 33.3% and 28.9% in liver metastases and primary lesions respectively. This incidence was contradictory to what was detected in the other TMA series examined, and in line with other studies (Horst, Kriegl et al. 2008; Horst, Kriegl et al. 2009). The difference in the incidence of CD133 high expression between the two TMA series

examined could be attributed to different sample size (45 cases compared to 449 cases), and different histologic types of colorectal cancer included in both studies and different stages in the larger TMA series.

Cytoplasm expression of CD133 was detected in few cases in both TMA series examined in this study. Similar to these findings, cytoplasmic expression of CD133 was reported in other solid tumours such as hepatocellular carcinoma (Song, Li et al. 2008), pancreatic cancer (Maeda, Shinchi et al. 2008) and brain tumors (Zhang, Song et al. 2008). Recently, a cytoplasmic staining was also detected in colorectal cancer (Takahashi, Kamiyama et al. 2010). The shift from membranous to cytoplasmic localization has been also reported in CD24 which was defined as a CSC surface marker (Al-Hajj, Wicha et al. 2003; Weichert, Denkert et al. 2005). Weichert *et al.* postulated that CD24 relocalization may reflect overproduction or disturbance of distribution of CD24 or the transition of cells to more invasive phenotype. (Weichert, Denkert et al. 2005). On account of these speculations, the shift of CD133 localization from the membrane to the cytoplasm may also reflect different cellular function or transition of cells to more invasive phenotype. The later speculation was supported with different studies which demonstrated that CD133 cytoplasmic staining correlated with tumour aggressiveness such as vascular invasion, lymph node metastasis, and high grade tumours (Sasaki, Kamiyama et al. 2010; Takahashi, Kamiyama et al. 2010). Although, cytoplasmic staining was detected in a small number of cases in

this study, it is worth considering that a larger study with more number of cases might help to identify such correlations more obviously.

To sum up, the current study demonstrated that CD133 is an independent prognostic factor for short overall survival in colorectal cancer, but with the same expression level in both primary tumours and corresponding liver metastases cases. On account of these findings, improved understanding of the functional role of CD133 and its mechanistic basis and/or regulation may disclose a role as a therapeutic target in colorectal cancer. Therefore, it is of utmost important to study the characteristics of CD133 expressing cells and try to shed some light on the mechanistic basis or regulation of CD133. This will be the context of next chapters.

4 Chapter 4: Evaluation of CD133 expression in colorectal cancer cell lines

4.1 Abstract

Background and aims: Based on the results presented in the previous chapter suggesting that CD133 may have a role in the prognosis of colorectal cancer patients and that it was an independent prognostic factor, it was of interest to investigate the biological characteristics of CD133 expressing cells *in vitro*, and try to shed light on some details of the mechanisms that govern CD133 action. The aim of this part of the project was to evaluate CD133 expression in a series of colorectal cancer cell lines both on CD133 mRNA and protein expression levels. Furthermore, since the origin of many of these was known, it was of interest to evaluate differences in CD133 expression between those derived from primary tumours and those derived from metastases.

Methods: 29 colorectal cancer cell lines were assessed for CD133 mRNA level using QRT-PCR, while the protein level was evaluated in 10 selected colorectal cancer cell lines based on their CD133 mRNA expression using flow cytometry.

Results: CD133 mRNA expression was detected in 24/29 colon cancer cell lines with variable levels of expression. CD133 protein expression was detected in seven out of ten selected colorectal cancer cell lines that showed variable levels of CD133 mRNA expression. Interestingly, CD133 mRNA expression was statistically significantly higher in CRC cell lines derived from metastases than those derived from primary tumours (p = *0.009, Mann-Whitney test*). Moreover, correlating CD133 mRNA levels with

CD133 cell surface protein expression by flow cytometry was found to be statistically significant (r_s = *0.831; p = 0.003, Spearman rank correlation coefficient test*).

Conclusion: CD133 was variably expressed in cell lines and may be associated with the process of metastasis.

4.2 Introduction

Growing evidence supports the new concept of cancer stem cells that may have implications in terms of developing novel diagnostic and therapeutic approaches. In this context, several molecules have been used to isolate tumor initiating cells in haematopoietic and several solid tumors (Singh, Clarke et al. 2003; Richardson, Robson et al. 2004; Suetsugu, Nagaki et al. 2006)

CD133, is a cell surface molecule, has been recently used to identify the potential tumor initiating cells in colon cancer (O'Brien, Pollett et al. 2007; Ricci-Vitiani, Lombardi et al. 2007). O'brien *et al.* and Ricci-Vitiani *et al.* demonstrated that CD133+ population were highly enriched with tumor initiating cells and had the abilities to self-renew and were highly tumourigenic (O'Brien, Pollett et al. 2007; Ricci-Vitiani, Lombardi et al. 2007). Furthermore, these cells were resistant to chemotherapy and were able to retain tumourigenicity when growing as tumor spheres *in vitro* (Ricci-Vitiani, Lombardi et al. 2007; Todaro, Alea et al. 2007).

Results presented in chapter 3, together with data from other groups, suggested a prognostic role of CD133 expression in colon cancer (Horst, Kriegl et al. 2008; Kojima, Ishii et al. 2008) as well as other tumors (Tae Lee, Ho Jang et al. 2001; Zeppernick, Ahmadi et al. 2008). Therefore, it was of interest to evaluate the biological characteristics of CD133 expressing cells in colon cancer and try to delineate the mechanistic basis of control of CD133 and its activity. For achievement of these aims,

colorectal cancer cell lines were used for assessment of characteristics of CD133 expressing cells. As a preliminary step for this study, colorectal cancer cell lines were first evaluated for CD133 mRNA and protein expression levels in order to choose the cell lines suitable for functional studies.

In this part of the project, RNA extraction from 6 cell lines (SW480, SW620, SW837, DLD1, SKCO1, LS1034) was performed, while RNA of the other 23 cell lines had previously been extracted in the Laboratory. Then, QRT-PCR using cDNA synthesized from the extracted RNA from all 29 CRC cell lines, and 10 normal colon mucosa was applied using CD133 primers, and data were normalized to *HPRT1* (a reference gene). Thereafter, 10 colon cancer cell lines were selected on the basis of CD133 mRNA levels to assess their protein expression levels using flow cytometry analysis. For flow cytometry analysis CD133/1 antibody (AC133, which can detect the glycosylated epitope of CD133) conjugated to R- phycoerythrin (PE) was used.

For detailed reading about RNA extraction, cDNA synthesis, QRT-PCR, labeling of the cells for flow cytometry analysis, refer to material and methods section. Mann-whitney test was used to compare means of CD133 mRNA expression level between the CRC cell lines.

4.3 Results

4.3.1 Colon cancer cell lines characteristics

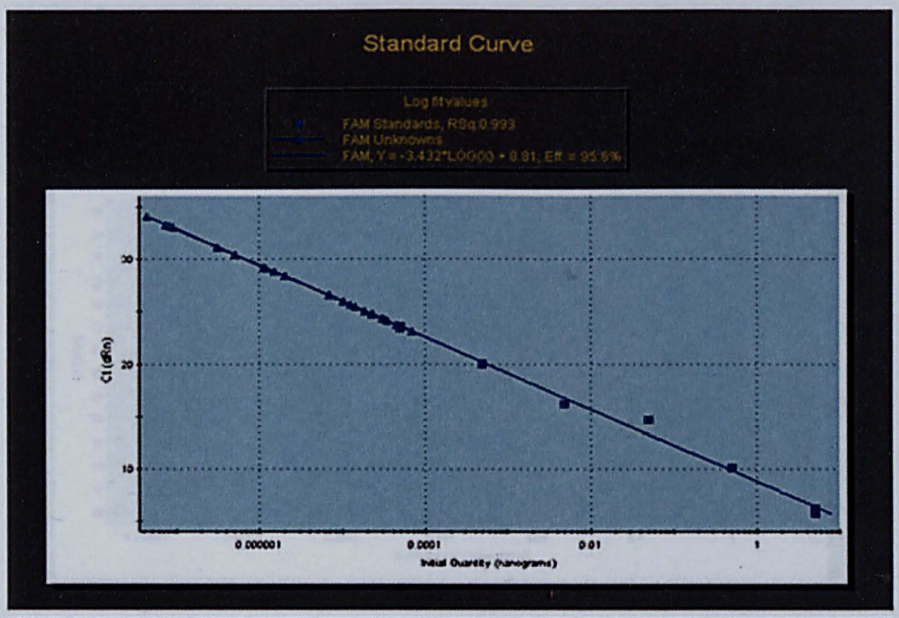
All 29 CRC cell lines were derived from colon cancer patients; six were derived from metastatic sites, 17 were derived from primary tumors, while for the remaining six cell lines no information available regarding the site it was derived from. All information for cell lines used in this study regarding site of origin is collected in Table 4-1.

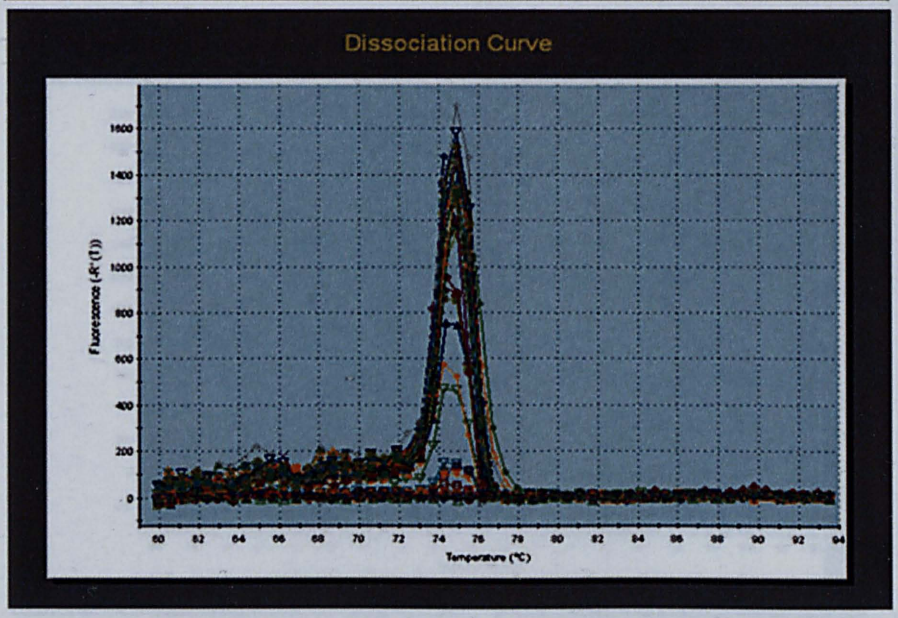
4.3.2 CD133 mRNA expression in colorectal cancer cell lines

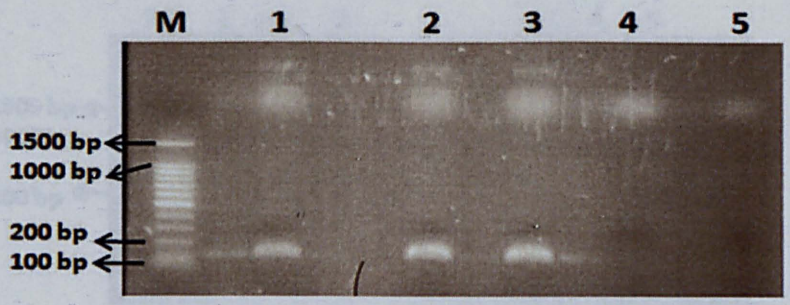
After designing CD133 and HPRT1 primers, their specificity was checked using primer blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). According to primer blast results, it was found that CD133 primer could detect all transcript variants of CD133. Prior to QRT-PCR experiments, CD133 and HPRT1 annealing temperatures were optimized first, and then the temperatures which produced high quality amplicon were used for generating standard curves to obtain the best PCR efficiency for CD133 and HPRT1 primers (refer to material and methods section 2.8 for optimum annealing conditions). For standard curves, serial 1:10 dilution of CD133 IMAGE clone (accession No.BC012089, IMAGE:4644690), and HPRT1 plasmid constructed by Dr. AbdulKadder Albasri (PhD student, Pathology Department) were used (figure 4-1). Employing QRT-PCR evaluation of CD133 mRNA level and its normalization to the reference gene HPRT1 mRNA levels revealed that 29 colon carcinoma cell lines showed variable levels of CD133 mRNA expression ranging from high, moderate to low and

negative expression relative to each other. A CD133 mRNA/HPRT mRNA ratio of < 0.2 was referred to as low/negative expression; a ratio between 0.2-0.8 was referred to as moderate expression, whilst a ratio > 0.8 was considered as high expression (Figure 4-2). Collectively, CD133 mRNA expression in colorectal cancer cell lines was lower than that in normal colonic mucosa samples. Table 4-2 showed the normalization data for CD133 mRNA in all cell lines. Moreover, it was noted that CD133 expression was more abundant in the cell lines derived from metastases than those derived from the primary cell lines. Histograms showing the expression of CD133 mRNA expression plotted against the number of cases for primary and metastatic CRC cell lines are shown in figure 4-3A. Relationship amongst expression of CD133 mRNA levels and site of origin of CRC cell lines was statistically significant (*p=0.009; Mann-whitney test*), such relationship was illustrated using box plot in figure 4-3B.

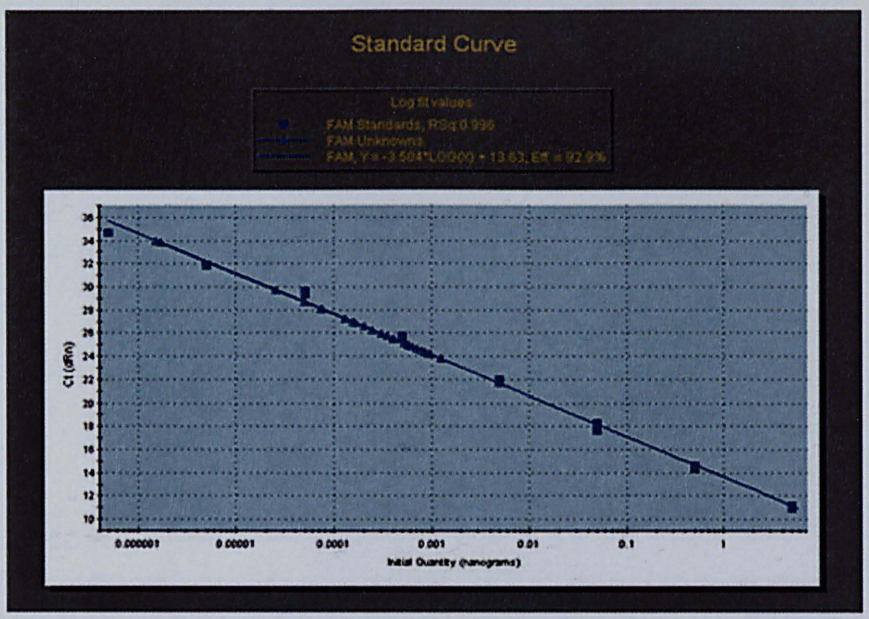
CD133

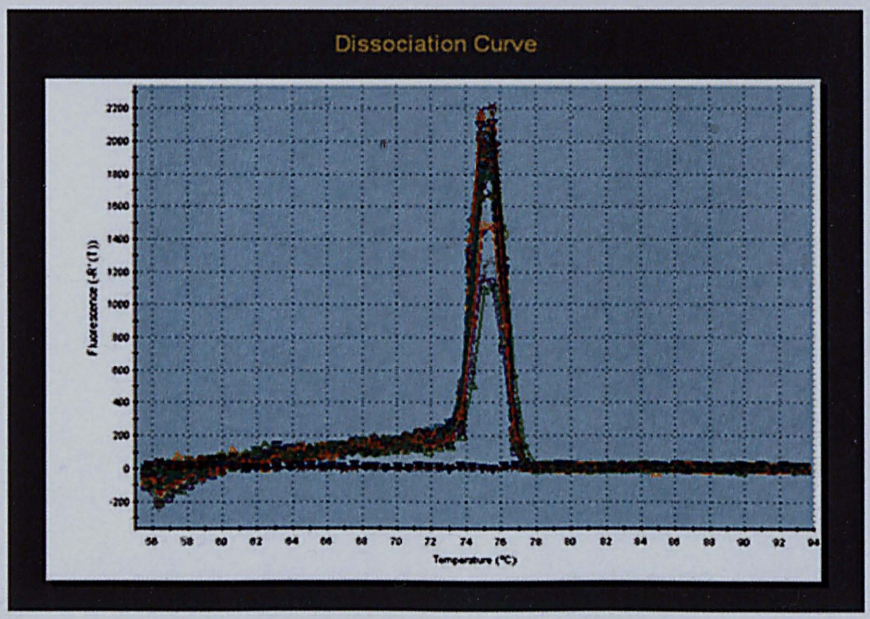

Standard Curve
Log fit values
FAM Standards, RSq:0.993
FAM Unknowns
FAM, Y = -3.432*LOG(X) + 8.81, Eff = 95.6%
Ct (dRn)
Initial Quantity (nanograms)


Dissociation Curve
Fluorescence (-R'(T))
Temperature (°C)


M
1
2
3
4
5
1500 bp
1000 bp
200 bp
100 bp

HPRT1


Standard Curve
Log fit values
FAM Standards, RSq 0.996
FAM Unknowns
FAM, Y = -3.504*LOGQ + 13.63; Eff = 92.9%
Ct (dRn)
Initial Quantity (nanograms)


Dissociation Curve
Fluorescence (-R' (T))
Temperature (°C)

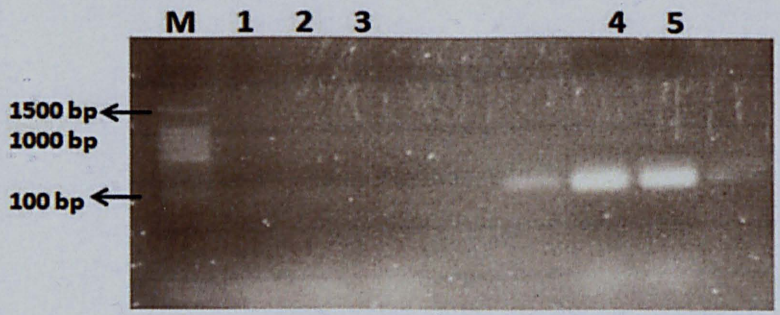

M
1
2
3
4
5
1500 bp
1000 bp
100 bp

Figure 4-1: Efficiency and specificity of CD133 and HPRT1 primers.
The upper row of this graph represents the efficiency of CD133 and HPRT1 primers which has been found 95.6% and 92.9% for CD133 and HPRT1, respectively. The middle row represents the melting curve which shows a single peak indicating one PCR product. The lower row showed agarose gel electrophoresis illustrating PCR product of both CD133 and HPRT1 was at the correct expected size which is 119 bp for CD133 and 122 bp for HPRT1. In agarose gel for CD133 (Lanes 1-3) sample containing DNA template, and (Lanes 4-5) were non-template control (NTC), while in case of HPRT1 (lanes 1-3) were NTC, and Lanes 4-5 were containing DNA template. 100 bp DNA ladder was used in both cases (M).

Table 4-1: Origin of cell lines used in this study

Cell line	Derived site
C84	UNK
C80	UNK
C32	UNK
C125	UNK
C106	UNK
SW620	Metastatic site (lymph node)
SKCO1	Metastatic site (ascites)
Lovo	metastatic site (left supraclavicular region)
GP2D	Recurrent tumor
Colo205	Metastatic site (ascites)
SW837	Primary
SW480	Primary
SW1222	primary
SW1160	Primary
RKO	Primary
LS1034	Primary
HUTU80	Primary
HT55	Primary
HT29	Primary
HRA19	Primary
HCT116	Primary
HCA7	Primary
HCA46	Primary
DLD1	Primary
Colo320	primary
CACO2	Primary
VACO5	Primary
SW948	Primary

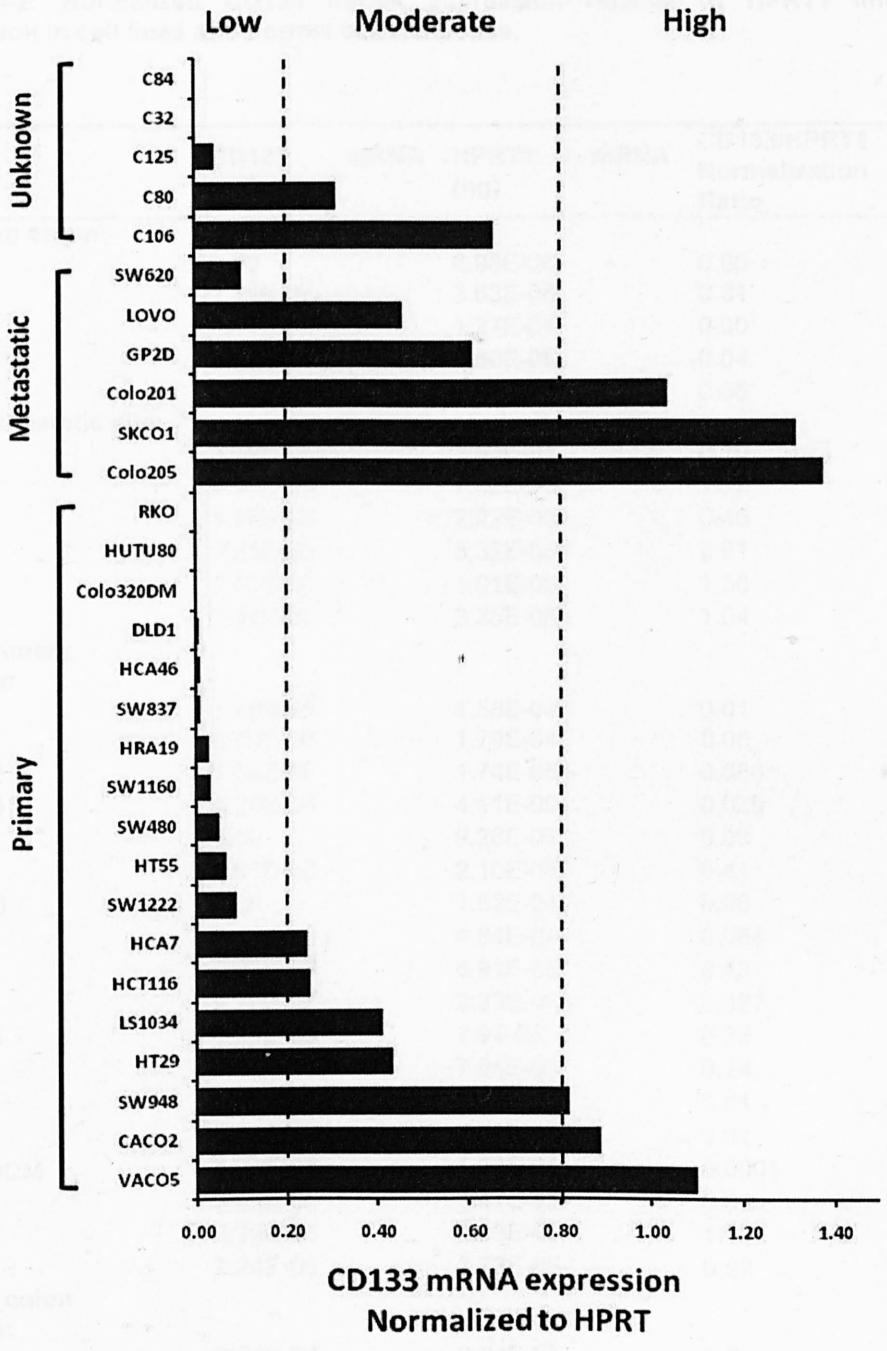


Figure 4-2: CD133 mRNA expression in different cell lines.
QRT-PCR experiment was employed to assess the CD133 mRNA expression level in 29 colorectal cancer cell lines. After normalization to HPRT1, data revealed that CD133 mRNA expression was varied among cell lines, and ratio of < 0.2 was referred to as low/negative expression; a ratio between 0.2-0.8 was referred as to moderate expression, whilst a ratio > 0.8 was considered as high expression, CD133 mRNA was higher in cell lines derived from metastatic sites compared with those derived from primary lesions.

Table 4-2: Normalized CD133 mRNA expression relative to HPRT1 mRNA expression in cell lines and normal colon mucosa.

