Isolation and Characterisation of Putative Colorectal Cancer Stem Cells

A thesis submitted to the Imperial College of London for the degree of Doctor of Philosophy in the Faculty of Medicine

by

Elodie du Potet

Department of Surgery and Cancer
Division of Surgery
Imperial College London
Hammersmith Hospital Campus
Du Cane Road
London
W12 0NN

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Abstract

The isolation and characterisation of cancer stem cells (CSCs) remain a major challenge. The ‘traditional’ (or ‘stochastic’) model of cancer suggests that cancer cells progress through clonal evolution and therefore, all cancer cells must be destroyed. The alternative model, the ‘hierarchical’ (or CSC) model proposes that a subpopulation of cancer cells, referred to as CSCs, initiates and sustains the continuous expansion of cancer growth. Studies have shown that CSCs are similar to normal stem cells as they are able to self-renew and to differentiate into a non-CSC progeny. However, their proliferation pathways are deregulated, they can form metastasis, trigger recurrences after chemotherapy and are tumourigenic. In the CSC model, the CSCs essentially need to be targeted and eradicated.

Although it is recognised that CSCs do exist, there is still a large gap in defining their molecular and functional characteristics. In this study, we have explored different approaches to isolate colorectal CSCs.

In the first approach, CSCs were isolated using a putative CSC marker, CD133. Cancer cell suspensions were obtained from patient tumour specimens. Several conditions were used to isolate CD133^+ cells by immunoaffinity. Technical difficulties were encountered throughout the procedure that prevented the isolation of CSCs using the CD133 antibody. In the second approach, we determined whether CSCs could be isolated using chemotherapeutic drugs since it has been shown that CSCs are resistant to such treatments. Cancer cells resistant to a short-term exposure with the chemotherapy drug oxaliplatin were isolated from two colorectal cancer cell lines. Data from in vivo tumourigenicity, expression of putative CSC markers and quiescence indicated that the intrinsically resistant cells did not exhibit CSC properties when compared with the untreated population. In the third approach, CSCs were isolated based on the aldehyde dehydrogenase 1 (ALDH1) activity. A high ALDH1 activity has been shown to be a marker of stem cells/CSCs. Immunohistochemistry revealed that ALDH1^{hi} cells expressed more putative CSC markers CD44, CD166, ABCG2 and Lgr-5 than ALDH1^{lo} cells. However, both populations were similarly clonogenic in vitro, they were equally invasive, as resistant to chemotherapeutic regimens and their cell cycle status was similar.

In conclusion, the approaches taken to isolate CSCs from cancer tissue samples or cell lines generated limited success. The data suggest that more refined techniques are required to isolate CSCs. On the other hand, they highlight the techniques that should be avoided in future studies. The results also question several concepts of the CSC theory, such as the intrinsic resistance of CSCs, and therefore emphasize on the need of gathering more evidence to validate the CSC model.
Declaration of Originality

I declare that this thesis is the result of my own work carried out between May 2007 and April 2010 in the Division of Surgery, Imperial College London, at the Hammersmith Hospital, Du Cane Road, London. All of the work, analysis of the results and graphic illustrations were carried out by myself, unless otherwise specified in the text.

Elodie du Potet
Acknowledgements

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Abbreviations

AACR - American Association for Cancer Research
A(SP)A - Animals (Scientific Procedures) Act
ABC(B1,B2,G2, C1 or C5) - Adenosine triphosphate-Binding Cassette (B1,B2,G2, C1 or C5)
ALDH – ALdehyde DeHydrogenase
AP – Alkaline Phosphatase
APC – Adenomatosis Polyposis Coli
AR- Androgen Receptor
ASC – Adult Stem Cell (s)
ATCC – American Type Culture Collection
BAA - Bodipy®-AminoAcetate
BAAA - Bodipy®-AminoAcetAldehyde
BCNU - 1,3-bis(2-chloroethyl)-1-nitrosourea
BCRP1 – Breast Cancer Related Protein 1
BMSC – Bone Marrow Stem Cell(s)
BSA – Bovine Serum Albumin
BTSC – Brain Tumour Stem Cell(s)
CaCO-2-OR - CaCO-2 Oxaliplatin Resistant
CAF- Cancer Associated Fibroblast(s)
CCK-8 - Cell Counting Kit 8
CD – Cluster of Differentiation
CEA - CarcinoEmbryonic Antigen
CM1 – Colorectal Metastasis 1
CM1-OR – CM1 Oxaliplatin Resistant
CPA – CycloPhosphAmide
CSC(s) – Cancer Stem Cell(s)
DAPI - 4’,6-DiAmidino-2-PhenylIndole
DEAB – DiEthylAminoBenzaldehyde
DMEM – Dubelcco’s Modified Eagle Medium
DNA – DeoxyriboNucleic Acid
ECM – ExtraCellular Matrix
EDTA – EthyleneDiamineTetra-acetic Acid
EGF – Epidermal Growth Factor
EGFR - Epidermal Growth Factor Receptor
EMT – Epithelial to Mesenchymal Transition
EpCAM – Epithelial Cell Adhesion Molecule
ES – Embryonic Stem cell (s)
ESA – Epithelial Specific Antigen
FACS – Fluorescence Activated Cell Sorting
FAP – Familial Adenomatous Polyposis
FBS – Fetal Bovine Serum
bFGF – basic Fibroblast Growth Factor (Also known as FGF-2)
FITC - Fluorescein IsoThioCyanate
FOLFOX – FOLinic acid – Fluorouracil-OXaliplatin
5-FU – 5-FluoroUracil
G6DP – Glucose-6-Phosphate Dehydrogenase
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<tr>
<td>GFAP</td>
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<td>TGF-β</td>
<td>Transforming Growth Factor β</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumour-Node-Metastasis</td>
</tr>
<tr>
<td>WST-8</td>
<td>Water-Soluble Tetrazolium 8</td>
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1 Introduction
1.1 Colorectal cancer

Colorectal cancer is the second most frequent cause of cancer death in the developed countries and more than 500,000 deaths a year are attributable to colorectal cancer across the world.

Early diagnosis has been successfully developed for colorectal cancer. Colorectal cancer first develops slowly as a benign adenoma, and only after a number of years does the adenoma transform to malignant adenocarcinoma. This sequence provides a good opportunity to detect and remove benign polyps before the disease develops further. Moreover, treatments at an early stage of the neoplasm are often successful and survival rates are high (5 year survival is 83% when disease is detected at Dukes’ stage A) [1].

However, recurrent disease or resistant metastases still kill more than one third of the patients affected. Despite decades of effort, the 5 year survival for stage IV is still only about 10% [2]. There is obviously an urgent need for novel and improved treatment.

1.1.1 Aetiology and epidemiology

Several parameters are thought to be involved in colorectal cancer genesis. Age is a determinant factor since most of the patients with colorectal carcinoma are diagnosed after age 50 years [3]. Low physical activity [4-6] and a high fat diet [7, 8] have been shown to increase the incidence of colorectal cancer. Sex distribution is more or less balanced, even though in Western countries rectal cancer affects slightly more men, whereas colon cancer is more prevalent in women [9].

The genesis of colorectal cancer is also influenced by environmental factors as suggested by its spread in economically developed countries. Higher incidence rates have been found in North America and Western Europe, whilst the lowest rates are in Sub-Saharan Africa [10]. This geographic shift directly correlates colorectal cancer incidence with the modern lifestyle.
Genetic predisposition is another parameter involved in colorectal cancer. All of the patients with familial adenomatous polyposis (FAP) develop cancer by the age of 50 [11]. FAP is an autosomal inherited condition, in which Adenomatosis polyposis coli (APC) gene is mutated on the long arm of chromosome 5, suggesting that mutation of APC gene is an early event in colorectal carcinogenesis. Gardner’s syndrome (a variant of FAP) is also associated with high predisposition to large number of polyps. Other familial syndromes, for example the hereditary non-polyposis colorectal cancer syndromes (HNPCC), predispose to adenomatous polyps with high malignancy potential [12].

1.1.2 Colorectal cancer staging

Dukes was the first to establish a system of international terminology for colorectal cancer [13]. It was introduced more than 60 years ago and divided the malignancies into three stages (A to C). It was later modified by the addition of a fourth stage and other subsets. In the first stage, A, tumour growth is contiguous with the bowel wall but does not penetrate it. At stage B, the tumour invades the surrounding muscularis and advential layers, and then reaches the lymph nodes (stage C). Eventually, the tumour forms metastases (stage D).

This first classification was not the most correct due to the large variations in survival rates among individuals at each stage (Table 1). However it has been, and still is, widely used because of its simplicity for pathologists.

<table>
<thead>
<tr>
<th>Dukes’ stage</th>
<th>5 year survival</th>
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<tbody>
<tr>
<td>A</td>
<td>80 %</td>
</tr>
<tr>
<td>B</td>
<td>48-62 %</td>
</tr>
<tr>
<td>C</td>
<td>22-33 %</td>
</tr>
<tr>
<td>D</td>
<td>10 %</td>
</tr>
</tbody>
</table>

Table 1. Five year survival rates for patients with different stages of colorectal carcinoma [14].
More recently, the Tumour-Node-Metastasis (TNM) system was developed by the American Joint Commission on Cancer in 1987 to classify all tumours using the same criteria [15]. It is more specific, but also more complex, and presents variants for each type of cancer. Four stages I (curable) to IV (metastasis/inoperable) are defined according to the combination of the sub stages T, N and M. T describes the size of the original tumour and its spread in the nearby tissues with 0 to 4 sub stages of invasion. N represents the involvement of lymph nodes (0 to 3 sub stages) and M the presence or not of metastasis (0 or 1). This classification also encompasses other parameters such as grade of cells (= refers to the amount of differentiation of the cells), invasion to veins, and clinical conditions (chemotherapy, resection).

1.1.3 Current treatments

Surgery has always been the treatment of choice for colorectal cancer. Surgical techniques are more or less invasive (local excision, resection or even colostomy) depending on the stage of the cancer, and their efficiency has been proven. However, they do not completely prevent recurrences and 10-15% of the patients undergoing surgery will develop local recurrences [16]. Usually, radiotherapy as well as chemotherapy are used as adjuvant therapy at the time of surgery, in order to remove any residual cancer cells.

Radiotherapy is restricted to rectal carcinomas. The difficulties in the accuracy of dosimetry and targeting render colon radiotherapy impossible since radiosensitive organs such as liver and kidneys surround the colon.

Historically, chemotherapy against colorectal cancer first started with the administration of 5-fluorouracil (5-FU) as an adjuvant therapeutic agent against metastatic disease. Progressively, new generations of agents have appeared, with the introduction of irinotecan and oxaliplatin as part of standard chemotherapy, and even more recently bevacizumab, cetuximab and panitumumab. The use of these chemotherapeutic agents is relatively successful and nowadays patients suffering from metastatic colorectal cancer have an increased overall survival of nearly 2 years [17].
1.1.4 New strategies for cancer treatment

Despite more than 50 years of intense research, the war on cancer is not over yet. Early detection is still the best way to prevent cancer death. In terms of prognosis, huge efforts on screening programs have allowed the prolongation of life, however we still do not know yet how to eradicate cancer completely. New strategies are now being developed in order to alleviate the limitations of the current treatments discussed above. Immunotherapy for instance, is an expanding field with the emergence of tools such as the anti-cancer vaccine TroVax [18].

Cell therapy is also another promising field of cancer research. Cell therapy aims at replacing cells that have been lost through physical, chemical or ischaemic injury, or as a result of degenerative diseases. The field covered by cell therapy is vast, and has already proven itself for a large range of diseases: spinal cord injury [19] and cartilage joint injury repair [20], multiple sclerosis [21], Parkinson’s disease [22], and, to a certain extent, cancer [23].

Besides the exploration of new treatments, cancer research seems to undergo a shift. After all, if during the previous decades research has only helped in the limited understanding of certain mechanisms regarding the molecular behaviour of cancer, then an essential question still remains: what is cancer? The traditional explanation for the origin, development and progression of cancer may be incomplete as the current therapeutic strategies do not lead to complete remission. An alternative model has been proposed and is based on the similarities between stem cells and a subpopulation of cancer cells that have the ability to initiate tumours. This model has fast become a popular view into defining the putative mechanisms of cancer development.

The cancer stem cell (CSC) theory will be presented after a brief account of stem cells and their biology.
1.2 Stem cells and intestinal stem cells

Stem cells are defined as undifferentiated cells, possessing an unlimited capacity of self-renewal, and the ability to generate differentiated cells [24]. Embryonic stem (ES) cells are chronologically the first source of stem cells, but also the most controversial due to ethical issues concerning their harvesting, which involves the destruction of an embryo [25]. The whole organism and all of its components are derived from them and they possess the unique capacity to give rise to any cell type. Adult stem cells (ASCs) have been said to be the remnants of the primitive embryonic stage. They reside in most of the organs at a low percentage throughout life, and their high proliferation capacity allows the organism to compensate for the loss of cells in tissues due to wear and tear (especially in blood, intestine and skin lining), or accidental loss due to injuries or degenerative diseases [26]. The range of cell types that ASCs can produce is limited. Important sources of ASC have been detected in the bone marrow and peripheral blood, liver, skin and gut, brain and skeletal muscle [27]. Besides ES and ASCs, stem cells have also been discovered in foetus, placenta, amniotic fluid and umbilical cord tissues [28-30]. All stem cells are defined by two undeniable hallmarks: self-renewal and the ability to differentiate.

1.2.1 Self-renewal and differentiation

Self-renewal is generally recognised as the cell’s capacity to generate two daughter cells, of which at least one remains identical to the mother cell. According to the prevailing stem cell model, self-renewal is a property of the undifferentiated cells. A cell committed to a differentiation pathway progressively loses its self-renewal capacity as it acquires a higher level of differentiation. However, this rule is not universal. Cells such as T and B immune cells maintain their renewal capability. They both possess a high clonogenic potential, although they are terminally differentiated into lymphoid cells [31]. Nevertheless, self-renewal remains essential to define stem cells, as it allows them to preserve a constant pool in tissues.
Stem cell homeostasis is maintained by a balance between self-renewal and differentiation. According to the current model, homeostasis is tightly regulated by symmetric or asymmetric division [32]. When stem cells undergo division, the fate of the two daughter cells may be similar or different. In the first case (symmetrical division), the two daughter cells both retain the stem cell features (Figure 1A) or are committed to a differentiation pathway (Figure 1B). In the latter case (asymmetric division) one cell retains the potential for self-renewal whereas the other becomes a progenitor cell committed to differentiation (Figure 1C). Its progeny consists essentially of transit-amplifying cells, a rapid cycling population of committed precursors capable of producing large numbers of terminally differentiated progeny. This differentiated progeny eventually undergoes terminal division arrest [33].

Figure 1. Symmetric (A,B) versus asymmetric (C) division. Stem cells can divide symmetrically, i.e. generate similar daughter cells, two stem cells (A) (self-renewal) or two differentiated cells (B). They can also divide asymmetrically (C) and manage in a single division both self-renewal and differentiation. SC : stem cell. PC : progenitor/differentiated cell. (Adapted from Lessard et al. (2004) [34]).

Theoretically, homeostasis and regeneration can be accounted for by symmetrical division alone [35]. Symmetric division is the only process through which stem cells can expand their number. Symmetric division is, for instance, responsible for the germline expansion of Caenorhabditis elegans [36], and is involved in the larval development and the adult germline stem cells of Drosophila [37, 38] as well as in mammalian stem cells during the embryonic and foetal stages [39].

Asymmetric division is a very attractive model, as it helps to understand how the stem cell pool can remain constant throughout life. In a single division, the necessary maintenance and renewal of differentiated tissues are ensured and, at the same time, stem cells preserve their numbers and phenotype. When stem cell proliferation is
required especially after injury, stem cells can potentially switch to symmetric division, since asymmetric division cannot sustain a stem cell expansion on its own [35]. This double and powerful tool renders stem cells perfectly flexible and able to dynamically prevent their depletion.

Intrinsic mechanisms of asymmetric division

In many organisms there is strong evidence that stem cells are capable of asymmetrical division in order to provide cells with different properties during proliferation. It has been shown in models such as the Caenorhabditis elegans germ lines, that the positioning of the mitotic spindle is an important factor that determines the process of asymmetric division [40]. Asymmetric division in that case divides the zygote into a smaller blastomere (mesoderm and endoderm precursor) and a larger blastomere (ectoderm precursor) [41].

Asymmetric division can also generate cell type diversity when the cell fate determinants are asymmetrically localized and unequally distributed to the daughter cells [42]. For instance, in the developing mammalian nervous system, Notch-1, a cell fate determinant associated with modulation of the stem cell self-renewal, is basally polarised in the dividing cells, and therefore unequally inherited during horizontal division. Through this process, one single division contributes to the production of two distinct cell types [43].

The mechanisms described above (cell polarization and mitotic spindle position) can be considered as intrinsic mechanisms [35]. But there also exist extrinsic mechanisms, and stem cell homeostasis cannot be fully understood without considering the importance of the cells neighbouring the stem cells.
Extrinsic mechanisms of asymmetric division

Maintaining a stem cell state *in vitro* is one of the main challenges in stem cell biology. *In vivo*, this state is regulated by the microenvironment and neighbouring cells. The site where stem cells reside has been defined as their “niche”, and is essential for the maintenance of a life-long stem cell reservoir [44]. Although little is known about the regulation of the microenvironment, it is now established that the stem cell environment is made of various types of differentiated cells. Secreted molecules like cytokines, and direct cell-cell interactions allow the stem cell characteristics to be maintained and protected [44]. One of the best-known models illustrating the influence of the microenvironment on stem cells is the *Drosophila germarium*. It has been demonstrated that the germ line stem cells are maintained by cell to cell interactions within their niche. If a division involves a loss of this contact then the daughter cell becomes a cytoblast (transit-amplifying cell) and differentiates to produce the cyst [45]. In vertebrates, haematopoietic stem cells (HSCs) are a good illustration of this phenomenon. In the bone marrow, HSC are surrounded by macrophages, adipocytes and fibroblasts, and are in direct contact with the osteoblasts located along the inner surface of the trabecular bone. This environment is adequate to maintain their stem cell state throughout life. However, migration of HSCs out of this zone and loss of interaction with the osteoblasts induces their differentiation towards myeloid and lymphoid progenitors [45]. The niche therefore seems to have a major role in regulating the balance between symmetric and asymmetric divisions. A better understanding of the niche and of relations between stem cells and their neighbours could be of primary importance in developing therapeutic solutions.

More studies are needed to assess the modes of division used by mammalian stem cells *in vivo*. It is difficult to study adult stem cells because they are not abundant and biologically quiescent. If the regulation of stem cell homeostasis may be controlled by both asymmetric and symmetric divisions, the process through which stem cells can switch from one to the other needs to be further elucidated.
1.2.2 Stem cell plasticity

Any stem cell possesses the capacity to differentiate into terminally differentiated cell types. However, stem cells are more or less plastic, according to the variety of cell types they can generate. An arbitrary hierarchy of stem cells has been established as a function of their degree of plasticity.

Totipotent cells
Totipotent cells are able to produce a whole organism and even the adjacent tissues necessary for their development. This class of stem cells corresponds to the zygote (and by extension to the daughter cells of the first two divisions), which not only forms the embryo but also the different adjacent tissues such as the chorion and the placenta [46].

Pluripotent cells
Pluripotent cells are able to give rise to a variety of mature progeny. ES cells are pluripotent cells isolated by immunosurgery from the inner cell mass (ICM) of the blastocyst stage of an embryo at about 5 days after fertilization [47]. After isolation, ES cells are cultured as cell lines capable of continuous growth in culture (almost immortal cell lines), under certain in vitro conditions. Human ES cell lines all express high levels of active telomerase, a key enzyme that ensures chromosome stability despite great numbers of cell divisions [48]. They express the transcription factor Oct-4, which allows them to maintain a self-renewal capacity and pluripotency [49]. However, under certain conditions, differentiation can be induced and ES cells may generate a broad range of cell types from ectodermal, mesodermal, and endodermal tissues [50].

The first cell line derived from a human embryo was achieved in 1998 [51]. To date, about 50 well-characterised human ES cell lines have been reported worldwide.

ES cells possess the highest degree of plasticity among the stem cells that have been studied up to now. Unlike the ASCs, which are derived from an adult organism, no clinical trials on human ES cells have been conducted to date. This delay may be explained by two major hurdles. First, the ethical issues raised by the research on the
embryo and the debate over the use and destruction of embryos for clinical purposes [25]. On the technical front, the tumourigenic potential of the ES cells hinders further clinical applications. It is really important to emphasise that ES cells and cancer cells have many common characteristics, including unlimited proliferation [52] and high expression of telomerase [48]. Moreover, ES cells have been shown to generate teratomas when injected in immunocompromised mice [50]. To avoid the formation of teratomas, one possibility would be to transplant fully differentiated ES cells and/or use the appropriate growth factors to induce cells into the desired committed lineage [53].

**Multipotent to unipotent cells**

Multipotent cells have a more restricted lineage differentiating capacity than ES cells and are usually referred as ASCs. They can produce a large range of differentiated cells but are restricted to a particular tissue. HSCs for instance are multipotent, since they are the source of all blood cell types [54]. ASCs such as spermatogenic stem cells can produce only one cell type (spermatozoid) and are therefore unipotent. Although less plastic than ES cells, ASCs can self-renew and differentiate. They remain in a quiescent state within their niche until they receive a stimulus to differentiate [45].

ASCs present several advantages in comparison to ES. Firstly, they are not associated with ethical issues and possess the advantage of being easily available. HSCs can be harvested by simple blood collection or bone marrow aspiration. Importantly, large amounts are available at the same time which tends to shorten the *in vitro* period preceding the transplantation in a clinical assay. Using ASCs also avoids heterograft complications, as autologous transplantation is possible. Moreover, the issue of tumourigenicity seems to be more relevant with ES rather than ASCs [55].

Recent discoveries have also reinforced the advantage of using ASCs over ES cells. The plasticity of bone marrow stem cells (BMSCs) is more extensive than initially thought, and HSCs have been reported to give rise to cell types of endo-, meso- and ectodermal origin, including myocytes, neurons, liver and pancreatic cells [56-60].
1.2.3 Intestinal stem cells

Intestinal crypts and villi are covered with epithelial cells renewing on average every 5 days in mice [61]. Intestinal crypts house the stem cells responsible for the high turnover of the epithelium. Stem cells reside at the base of the crypts, and produce transit-amplifying cells, able to divide at a very high frequency (12-16 hours [62]). In 48 hours, these multipotent cells undergo up to 5 rounds of divisions. Their progeny migrate upwards, and once they reach the villi, they start differentiating into Goblet cells (in the colon), Paneth cells (in the small intestine), entero-endocrine or absorptive epithelial cells. All of the cells migrate from the crypts towards the villi, except the Paneth cells which move down to the base of the crypt to support maintenance of the stem cell niche. Goblet cells are their counterpart in the colon but remain on the sides of the crypts. As indicated from the proliferative index from Figure 2, there is an inverse relationship between proliferation and differentiation of cells along the length of the crypts.

Figure 2. Stem cells, transit-amplifying and differentiated cells in the small intestine (A) and colon crypts (B). Along the length of the crypt, there is an inverse relationship between proliferation and differentiation. Source: [63].
The lack of specific stem cell markers renders the identification of intestinal stem cells tedious. Stem cells within the proximal small intestine have been detected at position +4 at close vicinity to the crypt, immediately above the Paneth cells [64]. These self-renewing and pluripotent cells have recently been shown to express Bmi1 protein [65]. The ablation of Bmi1 expressing cells led to the disruption of crypts, thus indicating the implication of these cells in crypt maintenance, and the possible role of Bmi1 as an intestinal stem cell marker.

Recent reports have now detected another putative intestinal stem cell. The cell surface marker Lgr-5 (leucine-rich-repeat containing G-protein-coupled receptor 5), a Wnt pathway target gene, is specifically expressed in the crypt base columnar cells, disposed in between the Paneth cells. Using in vivo lineage tracing, Barker et al. (2007) have proposed that Lgr-5 expressing cells are the stem cells of the small intestine and colon [66]. More recently, studies on Lgr-5+ cells have proved that these basal columnar cells are also those from which colorectal tumours preferentially originate [67]. In knock-in mouse models, deletion of the apc gene (leading to the activation of the Wnt proliferation pathway) in the Lgr-5+ columnar cells initiates their transformation. While remaining in the bottom of the crypts, the transformed columnar cells generate a macroscopic adenoma in less than 5 weeks. In other knock-in mouse models, the same deletion in transit-amplifying cells only generated rare adenomas, even after 30 weeks, suggesting that columnar cells, i.e. stem cells, and not transit-amplifying cells, are preferentially the cells in which mutated APC leads to cancer.
1.3 The CSC theory and the origin and development of cancer

1.3.1 The traditional view of cancer

Clonality of cancer
It is generally accepted that most, if not all tumours are clonally derived, i.e. they initially are derived from the proliferation of one abnormal cell. Evidence in favour of this concept is mainly supported from studies that investigated the distribution of genetically distinct types of X-linked isoenzymes with, in particular, the study of glucose-6-phosphate dehydrogenase (G6PD). 30% of African women are heterozygous [68] at this allele where, typically, the same member of the X chromosome pair is functional in all malignant cells. This phenomenon has been studied specifically in blood cancers as a tool to prove the clonality of neoplasms [69]. Additionally, many cancer cells possess the same abnormal karyotype and blood cancer cells have been shown to secrete a homogenous panel of cytokines, which suggests a common ancestry of all the cancer cells [69, 70].

Cancer progression is a multi-step process
The initial step towards carcinogenesis (termed ‘initiation’) is the process by which a cell undergoes irreversible genetic changes. Initiators (or carcinogens) are able to interact with deoxyribonucleic acids (DNA) directly or after their activation via drug metabolizing enzymes [71, 72]. The mutations they cause are irreversible and any daughter cells produced from the division of the mutated cell will also carry the mutation, thus creating a pre-neoplastic clone of cells. Once a particular cell has been affected by an initiator it is susceptible to promotion (see below) until its death.
In the second step towards carcinogenesis (termed ‘promotion’), continuous, epigenetic and reversible changes alter the ‘initiated’ cells, which then go on to
expand and take over the population of normal cells through clonal selection. At this stage, the cancer phenotype is expressed. The advanced steps towards carcinogenesis (termed ‘progression’) include further phenotypic changes, as well as the passage from a benign to a malignant tumour.

Colorectal cancer is one of the best models illustrating the subsequent events occurring in carcinogenesis. Due to the availability of tissue from each of the stages displayed in Figure 3, DNA alterations contributing to the different steps of development have been analysed and chronologically defined as follows:

Figure 3. The adenoma-carcinoma sequence in sporadic colorectal cancer and in HNPCC. Adapted from Fearon and Volgestein (1990) [73].

The *apc* gene, which is mutated in more than 70% of colorectal cancers, has been called the ‘gatekeeper’ gene as it seems to be the rate-limiting step in colorectal carcinogenesis [73]. This gene is a key member of the Wnt pathway, which is a cascade of signalling events involving a series of intracellular molecules. Its role is essential in development as deregulation of this signalling pathway results in carcinogenesis. In patients with FAP familial cancers (i.e. with a genetic predisposition), where *apc* is mutated in the germline cells and therefore transmitted to progeny [74], cancer appears earlier than in patients with sporadic cancers, in whom mutations affect random somatic cells, and therefore forms tumours over a longer period of time.
Nature of the genetic alterations

Genetic alterations responsible for carcinogenesis result in the aberrant regulation of growth control.

