ENRICHMENT OF COLORECTAL CANCER STEM CELLS BY SPHEROIDAL CULTURE AND GENE EXPRESSION ANALYSIS OF SELECTED ABC TRANSPORTERS

By

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ABSTRACT

ENRICHMENT OF COLORECTAL CANCER STEM CELLS BY SPHEROIDAL CULTURE AND GENE EXPRESSION ANALYSIS OF SELECTED ABC TRANSPORTERS

Vimalan Rengganaten

Colorectal cancer (CRC) is the second most common cancer in Malaysia. Recent reports hypothesise the recurrence of CRC could be linked to the existence of colorectal cancer stem cells (CrCSC), leading to chemoresistance. One of the proposed mechanisms of chemoresistance by CrCSCs is the involvement of ATP-binding cassette (ABC) transporter proteins. In the present study, CrCSC population was enriched by prolonged spheroidal culture with the aim to identify the ABC transporter subfamily members that are linked to the development of chemoresistance in CrCSC population. CRC spheroid-cultured cells showed increased expression levels of some CrCSC-associated surface markers, enhanced clonogenic ability, slower proliferation and cell division rate as compared to their parental cells. The gene expression analysis using real-time qRT-PCR revealed an up-regulation of pluripotency and tumourigenicity-associated genes in spheroid-cultured cells at increased passages in spheroidal culture, and down-regulation upon re-differentiation. The drug sensitivity assay using CRC chemotherapeutic drugs, 5-fluorouracil

and oxaliplatin showed that the CRC spheroid-cultured cells were more chemoresistant than their parental counterpart, with the prolonged spheroid-cultured cells showed increasing values the IC_{50} in passage-dependent manner. Collectively, CRC spheroid-cultured cells exhibited enhanced properties associated with CrCSC population. Gene expression analysis of 14 selected ABC transporter genes via real-time qRT-PCR showed distinct regulation of ABC transporter genes in CRC spheroid-cultured cells, where *ABCA3* and *ABCC2* were consistently up-regulated in all CRC spheroid-cultured cells. *ABCB5*, *ABCC4* and *ABCG2* were consistently down-regulated. Collectively, the results indicate that prolonged spheroidal culture is an effective method to enrich the population with CrCSCs. The up-regulated expression of *ABCA3* and *ABCC2* genes could indicate the involvement of these genes in the development of chemoresistance in CrCSCs, which could be used as potential biomarkers in the identification and isolation of chemoresistant variant of CrCSCs.

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"Success is not created by a person, but by a team that comes together"

– Jillian Farrar.

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APPROVAL SHEET

This dissertation entitled **"ENRICHMENT OF COLORECTAL CANCER STEM CELLS BY SPHEROIDAL CULTURE AND GENE EXPRESSION ANALYSIS OF SELECTED ABC TRANSPORTERS"** was prepared by VIMALAN RENGGANATEN and submitted as partial fulfilment of the requirements for the degree of Master of Medical Sciences at Universiti Tunku Abdul Rahman.

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SUBMISSION OF DISSERTATION

It is hereby certified that **Vimalan Rengganaten** (ID No: **14UMM07871**) has completed this dissertation entitled "ENRICHMENT OF COLORECTAL CANCER STEM CELLS BY SPHEROIDAL CULTURE AND GENE EXPRESSION ANALYSIS OF SELECTED ABC TRANSPORTERS" under the supervision of Prof. Dr. Shelly Soo @ Soo Shelly (Supervisor) from the Department of Medicine, Faculty of Medicine and Health Sciences, and Senior Prof. Dr. Choo Kong Bung (Co-Supervisor) from the Department of Pre-Clinical Sciences, Faculty of Medicine and Health Sciences.

I understand that the University will upload softcopy of my dissertation in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

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DECLARATION

I VIMALAN RENGGANATEN hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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(VIMALAN RENGGANATEN)

Date _____

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LIST OF ABBREVIATIONS

2D	2-dimensional
3D	3-dimensional
5-FU	5-fluorouracil
A260	Absorbance at 260nm wavelength
A280	Absorbance at 280nm wavelength
ABC	Adenosine triphosphate-binding cassette
ALD	Adrenoleucodystrophy
ALDH1	Aldehyde dehydrogenase 1
AML	Acute myeloid leukaemia
APC	Adenomatous Polyposis Coli
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BAD	B-cell lymphoma 2-associated death promoter
Bax	B-cell lymphoma 2-associated X apoptosis regulator
Bcl-2	B-cell lymphoma 2
Bcl-xl	B-cell lymphoma-extra large
BCRP	Breast cancer resistance protein
bFGF	Basic fibroblast growth factor
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
BSC II	Biological safety cabinet class II
CCK-SK	Cell Counting Kit - SK

cDNA	Complementary deoxyribonucleic acid
CHK1	Checkpoint kinase 1
CIC	Cancer initiating cells
c-MYC	Myc proto-oncogene protein
CO_2	Carbon dioxide
CRC	Colorectal cancer
CrCSC	Colorectal cancer stem cells
CSC	Cancer stem cells
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMEM F-12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ESA	Epithelial specific Antigen
FAP	Familial adenomatous polyposis
FBS	Foetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCN20	General control nonderepressible 20
HNPCC	Hereditary non-polyposis colorectal cancer
IC_{50}	Inhibitory concentration of 50%