Cell line	CD133 mRNA (ng)	HPRT1 mRNA (ng)	CD133/HPRT1 Normalization Ratio
Unknown origin:			
C84	0.00	6.98E-06	0.00
C80	1.14E-06	3.63E-06	0.31
C32	3.13E-07	1.27E-04	0.00
C125	2.03E-06	4.80E-05	0.04
C106	6.87E-05	1.04E-04	0.66
From metastatic site:			
SW620	3.59E-05	2.93E-04	0.10
SKCO1	9.90E-05	7.52E-05	1.32
LOVO	1.02E-05	2.22E-05	0.46
GP2D	3.25E-05	5.32E-05	0.61
Colo205	1.40E-05	1.01E-05	1.38
Colo201	3.48E-05	3.35E-05	1.04
From primary tumours:			
SW837	1.76E-06	1.58E-04	0.01
SW480	8.16E-06	1.79E-04	0.05
SW1222	1.50E-06	1.74E-05	0.086
SW1160	1.20E-06	4.11E-05	0.029
RKO	0.00	9.28E-05	0.00
LS1034	8.61E-06	2.10E-05	0.41
HUTU80	0.00	1.62E-04	0.00
HT55	3.10E-05	4.64E-04	0.068
HT29	3.00E-05	6.91E-05	0.43
HRA19	6.30E-07	2.33E-05	0.027
HCT116	1.99E-05	7.94-05	0.25
HCA7	1.86E-05	7.64E-05	0.24
HCA46	8.96E-08	1.04E-05	0.01
DLD1	5.14E-07	7.63E-05	0.01
Colo320DM	4.34E-08	1.26E-04	0.0001
CACO2	3.09E-05	3.47E-05	0.89
VACO5	2.29E-05	2.08E-05	1.10
SW948	2.24E-05	2.73E-05	0.82
Normal colon mucosa:			
N	2.67E-04	2.74E-05	9.7
N	1.85E-04	3.15E-05	5.9
N	1.15E-04	2.30E-05	5.0
N	6.54E-04	3.01E-05	21.8
N	3.19E-04	5.28E-05	6.0
N	5.24E-04	2.84E-05	18.4
N	1.22E-03	2.55E-05	47.8
N	1.63E-03	2.23E-05	73.1
N	2.13E-04	2.26E-05	9.4
N	2.56E-04	3.32E-05	7.7

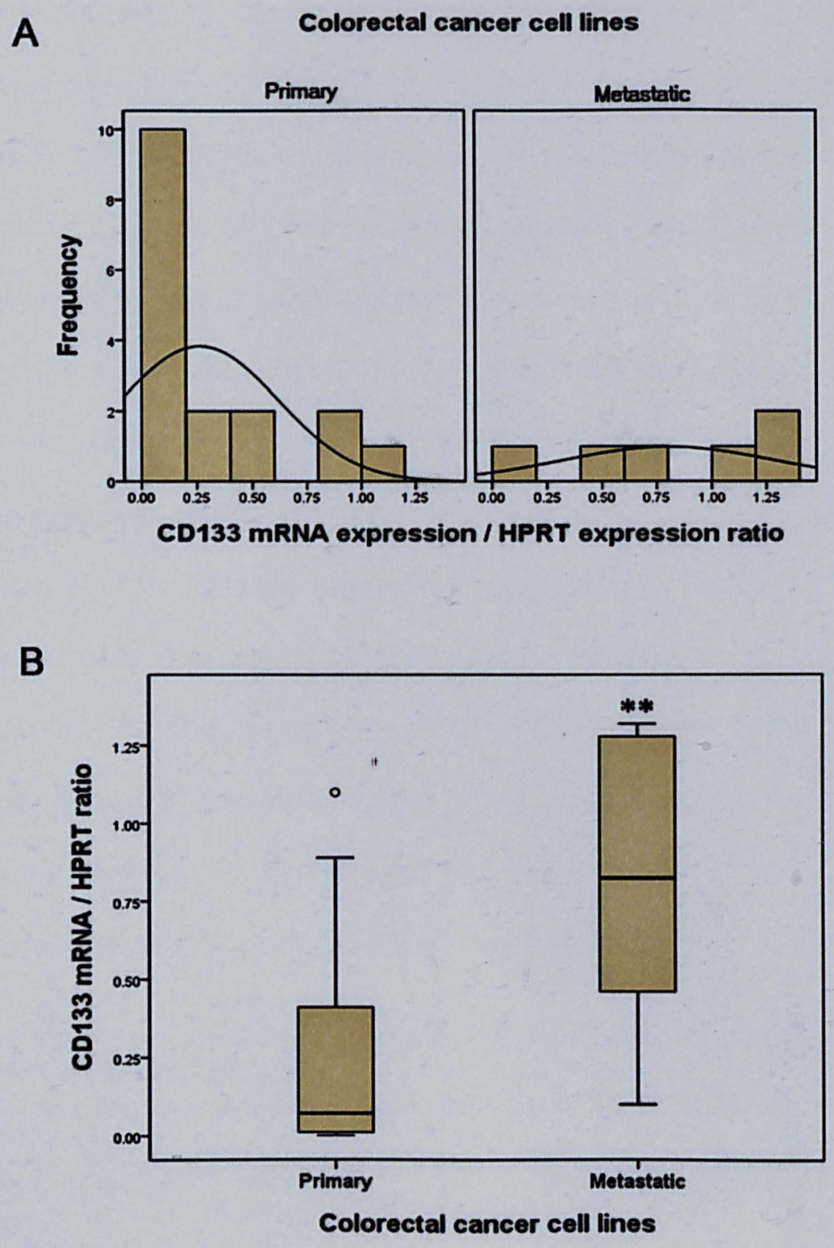


Figure 4-3: Distribution and relationship of CD133 mRNA expression in both primary and metastatic colorectal cancer cell lines.

(A) A histogram showing the distribution of CD133 mRNA / HPRT1 mRNA ratio plotted against the number of primary and metastatic colorectal cancer cell lines, (B) A box plot showing the relationship between primary and metastatic colorectal cancer cell lines and relative CD133 mRNA expression *(** p = 0.009; Mann-whitney test)*, the middle line in each box representing the median value.

4.3.3 Flow cytometry analysis of CD133 expression

Fifty thousand events were collected using flow cytometry to quantify the proportion of CD133 expressing cells in 10 CRC cell lines selected on the basis of CD133 mRNA expression level (which represent low, medium and high expression) in order to assess whether CD133 protein expression conforms to that of mRNA expression. Flow cytometry analysis revealed that three cell lines (HRA19, DLD-1, and SW837) were negative for CD133 expression, while in Caco2 and HT-29 cell lines, CD133 + populations were more than 90%. In the remaining cell lines, a bimodal population was present with a CD133+ populations ranging from 32% - 64% (Table 4-3), and (figure 4-4, and 4-5). Cell surface expression of CD133 protein mirrored by the percentage of CD133 positivity measured by flow cytometry in 10 colorectal cancer cell lines was found to be correlated with CD133 mRNA level measured by qRT-PCR (r_s = *0.831, p= 0.003, spearman rank correlation coefficient test*) (figure 4-6).

Table 4-3: Flow cytometry analysis of CD133 cell surface protein expression in 10 colorectal cancer cell lines.

The percentage represents means of two independent experiments with three replicates each ± SD

Colorectal cancer cell line	CD133 expression (%) ± SD
SW480	42 ± 0.19
SW620	67 ± 0.31
Caco2	97 ± 0.35
HT29	95 ± 0.25
LoVo	64 ± 0.26
LS1034	32 ± 0.31
DLD1	0
SW837	0
HCT116	40 ± 0.25
HRA19	0

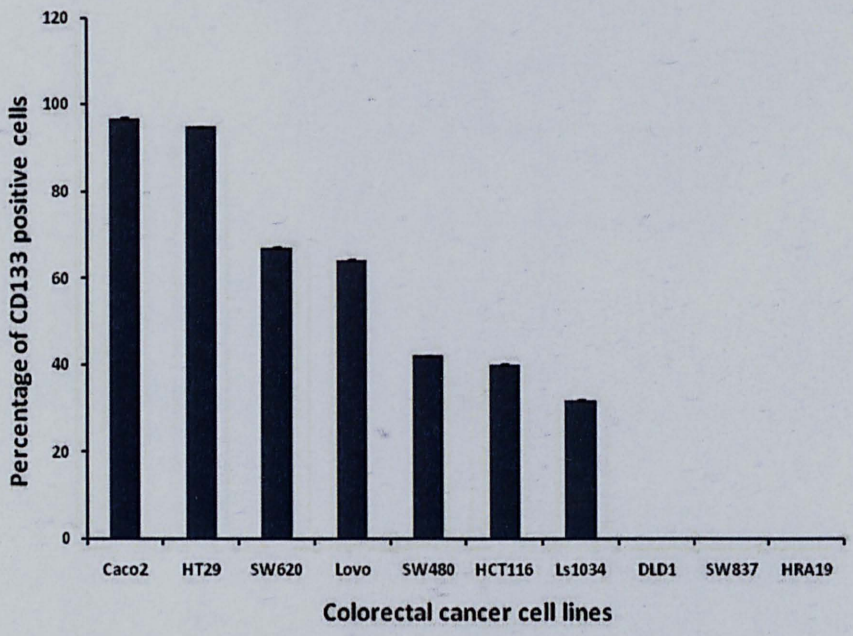


Figure 4-4: Histogram illustrating CD133 expression.
Evaluation of CD133 expression by flowcytometry in 10 colorectal cancer cell lines showed that three cell lines were negative for CD133 expression whilst the remainder showed variable levels of expression.

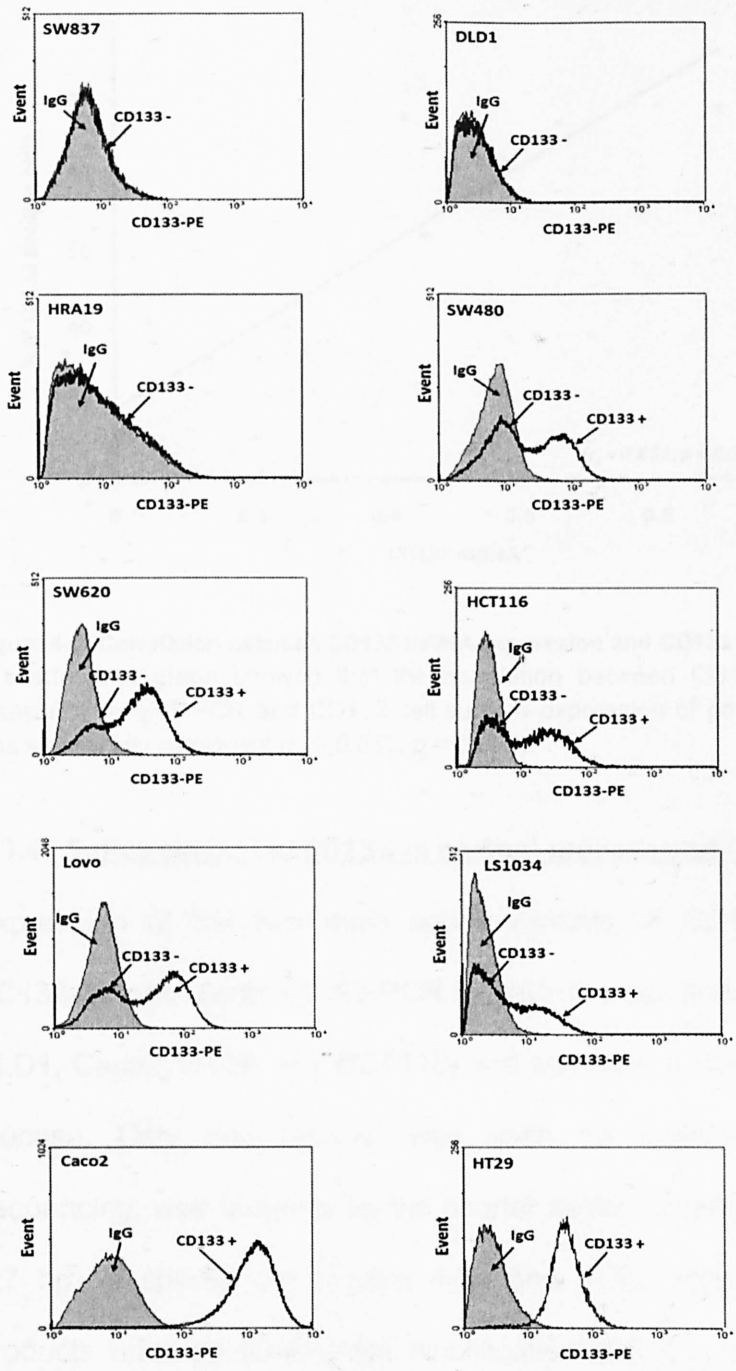


Figure 4-5. Cell surface positivity of CD133 in colorectal cancer cell lines.
Results of flow cytometry showed variable CD133 positivity in colorectal cancer cell lines where DLD1, SW837, and HRA19 (CD133-), HT29 and Caco2 (CD133+), and SW480, SW620, Lovo, HCT116, LS1034 (both CD133+/-). Filled histogram (gray) represents IgG isotype control, and CD133-PE labeled cells illustrated with solid black line. Each is a representative graph of two independent experiments with three triplicate each.

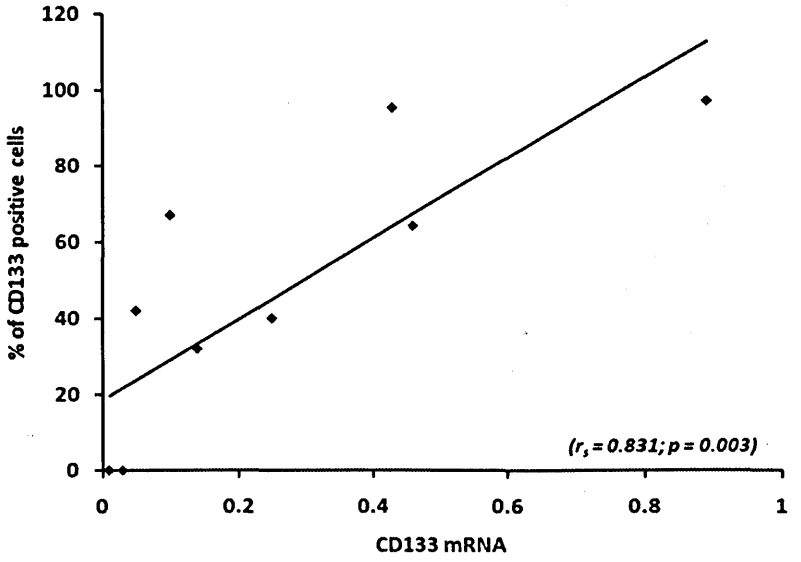


Figure 4-6: Correlation between CD133 mRNA expression and CD133 protein.
A scatter blot graph showed that the correlation between CD133 mRNA expression measured by qRT-PCR and CD133 cell surface expression of protein by flow cytometry was statistically significant ($r_s = 0.831$; $p = 0.003$)

4.3.4 Splice variant of CD133 in normal mucosa and CRC cell lines

Expression of the two main splice variants of CD133 (AC133-1 and AC133-2) was tested by RT-PCR in both the cell lines (SW480, SW620, DLD1, Caco2, HT29, and HCT116) and samples of Human normal colonic mucosa. Only one product was seen on agarose gels which, on sequencing, was found to be the shorter splice variant from which exon 4 (27 bp) is spliced out (Figure 4-7a and 4-7c). However, shorter PCR products undergo preferential amplification and it is possible that larger products may be missed by both agarose gel electrophoresis and sequencing (especially if present at low levels). The analysis was refined by performing PCR using SYBR green as a reporter dye to provide a more sensitive method of determining whether a single or multiple PCR products

are present. Evaluation of the dissociation curve showed the presence of a single PCR product only (Figure 4-7b).

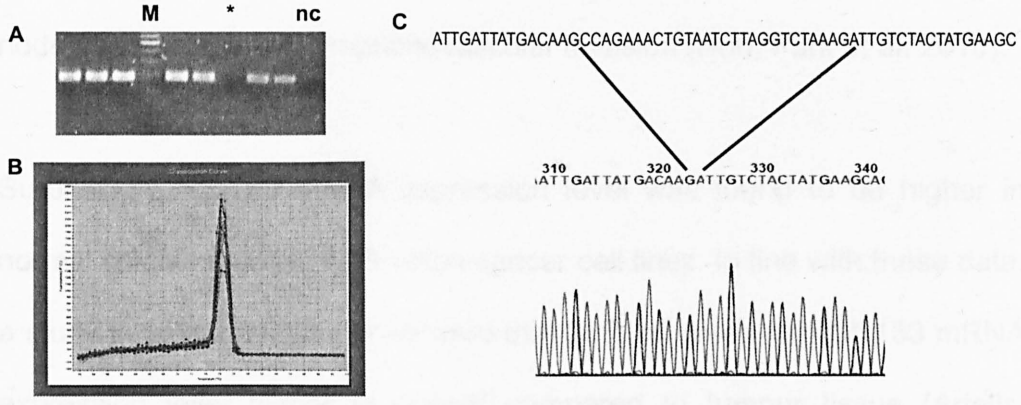


Figure 4-7: Splice variants of CD133.
Cell lines and normal mucosal samples were tested for expression of the two main CD133 splice variants by RTPCR with primers anchored on either side of the spliced out exon (exon 4). Sample data are shown which demonstrate only a single product was identified when PCR products were analysed on both (a) agarose gel and (b) the more sensitive Q-PCR technique using Sybr green as a reporter.M = size marker, nc = negative control, * represents DLD1. (c) Sequencing of the products showed that exon 4 was spliced out of the coding sequence; thus only the shorter splice variant that lacks exon 4 was detected. The sequence above the electropherogram is of AC133-1 with exon 4 shown between the lines.

4.4 Discussion

The present study evaluated CD133 mRNA and protein expression in human colorectal cancer cell lines. Assessment of CD133 mRNA expression in the cell lines by QRT-PCR revealed that CD133 expression was variable ranging from high, medium, to low and negative expression relative to each other. These findings are consistent with previous studies that examined CD133 expression in a different number of CRC cell lines by QRT-PCR, and showed the same variable expression levels amongst cell lines (Ieta, Tanaka et al. 2008; Jeon, Kim et al. 2010). It is of interest that a

significant difference in CD133 mRNA expression amongst primary and metastatic colon cancer cell lines was noted. In line with these data, Huh *et al.* demonstrated that CD133 mRNA expression was correlated with lymph node involvement and lymphohovascular invasion (Huh, Park et al. 2010).

Surprisingly, CD133 mRNA expression level was found to be higher in normal colonic mucosa than colon cancer cell lines. In line with these data, a study in colorectal cancer showed that 30% of cases had CD133 mRNA expression level higher in normal compared to tumour tissue (Artells, Moreno et al. 2010). Indeed, the higher level of CD133 mRNA in normal tissue compared to the CRC cell lines could be attributed to several plausible explanations. First, these normal colon samples could contain endothelial cells, and hematopoietic cells in addition to epithelial cells that might express CD133 mRNA, where CD133 was reported as a marker for haematopoietic stem cells and endothelial progenitor cells (Bonnet and Dick 1997; Fan, Li et al. 2003). However, if it is not the case, a second plausible explanation is that normal colon mucosa during differentiation could generate a small truncated variant of CD133 mRNA transcript in addition to the normal full length CD133 transcript, and this variant was lost during the process of tumorigenesis. Supporting this postulation, Osmond *et al.*, demonstrated that in Glioblastoma cell line anti-CD133 antibody (Ç24B9) which detect epitope on the second extracellular loop could detect a small truncated variant of the CD133 protein of molecular weight about 16kD in addition to the full length protein of molecular weight about 120 kd

by western blot analysis (Osmond, Broadley et al. 2010). Further support this postulation came from Corbeil *et al.* study who reported a small increase in CD133 mRNA level associated with down-regulation of AC133 antigen upon differentiation of Caco2 cell line (Corbeil, Roper et al. 2000), which also could be explained by the findings of Osmond *et al.* study (Osmond, Broadley et al. 2010). A third reasonable explanation of the higher level of CD133 mRNA in normal colon samples compared to CRC cell lines is the presence of unmethylated *CD133* promoter region in normal colon whilst the CRC cell lines contain hypermethylated *CD133* promoter area, where hypermethylaton of *CD133* promoter suppress its expression (Shmelkov, Jun et al. 2004). Supporting this speculation, Yi *et al.* revealed the absence of *CD133* promoter methylation in normal brain and colon samples, whereas a higher incidence of hypermethylation of *CD133* promoter was detected in (10/16 cases-62%) of CRC cell lines and (14/15; 93%) of GBM cell lines (Yi, Tsai et al. 2008).

Flow cytometry analysis of human colorectal cancer cell lines displayed variable patterns of cell surface CD133 protein expression with some cell lines lacking CD133 expression, others scoring as predominantly positive, and others as a mixture of CD133 positive and CD133 negative cells. Similar patterns have been reported in colon (Ieta, Tanaka et al. 2008), ovarian (Baba, Convery et al. 2009) and liver cancer cell lines (Ma, Chan et al. 2007). In addition, CD133 protein expression was also higher in metastatic cell lines (SW620, and LoVo) than the primary cell lines,

confirming the data obtained by QRT-PCR. Flow cytometry analysis of CD133 expression using human clinical colon cancer samples in two previous studies revealed that the percentage of CD133+ cells within the human clinical samples ranged from 2-25% (O'Brien, Pollett et al. 2007; Ricci-Vitiani, Lombardi et al. 2007), whilst, in the current study using colon cancer cell lines, cell surface expression of CD133, mirrored by the percentage of positive cells, ranged from negative, 32-64%, and > 90%. Such a difference in results may be due to different types of samples used; in this study human colorectal cancer cell lines were used while in other studies clinical samples were used. Moreover, the difference in expression level between cell lines and clinical samples could be attributed to passaging selection of either CD133+ or CD133 negative cells leading to remaining of the selected progeny in cell lines than in clinical samples or to the gene expression changes as a result of long term culture.

Although a wide variety of splice variants have been described for CD133, only one (termed AC133-1 and which was first to be cloned) has been allocated a Reference Sequence number. This contains the full length coding sequence whilst a second splice variant (AC133-2, the second to be cloned) appears to splice out exon 4. Our analysis of 8 CRC cell lines and 10 samples of normal mucosa, using both end-point and real-time methodologies would suggest the presence of a splice variant that lacked 27 base pairs, indicating that the full length CD133 splice variants (AC133-1), Sv6 and SV7 were not expressed in colon and the other splice variants

(Sv2, Sv3, Sv4, and Sv5 that lacks exon 4) were. Given that shorter PCR products are preferentially amplified , the primers used could be amended in a way that preferentially amplifies the splice variant that contains exon 4. This could be done by designing a primer pair in such a way that one of the primer pair spans exon 4 or completely hybridizes to exon 4. Another alternative way is by the use of target specific probes. TaqMan probes targeting exon 4 could be used to specifically detect the presence or absence of exon 4. In such ways we will be more accurate as regarding its detection. A support of our preliminary data comes from the study by Yu *et al.* in which AC133-2 was reported as being the splice variant which is present in many stem cell compartments whilst AC133-1 is limited to foetal brain and skeletal muscle (Yu, Flint et al. 2002). Recently, a study goes online with our finding showed that in colon cancer samples several splice variants were expressed at the mRNA level, but the majority of these mRNA encoded Sv2 (Kemper, Sprick et al. 2010).

To sum up, it appears from this study of human colorectal cancer cell lines, as has been reported in other studies, CD133 mRNA and protein expression was variable among cell lines. Moreover, a significant correlation was found between CD133 mRNA expression level and CD133 protein expression. It was also noted that in metastatic colorectal cancer cell lines, CD133 expression was higher than their primary counterparts. Nevertheless, in clinical samples, CD133 protein expression level was the same in primary tumours and liver metastases cases. Interestingly, CD133

splice variants that lacks 27 bp; not the full length CD133 splice variant 1, Sv6 and Sv7 were found to be expressed in CRC cell lines and normal colon mucosa. The results from this chapter together with the results suggesting CD133 as an independent prognostic factor from chapter 3 highlight the possible role of CD133 in tumor progression. Therefore, it was of interest to study the characteristics of CD133 expressing cells and to shed some light on the mechanistic role for CD133. These characteristics will be studied in the next chapter.

5 Chapter 5: Biological characteristics of CD133 expressing cells in colorectal cancer cell lines

5.1 Abstract

Background and aims: Data in chapter 3, together with other studies revealed that CD133 was of prognostic importance for patients' survival, but the main function of CD133 was still unknown. The previous chapter characterized the expression of CD133 in a series of CRC cell lines. This allowed us to select the appropriate cell lines for this part of the project which was to study the biological characteristics of CD133 expressing cells in colorectal cancer cell lines.

Methods: Two approaches were used to produce CD133+ (test) and CD133- (control) populations. SW480 – a CRC cell line shown to have a bi-phasic population of about 40% CD133+ cells- was isolated by FACS cell sorting into CD133+ and CD133- populations. CD133 was knocked down using small interfering RNA (siRNA) in the CRC cell line HT29 (a CRC cell line shown to have 95% CD133+ cells). Functional studies such as proliferation, migration, colony forming, and staurosporine induced apoptosis assays were then undertaken.

Results: A time course assay showed that CD133 knockdown in HT29 had no significant effect on cell proliferation when compared with scrambled controls. However, CD133 knockdown did result in greater susceptibility to staurosporine-induced apoptosis ($p = 0.01$) and reduction in cell migration (*$p=0.04$, unpaired t-test*). In concordance with knockdown experiments, time course assays performed on the sorted populations of SW480, revealed no significant proliferative differences between the

CD133+ and CD133- groups. Also greater resistance to staurosporine-induced apoptosis (*p = 0.008, unpaired t-test*), greater cell migration ability (*p = 0.03, unpaired t test*) and greater colony forming efficiency was seen in the CD133+ sorted population of SW480 cell line than the CD133- population in both 2D and 3D culture (*p=0.0001 and p=0.003 respectively, unpaired t- test)*. The plasticity of CD133 expression in tumour cells was tested and showed that prolonged culture of a pure CD133- population resulted in re-emergence of CD133+ cells.

Conclusion: Using two separate approaches, the data show that CD133 plays a role in cell motility and colony formation. These characteristics of CD133 expressing cells may be relevant to a role as tumour initiating cells and may support tumour metastasis in colorectal cancers.

5.2 Introduction

A number of studies claim to have isolated CSCs from several different tumour types such as brain (Singh, Clarke et al. 2003; Yuan, Curtin et al. 2004), breast (Al-Hajj, Wicha et al. 2003), colon (O'Brien, Pollett et al. 2007; Ricci-Vitiani, Lombardi et al. 2007), hepatocellular carcinoma (Chiba, Kita et al. 2006) and pancreatic cancer (Li, Heidt et al. 2007). These studies have used putative CSC markers to separate stem cells from differentiating cells within a tumour. One common method of separation is the dye elimination method (i.e. side population (Addla, Brown et al. 2008)) although this has recently been thrown into doubt as a marker for stem cells (Burkert, Otto et al. 2008). Identification of a number of cell surface markers (such as CD24, CD44, and CD166) has allowed use of fluorescence activated cell sorting (FACS) to isolate CSCs (Wright, Calcagno et al. 2008). Recently, CD133 gained attention as marker for isolation of CSCs in colorectal cancer as well as many other human solid tumours (Li, Deng et al. 2006; Suetsugu, Nagaki et al. 2006; Dou, Pan et al. 2007; O'Brien, Pollett et al. 2007; Ricci-Vitiani, Lombardi et al. 2007; Wei, Zhou et al. 2007).