Essentially, alterations involve the hyperactivation of proliferation pathways such as the notch [75], sonic hedgehog (shh) [76], and Wnt pathways as previously mentioned in colorectal cancer initiation [77] (Figure 4). Other alterations can affect the expression of oncogenes such as c-myc, or result in the down regulation of tumour suppressor genes such as p53 [78].

Figure 4. Schematic diagram of the signalling pathways involved in cancer biology. In a number of carcinomas, cancer cells are affected in their proliferation pathways. Mutations in Wnt, Shh and Notch 1 pathways can lead to carcinogenesis [79].
The proliferation pathways are predominantly active at the embryonic stage and are also functional in stem cells, as they seem to regulate their development and maintain their self-renewal capacity [80-83]. Any change in the tight regulation of stem cell self-renewal can easily lead to tumourigenesis.

*The heterogenic nature of cancer cells/tumourigenicity*

Heterogeneity of cells in the tissues is observed at the morphological and functional levels. They display differential expression of secreted factors and cell surface markers. All tissues are comprised of architecturally distinct layers each with a functional role, as illustrated by Figure 2 in the intestinal epithelium.

Similarly to normal tissues, solid cancers are also comprised of heterogeneous populations of cells. Most tumours usually reproduce the patterns of differentiation seen in the primary tissues from which they are derived, even though their processes of differentiation may follow alternative pathways (cf. ability for invasion). At the functional level, one important criterion of heterogeneity is the capacity to regenerate a tumour. Large numbers of cancer cells are required in order to produce tumours in xenotransplanted immunodeficient mice [84], which suggests that, amongst the population of injected cancer cells, only a small fraction of them possesses tumour-initiating potential.

To explain cancer cell heterogeneity, the traditional (stochastic) model of cancer suggests that the behaviour of tumour cells is variable, unpredictable, reversible - depending on various factors, be they intrinsic or extrinsic [85]. Heterogeneity of cancer cells is based on a stochastic model. A stochastic model is a non deterministic model, whereby, besides its predictable process, its development includes a random element. The stochastic model of cancer follows that any cancer cell can hypothetically acquire a function such as tumourigenicity, provided it is influenced by a combination of factors (signalling pathways, microenvironment, intracellular levels of transcription factors, genetic or epigenetic alterations, etc.). Unpredictability therefore makes the separation between tumourigenic and non-tumourigenic cells very difficult to achieve.
1.3.2 The CSC model as an alternative to the traditional model

The idea that cancer cells possess the same properties as stem cells has been suggested many years ago, and is well established now. For instance, ES cells resemble cancer cells in surprising ways. They possess intrinsic cancer cell-like properties and they develop teratomas when transplanted at an undifferentiated stage. Histologically, similarities between developing foetal cells and teratocarcinoma cells have been recognised more than 150 years ago [86].

The CSC view of cancer as a multi-step process

The relationship between the turnover rate of a tissue and the risk of developing tumour is difficult to establish. How can multiple alterations alter tissues with short life span cells, such as the bowel epithelial cells that are regenerated on average every 5 days? The role that CSCs may play in this setting is providing new insights into the mechanisms of cancer progression. In colorectal cancer for instance, the multiple alterations which are required for a normal epithelium to degenerate into carcinoma have been described extensively (cf. Figure 3) [87]. The idea that multi-step genetic mutations leading to cell malignancy can occur in cells with a long life span fits appropriately with the concept of CSCs [88]. It is argued that in many tissues the only cells present for a sufficiently long duration to accrue the requisite number of molecular abnormalities leading malignancy are stem cells.

The CSC explanation of cancer origin and heterogeneity

Use of the CSC model to explain the origin of cancer differs from the traditional view. CSCs have acquired mutations leading to uncontrolled growth and retain (or regain) their capacity for self-renewal and pluripotency. Like stem cells in normal tissues, CSCs lose some of their characteristics upon differentiation, through epigenetic changes, hence resulting in the heterogeneous cell population found within tumours [89]. Therefore, in the CSC model, heterogeneity is synonymous to intrinsic biological differences that occur through epigenetic changes. Epigenetic changes...
involve all the mechanisms responsible for the modification of the gene activation status other than changes (e.g. mutations) in the underlying DNA sequence.

The nature of the original cell has not been stated by the CSC model, as explained by Dick (2008) [90]. Stem cells may be preferentially the target of cancer initiation, but it is not excluded that tumours may also stem from differentiated cells that have regained the capacity to divide, self-renew and organize tissue-specific differentiation through genetic alterations. It is nevertheless intrinsic to the CSC theory that the originating cell is/becomes capable of self-renewal, differentiation and extensive proliferation.

*CSC and tumourigenicity*

Previous studies on clonogenic assays have shown that it is only a minority of cancer cells that can proliferate and form new tumours in transplanted mouse models. The CSC model posits that these cells are the CSCs. Non-CSCs in comparison only have a limited proliferation potential. Since CSCs possess the ability for self-renewal and proliferate extensively, it may explain the associated heterogeneity of cells found within a tumour mass regarding to tumourigenicity. In the traditional evolution model, differences between tumourigenic and non tumourigenic cells are the result of unstable, genetic or epigenetic changes. The potential for each cell to form a tumour depends on a stochastic probability [90]. In the CSC model, this potential is restricted to a specific population, with stem cell properties. The tumourigenic potential is lost upon differentiation and is the result of epigenetic changes [85] (Figure 5). Therefore, even though the term CSC has been used extensively, a CSC is intrinsically defined as a rare cell amongst other cells within the bulk of a tumour that is able to reconstitute a tumour when injected into an immuno-compromised mouse.
Figure 5. Heterogeneity and tumourigenicity in cancer. The hierarchical (=CSC) and traditional (=stochastic) models of cancer have different hypotheses with regard to the functional heterogeneity of cancer cells. Initiating a tumour in the CSC model is considered to be the exclusive property of cells capable of self-renewal and differentiation. Cells are therefore not biologically equivalent, and have different functions according to their position in the hierarchy. CSCs can self-renew and also generate non-tumourigenic cells. In the traditional (stochastic) view of cancer, tumour cells are biologically equivalent, but intrinsic and extrinsic factors also influence their behaviour, and the tumourigenic properties can be randomly acquired by any cell in the bulk. Adapted from Dick (2008) [90].
Such cells were first identified in leukaemic patients in 1997 by Bonnet and Dick [91]. They showed that as few as 100 of these rare CD34+/CD38− cancer cells were able to initiate leukaemia in non-obese diabetic severe combined immunodeficiency (NOD/SCID) mice and that these self-renewing cells were able to proliferate and differentiate into leukemic blasts.

It took several years before CSCs or tumour-initiating cells were identified in solid tumours. Al-Hajj et al. (2003) [92] succeeded in identifying CSCs in breast cancer, followed by Singh et al. [93], who isolated CSCs from brain tumours [94]. Subsequently, CSCs were discovered in prostate [95], liver [96], skin [97], and colon [98-100] cancer.

Clinical implications

The identification of CSCs brings about important therapeutic implications in terms of selective drug targets and recurrence/metastasis prevention essentially. If tumours truly follow a CSC model of development, therapy should aim at targeting the CSCs and not the cells that form the main mass of the tumour [101]. Similarly to normal stem cells, CSCs are predicted to possess enhanced mechanisms of resistance due to high levels of transporters [102-104], of anti-apoptotic proteins (bcl-2 family members [105]), slow cell cycling [106], and enhanced DNA damage repair [107]. These features explain how CSCs may escape the conventional chemotherapeutic treatments, and thus be responsible for recurrence and metastases.

Current treatments are characterised by ineffectiveness and serious adverse effects. The investigation of markers that may distinguish CSCs from the rest of the tumour and normal stem cells should give way to targeted therapies that may limit the damage caused to healthy tissues, while being more efficient.

The successful eradication of tumours will therefore require the successful isolation of CSCs, the identification of specific markers such as cell surface markers, and the understanding of the molecular mechanisms involved in the surviving, self-renewal and differentiation pathways of CSCs. A comparison with the rest of the tumour cells and cells from healthy tissues will be required.
1.4 The biology of CSCs

1.4.1 CSCs versus normal stem cells – proliferation pathways

Regardless of the origin of CSCs, they share several properties with normal stem cells.

The CSC model predicts that, as in any organ, cells within a tumour bulk are hierarchically organised, and derived from a rare population of CSCs, or their direct progeny (Figure 6) [91]. In order to ensure the continued expansion of malignant cells, CSCs can self-renew, i.e. maintain their pool, and in the meantime differentiate into progenitor and transit-amplifying cancer cells.

![Diagram of CSC model](Image)

**Figure 6.** In the CSC hierarchical model, the origin of cancer is restricted to CSCs. Like their stem cell counterpart, CSCs can self-renew and/or differentiate into progenitor cells which possess a high rate of division. Mature cells are not able to proliferate anymore. The question marks denote that the cellular origin of cancer is not yet defined. (adapted from Sagar *et al.* (2007) [108]).
Transit-amplifying cells are cells that are capable of extensive proliferation and differentiation but which progressively lose the tumourigenic potential upon differentiation [83].

The mechanisms of CSC self-renewal are suspected to be similar to those regulating stem cells in normal organs. Asymmetric division has been suggested to play an important role in maintaining the CSC pool, and producing a hierarchically organised tumour bulk [35]. Disruptions affecting the mechanisms regulating the asymmetric and symmetric modes of division have been shown to lead to tumourigenesis in Drosophila melanogaster. This observation has led to the idea that cancer may originate from stem cells [35, 109, 110]. The essential difference between normal stem cells and CSCs is that proliferation is no longer controlled in CSCs [111]. The ratio of CSCs/cancer cells is retained, but the total number of cells is constantly increasing, whereas, in normal stem cells, proliferation occurs only on demand. The hypothesis is that CSCs have undergone a series of mutations in their proliferation pathways such as Notch [75], shh [112], Wnt [113], which disrupt the proliferation balance in CSCs [83].

1.4.2 Other attributes of CSCs

CSCs are thought to be more invasive and form metastases more frequently than non-CSCs. In breast cancer for instance, the invasive property has been identified in a subpopulation of CSCs expressing the CD44+/CD24– CSC phenotype [114].

CSCs are also suspected to be more resistant to chemotherapy than the other cancer cells [115] although the link between CSCs and chemoresistance is still not well defined. In the conventional model of drug resistance, mutations occur randomly in one or several cells, according to a stochastic model here again, and confer on them a selective advantage during chemotherapy. The CSC hypothesis assumes that in most cases, resistance is driven through a rare population of CSCs, mainly as a consequence of their resting stem cell phenotype, high levels of transporters (Multidrug resistance 1 (MDR1), Adenosine triphosphate-binding cassette G1
(ABCG1) [102-104], of anti-apoptotic proteins (bcl-2 family members [105]), slow cell cycling [106], or enhanced DNA damage repair [107], thus providing them with innate resistance. Conversely to CSCs, differentiated tumour cells lose their intrinsic resistance upon differentiation, although they can always acquire resistance through the processes of gene amplification or chromosomal rearrangements [116].
1.5 Isolation of CSCs

Controversy still exists as to the true nature of CSCs. Their isolation has still not been conclusively derived and their use as a clinical tool is still undefined. To date, the isolation of putative CSCs has been based on intrinsic and stable properties that are believed to be unique to these cells.

1.5.1 Current strategies to isolate CSCs

Side population

Hoechst-dye is a tool primarily used in a strategy to enrich a subpopulation of HSCs. The cells that are able to expel the dye represent a distinct subset of cells identifiable by flow cytometry, and have been called “side-population” (SP) cells. This ability to exclude the dye relies essentially on the overexpression of transporters of the ABC family [117]. SP cells were identified in murine HSC by Goodell et al. [118] and their potential to repopulate lethally irradiated mice was 1000 fold higher in comparison to the whole bone marrow cells. Although there is controversy on the SP content in CSCs [119], SP cells have been identified among the human gastrointestinal, liver, nasopharyngeal and lung cancer cells [103, 104, 120, 121]. In several occasions, the SP cells have proven to be enriched with cancer-initiating cells [104] and to have stem-cell like properties such as increased invasiveness, high levels of telomerase activity [122], increased expression of drug resistance associated genes and evidence for self-renewal [103]. A critical limitation to the use of SP is that the Hoechst dye is cytotoxic depending on the dose used and the type of cells being treated.

Cell markers

Several studies to date have reported the existence of CSC specific markers, as shown in Table 2. Based on their similarities with normal stem cells, CSCs were isolated with corresponding stem cell surface markers. CD133, initially referred to as AC133, was chosen as the most relevant candidate marker since it is an important hallmark of ‘stemness’ state in primitive cells from the neural, epithelial, endothelial and haematopoietic tissues [123-125]. CD133 has also been reported to be present on CSCs from different haematopoietic and solid tumours such
as leukaemia, brain [93], colon [98, 99], liver [96] and prostate cancer [95]. CD133 thus appears to be the most likely candidate for a universal ‘stemness’ marker in cancer. Subsequently, other markers for the identification of CSCs have also been identified, importantly the marker CD44 in breast cancer [92]. CD44 in combination with CD24 and Epithelium specific antigen (ESA)\(^+\) allows the purification of breast tumour-initiating cells. CD44 is also expressed in pancreatic [111], breast [92], prostate [95] and colorectal CSCs [100]. In colorectal cancer, the proposed combination of markers CD44\(^+/CD166^+/ESA^{hi}\) is expressed by a highly tumourigenic population. Other markers used to isolate CSCs are ABCB5 and CD90. They are involved in CSC isolation, in melanoma [126] and liver cancer [127], respectively.

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>CSC markers</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukemia</td>
<td>CD34(^+/CD38^-)</td>
<td>[91]</td>
</tr>
<tr>
<td>Breast</td>
<td>ESA(^+)/CD44(^+/CD24^{low})</td>
<td>[92]</td>
</tr>
<tr>
<td>Brain</td>
<td>CD133(^+)</td>
<td>[93, 128]</td>
</tr>
<tr>
<td>Prostate</td>
<td>CD44(^+)</td>
<td>[129]</td>
</tr>
<tr>
<td></td>
<td>CD44(^+/\alpha_2\beta_1^{hi}/CD133^+)</td>
<td>[95]</td>
</tr>
<tr>
<td></td>
<td>CD44(^+/CD24^-)</td>
<td>[130]</td>
</tr>
<tr>
<td>Colorectal</td>
<td>CD133(^+)</td>
<td>[98, 99]</td>
</tr>
<tr>
<td></td>
<td>CD44(^+/ESA^+/CD166^+)</td>
<td>[100, 131]</td>
</tr>
<tr>
<td></td>
<td>CD44(^+)</td>
<td>[132]</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>CD133(^+)</td>
<td>[102, 133]</td>
</tr>
<tr>
<td></td>
<td>ESA(^+)/CD44(^+/CD24^+)</td>
<td>[134]</td>
</tr>
<tr>
<td>Head&amp;neck</td>
<td>CD44(^+)</td>
<td>[135]</td>
</tr>
<tr>
<td>Liver</td>
<td>CD133(^+)</td>
<td>[136, 96, 137]</td>
</tr>
<tr>
<td></td>
<td>CD90(^+)</td>
<td>[127]</td>
</tr>
<tr>
<td></td>
<td>EpCAM(^+)</td>
<td>[138]</td>
</tr>
<tr>
<td>Retinoblastoma</td>
<td>CD133(^+)</td>
<td>[139]</td>
</tr>
<tr>
<td>Lung</td>
<td>CD133(^+)</td>
<td>[122, 140]</td>
</tr>
<tr>
<td>Kidney</td>
<td>CD133(^+)</td>
<td>[141]</td>
</tr>
<tr>
<td>Ovary</td>
<td>CD133(^+)</td>
<td>[142]</td>
</tr>
<tr>
<td></td>
<td>CD44(^+/MyD88^+)</td>
<td>[143]</td>
</tr>
<tr>
<td>Melanoma</td>
<td>CD90(^+)</td>
<td>[126]</td>
</tr>
<tr>
<td>Larynx</td>
<td>CD133(^+)</td>
<td>[144]</td>
</tr>
</tbody>
</table>

**Table 2.** Summary of the most common markers used for CSC isolation
In most cases, these markers have not yet shown any functional activity. Some of them have been correlated with clinicopathological parameters. Essentially, CD133 and CD166 are associated with low patient survival in colorectal cancer [145-147], whilst the function of CD44 in this setting is still contradictory [148-151]. CD133 and CD24 may correlate with invasiveness and differentiation [152], whilst CD44 has also been reported to be involved in invasion and migration [151, 153, 154].

Sphere formation

The isolation of CSCs from the rest of a cancer cell population can be performed by reproducing a neurosphere assay set up by Reynolds and Weiss [155, 156]. This assay was used to isolate neural stem cells and relies on the unique ability of stem cells to form spheres. Expanding cells within these spheres seems to favour self-renewal rather than differentiation, thus enhancing the number of stem cells. Such a method has been adapted to purify putative CSC populations in brain cancer [157-161] as well as in breast [162], colorectal [163], pancreatic [164], ovarian [165], and prostate cancer [166], in order to isolate cells with a self-renewing capacity.

Aldehyde dehydrogenase (ALDH)

Another strategy to isolate stem cells and CSCs is based on the targeting of cells with high Aldehyde dehydrogenase (ALDH) activity, in particular ALDH1. This enzyme mediates the oxidation of intracellular aldehydes to carboxylic acids in the cytosol and is involved in retinoid metabolism [167]. It has been shown in mice HSCs that retinoids are responsible for the terminal differentiation of late progenitors and the self-renewal of early precursor cells [168, 169]. ALDH1 activity is hence implicated in maintaining a stem cell phenotype, and is said to be upregulated in HSC and neuronal stem cells. ALDH1 has been used successfully to isolate HSC, neural and breast stem cell populations [170-172]. Different groups have also isolated ALDH1+ populations from leukaemia [173], breast [172, 174], colorectal [100], liver [175] and lung [176] cancers.
1.5.2 Current knowledge on existence of CSCs in solid tumours

Brain tumours

Brain tumourigenic stem cells (BTSCs) have been detected in primary brain tumours, including glioblastoma multiform (GBM), medulloblastoma, ganglioglioma and pilocytic astrocytoma [93, 128, 160, 161, 177, 178]. They have also been identified in several brain cancer cell lines [157, 179-181].

Using the neurosphere assay, CSCs are grown to clonal density on uncoated plastic in serum free medium supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) [155, 156]. Cells that are responsive to these growth factors form floating spheres, and are thereafter considered as CSCs if they can maintain this sphere forming property after several passages to give rise to a large number of differentiated progeny [182]. Neurospheres grown from primary brain tumours and brain cancer cell lines express CSC-like properties including higher levels of stem cell markers CD133 and nestin, which is a cytoskeletal protein associated with neural stem cells [157, 160, 183]. Neurospheres originating from glioma have been shown to differentiate into neurons and astrocytes [184], or when derived from oligoastrocytoma, they have been shown to express glial fibrillary acidic protein (GFAP) and myelin basic protein, which suggests their capacity for multilineage differentiation [161]. Neurospheres themselves possess an enhanced sphere forming potential in vitro and an increased cancer-initiating capability in vivo compared to the initial population with only 1000-5000 cells from neurospheres necessary to reinitiate a tumour [157, 159-161, 183].

Besides neurosphere formation, brain CSCs have been targeted by virtue of the stem cell surface marker CD133. This marker is expressed at higher levels in the neurospheres isolated from brain tumours rather than in the primary tumour tissues [157, 160, 183]. Uchida et al. first showed that CD133+ cells isolated from paediatric brain tumours presented stem cell characteristics in vitro with high capacity for
sphere formation and a certain potential for engraftment in neonatal rat brain [124]. Later on, Singh *et al.* (2003) succeeded in isolating a CD133\(^+\) population from medulloblastomas and GBM. As few as 100 CD133\(^+\) cells could re-initiate a tumour in NOD/SCID mice even after several passages, whilst this was not achieved with CD133\(^-\) cells even when 1000 times more cells were used [93, 94]. In addition to this, CD133\(^+\) cells could generate clusters, they self-renewed and differentiated to recapitulate the original phenotype of the tumour they were derived from.

A few studies confirmed the stem cell-like phenotype of CD133\(^+\) glioma cells *in vitro* [185] and *in vivo* [107]. In this latter study, Bao *et al.* (2006) showed that 10,000 isolated CD133\(^+\) cells were able to reinitiate a tumour in immunocompromised mouse brains, while \(2 \times 10^6\) CD133\(^-\) cells could not form tumours in 3 out of 5 animals [107]. *In vitro* studies on CD133\(^+\) cells also include resistance assays to chemotherapeutic agents. In cell lines derived from primary cultures of glioblastomas, CD133\(^+\) cells were significantly more resistant than CD133\(^-\) cells when exposed to a panel of four agents including paclitaxel, carboplatin, temozolomide and etoposide. These CD133\(^+\) expressed higher levels of the drug resistance gene BCRP1 [105].

The use of CD133 as a universal BTSC surface marker has however recently been reassessed due to conflicting data. Studies reported that CD133\(^-\) cells were able to form neurospheres and were also tumourigenic in nude rats [186, 187]. Moreover, these cells were capable of generating CD133\(^+\) cells after their re-implantation in rat brains [187]. It thus appears that CD133 may not be as essential in brain tumour initiation as originally expected. Its role might be more critical for tumour progression rather than initiation.

**Breast cancer**

In primary breast tumours, the combination of the two markers CD44\(^+\)/CD24\(^-\) enabled Al Hajj *et al.* (2003) to isolate breast tumour-initiating cells. Additionally, these isolated cells that expressed ESA had a reinforced tumourigenic potential in NOD/SCID mice [92]. ESA has previously been used to identify a breast stem cell population defined as MUC/ESA\(^+\) [188]. As few as 200 cells presenting the CD44\(^+\)/CD24\(^-\)/ESA\(^+\) phenotype generated a tumour in all of the mice tested when injected into mammary fat pad of NOD/SCID mice. Furthermore, only the cells with
CD44+/CD24− expression progressed into the other phenotypes CD44+/CD24+, CD44−/CD24+ and CD44+/CD24− after injection in the mice. This indicates that if CD44+/CD24− phenotype can differentiate and reconstitute all the phenotypes present within the tumour bulk, it might be the most primitive phenotype among them. Similarly, in breast cancer cell lines SUM149 and SUM159, the cells with CD44+/CD24+/ESA+ phenotype showed higher tumourigenicity when as few as 100 cells were injected into NOD/SCID mice. This confirms that CD44+/CD24+/ESA+ might be the phenotype of breast CSCs in cell lines as well as in fresh tumour samples [189]. Additionally, in two clinical studies where patients received neoadjuvant chemotherapy, there was a marked increase in the expression levels of CD44+/CD24− compared to untreated patients. This supports the hypothesis that CSCs may be more resistant to chemotherapy than the rest of the cancer cells [190, 191].

Further work on breast cancer involved in vitro studies with assays primarily set up to culture breast stem cells as non-adherent “mammospheres” [192]. Like the neurosphere assay, mammary tissue can generate mammospheres with large populations of stem and progenitor cells capable of differentiating into the three types of mammary epithelia [192]. Interestingly, when such cultures were performed on breast tumours, mammospheres were obtained, and 95% of the cells showed CD44+/CD24− phenotype and were highly tumourigenic in vivo [162]. Moreover, CD44+/CD24− cells in breast cancer cell lines were associated with invasive properties, as confirmed by their efficiency of invading matrigel and by their gene expression profiling [114]. Resistance to radiation [193] and to chemotherapy [194] were suggested to be the selected advantages of this population according to preliminary studies on breast cancer cell lines.

In parallel to the CD44+/CD24−/ESA− phenotype, the enzymatic assay that targets cells with high ALDH1 activity has been confirmed as an alternative tool to isolate breast CSCs. In breast cancer primary cultures, an isolated ALDH1+ cell population representing 5% of the total population possessed the potential to form mammospheres whereas ALDH1− cells could not [172]. ALDH1+ cells were also highly tumourigenic in vivo and able to regenerate tumour diversity with only 500 cells injected. However, the overlap with CD44+/CD24− population was less than 1.2%, which emphasizes the fact that isolating CSCs by a single cell surface marker...
may not comprise the whole stem cell population. Cells combining CD44+/CD24- and ALDH1+ phenotypes were more tumorigenic with an encouraging number of just 12 cells being able to initiate the tumour, versus 1500 CD44+/CD24- cells. In breast cancer cell lines, a combination of the markers ALDH1+ and CD44+ also reinforced the tumourigenic and metastatic potential of these cells [195]. Indeed, a multi-step evolution of cancer predicts that several different markers may be co-expressed in CSCs. It is therefore to be expected that, in order to refine CSC isolation, a greater number of markers is required.

Recently, the role of ALDH1 was elucidated in a study involving an analysis of 33 breast cancer cell lines. An ALDH1+ population with obvious stem cell properties was detected in 23 of these cell lines. These putative CSCs proved to be an important mediator of invasion \textit{in vitro} and \textit{in vivo}. ALDH1+ cells developed metastases in several distant sites, while ALDH1- developed metastasis only once and it was limited to the lymph nodes [174].

\textit{Colorectal cancer}

CD133 was the first marker used to isolate a population of colorectal cancer cells with high tumourigenic potential [98, 99]. O’Brien \textit{et al.} (2007) reported that on average 1 in 262 CD133+ colorectal cancer cells was able to initiate cancer [99]. The CD133+ population, as compared to the total tumour cell population, would represent a 200-fold enrichment in CSCs. Ricci-Vitiani \textit{et al.} (2007) also confirmed the high tumourigenic potential of CD133+ colorectal cancer cells when compared to CD133- cells [98]. The CD133+ population isolated directly from primary colorectal specimens [98] possessed an exclusive sphere formation capability compared to the CD133- fraction, and could retain the ability to initiate tumours for more than one year when cultivated under serum free conditions.

Cells isolated directly from colorectal specimens have been propagated in serum free conditions [163]. The resulting spheres expressed the stem cell marker CD133 and were tumourigenic in NOD/SCID mice, whereas cells grown in the presence of serum
were not. Moreover, upon deprivation of growth factors, the spheres differentiated and lost both CD133 expression and their tumourigenic potential.

In addition to CD133, a combination of the markers CD44+/ESA^{hi} with CD166^{+} have been proposed to also identify a colorectal tumour-initiating population of cells [100, 131]. It has been shown recently that CD44^{+} cells were tumourigenic \textit{in vivo} [132]. In all the cases, these populations have been claimed to have an exclusive tumourigenic property when compared to their negative counterpart.

Amongst the populations used to isolate colorectal CSCs, it is unknown whether they differ in maturity. Little is known yet as of the nature of CSCs and their progeny. In an effort to classify colorectal CSCs, Todaro \textit{et al.} (2008) recently proposed a hierarchy model where CD133^{+} cells are seen as the stem cells, and where another important stem cell marker Musashi-1 (Msi-1) is likely to be the marker of progenitor cells [163]. In this study, it was also shown that amongst a CD133^{+}/Msi-1^{+} enriched population, CD44^{+} cells are those which possessed the highest metastatic potential.