IgG	Immunoglobulin G
KLF4	Kruppel-like factor 4
L-OHP	Oxaliplatin
MDR	Multidrug resistance
MEM	Minimum Essential Medium
mRNA	Messenger RNA
MRP	Multidrug resistance protein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCBI	National Center for Biotechnology Information
NTC	No template control
OABP	Oligoadenylate binding protein
OKSMN	OCT4, KLF4, SOX2, c-MYC, Nanog
P	Passage
PBS	Phosphate buffered saline
Penstrep	Penicillin-Streptomycin
PFA	Paraformaldehyde
PI	Propidium iodide
PI3K-AKt	Phosphoinositide 3-kinase
PolyHEMA	Polyhydroxyethylmethacrylate
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
SCM	Serum-containing medium
SFM	Serum-free medium

TIC	Tumour initiating cells
TP53	Tumour protein p53
TPC	Tumour propagating cells
$\Delta\Delta C_T$	Delta delta cycle threshold

CHAPTER 1

INTRODUCTION

Decades of research have yet to uncover the complexity of the pathogenesis and treatment of cancer. However, it has become evident that cancer arises as consequence of accumulation of mutations which results in genetic and epigenetic alterations (Marotta and Polyak, 2009). Despite the advancement in medical research, cancer still remains a major health problem worldwide (Biemar and Foti, 2013). In Malaysia, cancer is listed as the third common cause of death, after cardiovascular disease and septicaemia (Zainal and Nor Saleha, 2011).

Globally, colorectal cancer (CRC) is the third most common cancer-related death while is the second most common cancer in Malaysia (Zainal and Nor Saleha, 2011; Pourhoseingholi, 2012). Chemotherapy remains the most common therapeutic treatment among CRC patients. However, the current chemotherapeutic approach is reported to have major limitations which resulted in high cancer recurrence rate (Kanwar et al., 2012). Recent reports hypothesise the recurrence of CRC could be linked to the existence of colorectal cancer stem cell (CrCSC) population, leading to chemoresistance (Lundberg et al., 2016).

The theory of CSC suggests that tumours consist of a subset of cells, which drives the tumour initiation and progression (Chen et al., 2016). The rare subset of cancer cells has enhanced self-renewal ability and functional heterogeneity (Lee et al., 2016). More importantly, the enhanced chemoresistance in CSC population is held accounted for the ineffectiveness of the many chemotherapeutic drugs. The current approach of eradicating the entire tumour bulk is believed to only eliminate the non-stem cell components of the tumour, leaving the CSC population to survive and subsequently metastasise (Li and Li, 2014).

The isolation and enrichment of CrCSC population still remain a challenge. Spheroidal culture is a complementary *in vitro* assay that has been used in the enrichment of CSC population (Shaheen et al., 2016). However, contradicting reports on the characteristics of the population derived from spheroidal culture warrants more investigation. Furthermore, the lack of specific CrCSC markers is a major limitation in the identification of CrCSC population.

Various CSC-mediated chemoresistance mechanisms have been proposed such as enhanced altered DNA repair mechanism, cellular dormancy and high expression levels of pro-survival and low expression levels of anti-apoptotic factors (Paldino et al., 2014). However, ATP-binding cassette (ABC) transporters are postulated to be the major contributor to the development of chemoresistance in CSC population (Hu et al., 2016).

ABC transporters are a group of a family consisting of 49 different transmembrane proteins, mediating the movement of substances across the cells. Despite the association of some of the ABC transporters with the development of chemoresistance in cancers, the involvement of the ABC transporters in the development of chemoresistance CrCSCs remains to be elucidated (Zhao, 2016). Identification of specific members of ABC transporter superfamily could be used as potential biomarkers for isolate CrCSC population.

The present study hypothesised that the prolonged culture of CRC cells in spheroidal culture will be more effective in enriching CSC population. We anticipated that the population derived from the prolonged spheroidal culture will exhibit enhanced CrCSC-associated properties, including higher chemoresistance. We predicted a unique ABC transporter gene expression profile will be expressed in the CRC spheroid-cultured cells and the parental cells.

The objectives of the present study were:

1. To enrich colorectal cancer stem cells (CrCSC) using prolonged spheroidal culture
2. To characterise the putative properties of the enriched CrCSCs, including chemoresistant properties
3. To investigate the gene expression profile of selected ABC transporters in the established CrCSCs

CHAPTER 2

LITERATURE REVIEW

2.1 Colorectal Cancer (CRC)

Globally, CRC is the third most common form of cancer and fourth leading cause of cancer deaths (Arnold et al., 2016). In Malaysia, CRC is the most common form of cancer in males and third most common in females, which accounts for prevalence rates of 9.7% and 8.1%, respectively (Lim, 2014). In the multi-racial population of Malaysia, Chinese ethnics recorded the highest incidence, followed by the Malay and Indian ethnics (Bardhan and Liu, 2013).

The development of CRC generally occurs in a multistage stepwise fashion. CRC occur as a result of interaction between genetic, epigenetic and environmental factors. CRC begins as the uncontrolled proliferating colonocytes in the crypt of the intestine form small polyps. Over years, the small polyps progress into the bigger polyps and some would further develop into malignant tumours and advanced CRC, which might metastasise to other parts of the body (Goel and Boland, 2010; Bardhan and Liu, 2013; Grady and Markowitz, 2015).