The aim of this study is to further clarify the role of CD133 in CRC. Two approaches were used: (i) CD133 expression was functionally evaluated in HT29 after gene knockdown and (ii) SW480 underwent cell sorting into a

CD133+ population and a CD133- population followed by comparative functional analysis of the two populations.

5.3 Results

5.3.1 CD133 knockdown in HT29 cell line

Gene knock down was achieved by transfecting HT29 (shown to have 95% CD133 positive cells) with CD133 specific synthetic small interfering RNA (siRNA) using the stealth RNAi type. These are henceforth annotated as $HT29^{CD133-}$. Controls consisted of cells transfected with scrambled control stealth RNAi, and henceforth annotated as $HT29^{SSC}$. 72 hours after gene knockdown, flow cytometry analysis revealed that CD133 was 50% downregulated compared to scrambled control (Figure 5-1A). In order to improve the knockdown more than 50%, we compared different amounts of siRNA with final concentrations of 30, 50 and 100 nmol. We found that all different concentration resulted in the same knockdown level of 50%. Therefore, we used the final concentration of 100 nmol for all gene silencing experiments. Evaluation of these data by western blot, in contrast, showed that the protein was virtually undetectable when CD133 specific stealth RNAi was used. In contrast, transfection of the control stealth RNAi had no effect on protein expression (figure 5-1B). Such discrepancy between flow and western data comes from the fact that flow cytometry detects the percentage of positive cells rather than quantification of the amount of protein. Moreover, confirmation of the knockdown was

conducted by applying qRT-PCR analysis of CD133 mRNA level after knockdown (Figure 5-1C)

5.3.2 FACS of SW480 cell line

The SW480 cell line (shown to have 40% CD133 positive cells) was sorted into CD133+ and CD133- populations using FACS technique and underwent immediate re-analysis by flow cytometry to check the purity of the sorted cells. Analysis showed that the CD133+ and CD133- populations were 97.6% and 99.9% pure, respectively (Figure 5-2A). Quantitative PCR showed transcriptional repression of CD133 in the CD133- population and although CD133 mRNA was still detectable, it was only at 15% of the level seen in the CD133+ population (Figure 5-2B).

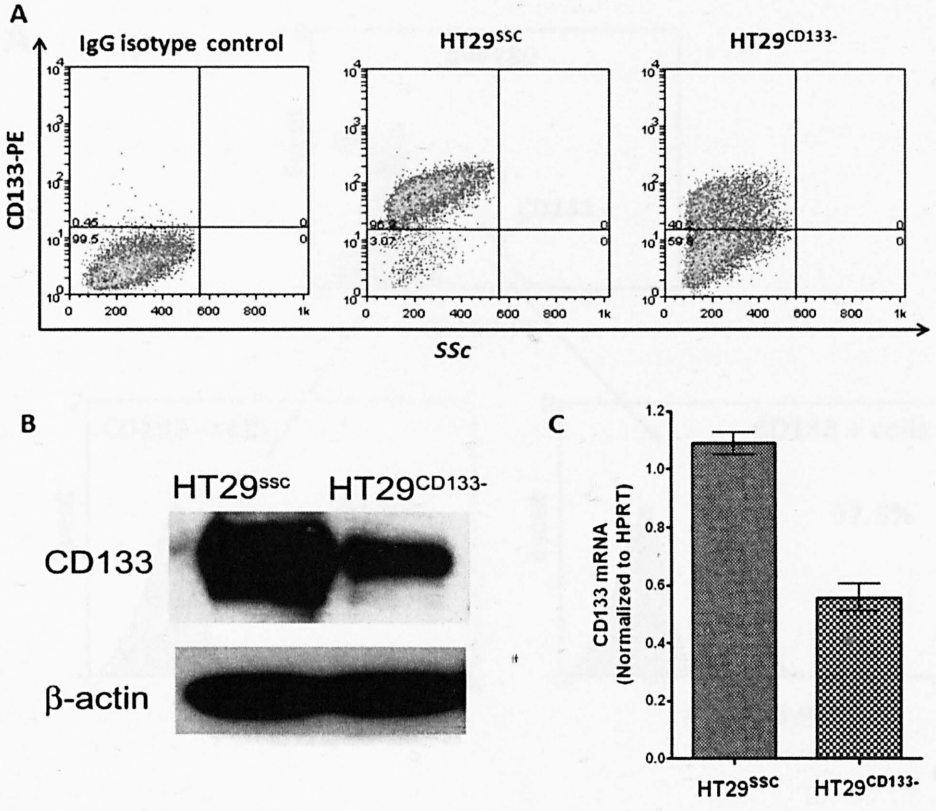


Figure 5-1: CD133 knockdown in HT29 cell line.
HT29 was transfected with either specific stealth RNAi ($HT29^{CD133-}$) or scrambled control stealth RNAi ($HT29^{SSC}$). 72 hours later, (a) Flow cytometry analysis showed down regulation of CD133 expression in $HT29^{CD133-}$ compared to $HT29^{SSC}$ (*SSC* on the dot blot = side scatter). Each graph represent a sample graph of two independent experiments with triplicate each. (b) Western blotting confirms the down regulation of CD133 by gene knock down (β-actin used as an indicator of equal protein loading). (c) QRT-PCR analysis confirm down-regulation of CD133 at the transcriptional level (data were normalized to HPRT1).

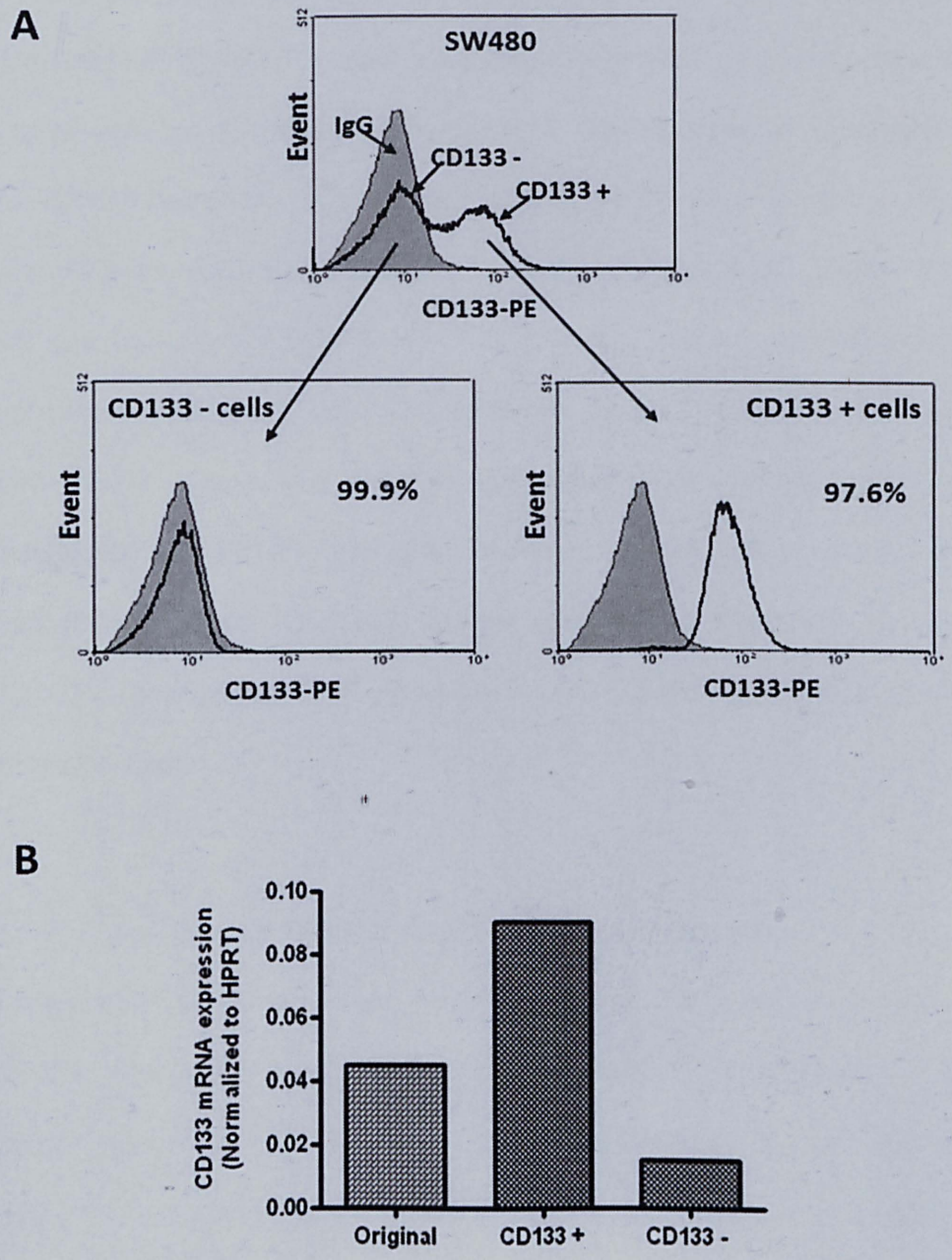


Figure 5-2. Sorting of SW480 cell line.

(a) SW480 was sorted into CD133 positive and negative populations which, on immediate analysis, were shown to be pure populations 97.6% and 99.9% for CD133+ and CD133- populations respectively (filled grey histogram represent IgG isotype control, while CD133-PE labeled cells presented by histogram with solid black line) **(b)** QRT-PCR analysis of the sorted CD133+/- populations showed that low level of CD133 transcript were still detectable in CD133- cells (data were normalized to HPRT1).

5.3.3 Association of CD133 with proliferation

The effect of CD133 expression on the proliferation was tested in a time course experiment using two experimental approaches. In HT29 cell line the CD133 protein was knocked down and cell numbers (as measured by methylene blue staining) monitored for 5 days (Figure 5-3A); whilst SW480 cell line was sorted into CD133+/CD133- populations and cell numbers were monitored over 9 days (Figure 5-3B). Both experiments showed the same results. There was no difference in the proliferation between those transfected with stealth RNAi and scrambled control RNAi. Similarly, there was no significant difference in the proliferation between the sorted CD133+/- populations. This showed that CD133 expression has no effect on proliferation

5.3.4 Association of CD133 expression with cell migration

Comparative analysis of cell motility between cells with high and low CD133 expression was tested by transwell migration assays. Both experimental conditions produced concordant results. Significantly fewer $HT29^{CD133-}$ cells migrated across the membrane than $HT29^{SSC}$ cells (Figure 5-4A, *p= 0.04, unpaired t-test*). Conversely, significantly larger numbers of CD133+ cells migrated than CD133 - cells (Figure 5-4B, *p = 0.03, unpaired t-test*). In order to validate the transwell migration assay a cell wounding assay was also employed following gene knockdown in HT29 which showed that the wound edges were significantly closer in $HT29^{SSC}$ cells

than in HT29^{CD133-} cells (*p<0.001, unpaired t-test*) thereby confirming the relationship between high CD133 expression and increased cell motility (Figure 5-4C).

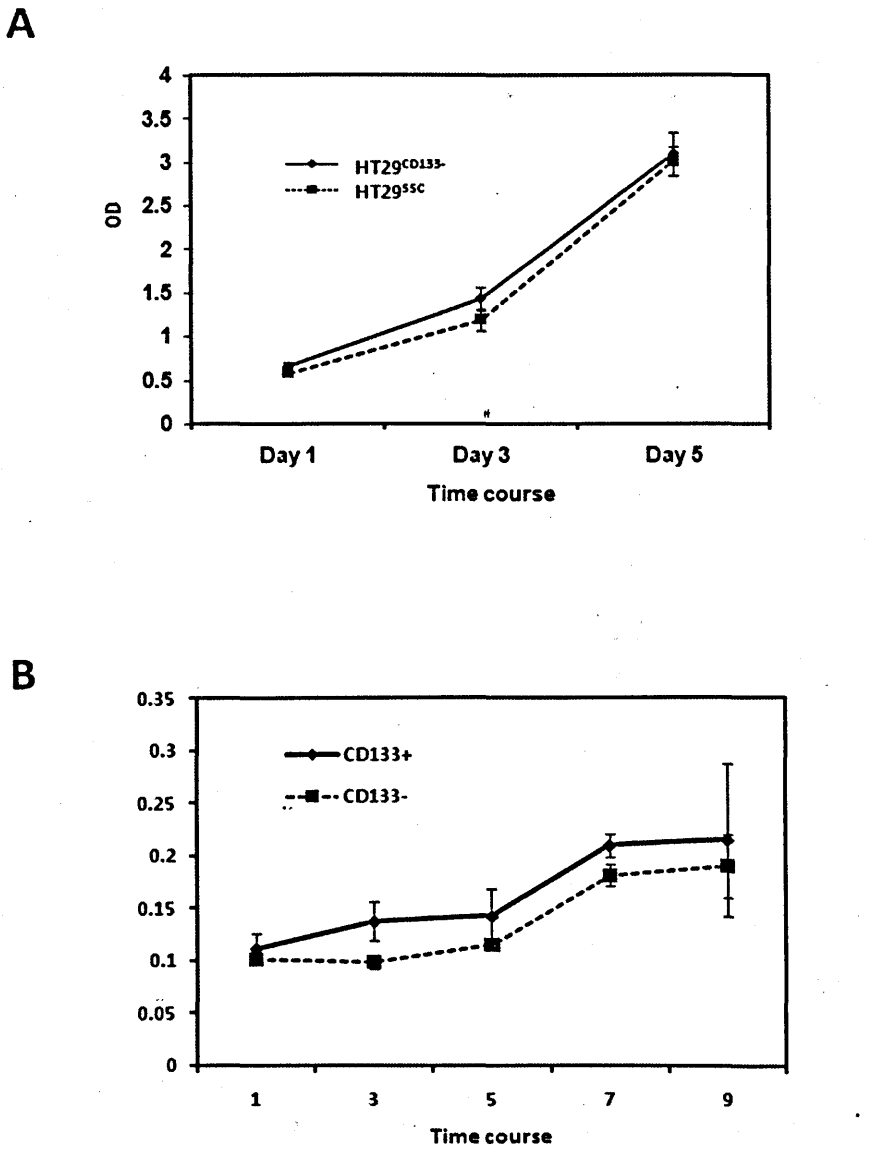


Figure 5-3. Association of CD133 expression with proliferation.
Cell proliferation was evaluated after knockdown of CD133 in HT29 (A), and sorting of SW480 into pure CD133+ and CD133- populations (B) A time course assay was performed over several days with no association seen between CD133 and cell numbers. Error bars represent mean ± SD.

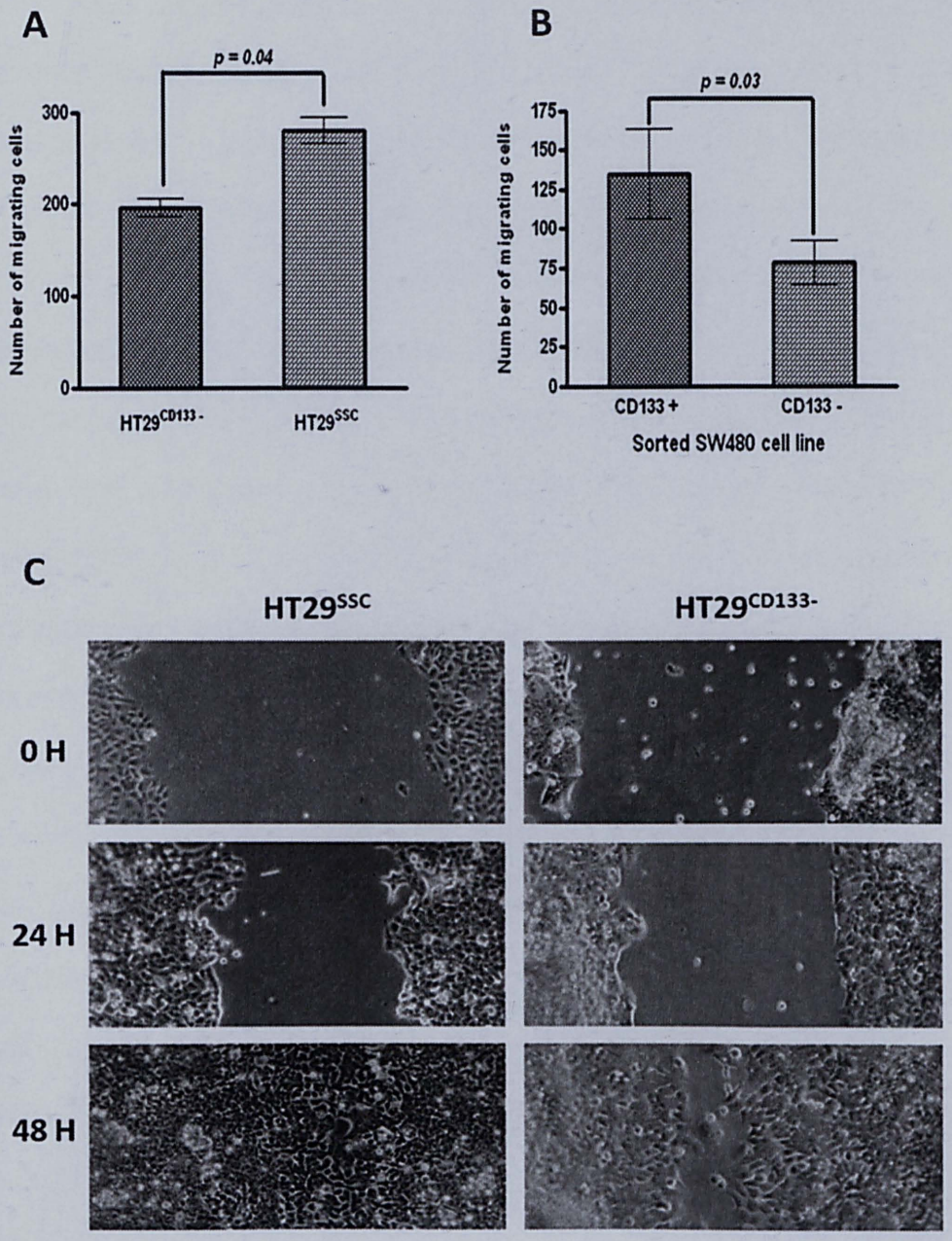


Figure 5-4. Association of CD133 with cell motility.

Transwell migration was performed 72 hours after knockdown of CD133 in HT29 and sorting of SW480 into CD133+/- populations. Significantly fewer $HT29^{CD133-}$ cells migrated across the membrane than $HT29^{SSC}$ cells (Figure 5-4a, $p = 0.04$, *unpaired t-test*). Conversely, larger numbers of sorted CD133+ cells migrated than CD133- cells (Figure 5-4b, $p = 0.03$; *unpaired t-test*). Error bars represent mean±SD of two independent experiments with triplicate each. A wounding assay was also undertaken and gene knockdown was associated with marked delay in closure of the wound which was visiually perceptible after 24, and 48 hours (Figure 5-4c) and statistically significant ($p < 0.001$, *unpaired t-test*).

5.3.5 Association of CD133 expression with colony formation

Another feature of "stemness" is the ability to form colonies. Colony forming assays were done only in the sorted populations because the effect of stealth RNAi just lasts for a short time as was stated by the providing company. Sorted CD133+/- populations were tested by growing single cells in tissue culture plates and DMEM medium for 10-14 days. In this assay, CD133+ cells gave rise to large adherent colonies growing as dense cell monolayer (Figure 5-5C) while, CD133- cells developed as small colonies of loose cell monolayer (Figure 5-5C). The number of colonies was significantly greater in CD133+ cells compared with CD133- counterpart (*p=0.0001, unpaired t-test*) (Figure 5-5b). Soft agar colony formation (anchorage-independent growth assay) was also performed. Consistently, sorted CD133+/- populations were seeded as single cells in soft agar for two weeks. After two weeks, figure 5-5d showed larger colonies were generated by CD133+ cells than those by CD133- cells. In addition, the number of colonies was significantly greater in CD133+ cells (*p=0.003, unpaired t-test*) (Figure 5-5a).

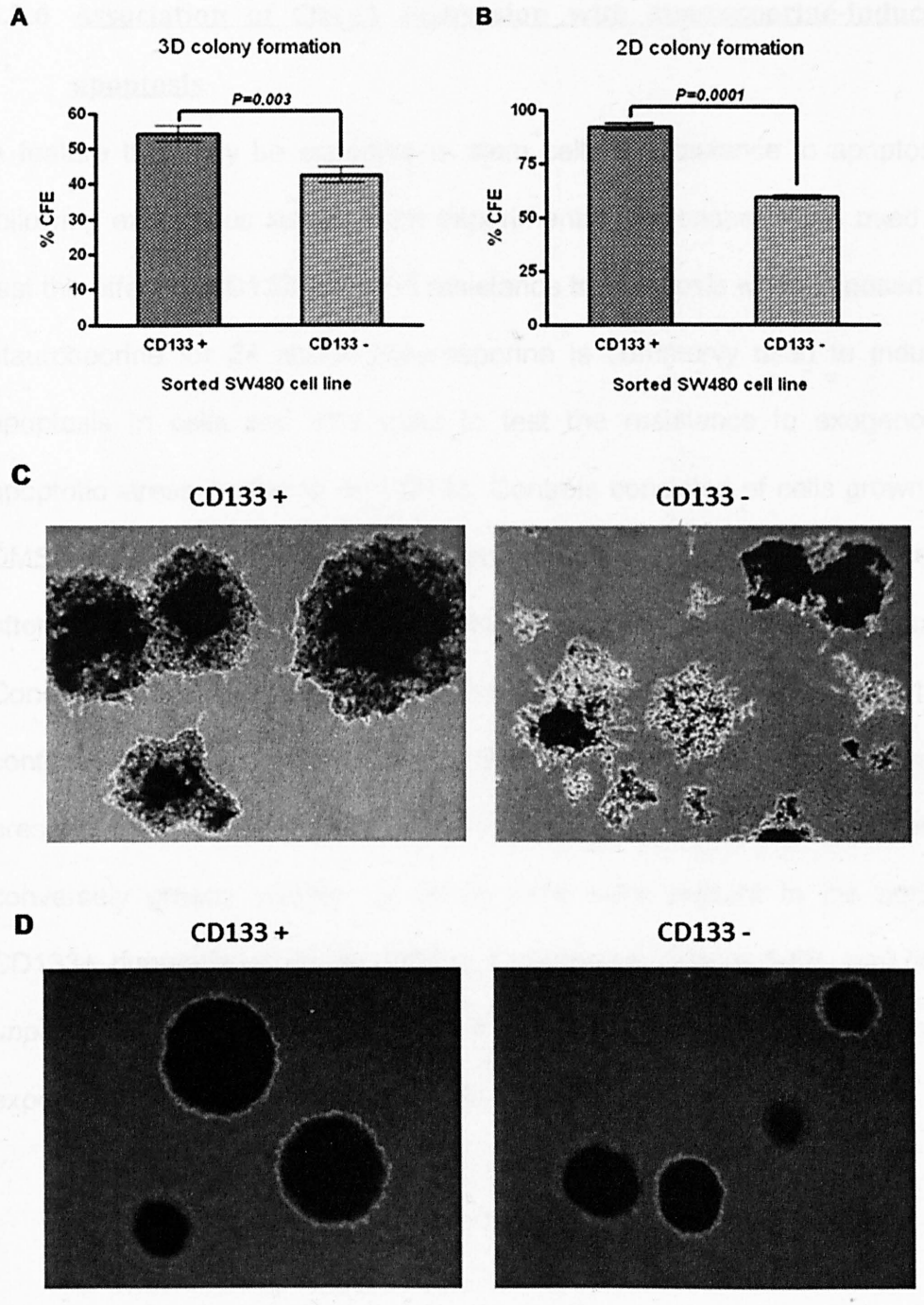


Figure 5-5. Asociation of CD133 with colony formation.

Figure 5-5A and 5-5B show CD133+ cells were significantly more clonogenic than CD133- cells (*p=0.0001 and p=0.003, unpaired t-test*) in 2D and 3D culture respectively (CFE = colony forming efficiency). Error bars represent mean ± SD of two independent experiments with triplicate each. Figures 5-5C and d (colonies formed by CD133+ cells and CD133- cells) show that both CD133+ and CD133- populations of SW480 were able to form colonies from single cells (typical colonies are shown).

5.3.6 Association of CD133 expression with staurosporine-induced apoptosis

A feature that may be expected in stem cells is resistance to apoptosis following exogenous stress. Both experimental approaches were used to test the effect of CD133 levels on resistance to apoptosis when exposed to staurosporine for 24 hours. Staurosporine is commonly used to induce apoptosis in cells and was used to test the resistance to exogenous apoptotic stress conferred by CD133. Controls consisted of cells grown in DMSO only without staurosporine were performed. Numbers of viable cells after 24 hours were measured using methylene blue staining assay. Concordant results were obtained indicating that high levels of CD133 conferred staurosporine resistance. Fewer viable $HT29^{CD133-}$ cells were present than $HT29^{SSC}$ cells (Figure 5-6A, *p=0.01; unpaired t-test)*; conversely greater number of viable cells were present in the sorted CD133+ population than the CD133 - population (Figure 5-6B, *p=0.008, unpaired t-test).* There was however no difference between cells when exposed to DMSO alone as a control.