In addition to cell surface markers, studies on the role of ALDH1 in colorectal CSCs have characterised its role when combined with the ESA^{+}/CD44^{+} phenotype. The potential of the ESA^{+}/CD44^{+}/ALDH1^{+} cells to enhance tumourigenicity \textit{in vivo} in comparison to their counterpart ESA^{+}/CD44^{+}/ALDH1^{-} [100, 132] has not been clearly defined yet.

\textit{Pancreas}

The existence of tumour-initiating cells has been reported among most organs, in particular liver, pancreas, prostate and ovary.

In primary pancreatic cancer cells, a population rich in tumour-initiating cells was discriminated using the same guidelines as for breast CSCs [134]. This subpopulation comprised 0.2 to 0.8\% of the original tumour population. As few as 100 cells isolated from this phenotype were able to regenerate a tumour comprising a heterogeneous population of cells with the phenotype present in the original tumour (ESA^{+}/CD44^{+}/CD24^{+}, ESA^{+}/CD44^{+}/CD24^{-}, ESA^{+}/CD44^{-}/CD24^{+} etc.). In parallel,
work has also been carried out using primary tumours and pancreatic cancer cell lines to isolate CD133+ cells [102, 133]. In fresh primary tumour samples, only 1-3% of the isolated cells were CD133+. Injecting just 500 of these cells into NOD-SCID mice was sufficient to reinitiate a tumour [133]. Only 14% of the isolated CD133+ cells stained for CD44+/CD24+/ESA+. This suggests that, similar to colorectal cancer, different sets of markers can discriminate different populations of cells bearing tumour-initiating potential.

Using PANC-1 pancreatic cancer cell line, a colony forming assay was established that allowed the detection of cells able to form adherent spheres over several generations in serum free medium [164]. The cells isolated from the spheres were capable of excluding Hoechst dye, and when cultured in medium containing serum, two types of cells were generated - one with and one without the Hoechst dye exclusion capability [164]. 5×10^5 PANC-1 cells that were dissociated from spheres were injected into nude mice to form a visible tumour nodule after 4 weeks. This was in contrast with using 20 times more cells (10^7) from the original population to form tumours. The SP that was isolated from PANC-1 [196, 197] showed a high resistance to the anti-tumour agent gemcitabin [196]. The CSC nature of this population however was not defined.

**Liver**

In the liver, early investigations have been carried out using the marker CD133 to isolate putative CSCs. Highly tumourigenic CD133+ cells were discovered in vivo from fresh primary tumour samples [137] and from several hepatocellular carcinoma (HCC) cancer cell lines [96, 136, 137]. In PCL8024 HCC cell lines, ALDH1 used in combination with CD133 [175] seemed to target an even more tumourigenic population, since significantly fewer CD133+/ALDH1+ cells (500) were needed for tumour formation in comparison to CD133+/ALDH1− cells (10 000) and CD133−/ALDH1− cells (300,000). The marker CD90 (also known as Thy-1) has also been associated with tumourigenicity. CD90+ cells were more tumourigenic than their counterpart in HCC cell lines, which was also validated from primary HCC [127]. In primary tumours, CD90+ cells were 200 times more tumourigenic than the rest of the...
population. The same paper also reported that the CD44 marker is necessary for tumour engraftment and tumour metastasis, which has been confirmed in other cancers [163]. The marker EpCAM (epithelial cell adhesion molecule, =ESA (epithelial specific antigen) also discriminates a highly tumourigenic and metastatic liver CSC population, with an interesting highlight on the role of the Wnt/β-catenin pathway in the survival of EpCAM$^+$ cells [138].

In another attempt to isolate liver CSCs, an SP in the HCC cell line Huh7 promoted tumours in NOD/SCID xenograft experiments with as few as $10^3$ SP cells, whereas $10^6$ cells of the non-SP population were necessary to obtain the same result [104].

Prostate and ovarian cancers have been also investigated. Mainly, the phenotypes CD44$^+$, CD133$^+$, α2β1$^{hi}$ as well as androgen receptor (AR)$^-$ are expressed by cells presenting increased clonogenic, metastatic and tumour-initiating potential [95, 129, 198]. The combination of CD44$^+$/CD24$^-$ [130], as described previously in breast cancer, may also discriminate a prostate CSC population. In ovarian cancer, the main markers to isolate a CSC-like populations were reported to be CD133$^+$ [142] and CD44$^+$/MyD88$^+$ [143]. In several prostate and ovarian cancer cell lines, sphere formation assays have been established [165, 166], and the resulting spheroids were able to expand exponentially after several passages and to establish tumours in mouse engraftment experiments.
1.6 Traditional or hierarchical model for cancer?

In order to fully characterise and establish the role of CSCs, their successful isolation must be achieved. Currently putative CSCs are isolated based on the techniques described above, i.e. surface marker targeting, sphere formation, ALDH assay, and chemoresistance selection.

The efforts invested in CSC isolation during these past few years have reinforced at least the evidence for the existence of tumour-initiating cells in nearly all types of cancers including colorectal cancer, as reviewed here. The fact that a small population of cancer cells express a specific combination of markers to indicate a tumourigenic ability, whilst the majority of cancer cells lose these markers upon differentiation, is in itself evidence in favour of a hierarchical model of cancer progression [85].

To account for this model, it seems that the number of cells with tumourigenic properties would be lower relative to the number of the other cancer cells. If the majority of cancer cells is capable of tumourigenicity, then the concept of a hierarchy becomes less meaningful and it therefore is more likely that a common clonal genetic disposition is in effect rather than a specific epigenetic state. In such a case, tumour heterogeneity may be explained according to a clonogenic model. Recently, the immunodeficient in vivo models used to test cancer cell tumourigenicci have been questioned. Indeed, mouse models with improved immunodeficiency have revealed more tumourigenic cells than obtained with Nude or NOD/SCID mice [199].

Other caveats in the hierarchical model are now being raised, and the debate is evolving towards a compromise which accepts that tumours may follow either a traditional or a hierarchical model [200], and that clonal evolution can also intervene for CSCs [201].
1.7 Aims of the study

The aims of this study were the isolation and the characterisation of putative CSCs from colorectal cancer liver metastases. Colorectal cancer has been used as a model for this study. After brain and breast cancer, it is one of the first solid tumours in which the existence of CSCs has been claimed [98, 99]. Colorectal cancer is directly relevant with the CSC theory, as recurrence and metastases are common occurrences in patients. Indeed, more than 40% of patients with stage IV disease develop liver metastases [202]. If CSCs are proven to be responsible for metastasis, then there will certainly be a clinical interest into characterising and targeting these cells. Although the ultimate aim of CSC research is the discovery of more specific therapeutic targets, the isolation of CSCs remains a major hurdle and the best method to isolate these cells has not been defined yet. We therefore compared the efficiency of various techniques to isolate CSCs:

- using CD133 surface marker expression. The efficiency of this marker to isolate tumourigenic cells in colorectal cancer has been claimed by two different groups [98, 99], however further characterisation of CD133+ cells is needed in order to validate CD133 as a colorectal CSC marker and to explore their potential as a therapeutic target.

- With techniques other than cell surface markers:
  → using a chemoresistance functional assay. Resistance is considered to be a CSC intrinsic characteristic. It is one of the most important parameters in a clinical point of view, however little is known yet on the relationship between cancer and resistance. Using the resistance properties of CSCs may be a means to attain this population in colorectal cancer as it has been shown in brain tumour, pancreatic cancer and leukaemia [203-205].
  → testing the Aldefluor® assay as a marker for colorectal CSC isolation. The high expression of ALDH1 in pre-selected CD44+/ESA+ cell populations has been reported in two studies [100, 131]. More work is needed to analyse the possibility of ALDH1 as a single marker for colorectal CSC isolation.
- to characterise whether or not the isolated cells are potential CSCs and,
- to describe CSC potential therapeutic targets for therapy if CSCs are successfully isolated.
2 Materials and methods
2.1 Source of cells

2.1.1 Cancer Cell lines

The colorectal cancer cell line CaCO-2 was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). Colorectal cancer cells CaCO-2 were grown in Minimal Essential Medium (MEM) (Gibco, UK) supplemented with 20% fetal bovine serum (FBS) (Biosera, UK) and 1% penicillin-streptomycin (PS) (Gibco, UK). All cells were incubated at 37°C in 5% CO₂ / 95% air.

2.1.2 Tumour dissociation and primary culture from colorectal cancer liver metastases

Informed consent and local research ethics committee approval were obtained for the use of colorectal liver metastases specimens obtained after operative removal of the tumour (Reference Ethical Committee : 07/Q0406/45).

Fresh samples (20) of metastases of colorectal cancer to the liver were collected in a sterile tube immediately after surgery and directly processed for cell culture or cell separation. All samples were initially rinsed three times in Hanks’ balanced salt solution (HBSS) (Gibco, UK) containing 1% PS. Tumours used for tumour growth were mechanically sheared using scalpels and scissors. 1mm³ pieces of minced tumour tissue were carefully placed on the surface of small Petri dishes (35mm²) (Nunc™, Denmark). Explants were allowed to adhere for 2-3h in a drop of culture medium in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, UK) supplemented with 10% FBS and 1% PS. After that, extra medium was carefully added as not to dislodge the explants. All cultures were incubated at 37°C with 5% CO₂ / 95% air. Culture medium was changed twice a week. When the cells were confluent (after 4-5 weeks), they were subcultured and grown in T25 flasks. To remove fibroblasts from the cultures, 1% of trypsin/ethylene-diamine-tetra-acetic acid
(EDTA, T/E) was added to the flasks (0.5mL), and cell detachment was observed under light microscopy. Detachment of fibroblasts is quicker than epithelial cells at that concentration. Once they rounded up, fibroblasts and T/E were removed from the flask with HBSS and replaced with fresh medium. Culturing in DMEM supplemented with 2% FBS was another strategy to give a survival advantage to the epithelial cells over the fibroblasts. Only one colorectal metastasis primary culture developed as a cell line and was called colorectal metastasis 1 (CM1). It was grown in DMEM supplemented with 10% FBS and 1% PS.

Samples used for CSC isolation were mechanically dissociated as described above and incubated for 1h in 15mg/ml of collagenase I (Sigma, UK) and 133units/ml of DNAse I (Sigma, UK). The resulting cell suspension was then sequentially filtered through 100µm, 70µm and 40µm strainers to remove debris. To remove red blood cells, the tumour cell suspension was incubated on ice for 10min in 30ml of red blood cells lysis buffer (10.375mg/ml of NH₄Cl, 1.25mg/ml of KHCO₃ and 0.01% of (EDTA)), and spun down for 7min at 1800rpm (Heraeus, UK). The resulting pellet was resuspended in HBSS and centrifuged for 10min at 800rpm to remove additional cell debris and dead cells. The pellet was resuspended in DMEM supplemented with 10% FBS and cell number and viability were determined by trypan blue exclusion. The cell suspension was used for the isolation of CSCs.
2.2 Isolation of CD133\(^+\) cells

Single cell suspensions from 5 colorectal cancer metastases were labelled with anti-CD133 antibody using commercially available CD133\(^+\) MicroBead Kit isolation kit (Miltenyi Biotec, Germany) as recommended by the manufacturer. Briefly, \(10^8\) tumour cells were incubated in 350\(\mu\)l MACS buffer (phosphate buffer saline (PBS) supplemented with 0.5% bovine serum albumin and 5mM EDTA), 100\(\mu\)l FcR blocking agent, 50\(\mu\)l CD133/1 (AC133)-Biotin antibody at 4\(^\circ\)C for 10min. Labelled cells were then washed twice in MACS buffer and centrifuged at 1800rpm for 3min. \(10^8\) cells were resuspended in 400\(\mu\)l of MACS buffer with 100\(\mu\)l of anti-biotin microBeads at 4\(^\circ\)C for 15min. Excess of secondary antibody was washed by adding 10-20 times the labelling volume buffer and resuspended in 500\(\mu\)l MACS buffer. Magnetic cell separation was performed using either a VarioMACS or a MiniMACS separation column (Miltenyi Biotec, Germany). The column was placed in the magnetic field of a suitable MACS separator (Miltenyi Biotec, Germany) and washed with 3ml of MACS buffer. The cell suspension was then placed into the column. Unlabelled cells which passed through were collected and the column was washed three times with 3ml of MACS buffer. The column was removed from the magnetic field and 5ml of MACS buffer was used to flush out the fraction of magnetically labelled CD133\(^+\) cells. To ensure an optimal separation and high purity, both labelled and unlabelled fractions were processed a second time through new columns. The purity of each fraction was then determined by flow cytometry and the cells were directly used for further characterisation.
2.3 Isolation of chemoresistant putative CSCs

2.3.1 Determination of cell viability by Cell Counting Kit-8 upon oxaliplatin exposure

CaCO-2 cancer cells (20000 cells/well) were plated in 96-well plates (Corning Life Sciences, USA). 24h later, oxaliplatin was added at various concentrations (0 to 50µg/ml) to determine its effect on cell growth and survival. The number of surviving cells was determined after 24, 48, 72 and 144h with Cell Counting kit-8 reagent (CCK-8, Axxora, UK). In this convenient colorimetric assay, the tetrazolium salt WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H) is cleaved to formazan dye by cellular mitochondrial dehydrogenase. The amount of dye generated is directly proportional to the number of living cells and is detectable on a spectrophotometer at 450nm. 10µg/ml of the CCK-8 reagent was added to the wells, and cells were incubated for 2h at 37°C. Optical density (OD) was directly measured using a µQuant microplate spectrophotometer (BioTek, UK). A calibration curve had been previously prepared to correlate OD to number of live cells.

2.3.2 Determination of cell viability by trypan blue upon oxaliplatin exposure

CaCO-2 cancer cell line (240000 cells/ml)) or CM1 primary culture cells (250000 cells/ml) were plated in Petri dishes (35mm²) (Nunc™, Denmark). To determine the appropriate dose, oxaliplatin was added 24h later at different concentrations (0 to 10µg/ml) and incubated at 37°C for 48h. Dishes were rinsed three times with PBS and medium (DMEM or MEM) supplemented with 10% FBS was added to the cells. The number of cells was then counted every 3 to 5 days for CaCO-2, and every 5 to 10 days for CM1. Cells were harvested using Trypsin (Gibco, UK), and cell viability was evaluated by trypan blue exclusion. The dose sparing 10% or less of cells was chosen. It was determined to be 5µg/ml for both cell lines.
2.3.3 *Isolation of oxaliplatin-resistant cells*

CaCO-2 cells were plated in T150 cell culture flasks. At 70% confluency, 5µg/ml of oxaliplatin was added to the cells for 48h. After 3 washes in PBS, DMEM supplemented with 10% FBS was added to the cells. Surviving cells were harvested at day 10 or 11 with trypsin after drug incubation, and used directly for characterisation.

CM1 cells were similarly plated in T150 cell culture flasks, and exposed to oxaliplatin in the same way as CaCO-2. However, cells were harvested at day 21-22 after drug removal.

2.4 *Isolation of ALDH1$^{hi}$ putative CSCs*

The Aldefluor® kit (StemCell Technologies, Durham, NC, USA) was used to identify a population with a high ALDH enzymatic activity. Reagents from this kit allow the oxidation of an ALDH substrate BAAA (BODIPY®-aminoacetaldehyde) into a fluorescent product BAA (BODIPY®-aminoacetate) [206], detectable by flow cytometry. The staining was processed according to the manufacturer’s protocol. Harvested cells were re-suspended in 1ml Aldefluor® assay buffer containing 5µl of the ALDH substrate solution, and incubated at 37°C for 1h. As a negative control, an aliquot of cells was treated with 15µmol/l diethylaminobenzaldehyde (DEAB), a specific ALDH1 inhibitor. After incubation, cells were spun down at 1800 rotations per minute for 3min and re-suspended in 400µl of Aldefluor® assay buffer supplemented with 1% bovine serum albumin (BSA) (PAA Laboratories GmbH, Austria). Cells were immediately sorted into ALDH1$^{hi}$ and ALDH1$^{lo}$ cell fractions using fluorescence activated cell sorting (FACS) performed by a FACSdiva (BD Biosciences, UK).

Cell viability was evaluated by trypan blue exclusion after the sorting process.
2.5 Flow cytometry staining

2.5.1 Cell marker analysis

To analyse cell markers, $10^5$ to $5 \times 10^5$ cells were harvested and stained with the appropriate antibodies (details and dilutions in Table 3). Prior to staining with internal antibodies, cells were fixed by adding 25µl 1% paraformaldehyde (PFA, Sigma, Poole, UK) for 10min after which cells were washed with PBS. Primary antibodies for internal staining were diluted in cell permeabilisation agent (FIX & PERM, Invitrogen, UK), and primary antibodies for external staining were diluted in PBS supplemented with 1% FBS. The cells were incubated with primary antibodies for 45min at 4°C and then washed with PBS. Cells were then incubated with a secondary antibody conjugated with a fluorescent dye (fluorescein isothiocyanate (FITC) or phycoerythrin (PE)) for 30min at 4°C. Cells were washed with PBS and analysed by flow cytometry.

PE- or FITC-conjugated primary antibodies were diluted in PBS supplemented with 1% FBS. Cells were incubated for 30min at 4°C with these antibodies, washed with PBS and analysed by flow cytometry.

2.5.2 Cell cycle analysis

$10^5$ to $10^6$ cells were fixed overnight at 4°C in 70% ice cold ethanol. Cells were then washed twice in 2ml PBS and 400µg/ml of propidium iodide (PI) solution (Sigma, UK) was added for 1h on ice in the dark. 100µl of PBS was then added to each tube prior to flow analysis.

Incorporation of PI is proportional to the quantity of DNA content, and thus provides information on the cell cycle stage of the cells. During G0 or G1 phases, cells possess
a normal diploid chromosomal (DNA content (2n)) whereas cells in G2 and just prior to mitosis (M) contain exactly twice this amount (4n). As DNA is synthesised during S-phase, cells are found with a DNA content ranging between 2n and 4n. Histogram plot (Figure 7) shows the following profile:

![Histogram plot](image)

**Figure 7.** Dot plot representing CaCO-2 cell distribution in the cell cycle after PI incorporation.

### 2.5.3 ALDH1 activity measurement

Cells were labelled in the same way as previously mentioned in paragraph 2.4.

### 2.5.4 Flow cytometry analysis

Cells were analysed using a FACS Calibur Flow Cytometer (BD Biosciences, UK) and CellQuest Pro software (BD Biosciences, UK).
2.6 **Immunocytochemistry**

2.6.1 **Cell marker analysis**

ALDH1$^{hi}$ and ALDH1$^{lo}$ sorted populations were plated on to sterile glass slides (Sigma, Poole, UK) and left overnight in a 37°C incubator to allow them to adhere. They were fixed in 2% PFA for 10 min. In the case of intracellular markers such as: Oct-4, Lgr-5, ABCG-2, alkaline phosphatase (AP), sucrase isomaltase (SI) and Msi-1, cells were permeabilised with 0.1% Triton-X 100 (Sigma, Poole, UK) for 10 min. Post permeabilisation or for extracellular markers such as CD24, CD44, CD133 and CD166, non-specific binding was blocked using 5% FBS and 3% BSA for 45 min at room temperature. The primary antibodies were added immediately afterwards, incubated for 1 h at room temperature and then washed three times with the blocking solution for 5 min. Fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated secondary antibodies were then added and incubated for 1 h, in the dark, at room temperature and then washed again three times in PBS for 5 min. PE- or FITC-conjugated primary antibodies were incubated for 1 h, in the dark, at room temperature and washed three times in PBS for 5 min. Antibodies were prepared to the specified dilution, determined by preliminary testing, using the blocking solution. The details of the antibodies used and their dilutions are detailed in Table 4. 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, US) was used to label the nuclei and mount the slides. Slides were then wrapped in the aluminium foil and kept in the fridge for analysis within a week of staining.
2.6.2 *Microscopy*

Immunofluorescent stained cells were visualised on Zeiss fluorescent microscope and analysed using the Metamorph program. At least 250 cells were counted in 5 random fields of view to assess the approximate percentage of cells with positive expression of the marker of interest. Percentage positivity was calculated as shown below:

\[
\text{Percentage Positivity} = \frac{\text{Number of Positive Cells}}{\text{Total Number of Cells}} \times 100
\]

The cells were counted, at 40x or 20x magnification, in the control, ALDH1\textsuperscript{hi} and ALDH1\textsuperscript{lo} cell populations for each marker. All experiments were repeated 3 times for both the CaCO-2 and CM1 cell lines.

2.7 *Colony forming assay*

ALDH1 sorted cells were diluted so as to obtain 10 cells/mL. They were then plated onto 96-well plate, and checked under the microscope so as to pinpoint the wells containing 1 cell only.
For each experiment, 80-100 wells with one unique cell were scored and followed-up over 5 weeks for CaCO-2, and 8 weeks for CM1.

After that time, the number of mature colonies was determined, and percentages of colony forming potential were determined as follows:

\[
\text{Percentage Positivity} = \frac{\text{Number of mature colonies}}{\text{Total Number of wells containing one cell}} \times 100
\]
2.9 Migration assay

Migration assay was conducted using polycarbonate membrane inserts with 8-µm pores (Nunc, Roskilde, Denmark) which were used in standard 24-well cell culture plates. Polycarbonate membrane inserts were placed above the plated cells and 500µl of cell suspension (1x10^5 cells/ml) were added within the insert, in the absence of serum. 1mL of 20% FCS medium + 50ng/ml EGF was added in the bottom wells (to create a chemo-attractant gradient). No FCS and EGF were added to the control bottom wells. The entire chamber system was incubated at 37°C for 72 hours. After incubation, cells on the membrane were washed and fixed in PFA for 15min at 37°C. The filters were then washed twice with PBS and stained with 0.5% crystal violet for 50min. After several washes with water, the non-migrating cells were removed from the upper surface of the membrane by using a cotton tipped swab to scrape the cells off the membrane with firm but gentle pressure. The membrane was cut out using a scalpel and placed into a 96-well plate well containing 200µL of 0.1% acetic acid for solubilisation of the crystal violet. After 20min, OD was directly measured at the wavelength of 470nm using the µQuant microplate spectrophotometer.

Analysis

To calculate the percent invasion of cells, the following formula was used:
Migration rate = (mean # OD cells migrating across the pores towards the gradient / mean # OD cells migrating across the pores without gradient).

Three independent (single) experiments were performed.
2.10 Oxaliplatin resistance experiment

ALDH1\textsuperscript{hi} and \textsuperscript{lo} cells (15000 CM1 or 5000 CaCO-2 cells) were plated in 96-well plates. They were left overnight to adhere, and were then exposed to oxaliplatin at concentrations of 0, 1, 2, 5 and 10 µg/mL. After 96 h incubation, 10 µL of CCK-8 were added in each well, and the plates were incubated for 2 h and read on the spectrophotometer.

2.11 Animal study

All animal studies were carried out in accordance with the Animals (Scientific Procedures) Act 1986 (A(SP)A 86) and local institutional guidelines. 12 NOD-SCID mice (Harlan Ltd., UK) were injected subcutaneously in the right flank with $10^2$, $5 \times 10^2$, $10^3$, $10^4$ or $10^5$ oxaliplatin chemoresistant CaCO-2 cells, or with $10^7$ CaCO-2 or LS174T untreated cells. Animals were assessed every 2 days for the presence of tumour and were maintained and cared for in accordance with institutional and UK guidelines.

2.12 Statistical analysis

The Student’s t test was used when experiments were repeated 3 to 5 times. For a greater number of experiments, we used the Wilcoxon test. Data are presented as the mean±standard deviation (SD). The student t test (2-sided) was used for paired samples. Differences of $p<0.05$ were considered as statistically significant.
Table 3. Antibodies dilutions and origin for flow cytometry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
<th>Details</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44-FITC</td>
<td>Abcam</td>
<td>Mouse monoclonal IgG1</td>
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</tr>
<tr>
<td>CD133-PE</td>
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<td>Mouse monoclonal IgG2b Clone 293C2</td>
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<td>Mouse monoclonal IgG1 Clone AC133</td>
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</tr>
<tr>
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<td>Santa-Cruz</td>
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<td>1:25</td>
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<td>Abcam</td>
<td>Mouse monoclonal IgG1</td>
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</tr>
<tr>
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<td>Abcam</td>
<td>Mouse monoclonal IgG2a</td>
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</tr>
<tr>
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<td>Abcam</td>
<td>Mouse monoclonal IgG2a</td>
<td>1:20</td>
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<td>Msi-1</td>
<td>R&amp;D Systems</td>
<td>Goat IgG</td>
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<td>Lgr-5 (GRP49)</td>
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<td>Abcam</td>
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</tr>
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<td>Oct-4</td>
<td>Abcam</td>
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</tr>
<tr>
<td>Mouse IgG1-PE</td>
<td>Miltenyi Biotech</td>
</tr>
<tr>
<td>Mouse IgG2b-PE</td>
<td>Miltenyi Biotech</td>
</tr>
<tr>
<td>Mouse IgG1</td>
<td>Santa-Cruz</td>
</tr>
<tr>
<td>Mouse IgG2b</td>
<td>Santa-Cruz</td>
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<tr>
<td>Mouse IgG2a</td>
<td>Santa-Cruz</td>
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<td>Goat IgG</td>
<td>Santa-Cruz</td>
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<td>Rabbit IgG</td>
<td>Santa-Cruz</td>
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<td>Rabbit anti-mouse FITC</td>
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<td>Santa-Cruz</td>
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<td>Swine anti-rabbit FITC</td>
<td>Dako-Cytomation, Denmark</td>
<td>1:100</td>
</tr>
<tr>
<td>Antibody</td>
<td>Supplier</td>
<td>Details</td>
</tr>
<tr>
<td>----------</td>
<td>----------</td>
<td>-----------------------------</td>
</tr>
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<td>Mouse monoclonal IgG1</td>
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<td>Miltenyi Biotech</td>
<td>Mouse monoclonal IgG1 Clone 293C2</td>
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<td>CD166-PE</td>
<td>Abcam</td>
<td>Mouse monoclonal IgG1</td>
</tr>
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<td>CD24</td>
<td>Abcam</td>
<td>Mouse monoclonal IgG1</td>
</tr>
<tr>
<td>ABCG2</td>
<td>Abcam</td>
<td>Mouse monoclonal IgG1</td>
</tr>
<tr>
<td>Msi-1</td>
<td>R&amp;D Systems</td>
<td>Goat IgG</td>
</tr>
<tr>
<td>Lgr-5 (GRP49)</td>
<td>Abcam</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>Oct-4</td>
<td>Abcam</td>
<td>Rabbit polyclonal</td>
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<tr>
<td>Mouse IgG1-FITC</td>
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</tr>
<tr>
<td>Mouse Ig1-PE</td>
<td>Miltenyi Biotech</td>
</tr>
<tr>
<td>Mouse IgG2b-PE</td>
<td>Miltenyi Biotech</td>
</tr>
<tr>
<td>Mouse IgG1</td>
<td>Santa-Cruz</td>
</tr>
<tr>
<td>Mouse IgG2a</td>
<td>Santa-Cruz</td>
</tr>
<tr>
<td>Goat IgG</td>
<td>Santa-Cruz</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Santa-Cruz</td>
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<thead>
<tr>
<th>Secondary antibodies</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlexaFluor 488 anti-mouse</td>
<td>Invitrogen</td>
<td>1:500</td>
</tr>
<tr>
<td>AlexaFluor 594 anti-goat</td>
<td>Invitrogen</td>
<td>1:250</td>
</tr>
<tr>
<td>AlexaFluor 488 anti-rabbit</td>
<td>Invitrogen</td>
<td>1:500</td>
</tr>
</tbody>
</table>

**Table 4.** Antibodies dilutions and origin for immunohistochemistry.
3 Isolation of putative colorectal CSCs according to CD133 expression
3.1 Background

The use of cell surface markers has been extremely useful in the isolation of various types of stem cells from organs such as bone marrow, brain and breast. Since it is hypothesised that CSCs are altered stem cells or stem cell-like cells that retain some of their original markers, then it is reasonable to attempt to isolate CSCs in the same way as normal stem cells.