Many cancers occur as a result of mutations in oncogenes and tumour suppressor genes. However, recent genome-wide studies on colorectal cancer revealed the existence of thousands of mutations in colon cells that could have subsequently affected the initiation and progression of CRC (Lao and Grady, 2011). Adenomatous polyposis coli (*APC*) is the most commonly mutated tumour suppressor gene in CRC. Mutated *APC* in colon cells results in dysregulation of Wnt pathway, which has been linked with the progression of non-cancerous polyps to cancerous polyps (Stigliano et al., 2014). In addition to *APC*, mutations in proto-oncogenes such as *KRAS*, *TP53* and *BRAF* occurs up to 60% of CRC, have been reported to promote proliferation and suppress apoptosis in CRC (Bardhan and Liu, 2013).

Besides genetic dysregulation, the overall pathogenesis of CRC includes chromosomal and microsatellite instability which contributes to the aneuploidy and dysregulation of DNA mismatch repair mechanisms (Stigliano et al., 2014). Changes at epigenetic level, such as DNA methylation state and dysregulation of microRNAs have also been linked with the development of CRC (Goel and Boland, 2010). Despite decades of research on CRC, many aspects of the pathogenesis are poorly understood, prompting for more comprehensive investigation which comprises the interaction between genetics and epigenetic modification in colon cells.

2.1.1 Risk Factors of CRC

Approximately up to 3% of the total CRC cases are hereditary while the vast majority of the cases are sporadic (Wills and Burt, 2002). Various risk factors have been identified that contribute to the development of sporadic CRC. Smoking, sedentary lifestyle, poor fibre diet, obesity and alcohol consumption have been listed as risk factors for CRC development (Jasperson et al., 2010; Deen et al., 2016). Furthermore, the likelihood to develop CRC is higher after the age of 50. Personal history with inflammatory bowel disease increases the risk of developing CRC from 4- to 20- fold (Haggar and Boushey, 2009). Most of the risk factors of sporadic CRC can be prevented by maintaining a healthy lifestyle.

The common syndromes of hereditary-linked CRC include familial adenomatous polyposis (FAP) and hereditary non-polyposis CRC (HNPCC) (Deen et al., 2016). Germline mutation in APC results in the development of FAP, while the mutation in the DNA mismatch repair genes results in the development of HNPCC (Lim, 2014). Therefore, the risk of developing CRC is increased with positive CRC family history, can be up to several folds (Jasperson et al., 2010). The availability of genetic and colonoscopy screening can help with the early diagnosis of some hereditary CRC (Dyson and Rutter, 2012).

2.1.2 Current CRC Treatments

The treatment regimen for CRC varies according to the stage of cancer. Patients with early-stage CRC (stage I and II) are commonly subjected to surgical removal of colon region (colectomy) while chemotherapy remains as the primary option for patients with advanced CRC (Chibaudel et al., 2012). Two major chemotherapeutic agents used in CRC treatment, 5-fluorouracil (5-FU) and Oxaliplatin (L-OHP), are administered with an adjuvant (leucovorin) in a regimen called FOLFOX (Healey et al., 2013).

5-FU has been a key component in the first-line CRC chemotherapy since 1957 (Hammond et al., 2016). 5-FU shares structural similarities with the pyrimidine molecule of DNA and RNA. As an analogue of uracil, 5-FU is actively uptake by the rapid proliferating CRC cells. The active metabolites of 5-FU will inhibit thymidylate synthase which is responsible for converting uracil to thymine (Longley et al., 2003; Singh et al., 2015). Consequently, the imbalance DNA and RNA precursors result in inhibition of DNA synthesis (Hammond et al., 2016). However, 5-FU has a poor response in advance CRC, with only up to 15% of response rate (Bracht et al., 2010).

L-OHP is a platinum analogue that has been actively in use since 1996 (Hammond et al., 2016). L-OHP causes DNA intra-strand linkages which block the action of RNA polymerase. Furthermore, L-OHP is also reported to inhibit DNA synthesis via targeting thymidylate synthase similar to the mechanism of action of 5-FU (Alcindor and Beauger, 2011). L-OHP showed higher response

rate as compared to 5-FU, up to 24% (Comella et al., 2009). However, the combined synergistic effect of 5-FU and L-OHP revealed higher response rate, as high as 50% (Hammond et al., 2016). Therefore, the combined treatment of 5-FU and L-OHP under the treatment regimen FOLFOX remains the most common CRC chemotherapy option (Hammond et al., 2016). Despite the advancement of chemotherapy approaches, the development of chemoresistance evident by the high recurrence rate suggest a need to review the current chemotherapeutic approach (Kanwar et al., 2012).

2.2 Cancer Stem Cells (CSCs)

2.2.1 Definition and Origin of CSCs

The conventional stochastic model of cancer postulates that all cells within a tumour have equal tendency to form new tumours (Puglisi et al., 2013). However, recent studies dispute the conventional model and further suggest an existence of a cellular hierarchy in a tumour. The new hierarchical model of cancer postulates that only a small population of cells within the tumour has the ability to form a new tumour (Nguyen et al., 2012; Bradshaw et al., 2016).

The small subpopulation of cells within a tumour has been described using various terms, including tumour-initiating cells (TIC), cancer-initiating cells (CIC) and tumour propagating cells (TPC). However, the subpopulation is most commonly known as cancer stem cells (CSCs) (Wang et al., 2013; Safa et

al., 2015). Besides being involved in tumour initiation, CSCs have been proposed to be involved in oncogenesis, metastasis and cancer recurrence (Borah et al., 2015). The widely accepted definition of CSC is, a cell within a tumour that is able to produce a heterogeneous population with enhanced self-renewal ability (Clarke et al., 2006). Physiologically, CSC populations are described as cancer cells with unlimited self-renewal ability and enhanced capacity to produce matured specialised cells (Frank et al., 2010).