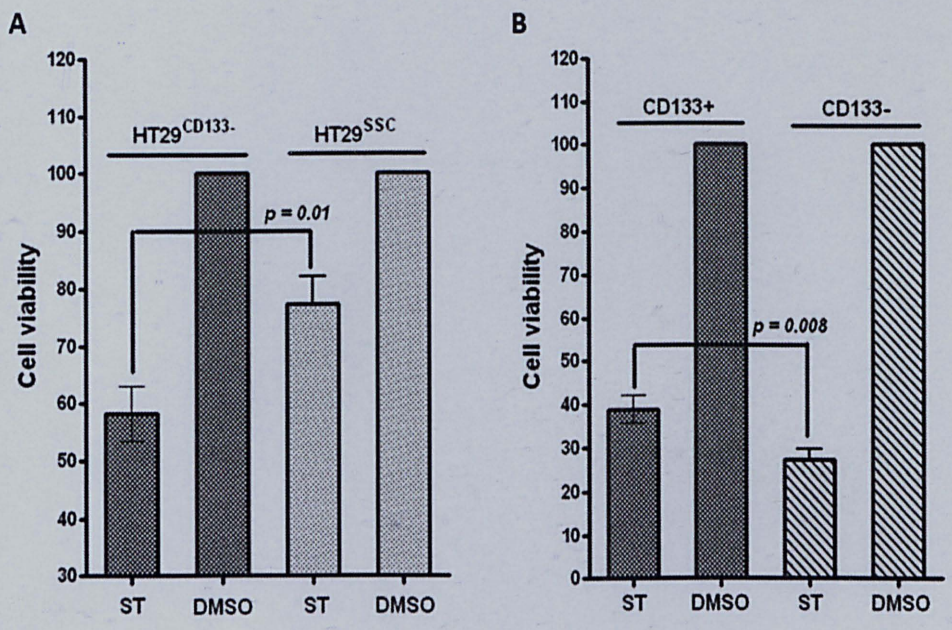


Figure 5-6. CD133 gave resistance to staurosporine induced apoptosis.
Figure 5-6A shows that after exposure to staurosporine there were fewer viable cells after transfection with CD133 specific siRNA ($HT29CD^{133-}$) than with scrambled control ($HT29^{SSC}$) (*p = 0.01; unpaired t-test*). Figure 5-6B shows that the CD133+ population of SW480 showed cells showed significantly greater resistance than the CD133- population (*p = 0.008; unpaired t-test*). One hundred percent represented cell viability in each control cells treated with DMSO which has the same optical density (OD) reading;Staurosporine (ST). Error bars represent mean ± SD of two independent experiments with triplicate each.

5.3.7 Long-term culture changes on sorted population

Sorted CD133+/- population were allowed to grow in culture medium to investigate the time-dependent changes in CD133 expression. Long-term culture of sorted populations resulted in both populations reverting to a bimodal profile. The CD133+ population, after 3 weeks, consisted of 70% CD133+ cells and 30% CD133- cells, frequencies which remained stable thereafter for at least 3 months. The CD133 - cells developed a population of CD133+ cells which, after 3 weeks, reached 17% but did not increase thereafter (Figure 5-7A and 5-7B).

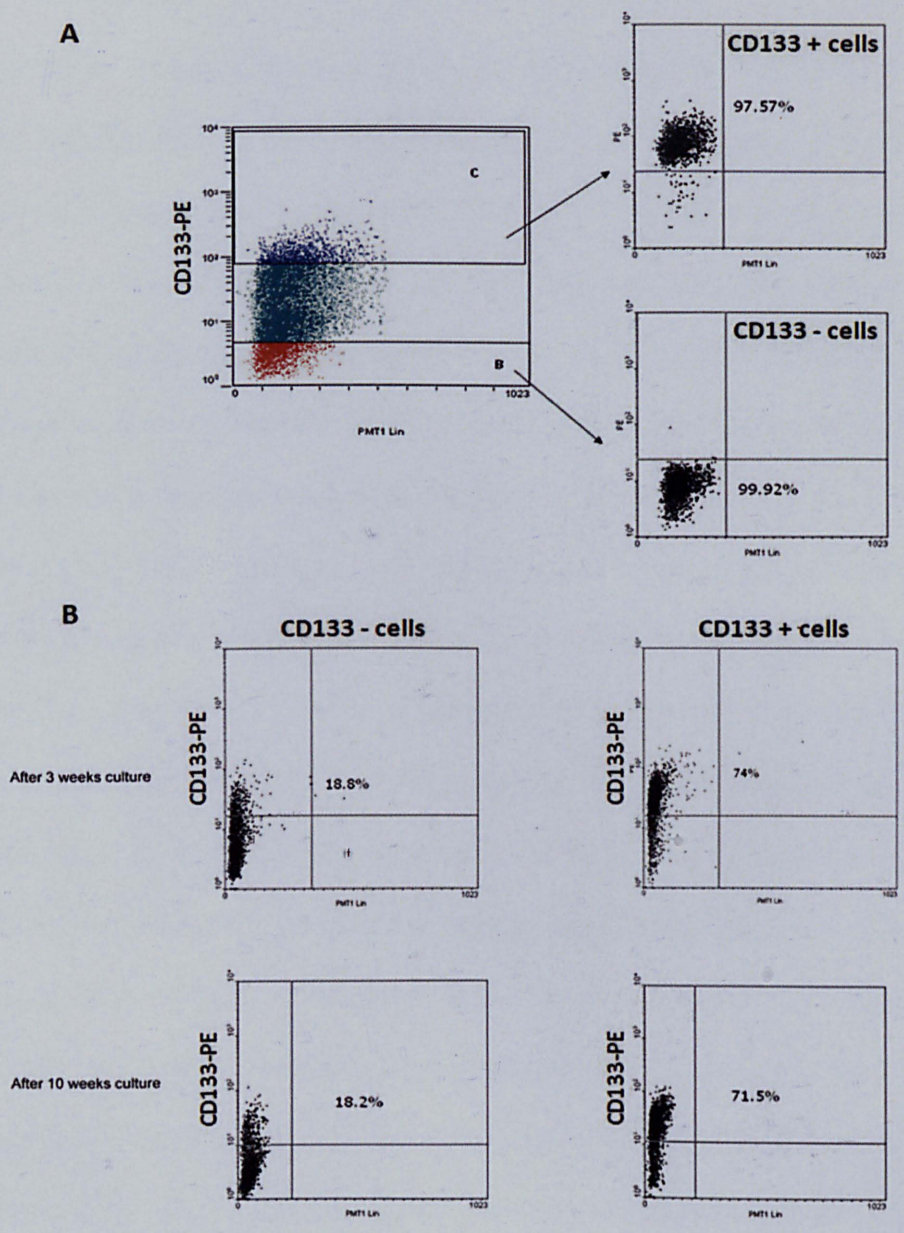


Figure 5-7. Effect of long term culture on sorted CD133+/- cells.
SW480 was sorted into CD133 positive and negative populations which were then cultured separately. (a) Gating was set so that only the extreme populations were collected which, on immediate retesting, were shown to be very pure populations. (b) After prolonged culture, both of the sorted populations became biphasic. The ratios of the CD133+ and CD133- cells became stable after three weeks and did not change after that (although they were not the same as the original parental cell line)

5.4 Discussion

In order to evaluate the function of CD133, the cell line HT29 was tested after knockdown of CD133 by RNA interference. Another complementary approach used was to separate SW480–a cell line with a CD133+ population of 40% - into pure CD133+ and CD133 - populations. Both types of experiments yielded similar results. Firstly, levels of CD133 did not appear to alter cell proliferation. Second, there were however significant differences in the features which may be regarded as part of the stem cell phenotype. Thus high levels of CD133 were associated with increased clonogenicity and resistance to staurosporine induced apoptosis. The latter may be due to an innate resistance to apoptosis possibly due to preferential activation of certain pathways or molecules that gives an advantage to the CD133+ expressing cells to resist apoptosis. Such pathways or molecules included in the resistant phenotype of CD133+ cells include DNA damage checkpoint (Bao, Wu et al. 2006), FLIP (caspace 8 inhibitor) (Zobalova, McDermott et al. 2008), BCL-2, BCL-XL , inhibitors of apoptosis protein (IAP) (Liu, Yuan et al. 2006; Chiou, Kao et al. 2008), and AKT/PKB pathway (Ma, Lee et al. 2008). Alternatively, it may be due to enhanced cytoprotective strategies such as the ability to actively extrude toxic substances from the cytoplasm and the enrichment of these cells with ABC transporter members (Jin, Bin et al. 2009; Hostettler, Zlobec et al. 2010).

Another feature we found to be associated with CD133 expression was enhanced cell motility. Other studies have suggested a role for CD133 in cell motility since it is classically expressed in membrane protrusions (Corbeil, Roper et al. 2000; Giebel, Corbeil et al. 2004). In certain situations, such as embryogenesis and wound healing, stem cells need to acquire features of motility. In CSCs, features of enhanced motility may allow invasion and metastasis to occur–a notion supported by the study of Rappa *et al.* which found CD133 expression was associated with metastasis in melanoma cells (Rappa, Fodstad et al. 2008). Our data do, however, contradict those of Horst *et al.* (Horst, Scheel et al. 2009) who did not find that CD133 expression was associated with cell motility in Caco2. We are uncertain of the cause for this discrepancy but a plausible explanation for this contradiction is the utilization of different cell lines that may harbour genetic evolution during *in vitro* growth for a long time that may affect the function of CD133 (Jiang, Gwye et al. 2010). Alternatively, CD133+ cells in a given cell line may harbour a heterogenous colony forming, migrating and resistance ability different from that in other cell line even within CD133+ cells of the same cell line. In support of the latter speculation, Li *et al.* reported that CD133+ cells of SW480 cell lines had a different invasive and migration ability and attributed this to heterogeneity of CD133+ cells with different invasive and migration abilities (Li, Liu et al. 2010). Also, technical differences between laboratories, or differences in the duplex sequence used for gene knockdown could be considered.

We used FACS to sort SW480 into pure CD133+ and CD133 - populations and, by mRNA quantification, showed that this was due to transcriptional repression rather than changes in glycosylation. The mechanistic basis of inhibiting CD133 expression is uncertain. However, epigenetic silencing due to hypermethylation of CD133 promoter has been reported as a means of controlling CD133 expression (Yi, Tsai et al. 2008). Alternatively other mechanisms such as mRNA degradation may be involved.

Prolonged culture of pure CD133+/ CD133 - populations resulted in both populations reverting to a bimodal pattern although in neither case did the pattern revert to that of the original cell line. Thus, after 3 months, the cultured pure CD133+ population developed a CD133+/CD133 - split of 70%/30% respectively. These data are consistent with other studies showing that tumours derived from CD133+ populations ultimately become bimodal (Balla, Vemuganti et al. 2009). However, it is uncertain why the CD133+ population did not revert to 40% as seen in the original SW480 cell line. The cultured pure CD133 - population developed a stable CD133+ population of around 17%. The study by O'Brien found that CD133 - sorted populations could induce tumours containing a CD133+ population and attributed this to contamination of the CD133 - cells by CD133+ cells. We think that contamination is unlikely to be an explanation in our case as our initially sorted populations were very pure and our time-course studies did not show any difference in rates of proliferation between CD133+ and CD133 - cells. Another possibility is that CD133 was re-induced in these

cells. Since CD133 mRNA was detected in the CD133 - cells, it shows that a low level of transcriptional activity may still have been occurring in cells which were negative for protein expression. This would fit with data from animal studies demonstrating that transit amplifying cells (which lie beyond the stem cell compartment) can re-acquire stem cell properties in the small intestine under appropriate conditions (Potten 1998). If this is the case, then it would question the existence of CSCs since, by extension, non-stem cells within a tumour could theoretically acquire stem cell features.

To sum up, in this study, the biological characteristics of CD133 expressing cells were identified such as enhanced migratory, clonogenic abilities as well as resistance to stress induced apoptosis. Furthermore, CD133 + cells have the ability to induce both populations after long time culture indicating plasticity of these cells, and surprisingly, CD133 – cells did as well. CD133 may, at least in part, play a role in tumour metastases and tumour progression. The mechanistic bases that regulate the expression or the function of these cells warrant further investigation. This will be discussed in the next chapter.

6 Chapter 6: Evaluation of upstream and downstream targets of CD133 in colorectal cancer

6.1 Abstract

Background and aim: Recent data together with data presented in previous chapter indicated that CD133 expression was associated with enhanced colony forming abilities, enhanced motility and resistance to stress induced apoptosis. The aim of this study is to test the downstream targets of CD133 which mediate these effects and to investigate the mechanisms of CD133 regulation. Specifically, the aims are to test the hypotheses that 1) CD133 interacts with proteins of the adherence junctions (E-cadherin and β-catenin) and the focal adhesions (Cten) during cell motility and, 2) CD133 activity is regulated by Wnt signalling or STAT3 (functional pathways deregulated in colorectal cancer).

Methods: Expression of specific genes was manipulated and the effect on downstream targets tested by Western blot or flow cytometry. Reduction of gene expression was achieved by gene knockdown using siRNA targeted to genes of interest (i.e. CD133 and STAT3). Increase in gene expression was induced by forced ectopic expression of cloned cDNA (CD133 and Cten). Wnt signalling pathway was blocked by forced ectopic expression of dominant negative (DN) TCF4. Finally the effect of promoter hypermethylation on CD133 gene regulation was tested by demethylation using 5-Aza-deoxycytidine.

Results: Knockdown of STAT3 resulted in down-regulation of CD133 whilst alterations of CD133 levels did not affect the levels of E-cadherin, β-catenin. Alternatively, alteration of Cten showed a modest effect on the

level of CD133 and inhibition of Wnt with dnTCF4 did not affect CD133 level. Promoter demethylation did affect CD133 levels in cell lines showing low CD133 expression.

Conclusion: These data indicate that STAT3 signalling is, at least in part, a potential regulator of CD133 expression in colorectal cancer cell lines. Furthermore, CD133 might enhance the motility through other molecules rather than E-cadherin, β-catenin, however, its association with Cten warrants further investigations. In this study, although Wnt signalling inhibition using dnTCf4 might have no effect on CD133 level, further optimization of dnTCf4 transfection should be warranted to verify the absence of such a relationship between Wnt pathway and CD133. Finally, methylation status of CD133 promoter may, at least partially, play a role in regulation of CD133 expression.

6.2 Introduction

Consistently, with published studies in colorectal cancer (Ieta, Tanaka et al. 2008; Puglisi, Sgambato et al. 2009) and other solid tumours (Rappa, Fodstad et al. 2008; Yanagisawa, Kadouchi et al. 2009; Sato, Sakurada et al. 2010), the data in the previous chapter demonstrated that CD133 expressing cells have enhanced migratory and colony forming ability, implying that CD133, might play a role in both primary tumour formation and migration of cancer cells.

Epithelial-mesenchymal transition (EMT) is a process through which tumours are thought to be able to acquire the ability to metastasize (Thiery 2003). It is associated with E-cadherin downregulation and increased nuclear β-catenin. Recently, two studies reported a connection between EMT and cancer stem cells. Mani and Morel and their co-workers reported that activation of EMT was associated with the acquisition of stem cell properties in breast cancer (Mani, Guo et al. 2008; Morel, LiÃ¨vre et al. 2008). Another study in hepatocellular carcinoma (HCC), where CD133 used as a marker for isolating CSCs, E-cadherin was downregulated in CD133+ compared to CD133- cells (Lee, Han et al. 2010). Moreover, Snail, which is involved in EMT and leads to downregulation of E-cadherin, was only expressed in CD133 positive glioblastoma cells (Liu, Yuan et al. 2006). Consistently, CD133+ cells have been detected at the invasive front of primary tumours of the pancreas and colorectal cancer (Hermann, Huber et al. 2007; Li, Li et al. 2009). In addition, cells at the invasive sites

showed nuclear localization of β-catenin compared to the central area which showed membranous expression (Brabletz, Jung et al. 1998). Taken together, these circumstantial data raise the possibility of a role for CD133 in the process of EMT and initiation of metastasis.

Boivin *et al.* reported that CD133 was phosphorylated at the tyrosine residue found on the C-terminal cytoplasmic end (tyrosine-828) by Src kinase creating a binding site for interaction with proteins that contain phosphotyrosine binding Src- homology 2 (SH2) domain (Boivin, Labbe et al. 2009). Recently, it has been reported that Cten (C-terminal tensin-like), a member of Tensin gene family that lacks N-terminal acting binding domain and contains an SH2 domain, affects cell motility and migration through repression of E-cadherin (Albasri, Seth et al. 2009). Taken together, it was hypothesised that Cten through its SH2 domain could regulate CD133 expression.

Several pathways are involved in normal stem cell self-renewal and decisions about differentiation. The Wnt signalling pathway has been reported to play a major role in the maintenance of normal and cancer stem cells (Vermeulen, De Sousa E Melo et al. 2010). Furthermore, signal transducer and activator of transcription 3 (STAT3) activation supports self-renewal and the undifferentiated state of mouse embryonic stem cells (mouse ESCs) (Matsuda, Nakamura et al. 1999), although it fails to prevent differentiation of Human embryonic stem cells (Boiani and Scholer 2005). STAT3 was reported to be activated in many human tumours

including colorectal cancer (Corvinus, Orth et al. 2005), and gliomas (Abou-Ghazal, Yang et al. 2008). A study in colorectal cancer reported that STAT3 and other signalling molecules were activated in $CD133^{high}/CD44^{high}/progastrin^{high}$ cells (Ferrand, Sandrin et al. 2009). However its role in regulating cancer stem cells is still under investigation.

In a study that investigated the transcriptional regulation of the *CD133* gene, it was found that Exon 1A, 1B, 1C, promoter 2, promoter 3 and partially promoter 1 were located within a CpG island suggesting a speculation that methylation of CpG islands in the promoter areas may suppress the expression of the *CD133* gene (Shmelkov, Jun et al. 2004). Furthermore, induction of methylation of CD133 promoter 1 and promoter 2 resulted in suppression of activity of the promoters in Caco2 cell line (Shmelkov, Jun et al. 2004). In support, Tabu *et al.* reported that hypomethylation of the CpG island was associated with increased level of CD133 mRNA expression in glioma (Tabu, Sasai et al. 2008). Another study in CRC and brain tumours revealed a higher incidence of methylated *CD133* promoter region in CRC and GBM cell lines compared to the normal colon and brain samples which showed unmethylated *CD133* promoter (Yi, Tsai et al. 2008). These data showed that CD133 could be epigenetically regulated by the methylation status of its promoter.

The aim of this part of the project is to (1) test the hypothesis that CD133 could enhance the migration ability of colorectal cancer cells through EMT,

by studying the effect of CD133 expression on both E-cadherin and β-catenin (a key regulator of EMT) and effect of Cten expression on CD133, and (2) to test the hypothesis of controlling CD133 through Wnt signalling, STAT3 and DNA hypermethylation.

The following protocols were used:

(1) CD133 was knocked down in Caco2 and HT29 (both high expressers of CD133) whilst CD133 was forcibly expressed in DLD1 and Sw837 (negative for CD133 expression). E-cadherin and β-catenin expression were evaluated using western blotting.

(2) Green fluorescent protein tagged Cten (GFP-Cten) was forcibly expressed into HCT116, SW837, and HT29 cell lines and CD133 expression was evaluated using western blotting and flow cytometry.

(3) Wnt signalling was inhibited using DN-TCF4 (kindly gifted by Dr Nateri AS, Division of Pre-clinical oncology, University of Nottingham) whilst the, STAT3 signalling pathway was inhibited by gene knockdown. CD133 expression was evaluated by flow cytometry.

(4) DNA hypermethylation was reversed using 5'aza treatment and CD133 expression was evaluated by flow cytometry.

Details for transfection protocols; refer to material and methods section.

6.3 Results

6.3.1 Cloning of CD133 into pcDNA™3.1D/V5-His-TOPO® vector

Manually designed primers for PCR amplification of the full length CD133 coding sequence were used with the help of Pfu DNA polymerase enzyme. Visualizing the amplified PCR product using 1% agarose gel electrophoresis demonstrated an amplicon of the same size of full length CD133 at ~ 2.6 Kbp (figure 6-1).

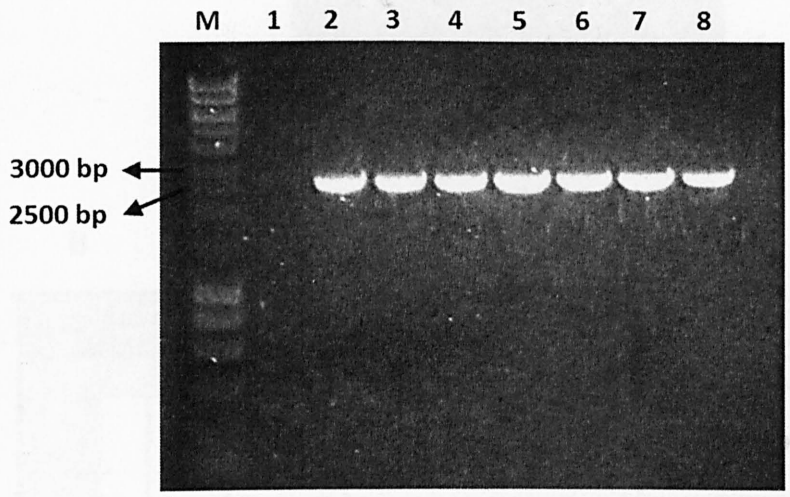


Figure 6-1. Amplification of full length CD133.
Gel electrophoresis showed a PCR amplification of full length CD133 using CD133 forward and reverse primers. The CD133 product; which is 2600 bp, was detected in lanes 2-8. Lane 1 is a negative control containing no DNA template. A 1kb DNA ladder (DNA size marker) (M) is shown on the left

Purification of the PCR product from any excess primers, nucleotides, polymerase enzyme, and salts were conducted using QIAquick PCR purification Kit (Qiagen). Measurement of the DNA amount after

purification using Spectrophotometer Nanodrop was done and revealed an amount of 104 ng/µl (figure 6-2A, B).

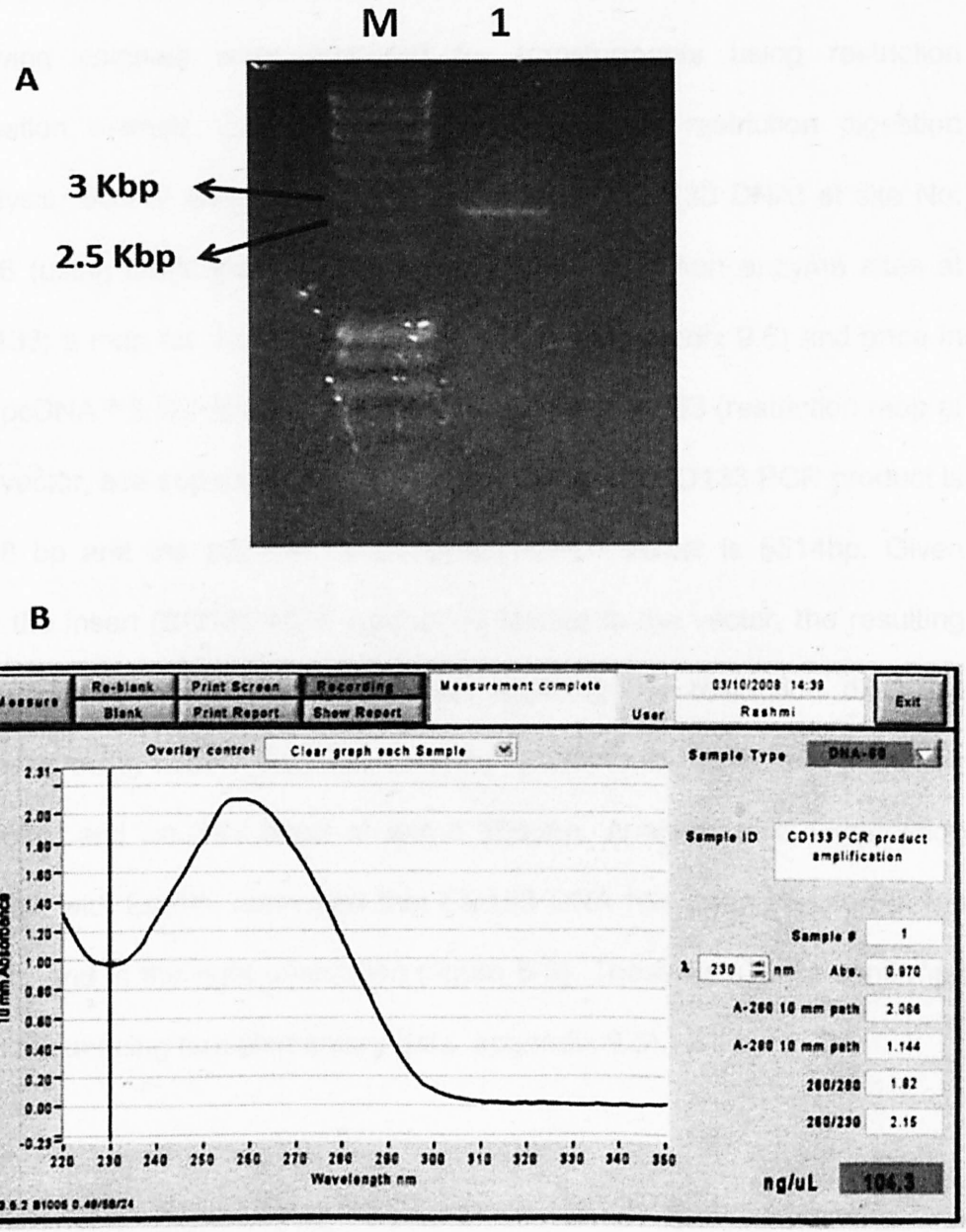


Figure 6-2. PCR product after purification.
Purified PCR product visualizaed by agarose gel electrophoresis (a) showed CD133 product of corrected size in lane 1, and 1kb ladder is shown on the left **(b)** illustrate the quantity of CD133 DNA after purification using Nanodrop technology (nanodrop spectrophotometer).