3.1.1 CD133 cell surface marker and stem cells

CD133 is a pentaspan membrane protein and part of the prominin family. Its role is involved in organising the plasma membrane [207]. Its functional link with stem cell biology has not been completely assessed, but several observations have been made which associate its presence with a ‘stemness’ state. CD133 is present in the apical membrane protrusions of embryonal epithelial structures. It has also been previously observed that the differentiation of CaCO-2 cells is associated with the release of membrane bound CD133 [208]. Maintaining CD133 expression in the membrane may therefore sustain a stem cell phenotype.

The antibody recognising a glycosylated epitope of the CD133 protein, the AC133 antigen, detected a subpopulation of CD34\(^+\) HSC with long-term repopulating capacity in mouse xenografts [123]. AC133 expression is restricted to the CD34\(^+\) compartment in blood cells and is also a characteristic of endothelial [209] and brain stem cells [124].
3.1.2 CD133, a universal cell surface marker for CSCs?

CSCs were initially isolated using CD133 expression. With an antibody that detects the glycosylated AC133 epitope, tumour-initiating cells were successfully isolated from neural malignancy (in combination or independently of nestin) [93, 94, 105, 161], prostate cancer (combined with CD44 and α1β2 integrin) [95], pancreatic adenocarcinoma [102], HCC [96, 136], and, importantly for this project, colon carcinoma [98, 99].

Focusing on colorectal cancer, CD133 was first chosen by O’Brien et al. (2007) in an attempt to identify a tumour-initiating population, using the same approach as in previous brain CSC extraction [99]. Out of 17 primary and metastatic colonic tumour samples, O’Brien et al. (2007) found that the number of CD133 expressing cells comprised between 1.8 and 24.5% of the total cell population. By limiting dilution assay, they predicted that when transplanting the total number of colon tumour cells in mouse xenografts, one cell out of $5.7 \times 10^4$ was a tumour-initiating cell. When CD133+ cells were injected into mice, they established that there was a 200-fold enrichment of tumour-initiating cells. Since CD133− cells were never able to induce a tumour, it meant that CD133+ cells contained the tumour-initiating population. However, not all of them were equally tumourigenic (only one CD133+ cell in 262).

A study by Ricci-Vitiani et al. (2007) extended the work of O’Brien [98]. They isolated CD133+ cells from primary colon cancer (the average CD133 expression was 2.4%) and also showed that tumourigenicity was restricted to the CD133+ fraction. They were able to maintain CD133 expression by culturing CD133+ cells in a serum-free medium, and the resulting colonies that formed in these culture conditions maintained and even increased their tumourigenic potential following in vivo and in vitro passages.

These results encouraged us to attempt to isolate a putative CD133+ CSC population from metastatic colon carcinoma for their characterisation and potential development as a new therapeutic target.
3.2 Isolation of putative colorectal CSCs according to CD133 expression

3.2.1 CD133⁺ cells isolation

A putative CSC population was isolated from colorectal metastases resected from the liver, using a CD133 immuno-affinity assay. The tumour samples that were collected from the operating theatre were used to optimise the cell dissociation process with the aim of minimising cell death. After optimisation of the protocol (as discussed in Material and Methods) five tumour samples were dissociated and incubated with a primary biotinylated anti-CD133 antibody (clone AC133, which recognizes epitope CD133/1), and then with anti-biotin Microbeads, according to the manufacturer’s protocol (Miltenyi Biotech). Labelled cells were isolated by being passed through an LS magnetic column twice. Cells eluted by the column were defined as the negative fraction while the magnetically labelled cells were retained by the column and constituted the positive fraction. The total number of cells before labelling and the number in the positive fraction were counted with a haemocytometer. Dead cells (trypan blue positive) were excluded. The percentages of cells in the positive fraction (7.52±6.36% ; n=5) isolated from the total tumour cell population are listed in Table 5.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percentages of cells in the positive fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17.3%</td>
</tr>
<tr>
<td>2</td>
<td>0.9%</td>
</tr>
<tr>
<td>3</td>
<td>2.9%</td>
</tr>
<tr>
<td>4</td>
<td>8.4%</td>
</tr>
<tr>
<td>5</td>
<td>8.1%</td>
</tr>
</tbody>
</table>

Table 5. Percentages of cells isolated from the total tumour cell population and retained by the magnetic column after labelling with anti-CD133 antibody conjugated to magnetic beads.
3.2.2 Assessment of cell purity

To assess the efficiency of the magnetic separation, we stained the positive and negative fractions with CD133 antibody-FITC conjugated (clone 293C3, that recognizes epitope CD133/2 as recommended by the manufacturer), and analysed CD133 expression by flow cytometry.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percentage of CD133&lt;sup&gt;+&lt;/sup&gt; cells in total population</th>
<th>Percentage of CD133&lt;sup&gt;+&lt;/sup&gt; cells in negative fraction</th>
<th>Percentage of CD133&lt;sup&gt;+&lt;/sup&gt; cells in positive fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NA</td>
<td>43%</td>
<td>65.8%</td>
</tr>
<tr>
<td>2</td>
<td>1.02%</td>
<td>81.4%</td>
<td>87.31%</td>
</tr>
<tr>
<td>3</td>
<td>67.79%</td>
<td>55.17%</td>
<td>94.4%</td>
</tr>
<tr>
<td>4</td>
<td>29.4%</td>
<td>12.78%</td>
<td>46.6%</td>
</tr>
<tr>
<td>5</td>
<td>1.52%</td>
<td>1.6%</td>
<td>10%</td>
</tr>
</tbody>
</table>

Table 6. FACS analysis for purity check of the total population of samples, the fraction eluted by the column (negative fraction), and the fraction retained by the column (positive fraction) (use of antibody anti-CD133 clone 293C3).

According to the FACS analysis data in Table 6 and Figure 8, the percentages of CD133<sup>+</sup> cells amongst the total population were only 24.9±31.5% ; n=4. There was an enrichment of the positive fraction in CD133<sup>+</sup> cells when compared with the negative fraction.

On average, 22% more CD133<sup>+</sup> cells were retained in the positive fraction than in the negative one. This result indicates that the system did select for CD133, however the quality of separation was not optimal. Positive fractions were anticipated to be at least 90% positive for the CD133 marker after a two-column separation. Only samples 2 and 3 showed the expected percentage of positivity for CD133<sup>+</sup> (87.31% and 94.4%, respectively). In samples 1, 4 and 5, percentages of CD133<sup>+</sup> cells in positive fractions were only between 10 and 68%. The positive fractions were therefore contaminated with CD133<sup>-</sup> cells. The fractions expected to be negative for CD133 expression contained a high frequency of CD133<sup>+</sup> cells in 4 out of 5 samples tested (up to 81.4% in sample 2), indicating that not all CD133<sup>+</sup> cells were retained in the column.
Figure 8. Representative flow charts indicating the poor purity of CD133 magnetic separation by flow cytometry. Blue : IgG. Green : CD133. Total population for sample 1 is not available. The charts reveal an enrichment of the positive and negative fractions in CD133\(^+\) cells, indicating that all CD133\(^+\) were not retained by the column. Although contaminated with CD133\(^-\) cells, the positive fractions contained more CD133\(^+\) cells than the negative fractions.
3.3 Understanding and overcoming the bad quality of separation

To determine why the separation process was not optimal, we considered that the problem might occur at different stages:

- the antibody used to check purity is not specific,
- the microbead labelling is not specific, or
- the separation process is not efficient.

1) To check the specificity of the antibody (clone 293C3) used to confirm the purity of positive and negative fractions, we used the leukemic cell line KG1a as a negative control. It has been reported that AC133 does not react to this cell line [123]. KG1a cells were labelled with 3 different anti-CD133 antibodies produced by Miltenyi Biotech (clones 293C3, AC141 and AC133). The results were indeed negative on the flow cytometry, thus eliminating the possibility of non-specific staining at this level (Figure 9).

![Figure 9](image)

**Figure 9.** Representative flow charts indicating the expression of CD133 in KG1a cells (negative control). The three anti-CD133 antibodies we tested did not react with KG1a cells that have been reported to not express CD133. This experiment eliminated the possibility of non-specific staining when using these antibodies to confirm the purity of the separated cells. Blue : IgG. Green : CD133.
2) If the problem was at the microbead labelling step, one hypothesis was that adding the immunomagnetic beads could trigger a change in the cell membrane, and make them more reactive to the anti-CD133 antibody. We decided to compare by flow cytometry the percentage of CD133\(^+\) positive cells among the total population before and after the incubation with the magnetic beads. However, percentages of CD133\(^+\) cells in sample 3 and the cell line CaCO-2 (known to express CD133) were similar before and after the incubation, suggesting that adding the beads to the cells did not influence their reactivity to anti-CD133 antibody.

3) As a control for the separation, CD133\(^+\) isolation was performed on CaCO-2, and on average, 7\% of the cells were retained by the column (n=2). Flow cytometry data revealed that the unseparated population, as well as the positive and negative fractions all contained a very high percentage of positivity (respectively 96.08\%, 98.27\% and 95.76\% as an average for two experiments) showing poor separation in the case of high CD133 expression (cells were separated twice in the column) (Figure 10). For one of these two experiments, we also used anti-CD133/2 clone 141 and anti-CD133 AC133 clone to confirm the FACS results obtained with anti-CD133/2 clone 293C3. The three antibodies gave similar results.

**Figure 10.** Representative flow charts indicating the strong expression of CD133 in CaCO-2 total, positive and negative fractions after magnetic separation. Blue : IgG, Green : CD133. After magnetic separation, the negative fraction still contained a high percentage of CD133\(^+\) cells, indicating that the kit might not be efficient when there was high number of CD133\(^+\) cells in the total cell population.
When Table 7 was analysed relative to Table 6 it appeared that the number of CD133$^+$ cells present in the total population was nearly equal to the number of CD133$^+$ cells present in both negative and positive fractions after separation, although a loss of cells was inevitable during the process. This was consistent for all samples except sample 2, in which the positive fraction contained 2 fold more CD133$^+$ cells than in the total population. If we consider sample 2 as a technical fault, it appears that the problem might be due to a contamination of the negative fraction. All CD133$^+$ cells may not be retained in the column as they should be.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of CD133$^+$ cells in total population</th>
<th>Number of CD133$^+$ cells in negative fraction</th>
<th>Number of CD133$^+$ cells in positive fraction</th>
<th>Total number of CD133$^+$ cells after separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NA</td>
<td>1.02 $\times 10^7$</td>
<td>3.29 $\times 10^6$</td>
<td>1.35 $\times 10^7$</td>
</tr>
<tr>
<td>2</td>
<td>0.09 $\times 10^7$</td>
<td>7.26 $\times 10^7$</td>
<td>0.07 $\times 10^7$</td>
<td>7.33 $\times 10^7$</td>
</tr>
<tr>
<td>3</td>
<td>2.3 $\times 10^7$</td>
<td>1.82 $\times 10^7$</td>
<td>0.09 $\times 10^7$</td>
<td>1.91 $\times 10^7$</td>
</tr>
<tr>
<td>4</td>
<td>5.6 $\times 10^7$</td>
<td>2.22 $\times 10^7$</td>
<td>0.74 $\times 10^7$</td>
<td>2.96 $\times 10^7$</td>
</tr>
<tr>
<td>5</td>
<td>1.67 $\times 10^8$</td>
<td>1.62 $\times 10^6$</td>
<td>0.9 $\times 10^6$</td>
<td>2.57 $\times 10^8$</td>
</tr>
</tbody>
</table>

**Table 7.** FACS analysis of purity of cells in the total population of samples, in the fraction eluted by the column (negative fraction), and in the fraction retained by the column (positive fraction) (use of antibody anti-CD133 clone 293C3).

The difficulty to obtain pure populations of CD133$^+$ and CD133$^-$ cells as well as the important number of dead cells contained in both fractions prevented us from pursuing further experiments with CD133 labelling.
3.4 Discussion

CSC research is still a newly emerging field, thus isolating CSCs still remains a major challenge. Since promising results have been reported throughout the literature, we decided to isolate CSCs from colorectal liver metastases based on their cell surface expression of CD133. However, after dissociation of the clinical specimen, our study was hindered. We have attempted to separate CD133\(^+\) and CD133\(^-\) fractions by immuno-affinity on a magnetic column, however both positive and negative fractions contained a high percentage of CD133\(^+\) cells in the majority of the samples. In one sample only, the negative fraction contained fewer than 2\% of CD133\(^+\) cells, but in this case, only 10\% of the corresponding positive fraction were CD133\(^+\).

The commercially available kit from Miltenyi Biotec for CD133\(^+\) cell isolation has been designed to isolate stem cells from haematopoietic tissues and not from solid tissues. After mechanical dissociation and enzymatic degradation of solid tissues, cell damage and debris might have resulted in a lack of specificity for the antibodies at the time of cell labelling.

It is important to add that, at the time we started CD133 isolation, Miltenyi Biotec had redesigned their labelling kit where the new protocol involved an indirect labelling step. Miltenyi Biotec refused to provide an explanation for this change. The indirect method involved a primary incubation with a biotinylated antibody against CD133, followed by a secondary incubation with anti-biotin microbeads. In the original kit, the anti-CD133 antibody was conjugated to the beads and only required one incubation. The use of a two-step process may therefore have increased the risk of unspecific staining. Although the direct kit is now available again, we did not have the opportunity to compare it with the indirect method.

To help interpret our results, we questioned the specificity of the antibody that was provided with the kit. The CD133 cell surface marker has two potential epitopes which are currently targeted by monoclonal antibodies. These epitopes are AC133 (epitope 1 = CD133/1)) and AC141 (epitope 2 = CD133/2)). Miltenyi Biotec provides four monoclonal antibodies: clones AC133 (mouse IgG1) and W6B3C1 (mouse
IgG2b) recognize epitope 1. Clones 293C3 (mouse IgG2b) and AC141 (mouse IgG1) recognize epitope 2. The clone used for magnetic separation is AC133, and the manufacturer advises the use of clone 293C3 (epitope 2) for checking the purity of the separated fractions. The location of these epitopes has not been described yet, but it is known they are spatially distinct [123]. A key point is that both of these epitopes are glycosylated, and the monoclonal antibodies we used are designed to recognize the glycosylated form of the CD133 molecule. Glycosylation is a post-translational process in which the neo-synthesized proteins are modified by being linked to a glucid. This process is site-specific, enzymatic (i.e. tightly regulated), and mainly involves cell surface and secreted proteins. Glycosylation confers stability to the proteins by protecting them from proteolysis, and has been shown to be highly involved in cell signalling and cell-cell adhesion [210]. Importantly, glycosylation can also affect the tertiary structure of proteins since some proteins cannot fold properly until they have been glycosylated. Any variation in the glycosylation regulation can easily alter the protein folding. In our case, the access of the antibody to the epitopes may therefore be impossible due to steric hindrance. Moreover, changes in the glycosylation pattern can alter specific sites on the protein, and therefore the antibodies might not be able to recognize their antigen [211].

According to our data, however, the system did select for CD133+ cells, as there was an enrichment of CD133+ cells in the fraction retained by the column, compared with the fraction eluted by the column. Moreover, the positive and negative controls (CaCo-2 and KG1a cells, respectively) confirmed the specific recognition of the CD133 marker by the antibodies used in the kit. Therefore the glycosylation issue may not be the key element to explain our data. We have also demonstrated with the CaCO-2 cell line, which expresses high levels of CD133, that the use of the Miltenyi Biotec kit for large amounts of CD133+ cells was not appropriate since CaCO-2 positive and negative fractions both contained more than 94% of CD133+ cells. We believe that an excess of labelled cells in the column might not be retained once all the binding sites have been occupied. The manufacturer indicates that a maximum of $10^7$ labelled cells can be retained in the small (MS) columns, and $10^8$ cells in the large ones (LS). We used an LS column to process $3 \times 10^7$ CaCO-2 cells, but were still not able to produce an optimal isolation as the negative fraction contained 97% of CD133+ cells (Figure 10). As the kit was designed for haematopoietic cells, the
maximum number of cells retained by the column may be different with colorectal cancer cells. It would be important to determine in future experiments whether the saturation of the column is responsible for the bad quality of separation. This experiment could be conducted using firstly a mixed population of KG1a and CaCO-2 cells, with an increasing percentage of CaCO-2 cells, to detect the threshold number of colorectal CD133$^+$ cells the column is able to retain. Similar experiments should be conducted with cancer cells extracted from fresh tumour samples, starting with a low number of total cells to prevent saturation in the column.

At the time we were facing technical difficulties with isolating CSCs according to their expression of CD133, the literature reported problems caused by using glycosylation-dependent CD133 antigens. Shmelkov et al. (2008) demonstrated that CD133 expression extended to a wide range of differentiated colonic cells, and not only to a low percentage of stem cells/CSCs as had previously been assumed using the CD133 antibodies [212]. In their study, Shmelkov et al. (2008) created a knock-in tumour mouse model, where CD133 expression could be detected by histochemistry using a LacZ reporter associated with the CD133 gene. To detect CD133 molecule, they chose an alternative method, avoiding the use of the anti-CD133 antibodies. Their method revealed a broad expression of CD133 in a large number of differentiated colonic cells, suggesting that the technique of using antibodies (anti-CD133) may not be sufficiently sensitive to detect all the CD133 expressing cells. The discordance between the results obtained by these different methods seems to be limited by the state of glycosylation in these samples. In the CaCO-2 cell line, it has been observed that there is a decrease of immunoreactivity of the AC133 antigen upon differentiation, while the levels of mRNA (ribo-nucleic acid) were still elevated [213]. This observation therefore suggests that the antibodies directed to AC133 were not able to bind to their antigen while the CD133 protein was expressed. A loss of glycosylation could be the main reason for this lack of recognition, and would be consistent with the fact that in HT-29 colorectal cancer cell line (a model for enterocytic differentiation) the glycosylation pattern is variable and correlates with the stage of differentiation [214]. Therefore, if the CD133 pattern of glycosylation is dependent on the cell status of differentiation then variations in the antigen affinity
will be expected. These data therefore directly question the specific targeting of CD133 with the current antibodies that are commercially available.

In addition to the limiting factors caused by the state of glycosylation, doubt still remains as to whether CD133 is as essential as initially expected in brain tumour and colorectal cancer initiation, and *ipso facto* in CSC isolation. In brain cancer, CD133 was thought to be a CSC marker [93]. A recent study has shown that CD133− cells also presented CSC characteristics as they were able to form neurospheres and were tumourigenic in nude rats [186, 187]. Moreover, CD133− cells were capable of generating CD133+ cells after their re-implantation in rat brains, suggesting that the CD133− fraction contained precursors of CD133+ cells [187]. Another study showed that GBM tumours negative for the CD133 marker still contained CSCs and were still able to form tumour spheres *in vitro*, as well as maintaining tumourigenicity *in vivo* [186]. Shmelkov et al. (2008) also reported that, in their hands, CD133− cells from metastatic colon cancer were as tumourigenic as CD133+ cells. Like the two studies quoted above for colorectal CSC isolation [98, 99], they used the Miltenyi Biotec kit with the anti-CD133 antibodies to isolate CD133+ cells, but obtained opposite results. Other compromising results obtained by Horst et al. (2008) indicate that although CD133 maybe an important prognostic factor in colorectal cancer, it is not essential for cancer initiation or metastasis [145].

These studies question the use of CD133 itself as a marker of choice for CSC isolation as it does not seem to be essential for CSC characteristics. Its expression still continues in differentiated colonic cells. It is possible that these controversial data may be the result of a lack in specificity from the glycosylated epitopes used for CD133 isolation. Perhaps if a new epitope was chosen it may change the results. Indeed, other anti CD133-antibodies are commercially available from other companies (Santa Cruz Biotechnology, Abcam and Cell signalling Technology) and recognise non glycosylated extracellular epitopes. Their use has not been reported yet throughout the literature and it would be interesting to compare the results obtained with these antibodies.
The role of CD133 has to be reconsidered in the light of these observations. CD133 might be an appropriate marker to isolate CSCs, but only when it is in its glycosylated form. In this case, the fact that only a glycosylated variant of CD133 is required for positive CSC recognition brings in numerous technical hurdles specifically for antibody recognition. However, this still does not explain how tumours that do not express CD133 at all [100, 215], still contain a subpopulation of tumour-initiating cells [186]. It does however confirm that CD133 is not an absolute marker of CSCs.

It is important to consider the origin of cancer cells in order to define the best strategy to isolate them. The stage of differentiation at which the mutations leading to malignant transformations happen is not known yet, and it is quite possible that early or even late progenitor cells as well as stem cells can function as CSCs, and express a different panel of markers. The evidence that in the same organ two sets of CSC populations can exist, such as CD133$^+$ and CD44$^+/CD166^+$ in colorectal cancer [98, 100], and are similarly able to initiate tumours, proves that none of these markers is exclusively expressed by CSCs. Moreover, the proposed CSC markers are not expressed in 100% of the primary tumours examined, which seems to indicate that the search for one single marker or even a combination of markers might be the wrong strategy. It is therefore necessary to use other complementary techniques to isolate a true CSC population.

Based on all these considerations, and as we were limited on the technical front, we made the decision not to pursue on further experiments with the CD133 cell surface marker.
4 Establishment of primary culture from colorectal cancer metastases
4.1 Background

Several experimental models are commonly used to understand cancer biology. Pathological specimens freshly isolated from patients represent the best available sources for isolation and characterisation of CSCs. Indeed, if CSCs are truly present in tumours, it is more relevant to analyse them immediately after their isolation to prevent any potential alterations in their state following \textit{in vitro} culturing (division and differentiation, number, mutations, etc.). However, these models are not ideal to investigate the origin of tumours since they reflect the real state of tumours \textit{in vivo} only at the time of resection which is already at a late stage of tumour growth. Additionally, the direct isolation of stem cells from solid tumours raises technical hurdles. During the process of mechanical and/or enzymatic dissociation, tissues are severely damaged, and large numbers of cells are destroyed. It is extremely difficult to limit the number of dead cells, and to separate them from the viable ones. Lastly, initiating cancer cell growth \textit{in vitro} after a dissociation process is another limiting step as less than 10\% of the viable cells in a primary colorectal culture preparation are able to attach and to grow [216].

In order to circumvent these difficulties and the problem of specimen availability, other models have been explored.

Cancer cell lines have been widely used, even though their validity is debatable. They are easily available and propagate to many passage numbers. However, they may not necessarily represent the original cancer cells in a reliable way. There are several reasons for this uncertainty.

Cell lines represent a clonal selection of a heterogeneous population of cells [217]. For instance, different metastases from the same patient have been shown to give rise to cell lines that are genetically different [218]. Each particular lineage constitutes a distinct model of the same disease and does not necessarily reflect the initial heterogeneity of the neoplasm [218]. The lineage selected for may simply represent a subtype of cell that grew more favourably in the \textit{in vitro} culture environment.
Culture conditions are critical since infections, genome instability and even cross contamination can alter the cell lines. A high number of passages considerably increases those risks [219].

A change in the genome as a result of selective pressure from a growth condition is an important issue as many studies have shown that the pattern of gene expression changes in cell lines grown in different culture conditions. The longer the cells are kept *in vitro*, the greater is the likelihood that they will undergo epigenetic or genetic changes. When comparing individual cancers from various organs and the corresponding cell lines from different origins, the genetic evolution is such that cell lines surprisingly tend to genetically group together and to display more similarities between each other than with the original tumours [220-222]. Moreover, in many cases, genetic profile has been shown to be less distinct between the normal tissues and their corresponding tumours than between the tumours and the derived cell lines [221, 223, 224]. This indicates that the processes of genetic alterations that would have started *in vivo* continue and are even amplified *in vitro* as cells are passaged.

Some cell lines clearly segregate from the tumour from which they derive whereas some of them still evolve slowly and can retain several properties of the original tissue [225]. Analysing cell lines newly isolated from primary tissues therefore seems to be the best option for representing a particular cancer cell lineage, as suggested by a recent study [226]. Colorectal cancer cells cultured *in vitro* are an encouraging example. In a recent paper, it has been shown that, out of 389 mutations analysed in the cell lines derived from colorectal cancers *in vitro* and passaged for six months to one year maximum, 99.3% were present in the primary tissue of colorectal cancers [227].

Collecting and isolating CSCs from fresh patient specimens was technically challenging in our hands. Based on all the above considerations, we sought to grow cancer cells prior to analysis and CSC isolation. We herein describe the method we used to establish a cancer cell line, in order to analyse it for the presence of CSCs.
4.2 Results

4.2.1 Culturing and developing CM1 primary cell line

Fresh samples of colorectal cancer liver metastases were processed as detailed in Materials and Methods. In total, 15 samples were processed. Cells from 33% of the samples did not reach confluence in culture and did not survive to the first passage. In these cases, the cells did not adhere on to the plastic culture dishes and subsequently died. In 66% of the samples, cells were able to adhere and to survive. After 1 to 2 weeks, adherent fibroblasts were observed, followed by adherent epithelial cells which started to spread as dense islands, typical of colorectal cancer cells (Figure 11). Once confluent (usually after a month), cells were passaged in T25 flasks. However, fibroblasts predominantly overtook the culture space since epithelial cells failed to develop after passage (P)1. After P3, cells were grown in DMEM supplemented with 2% FBS to favour epithelial cell growth. We also used 1% T/E to help remove the fibroblasts. Cells started to grow faster after P4.

A primary cell line (CM1) was developed from one colorectal metastasis. Thus, the efficacy of obtaining cell lines from colorectal cancer metastases was 6%.
Figure 11. Representative pictures of colorectal liver metastases in culture. After 2 weeks in Petri dishes, epithelial outgrowth appeared and spread from the tissue explants (A, B, C, D). CM1: epithelial cells started to grow after passage 2 (E,F) and the fibroblast population appeared to diminish as epithelial cells spread.
4.2.2 Patient history

The CM1 cell line was established from the liver metastasis of a patient, aged 65, with colorectal cancer. This patient first underwent a right hemicolectomy, and received adjuvant FOLFOX (FOLinic acid, Fluorouracil and OXaliplatin [228]) postoperatively for 5 months. Nevertheless, 18 months later, the disease had metastasised to the liver thus requiring liver resection and cholecystectomy.

4.2.3 Brief characterisation of the CM1 cell line

To confirm the nature of our CM1 cells, we analysed them for the expression of two markers: carcino-embryonic antigen CEA and villin (Figure 12). CEA is a tumour-associated antigen, specifically expressed by colorectal cancer cells [229]. FACS analysis revealed that 69% of the cells were positive. CM1 cells were also positive (>70%) for villin, a gastrointestinal-associated cytoskeletal protein expressed specifically in the microvilli of epithelial cells. This confirmed the epithelial nature of these cancer cells.