Despite intense research on CSCs, an important central question on CSCs has yet to be unanswered; what is the cell-of-origin for CSCs (Ciurea et al., 2014). Some argue that CSCs are the result of transformed normal stem cells that lost growth control and proceeded to malignancy. Others, postulate that CSCs are somatic cells that acquired gain of function mutations that de-differentiated the cells which resulted in adapting stem-like features (Wu, 2008; Huels and Sansom, 2015). All cells, including stem cells, progenitors and differentiated cells can accumulate mutations and initiate cancer progression as the cell of origin. Nonetheless, it is widely accepted that the terminology "cancer stem cell" is a reflection on the stem-cell-like phenotype rather than the true "stemness" of the cells (Marotta and Polyak, 2009).

2.2.2 Characteristics and Features of CSCs

The first evidence that suggests the existence of CSCs was from a study conducted by John Dicks and group in 1994 on isolation of haematopoietic stem cells from acute myeloid leukaemia (AML) (Lapidot et al., 1994). Dicks initially focused on the proliferative and self-renewal potential of transplanted human AML cells *in vivo*, where the AML cells were isolated based on surface markers. AML cells expressing $CD34^{+}CD38^{-}$ were highly tumourigenic and the transplanted cells were in various stages of differentiation while closely resembling the disease of the original AML patients. These cells were known as leukaemia-initiating cells or CSCs as what we know it now (Lapidot et al., 1994; Todaro et al., 2010).

CSC population within a tumour is reported to make up between 0.1 – 10% of the total cancer cell population (Garza-Treviño et al., 2015). The microenvironment or "niche" plays an important role in maintaining the survival of CSCs. The niche refers to the physical environment in which the stem cell resides (Todaro et al., 2010). The niche is responsible for maintaining the stem-cell-like properties of CSCs, which is made up of a heterogeneous population of CSCs and differentiated cells (non-CSCs). The non-CSCs population is postulated to control the intrinsic factors that determine the fate of the CSCs, such as stromal support cells, soluble factors, extracellular matrix proteins and blood supply (Shah et al., 2014; Jones and Wagers, 2008).

The growing body of evidence supports that normal stem cells and CSCs share similarities in their self-renewal and differentiation ability (Borah et al., 2015). The self-renewal ability is one of the key features of stem cells and CSCs, which ensures the stem cell pool maintenance (Verga Falzacappa et al., 2012; Yoo and Kwon, 2015). Cells with enhanced self-renewal ability undergo asymmetric and symmetric cell division. In asymmetric cell division, stem cells divide producing one copy of the parental cells with self-renewal ability and another daughter cell for differentiation. In symmetric cell division, stem cells divide to produce two identical daughter cells with self-renewal ability (Bu et al., 2013; Yoo and Kwon, 2015). In CSCs, the balance in asymmetric and symmetric cell division is reported to maintain the self-renewal capacity and stem pool of cell population (Yoo et al., 2013).

Cellular dormancy is also a common similarity between CSCs and normal stem cells (Kleffel and Schatton, 2013). Stem cell population has slow cell cycle progression as part of the preservation of stem pool and functionality (Viale and Pelicci, 2009). Dormancy is a form of adaption of stem cells and CSCs when the cells are residing in adverse futile environments (Patel and Chen, 2012). Similar to stem cells, CSCs have high DNA repair mechanism and anti-apoptotic protein expression levels, which suppresses the apoptotic levels of the cells under futile environment. Upon entering a suitable environment, the cells exit dormancy and fosters cellular proliferation (Giancotti et al., 2013). Cellular dormancy has been associated with enhanced metastatic ability of CSCs and evasion of chemotherapy (Borst, 2012; Patel and Chen, 2012).

The full differentiation potential of CSCs with regards of potency still remains unknown. Theoretically, the plasticity of CSCs is postulated to mimic the plasticity of multipotent stem cells (Lee et al., 2016). This concurs with *in vitro* findings of single-sorted CSC producing multi-lineage differentiated cell populations (Beier et al., 2007). Furthermore, CSCs from glioblastoma, melanoma and prostate cancer had "transdifferentiated" to endothelial cells while some re-organised into blood vessel-like resembling structure (Hendrix et al., 2003; Casal et al., 2010; Wang et al., 2013).

Reports of inducing differentiation in CSCs have shown to decrease in the tumourigenicity of the cells (Azzi et al., 2011; Pham et al., 2011). Induction of differentiation causes CSCs to lose their self-renewal properties, and "stemness" factor. Several signalling pathways have been linked to the maintenance of potency in CSCs such as Wnt, Hedgehog and Notch signalling. Inhibiting the signalling pathways have been postulated as a possible CSCs eradicating approach (Cochrane et al., 2015; Evans et al., 2015; Takebe et al., 2015).

2.3 Colorectal Cancer Stem Cells (CrCSC)

Intestinal stem cells in the crypts of the colon are responsible for regenerating differentiated intestinal epithelium cells including enterocytes, goblet cells and endocrine cells, which are involved in the physiological function of the colon (Vaiopoulos et al., 2012). As the differentiated intestinal

cells reach senescence, intestinal stem cells will divide asymmetrically to restore the differentiated cell pool. The microenvironment in the colon is reported to maintain the balance of propagation and differentiation of intestinal stem cells (Sailaja et al., 2016).