TOPO cloning reaction between PCR product (CD133 full length DNA) and pcDNA™3.1D/V5-His-TOPO® vector and transformation into competent cells (E-coli) was conducted (see supplementary data for vector map). The growing colonies were analyzed for transformants using restriction digestion method. EcoRV enzyme was used for restriction digestion analysis. EcoRV enzyme cut once in the insert (CD133 DNA) at site No. 1176 (using DNADynamo software, to detect restriction enzyme sites at CD133; a map for EcoRV site on CD133 , see appendix 9.6) and once in the pcDNA™3.1D/V5-His-TOPO® vector at site No. 963 (restriction map of the vector, see supplementary data). The full length CD133 PCR product is 2598 bp and the pcDNA™3.1D/V5-His-TOPO® vector is 5514bp. Given that the insert (CD133 PCR product) is ligated to the vector, the resulting plasmid size will be 8112bp. When applying the restriction digestion analysis using EcoRV enzyme, it should result in two bands; one band is ~ 1446bp, and another band of about 6666bp. Analysis of the restriction digests with EcoRV confirmed that CD133 DNA has been inserted in the vector and in the right orientation (figure 6-3). These data were confirmed with sequencing (supplementary data, appendix 9.3).

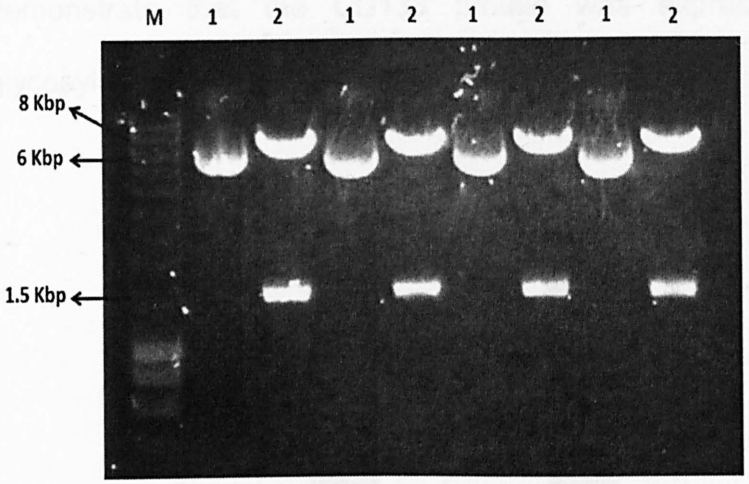


Figure 6-3. Restriction digestion analysisof CD133 plasmid.
Analysis with EcoRV showed two bands, one ~ at 6.6 Kbp, and the second at 1.5 Kbp, confirming the insertion of CD133 DNA into the vector. M; 1 Kbp DNA ladder, 1; Uncut CD133 plasmid, 2; restriction enzyme digestion with EcoRV.

6.3.2 Transfection of pcDNA 3.1-CD133 plasmid into SW837 and DLD-1

Transfection efficiency of pcDNA3.1-CD133 plasmid into SW837 and DLD1 was verified by qRT-PCR. Data revealed increased CD133 mRNA expression (figure 6-4), which *per se* does not indicate that it is translated to the corresponding CD133 protein or that CD133 protein is expressed on the cell surface. Therefore, flow cytometry analysis of CD133 protein expression on the cell surface was employed and showed heightened numbers of CD133 expressing cells from 0% in both SW837 and DLD1 to 57% and 39% respectively (figure 6-5). Moreover, western blot was performed and revealed that the protein was detected when pcDNA 3.1-CD133 plasmid was used, whilst transfection with the corresponding empty vector has no effect on the protein expression (figure 6-6). These data

demonstrate that the CD133 protein was expressed, appropriately glycosylated and trafficked to the cell membrane.

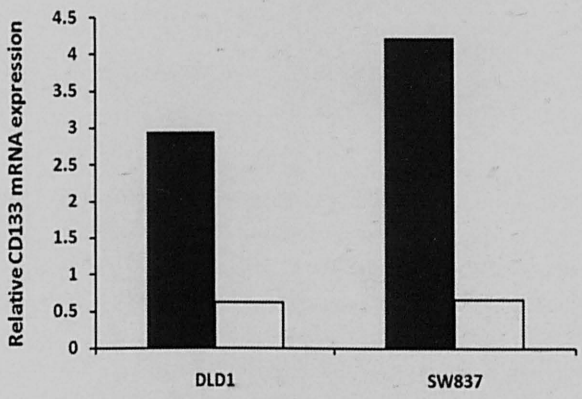


Figure 6-4: Transfection of CD133 gene in both Sw837 and DLD1 cell lines.
The efficiency of CD133 transfection was assessed by qRT-PCR. CD133 mRNA expression (normalized to HPRT1) in transfected cells with pcDNA3.1-CD133 plasmid (black bars) compared to transfected cells with corresponding empty vector (white bars).

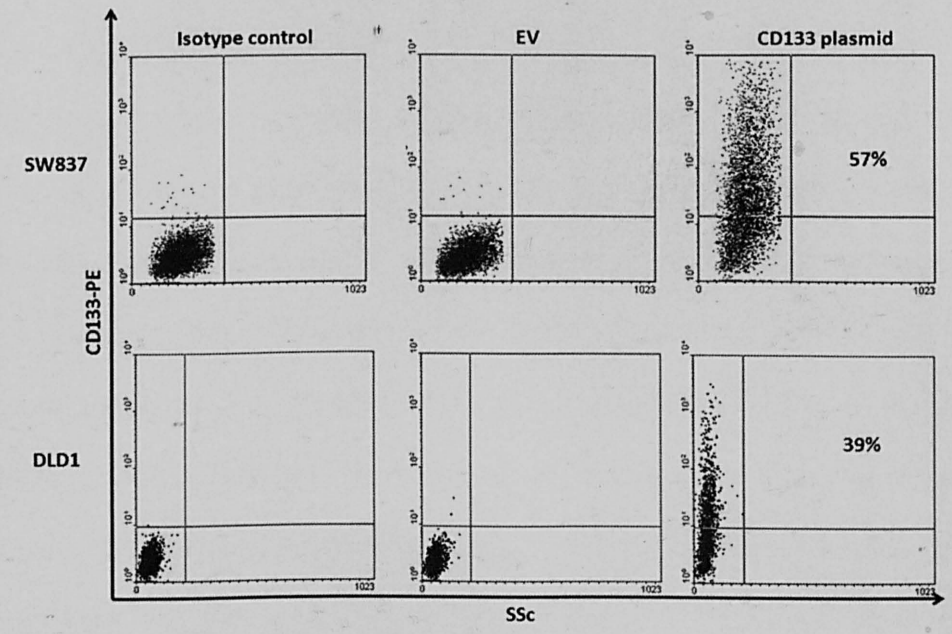


Figure 6-5. Forced expression of pcDNA 3.1-CD133 plasmid.
Transfection of SW837 and DLD1 (negative expressers of CD133) with pcDNA 3.1-CD133 plasmid resulted in upregulation of CD133 on protein level which was detected by flowcytometry as increased number of CD133+ cells, when compared with cells transfected with the corresponding empty vector (EV).

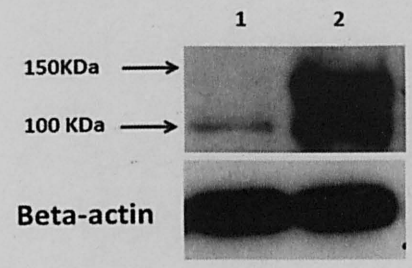


Figure 6-6. Evaluation of forced expression of pcDNA 3.1- CD133 plasmid.
Transfection of SW837 cell line with pcDNA 3.1-CD133 plasmid resulted in upregulation of the protein level (lane 2), compared to transfection with the corresponding empty vector (lane 1). With the use of anti-CD133 antibody (C24B9) which detect both glycosylated and non-glycosylated epitopes of CD133, western blot showed a small faint band at a size ~ 100 kDa which is the expected size of non-glycosylated epitope of CD133 protein (97 kDa) in lane 1. While, in Lane 2, there are two dense bands, one at a size ~ 100 kDa (represent non-glycosylated epitope), and another dense band at ~ 120 kDa representing the glycosylated epitope of CD133. B-actin showed equal loading of protein.

6.3.3 Assessment of E-cadherin and β-catenin following manipulation of CD133 expression

HT29 and Caco2 cell lines were transfected with CD133 SiRNA and its corresponding scrambled control (SSC), and CD133 protein expression in knocked down cells was down-regulated in 72 hours after transfection as evaluated by flow cytometry (Figure 6-7B). Meanwhile, SW837 and DLD1 were transfected with pcDNA 3.1-CD133 plasmid, and the expression of CD133 mRNA and protein was heightened after 48 hours as shown in figure 6-7A. Next, western blot analysis was employed to examine E-cadherin, and β-catenin expression. As shown in figure 6-8, there are no significant changes in E-Cadherin, β-catenin, expression between the knocked-down cells and their control, and cells transfected with pcDNA

3.1-CD133 plasmid compared to cells transfected with the corresponding empty vector (EV).

6.3.4 Assessment of relation between CD133 and Cten

Cten localizes to focal adhesions where it is found in complex with the cytoplasmic domains of integrins. It has been shown to modulate cell motility and in order to determine whether this was affected through CD133, GFP-Cten was forcibly expressed in HCT116 (a cell line showing both moderate CD133 expression and low Cten expression). Surprisingly, Western blot analysis showed an increase in CD133 protein expression in cells treated with GFP alone compared to the nontransfected cells. Also, cells transfected with GFP-Cten plasmid showed a slight increase of CD133 protein expression compared to cells transfected with GFP plasmid. (Figure 6-9). Later, SW837 and HT29 (both are negative expressers for Cten) were transfected with GFP-Cten plasmid. Following transfection, CD133 expression was assessed by flow cytometry and revealed a limited increase in CD133 expression (figure 6-10)

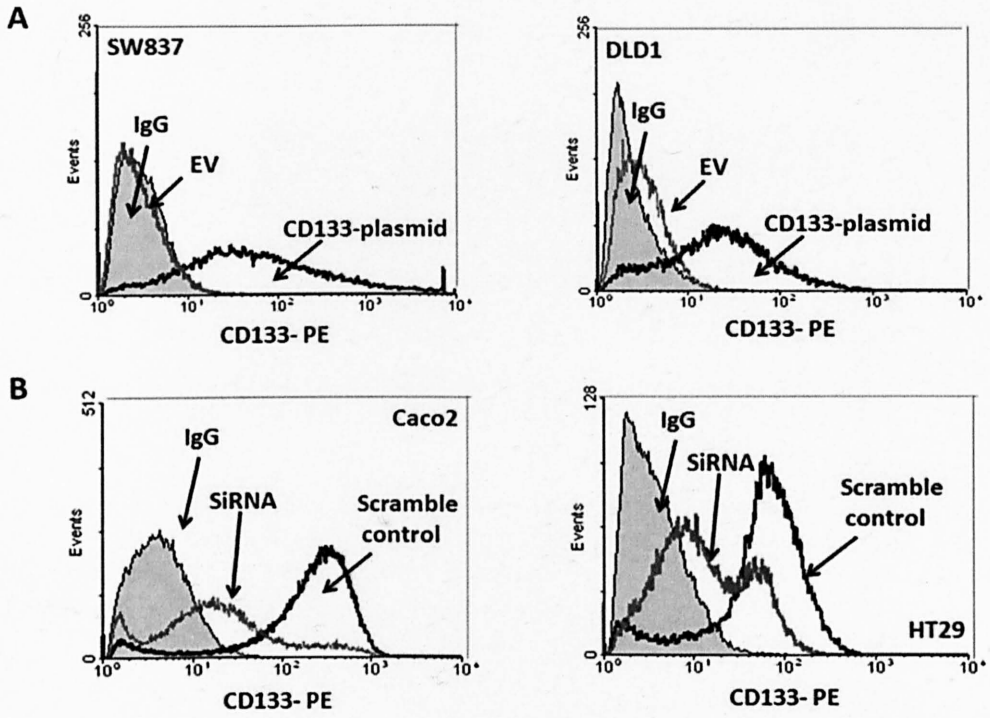


Figure 6-7: Knock down and forced expression of CD133 assessed by flow cytometry.

(A) Flow cytometry analysis shows that CD133 was significantly expressed in SW837 and DLD1 cell lines (negative expressers of CD133) after forced expression of CD133-plasmid, filled histogram (gray) represents IgG isotype control, blue line histogram represents CD133-PE labeling of cells transfected with empty vector, black line histogram represent CD133-PE labeling of cells transfected with CD133-plasmid, (B) a flow cytometry histogram confirming the occurrence of knock down of CD133 in HT29 and Caoc2 cell lines (high expressers of CD133), filled histogram (gray) represent IgG isotype control, Black line histogram represent CD133-PE labeling cell transfected with scrambled control siRNA, and blue histogram represent CD133-PE labeling of cells transfected with CD133 siRNA.

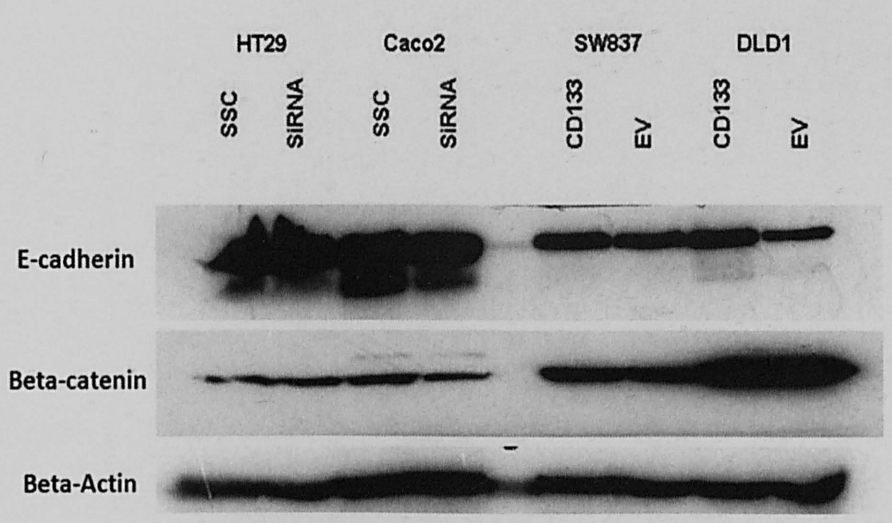


Figure 6-8: E-cadherin and β-catenin expression association with CD133. Western blot analysis of E-cadherin and β-catenin expression after CD133 knockdown and CD133 transfection showed no association between these two molecules and CD133 expression. SSC (SiRNA scrambled control), EV (empty vector).

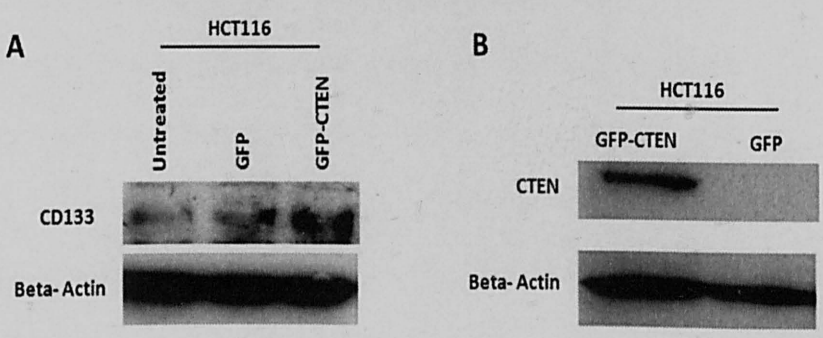


Figure 6-9: Cten and CD133 in colorectal cancer.
CTEN mediated metastasis was hypothesized to be induced through CD133. A protein lysate of HCT116 that forcibly expressed Cten was assessed using western blotting for CD133 expression. Western blotting revealed (a) an increase of CD133 protein expression in both GFP alone and GFP-Cten plasmid treated cells which is slightly more in the latter, (b) western blotting showed Cten protein expression in cells transfected with GFP-Cten plasmid compared to cell transfected with GFP vector alone. Beta-actin showed equal protein loading.

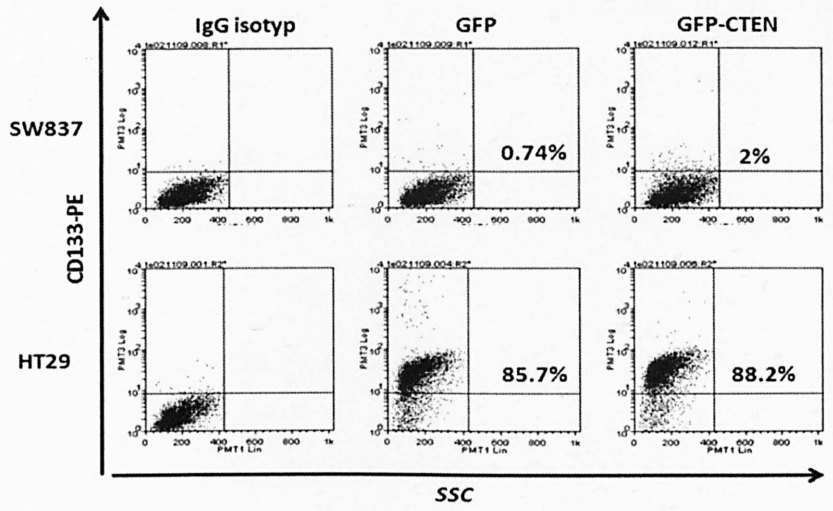


Figure 6-10: CD133 expression and Cten.
GFP-Cten plasmid was forcibly expressed into SW837 and HT29 cell lines. Flow cytometry analysis of CD133 protein expression showed a minimal increase in CD133+ cells in cell transfected with GFP-Cten plasmid compared to cells treated with GFP vector alone which also showed increased CD133+ cells compared to isotype control , gating was performed using isotype control . (SSC = side scatter)

6.3.5 Wnt signalling pathway and CD133 expression

In order to test the hypothesis whether CD133 is regulated by Wnt signalling pathway or not, HT29 (high expresser of CD133) SW837 (negative expresser for CD133), and SW480 (showed 40% positivity for CD133) cell lines were transfected with dominant negative TCF4 (NTCF-4), and CD133 expression level was assessed. Dominant negative TCF4 has a deletion of N-terminal B-catenin binding site allowing no binding between B-catenin and TCf/LEf complex leading to inhibition of transcription of downstream targets of Wnt signalling pathway (Kolligs, Nieman et al. 2002). Flow cytometry analysis revealed neither increase nor decrease in the number of CD133 expressing cells (figure 6-11). In order to validate that dnTCF-4 has been efficiently transfected, c-myc protein level

was evaluated by western blot. Minimal decrease in the level of c-myc protein was detected (figure 6-12A). QRT-PCR analysis of c-myc mRNA level revealed a change when comparing cells transfected with dnTCF-4 to cells transfected with corresponding empty vector (figure 6-12B). This minimal change in c-myc level may indicate that the transfection of dnTCF4 was not efficient making it less likely that we could see a change in CD133 protein level.

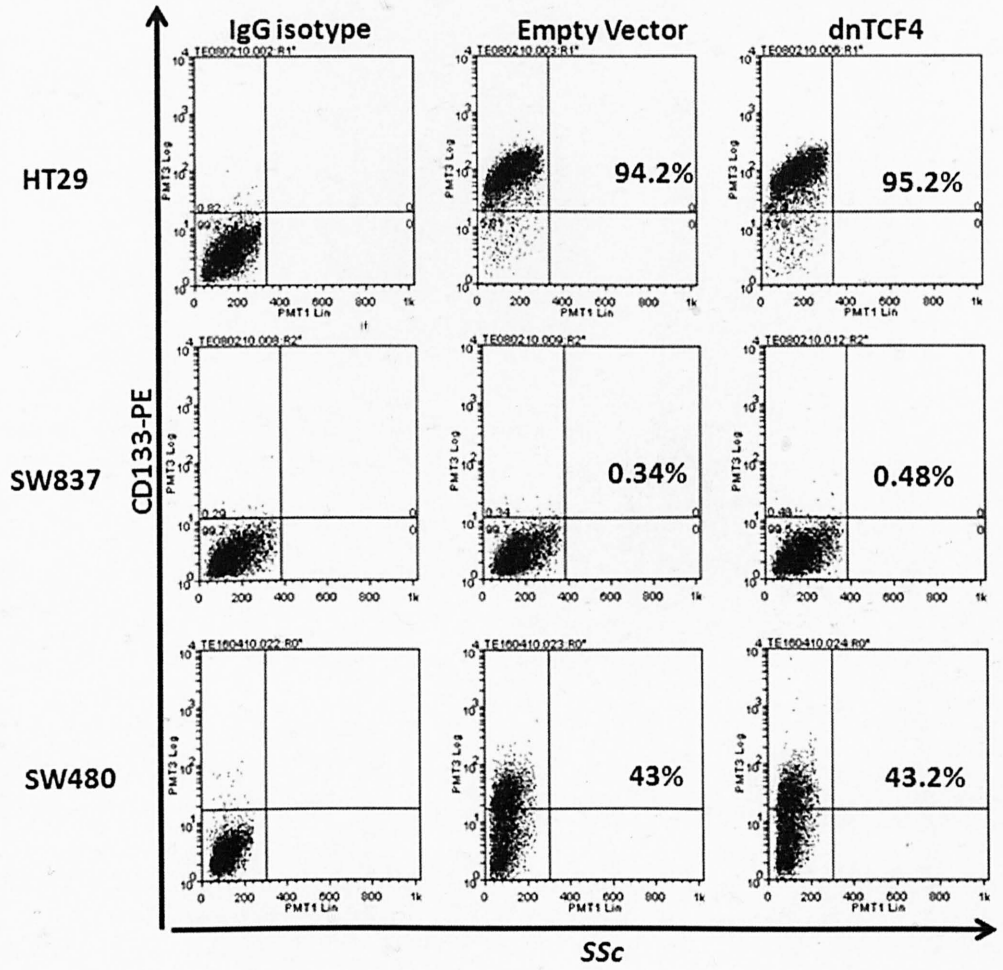


Figure 6-11: dnTCF4 and CD133.
Flow cytometry analysis showed that cell surface expression of CD133 protein neither increased nor decreased when comparing cells transfected with dnTCF4 with cells

transfected with the corresponding empty vector. Gating was done based on the IgG isotype control.

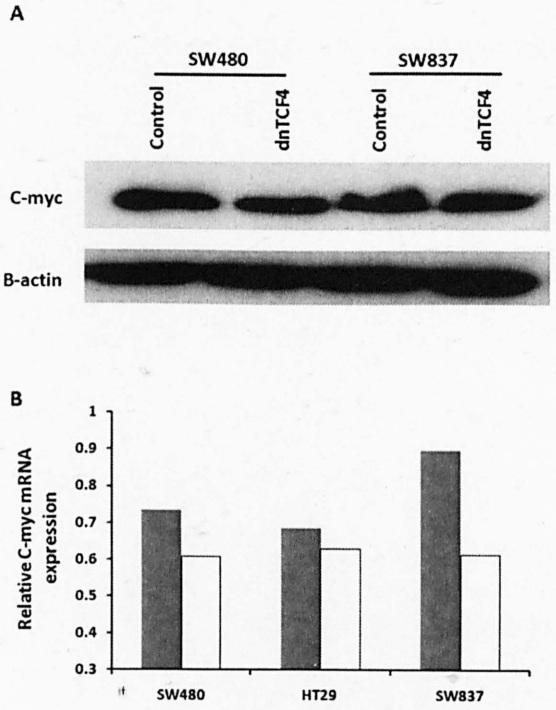


Figure 6-12: Effect of dnTCF4 on C-myc expression.
(a) Western blot analysis showed minimal change in the level of C-myc protein when cells transfected with dnTCF4 compared to control cells. B-actin showed equal protein loading. (b) qRT-PCR analysis of showed decrease in the level of C-myc mRNA when comparing cells transfected with empty vector (gray bars) and cells transfected with dnTCF4 (white bars).

6.3.6 STAT3 controls CD133 expression in CRC

STAT3 has been identified to be associated with poor clinical outcome and drug resistance. Also, it is involved in self-renewal of stem cells. Several studies have demonstrated that CD133 expressing cells have features associated with stemness such as resistance to chemotherapy; in addition it was widely used as a marker for isolating cancer stem cells in many

tumours. Hence, we wanted to test the hypothesis that CD133 is one of the targets of STAT3 signalling. In order to assess the role of STAT3 in governing CD133 expression, RNAi was employed and a STAT3 specific stealth siRNA was used (see Material and Methods). SW837 (negative expresser for CD133), SW480, and SW620 (which contains 40% and 60% of CD133+ cells respectively) were transfected with STAT3 stealth siRNA and its corresponding control to evaluate the effect of STAT3 on CD133 expression level. Quantitative measurement of STAT3 silencing was conducted by western blot which confirm STAT3 knockdown (figure 6-13A). As shown in figure 6-13B, flow cytometry analysis revealed that numbers of CD133+ cells in both SW480 and SW620 was decreased by ~27% - 30% following STAT3 knockdown. Furthermore, QRT-PCR demonstrated that STAT3 gene silencing associated with reduction in the level of CD133 mRNA (figure 6-13C). This could indicate that STAT3 control CD133 expression is, at least in part, at the transcription level.

6.3.7 Re-expression of CD133 after treatment with DNA demethylating reagent

To find out whether CD133 expression is partially regulated by methylation or not, SW837 cell line (negative expresser of CD133) and DLD-1(known previously to be methylated for CD133) were treated with 5-aza-2'-deoxycytidine. Control cells were treated with DMSO. Following treatment, flow cytometry analysis of cell surface expression of CD133 protein revealed an increase in CD133+ cells from 0% in both SW837 and DLD-1

to 10.1% and 17.3% respectively (figure 6-14). This could indicate that CD133 expression is, at least partially, regulated by DNA methylation.