**Figure 12.** Representative flow charts indicating the strong expression of CEA (A) and villin (B) by CM1 cells. Blue: isotype. Green: CEA or villin. This FACS analysis was conducted to check the CM1 cells express markers associated with epithelial and carcinogenic characteristics.
This experiment was performed using cells at passages 14 and 15.

4.2.4 Growth behaviour of the CM1 cell line

In all the experiments, we decided to use the CM1 primary cell line at low number of passages, up to a maximum of 15 passages. We observed a change in the cell behaviour. In particular, cells increased their proliferation rate after passage 3-4 (data not available, and based only on observation), with a doubling time of 1 to 2 days. As passages increased, the cells seemed to become more resilient as we also noticed a better recovery after freezing-thawing when cells were frozen at later passages.
4.3 Discussion

Specimens collected from the patients were metastases of colorectal cancer in the liver. Out of 15 samples, we were able to establish only one primary cell line, CM1, which represents a 6% success rate.

The difficulty of growing and expanding such colorectal carcinoma tissues in vitro has previously been reported [216, 230]. The use of collagen-coated flasks is also recommended as it might be beneficial to facilitate the adherence of the cells to the dishes. We tried this method but did not obtain much success, and therefore kept our initial protocol, i.e. with uncoated dishes.

In all our cultures, the rapid expansion of fibroblasts constituted a limiting step. It was necessary to constantly control their growth, essentially by reducing the percentages of FBS from 10% (ideal for fibroblastic cultures) to 2% in the culture medium, or by proceeding to differential trypsinisation (cf. Materials and Methods). Fibroblasts are major constituents of the tumour stroma and play a very important role in cancer initiation and expansion. Malignant cells cannot expand without the help of the surrounding stroma as their growth also depends on angiogenesis, inflammatory cells and fibroblasts [231]. In healthy tissues, fibroblasts are responsible for the deposition of the extracellular matrix (ECM), they regulate inflammation and epithelial differentiation by secreting cytokines and growth factors, and are involved in wound healing [232]. Fibroblasts are recruited and activated by cancer cell secreted factors (transforming growth factor β (TGF β), platelet-derived growth factor (PDGF) and fibroblast growth factor-2 (FGF-2)) into an activated phenotype. Once activated, they are referred to as cancer associated fibroblasts (CAF) and are able to secrete indispensable cancer growth factors (TGFß and stromal derived factor-1 (SDF1)) and enzymes such as the matrix-metalloproteinases (MMP) to help cancer progression [233]. Some of the CAF might have a malignant origin as it has been shown in some cases that cancer cells have a potential to turn into fibroblasts, through a process referred to as epithelial-to-mesenchymal transition (EMT). Epithelial and cancer cells can acquire through EMT a mesenchymal phenotype, and this phenomenon seems to be involved in metastasis and in the
production of additional CAF [234]. We are not sure whether EMT is the reason why so many fibroblasts developed in our culture dishes as fibroblasts were present for a limited number of passages only. In the CM1 culture dishes, the fibroblasts started to disappear as the epithelial islets expanded. After P6, we no longer observed any fibroblasts in our culture flasks. Therefore it seems that the fibroblasts were not of tumourigenic origin, but died progressively as happens in the case of non-immortalized healthy tissue cultures. Whatever the origin of the fibroblasts in our cultures was, it is important to keep in mind that in vitro culture conditions determine the behaviour of the cultured cells.

For CM1 cell line, the epithelial cells began to increasingly expand amongst the fibroblasts and formed several growth islands after 2 passages. This clearly indicates that only a low number of cancer cells was able to survive and proliferate and provides evidence that only the most resistant clones are able to adapt to in vitro culture conditions. Additionally, the prolonged duration required by these cells to initiate their expansion may be due to adaptative epigenetic/genetic changes. These changes seem to have persisted and even amplified since the cell growth behaviour progressively improved over time.

Obtaining a cancer cell line from colorectal primary culture is a tedious and time consuming process, and therefore we did not establish any other short-term cell line after CM1. Rather, we decided to extract CSCs from CM1 cells. These cells are derived from a colorectal cancer metastasis in the liver that appeared after a FOLFOX chemotherapeutic regimen, and it would have been interesting to compare the presence of CSCs in the primary tumour to the corresponding metastasis. Indeed, the CSC theory predicts that CSCs should be present in metastases as they have the potential 1) to escape chemotherapy, 2) to migrate and initiate a tumour at distant sites and 3) to sustain tumour growth. Moreover, the presence of CSCs has been reported in colorectal cancer metastases [99, 100, 215]. Unfortunately, we were unable to obtain both primary and metastatic tumour specimens together.
In the following chapters, we have tested various methods to isolate CSCs from the CM1 cell line and from a long-term cancer cell line, CaCO-2, derived 40 years ago from a moderately differentiated primary colon adenocarcinoma [235]. The CaCO-2 cell line has been shown to differentiate spontaneously upon culture into mature enterocyte-like cells [236]. Cell lines are not the most appropriate model of cancer as they are not the exact representation of the in vivo tumour cell population. They only represent a late stage of cancer. They do not reproduce the whole tumour heterogeneity, and they undergo genetic/epigenetic changes depending on the in vitro culture conditions. That is why, in addition to CaCO-2, we used a cell line as close as possible to its corresponding original tumour, by limiting the number of passages (up to passage 15 maximum) and keeping the in vitro procedures as simple as possible.
5 Isolation and characterisation of putative CSCs from CaCO-2 and CM1 cell lines using a chemoresistance assay
5.1 Background

As mentioned in the introduction, the choice of an appropriate method is a critical point for isolating CSCs. The methodological and theoretical concerns raised by the isolation of stem cells using cell surface antigens have been discussed in the first chapter. To bypass the problem caused by CD133 targeting, we decided to tackle CSC isolation differently, independently of cell surface markers and on the basis of a putative functional characteristic of CSCs, in particular chemoresistance.

5.1.1 Cancer and chemoresistance

From a clinical perspective, understanding the basis of drug resistance is a principal goal. Besides surgical intervention, other regimens such as chemo- or radiotherapy have been developed to treat cancer. However, the efficacy of such methods is limited since cancer cells sometimes display or are able to acquire a resistant phenotype, usually following long term chemotherapy.

Molecular mechanisms of resistance have been at the centre of extensive work [237]. They reveal that cancer cells can undergo alterations in various signalling elements, culminating in the elevated expression or activity of ABC multidrug efflux pumps or DNA-repair enzymes [238, 239]. Genetic alterations can affect growth factor signalling elements such as EGF receptor (EGFR), hedgehog and Wnt/ß-catenin expression [240-242]. Resistance to drugs or radiation may also be the consequence of deregulation within the apoptotic cascade [243], in particular through the ceramide signalling pathway [244].

Additionally, when cancer cells are exposed to a single drug, they are prone into acquiring a multidrug resistance (MDR) phenotype. As its name implies, MDR confers resistance to other functionally and structurally unrelated anticancer drugs via the overexpression of genes of the ABC super family [245]. ABCB1 (Adenosine triphosphate-binding cassette B1, also called MDR1 and encoding for P-glycoprotein (P-gp)), ABCG2 (or breast cancer resistance protein1 (BCRP1) and ABCC1 (also
known as MDR associated protein 1 (Mrp1)) are the best defined representatives of this super family. These genes encode membrane bound ATP-transporters, which play a key role in the efflux of cytotoxic compounds from the cells. They are expressed in the placenta and other physiological barriers such as the intestinal barrier. For instance, on the apical membrane of enterocytes, the expression of P-gp and BCRP1 determines the absorption, distribution and excretion of drugs, xenobiotics and their metabolites, and prevents their entry into the circulation [246].

5.1.2 Innate versus acquired resistance

The distinction between innate and acquired resistance is an essential concept to understand the CSC model of chemoresistance. Essentially, cancer cells can be inherently (or intrinsically) resistant to a drug, or they can develop (acquire) resistance after exposure to the drug.

In the traditional model of cancer, resistance in tumour cells is thought to arise from the clonal selection of genetically transformed cells within a heterogeneous population of cells. Intrinsic resistance is therefore the result of random genetic alterations. Several cells are able to obtain a transformed growth advantage and maintain this even in the presence of chemotherapeutic compounds to ultimately repopulate the tumour mass with drug resistant cells.

If cancer cells are initially responsive to chemotherapy, they may acquire resistance through genetic mutations and clonal selection of the transformed cells, subsequently leading to an MDR phenotype.

Alternatively, in the CSC model, resistance is considered to be the monopoly of the small subset of CSCs. It is hypothesised that CSCs are intrinsically resistant as a result of their inherent stem cell characteristics. Since the CSC status is an epigenetic phenomenon, CSCs lose this resistant phenotype upon differentiation [85]. Therefore, the majority of non-CSC cancer cells would be more sensitive to chemotherapeutics. Intrinsic (or innate) resistance allows CSCs to circumvent
treatment, and subsequently, these CSCs repopulate the tumour since they also possess the tumourigenic ability, thus establishing a resistant lineage [247]. The CSC model does not exclude the possibility that, under treatment pressure, genetic alterations may occur and confer an MDR phenotype on cancer cells, but this is not the main reason why cancer can recur following therapy.

5.1.3 Factors responsible for CSC intrinsic resistance

It is necessary for stem cells to be preserved for the entire life of the organism as they ensure the long-term maintenance of tissues throughout life. To ensure their longevity, several mechanisms protect them from death and toxin-induced damage.

In stem cells, innate resistance is associated with the upregulation of the ABC transporters. ABCG2 expression, for instance, has been shown to be upregulated in HSC, whilst it is downregulated in progenitor and fully differentiated cells [248]. With regard to CSCs, many studies in blood and diverse solid tumours report that the SP, characterised by cells with high capacity for pumping out Hoechst dye through ABC transporters possess stem cell properties [103, 104, 120, 121]. In several solid tumours CSCs have also displayed a resistant phenotype with high expression of MDR genes (MDR1, Mrp1, BCRP1) [105, 249, 250] and apoptosis inhibition [251].

Stem cells and by extension CSCs have also been described as having slow cell cycle rates [252]. For instance, whilst the expression of cell cycle initiating factors such as cdk4 is barely detected in long term HSC, these factors are highly expressed in progenitor cells [252]. Similar to stem cells, quiescence may be a characteristic of CSCs that protects them from drugs inhibiting cell cycle-initiating factors. Although the quiescent nature of CSCs has been extensively cited in the literature as a possible feature, it has not yet been confirmed.
Several clues for CSC chemoresistance have been reported by *in vitro* studies showing that putative CSCs display a higher resistance to chemotherapeutic drugs compared with somatic cancer cells. Liu *et al.* (2006) demonstrated that glioma CD133+ cells were significantly more resistant to 4 chemotherapeutic reagents, possibly through an upregulation of anti-apoptotic proteins [251]. Similarly, CD133+ cells freshly isolated from GBM clinical specimens were also less sensitive to radiation-induced apoptosis than the rest of the population, and identical results were obtained in medulloblastoma cell lines [253]. Other cancer cells with CSC markers from pancreas, liver, breast and colorectal cancer might also display resistant properties against chemo/radiotherapeutic regimens [191, 254-257]. In colorectal cancer, higher resistance to oxaliplatin and 5-FU treatments was shown in putative CSCs such as CD133+ cells and spheres grown in serum-free medium [258].

An interesting *in vivo* study seems to confirm the chemoresistant nature of CSCs. Breast cancer cells isolated from chemotherapy-naïve patients were compared with cancer cells isolated from patients who received chemotherapy [190]. Cells were analysed for their potential to form mammospheres and to express the putative breast CSCs CD44+/CD24- phenotype. Results showed that a higher percentage of cells obtained from chemotherapy-patients were able to self-renew and to display the CSC phenotype. Thus in an *in vivo* setting, chemotherapy appears to be adding a selective pressure for the enrichment of tumour-initiating cells.

### 5.1.4 Isolation of CSCs using a chemotherapeutic assay

Developing an assay in which putative CSCs are isolated by virtue of their chemoresistant phenotype has been previously attempted. For instance, resistant cancer cells have been isolated *in vitro* from L3.6pl and AsPC-1 pancreatic cancer cell lines following long-term gemcitabine exposure. Interestingly, the long term resistant cells displayed more invasive and migratory properties than their non resistant counterpart and CSC markers CD44, CD24 and ESA were also expressed in higher proportion in these resistant cells (about 10-15% more) [204].
Other studies have isolated intrinsically resistant cancer cells after short but lethal exposure to a chemotherapeutic drug. GBM cells resistant to a lethal dose of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) showed cancer stem-like cell properties [203]. They were enriched in CD133^+ cells, were multipotent and able to generate neuron- and glial-like cells, and formed tumours when transplanted in brain of NOD/SCID mice.

Isolating resistant cells should therefore be a means of obtaining CSCs. Little work has been done yet to confirm this hypothesis. To our knowledge, only two studies on brain glioma (as mentioned above) and on leukaemia have explored intrinsically resistant cells [203, 205] and shown they possessed CSC-like properties.

To explore a method of CSC isolation independent from cell surface marker expression, we chose to isolate and characterise a population of cells intrinsically resistant to oxaliplatin, a chemotherapeutic drug commonly used to treat colorectal cancer. Oxaliplatin is a third generation platinum compound. This drug has shown efficacy in colorectal metastatic cancer as it potentiates the effects of 5-FU, and it is generally used in combination with 5-FU and folinic acid (FOLFOX) [259]. Mechanisms of action of the drug are still hypothetical, but it is known that like other platinum agents such as cisplatin, oxaliplatin triggers direct damage to DNA leading to cell death [260].
5.2 Isolation of oxaliplatin-resistant CaCO-2 cells

We first set out to establish the ideal dose of oxaliplatin that would select for a resistant population of CSCs. We found that the optimal condition for selection of the putative population of CSCs was at a concentration of 5µg/ml for 48h. We chose the shortest incubation time, as surviving cells should possess an intrinsic resistance to chemotherapy rather than acquiring a resistant phenotype upon long term exposure with the drug. The concentration of oxaliplatin used was chosen to eliminate about 90% of cells since we wanted to attain the reported percentage of CSCs (2-3% of the total cancer cell population). Our other criterion was that surviving cells should be able to regrow after drug exposure.

5.2.1 Establishment of an oxaliplatin dose

An initial experiment was set to establish the concentration of oxaliplatin necessary to have an effect on cell viability (Figure 13). In this experiment, cells were incubated with oxaliplatin for 24, 48, 72 or 144h. Although oxaliplatin half-life has been reported to be short (10-25 mn in human blood in vitro [261]), the effect of oxaliplatin increased with the length of incubation in our setting. There was more cell death with longer incubations. We found that the growth of cells was slower at low drug concentrations (0.2 to 1µg/ml) as compared to cells not treated with the drug (0µg/ml). Cell growth was inhibited at 2µg/ml, and the number of live cells was lower for doses higher than 2µg/ml, after 48h of incubation.
Based on this preliminary evaluation, the optimal dose was then defined more accurately (Figure 14). Cells were plated in Petri dishes and incubated with oxaliplatin for 48h at 3, 4 and 5µg/ml. The number of surviving cells was counted according to trypan blue exclusion. We counted the number of live cells over three weeks and, for each dose, cell kinetics followed a similar pattern. The number of cells decreased, to plateau at around day 12 after incubation, and cells started to re-grow after day 17. A dose of 5µg/ml was chosen as the percentage of surviving cells fell below the 10% mark after day 9, after which cells displayed resistance to treatment by recovering growth.

Oxaliplatin resistant CaCO-2 cells (CaCO-2-OR cells) were harvested at day 12 or 13 after incubation for further characterisation.
5.2.2 Characterisation of CaCO-2-OR cells

5.2.2.1 Morphology of the drug resistant cells

CaCO-2 cells are morphologically heterogeneous. As represented in Figure 15 (A), the majority of the cells have a similar diameter, whereas a certain percentage of cells have a much larger diameter.

Under the microscope, CaCO-2-OR cells appeared rounded and “spindle-shaped” as illustrated in Figure 15 (B).
We found that CaCO-2-OR had on average a larger diameter than untreated CaCO-2 cells.

We hypothesised that cells with a significantly larger diameter might be more resistant and we therefore established an indicative dose response curve to assess the

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**Figure 15.** Morphology of CaCO-2 and CaCO-2-OR cells. (A-B) : CaCO-2 cells. The arrow indicates cells with a larger diameter. (C-D) : CaCO-2-OR cancer cells, 12 days after exposure to the drug. (×20). Arrows indicate the spindle-shaped cells of CaCO-2-OR cells.
percentages of surviving cells with a large diameter in comparison to the rest of the cells (Figure 16).

Cells were seeded at a density of 270,000 cells/Petri dish. Oxaliplatin was then added at various concentrations (0-10µg/ml) and cells were incubated for 12 days.

At day 0, the percentage of cells with a large diameter was 5%.

At day 12, live cells were counted using a haemocytometer. Results are shown in Figure 10. The percentages of larger cells were established as follows (based on observation):

\[
\text{% of larger cells} = \frac{\text{Number of larger cells at day 12}}{\text{Number total of surviving cells at day 12}} \times 100
\]

Figure 16 clearly shows that the proportion of cells with a large diameter increases when cells are exposed to oxaliplatin, suggesting that they might be more resistant to oxaliplatin. Although this experiment was based only on visual assessment of the cell diameter, and was conducted only once, this phenomenon was observed for each dose of oxaliplatin tested.
5.2.2.2 Cell marker analysis

To assess an eventual enrichment of CSCs among CaCO-2-OR cells, we analysed by flow cytometry the expression of markers related to MDR (ABCG2, Mrp1), putative stem cell and CSC markers (CD44, CD24, Msi-1, Lgr-5) and genes that functionally contribute to a ‘stemness’ state by regulating self-renewal and pluripotency (Oct-4, Nanog).

CaCO-2-OR cells did not show upregulation in markers of drug resistance. On the contrary, expression of Mrp1 and ABCG2 was lower in resistant cells (p = 0.023 and p = 0.001, respectively). Expression of stem cell markers Msi-1 and CD24 was below 10% in both population and Lgr-5 was highly expressed but without any significant difference between the two populations. There was however a significant difference in CD44 expression between both populations. Whilst it reached a level of 46.8±14.5% in CaCO-2 cells, there was almost no expression of CD44 in CaCO-2-OR cells (p = 0.005).

CaCO-2-OR cells expressed significantly less ‘stemness’ genes Oct-4 and Nanog (p = 0.011 and p = 0.019, respectively) compared with the parent cell line. For all markers, results are displayed in Figure 17.
Figure 17. Flow cytometry analysis of CaCO-2-OR cells as compared to the untreated CaCO-2 cells. Markers analysed were (A) Abcg2 and Mrp1 (drug resistance related), (B) CD44, CD24, Lgr-5 and Msi-1 (stem cell markers) and (C) Nanog and Oct-4 (‘stemness’ state). * = p <0.05 ; ** = p <0.005 (t test).
5.2.2.3 Cell cycle analysis

Since stem cells are described as slowly cycling cells, the cycling status of CaCO-2 and CaCO-2-OR cells was assessed. Cells were incubated with PI, a reagent able to intercalate with DNA (Figure 18). Proportions of cells in the G0/G1 and S/G2/M phases are reported in Figure 19 for both populations. No significant difference in cell cycle distribution was noted between CaCO-2 and CaCO-2-OR cells.

Figure 18. Representative charts of PI integration for cell cycle analysis of CaCO-2 cells (A) and CaCO-2-OR (B) cells. Results show no significant difference between the two populations.

Figure 19. Distribution of CaCO-2 and CaCO-2-OR cells in the cell cycle. A) represents number of cells in G0/G1 phase, B) represents number of cells in S/G2/M phase. Number of quiescent cells was not significantly different in the two populations (n=6, Wilcoxon test).
5.2.2.4 Measurement of ALDH1 activity

In order to further characterise the CaCO-2-OR cell population, we measured its ALDH1 activity relative to non treated CaCO-2 cells. ALDH1 is responsible for the oxidation of intracellular aldehydes. Cells with high ALDH1 activity are believed to represent a CSC enriched population in blood and also solid tumours, particularly in the brain and colon [100, 171]. As described in Chapter 2, an Aldefluor® kit was used and flow cytometry analysis showed that 18.2±6.6% of CaCO-2 cells had a positive ALDH1 activity versus 7.7±2.7% cells in CaCO-2-OR group (p = 0.04) (Figure 20).

![Measurement of ALDH1 activity in CaCO-2 and CaCO-2-OR cells](image)

Figure 20. ALDH1 activity was measured using Aldefluor® kit and cells were analysed by flow cytometry. Fewer CaCO-2-OR cells than CaCO-2 cells were positive for ALDH1 activity.

* = p value<0.05, n= 5 (Wilcoxon test).
5.2.2.5 *In vivo experiment*

Since CSCs are believed to harbour an innate tumourigenic potential, which implies that injection of a small population of isolated CSCs in immunocompromised mice should develop tumour growth. CaCO-2 and CaCO-2-OR cells were injected subcutaneously in the right flank of NOD/SCID mice (Table 8). LS174T colorectal cancer cells (10^7 cells) were used as a positive control and formed a detectable tumour after 3 weeks. 10^7 CaCO-2 cells were also tumourigenic and the tumour was detectable 12 weeks after injection (Figure 21). 100 to 100 000 CaCO-2-OR cells did not trigger a tumour after 12 weeks and the animals were sacrificed.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Number of cells injected</th>
<th>Tumour incidence</th>
<th>Detection of tumour (weeks after inoculation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS174T</td>
<td>10 000 000</td>
<td>1/1</td>
<td>3 weeks</td>
</tr>
<tr>
<td>CaCO-2</td>
<td>10 000 000</td>
<td>1/1</td>
<td>12 weeks</td>
</tr>
<tr>
<td>CaCO-2-OR</td>
<td>100 000</td>
<td>0/2</td>
<td>-</td>
</tr>
<tr>
<td>CaCO-2-OR</td>
<td>10000</td>
<td>0/2</td>
<td>-</td>
</tr>
<tr>
<td>CaCO-2-OR</td>
<td>1000</td>
<td>0/2</td>
<td>-</td>
</tr>
<tr>
<td>CaCO-2-OR</td>
<td>500</td>
<td>0/2</td>
<td>-</td>
</tr>
<tr>
<td>CaCO-2-OR</td>
<td>100</td>
<td>0/2</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 8. Summary of the *in vivo* tumour development experiments.

**Figure 21.** *In vivo* tumourigenicity of CaCO-2 and CaCO-2-OR cells. 12 weeks after injection, 10^7 CaCO-2 cells were able to grow a tumour nodule in NOD/SCID mouse. A low number of CaCO-2-OR cells (up to 10^5) was not tumourigenic *in vivo.*
5.2.2.6 Markers of differentiation

According to the *in vivo* and *in vitro* analysis, CaCO-2-OR cells did not appear to exhibit CSC characteristics. It may be that, instead of oxaliplatin selecting a CSC population, drug treatment might rather be inducing a more differentiated phenotype in the majority of the resistant cells. This has recently been shown in HT-29 colorectal cancer cell line after exposure to oxaliplatin [262].

CaCO-2 cells naturally differentiate to an enterocytic phenotype upon confluence in culture. Morphologically, CaCO-2 cells develop microvilli on the apical side and tight junctions between adjacent cells. Functionally, an enterocytic differentiation is characterised by a higher activity of enzymes normally present on the brush border of the intestine including small hydrolase enzymes such as SI, lactase and aminopeptidase [263], with a peak of secretion 9 days after confluence [264]. In parallel, the activity of AP enzyme has been shown to be elevated upon differentiation and confluence in CaCO-2 cells [265]. AP activity reached a peak 18 days after confluence [264].

We chose two relevant markers of the enterocytic differentiation pathway: the intestinal AP and SI. We compared their expression in resistant CaCO-2-OR and non-resistant CaCO-2 cells (Figure 22).
On average, none of these enzymes were significantly upregulated in the resistant CaCO-2-OR population. AP expression was more elevated in the CaCO-2-OR cells (34.85±12.30% vs. 32.0±15.5% in the CaCO-2 cells), but with no significant difference. SI was expressed at 8.33±2.18% in CaCO-2 cells and 5.35±4.62% in CaCO-2-OR cells.

CaCO-2-OR cells did not exhibit a more differentiated phenotype than CaCO-2 cells after oxaliplatin exposure, although more differentiation markers should be analysed to confirm our result.
5.3 Isolation and characterisation of oxaliplatin-resistant CM1 cells

To validate the results obtained with the CaCO-2 cell line, we repeated the same isolation and characterisation of oxaliplatin resistance on CM1 cells to see whether cells cultivated at low passage numbers behaved in the same way as the established cell lines.

5.3.1 Choice of a therapeutic dose

Although we had previously established that 5µg/ml was the appropriate dose for CaCO-2 cells, we did not know whether the same applied to CM1 cells. We therefore performed a dose response assay to determine viability of CM1 cells to oxaliplatin (Figure 23).

CM1 cells responded similarly to CaCO-2 cells with 5µg/ml of oxaliplatin after 48h. However, CM1 cells appeared more resistant, since more than 20 days were necessary to reach a 10% viability of the original number of seeded cells, vs. 12 days for CaCO-2 cells.

The number of surviving oxaliplatin resistant CM1 (CM1-OR) cells slowly started to grow after day 30.

CM1-OR cells were therefore harvested at days 22 or 23 after incubation with oxaliplatin, and consecutively characterised.
Figure 23. Dose response of CM1 cells to oxaliplatin treatment. Cell viability reached a minimum 22 days following treatment but recovered thereafter. A dose of 5µg/ml of oxaliplatin was chosen, and cells were harvested at days 22-23. Percentages are relative to the number of cells present at day 0.
5.3.2 Analysis of CM1 oxaliplatin resistant cells

5.3.2.1 Morphologic observation

Similar to our observations with CaCO-2-OR cells, CM1-OR cells also exhibited cells with a spindle shape and a larger diameter. This was observed under the microscope (Figure 24).

![CM1 cancer cells (no treatment)](A-B) CM1 cells. (C-D). CM1-OR cancer cells, 22 days after exposure to the drug. (×20). The arrows indicate the spindle-shaped cells, similar to those observed in CaCO-2-OR cells. Like in CaCO-2 cell line, CM1-OR cells showed a larger diameter than CM1 untreated cells.

**Figure 24.** Morphology of CM1 and CM1-OR cells. (A-B) CM1 cells. (C-D). CM1-OR cells, 22 days after exposure to the drug. (×20). The arrows indicate the spindle-shaped cells, similar to those observed in CaCO-2-OR cells. Like in CaCO-2 cell line, CM1-OR cells showed a larger diameter than CM1 untreated cells.
5.3.2.2 Cell markers analysis

We performed FACS analysis with CM1 and CM1-OR cells to determine the expression of Oct-4, msi-1 and Lgr-5 stem cell markers, of the putative CSC markers CD44, CD166, CD24 and CD133, and of the marker of chemoresistance ABCG2 (Figure 25).