Alterations in the microenvironment such as uncontrolled growth-promoting signals and accumulation of CRC-linked mutations such as *APC* and DNA mismatch repair genes in the intestinal crypt, have shown to affect intestinal stem cells (Abdul Khalek et al., 2010; Huels and Sansom, 2015). The transformed intestinal stem cells are said to initiate the CRC carcinogenesis. The likelihood that the cell of origin of CSCs in most CRC is derived from intestinal stem cells is high (Sanders, 2011; Vaiopoulos et al., 2012). The probability of a differentiated colon cell to acquire multiple mutations such as *APC* and *KRAS* is lower because of the short lifespan of a differentiated cell. Therefore, a colorectal cancer stem cell (CrCSC) carcinogenesis model with a transformed intestinal stem cell as the cell-of-origin of CSCs would be more representative in reflecting the CRC carcinogenesis (Huels and Sansom, 2015). However, the exact mechanism of the involvement of CSCs in the CRC carcinogenesis remains to be investigated.

2.3.1 CrCSCs Biomarkers

The functional roles of CSCs in various carcinogenesis are being established in recent years. However, lack of effective and standardised strategies in identifying and isolating CSC population limited the investigations on the properties of CSC (Vaiopoulos et al., 2012; Templeton et al., 2014). CSC research is moving towards the identification of exclusive fundamental properties of CSC such as epigenetic alterations and differential gene expression profiles for isolation purposes. However, isolation based on immunohistochemistry analysis using surface marker antigens still remains prominent (Abdul Khalek et al., 2010; Fanali et al., 2014). It is important to note that the most of reported CSCs markers are highly expressed in non-CSC population and display variation in the expression levels across independent studies, indicating the lack of reliability in the markers (Garza-Treviño et al., 2015).

Several potential markers have been proposed as potent CrCSC markers including CD133, CD44 and Aldehyde dehydrogenase 1 (ALDH1) (Sahlberg et al., 2014; Kozovska et al., 2014). Other markers such as CD24, CD29, CD166 and Lgr5 have been previously associated with CrCSC population, however, further investigation is required to study potential use as CrCSCs isolation markers (Fanali et al., 2014).

CD133, a five-transmembrane glycoprotein with the function remains unclear to date (Ren et al., 2013). CRC expressing $CD133^+$ cells were reported to form tumours *in vivo* as compared to $CD133^-$ counterpart (Ricci-Vitiani et al., 2007; O'Brien et al., 2007). In another study, the $CD133^+$ derived CRC tumours displayed morphological similarity to their parental derivatives (Horst et al., 2008). $CD133^+$ cells have been also associated with chemoresistance, enhanced metastatic and invasive ability (Fanali et al., 2014).

On the other hand, it was reported that only one out of 262 CRC $CD133^+$-expressing cells could be a true CSC (O'Brien et al., 2007). It is also demonstrated that CD133 lack of restriction to CSC and stem cell population as it is expressed in various types of differentiated cells. Furthermore, both $CD133^+$- and $CD133^-$-expressing CRC cells have been reported to form tumours *in vivo* (Shmelkov et al., 2008). The contradicting findings of CD133 further questions the candidacy of CD133 as a potent CrCSC marker.

CD44 has been commonly associated with CSC population derived from CRC, breast, pancreatic and gastric cancer (Hurt et al., 2008; Li et al., 2009; Takaishi et al., 2009). CD44 is reported to be involved in cell adhesion, motility, proliferation and cell survival (Keysar and Jimeno, 2010). Knockdown of CD44 in primary CRC showed a reduction in clonogenicity and tumourigenicity suggesting CD44 plays a role in regulating the "stemness" in CrCSCs (Du et al., 2008).

Conversely, several reports showed contradicting findings on the role of CD44 in the involvement of CrCSCs. The loss in CD44 expression in CRC has been linked with CRC tumour progression (Lugli et al., 2010). There is a lack of significant correlation of CD44 and survival of CRC patients (Choi et al., 2009). The differences in the findings could be contributed due to the expression of various splice variants of CD44 (Ozawa et al., 2014).

ALDH1 is an endogenous enzyme that is involved in protecting stem cells against oxidative insults. ALDH1 converts retinol to retinoic acid, an important modulator in cell proliferation and differentiation (Huang et al., 2009; Shenoy et al., 2012). ALDH1 activity is often used in refining CSCs population as the high expression of ALDH1 is linked with the "stemness" properties of the cells. Huang et al. (2009) reported that *in vivo* transplantation of $ALDH1^+$ CRC cells were able to generate xenograft tumours while $ALDH^-$ counterpart cells were not able. The high expression of ALDH1 has been correlated with a poor CRC prognosis (Zhou et al., 2014). However, there is a lack of relationship between ALDH1 and the survival time for CRC patients (Hostettler et al., 2010).

The inconsistency in the roles of the CrCSC markers has prompted many reports on the analysis of co-expression of multiple CrCSC markers. CRC cells co-expressing $CD133^+/CD44^+$ were more tumourigenic (Haraguchi et al., 2008). Similarly, $CD44^+/CD166^+$ CRC cells displayed higher tumourigenic ability compared to $CD44^+/CD166^-$, $CD44^-/CD166^-$ and $CD44^-/CD166^+$-expressing CRC cells (Dalerba et al., 2007). Together, the reports suggest that the usage of multiple markers would be a useful tool in

identifying CrCSCs. Despite this, the identification of CrCSC population remains a great challenge due to lack of specific markers (Cherciu et al., 2014). Therefore, novel specific CrCSC biomarkers are in need to further understand the characteristics and regulation of CSCs (Hung et al., 2015).