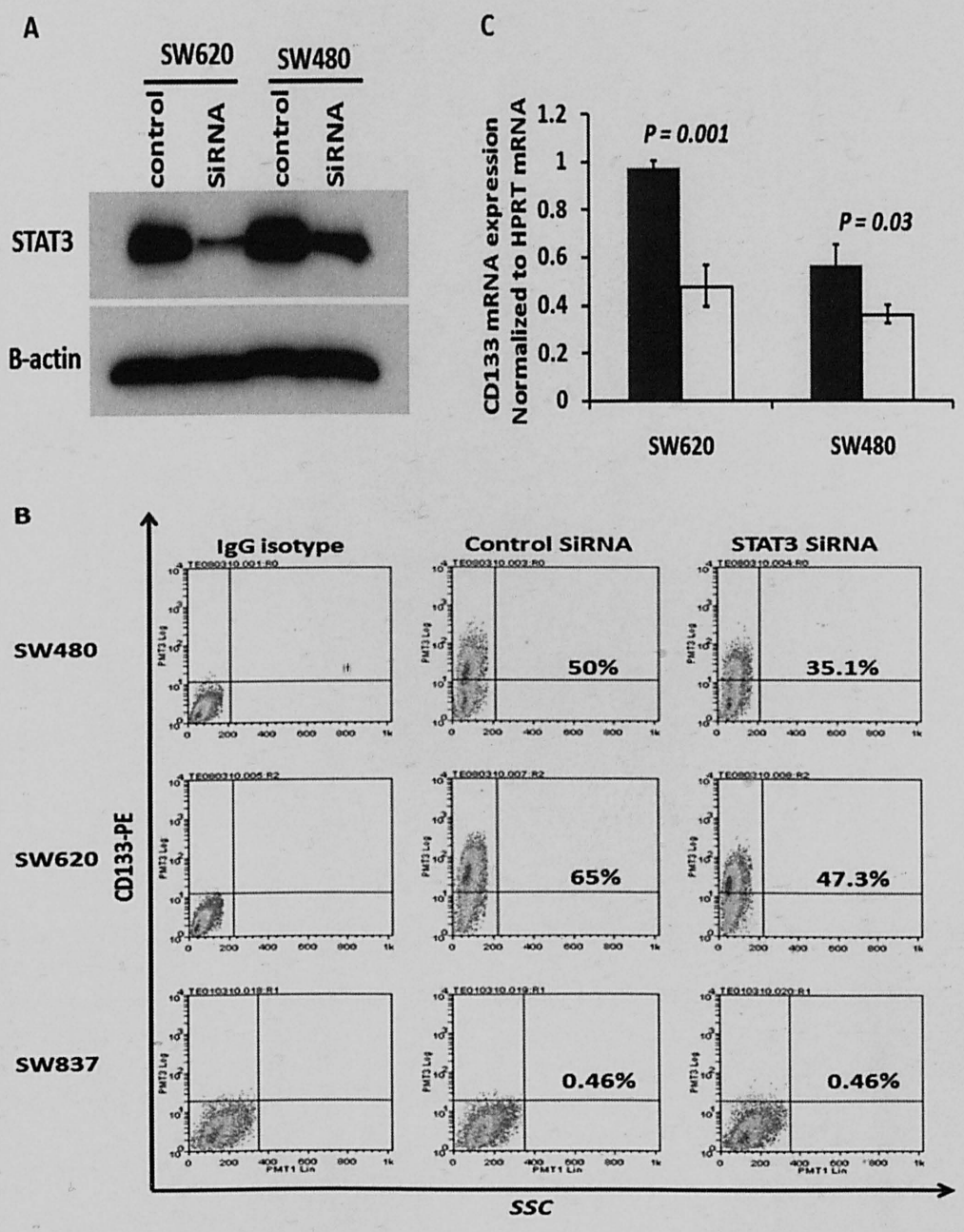


Figure 6-13: STAT3 gene silencing and CD133 expression.
SW480, SW620 and SW837 were transfected using siRNA specific for STAT3 and with scrambled siRNA as a control. Gene knock down significantly down-regulated STAT3 on the protein level as evaluated by (A) wetstern blotting, beta-actin showed equal loading of

protein. Transfected cells with STAT3 siRNA showed decrease in CD133 expression on protein level using flow cytometry , each graph is a sample graph of two independent experiments (B) and on mRNA level by qRT-PCR, the difference was statistically siginificant (*p= 0.001, and p= 0.03; unpaired t-test*) in both SW620 and SW480 cell lines respectively. Error bars represent mean ± SD of two independent experiments (C), when compared with cells transfected with scrambled siRNA control.

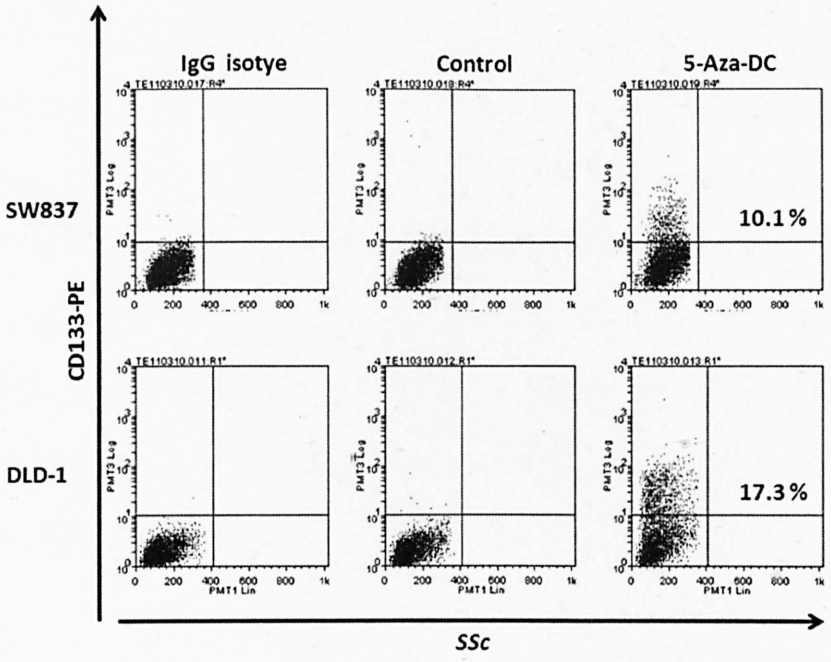


Figure 6-14: CD133 expression analysis after treatment with 5-aza-2'deoxycytidine (5-aza-dc).

Flow cytometry analysis of cell surface expression of CD133 protein in SW837 and DLD-1 (negative expressers of CD133) with and without 5-aza-dc treatment. Representative data revealed that CD133 expression was up-regulated after 5-aza-dc treatment. (SSc= side scatter). Each graph is a sample graph of two independent experiments.

6.4 Discussion

In recent years, large numbers of researchers have tried to identify colorectal cancer stem cells and to elucidate their roles in developing and

maintaining the tumour, as well as their resistance to the conventional chemo-radiotherapy. CD133 is one of several markers that have been used to isolate colorectal CSCs in an attempt to study their characteristics and clarify the pathways associated with their characteristics.

Consistent with Rappa *et al.* who reported in malignant melanoma that CD133 knockdown resulted in impaired cell motility *in vitro*, and decreased metastasis specially to spinal cord *in vivo* (Rappa, Fodstad et al. 2008), our data showed that SiRNA–mediated downregulation of CD133 was associated with reduced cell motility and invasion *in vitro*. Therefore, we sought to clarify the impact of CD133 on metastasis by studying its association with EMT, an initial step in the process of metastasis. This was achieved by studying the association between CD133 expression and E-cadherin and β-catenin.

Two approaches were conducted to assess the impact of CD133 on both E-cadherin and beta-catenin. First, gene expression silencing using the stealth RNAi technique. Second, forced expression of CD133 gene using the cloned pcDNA3.1-CD133 plasmid, and hence exploring CD133 gene functionality on expression of E-cadherin and beta-catenin. Knockdown and forced expression data revealed that CD133 has neither effect on E-cadherin nor β-catenin. In agreement with our data, Horst et al reported that no correlation was found between CD133 and nuclear β-catenin in

stage IIA colon cancer using immunohistochemistry approach (Horst, Kriegl et al. 2009).

Surprisingly, when evaluating the role of Cten as an upstream regulator of CD133, GFP alone treated cells revealed a minimal increase of CD133 protein expression detected on both western blot and flowcytometry analysis, In addition, GFP-Cten plasmid treated cells showed a modest increase of CD133 protein expression compared to cells treated with GFP alone. In a study in breast cancer, it was reported that Cten induction was mediated by Interleukin-6 (IL-6) was dependent on STAT3 activation (Barbieri, Pensa et al. 2010). Recently, other observations revealed that IL-6 could induce CD133 expression through loss of methylation of *CD133* promoter (D'Anello, Sansone et al. 2010). This could lead to a speculation that Cten may induce CD133 expression through loss of methylation of *CD133* promoter. Supporting this speculation, CD133 induction occurred in SW837 cell line (cell line negative for CD133) after 5-aza-2' deoxycytidine treatment suggesting either a methylation of *CD133* promoter or methylation of promoters of molecules regulating CD133. In addition, Liao *et al.* found that Cten was detected in both the cytoplasm and the nucleus of colon cancer cells suggesting different cellular functions of Cten (Liao, Chen et al. 2009). Given that SW837 cell line that appears to be negative for CD133 and transfection with GFP vector alone lead to a minimal increase of CD133 protein expression, caution should be considered during interpretation of these data as regarding Cten an upstream regulator of CD133. Such a relationship could be further investigated by either using

another vector type or doing gene silencing for Cten and evaluate CD133 protein expression. At this stage, it will be difficult to give a clear statement regarding the mechanistic role by which CD133 promotes motility, but a possible mechanism may include that CD133 enhances cancer cell motility through induction of metalloproteinases as stated in hepatocellular carcinoma (Kohga, Tatsumi et al. 2010).

In human tumours, Wnt signalling pathway is dysregulated in diverse tumours (Ilyas 2005). Brabletz *et al.* proposed that colon CSCs are characterized by Wnt signalling pathway activation (Brabletz, Jung et al. 2005). This proposition was supported by the finding that spheroids resulted from culturing a single colon CSC expressed a subset of stem cell marker including CD133, CD166, CD44 and CD24 beside nuclear beta-catenin (Vermeulen, Todaro et al. 2008). Despite what has been stated above, information about direct impact of Wnt signaling activation on CD133 expression was not yet known.

Hence, we blocked Wnt signalling using dnTCF4 in order to assess the relation between Wnt signalling and CD133. Our data revealed that the dnTCF4 resulted a small decrease on the level of c-myc that was detected on the level of mRNA and this small effect has no impact on the level of CD133 protein expression - neither increased nor decreased. Therefore, further work is needed to improve the transfection efficiency of dnTCF4

and demonstrate its effect on c-myc level, so that we will be able to prove or disprove the relation between Wnt signaling pathway and CD133.

STAT3 is important in tumorigenesis and embryonic stem cell development. STAT3 target genes are involved in many cellular process including proliferation, tumour growth and metastasis (Corvinus, Orth et al. 2005; Huang 2007). A study by Ferrand *et al.* reported that holoclone (a type of colony morphology) showed higher expression of CD133, active forms of STAT3, Jak2, ERk and AKT compared to other types of colony morphology in a cancer cell line (Ferrand, Sandrin et al. 2009). In viewing of these data, we sought to assess the role of STAT3 in regulating CD133 expression. The stealth RNAi technique was employed to evaluate the STAT3 gene silencing function on CD133 expression. Our data revealed that down-regulation of CD133 expression followed STAT3 knockdown, implying that CD133 could be a downstream target of STAT3. In line with our data, STAT3 knockdown in glioblastoma is associated with initiation of apoptosis, a reduction in BCL-2 and cyclin D1 expression as well as decrease in the number of CD133+ cells (Li, Wei et al. 2010). Taken together, STAT3 appears to act as a regulator of CD133 expression in colorectal cancer.

While screening cell lines for an effect of STAT3 Knockdown, it was noticed that STAT3 was expressed in SW837 (appendix 9.6) but CD133 is not expressed in this cell line. A plausible explanation is that CD133 expression could be inhibited by epigenetic modification. This notion was

supported by the finding that DNA methylation suppressed CD133 expression in CD133 negative progeny of CD133 positive cells (Baba, Convery et al. 2009). In line with these findings, this study revealed that treatment of SW837 cell line (negative expresser of CD133) with 5-aza-2'-deoxycytidine recovered CD133 expression. The later data are corroborated with other studies in colorectal cancer which showed that CD133 expression was modified by epigenetic regulation through promoter methylation status. For instance, a study showed that CD133 promoter hypermethylation was only found in colon cancer tissue compared to normal colon samples, furthermore hypermethylation was associated with repression of CD133 expression (Yi, Tsai et al. 2008). Similarly, other studies showed the influence of CD133 promoter methylation on CD133 expression in colon cancer (Jeon, Kim et al. 2010), ovarian cancer (Baba, Convery et al. 2009), breast cancer (D'Anello, Sansone et al. 2010), and glioma (Tabu, Sasai et al. 2008).

As a conclusion, this study revealed that CD133 might be controlled by STAT3 or DNA methylation under unique circumstances. Furthermore, CD133 might not be a Wnt signalling target. Although CD133 enhances the motility of cancer cells, this appears not to be through regulation of either E-cadherin or β-catenin. In addition, according to this study the relation between CD133 and Cten in colorectal cancer is still unclear and warrants further investigations.

7 Chapter 7: CD133+ colorectal cancer cells are associated with stem cell markers

7.1 Abstract

Background and aim: Data in chapter 5 showed that CD133 might be associated with features of stemness; however, CD133 expression in the cell lines ranged from 0-95%, so, it is unlikely that CD133 can be considered as an absolute marker for CSCs. We hypothesized that CD133 expression may be associated with other stem cell markers.

Methods: SW480 cell line was sorted using MACS, and then qRT-PCR for assessment of expression of five stem cell-related genes (*Musachi-1, Oct4, Lgr5, KLF-4*, and *Nanog*) in the sorted CD133+/- populations was conducted.

Results: All stem cell related genes were highly expressed in CD133+ than in CD133- populations and this difference was statistically significant with the exception of Lgr5 (*p=0.03, p=0.008, p=0.004, p=0.006, p=0.001,* and *p=0.11;unpaired t-test*, for *CD133, KLF-4, Musashi-1, Oct4, Nanog,* and *Lgr5* respectively)

Conclusion: CD133+ cells were enriched with cancer cells that harbor stem cell related genes.

7.2 Introduction

Our data have shown that CD133 expressing cells were associated with greater colony forming efficiency, and are resistant to apoptotic stress compared to their CD133 negative counterparts (Ieta, Tanaka et al. 2008; Zobalova, McDermott et al. 2008). Thus, by these criteria, CD133 might be considered as a marker associated with features of stemness. However, the percentage of tumour cells showing CD133 expression in the CRC cell lines was variable ranging from 0 – 95%. From this, it is unlikely that CD133 is an absolute marker of CSC. This led us to wonder whether CD133 expression was associated with other stem cell markers and whether, in future, a panel of markers could be developed to define more precisely CSCs.

In recent years a number of markers have been identified which, when induced in terminally differentiated cells, are able to re-program the cells into pluripotent stem cells (Takahashi and Yamanaka 2006; Yu, Vodyanik et al. 2007). These markers of induced pluripotent stem cells (iPS) include Krüppel-like factor 4 (*KLF-4*), Octamer binding transcription factor 4 (*Oct4*), and *Nanog* – these are transcription factors that are important for development and maintenance of pluripotent cells. It has been stated that *KLF-4*, which plays an important role in a number of cellular processes such as cellular proliferation, differentiation and maintenance of stem cells, is involved in the process of carcinogenesis through its function as both

tumour suppressor and oncogene depending on tissue type and cellular context (Rowland and Peeper 2006).

Oct4 (*POU5F1*) is a transcription factor that is involved in maintenance of self renewal and pluripotency of human embryonic stem cells (Pesce and Schöler 2001). Moreover, Oct4 was expressed in germ cell tumours and used as a diagnostic marker for subtypes in germ cell tumours (Cheng, Sung et al. 2007), suggesting that *Oct4* contribute to malignant transformation of primordial germ cells (Kehler, Tolkunova et al. 2004). Several studies have reported that *Oct4* expression can be detected in adult somatic stem cells and different cell lines (Steingart, Heldenberg et al. 2002; Tai, Chang et al. 2005).

Similarly, *Nanog* is a transcription factor that is also involved in maintenance of self renewal and undifferentiated state of embryonic stem cells (Pan and Thomson 2007). Along with *Oct4* and *Sox2*, it is involved in the reprogramming of differentiated cells into stem cell state (Takahashi and Yamanaka 2006; Yu, Vodyanik et al. 2007)

Other proposed markers for CRC cancer stem cells include *Musashi-1* (Msi-1). This is an RNA binding protein that is involved in the asymmetric divisions of neural stem/progenitor cells (Kaneko, Sakakibara et al. 2000). In addition, it regulates the expression of several genes involved in cell cycle regulation, proliferation (de Sousa Abreu, Sanchez-Diaz et al. 2009). In mammary epithelium, expression of *Msi-1* leads to inhibition of

Dickkopf3 (Wnt inhibitor) and activation of *proliferin1*, which in turn activates the *Wnt* pathway and *Notch* in mammary stem/progenitor cells (Wang, Yin et al. 2008). In mice and humans, it has been found that Msi-1 is preferentially expressed in the intestinal stem / progenitor cell regions, suggesting its role as a marker for stem/ progenitor cells (Kayahara, Sawada et al. 2003; Potten, Booth et al. 2003)

Leucine-rich repeat-containing G-protein coupled receptor 5 (*Lgr5*), which is a G-protein couple receptor and a downstream target gene of Wnt signalling pathway, was identified as a marker for intestinal stem cells (Barker, van Es et al. 2007) and hair follicles stem cells (Morris, Liu et al. 2004). In colon cancer, Zhu *et al.* stated that lgr5 positive intestinal stem cells also expressed CD133 that is susceptible to neoplastic transformation (Zhu, Gibson et al. 2009).

The plasticity of CD133+ cells and their ability for self renewal would predict that CD133+ cells harbouring genes related to stem cell maintenance. Therefore, the aim of this study is to assess the expression of stem cell related genes involved in stem cell maintenance and reprogramming of differentiated cells in CD133+ and CD133- cells by qRT-PCR.

7.3 Results

7.3.1 MACS sorting of SW480 cell line

In order to investigate the relationship between expression of CD133 and other stem cell markers, we sought to test the CD133+/CD133- populations derived from the bimodal cell lines. We have shown, by flow cytometry, that in SW480 there is a CD133+ population comprising approximately 40% of the total tumour cell population. Because MACS is less time consuming than FACS in collecting large volume of either positive or negative populations, SW480 was sorted into CD133+/- populations by MACS (see Materials and Methods). The purity of CD133+ cells was 88%, while CD133- population was 76% as shown by post-sorting flow cytometry analysis (figure 7-1).

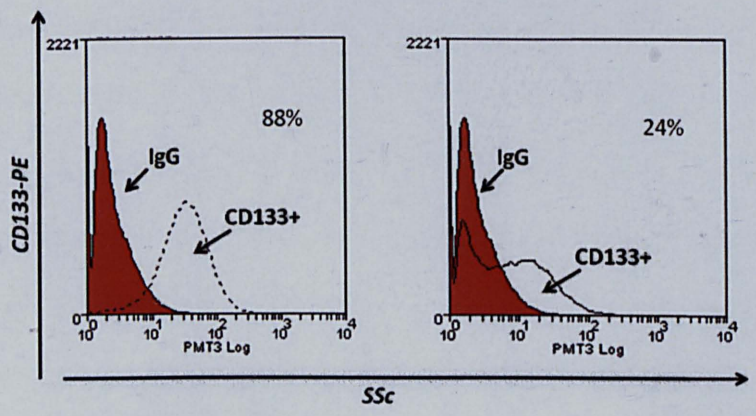


Figure 7-1: Purification of Sorted populations of SW480 cells
Sw480 cell line was sorted using MACS, and the sorted populations were subjected to post sorting flow cytometry analysis. FACS analysis showed that sorted CD133+ cells were 88% pure (left histogram), while the CD133- cells still have 24% positivity (right histogram). Filled histogram (red) represents IgG isotype control.

7.3.2 PCR optimization and data analysis

The expression of genes involved in the stem cell maintenance pathways such as *Oct4, Nanog, Klf-4, Musashi-1* and *Lgr5* were scrutinized by qRT-PCR. First, primers for these genes were designed with the help of Primer3-web (v. 0.4.0) (http://frodo.wi.mit.edu/primer3/input.htm). Then, the specificity of the primers was checked with primer blast (http://www .ncbi.nlm.nih.gov/tools/primer-blast). For qRT-PCR, primers annealing temperature was first optimised. For the purpose of primers annealing temperature optimisation, a wide range of annealing temperatures from 53°C - 62°C were used to obtain a high quality PCR amplified product. The PCR products were then subjected to 1% agarose gel electrophoresis to discover the effect of different annealing temperature and to further validate the specificity of the primers (Figure 7-2). Then, cDNA of 6 different cell lines (SW480, SW620, Lovo, HCT116, SW837, and HT55) was screened for the different levels of the stem cell related genes, and the cell line with the lowest C_t (cycle threshold) value which indicates high expression was used as a template for initiation of standard curve for each of these genes. This experiment showed that HCT116 was high in case of *Musashi-1* (27.28), and *Oct4* (28.38), while SW620 was high in *Lgr5* (27.17), and SW480 high in *Nanog* (26.66) and *KLF-4* (23.73) based on C_t value shown between brackets.

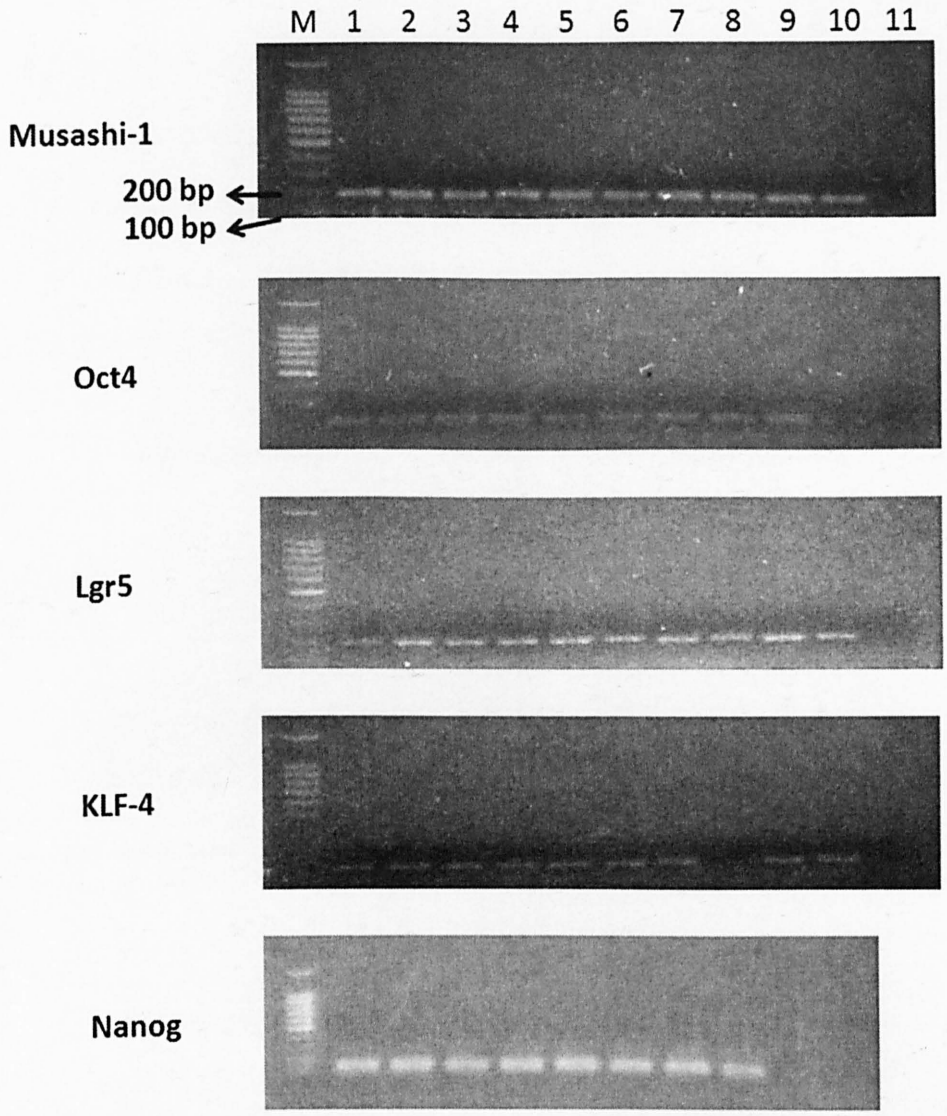
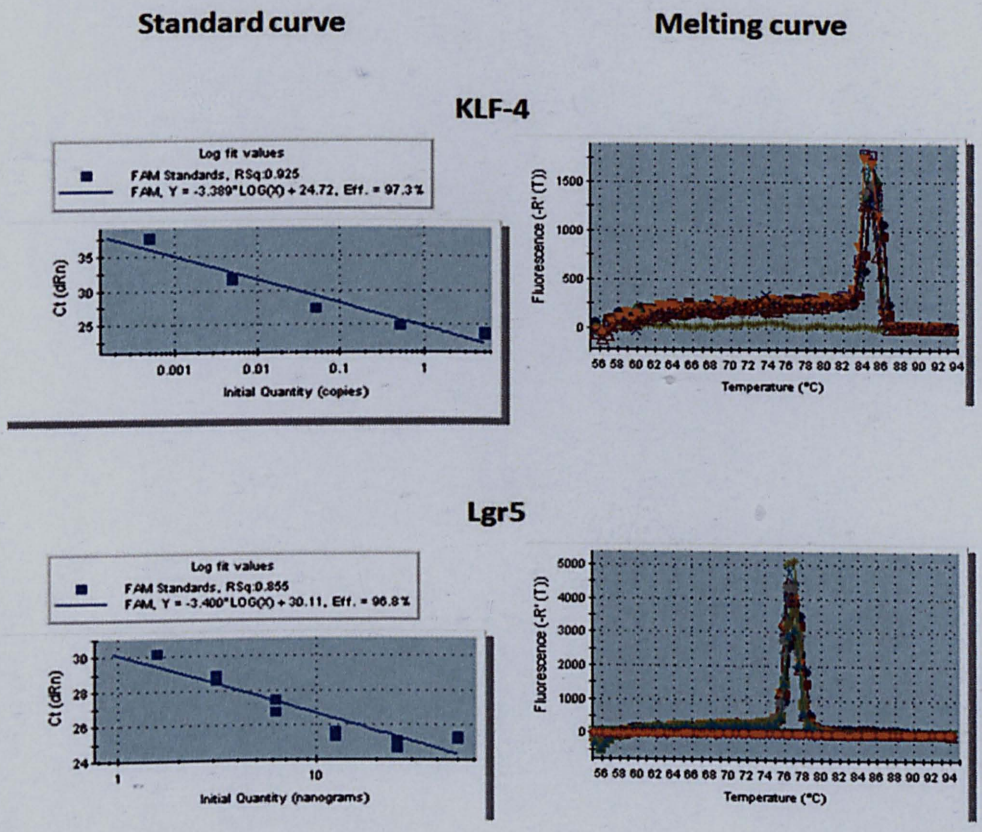


Figure 7-2: Agarose gel electrophresis for gradient annealing temperature.
Annealing temperature was optimised by doing gradient temperature in order to yield a high quality PCR product. A temperature range from 53°C-62°c was used (lane 1-10), lane 11 represent no template control (NTC). Agarose gel electrophoresis showed PCR products at the correct expected size which are 191, 183, 181, 169, and 213 bp for *Musashi-1, Oct4, Lgr5, KLF-4* and *Nanog* respectively. A 100 bp DNA ladder was used (M).