Figure 25. Flow cytometry analysis of CM1-OR cells as compared to the untreated CM1 cells. Markers analysed were (A) ABCG2 (drug resistance related), (B) CD44, CD133, CD166, CD24 and (C) Msi-1 (stem cell markers) and Oct-4 (stemness state). * = p <0.05 (t test). The error bars represent the standard deviations for n=3 independent experiments. For markers without error bars, n=2. None of the markers was upregulated in the resistant CM1-OR cells.
5.3.2.3 Cell cycle analysis

When analysing the cell cycle status of CM1 and CM1-OR cells, a similar distribution was observed between the two populations, with on average 85.4% and 83.8% of quiescent cells respectively (Figure 26). This experiment has been conducted twice only, and more repeats are needed to confirm our observations.

Figure 26. Distribution of CM1 and CM1-OR cells in the cell cycle. (A) represents number of cells in GO/G1 phase, (B) represents number of cells in S/G2/M phase. The proportion of quiescent cells was similar in the two populations (n=2).
5.4 Discussion

In our study, cancer cells that potentially possess an intrinsic resistance to chemotherapy were isolated and characterised for the presence of CSCs. We chose oxaliplatin as the treatment compound since it is commonly used in chemotherapeutic regimens to target colorectal cancer. CaCO-2 and CM1 cells were exposed to 5µg/ml oxaliplatin only for a short period of time (48h). Resistance to oxaliplatin has previously been reported, and in patients with metastatic colorectal cancer, the median time of progression to resistance is about 8 months [266]. To avoid the events leading to acquired resistance, we chose to apply a lethal dose of oxaliplatin for 48h. This allowed us to isolate cells with an intrinsic resistance. The dose of oxaliplatin at 5µg/ml eliminated more than 90% of the CaCO-2 and CM1 cells. We have shown that under the conditions we defined, resistant cells were later able to re-grow. This was an important parameter as our aim was to mimic the events in patients where recurrence occurs after apparent remission. Comparative characterisation of treated and untreated cells was performed with CaCO-2-OR cells harvested 12 to 13 days after exposure to the drug (Figure 14), and CM1-OR cells 22-23 days after exposure to the drug (Figure 23), as those were the points where cells with intrinsic resistance would be selected for. CM1 cells are derived from the tumour of a patient that followed a chemotherapy regimen including oxaliplatin, which might be an explanation as to why CM1 cells were more resistant to the drug than CaCO-2 cells.

*In vivo* characterisation of xenotransplanted CaCO-2-OR cells did not show potential for high tumour growth in animals. CaCO-2-OR population apparently did not contain an increased number of cancer-initiating cells since 12 weeks after injection in NOD/SCID mice, $10^7$ untreated CaCO-2 cells were able to form a tumour whilst a comparatively reduced number of CaCO-2-OR injected cells ($10^5$ to $10^6$) were not tumourigenic.

*In vitro* analysis of the resistant population confirmed the *in vivo* experiment, where there were no detectable signs of CSC characteristics.
We first looked at expression of the markers ABCG2 and Mrp1 as they are described as being associated with a resistant phenotype. These proteins are part of the super family of ABC transporters involved in the exclusion of drugs out of cells. ABCG2 is an important determinant in the SP phenotype of haematopoietic, muscle, neural and testicular stem cells [267] and has also been identified in populations of cancer-initiating cells [102]. Neither of these proteins were upregulated in the resistant population. This suggests that the intrinsic resistance of CaCO-2 cells to oxaliplatin is not linked to the expression/activity of these transporters. Indeed, resistance to oxaliplatin seems to be mainly associated with defects in apoptosis, impaired DNA adduct formation and increased expression of copper efflux transporters ATP7A and ATP7B [260, 268]. In contrast, expression of ABCG2 and Mrp1 was down-regulated in CaCO-2-OR cells according to the FACS analysis data. ABCG2 expression was also lower in CM1-OR cells when compared to CM1 cells. Another important transporter involved in MDR is the MDR1 (ABCB1) protein, and it would be interesting to analyse its expression in further studies, as well as ATP7A and ATP7B, as little is known yet on the role of these transporters in colorectal resistance to oxaliplatin [268].

We also studied the expression of markers previously used to isolate CSC populations from several types of cancer (breast, colorectum, prostate): CD44, Msi-1 and CD24, none of them was strongly expressed in CaCO-2-OR cells or CM1-OR cells. Unexpectedly, CD44 was significantly down-regulated in CaCO-2-OR cells and CM1-OR cells, as well as CD166 in CM1-OR cells. CD44 is known to associate with CD166 and has previously been used as a marker for colorectal CSCs [100].

Interestingly, Lgr-5, a marker of stem cells in small intestine and colon [66] was equally expressed in more than 50% of CaCO-2 cells and CaCO-2-OR cells. In normal intestine, this marker is expressed only in the basal columnar cells of the crypts. It has recently been shown that Lgr-5 positive cells in the intestine may be the cells from which colorectal tumours originate [269]. Although Lgr-5 was not overexpressed in the resistant population compared to the non treated cells, its upregulation in the CaCO-2 cell line may indicate the importance of this marker in the development of cancer.
Beside cell surface markers, we also investigated the cell cycle status of treated and untreated cells. We performed a test with PI integration, which can discriminate the number of cells in G0/G1 phases from the number of cells in the other phases S/G2/M. We did not find any difference in the cell cycle status of both CaCO-2/CM1 cells and their corresponding resistant cells. Although CSCs are often described as potentially quiescent [83, 106], i.e. maintained in G0 phase, the literature does not report any convincing evidence of this characteristic. In pancreas, for instance, CD44+/CD24+/ESA+ cells have been identified as the putative pancreatic CSCs population, but no difference in the PI incorporation was observed when compared with CD44-/CD24-/ESA- cells. Similarly, in breast cancer the CD44+/CD24-/ESA+ CSCs, and in nasopharyngeal cancer the CSC population defined as SP, were not enriched for cells at a particular stage of cell cycle when compared to the rest of the cancer population [92, 121]. The putative CSCs isolated thus far do not appear to exhibit the characteristics of quiescence.

In another functional test to investigate the potential CSC-like state of CaCO-2-OR cells, we wanted to see whether the enzyme ALDH1 was differentially regulated relative to the CaCO-2 untreated cells. We found high levels of ALDH1 activity in CaCO-2 cells, and negligible amount in treated CaCO-2-OR cells. Intrinsically resistant CaCO-2 and CM1 cells did not exhibit the expected CSC characteristics. This suggests that the attempt to isolate CSCs based on an ‘intrinsic’ resistant phenotype is not a sufficiently characterised attribute.

As an explanation of our results, we propose the following conclusions:

1) Several studies have reported that exposing cancer cells to chemotherapeutic agents could trigger numerous effects on them. This includes their transformation into a more aggressive and alternative phenotype, by rendering them either more invasive, or more differentiated.

It has been shown in colorectal cancer cells that treatment with oxaliplatin initiates a process similar to what usually happens in embryos to facilitate cell motility. This is referred to as epithelial-to-mesenchymal transition (EMT) [262]. As briefly mentioned in Chapter 4, EMT is a developmental process through which cells lose their epithelial characteristics such as adherence, and acquire mesenchymal properties in order to facilitate motility and invasion. EMT could therefore be involved in
metastasis [41, 42], although this theory is still largely controversial [270]. Both
KML24 and HT29 colorectal cancer cells that have acquired resistance to long term
oxaliplatin treatment begin to show transformation to mesenchymal-like features.
This includes phenotypic changes (intercellular separation, pseudopod formation and
loss of polarity) and functional changes (enhancement of migrating and invading
capabilities) [262]. These observations have also been reported following treatment
with 5-FU and methotrexate [271]. Further studies on our CaCO-2 and CM1
oxaliplatin resistant cells should take into account those observations, specifically as
we noted morphologic changes in the cells after incubation with oxaliplatin
(appearance of spindle-shaped cells).
Chemoresistance could be somehow linked to differentiation treatments involving
differentiating therapy. This therapy is based on the concept that cancer cells are
immature cells, unable to regulate their own proliferation. Such agents are able to
induce differentiation and therefore to resume the normal processes that have not
been engaged naturally. For instance, sodium butyrate, a differentiating agent, tested
on colorectal cancer cell line LS174T selects for cancer cells with enterocytic
differentiation [272]. Cancer cell growth is thus restrained, although these agents do
not induce cell death. The interesting point is that in some cases, differentiating
treatments also induce the expression of ABCB1. In the LS174T study, it was
demonstrated that butyrate generated an increase cell resistance towards the agents
cisplatin, 5-FU and methotrexate. Therefore, these studies could explain why the
resistant cells we have isolated seem to have been directed to a more differentiated
phenotype, rather than exhibit the CSC characteristics.
In our case, the two markers of differentiation we studied, AP and SI were not
upregulated in CaCO-2-OR cells. Further experiments to analyse a potential
differentiation process should be led, with for instance the assessment of the AP
activity rather than its expression, and the analysis of other markers of enterocytic
differentiation.

2) The model of intrinsic resistance of CSCs could be more complex than previously
expected. Firstly, the current literature does not yet include conclusive evidence that
CSCs possess an innate chemoresistance. If in brain tumour cells, expression of
CD133 seems more and more convincingly linked to a more resistant phenotype (be it
chemo- or radioresistance), in some other organs the differences in drug sensitivity
might be significant but not always marked enough to explain the escape of CSCs from chemotherapeutic treatments [258]. It is also important to consider carefully the results regarding chemoresistance. For instance, in a study on lung cancer, it was demonstrated that putative CSCs (lung cancer spheres) were resistant to drug concentrations conventionally used in chemotherapy. However, no comparison has been done with the rest of the non-CSC population [140]; therefore there is no evidence showing that chemoresistance is higher in the putative CSCs than in the rest of the population. Further work is needed to prove the innate chemoresistance of CSCs.

The CSC model of chemoresistance does not explain how sometimes chemotherapy in patients has no effect on somatic cancer cells or CSCs [247]. In those cases, all the cells in the tumour bulk possess the same degree of innate resistance, which contradicts the notion that CSCs are the only resistant cells.

It is important to add that intrinsic resistance of CSCs might not be the only explanation for the presence of cells able to survive drugs and able to repopulate the tumour. Cells might be less responsive for other reasons than a hierarchical organisation. Genetically determined differences or a protective environment could be some of these reasons. For instance, we observed that resistant cells looked morphologically larger in both cell lines. Our observations were preliminary and deserve further attention in order to prove whether the resistant cells are significantly larger, and to study whether, in that case, larger cells are simply more resistant due to their size or whether this phenotype is induced following exposure to the drug. In a non-CSC model of chemoresistance, in which all cancer cells have the same probability to be tumourigenic, it is conceivable that any cell, having survived chemotherapy for reasons independent of intrinsic resistance, might be responsible for recurrence without any interference of so-called CSCs. The traditional model of cancer predicts the existence of genetic and epigenetic changes that could be sufficient to account for the unequal degrees of chemoresistance among cancer cells [72].

3) Our model to isolate innate chemoresistant cells may not be appropriate. In the hierarchical model of cancer, CSCs are predicted to be intrinsically resistant. For this reason, we chose to analyse the cells surviving a short dose of oxaliplatin, assuming that they may contain intrinsically resistant cells. This strategy has proven efficient to
isolate cells with CSC characteristics in brain tumour and leukemic cells [203, 205]. It is conceivable that, in our setting, the resistant populations we isolated did contain the target CSCs, however their number was too low for detection. ABCG2 and Mrp1 were less expressed in the CaCO-2-OR and CM1-OR populations than in their respective counterpart. These drug resistant genes may be downregulated following an overexpression at the time we added the drug. This may also be an indication that the majority of the isolated cells have not survived due to an intrinsically resistant phenotype, as mentioned above. Comparative studies on the resistance capacity of CaCO-2 and CaCO-2-OR cells (and CM1/C1-OR cells) following isolation will be a means to ascertain the resistance degree in both populations and the validity of our initial hypothesis. Besides ABCG2 and Mrp1, CD44 and ALDH1 were also strongly downregulated in CaCO-2-OR and CM1-OR cells. Cross-experiments involving the isolation of cells with the phenotype expressed by CaCO-2-OR cells (ABCG2\textsuperscript{lo}, Mrp1\textsuperscript{lo}, CD44\textsuperscript{lo} or/and ALDH1\textsuperscript{lo}) and CaCO-2 cells (ABCG2\textsuperscript{hi}, Mrp1\textsuperscript{hi}, CD44\textsuperscript{hi} or/and ALDH1\textsuperscript{hi}) and the comparison of their resistance capacity will allow us to test our initial hypothesis and to determine whether cells surviving a short dose of oxaliplatin truly are resistant.

The idea of culturing cells over a long period of time in the presence of oxaliplatin maybe another strategy to select for resistant clones and obtain a good number of resistant cells. A recent paper on colorectal cancer cells has studied the effects of long term exposure to the drug [273]. They used the human colorectal HT29 cancer cell line, and exposed it to increased concentrations of oxaliplatin, starting from 0.5\textmu mol/L up to the clinically relevant plasma concentrations (2\textmu mol/L). Their results are very interesting since they show that their resistant cells are 22 times more enriched in CD44\textsuperscript{+}/CD133\textsuperscript{+} compared to the original cell line. The potential of those resistant cells to form colonies in anchorage-dependant conditions and to form spheres was also enhanced. Oxaliplatin resistant cells were as expected more resistant to oxaliplatin but were additionally cross-resistant to 5-FU, compared to the non resistant cells. Therefore, studying these long term rather than intrinsically resistant cells may be more promising to isolate cells with CSC-like attributes.
6 Isolation and characterisation of putative CSCs according to their ALDH1 activity
6.1 Background

Since the full compendium of CSC specific surface markers has yet to be fully characterised, their use as a target for isolating specific subtypes of CSCs is still open to criticism. Whilst numerous groups are still involved in this area of research, others are attempting to characterise CSCs independently of stem cell surface markers. The use of ALDH is one such example.

ALDH and ‘stemness’ state

ALDH enzymes are part of the ALDH superfamily; in the human genome, 19 genes and 3 pseudogenes have been described [274, 275]. In mammals, they are divided in 3 sub-classes. Enzymes of class 1 and 3 are cytosolic, whereas class 2 is mitochondrial. In all cases, their main activity is the oxidation of various substrates, be they endogenous (substances derived from endogenous metabolism, essentially aliphatic and aromatic aldehydes) or exogenous (related to the xenobiotic metabolism) [276].

Among all the isotypes of ALDH, ALDH1 seems to be predominantly involved in stem cell biology as it is expressed at elevated levels in haematopoietic, neuronal and breast stem cells, and also in intestinal crypts [170, 171, 277]. Additionally, ALDH1 is mainly responsible for the oxidation of cytosolic aldehydes into carboxylic acids [278]. Aldehydes have important roles in eye development where they are linked in retinoid metabolism where ALDH1 biosynthesizes retinol (vitamin A) into retinoic acid [167, 278]. The retinoids influence the balance of self-renewal/differentiation either way depending on the stage of cell differentiation. In murine HSC, they are responsible for the terminal differentiation of late progenitors whereas they maintain the self-renewing function of the early precursor cells [168, 169]. Therefore, ALDH1 is involved in SC self-renewal through regulation of retinoid metabolism.

Aldehydes can also mediate deleterious effects on cells (essentially cytotoxicity and mutagenicity). The oxidation of these aldehydes is therefore a strategy to preserve the
cell integrity. Hence, elevated levels of ALDH1 in stem cells reflect its function in mediating the long term survival of stem cells and CSCs.

**ALDH and tumourigenesis**

It is noteworthy that ALDH also plays a role in carcinogenesis. It has been observed that the ALDH activity changes during tumourigenesis, in rodents and human colon and liver primary neoplasms, leukemic cells, and in human mammary adenocarcinoma cell lines [71]. The activity of classes 1 and 3 ALDH is enhanced, where this increase has been related to resistance against the anti-cancer agents oxazaphosphorines (cyclophosphamide (CPA) is the best known [279, 280]). Sladek et al. (2002) demonstrated that exposing primary and metastatic breast cancer cells to CPA increases ALDH1 activity [257]. Additionally, ALDH3A1 modulates the response of cells under oxidative stress, and is involved in mediating resistance to other chemotherapeutic drugs including etoposide [281].

In vitro studies revealed that inhibiting ALDH in a panel of cancer cells induced apoptosis, reduced tumour growth and invasiveness [282].

The biological properties of ALDH enzymes therefore make them an important factor that maintains resistance of cancer cells (and possibly CSCs) to chemotherapy by preventing apoptosis and oxidative stress, thus facilitating tumour growth and development.

**ALDH1 and CSCs**

To isolate a population of cells with high ALDH1 activity, Storms et al. (1999) developed an Aldefluor® kit in which ALDH1 activity can be quantified through the degradation of a fluorescent substrate in murine and human species [206]. ALDH1 is the main enzyme detected by this kit, and human HSC enriched for CD34+/CD38- could thus be isolated by FACS sorting. The emergence of this kit permitted the expansion of ALDH1<sup>hi</sup> cell detection in various malignant and non malignant tissues.
Among solid tumours, breast tumour ALDH1\textsuperscript{hi} positive cells are the first to have been described as a functional marker of cancer stem and progenitor cells [277, 283]. At present, ALDH1 shows increasing potential as a CSC marker since various studies have also isolated putative CSCs from ALDH1\textsuperscript{hi} populations in leukaemia [173], colorectal [100], liver [175], pancreatic and lung cancers [176].

In colorectal cancer, the role of ALDH1 in the CSC population is still being studied. Preliminary data showed that putative colorectal CSCs ESA\textsuperscript{+}/CD44\textsuperscript{+} cells were characterised by a higher activity of ALDH1, but in their hands, ESA\textsuperscript{+}/CD44\textsuperscript{+}/ALDH1\textsuperscript{hi} cells were not significantly more tumourigenic than ESA\textsuperscript{+}/CD44\textsuperscript{+}/ALDH1\textsuperscript{lo} cells [100].

In another study, Dylla \textit{et al.} (2008) confirmed an increase of ALDH1 activity in ESA\textsuperscript{+}/CD44\textsuperscript{+} cells, and showed a possible role of ALDH1 in tumourigenicity in combination with CD44 marker. Indeed, CD44\textsuperscript{+}/ALDH1\textsuperscript{hi} cells were tumourigenic whereas CD44\textsuperscript{+}/ALDH1\textsuperscript{lo} cells and CD44\textsuperscript{+}/ALDH1\textsuperscript{+} cells were not [131]. The combined role of ALDH1 and CD44 in tumourigenicity was recently confirmed by Chu \textit{et al.} (2009) who reported that, for two patients, xenografted CD44\textsuperscript{+}/ALDH1\textsuperscript{hi} tumour cells produced a 10-fold increase of the tumourigenic potential of CD44\textsuperscript{+} cells [132].

Recently, ALDH1 was shown to be expressed specifically in the intestinal crypts of malignant and non malignant colonic epithelia [284]. Using ALDH1 as a single marker for CSC isolation, they confirmed the exclusive tumourigenicity of ALDH1\textsuperscript{hi} cells and their functional capacity for self-renewal compared to ALDH1\textsuperscript{lo} cells. CD44\textsuperscript{+}/ALDH1\textsuperscript{hi} cells were not consistently more tumourigenic than ALDH1\textsuperscript{hi} cells, indicating that ALDH1\textsuperscript{hi} could be used as a single marker.

The use of ALDH1 as a CSC marker therefore needs to be further elucidated in colorectal cancer. Hence, the aim of this study was to characterise putative colorectal CSCs via ALDH1 staining of a colorectal cancer cell line (CaCO-2) and primary cell culture of colorectal metastasis in the liver (CM1).
6.2 Isolation and characterisation of CaCO-2 and CM1 ALDH1\textsuperscript{hi} cells

6.2.1 ALDH1\textsuperscript{hi} cells labelling and FACS sorting

6.2.1.1 Percentages of ALDH1\textsuperscript{hi} cells in both cell lines

CaCO-2 and CM1 cell populations were labelled with the Aldefluor® kit and sorted using FACS. Doublets of cells were excluded.

Results showed that compared to controls, on average 15\% of CaCO-2 cells were ALDH1\textsuperscript{hi}, whereas 26\% showed ALDH1\textsuperscript{hi} in the CM1 cell population (Figure 27).

6.2.1.2 Viability of CaCO-2 cells and CM1 cells after FACS sorting

Cell viability was evaluated by trypan blue exclusion after ALDH1 sorting. In the first trials, the viability of ALDH1\textsuperscript{hi} cells was generally higher than that of ALDH1\textsuperscript{lo} cells, specifically in the CM1 primary cell line where the ALDH1\textsuperscript{hi} cells (70±12\% viability) were more viable than the ALDH1\textsuperscript{lo} cell population (51±8\% viability). However, we could later increase the viability with more than 90\% for CaCO-2 and 80\% for CM1 each time in each fraction, by making sure the cells were in optimal conditions through the addition of fresh medium 24\text{h} prior to harvesting and by supplementing the buffer with 1\% BSA during the sorting process.
Figure 27. Flow cytometric isolation of putative colon CSC on the basis of ALDH1 activity. 
CaCO-2 (A, B) and CM1 (C, D) cells were labelled with the Aldefluor reagent in the presence (A, C) or absence (B, D) of DEAB, an ALDH1 activity inhibitor. ALDH1<sup>lo</sup> cells were gated in P3, and ALDH1<sup>hi</sup> cells in P4 with an indication of their respective percentages. On average, ALDH1<sup>hi</sup> cells represented 15% of the CaCO-2 cells and 26% of the CM1 cells.
6.2.1.3 Assessment of the purity of ALDH$^{hi}$ and $^{lo}$ fractions obtained after FACS sorting

Cells that were sorted and collected were gated so as to avoid any contamination between the negative and positive fractions.

The purity of each fraction was checked after FACS sorting. Purity was usually good, considering that no method achieves perfect separation, as illustrated in Figure 28.

If negative fractions were ≥98 % pure, the positive fractions for both CM1 and CaCO-2 were less pure (around 90 and 84%, respectively), which, besides the sorting which is never 100% efficient, might certainly be due to the dye leaking out of the cells. Indeed, we observed a slight decrease of the dye intensity over the time, sometimes even during the sorting process.

ABC transporters are responsible for the extrusion of fluorescent substrates out of cells, and this process can be extremely rapid if they are not inhibited as indicated in the manufacturer’s instructions from the Aldefluor® kit. The buffer in which cells are incubated contains the inhibitors specific to the human haematopoietic cells for which it has been designed, but these inhibitors may not necessarily prevent active efflux in other cell types.

In order to prevent efflux, the manufacturer recommends storing cells in the buffer and on ice after incubation (to prevent the transporters enzymatic activity), and proceeding to FACS sorting shortly after incubation. Although we always proceeded to FACS sorting immediately the incubation, the cells were not kept at 4°C during and after the procedure. This may account to why some leakage must have occurred.
Figure 28. Purity sort check of the ALDH1 high and low fractions after FACS sorting. (A, B) = CaCO-2 cells, ALDH1\textsuperscript{hi} and ALDH1\textsuperscript{lo} cells respectively. (C, D) = CM1 cells, ALDH1\textsuperscript{hi} and ALDH1\textsuperscript{lo} cells respectively. ALDH1\textsuperscript{lo} cells were gated in P3, and ALDH1\textsuperscript{hi} cells in P4, with an indication on their respective percentage. In CaCO-2 and CM1 cells, the ALDH\textsuperscript{hi} cells did not show 100% purity (84.5 and 91%, respectively), which might be due to the dye leaking out of the cells.
6.2.2 Decrease of Aldefluor® intensity over the time

As mentioned above, Aldefluor® fluorescent substrates leaks out of the cells rapidly if cells are not kept on ice in the buffer containing inhibitors of the ABC transporters. As we wanted to use the cells derived from FACS sorting for other staining experiments involving fluorescence, we thought it necessary to check first whether the dye was fading rapidly enough so as not to interfere with other dyes. Cells were cultured in fresh medium at 37°C and the fluorescence measured 3h and 24h after sorting. Results are shown in Figure 29.

<table>
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<tr>
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<th>0 H</th>
<th>3 H</th>
<th>24 H</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CaCO-2</strong></td>
<td>100 %</td>
<td>32.6 ± 9.9%</td>
<td>2.8 ± 2.4%</td>
</tr>
<tr>
<td><strong>CM1</strong></td>
<td>100 %</td>
<td>25.1 ± 4.4%</td>
<td>0.5 ± 0.4%</td>
</tr>
</tbody>
</table>

**Figure 29.** Measurement of Aldefluor® fluorescence in ALDH1hi cells 3 and 24h after FACS sorting. Data are summarized in (A). Representatives charts (B) indicate clearly the rapid drop of fluorescence of the Aldefluor® dye. The values at t=0h were considered to be 100%, although we did not check the exact value each time. n=3.
After 24h, the fluorescence of ALDH1\textsuperscript{hi} cells for both cell lines were almost 0 % (2.8 % for CaCO-2 ALDH1\textsuperscript{hi} cells, and 0.5 % for CM1 ALDH1\textsuperscript{hi} cells). Prior to fluorescent labelling for immunofluorescence of ALDH1\textsuperscript{hi} and \textsuperscript{lo} populations, we therefore cultured the cells for 24h, so as to avoid interference with the dyes.

We also did not want to leave the cells any longer in culture in order to preserve as much as possible the putative ‘stemness’ characteristics of the ALDH1\textsuperscript{hi} fractions. There is no well-defined cut-off point for this state of ‘stemness’. However, since differentiation is a process coupled to proliferation, it was preferable to analyse cells shortly after their isolation, i.e. before any potential CSCs have had time to commit to a differentiation pathway, thus losing their CSC characteristics.
6.3 Characterisation of Aldefluor® positive cells

6.3.1 Cell marker analysis

ALDH1\textsuperscript{hi} and ALDH1\textsuperscript{lo} cells isolated from CaCO-2 and CM1 cell lines were analysed for the expression of putative CSC markers by immunocytochemistry: cell surface markers conventionally chosen to proceed to CSC extraction, CD44, CD133, CD24 and ABCG2, and also markers highly expressed in embryonic stem cells such as Oct-4, and specific markers of colonic stem cells lgr-5 and msi-1 were investigated. Due to a very low number of cells resulting from the FACS sorting, we chose immunocytochemistry as the optimal method to proceed with this study.