2.4 Spheroidal Culture and CSCs

2.4.1 Principle of Spheroidal Culture

In vivo tumourigenic assay is the gold standard for functional assay to study CSCs, however spheroidal culture has been used as a complementary *in vitro* assay to culture CSC population (Alison et al., 2011). The spheroidal culture was first used by to investigate the potency of neuronal cells. Since the emergence of CSCs theory, spheroidal culture has gained popularity due to the ability of the culture to generate stem cell-like population (Pastrana et al., 2011).

The spheroidal culture involves the culturing of cells in an anchorage-independent manner (Qureshi-Baig et al., 2016). The normal 2D monolayer cell culture, where the cells are grown on a flat surface as an adherent population has limitations including unnatural microenvironment. As most of the cells *in vivo* are enclosed by other cells and complex extracellular matrix, thus the monolayer culture is often reported to provide misleading results (Edmondson et al., 2014).

On the other hand, spheroidal culture is able to mimic the microenvironment by facilitating the oxygen and nutrient delivery to the cells, and the cell-to-cell adhesion. The microenvironment created by the spheroidal culture provides a fair comparison of the *in vivo* microenvironment (Bielecka et al., 2016). It was also reported that cells derived from spheroidal culture are morphologically and physiologically different as compared derivatives of monolayer culture (Baharvand et al., 2006).

Spheroidal culture is an anchorage-independent culture which reduces the cell-matrix interaction. The anchorage-independent culture prompts the non-malignant or differentiated cells to be negatively selected and undergo anoikis-induced apoptosis (Cammareri et al., 2008; Weiswald et al., 2015). Anoikis is a programmed cell death which takes place when a cell detaches from their home ground. Therefore, spheroidal culture will serve as a preliminary positive selection of cells in higher level of hierarchy. Cells with higher "stemness" will proliferate and undergo clonal expansion which results in the formation of floating spheroids (Phung et al., 2011; Weiswald et al., 2015).

2.4.2 Enrichment of CrCSCs using Spheroidal Culture

Enrichment of CSCs from various type of cancers have been established using spheroidal culture, including prostate, lungs, ovarian, colorectal and breast cancer (Collins et al., 2005; Eramo et al., 2008; Zhang et al., 2008; Hwang et al., 2011; Boo et al., 2016). The cells derived from spheroidal culture

showed enhanced properties associated with CSCs such as self-renewal ability and chemoresistance, as compared to parental cells.

Ricci-Vitiani et al. (2007) reported that $CD133^{+}$-expressing CRC cells which were grown in spheroidal culture over a year, maintained the undifferentiated state of the cells and reproduce tumours that were morphologically similar to the parental. Emmink et al. (2011) demonstrated that CRC cells grown as spheroids were highly tumourigenic and chemoresistant. Besides that, the differentiated spheroid CRC cells yield a heterogeneous population of the various type of colon residing cells such as mucin-producing goblet cells. Similar findings were also reported by Rajcevic et al. (2014) where CRC cells grown in spheroidal culture produced a heterogeneous population of cells types and showed up-regulation of biological processes related with CSCs.

Some studies have reported that the population derived from spheroidal culture showed no CSCs enrichment (Kai et al., 2009; Wu et al., 2014; Calvet et al., 2014). The differences in the findings could be due to the lack of standardised protocol in establishing spheroidal culture to enrich CSC population. Medium composition, extracellular matrix and growth factors used in the spheroidal culture are postulated to affect the functional properties of the spheroid-cultured cells (Qureshi-Baig et al., 2016).

Despite inconsistency in the observation of the spheroid-cultured cells, it has been demonstrated that spheroidal culture is able to generate a population that resembles CSCs. More investigations are needed to reduce the variabilities

and inconsistencies observed, and subsequently, improve the quality of spheroidal culture.

2.5 CSC-Mediated Chemoresistance

The development of chemoresistance in cancers possesses a major limitation to chemotherapy (Abdullah and Chow, 2013). The current failure rate of chemotherapy is as high as 50% among all cancer patients and 90% metastatic cancer patients (Longley et al., 2003; Dallas et al., 2009). The strategies of most of the current chemotherapeutic drugs involve the inhibition of proliferation and induction of apoptosis in the rapidly-dividing cells, a hallmark of cancer. The effectiveness of treatment regimen is often measured by the ability of the drugs to reduce the tumour size. However, slow-dividing and quiescent cells will not be affected by the current treatment approach (Lind, 2011).

CSC population is hypothesised to be a key component in the development of chemoresistance against the current treatment regimen (Zhao, 2016). CSC population is protected by various chemoresistance mechanisms and survive the chemotherapy.The CSC population which survived the treatment could progress into a more malignant form of cancer with enhanced chemoresistance. Recurrent cancer often results in poor prognosis (Vinogradov and Wei, 2012). The chemoresistance in CSC population are attributed by two

factors; intrinsic and extrinsic factors. The factors will be discussed in the next subsections (Section 2.5.1 and 2.5.2).