With the use of the aforementioned cell lines, a standard curve was initiated for each gene primer using different annealing temperatures in

order to get the best PCR efficiency (Figure 7-3). After several experiments, the best efficiency for KLF-4 was 97%, Lgr5 was 96.8%, Msi-1 was 94.7%, nanog 111%, and Oct4 was 85.5%. Moreover, melting curve analysis revealed specificity of the primer (for primer sequence, amplicon size and optimum annealing temperature see material and methods section 2.8).



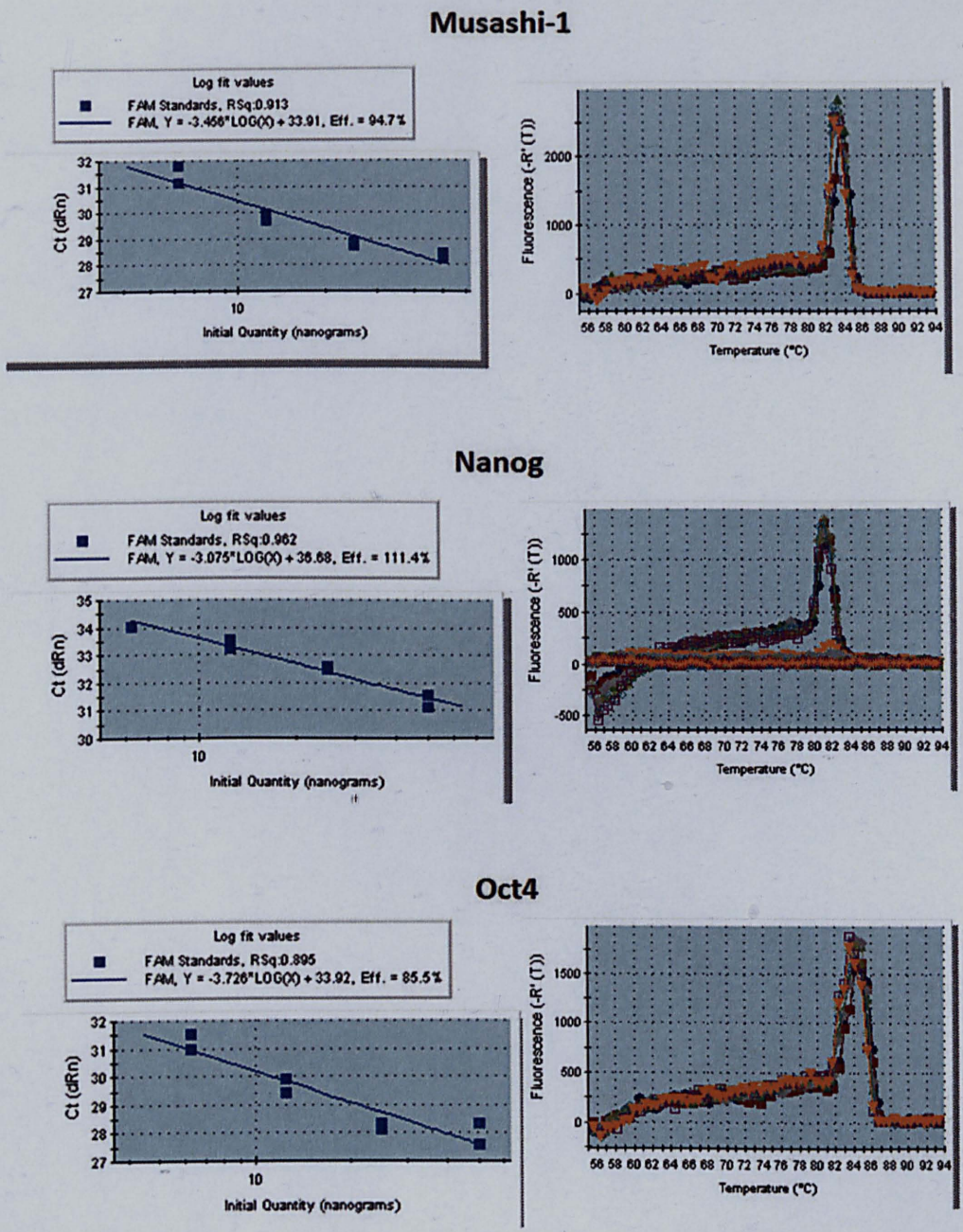


Figure 7-3: Standard curve efficiency for different gene primers

This figure illustrated the efficiency of standard curve for different gene primers studied. Also, melting curve analysis revealed a single product indicating the specificity of the primers used.

After obtaining a good PCR efficiency, cDNA from sorted CD133+/- populations was tested for each gene. Each experiment was done twice with triplicates for each sorted population. As shown in figure 7-4, the stem cell related genes such as *Oct4, Nanog, KLF-4, Msi-1, Lgr5* and *CD133* were more highly expressed in CD133+ than in CD133- populations and this difference was significant when performing unpaired t-test with the exception of *Lgr5*.

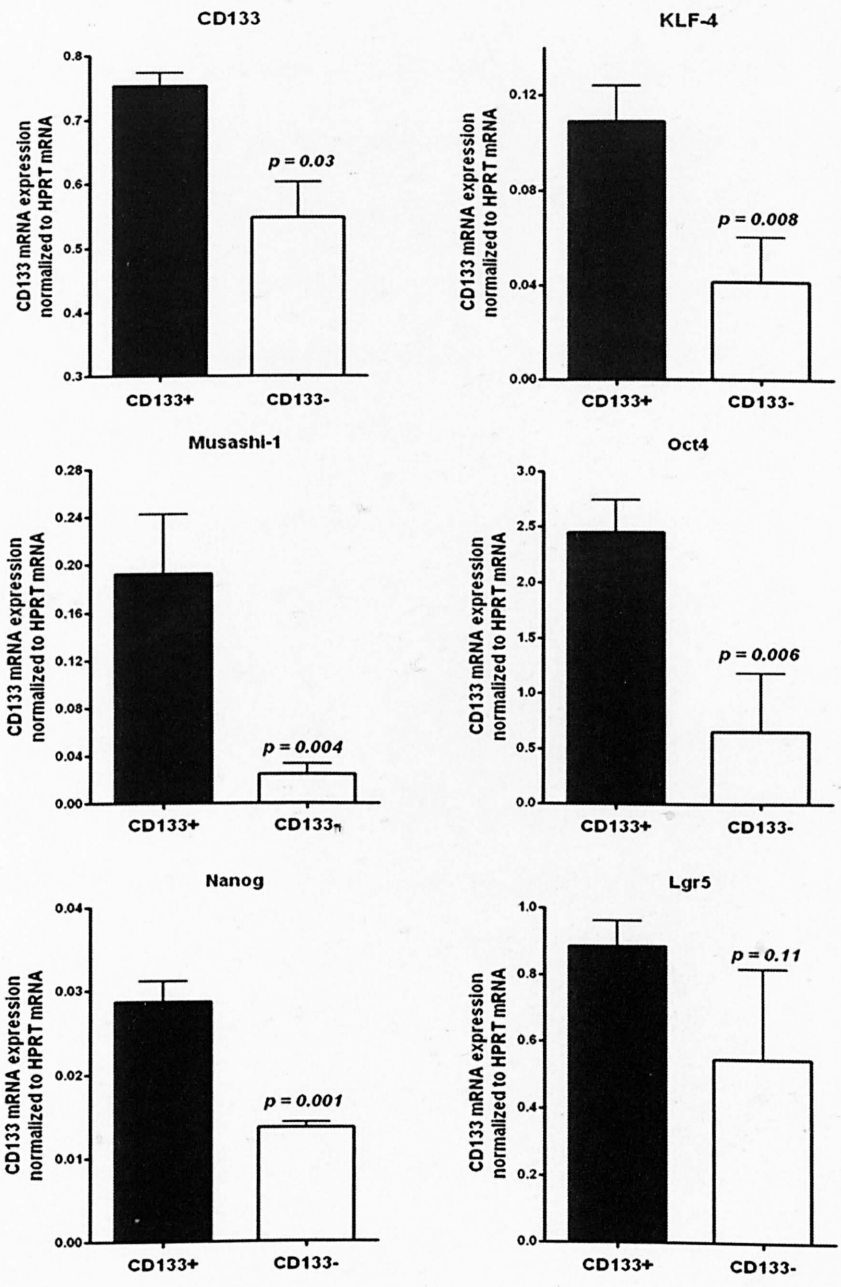


Figure 7-4: qRT-PCR analysis of different stem cell related genes in sorted CD133+/- cells

This figure showed expression of different stem cells related genes in sorted CD133+/- populations of SW480 cell line. The data revealed that Oct4, Nanog, Msi-1, Klf4, and Lgr5 were preferentially expressed in CD133+ cells compared to negative counterpart and this expression was statistically significant using unpaired t-test with the exception of Lgr5. Data represent mean of 2 independent experiments ± SD with triplicate for each condition.

7.4 Discussion

Recently, there has been accumulating evidence supporting the hypothesis that human cancer development could be attributed to a small population of cells with stem cell like features termed CSCs. In several cancer types; CD133 is used to isolate this group of cells (Huang, Dong et al. 2006; Suetsugu, Nagaki et al. 2006; Brown, Gilmore et al. 2007; Li, Heidt et al. 2007; O'Brien, Pollett et al. 2007).

Several studies support the notion that CD133 expression is associated with stem cell features such as self renewal capability and sphere formation. Consistently, several studies in many solid tumours have demonstrated that CD133+ cells, unlike CD133- counterparts, are able to grow as tumour spheres *in vitro*, and to develop tumours in a mouse model (Beier, Hau et al. 2007; Ferrandina, Bonanno et al. 2008; Friedman, Lu et al. 2009). In colon cancer, several studies support the fact that CD133 positive cells are able to induce tumour self-renewal and possess higher colony forming ability *in vitro* than CD133 negative cells (Ricci-Vitiani, Lombardi et al. 2007; Ieta, Tanaka et al. 2008; Li, Xiao et al. 2008) with the exception of Shmelkov *et al.* (Shmelkov, Butler et al. 2008)

Several studies have demonstrated the involvement of a number of transcription factors such as *KLF-4, Oct4,* and *Nanog*, in reprogramming the differentiated cells into pluripotent stem cells and maintaining their self renewal ability (Takahashi and Yamanaka 2006; Yu, Vodyanik et al. 2007).

In addition, other markers have been used to identify intestinal stem cells; *Lgr5* and *Musashi-1* (Potten, Booth et al. 2003; Barker, van Es et al. 2007).

On account of this information, SW480 cell line was purified into CD133+/- populations using MACS and the sorted populations were examined for the expression of *Oct4, Nanog, KLF-4, Lgr5,* and *Musashi-1*. Although the purity of CD133+ and CD133- populations was not as high as that obtained with FACS, it was still in line with another study in hepatocellular carcinoma where MACS sorting of hepatocellular carcinoma cell line resulted in 84% CD133+ cells and CD133- cells still containing 23% positivity of CD133 (Kohga, Tatsumi et al. 2010). However, this purity is not similar to other studies using MACS for isolation of CD133+/- populations. A plausible explanation for this discrepancy, in this study we used two step labelling of the cells (first cells were labelled with CD133 conjugated with PE, then anti-PE microbeads were used) rather than a single labelling step (using anti-CD133 conjugated to microbeads). This may cause escape of some of CD133-PE labelled cells for being attached to anti-PE microbeads resulting in escape of some of positive cells during magnetic sorting.

QRT-PCR data revealed that CD133 mRNA expression in CD133 + cells was double that in CD133- cells, which in contrast to what we found earlier after FACS. This contradiction might be due to the low purity of the sorted population. Moreover, PCR data showed that CD133 expression was associated with the expression of the other proposed CSC or iPS markers.

In contrast to these data, Saigusa *et al.* found that there was no correlation between CD133, OCT4 and Sox2 expression in colorectal cancer by immunohistochemistry (Saigusa, Tanaka et al. 2009). However, our data are in agreement with other studies in different tumour types which have demonstrated that CD133+ cells expressed stem cell related genes such as *OCT4, Sox2* and *Nanog* in liver (Kordes, Sawitza et al. 2007), breast (Wright, Calcagno et al. 2008), glioblastoma (McCord, Jamal et al. 2009), thyroid (Friedman, Lu et al. 2009), and lung (Bertolini, Roz et al. 2009). Moreover, in glioblastoma, qRT-PCR revealed that mRNA expression of stem cell gene such as *Nanog, OCT4* and *KLF-4* was higher in CD133+ than CD133- population (Zbinden, Duquet et al.).

To sum up, this study showed that CD133+ cells were enriched with cancer cells that are endowed with expression of stemness related genes.

8 General Discussion

Introduction

Colon cancer is a serious health problem worldwide and in the UK. In the UK, it is the third common cause of death (2010). Despite advances in chemotherapy, metastases still represent a common challenge in affecting the prognosis of patients (Ratto, Sofo et al. 1999). In recent years, a cancer stem cell theory has emerged and according to this theory, these cancer stem cells are responsible for growth, metastases and heterogeneity of the tumour (Reya, Morrison et al. 2001; Brabletz, Jung et al. 2005; Burkert, Wright et al. 2006). Currently, there has been a dramatic increase in the evidence supporting the cancer stem cell theory which led to a surge of interest towards isolation and characterization of these cells in different solid tumours.

Researchers have been challenged by the identification of normal stem cells in adult tissue and have used different techniques to identify these cells such as morphology (Karam 1999) or the dye-exclusion method (i.e side populations) (Chiba, Kita et al. 2006). Recently, cell surface markers have been identified on these cells with the help of fluorescence-labelled antibodies and flow cytometry (Ebener, Brinkmann et al. 2000). Generally these putative markers of normal stem cells have been used to identify possible tumour stem cells and to isolate them in order to assess the stem cell–like features (such as self renewal, and differentiation ability) of these cancer cells.

Within this context, several studies have used several cell surface markers such as CD44, CD24, ESA, α2β1 integrins, ALDH1, and CD133 to isolate the tumour stem cells. Cells expressing these markers have been found to be capable of initiating tumours *in vivo*, grow as spheres *in vitro* as well as have the ability to self renew when serially transplanted, hence supporting the concept of cancer stem cells (Al-Hajj, Wicha et al. 2003; O'Brien, Pollett et al. 2007; Prince, Sivanandan et al. 2007).

One of these markers is CD133, which is a five transmembrane glycoprotein molecule with a molecular weight of ~ 120 KD (Miraglia, Godfrey et al. 1997). In humans, CD133 has been shown to be located at the apical plasma membrane protrusions (Corbeil, Roper et al. 2000). Although the function of CD133 is not known, a role in cell polarity, migration, cell – cell interaction, and /or interaction with extracellular matrix has been suggested due to its localization (Shmelkov, St Clair et al. 2005). Initially, CD133 was reported to be a marker of normal haematopoietic stem cells (Yin, Miraglia et al. 1997) although now it is considered a putative marker of cancer stem cells in many solid tumours such as colon cancer (O'Brien, Pollett et al. 2007; Ricci-Vitiani, Lombardi et al. 2007), glioma (Singh, Clarke et al. 2003; Bao, Wu et al. 2006), hepatocellular carcinoma (Suetsugu, Nagaki et al. 2006), melanoma (Rappa, Fodstad et al. 2008), thyroid carcinoma (Zito, Richiusa et al. 2008), and pancreatic cancer (Olempska, Eisenach et al. 2007). However, its role as a sole cancer stem cell marker is still debatable.

The work conducted in this thesis was to (i) examine the frequency and distribution of expression of CD133 in colorectal cancer and its relationship to tumour clinicopathological characteristics and patients' clinical outcome, (ii) identify the biological characteristics associated with CD133 expression, (iii) delineate the mechanistic basis that could be possibly implicated in the regulation or the function of CD133 in colorectal cancer, and (iv) study the molecular features of stem cell related genes in CD133 expressing cells.

Clinical implications of CD133 expression

Apart from characterization of cells expressing stem cell markers, it is of utmost importance to study the impact of expression of these markers on the clinical outcome. However, this field of research could face a lot of challenges. For instances, several studies have shown that more than one marker may be required to identify these cancer stem cells which is technically easy in fresh tissue with the use of flow cytometry technique (Al-Hajj, Wicha et al. 2003; Li, Heidt et al. 2007; Yi, Zhou et al. 2007). However, in formalin-fixed paraffin embedded tissue, only a single antibody using immunohistochemistry technique can be applied on each section. Another challenge is that, in order to translate the cancer stem cell markers into clinical practice, these markers should support the cancer stem cell model in determining the prognosis of the patient and response to treatment (Woodward and Sulman 2008).

The impact of CD133 expression on clinical patients' outcome has been assessed in several solid tumours. These studies showed that CD133 is a prognostic factor for low patient survival (Klein, Wu et al. 2007; Horst, Kriegl et al. 2008; Maeda, Shinchi et al. 2008; Pallini, Ricci-Vitiani et al. 2008; Song, Li et al. 2008; Tong, Zheng et al. 2008).

In this study, the impact of CD133 expression on clinical outcome in colorectal cancer was evaluated by immunohistochemical staining of a larger series of 449 cases of primary colon cancer samples and 45 cases of matched primary and liver metastases constructed as TMAs. CD133 was found to be an independent prognostic factor for low patient survival in colorectal cancer but without any correlation with any of the clinicopathological variables and no difference in expression between primary and metastatic cases. Other reports have shown similar findings in colon cancer (Ferrandina, Bonanno et al. 2008; Horst, Kriegl et al. 2008; Kojima, Ishii et al. 2008; Liu, Li et al. 2009). Moreover, several studies revealed that CD133 was a predictive marker for non-response to treatment in colon (Yasuda, Tanaka et al. 2009; Nakamura, Iinuma et al. 2010; Ong, Kim et al. 2010), and lung (Salnikov, Gladkich et al. 2010). Taken together, our data and other studies, CD133 is an independent prognostic factor in colorectal cancer. Although promising as a prognostic and predictive marker, several issues should be taken into account before translating these laboratory studies into clinical oncology. Notably, these studies have used different cut-off points dividing the data into low and high, different antibodies, testing small sample size. All these issues

resulted in variation in judging whether CD133 of prognostic or predictive values or correlated with any of the clinicopathological variables.

CD133 mRNA expression is higher in metastatic than primary colorectal cancer cell lines

Since CD133 might play a role in predicting patient clinical outcome, it was of interest to study the biological characteristics of CD133 expressing cells. As gene silencing and forced expression techniques were difficult to be applied for primary clinical samples, it was reasonable to use colorectal cancer cell lines as a model for studying the characteristics of CD133 expressing cells. A number of colorectal cancer cell lines were first screened for the level of CD133 mRNA level. Interestingly, it was found that CD133 mRNA expression level was higher in colorectal cancer cell lines derived from metastatic sites compared to the cell lines derived from sites of primary tumours. In line with these data, recently, two studies in colorectal cancer showed that high CD133 mRNA was correlated with lymphovascular invasion, lymph node involvement and depth of invasion (Huh, Park et al. 2010) and associated with shorter survival (Artells, Moreno et al. 2010) suggesting that CD133 mRNA level could be used as a new diagnostic and therapeutic tools in CRC patients.

CD133 as stem cell marker

Given that, as reported in the literature, CD133 may represent a stem cell marker, it was reasonable to study the biological characteristics associated with CD133 expression in colorectal cancer.

Two approaches were used to compare functional characteristics of CD133+ cells with CD133- cells. Firstly gene silencing using RNAi technique was employed to inhibit gene expression. Secondly, the identification of biphasic populations allowed sorting of cells in this CD133+ and CD133- populations. Sorting technique of cells into CD133+/- populations was conducted as a complementary approach to explore CD133 biological functionality.

Data in this study showed that CD133 expressing cells had enhanced migrating ability, colony formation and resistance to apoptotic stress induced by staurosporine, compared to the negative counterparts in *in vitro* studies. Moreover, CD133+ cells were able to produce both progeny (CD133+/- populations) after prolonged culture, and surprisingly, CD133- cells similarly reverted to a biphasic phenotype. During the current project, many studies showed the same findings in colon cancer (Ieta, Tanaka et al. 2008; Puglisi, Sgambato et al. 2009; Kawamoto, Yuasa et al. 2010), and many different tumour types such as glioma (Singh, Clarke et al. 2003), hepatocellular carcinoma (Suetsugu, Nagaki et al. 2006; Yin, Li et

al. 2007) pancreatic (Moriyama, Ohuchida et al. 2010), thyroid (Zito, Richiusa et al. 2008) ovarian (Ferrandina, Bonanno et al. 2008; Curley, Therrien et al. 2009), endometrial cancer(Rutella, Bonanno et al. 2009), and lung cancer (Eramo, Lotti et al. 2008; Bertolini, Roz et al. 2009).

Having identified CD133 expressing cells were associated with some stem cell-like features, we next set up to investigate the expression of stem cell related genes such as *Oct4, Nanog, KLF-4, Musashi-1*, and *Lgr5.* First, we sorted one of the cell lines into CD133+/- population by MACS. However, the level of purity of CD133- populations was low; qRT-PCR analysis of CD133 mRNA expression level was higher in CD133+ cells than that in CD133- population. Then mRNA level of stemness related genes were assessed. Interestingly, data from this analysis showed that mRNA levels of stemness related genes were higher in CD133+ cells compared to their negative counterpart, indicating that CD133+ cells are enriched with associated with a stem cell-like signature.

O'Brien *et al.* reported that there was one colon CSC in 262 CD133 + cells (O'Brien, Pollett et al. 2007). In another study, it was shown that 1 in 20 CD133 + cells have a clonogenic ability, and this ability was increased into 1 in 5 when CD133 coexpressed with CD24 (Vermeulen, Todaro et al. 2008). As has been stated, and together with our findings that CD133 expressing cells were endowed with stem cell like features and expressed higher levels of stem cell related genes, we can conclude that not all

CD133 expressing cells were cancer stem cells, and CD133 alone could not identify all these cells.

One of the limitations of this study is the absence of an animal model to justify the tumourigenic and metastatic potential of CD133 expressing cells. To do an animal model study, either gene silencing or CD133 stably transfected cell lines should be used. In this study, the siRNA used for CD133 gene silencing is short lived and not suitable for the animal studies. However, constructs expressing small hairpin (sh) RNAs that are stably transfected into the cells will be suitable, but this technique was not available in the laboratory. Another possibility would be to stably express CD133 in a cell line which is CD133- (such as DLD1or SW837). Unfortunately, during this study several attempts to generate cell lines stably transfected with a CD133 expression vector failed which could be due to inability of the vector to be integrated into the cell genome as these cells are cancer cells. However, many cancer cells have been successfully stably transfected, and it is much more likely that due to their resistance to the drugs used for selection of cells with integrated DNA, wt cell-lines were able to survive the selection process. An alternative approach would be to use another system e.g lentiviral system which more efficiently integrates DNA into genome increasing chance of stable expression.

In summary, this study identified that CD133 may contribute to the observed migration, colony formation, and resistance to apoptosis abilities

in colorectal cancer. CD133 expressing cells are able to produce CD133+/- progeny indicating the plasticity of these cells. Furthermore, stem cell related genes are higher in CD133+ cells than in CD133- populations suggesting the presence of cancer cell with stem cell-like signature within the CD133+ population.

STAT3 and DNA methylation, but not Wnt signalling pathway regulate CD133 expression

Epithelial-Mesenchymal Transition (EMT) is a process of molecular and morphological changes that affects epithelial cells leading to gain of mesenchymal features, increase in cell motility and which may play a fundamental role in invasion of tumour cells (Lee, Dedhar et al. 2006). Recent studies have demonstrated a coincidence between EMT and CSCs, and initiation of EMT has been shown to result in up-regulation of stem cell related "stemness" genes (Mani, Guo et al. 2008; Morel, LiÃ¨vre et al. 2008). Altered expression of E-cadherin and β-catenin, key regulator elements of EMT, has been noticed when CD133+ cells were compared to CD133- cells (Brabletz, Jung et al. 1998; Lee, Han et al. 2010). Our data however revealed no association between CD133, β-actin and E-cadherin expression which was consistent with another study (Horst, Kriegl et al. 2009).