CaCO-2

In the CaCO-2 cell line, the expression of two markers was significantly different in the ALDH1\textsuperscript{hi} cell population when compared with the ALDH1\textsuperscript{lo} cell population, CD44 and Lgr-5. Lgr-5 was expressed by 46±8\% of the ALDH1\textsuperscript{hi} cells which is 2.5 times more than the 19±10\% expression by the ALDH1\textsuperscript{lo} cells (p<0.05). CD44 marker expression was almost 3 times more in the ALDH1\textsuperscript{hi} cells in comparison with the ALDH1\textsuperscript{lo} cell population (59±19\% and 20±4\%, respectively, n=3, p<0.05). All other putative CSC markers tested showed no statistically significant differences between ALDH1\textsuperscript{hi} and ALDH1\textsuperscript{lo} cells from the CaCO-2 cell line. These results are displayed in Figures 30, 31 and 31bis.
Figure 30. Mean expression of the putative CSC markers tested in ALDH1\textsuperscript{hi} and ALDH1\textsuperscript{lo} cell fractions of CaCO-2 cell line (A) and corresponding representative chart (B). Immediately after FACS sorting, cells were plated onto 96-well plates and immuno-stained for the different markers. Fluorescent cells were detected by fluorescent microscopy, and the percentages represent the number of fluorescent cells out of 250 counted cells. n=3. * = p <0.05 (t test). CD44 and Lgr-5 markers were significantly more expressed in ALDH1\textsuperscript{hi} cells than in ALDH1\textsuperscript{lo} cells.
Figure 31. Immunohistochemistry analysis for ABCG2, CD133, CD166, CD24 and CD44 CSC markers expression. Immediately after FACS sorting, ALDH1$^{hi}$ and $^{lo}$ CaCO-2 cells were plated onto 96-well plates for 24h, fixed in 4% PFA, and incubated with the primary and secondary antibodies when required. Nuclei were stained with DAPI. The fluorescent cells were detected under fluorescent microscopy. The experiment was repeated 3 times and 250 cells per field were counted. CD44 marker was significantly more expressed in ALDH1$^{hi}$ cells, whereas there was no difference in the expression of the other markers.
Figure 31bis. Immunohistochemistry analysis for Lgr-5, Msi-1 and Oct-4 CSC/stem cell marker expression. Immediately after FACS sorting, ALDH1\textsuperscript{hi} and \textsuperscript{lo} CaCO-2 cells were plated onto 96-well plates for 24h, fixed in 4\% PFA, and incubated with primary and secondary (when required) antibodies. Nuclei were stained with DAPI. The fluorescent cells were detected under fluorescent microscopy. The experiment was repeated 3× and 250 cells per field were counted. Lgr-5 marker was significantly upregulated in ALDH1\textsuperscript{hi} cells, whereas there was no difference in the expression of the other markers.
CM1

CM1 ALDH₁⁺hi cells showed a significant difference in the expression of three markers when comparing ALDH₁⁺hi and ALDH₁⁺lo CM1 cells (See Figures 32, 33 and 33bis). A significant difference was observed for the CD44 marker which showed almost twice the expression in ALDH₁⁺hi cells when compared to ALDH₁⁺lo cells (78±17% and 40±9%, respectively, n=3, p<0.05). ALDH₁⁺hi CM1 cells also expressed more CD166 and ABCG2 markers than ALDH₁⁺lo CM1 cells with a 50% increase (CD166=88±8% and 59±1%, ABCG2 =74±6% and 48±3%, respectively, n=3, p<0.005 for both). All other putative CSC markers tested demonstrated no statistically significant differences between the ALDH₁⁺hi and ALDH₁⁺lo cell populations in the CM1 primary cell line.

The combination CD44/CD166 has been reported to be expressed by colorectal CSCs [100]. In the samples that contained a higher number of CM1 ALDH₁⁺hi cells, we analysed the combined expression of CD44 and CD166 by FACS analysis in ALDH₁⁺hi cells (Figure 33ter). Out of 3 independent experiments, an average of 35.6±14.8% ALDH₁⁺hi cells was CD44⁺/CD166⁺. Nearly all CD44 positive cells (89.7±7.5%, n=3) were also positive for CD166 marker.
A

<table>
<thead>
<tr>
<th>CSC Marker Expression</th>
<th>ALDH1&lt;sup&gt;lo&lt;/sup&gt;</th>
<th>ALDH1&lt;sup&gt;hi&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Mean percentage positivity ± 1 standard deviation)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD24</td>
<td>25±10%</td>
<td>25±11%</td>
</tr>
<tr>
<td>CD44</td>
<td>40±9%</td>
<td>78±17%</td>
</tr>
<tr>
<td>CD133</td>
<td>45±15%</td>
<td>61±17%</td>
</tr>
<tr>
<td>CD166</td>
<td>59±1%</td>
<td>88±8%</td>
</tr>
<tr>
<td>ABCG2</td>
<td>48±3%</td>
<td>74±6%</td>
</tr>
<tr>
<td>Oct-4</td>
<td>16±9%</td>
<td>22±13%</td>
</tr>
<tr>
<td>Msi-1</td>
<td>36±16%</td>
<td>42±30%</td>
</tr>
<tr>
<td>Lgr-5</td>
<td>34±11%</td>
<td>48±17%</td>
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B

**Figure 32.** Mean expression of the putative CSC markers tested in ALDH1<sup>hi</sup> and ALDH1<sup>lo</sup> cell fractions of CM1 cell line (A) and corresponding representative chart (B). Immediately after FACS sorting, cells were plated onto 96-well plates and immuno-stained for the different markers. Fluorescent cells were detected by fluorescent microscopy, and the percentages represent the number of fluorescent cells out of 250 counted cells. n=3. * = p <0.05 (t test). CD44, CD166 and ABCG2 markers were significantly more expressed in ALDH1<sup>hi</sup> cells than in ALDH1<sup>lo</sup> cells.
Figure 33. Immunohistochemistry analysis for ABCG2, CD133, CD166, CD24 and CD44 CSC markers expression. Immediately after FACS sorting, ALDH1$^{hi}$ and $^{lo}$ CM1 cells were plated onto 96-well plates for 24h, fixed in 4% PFA, and incubated with the primary and secondary antibodies when required. Nuclei were stained with DAPI. The fluorescent cells were detected under fluorescent microscopy. The experiment was repeated 3× and 250 cells per field were counted. CD44, CD166 and ABCG2 markers were significantly more expressed in ALDH1$^{hi}$ cells, whereas there was no difference in the expression of the other markers.
Figure 33bis. Immunohistochemistry analysis for Lgr-5, Msi-1 and Oct-4 CSC/stem cell markers expression. Immediately after FACS sorting, ALDH1\textsuperscript{hi} and \textsuperscript{lo} CM1 cells were plated onto 96-well plates for 24h, fixed in 4% PFA, and incubated with primary and secondary antibodies when required. Nuclei were stained with DAPI. The fluorescent cells were detected under fluorescent microscopy. The experiment was repeated 3 times and 250 cells per field were counted. None of these markers was significantly more expressed in ALDH1\textsuperscript{hi} cells.

Figure 33ter. FACS analysis evaluating the co-expression of CD166 and CD44 CSC markers in ALDH1\textsuperscript{hi} CM1 cells. Cells were incubated with directly conjugated antibodies. The fluorescent cells were detected by flow cytometry. Out of three independent experiments, 65.7±22.7% ALDH1\textsuperscript{hi} CM1 cells were CD166\textsuperscript{+}, 38.38±13.7% were CD44\textsuperscript{+} and 35.6±14.8% co-expressed CD166 and CD44.
In summary, immunofluorescence analysis revealed that a high ALDH1 activity was related to a significant increase in several CSC markers in both cell lines. Interestingly, CD44, which has been used in several studies to isolate colorectal CSCs was over-expressed in both CaCO-2 and CM1 ALDH1<sup>hi</sup> cells. CD166, which was overexpressed in CM1 ALDH1<sup>hi</sup> cells was co-expressed with CD44. As the literature reports that CD44<sup>+</sup>/CD166<sup>+</sup> phenotype is expressed by cells with CSC characteristics [100], the overexpression of both these markers suggest an enrichment of CSCs in the ALDH1<sup>hi</sup> cell population. The Lgr-5 colorectal stem cell marker has recently been defined as a stem cell marker in the intestine, and is under investigation as a possible CSC marker. Interestingly, it was highly expressed in CaCO-2 ALDH1<sup>hi</sup> cells. In CM1 ALDH1<sup>hi</sup> cell populations, the levels of ABCG2 were higher than in the negative population. The fact that ALDH1<sup>hi</sup> cells express more CSC/SC markers suggested that isolating cancer cells with a high ALDH1 activity might select cells with a CSC phenotype. Further <i>in vitro</i> experiments were needed to observe whether ALDH1<sup>hi</sup> cells possessed other CSC characteristics.

6.3.2 Colony forming assay

CSCs essentially are cancer cells possessing stem cell characteristics. As mentioned in the introduction, a hallmark of stem cells is their capacity to self-renew and to differentiate. They have the capacity to regenerate themselves and to give rise to a fully differentiated progeny. Two assays are commonly used to explore this capacity. The most popular is the <i>in vivo</i> xenograft assay, where cancer cells are injected into mice. If these cells are able to reproduce a whole tumour containing cells hierarchically organised in the same manner as in the original tumour, then there is evidence for their self-renewing potential. There exists another assay designed for <i>in vitro</i> use, also called the colony forming assay. If one cell is able to self-renew and to differentiate, then it should be able to regenerate the whole cell line. The clonogenic cells are defined as those cells capable of forming a large family of descendants in an artificial <i>in vitro</i> environment.

To achieve this experiment and compare the colony forming efficiency of ALDH1<sup>hi</sup> to ALDH1<sup>lo</sup> cells, we plated one cell per well in 96-well plate (cf. Materials and Methods) and tracked its regenerative potential.
CaCO-2

The difficulty of this assay is based on the fact that it is rather subjective to determine what a mature colony with extensive growth capacity is. We proceeded with preliminary trials, and observed after 6 weeks 4 types of development:

1. the cell in the well had died without any division,
2. the cell had divided only a few times, but all died thereafter,
3. the cell had divided but was not able to repopulate the whole well, so it seemed that its capacity for repopulation was restrained, or
4. the well was confluent or nearly confluent.

We therefore decided to set up a cut-off point at week 6 after plating, with a well at 75% confluency. In these cases, we assumed that the original cell was able to regenerate the whole cell population. Results showed that no significant difference in colony forming potential existed between total population, ALDH1\textsuperscript{hi} and ALDH1\textsuperscript{lo} cells (4±2.82%, 6.6±1.52% and 8.6±3.78, respectively) (Figure 34).

Figure 34. CaCO-2 ALDH1\textsuperscript{hi} and \textsuperscript{lo} cells were plated in 96-well plate, at a rate of one cell per well. Each well was visualised and validated when it contained one cell only. Six weeks later, wells with 75% confluency were scored. The experiment was repeated three times, and percentages established out of 75-100 cells per category for each experiment. There was no difference in the colony-forming potential of ALDH1\textsuperscript{hi} and \textsuperscript{lo} CaCO-2 cells.
The same experiment was repeated with CM1 cells after FACS sorting. However, these cells did not grow in the same way as CaCO-2 cells as they grew in multilayers rather than spread as a monolayer, thus forming a piling-up structure. In preliminary trials we observed that some CM1 single cells lost viability without dividing or after a few divisions. Some of them formed small colonies, or large colonies by piling-up as multi-layers. We scored the colonies displaying the piling-up structure 8 weeks after plating (see Figure 35).

**Figure 35.** Morphology of CM1 cells colonies in 96-well plates, after 8 weeks in culture. (A) A single cell divided several times before dying. (B) The single cell divided and formed a colony but with a limited size and stopped growing. (C) Mature colony with obvious piling-up structure. (D) Detail of the piling-up structure. Mature colonies only were scored.
Similarly to CaCO-2 cells, the percentages obtained in Figure 36 do not reveal any significant difference in the colony forming potential of our ALDH1\textsuperscript{hi} and \textsuperscript{lo} CM1 cells (4.4±3.2% and 2.1±0.7% respectively, no significance). The total population reached 13.2±2.7%, which was significantly more than the negative ALDH1 population (p=0.01), but not the ALDH1\textsuperscript{hi} cells.

ALDH1\textsuperscript{hi} cells in this setting did not reflect a higher colony forming potential.

**Figure 36.** CM1 ALDH1\textsuperscript{hi} and \textsuperscript{lo} cells were plated in 96-well plates, at a rate of one cell per well. Each well was visualised and validated when it contained one cell only. 8 weeks later, one-cell wells with confluent colony (i.e. starting to pile up) were scored. The experiment was repeated 3 times, and percentages established out of 75-100 cells per category for each experiment. Like in the CaCO-2 cell line, ALDH1\textsuperscript{hi} cells were not enriched in colony forming cells.
6.3.3 Cell cycle analysis

A resting phenotype has been attributed to stem cells, and possibly to CSCs. ALDH\(^{hi}\) and \(^{lo}\) populations were stained with PI, and the DNA content of cells measured by FACS analysis.

Results in Figure 37 indicate that a higher number of CM1 cells is quiescent (i.e. in G0/G1 phase) than in CaCO-2 cells (74.3±4.3% and 80.4±6.4% for ALDH\(^{hi}\) and \(^{lo}\) CM1 cells, respectively, vs. 56.9±6.7% and 66.4±7.9% in ALDH\(^{hi}\) and \(^{lo}\) CaCO-2 cells).

When comparing G0-G1 phases of ALDH\(^{hi}\) and \(^{lo}\) cells in the same cell line, there was no significant difference, attesting that the ALDH1 high activity does not correlate with a higher quiescent state, although studies have shown that ALDH\(^{hi}\) phenotype correlated with a dormant phenotype, as revealed by slow growing colonies and a low proliferative rate of growth [285]. However, it has also been shown that tumour growth and development are impeded when using ALDH inhibitors [282]. Therefore, if ALDH1 facilitates expansion, ALDH\(^{hi}\) cells may therefore not be expected to be in a quiescent stage.
Figure 37. Distribution of ALDH1$^{hi}$ and $^{lo}$ CaCO-2 and CM1 cells in the cell cycle. Number of quiescent cells (G0/G1) was not significantly different between ALDH1$^{hi}$ and $^{lo}$ cells for both cell lines. n =5 for CaCO-2 cells and n=4 for CM1 cells.
6.3.4 Differentiation of ALDH1\textsuperscript{hi} and ALDH1\textsuperscript{lo} cells over the time

An important parameter that might help in recognising a cell population as a CSC population is its capacity to differentiate, and to regenerate the whole tumour population. CSCs are theoretically expected to give rise to more differentiated progeny.

One way of verifying the differentiation capability of putative CSCs is the analysis of markers of differentiation. An alternative idea is that a putative CSC phenotype should be able, after a certain time, to generate a progeny comprising of the original CSC phenotype, and also of the more differentiated non-CSC phenotype. On the other hand, a non-CSC phenotype should not be able to have a progeny with a more primitive phenotype.

We therefore attained short term cultures of ALDH1\textsuperscript{hi} and \textsuperscript{lo} populations, and analysed their progeny 2 weeks after isolation.

\textit{CaCO-2}

In CaCO-2 cells, we analysed the activity of ALDH1 2 and 4 weeks after the isolation of ALDH1\textsuperscript{hi} and \textsuperscript{lo} cells (Figure 38).

Results interestingly showed a decrease of ALDH1 activity in the ALDH1\textsuperscript{hi} fraction, but becoming more stable after 2 and 4 weeks to a value of 25% (26.9±7.9% and 26.55 ±2.9%, respectively). This suggested that after a month, some of ALDH1\textsuperscript{hi} cells had differentiated into ALDH1\textsuperscript{lo} cells, but the activity of ALDH1 remained beyond the initial value of 15%.

In the ALDH1\textsuperscript{lo} fraction, we were even more surprised to see that after 2 weeks, cells had recovered to the initial amount of ALDH1\textsuperscript{hi} cells (14.0±2.9% and 14.64±2.1%, respectively). This suggested that either the contaminating ALDH1\textsuperscript{hi} cells developed quickly enough to readjust their number up to the initial percentage, or ALDH1\textsuperscript{lo} cells could also regenerate ALDH1\textsuperscript{hi} cells, thus recapitulating the heterogeneity seen in the original population.
**Differentiation of CaCO-2 ALDH1\textsuperscript{hi} and ALDH1\textsuperscript{lo} cell populations over a month in culture**

![Graph showing differentiation over time](image)

**Figure 38.** CaCO-2 ALDH1\textsuperscript{hi} and \textsuperscript{lo} cells were left in culture immediately after FACS sorting isolation. 2 and 4 weeks later, each fraction was reanalysed for ALDH1 activity. Error bars represent the standard deviations for \(n=4\) experiments at week 2 and \(n=2\) at week 4. The values at week 0 were considered to be 100% in ALDH1\textsuperscript{hi} cells and 0% in ALDH1\textsuperscript{lo} cells, although we did not check the exact value each time. The green line indicates the ALDH1 percentages of cells with a high ALDH1 activity in the total cell population = 15%.

**CM1**

In CM1, ALDH1 activity was assessed 2 weeks after FACS sorting, and in both ALDH1\textsuperscript{hi} and \textsuperscript{lo} cells, the initial percentage (26%) of ALDH1 activity had almost been recovered (32.9±11.5% and 36.1±15.9%, respectively) as shown in Figure 39.

![Graph showing differentiation after 2 weeks](image)

**Figure 39.** CM1 ALDH1\textsuperscript{hi} and \textsuperscript{lo} cells were left in culture after FACS sorting isolation. Error bars represent the standard deviations for \(n=3\) experiments. The green line indicates the ALDH1 percentages of cells with a high ALDH1 activity in the total cell population (26%).
6.3.5 Resistance to oxaliplatin

An intrinsic property of CSCs is their resistance to chemotherapeutic drugs. ALDH has been implicated in resistance to the common chemotherapeutic agent CPA in colorectal cancer [131].

It was interesting to establish whether cells expressing ALDH1 would possess a higher capacity of resistance to other chemotherapeutic drugs, more commonly used in regimens directed against colorectal cancer. In our study, we chose oxaliplatin. The patient from whom the CM1 cells had been derived underwent chemotherapy with oxaliplatin. The metastatic cells were therefore cells that showed resistance to this drug. Since the putative CSC marker ALDH1 maybe involved in generating a chemoresistant phenotype, we established if this was the case by incubating both ALDH1$^{hi}$ and $^{lo}$ cells of both cell lines for 96h with various concentrations of oxaliplatin. The percentages of viable cells were then analysed by a colorimetric assay.

According to Figure 40, the response to oxaliplatin was similar for the ALDH1$^{hi}$ and $^{lo}$ cells in both cell lines. Thus resistance to oxaliplatin does not seem to be mediated through ALDH.

At 96 h, the percentage of resistant cells was higher in CM1 cells, which is consistent with the fact that CM1 cells are derived from an oxaliplatin resistant clone.
Figure 40. Oxaliplatin resistance of ALDH1<sup>hi</sup> and <sup>lo</sup> cells. CaCO-2 and CM1 cells were exposed to various concentrations of oxaliplatin over 96h. The number of surviving cells was normalised to the number of cells with no oxaliplatin exposure (0µg/ml). Error bars represent standard deviations of 3 experiments in triplicates.
6.3.6 Migration assay

A transwell migration assay was carried out to determine if there was any difference in the migration potential of ALDH1$^{\text{hi}}$ vs. $^{\text{lo}}$ cells. As CSCs are defined as being responsible for metastatic growth, we expected the CSCs to have increased capacities for migration and invasion. Indeed, in breast cancer, migrating and invading capabilities are enhanced in CD44$^+/\text{CD24}^+$ CSCs compared to the rest of the cancer population. In breast cancer cell lines, a high ALDH1 activity has also been correlated with higher invasive properties 	extit{in vitro} as well as 	extit{in vivo} [174].

It has also been reviewed that the CD44 marker was specifically associated with a highly invasive/migrating phenotype in cancer [151, 153, 154]. In both cell lines, we detected a high expression of CD44 in ALDH1$^{\text{hi}}$ cells in comparison to the ALDH1$^{\text{lo}}$ cells, and therefore expected them to be able to migrate more than their counterpart.

Preliminary experiments

Before beginning any assay with our sorted cells, we carried out a preliminary migration assay with the total cell populations. We chose PC3wtAR prostate cancer cell line as a positive control, since these cells are known to be invasive and migratory. Although the literature reports on CaCO-2 cell invasiveness, the same has not yet been assessed with CM1 cells.

Cells were seeded in medium without serum on 8µm pore transwells ($5 \times 10^4$ cells/transwell). A 20% FCS gradient was established in the lower wells (except in the control wells). After 48h incubation, PC3wtAR cells had migrated. However, CaCO-2 and CM1 did not, even after 72h.
We then checked whether the number of cells we had seeded was adequate and did not provoke contact inhibition to prevent the cells from growing. Migration and invasion events can only happen if they are conjugated to cell growth. If cells are sensitive to the gradient, then they should expand towards the gradient rather than parallel to it.
When plating $5\times10^4$ cells in a 96-well plate, the number of cells had doubled for each cell line after 72h (Table 9), thus showing at this seeding density there was no contact inhibition, and the number of cells obtained after 72h was still reasonable to allow analysis.

<table>
<thead>
<tr>
<th>Fold-expansion after</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
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<tbody>
<tr>
<td>PC3wtAR</td>
<td>1.3</td>
<td>1.4</td>
<td>2</td>
</tr>
<tr>
<td>CaCO-2</td>
<td>1.2</td>
<td>2.5</td>
<td>4.4</td>
</tr>
<tr>
<td>CM1</td>
<td>1.0</td>
<td>1.6</td>
<td>2.7</td>
</tr>
</tbody>
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**Table 9.** Proliferation rate of PC3wtAR, CaCO-2 and CM1 cell lines. $5\times10^4$, $2.5\times10^4$ and $7.5\times10^4$ cells of each cell line were plated in a 96-well plate, respectively. Cells were trypsinised and counted in a haemocytometer chamber after 24, 48 and 72h.

Once we optimised the seeding density of the cells, we then decided to increase the gradient by adding 50ng/ml EGF to 20% FBS in the lower wells. Under these conditions, CaCO-2 cells were able to migrate across the pores after 72h (Figure 41). However, CM1 cells, although their diameter was smaller than CaCO-2’s, were not able to migrate across the pores, even when we added 50ng/ml EGF and 50ng/ml hepatocyte growth factor (HGF), as suggested in the literature for colorectal cancer cells. PC3wtAR prostate cancer cells were used as a positive control and were able to migrate across the pores after 48h under a 20% FBS gradient.
Migration of ALDH1\textsuperscript{hi} and \textsuperscript{lo} CaCO-2 cells

ALDH1\textsuperscript{hi} and \textsuperscript{lo} CaCO-2 cells were isolated, and immediately after FACS sorting were seeded in the upper chamber of the transwell system with or with a gradient and incubated for 72h. After fixation and incubation with crystal violet, the membranes were removed from the insert housing using a scalpel, mounted on a microscope slide, and observed under light microscopy.

The membranes were then incubated in 0.1% acetic acid to dissolve the crystal violet. OD was read on a spectrophotometer.

\textbf{Figure 41.} Migration of ALDH1\textsuperscript{hi} and \textsuperscript{lo} CaCO-2 cells. CaCO-2 cells were able to migrate through an 8µm pore membrane after 72h under a gradient of 20% FBS and 50ng/ml EGF. (A) ALDH1\textsuperscript{lo} CaCO-2 cells with and without gradient. (B) ALDH1\textsuperscript{hi} cells with and without gradient. For each condition, $2.5\times10^5$ cells were seeded in the upper well of a transwell system. After 72h, transwells were washed in PBS, fixed in 4% PFA and stained with 0.5% methyl blue for 50mn. Membranes were washed, cut out with a scalpel and placed onto glass slides. Images were captured under light microscopy ($\times20$).
Results in Figure 42 indicate that a high ALDH1 activity did not correlate with an increased migratory phenotype in CaCO-2 cells. Although we expected that ALDH1<sup>hi</sup> cells migrate more across the pores towards the gradient, there was no statistical difference in the migration rates of ALDH1<sup>hi</sup> and <sup>lo</sup> cell populations. In our case, a high expression of CD44 and ALDH1 activity did not increase the migrating capacities of the cancer cells.

![Migration rate of ALDH1<sup>hi</sup> and <sup>lo</sup> CaCO-2 cells](image)

**Figure 42.** Migration rate of ALDH1<sup>hi</sup> and <sup>lo</sup> CaCO-2 cells. Cells migrated through an 8µm pore membrane, under a 20%FBS+50ng/ml EGF gradient, after 72h. The OD of ALDH<sup>hi</sup> and <sup>lo</sup> cells in the gradient inserts was normalised to the OD of cells in the control inserts (with no gradient). Error bars represent the standard deviations for 3 independent experiments. The migration rate of cells was similar in both populations.

Unfortunately, we could not conduct that experiment on CM1 cells, as we were not able to determine the right conditions for their migration across the pores. Analysing the invading properties of the cells through a matrigel membrane, to reproduce the *in vivo* degradation of a basal membrane by the cancer cells would be another interesting experiment to conduct.
6.4 Discussion

In solid tumours, ALDH1 appears to be a successful method to select a breast cancer cell population with CSC characteristics. This target for isolation has rapidly expanded and an increasing number of studies are now being conducted using this enzyme as a potential CSC marker. The simplicity of the Aldefluor® kit and, importantly, the high rate of cell viability after staining are two key parameters explaining the emerging use of this technique.

As mentioned in this chapter, the importance of ALDH1 has not been fully established yet in colorectal cancer and its role in tumourigenicity is still to be confirmed.

Two preliminary studies mention the high expression of ALDH1 in pre-selected CD44⁺/ESA⁺ cell populations, and the possible enhancement of tumourigenicity when ALDH1 is co-expressed with CD44 cell surface marker [100, 131].

In our study, we analysed the role of ALDH1 as a single marker, and assessed its impact on various cell processes relevant to a potential CSC state.

When comparing the phenotype of ALDH1$^{\text{hi}}$ and ALDH1$^{\text{lo}}$ populations, two markers were significantly overexpressed in CaCO-2 cancer cell line, Lgr-5 and CD44.

In the CM1 primary cell line, an increase of CD44 in ALDH1$^{\text{hi}}$ cells was noticed and, interestingly, as were CD166 and ABCG2.

Therefore, in both CaCO-2 cell line and CM1 primary cell line, CD44 was upregulated in the ALDH1$^{\text{hi}}$ fraction. This is consistent with reports which have revealed an increase of ALDH1 expression among CD44⁺ population [100, 131]. In the CM1 cell line, FACS analysis of ALDH1$^{\text{hi}}$ cells showed that more than 90% of CD44 cells were comprised in the CD166 fraction. The phenotype CD44⁺/CD166⁺ of our ALDH1$^{\text{hi}}$ CM1 cells is also consistent with the CD44⁺/CD166⁺/ESA⁺ phenotype proposed by Dalerba et al. (2007) as a colorectal CSC phenotype [100].

Looking further down into the functionality of ALDH1$^{\text{hi}}$ cells, a clonogenic assay was conducted in order to define the self-renewing capacity of ALDH1$^{\text{hi}}$ cells in comparison to ALDH1$^{\text{lo}}$ cells. Our data do not show any significant difference in the colony forming potential of positive and negative cell lines. To define a population as
self-renewing, more than one passage are ideally necessary in order to establish an obvious evidence of that potential. In our case, the technical difficulty of the method and the time required to conduct it did not allow us to achieve several passages. None of the studies on colorectal CSCs using ALDH1 as a possible marker has reported a test on \textit{in vitro} clonogenicity. Further work is therefore needed to confirm our results and to be able to compare them.

Chemoresistance linked to ALDH1 expression has been reported in various organs, as for example in breast cancer. In a study conducted on patients undergoing chemotherapy (paclitaxel and epirubicin), the number of ALDH1\textsuperscript{hi} breast cancer cells in the tumours significantly increased after neoadjuvant therapy [286]. Similarly, more ALDH1 cells were present in metastatic tumours insensitive to paclitaxel than in those that did respond to the drug [257]. In colorectal cancer, an enrichment in ALDH1\textsuperscript{hi} cells has also been reported when xenogeneic tumours were submitted to a treatment with CPA [131]. Based on these studies, we analysed the chemoresistance of ALDH1\textsuperscript{hi} and ALDH1\textsuperscript{lo} cells. When comparing the percentages of surviving ALDH1\textsuperscript{hi} and ALDH1\textsuperscript{lo} cells after exposure to oxaliplatin, no difference could be established between the two populations, indicating that ALDH1\textsuperscript{hi} cells are not more resistant to this drug. However, according to immunohistochemistry, data revealed an increase in ABCG2 expression in CM1 ALDH1\textsuperscript{hi} cells (not in CaCO-2 cell line), suggesting a possible resistant phenotype of these cells to other chemodrugs, but obviously not to oxaliplatin. Therefore, it seems that an upregulation of ABCG2 is not involved in the mechanisms of resistance to oxaliplatin, which is consistent with the results on chemoresistance obtained in Chapter 5.