2.5.1 Intrinsic Factors of CSC-Mediated Chemoresistance

Intrinsic factors that contribute to the chemoresistant properties in CSC population include high expression of ATP-binding cassette (ABC) transporter proteins, dysregulated DNA repair mechanism, loss of balance between pro- and anti-apoptotic proteins, over-expression of ALDH1 enzyme and cellular dormancy (Chu and Allan, 2011; Buhagiar and Ayers, 2015; Zhao, 2016). The chemoresistance conferred via ABC transporter proteins will be discussed in the next section (Section 2.6).

Cancer cells are reported to confer chemoresistance by altering the DNA repair mechanism. Most of the chemotherapeutic drugs aim to induce DNA damage in cancer cells which would subsequently lead to apoptosis. However, the altered DNA repair mechanism allows the cancer cells to survive and pass down the altered mechanism to their daughter cells (Thomas et al., 2014). It is reported that CSC population shows enhanced DNA repair mechanism (Zhao, 2016). The CrCSC population that expresses $CD133^{+}$ antigen marker was more chemoresistant as a result of over-expression DNA repair protein, CHK1 (Gallmeier et al., 2011). Knockdown of CHK1 in radio-resistant glioblastoma CSCs and chemoresistant pancreatic CSCs increased the chemo-sensitivity of the cells (Squatrito et al., 2010; Venkatesha et al., 2012).

Evasion of apoptosis is another hallmark of cancer. Chemoresistant cells are typically linked with over-expression of anti-apoptotic proteins such as Bcl-2 and Bcl-xl, and inhibition of pro-apoptotic proteins such as Bax and BAD (Rebucci and Michiels, 2013). The expression pattern of the apoptosis-regulating proteins which favours the proliferation of cancer cells renders the chemotherapy ineffective. Hepatocellular carcinoma CSC population showed increased Bcl-2 expression levels after treatment with doxorubicin and 5-fluorouracil. Subsequently, the sensitivity of the CSC population to the drugs was increased by inhibiting Bcl-2 protein expression (Ma et al., 2008). Similar findings were reported in CrCSC population (Todaro et al., 2007).

ALDH1 is a detoxification enzyme that oxidises aldehyde into carboxylic acid (Vinogradov and Wei, 2012). Although ALDH1 is commonly used as CSC identification marker in various type of cancers, the enhanced expression of ALDH1 in CSC population has been linked with the development of chemoresistance to multiple drugs such as cyclophosphamide and doxorubicin (Thomas et al., 2014). $ALDH1^{+}CD44^{+}ESA^{+}$ CrCSC survived cyclophosphamide treatment, however showed decreased survival when pre-treated with ALDH1 inhibitor (Dylla et al., 2008). Similarly, chemoresistant pancreatic CSC which expressed high ALDH1 levels showed increased sensitivity to gemcitabine after ALDH1 knockdown (Duong et al., 2012).

As mentioned earlier, most of the chemotherapeutic drugs target a specific characteristic of cancer cells, the rapid proliferation of cancer cells. Rapidly dividing cancer cells are more vulnerable against chemotherapeutic

drugs as the drug up-take rate will be higher due the high metabolism rate. Besides that, as the cells enter S-phase faster, the DNA damage from the drugs will be failed to repair, resulting in initiation of apoptosis (Chan et al., 2012). However, CSC population has been reported to be relatively quiescent or dormant compared to the non-CSC population. Therefore, CSCs are less likely to be affected by cell-cycle targeted chemotherapeutic agents (Vinogradov and Wei, 2012; Thomas et al., 2014). Induction of dormant leukemic CSC population to progress from cell cycle arrest showed increased chemo-sensitivity, linking the cellular dormancy of CSC population and chemoresistance (Saito et al., 2010).

2.5.2 Extrinsic Factors of CSC-Mediated Chemoresistance

The extrinsic factors of chemoresistance in CSCs are external signals that affect the regulations of the cells, including epithelial-mesenchymal transitions (EMT) and microenvironment (Verwey et al., 2016). EMT is a physiological process of epithelial cells that allows the cells to migrate during embryogenesis. The epithelial cells will undergo morphological transformation to acquire mesenchymal-like phenotype which causes the cells to lose cell-to-cell contact and gain motility. Similarly, EMT is involved in wound healing process which allows the cells to move to the region of wound (Zhao, 2016). Mesenchymal phenotype as compared to the epithelial phenotype has been linked with chemoresistance in pancreas, breast and colon cancers (Sabbah et al., 2008; Kim et al., 2009; Wang et al., 2011).

Cancer cells induce EMT process which allows the cells to migrate to distant region and acquire metastatic properties (Bagnato and Rosanò, 2012). EMT induction has been associated with CSC population and the development of chemoresistance. CSCs and EMT induced cells have been reported to share similar molecular profile (Mani et al., 2008; Ahmed et al., 2010). Notch signalling pathway which regulates the EMT induction, is an important pathway in CSC maintenance (Ranganathan et al., 2011). Besides maintaining CSC properties in various cancer and regulating EMT induction, Notch signalling activity is reported to be up-regulated in chemoresistant CSC population. The knockdown of Notch signalling showed a reduction in chemoresistance in pancreatic CSC (Hindriksen and Bijlsma, 2012; Capaccione and Pine, 2013).