It is worth noting that Cten forced expression in cell lines was associated with a modest increase in CD133 level. The significance of this is uncertain and warrants further investigation (such as doing gene silencing for *Cten* gene in cell lines which express both CD133 and CTEN) since Cten is also an inducer of cell motility (Albasri, Seth et al. 2009).

Previous studies have shown that STAT3 expression was high in CD133+ cells and it was assumed that STAT3 could contribute to the regulation of CD133 (Ferrand, Sandrin et al. 2009; Li, Wei et al. 2010) . In the current thesis, data revealed that gene silencing of STAT3 resulted in down-regulation of CD133 protein expression mirrored by decreased number of CD133+ cells by flow cytometry analysis. Moreover, CD133 mRNA level was also down-regulated using qRT-PCR. During the current study, and in line with the data from Li *et al.* , in glioblastoma stem cells, STAT3 down-regulation was associated with a synchronous down regulation of CD133 protein (Li, Wei et al. 2010). In order to confirm the correlation of CD133 with STAT3, STAT3 immunohistochemistry should be done and evaluate its correlation with CD133 expression.

Interestingly, we found that blockage of the Wnt signalling pathway using dnTCF4 resulted in no effect on CD133 expression. With caution taken into consideration as regarding the aforementioned results, further validation by using Wnt inhibitors is required to prove or disprove such a relation between CD133 and Wnt signalling pathway. In line with our preliminary data, a study in colon cancer showed upregulation of Dickkopf homolog1

(DKK1, a Wnt signalling inhibitor) in the CD133+ compared to CD133- counterpart (Ieta, Tanaka et al. 2008). Thus STAT3 signalling seems more important than Wnt signalling in regulating CD133 levels. STAT3 transduce signals for a variety of growth factors (such as Epidermal Growth Factor) as well as a number of cytokines. The mechanism of STAT3 activation which results in increased CD133 expression needs further investigation.

The CRC cell line SW837 was found to express STAT3 but not CD133; therefore, it was thought that another possible mechanism could be controlling CD133 expression. Promoter hypermethylation of CD133 has been reported in different tumour types such as glioma (Tabu, Sasai et al. 2008), glioblastoma and colon cancer (Yi, Tsai et al. 2008), and ovarian cancer. (Baba, Convery et al. 2009) Hence, we sought to test for hypermethylation of CD133 in SW837. Using 5-aza-DC, we found heightened expression of CD133 in SW837, indicating that either CD133 promoter is methylated or methylation affected an upstream regulator of CD133. However, methylation specific PCR and bisulfate sequencing analysis for recognition of methylated and unmethylated sites should be performed to further investigate this, but due to time limitations they were not undertaken.

Taken together from this study, CD133 expression was controlled, at least in part, by STAT3 and DNA methylation in a coordinated manner, but not with Wnt signalling pathway that may require further optimization of

dnTCF4 trasnfection efficiency to verify these results. Moreover, CD133 expression is not associated with E-cadherin, β-actin, and at least partially, associated with Cten expression which may warrant further investigation; however, it may contribute to the migration ability of cancer cell through a different pathway.

Concluding remarks

The data presented in this thesis provide a perception into the importance of CD133 expression in colorectal cancer as an independent prognostic factor of low patient survival and the contribution of CD133 to increased migratory, colony forming, and resistance to apoptosis. Also, the data provide a new insight into the regulatory mechanisms of CD133 by STAT3 which have a role in invasion, metastases and resistance of colon cancer, and epigenetic changes (such as DNA methylation), and not by Wnt signalling pathway which warrants further optimization to verify it. Furthermore, the stem cell molecular features were associated with its expression. In depth understanding of the mechanisms underlying CD133 expression in colorectal cancer will enable translation of laboratory researches into targeted rationale therapy

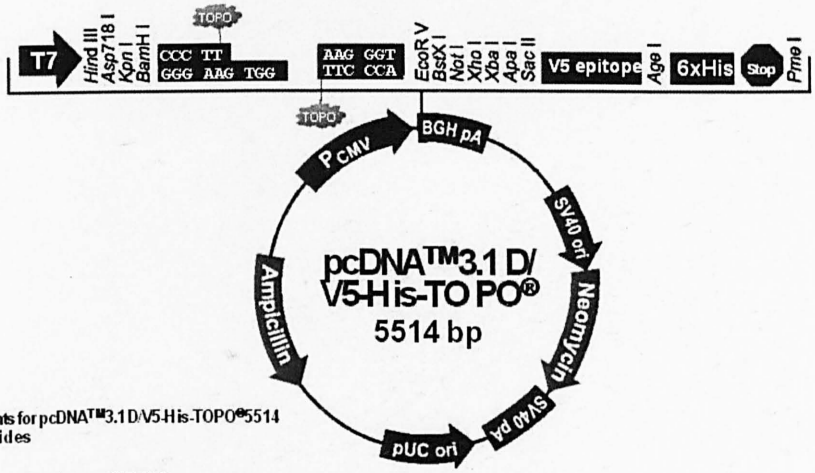
Future prospects

All these preliminary data in this thesis required more in depth studying in order to delineated the underlying mechanisms by which CD133 confer its migratory, colony forming, and apoptotic potentials.

- *In vivo* animal studies using stable transfected cell lines are required in order to validate the tumourigenic potential of CD133.
- Gene microarray analysis comparing cells either after CD133 knockdown or CD133 forced expression. This will result a large list of genes, selection of genes will be based on whether they belong to any signalling pathways involved in the migration or apoptosis or any signalling pathways involved in maintenance of stem cells. These genes will be validated by qRT-PCR.
- STAT3 immunohistochemistry in order to validate the correlation of CD133 and STAT3.
- Further optimization of dnTCF4 transfection efficiency to verify the relation between CD133 and Wnt signaling pathway.
- Methylation specific PCR and bisulfate sequencing analysis of CD133 promoter methylation status should be performed on cell lines with and without 5-aza-2' deoxycitidine treatment to further support the role of methylation in regulation of CD133.

9 Appendix

9.1 *pcDNA™3.1D/V5-His-TOPO® vector map*

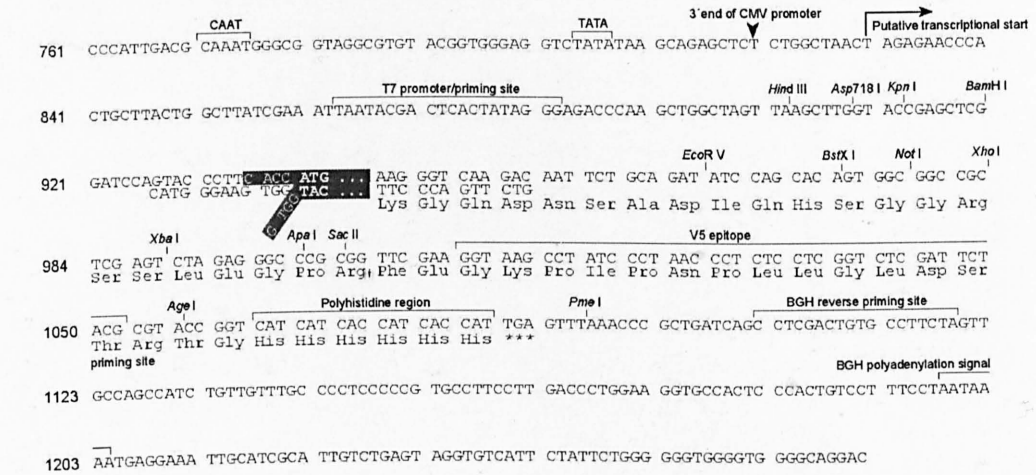


Comments for pcDNA™3.1D/V5-His-TOPO® 5514 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882 TOPO® recognition site 1: bases 930-934 Overhang sequence (complementary strand): bases 935-938
TOPO® recognition site 2: bases 939-943 V5 epitope: bases 1011-1052
Polyhistidine (6xHis) tag: bases 1062-1079
BGH reverse priming site: bases 1102-1119
BGH polyadenylation signal: bases 1108-1332
SV40 early promoter and origin: bases 1833-2142
Neomycin resistance gene: bases 2217-3011
SV40 early polyadenylation signal: bases 3189-3319
pUC origin: bases 3700-4373 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 4518-5378 (complementary strand)
bla promoter: bases 5379-5477 (complementary strand)

Invitrogen™
life technologies

9.2 *Polylinker of pcDNA™3.1D/V5-His-TOPO® vector*



9.3 Sequence of cloned CD133

Sequencing data of T7 forward primer:

CTGGCTGTTTAGCTTTGGTCCGAGCTCGGTCCAGTACCCTTCACCAT
GGCCCTCGTACTCGGCTCCCTGTTGCTGCTGGGGCTGTGCGGGAAC
TCCTTTTCAGGAGGGCAGCCTTCATCCACAGATGCTCCTAAGGCTTG
GAATTATGAATTGCCTGCAACAAATTATGAGACCCAAGACTCCCATAA
AGCTGGACCCATTGGCATTCTCTTTGAACTAGTGCATATCTTTCTCTAT
GTGGTACAGCCGCGTGATTTCCCAGAAGATACTTTGAGAAAATTCTTA
CAGAAGGCATATGAATCCAAAATTGATTATGACAAGATTGTCTACTAT
GAAGCAGGGATTATTCTATGCTGTGTCCTGGGGCTGCTGTTTATTATT
CTGATGCCTCTGGTGGGGTATTTCTTTTGTATGTGTCGTTGCTGTAAC
AAATGTGGTGGAGAAATGCACCAGCGACAGAAGGAAAATGGGCCCTT
CCTGAGGAAATGCTTTGCAATCTCCCTGTTGGTGATTTGTATAATAATA
AGCATTGGCATCTTCTATGGTTTTGTGGCAAATCACCAGGTAAGAACC
CGGATCAAAAGGAGTCGGAAACTGGCAGATAGCAATTTCAAGGACTT
GCGAACTCTCTTGAATGAAACTCCAGAGCAAATCAAATATATATTGGC
CCAGTACAACACTACCAAGGACAAGGCGTTCACAGATCTGAACAGTA
TCAATTCAGTGCTAGGAGGCGGAATTCTTGACCGACTGAGACCCAAC
ATCATCCCTGTTCTTGATGAGATTAAGTCCATGGCAACAGCGATCAAA
GGAGACCAAAGAGGCGTTGGGAGAACATGAAACAGCACCCTTGAAGA
GCTTGCACCAACAAAGTACACAGCTTAGCAGCAGTCTGACCAGCGTG
AAAACTAGCCCTGGCGGTTCATCTCTCAATGACCCCTCTGTGCTTGGT
GCCATCATCAAAGTGAATCTGCCACCAGCATCAGATTGTCTCTAGCCA
GCCTGGATTAGCATCCTGAACCTGAGCAGGATTCACCTGAATGCAGA
CTGGTACACACCGT

Sequencing results of BGH reverse primer:

AGGGTAACTAAAGGGTGATGGTGATGATGACCGGTACGCGTAGAATC
GAGACCGAGGAGAGGGTTAGGGATAGGCTTACCTTCGAACCGCGGG
CCCTCTAGACTCGAGCGGCCGCCACTGTGCTGGATATCTGCAGAATT
GTCTTGACCCTTTCAATGTTGTGATGGGCTTGTCATAACAGGATTGTG
AATACCATATACATGATCTTTATGATAACCATTATTACCATTTTCCATAT
TTTTCATGGGTATAGTTTCAACATCATCGTACACGTCCTCCGAATCCAT
TCGACGATAGTACTTAGCCAGTTTTACCGCAAAAATTAGAGCCGGAAG
TAAAAATACAGTAGCTTTTCCTATGCCAAACCAAAACAAATTCAAGGG
GTCGATAATGTAGCTACACAGAAAGACATCAACAGCAGTATCTAGAGC
GGTGGCCACAGGTTTGCACGATGCCACTTTCTCACTGATAGAGAACT
CGATCCACTGCAGATAATGTTCAAAATATCCTATTATTGTTCTCCCATA
CTTCTTAGTTTCCTCAATAATAACAGAGGAAGTATTGTTTGTGATGAAG
TTCTGAGCAAAATCCAGAGAAGCTAGAATCCTAGTTACTCTCTCCAAC
AATCCATTCCCTGTGCGTTGAAGTATCTTGACGCTTTGGTATAGAGTG
CTCAGTGATTGTTCTATAGGAAGGACTCGTTGCTGGTGAATTGTTTTA
ATAGTTTGTGCATCTCTTTTCAGGGAGTTCCTCAAATTTCCTGGGGGC
AAACTGTTTGCTTTTGCTTCTAGATCATATGCAAATGATAAAAGATTCA
CTCCTGCGGGGGATTTACCAGTCTGAGCCAAGTAGCTGTCATAATTCA
TTCTGTCTATTCCACAAGCAGCAAAATCCTGAAGGTTTTTTCTTCCTGC
TGCACCCAACAGAAGATATTAAGATTTACCTTCAGACTTTCCAATTCAC
TGCTTATGCTCAGTATGCTCATATGTGAGATGTCACTGATATGAGCTG
TCTGCAGTGAGAGTGCGTAGTGCTCTTATTTTTTTTTGCAGTCACTGTA
CCTGTTCAAGTGAGCTCAATTTTGATTATAATAGCTCCGAAGATAGATT
TCAGCTCATAGTAAGTAGGGGATCATCCGAAATCCTGGTCGGTAAGG
TTTCA

9.4 BCA protein Assay kit instruction

Preparation of Standards and Working Reagent (required for both assay procedures)

A Preparation of Diluted Albumin (BSA) Standards

Use Table 1 as a guide to prepare a set of protein standards. Dilute the contents of one Albumin Standard (BSA) ampule into several clean vials, preferably using the same diluent as the sample(s). Each 1 ml ampule of 2.0 mg/ml Albumin Standard is sufficient to prepare a set of diluted standards for either working range suggested in Table 1. There will be sufficient volume for three replications of each diluted standard.

Table 1. Preparation of Diluted Albumin (BSA) Standards

Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working Range = 20–2,000 µg/ml)			
Vial	**Volume of Diluent**	**Volume and Source of BSA**	**Final BSA Concentration**
A	0	300 µl of Stock	2,000 µg/ml
B	125 µl	375 µl of Stock	1,500 µg/ml
C	325 µl	325 µl of Stock	1,000 µg/ml
D	175 µl	175 µl of vial B dilution	750 µg/ml
E	325 µl	325 µl of vial C dilution	500 µg/ml
F	325 µl	325 µl of vial E dilution	250 µg/ml
G	325 µl	325 µl of vial F dilution	125 µg/ml
H	400 µl	100 µl of vial G dilution	25 µg/ml
I	400 µl	0	0 µg/ml = Blank
Dilution Scheme for Enhanced Test Tube Protocol (Working Range = 5–250 µg/ml)			
Vial	**Volume of Diluent**	**Volume and Source of BSA**	**Final BSA Concentration**
A	700 µl	100 µl of Stock	250 µg/ml
B	400 µl	400 µl of vial A dilution	125 µg/ml
C	450 µl	300 µl of vial B dilution	50 µg/ml
D	400 µl	400 µl of vial C dilution	25 µg/ml
E	400 µl	100 µl of vial D dilution	5 µg/ml
F	400 µl	0	0 µg/ml = Blank

B. Preparation of the BCA Working Reagent (WR)

1. Use the following formula to determine the total volume of WR required:

   (# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume WR required

   Example: for the standard test-tube procedure with 3 unknowns and 2 replicates of each sample:

   (9 standards + 3 unknowns) × (2 replicates) × (2 ml) = 48 ml WR required

   Note: 2.0 ml of the WR is required for each sample in the test-tube procedure, while only 200 µl of WR reagent is required for each sample in the microplate procedure.

2. Prepare WR by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). For the above example, combine 50 ml of Reagent A with 1 ml of Reagent B.

   Note: When Reagent B is first added to Reagent A, turbidity is observed that quickly disappears upon mixing to yield a clear, green WR. Prepare sufficient volume of WR based on the number of samples to be assayed. The WR is stable for several days when stored in a closed container at room temperature (RT).

Microplate Procedure (Sample to WR ratio = 1:8)

1. Pipette 25 µl of each standard or unknown sample replicate into a microplate well (working range = 20-2,000 µg/ml).

   Note: If sample size is limited, 10 µl of each unknown sample and standard can be used (sample to WR ratio = 1:20). However, the working range of the assay in this case will be limited to 125-2,000 µg/ml.

2. Add 200 µl of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.
3. Cover plate and incubate at 37°C for 30 minutes.
4. Cool plate to RT.
5. Measure the absorbance at or near 562 nm on a plate reader.

   Notes:
   - Wavelengths from 540-590 nm have been used successfully with this method.
   - Because plate readers use a shorter light path length than cuvette spectrophotometers, the Microplate Procedure requires a greater sample to WR ratio to obtain the same sensitivity as the standard Test Tube Procedure. If higher 562 nm measurements are desired, increase the incubation time to 2 hours.

9.5 *Western Blot protocol*

(1) Prepare the SDS polyacrylamide gel

- Clean the plates and the comb with mild detergent and soft cloth.
- Put the two plated together with the thinner one in front.
- Slide the plates carefully into the green clamp.
- Insert the comb and mark a fill level 1 cm below the bottom of the wells.
- Prepare the monomer solution for the running gel as per table. *Final gel concentration (1 0ml, 2 each, 0.75mm thick SE250 gels)*

	10%
Monomer solution (gel)	*3.3ml*
4x Running Gel Buffer (1.5M Tris-HCL PH	*2.5ml*
10% SDS	*0.1ml*
dH2O	*4 ml*
*APS**	*50 µl*
TEMED	*3.3µl*

* 10% APS should be prepared first

- Pour the gel into the plates, and then overlay the gel with 200µl of 1% SDS.
- While the gel is polymerizing, prepare the stacking gel as per table.

	0.75mm
Monomer solution	*0.44ml*
4x Stacking Gel Buffer(0.5M Tris-HCl Ph	*0.83ml*
10% SDS	*33µl*
dH2O	*2.03ml*
APS	*16.7µl*
TEMED	*1.7µl*

Stacking gel solutions (for two gels)

- Insert the gel into the electrophoresis unit with the larger thicker plate to the outside. If not running two gels insert the clear plastic plate against the gasket and close the tabs. Mark the location of the well by a permanent marker. Add tris-glycine electrophoresis buffer (***10% Tris-glycine buffer***).
- Remove the comb carefully.

- Make 4X loading buffer by adding ***95µl of loading buffer + 5µl β- mercaptoethanol.***
- Prepare the protein samples by adding ***5µl of loading buffer + 15µl sample***, then heat at 90°C for 5 minutes, and then put in ice for 5 minutes.
- Load the samples onto the gel, and run the gel at ***30mA per gel*** for 60-90 minutes. Electrophoresis is complete when the dye reaches the bottom of the gel.

(2) Western Blot

- Gently separate the glass plates, and discard the stacking gel.
- Equilibrate the gel in transfer buffer for 15 minutes.
  - Transfer buffer prepared as follow:
    - 10X TGS buffer 50ml
    - 20% ethanol 100ml
    - water 350 ml
- Prepare 8 filter paper s the same size as the gel.
- Prepare PVDF membrane, wet them with methanol, and then wash with transfer buffer for 5 minutes.
- Soak the filter paper in transfer buffer.
- Carefully place the gel onto the filters and smooth out, then the membrane and finally place the remaining filter papers on top of the membrane.
- This Gel filter sandwich needs inverting before placing onto the anode (base) of the semi-dry, the final arrangement being as follows.

Top
Cathode
Buffer soaked filter
paer
Gel
Membrane
Buffer soaked filter
paper
Anode ++++
Base

- Gently roll out any bubbles from the 'sandwich' using a universal tube.
- Run on Biorad semi-dry, ***max 25v (32 A) for one membrane*** for 1-2 hours.

(3) Immunodetection of protein

- Make the blocking solution which is either milk (5%)
  - 200 ml 1X PBS
  - 200µl Tween (thick, so cut the tip)
  - 10 g milk

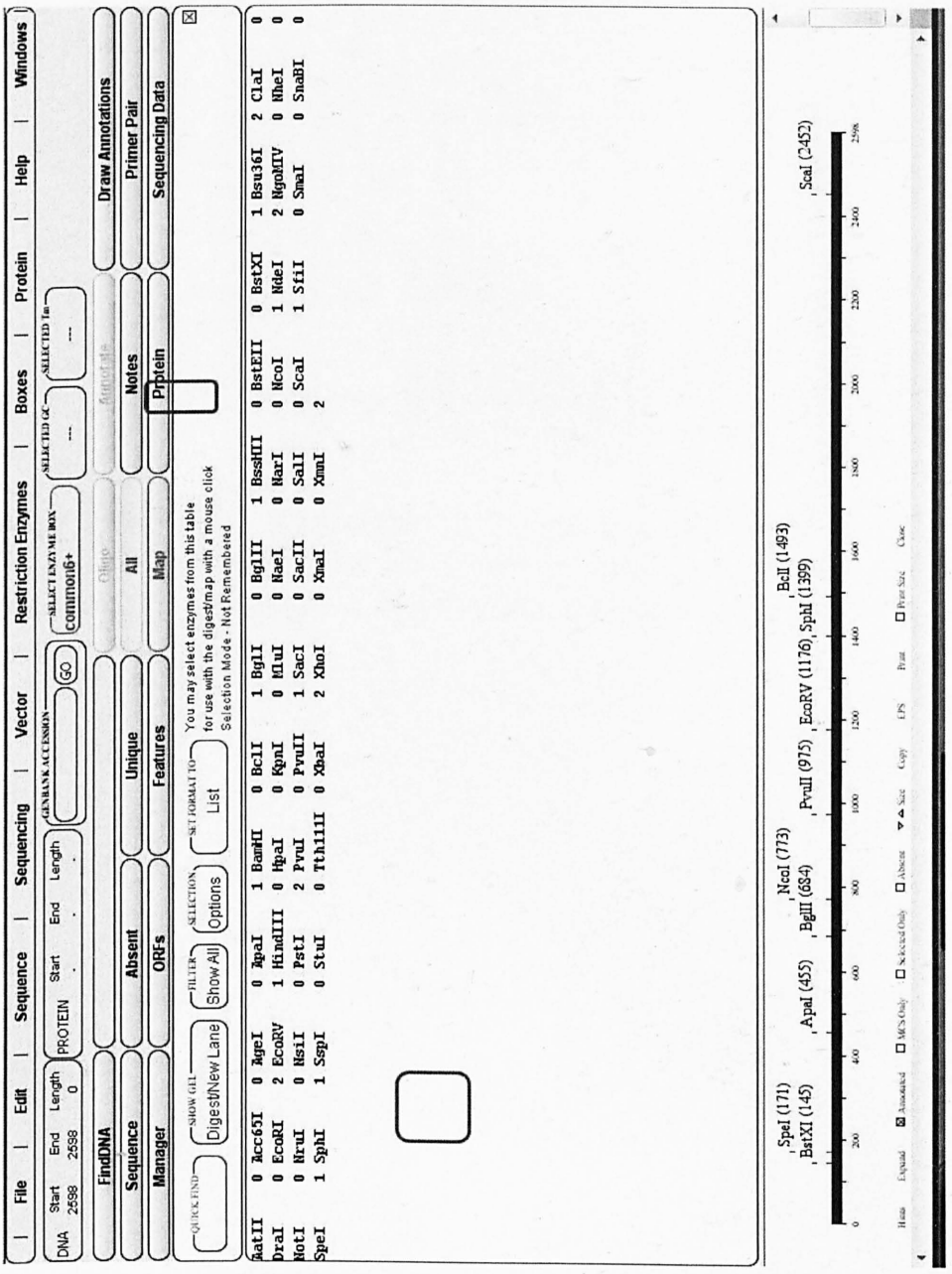
  Or Bovine serum albumin (5%) in case of CD133

  - 100 ml 1x PBS
  - 100µl Tween
  - 5 g bovine serum albumin

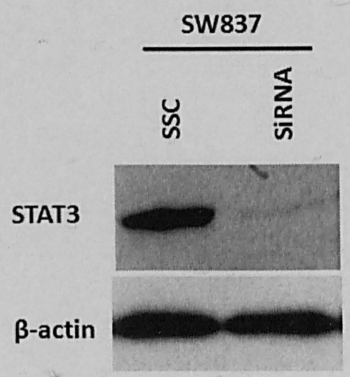
  Stir all of them well for 20 minutes

- Stain the gel with red dye first for 5 minutes, and then wash with distilled water.
- Put the membrane in the blocking solution for 1 hour on the shaker.
- Dilute the 1^{ry} antibody in 5% blocking solution (prepared before).
- Incubate the membrane with 1^{ry} antibody for overnight on the roller to mix either at RT or 4°C according to the antibody used.
- Pour off the 1^{ry} antibody and wash 3X with blocking solution for 5 minutes each time.
- Make the 2^{ry} antibody in blocking solution (1:10000), and leave it on the roller for 1 hour.
- Wash 2X with the blocking solution (PBS and Tween only) without milk or BSA, then once by PBS 1X only.
- Prepare the chemiluminescent mix (5ml of each bottle), and incubate for 5 minutes in the dark.
- Cut 2 pieces of plastic, seal it as a bag and the membrane should be inside the bag, and then incubate for 5 minutes.
- Remove excess liquid and dry the membrane, and put it in a new plastic bag.
- Put the membrane in the cassette.
- In the dark room expose the membrane to the film for 1 minute.
- Put the film in the developer first until the band appear, then transfer the film in the fixer for 30 seconds, and lastly wash the film with water and leave it to dry in the drying cupboard.

9.6 A map of EcoRV enzyme cut site at CD133



9.7 STAT3 knock down in SW837



Western blot analysis showed that STAT3 was knocked down in SW837 transfected with STAT3 siRNA compared with cells treated with scrambled control (SSC), β-actin used to show equal loading amount of protein in both lanes.

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