An argument in favour of the self-renewing/differentiating capabilities of ALDH1\textsuperscript{hi} cells is that short term \textit{in vitro} cultures of ALDH1\textsuperscript{hi} cells were capable of generating ALDH1\textsuperscript{lo} cells in a time-dependent manner. After two weeks of culture growth, the ALDH1 expression had already reached a steady state and returned to the ALDH1 expression level of the cell line from which it was originally sorted ((15% for CaCO-2, 26% for CM1), demonstrating that at least some cells were able to keep a self-renewing ability whilst others perhaps progressed along a differentiation pathway.
Such an observation had previously been made in a study on HCC CD133\(^+\) cells [137]. In this study, the expression of CD133 in CD133\(^+\) cells was followed-up over 6 weeks and it was observed that a steady state was reached after that period of time. The authors concluded that among CD133\(^+\) cells, which they assumed to be stem/progenitor cells due to their \textit{in vivo} tumourigenic potential, part of them did not possess the self-renewal capacity and therefore shortly developed into CD133\(^-\) cells. However, the fact that not all CD133\(^+\) cells developed into CD133\(^-\) cells, and that some persisted in culture, suggested to them that some CD133\(^+\) cells would therefore be more primitive progenitor/CSCs. Another explanation maybe that there is a regulation of the differentiation process of progenitor/CSCs, tending to maintain the proportions of putative CSCs at their initial level. This was also observed in SP cells extracted from human lung cancer, in which the hierarchy was re-established after a period of two weeks [122].

Similarly to our data, the two studies mentioned above report that non-putative CSCs also regenerate putative CSCs, and the authors attribute this phenomenon to the contamination of the negative population by the positive one. Contamination might be one issue, but another reason could be that ALDH1\(^{lo}\) cells are not less primitive and also contain progenitor/CSC with self-renewal capacity. This would be consistent with the negative results obtained in the clonogenic assay, however, the only way to determine this would be through \textit{in vivo} transplantation of our cells into xenogeneic model for a tumourigenicity test.

In summary, a high ALDH1 activity in CaCO-2 and CM1 colorectal cancer cell lines correlated with a high expression of CD44, CD166, Lgr-5 and ABCG2 colorectal stem cells and CSC markers. To confirm these results, experiments involving the isolation of cells expressing these markers (in combination preferentially) should be conducted in order to verify their high ALDH1 activity. The biological functions attributed to CSCs, such as clonogenicity, chemoresistance and migration, were not enhanced in cells with a highly active ALDH1 enzyme. The fact that the percentages of cells with high ALDH1 activity returned to the normal value after a couple of weeks suggests that ALDH1 activity may not be an appropriate marker for CSC isolation, as ALDH1\(^{lo}\) cells could regenerate ALDH1\(^{hi}\) cells.
An *in vivo* experiment would be highly recommended to confirm the *in vitro* work, but we did not have the opportunity to conduct this and therefore doubt remains on the use of ALDH1 for colorectal CSC isolation.
7 Discussion
This work was aimed at isolating and characterising putative cancer stem cells (CSCs) in the liver metastases of colorectal cancer. Over the past three years, the landscape on concepts around CSCs has considerably developed. Different approaches have been explored in order to achieve efficient isolation of these cells. Whilst several promising results have arisen, controversy exists as to the exact definition of the term CSC, the validity of the hierarchical model of cancer progression, and its putative implications in other concepts related to cancer, including chemoresistance and metastasis. Essentially the theory of CSCs still has to overcome two challenges. The first one is to confirm the existence of true CSCs. This can only be achieved through their specific isolation and characterisation. The second challenge would then be to provide evidence on whether targeting CSCs would be of any therapeutic benefit.

The primary challenge within the field of CSC research is efficacy of CSC isolation. Since the hierarchical model of cancer posits that CSCs are the only cells to possess tumourigenicity, then it is crucial that optimal isolation and eradication of these cells are achieved.

Although we were not successful in obtaining a pure isolation of CSC population from CaCO-2 and CM1 cell lines in our study, we have highlighted here that the accuracy of isolating tumour-initiating cells is not only dependent on the choice of techniques and the type of cells, but also that the specificity of each method is undoubtedly related to each system. In addition to the technical hurdles faced in isolating CSCs in our system, we also had to contend with the issue that the cells we used may have followed a traditional (stochastic) model of cancer progression rather than a hierarchical model. The traditional model of cancer predicts that the isolation of tumour-initiating cells is quasi impossible as the property of tumourigenicity can be randomly acquired by all cancer cells, under the influence of extrinsic or intrinsic factors. The lack of particular characteristics to discriminate the tumour-initiating cells therefore should prevent their isolation. On the contrary, according to the CSC model, tumourigenicity is the exclusive property of a distinct population of cells with specific characteristics (capacity of self-renewal, undifferentiated state, pluripotency). CSCs can differentiate into non-tumourigenic cells through irreversible epigenetic changes, and thus constitute a distinct population that can be isolated on the basis of their phenotypic and functional attributes.
7.1 Techniques

To date, several techniques have been used to isolate cells that are classed as possessing tumour-initiating potential.

7.1.1 Isolation of CSCs according to the expression of the cell surface marker CD133

For this project, we initially set out to use cell surface markers that have previously been described as the ‘gold standard’ for CSC isolation. In opposition to a traditional model of cancer, in which cancer cell properties vary randomly according to genetic or epigenetic transformations [72], the hierarchical model follows that cells with a tumourigenic potential differ from the rest of the cells on the basis of epigenetic differences only. According to this model, it has been proposed that tumourigenicity is exclusive of only a few cells expressing a specific marker or combination of markers that are progressively down-regulated during differentiation at a non-genetic level [85]. Using this principle, therefore CSCs may be feasibly isolated on the basis of particular cell surface markers that are expressed at a particular time point during CSC differentiation.

We initially chose to isolate putative colorectal CSCs through the expression of CD133, since its expression is reported to be associated with high tumourigenicity in cells from many organs, specifically brain and colon cancers [93, 99]. Unexpectedly, technical hurdles prevented us from obtaining a pure CD133 CSC population out of freshly isolated tumour samples. We therefore were not able to continue studying their characteristics. To improve the isolation, optimising the conditions of separation would have been necessary. However, further experiments should also take into account that, since the epitopes targeted by the commercially available CD133 isolation kit are directed against glycosylated domains of CD133, the success of the isolation may be dependent on the level of glycosylation present within these cells. Although this problem may not be directly involved to explain the
purity of our isolated fractions, it is extremely rate limiting for CSC research, as the state of glycosylation is dependent on the state of differentiation of the cells, as explained in Chapter 3. Recent investigations into CD133 biology report controversial results about its relevance to be a CSC marker, and glycosylation of the CD133 epitopes may be the key to inconsistent data. Indeed, latest studies have shown that CD133 expression profile was too broad to be used as a tool for CSC isolation. Shmelkov et al. (2008) have demonstrated that the expression of CD133 continues in differentiated colonic cells [212]. Moreover, CD133- cells from primary and metastatic colon cancer have been demonstrated to be as tumourigenic as CD133+ cells [212]. This observation has also been reported in a study on brain cancer [187]. In our setting, therefore, as well as in other recent publications, glycosylation of CD133 is a rate limiting step and questions the reliability of CD133 as a bona fide colorectal CSC marker [187, 212].

Future work needs to focus on antibodies that target non-glycosylated epitopes of a putative marker which demonstrates a narrower pattern of expression specific to undifferentiated CSCs.

Besides the practical and technical aspects involved when relying on cell surface markers to target CSCs, one of the caveats of the CSC hypothesis is the question of the cellular origin of cancer. Both the clonal and hierarchical models of cancer are based on the concept that the origin of cancer stems from a single cell [72], the nature of that cell however has yet to be determined. The American Association for Cancer Research (AACR) reports (2006) clearly state that stem cells do not have to be the cells from which a malignant progeny is derived [287]. There is indeed evidence that cancer may preferentially emerge from the stem cell compartment, as it was shown for instance with experiments in the intestine. A constitutive activation of the Wnt self-renewal pathway in Lgr-5+ intestinal stem cells initiates their transformation and consequently generates an adenoma [67]. Activating the same Wnt pathway in a stem cell progeny (transit-amplifying cells) does not have the same effect and only generates an adenoma in very rare cases. This therefore suggests that hyperactivation of the Wnt pathway triggers a tumour within the stem cell compartment rather than in their progeny. Despite this, however, there is evidence to show that cancer can arise from a population of differentiated cells. Transducing neural stem cells and/or mature astrocytes to express constitutively active EGFR induced a glioblastoma (GBM) in
both cell types, indicating that the deregulation of a specific pathway is able to induce GBM regardless of the cell origin [288].

If a cell with a cancerous phenotype can originate from any given cell population, and the expression of cell surface protein is specific for the stage of differentiation and cancer type, then the attempt to identify a CSC population based solely on cell surface marker is almost unachievable. It may be more accurate to extract CSCs based on the expression or activity of markers that are associated with maintaining a state of pluripotency. None of the currently targeted cell surface markers has an apparent functional role in the regulation of self-renewal and ‘stemness’ function. They may be associated with low survival in cancer patients (CD133 and CD166[145] [146] [147]), or migration, invasion, and metastasis of cancer cells (CD24 and CD44 [152]). CD44 has also been reported to be involved in invasion and migration. Even CD133 has not clearly proven to have a functional role in maintaining a stem cell function, although it has been reported to be expressed by CSCs from various types of cancer [93, 94, 105, 161] [95] [102] [96, 136] [98, 99].

### 7.1.2 Isolation of CSCs according to the activity of ALDH1

The literature reports on ALDH1 enzyme as a non-immunological approach for stem cell isolation. ALDH1 is known to be directly linked to the regulation of stem cell phenotype and by extension possibly that of CSCs [168, 278]. Although this enzymatic marker has proven efficient in the isolation of a primitive cell population from breast, colorectal, lung and liver cancer tissue [92, 172, 174, 175, 289], it is still too early to confirm whether or not ALDH1 can be used as a specific marker of CSCs [100]. To address this gap in data, we tested how specific ALDH1 would be in isolating CSCs from colorectal cancer cells.

Since ALDH1$^\text{hi}$ cells expressed more CD44, CD166 and Lgr-5 intestine CSC markers than ALDH1$^\text{lo}$ cells, we confirmed that ALDH1 cells may be enriched in putative CSC. ABCG2 was upregulated in our ALDH1$^\text{hi}$ cells but its expression did not appear to bear influence on resistance to oxaliplatin. ABCG2 is responsible for an MDR phenotype, and we expected ALDH1$^\text{hi}$ cells to be resistant to oxaliplatin. Our data in Chapter 6 showed that ALDH1$^\text{hi}$ cells were not significantly more resistant to
oxaliplatin than ALDH1\textsuperscript{lo} cells. This result was also consistent with data from Chapter 5 that shows that ALDH1 activity was higher in CaCO-2 cells than in CaCO-2-OR cells. Little is known on the therapeutic compound oxaliplatin, and we showed here that ABCG2 does not mediate cell resistance to oxaliplatin.

We did not find ALDH1\textsuperscript{hi} cells to bear additional \textit{in vitro} stem cell characteristics such as an enhanced clonogenicity and capacity to migrate. Since numerous factors could affect the biochemistry of cells in an \textit{in vitro} environment, a more accurate method to compare the biological behaviour of ALDH1\textsuperscript{hi} and ALDH1\textsuperscript{lo} cells would involve \textit{in vivo} models. This will undoubtedly form a future plan for the continuation of this project.

\textbf{7.1.3 Isolation of CSCs according to their innate resistance}

It is believed that CSCs possess an innate resistance to drugs due to the key features of stem cells which allow them to escape chemotherapy. Studies in brain tumours and leukaemia have explored the characteristics of intrinsically resistant cells and shown they possess CSC-like properties [26, 27]. Since chemoresistance of cancer cells has been attributed to CSCs and may be more relevant from a clinical point of view, we decided to focus our research on an assay based on the chemoresistance of cancer cells. We hypothesized that chemoresistance could therefore be used as a tool to select for a CSC-like population from the tumour bulk.

In our study, a subpopulation of putative CSC-like cells possessing an intrinsic resistance to the chemotherapeutic agent oxaliplatin was isolated from an established CaCO-2 colorectal cancer cell line, and from the shorter passage cell line CM1. We have shown that intrinsically resistant cells (CaCO-2-OR and CM1-OR cells) isolated after short term exposure to oxaliplatin (5µg/ml for 48h) did not exhibit enhanced CSCs characteristics when compared to the non-resistant CaCO-2 and CM1 cells. We analysed expression of CSC/stem cell markers, cell cycle status and \textit{in vivo} tumourigenicity, however all tests were consistent to show that there was no sign of a CSC phenotype in the resistant cells. It must be noted that the \textit{in vivo} experiments with the CaCO-2 cell line was inconclusive as time constraints meant that we were unable to meet the required number and also to proceed to the CM1 cell line \textit{in vivo}. 
experiments. We also noted that resistance to oxaliplatin was not associated with an upregulation of the ABCG2 or Mrp1 transporters.

Isolating CSCs through exploitation of their intrinsic resistant phenotype is not a\textit{ priori} a good way to achieve it. Further studies must be conducted for a better understanding of the role that CSCs have in chemoresistance as currently our knowledge of CSCs is only limited to a number of observations:

1) when comparing putative CSCs with the rest of the cancer cell population, published data support the observation that CSCs are more resistant to chemotherapeutic agents. [251] [191, 253-258]. If chemoresistance is the attribute of CSCs, then it is worth targeting them but as discussed in Chapter 5, further data comparing the capacity of chemoresistance in CSC and in non-CSC are necessary.

2) In order to prove that CSCs possess an intrinsic resistance to therapeutic compounds, these cells must be able to display their phenotype following short term exposure with the relevant drugs. Since our isolated cell populations did not show evidence of a primitive phenotype, we postulated in Chapter 5 that the ability of cells to survive short term exposure with the drug may involve factors other than a stem cell phenotype, such as a protective environment or the capacity to undergo EMT. There is therefore a need to test the initial hypothesis and to verify whether the isolated cells truly are resistant with a second exposure to the drug.

3) The literature reports of an experiment in which cancer cells were exposed to increasing concentrations of drugs over 8 months [273]. In this setting, chemoresistant clones were selected and propagated until they were able to survive the clinically relevant dose. Those cells with acquired resistance were shown to possess several CSCs characteristics \textit{in vitro}. The setting of this type of experiment, however, does not address the question whether CSCs are inherently more resistant than non-CSCs.

Further data are needed to establish a clinically relevant link between CSCs and chemoresistance, and to prove whether or not their primitive phenotype provides on them an advantage to escape chemotherapeutic treatment. This issue has to be addressed in order to confirm the relevance of targeting CSCs from a clinical point of view.
7.2 Choice of cells

In Chapter 4 the use of cancer cell lines to study the origin and development of cancer was discussed. Since there is a selection pressure on cancer cell lines to survive in an artificial *in vitro* environment, they are not always the best choice to study a particular cancer model. Progressive passages of these cell lines ultimately result in accumulated genetic changes which sometimes take them further away from their parental phenotype. In order to address these issues, we studied an established CaCO-2 cell line alongside a newly created cell line CM1 where we kept the passage numbers as low as possible.

In the CM1 cell line, we found a high proportion of the intestine stem cell marker Lgr-5 (up to 46%) and of CSC markers CD166, CD24 (more than 80% for both markers). CaCO-2 cells also expressed high levels of CD133 (98%), Lgr-5 (60%), CD166 (47%) and Nanog (40%) CSC/stem cell markers. It is important to bear in mind that, when using cell lines to study the CSC model, the CSC markers expressed in the primary tumours may not be similarly expressed in the cancer cell lines. In normal organs, and even in tumours, the CSC/stem cell markers have been shown to be expressed at a low level. It seems that the proportion of cells with CSC markers is increased in our cell lines, suggesting that *in vitro* culture conditions either artificially upregulate these markers, or maintain CSCs proliferation, thus increasing their proportions *in vitro*. This may account as to why we could not discriminate a putative CSC population from a non-CSC one.

A clue supporting the hypothesis that cancer cell lines are in fact a culture of CSCs, is that cancer cell lines not only retain their tumourigenic potential over decades, but also some of them have been shown to maintain their aptitude to differentiate, when cultured under certain conditions. As we have previously mentioned, the CaCO-2 cell line is known to be able to differentiate into enterocytic cells upon confluence [263]. HRA-19, another colorectal cancer cell line, is capable of differentiating into the three intestinal cell lineages (absorptive enterocytes, mucus-producing goblet cells and entero-endocrine cells) when cells are cultured in serum-free medium supplemented with insulin, transferrine and ascorbic acid [290]. When cultured in DMEM supplemented with 10% FBS, HRA-19 cells maintained an undifferentiated state.
When grown as xenografts, these cells formed tumours containing absorptive, mucous and endocrine cells [291], which is a reflection of their pluripotency. However, as an objection to the hypothesis that the majority of cells in a cancer cell line are CSCs, we found that in vitro clonogenicity was restricted to only 13.6% of the CM1 cells. We would expect a higher percentage if the majority of cells in a cell line was to be CSCs. It would be extremely interesting to test in vivo and in vitro the clonogenicity and tumour-initiating potential of these cells.

It is therefore not known yet whether cells in cancer lines should all be considered as CSCs, if they keep the initial hierarchy, or if they simply follow a traditional (non-CSC) model of development as it was recently suggested for the HCT116 colonic cancer cell line [292]. Since we were not able to extract CSCs from CaCO-2 and CM1 colorectal cancer cells, an alternative reason simply may be that our cell lines do not follow a CSC model, and rather develop according to a traditional model of cancer in which all cells progress through clonal evolution.
7.3 Cancer model

Our results suggest that the current techniques for isolating CSCs are not entirely satisfactory. There are indeed limitations to our study as discussed in this report, and it is possible that a single technique alone is not sufficient to attain isolation of a CSC population.

Despite the emergence of an established model that accounts for cancer initiation/progression through CSCs, there are still gaps in our understanding. The quiescence of CSCs for instance, as well as the mechanism of chemoresistance, have yet to be better characterised.

The gold standard in vivo xenogeneic transplant of CSCs designed to prove their tumourigenic potential is itself subject to limitations. Firstly, it seems that the growth of xenotransplanted cells is extremely variable depending on the conditions in which this assay is realised. The site of transplantation and the medium in which cells are injected (e.g. with or without matrigel) have shown to influence the results and to be partly responsible for the heterogeneity of the observed results [199, 293]. Secondly, the degree of immunodeficiency of the animal models may also influence the survival of the xenografted cells. A recent study has shown that a large proportion (25%) of unselected melanoma single-cells is capable of tumourigenic potential in vivo when using NOD/SCID mice strongly immunocompromised (with an additional interleukin-2 receptor gamma chain null (Il2rg/-)) [199]. When using a standard NOD/SCID mouse model, the average number of melanoma cells capable of forming a tumour was only one cell in a million.

This observation is reinforced by the fact that, when transplanting 10 cells to several thousands murine lymphoid and myeloid tumour cells into histocompatible mice, i.e. generating no immuno-reactivity at all, the number of cells being able to initiate a tumour has also been shown to be relatively large (>10%) [200]. Previous reports indicated that the average number of tumour-initiating cells was only one in one million when xenografting human AML cells into NOD/SCID mice [294]. Therefore, it is very likely that the number of tumourigenic cells detected depends on the cell capacity to survive the host environment.
If further studies reveal a number of tumour-initiating cells broader than expected, it may severely question the concept of hierarchy. Heterogeneity may thus be simply due to random genetic/epigenetic differences as it is predicted in the traditional model of cancer. Although the number of tumour-initiating cells within cancers has been claimed not to be an important parameter in the CSC theory [295], the existence of CSCs may nevertheless become irrelevant from a clinical point of view. Indeed, cancers in which a majority of cells are CSCs will be treated as any cancer that follows a traditional model of development [85].

If the numerous studies described in Chapter 1 indicate that some cancers must truly follow a hierarchical model, as demonstrated by a minority of cells able to sustain the tumour growth in several solid tumours, there are nevertheless other studies tending not to confirm this hypothesis. It seems that both models of cancer are not exclusive, and vary according to the type of cancer. For instance, a hierarchical model is not exclusive of the traditional model. It is important to keep in mind that CSCs can themselves undergo genetic alterations, following the traditional model of selective mutations, and transmit these alterations to their clones/progeny, thus creating heterogeneity in the same patient. Indeed, a study on colorectal cancer has reported chromosomal instability in the CSCs themselves [215]. One of the key study that will support the authenticity of the CSC model will be the investigation of the nature of the differences between tumour-initiating cells compared with the rest of the cells, as proposed by Shackleton et al. (2009) [85].
7.4 Future work

Based on the data we have obtained in this study, isolating colorectal CSCs in our hands would involve the improvement of some of the techniques we have been using here, and would also require the establishment of innovative assays.

1. Developing the isolation and culture of fresh tumour samples

Since *in vitro* cultured cancer cells are not entirely representative of the tumour they are derived from, then isolating CSCs from freshly resected tumours will be a more accurate approach. The major hurdle associated with the use of primary tissues is the presence of large amount of dead cells and debris obtained during mechanical dissociation of the samples. To eliminate this, several methods are conceivable. For instance a ficoll gradient traditionally used for mononucleocyte isolation could be adapted for our purpose. Ficoll is a medium widely used for density centrifugation and it exploits gradient centrifugation and cell size/density to enrich or fractionate cell populations. Using this principle, we may be able to separate live cells from the debris according to their density. Alternatively, a more expensive approach would be from commercially available kits which are designed to remove cell debris.

It is important to bear in mind that the choice of any method that reduces the amount of cell debris will also result in the potential loss of viable CSCs – thus limiting the number of downstream experiments.

2. Developing and confirming our results – need for cross-experiments

- The isolation of putative CSCs based on ALDH1 activity is a technique currently being investigated within the field of CSCs. Our observations that markers of CSCs were upregulated in isolated ALDH1<sup>hi</sup> population compared with ALDH1<sup>lo</sup> suggests a possible enrichment of CSCs. This technique, however, was subject to limitations as demonstrated from our inconclusive *in vitro* data. A more accurate characterisation of our ALDH1<sup>hi</sup> and <sup>lo</sup> cells would involve their xenotransplantation into an animal
model to further determine their status as CSCs. The direct isolation of cells with a CD44/CD166/Lgr-5 phenotype and the analysis of their ALDH1 activity would constitute a proof to confirm the results we obtained in immunofluorescence.

- Since we identified certain limitations when relying on cell surface markers as an isolating technique, other biological characteristics were approached as a possible target. We attempted to isolate CSCs based on their chemoresistant phenotype, but \textit{in vitro} results were inconclusive. It will be necessary to test our initial hypothesis, i.e. to verify that cells surviving a lethal dose of oxaliplatin possess true oxaliplatin resistance. The cells we isolated (CaCO-2-OR and CM1-OR) may contain CSCs, but they may be so rare that a majority of these surviving cells may not show enhanced chemoresistance when exposed to a second adjuvant treatment with oxaliplatin.

In preliminary experiments, we redesigned our existing protocol whereby CaCO-2-OR cells were now exposed to a second exposure to oxaliplatin. Our preliminary data showed that expression of the enterocytic marker AP was upregulated in CaCO-2-OR cells after two exposures to the drug (data not shown). To confirm whether these resistant cells were directed towards a more differentiated or undifferentiated state under the influence of oxaliplatin, other pluripotent genes and markers of CSC should be analysed. If the number of isolated cells allows it, it would also be ideal to investigate the biological behaviour of the resistant CaCO-2 cells when xenografted \textit{in vivo}.

The validation of this approach also requires further \textit{in vivo} characterisation. Essentially, the same numbers of CaCO-2 and CaCO-2-OR cells should be injected in mice for a comparative analysis of their tumourigenic potential.

3. \textbf{Exploration of other approaches}

- A non immunological assay that has previously proven successful in isolating CSCs from brain, breast and colon relies on the growth of spheres in serum-free medium. This technique is believed to maintain the self-renewal capacity of stem cells and to preserve their pluripotency. However, despite its usefulness, this assay is not exempt from limitations (as discussed in the Introduction). In particular, the capacity to self-
renew is not strictly restricted to stem cells as progenitor cells are also able to do so, albeit for a shorter period of time. Reynolds et al. (2005) reported the existence of cells able to form spheres for up to two or three passages maximum [182]. It is therefore never possible to assume that all spheres in a culture are derived from stem cells, and it is of major importance to define the number of passages necessary to isolate true stem cells.

Regardless of the fact that the neurosphere assay is so far one of the best in vitro tests to account for the existence of stem cells, it does not allow the isolation of a pure population of stem cells since spheres also comprise of differentiated cells. In brain cancer, the number of cells isolated from neurospheres necessary to reproduce tumours in vivo was generally not lower than 5000 cells, which is still far from the theoretical value of one cell being able to reconstitute the whole tumour [157-161]. Therefore, trying to characterise CSCs from a population of cells derived from a sphere will always be approximate as each sphere contains self-renewing and non self-renewing cells.

- The data from Chapter 3 led us to question the reliability on the use of surface markers to isolate CSCs. Further experiments using a wider range of CSC markers will have to take into account that the nature of the initial cancer cell has not been defined yet. Cancer cells emerging from a cell at the basis of the hierarchy may not possess the same surface marker pattern as cancer cells emerging from a cell at the top of the hierarchy, due to the epigenetic changes that occur as cells differentiate. For that reason, CSC markers are not only tissue-specific, but they may also be patient-specific. However, with regard to colorectal cancer, recent studies have shown that activation of the Wnt pathway triggers a tumour only from the Lgr-5⁺ cells [67]. If a majority of colorectal cancers are initiated from Lgr5⁺ cells, then the use of Lgr-5 as a putative CSC marker may prove relevant for CSC isolation.

- Since epigenetic changes have been suggested to occur when CSCs progress into a differentiation pathway responsible for the loss of their tumour-initiating capacity, it may be possible to screen for the validity of isolated CSCs based on their epigenetic profile [85]. Epigenetics involve all the mechanisms responsible for the modification of the gene activation status. These mechanisms predominantly include DNA
methylation and histone modification. Designing a technique allowing this analysis based on our small numbers of isolated CSCs will however make this challenging.

In conclusion, more work is needed to develop and confirm the validity of the techniques we have used for the isolation of CSCs. In the meantime, the current literature on CSCs keeps proving the difficulty of attaining this population. New methods of isolation need to be developed in order to improve on the specificity of CSC isolation. Although different techniques have proven efficient for isolating putative tumour-initiating cells in certain cancers, the issue of disease specificity comes into play as these techniques are not always transferable. There is an obvious need to redefine the studies in order to be able to unify the results and better understand the biology of cancer stem cells.
Bibliography