CSCs are hypothesised to be protected by microenvironmental factors such as hypoxia, extracellular matrix and cell mixture of a cancerous and non-cancerous population (Vinogradov and Wei, 2012). The specific niche created is advantageous to CSC population as protects the cells from the effect of chemotherapeutic agents (Paldino et al., 2014). Hypoxia or lack of oxygen is believed to be the primary feature of the microenvironment in where CSCs reside. Hypoxic-induced factors which respond to hypoxic conditions activates down-stream regulators which prevent cellular differentiation and apoptosis (Harada et al., 2007). Reports suggest that hypoxia favours the enrichment of CSC population (Lagadec et al., 2013). Glioblastoma CSC cells which were exposed to hypoxic condition showed higher chemoresistance to temozolomide as compared to the cells exposed to the non-hypoxic condition (Pistollato et al., 2010).

Many mechanisms, either inherited or acquired, by CSC population impose great challenges to the current chemotherapeutic approaches. There is a need to review the current cell-based chemotherapeutic drugs in an attempt to overcome the high recurrence rate due to the development of chemoresistance in cancer (Kanwar et al., 2012).

2.6 ATP-Binding Cassette (ABC) Transporters

Adenosine triphosphate (ATP)-binding cassette (ABC) transporters are transmembrane proteins responsible for regulating the movement of substance in and out of the cells. ATP hydrolysis by ABC transporters causes the conformational change in the ABC transporter protein structures which allows the movement of substance against concentration gradient. ABC transporters are ubiquitously expressed by all living organisms, including bacteria, plants and animals (Locher, 2016). Structurally, all ABC transporters have a common architecture with the core transporter made up of two nucleotide-binding domains and two transmembrane domains (Vasiliou et al., 2009). The presence of additional domains in ABC transporters and phylogenetic analysis have been used to classify the proteins in ABC transporter superfamily. However, most ABC transporters can be divided into two groups based on functionally; importers and exporters. Importers have remained exclusive to prokaryotes while exporters are common in all types of organisms (Wilkens, 2015).

2.6.1 ABC Transporter Genes in Humans

ABC transporters in humans facilitate the movements of various substrates including small molecules such as amino acids and large molecules such as complex sugar (Theodoulou and Kerr, 2015). To date, there are 49 members of ABC transporter superfamily found in the human genome which are divided into 7 subfamilies as shown in Table 2.1 (Vasiliou et al., 2009). The subfamilies of ABC transporters are ABCA, ABCB, ABCC, ABCD, ABCE, ABCF and ABCG.

Defects in the physiological function of ABC transporter proteins due to mutations have been associated with various human diseases. Tangier disease, a deficiency in high-density lipoprotein, is caused by mutation in *ABCA1*. Stargardt disease, a form of retinal degeneration results from mutation in *ABCA4*. Besides that, mutation in *ABCA7* is reported as a risk factor in the development of late-onset Alzheimer's disease (Dean et al., 2001; Theodoulou and Kerr, 2015). The most highlighted disease resulted from mutation in ABC transporter is cystic fibrosis. Cystic fibrosis is a fatal disease manifested at a young age due to the of accumulation of excess mucous in several organs which leads to deadly infections. Mutation in *ABCC7* or also known as cystic fibrosis transmembrane conductance regulator affects the balance of water potential and ions. Subsequently, the loss of homeostasis increases the viscosity of mucous (Dean et al., 2001; Cant et al., 2014).

Table 2.1 Members of human ABC transporter superfamily

(Adapted from Vasiliou et al. 2009)

ABC subfamily	Alias	Number of members
ABCA	ABC1	12
ABCB	MDR (Multidrug resistance)	11
ABCC	MRP (Multidrug Resistance Protein)	13
ABCD	ALD (Adrenoleucodystrophy)	4
ABCE	OABP (Oligoadenylate binding protein)	1
ABCF	GCN20 (General control nonderepressible 20)	3
ABCG	White	5
	Total	49

2.6.2 Association of ABC transporter with Chemoresistance

Besides being involved in the transport of substances across of the cells, ABC transporters also provide cellular protective against various harmful substances, as evident by the high expression levels in stem cells and vital regions of the human body (de Jonge-Peeters et al., 2007; Fletcher et al., 2010). For example, ABCB1 protein which is found in the blood-brain barrier, blood-testes barrier and blood-placenta barrier, which effluxes harmful substances out of these regions (Xiong et al., 2015). The high expression levels of ABCG2 protein in the mammary gland is reported to concentrate the toxins released into the milk (Jonker et al., 2005).

However, the protective features of ABC transporters have been linked with the development of chemoresistance in various cancers. Through the efflux ability, ABC transporters reduce intracellular drug concentrations, thus diminishing the effect and activity of drugs (Kim et al., 2014). Even though many members of the ABC transporter superfamily have been linked with chemoresistance in various cancers, only three protein members have been thoroughly studied; ABCB1, ABCC1 and ABCG2 (Kim et al., 2014; Zhao, 2016).

ABCB1 or also known as multidrug resistance protein 1 (MRP1) have been associated with chemoresistance in pancreas, colon and kidney (Zhao, 2016). In colon, ABCB1 is expressed in the epithelial cells of the intestinal lumens provide protection against diet-derived xenobiotics (Hlavata et al.,

2012). In a recent study, the over-expression of ABCB1 proteins is linked with the development of acquired chemoresistance of CRC (Liu et al., 2013; T. Wang et al., 2015). ABCC1 or multidrug resistance-associated protein 1 (MRP1), commonly found in lungs, testes and kidneys, is involved in efflux substance in the central nervous system (Xiong et al., 2015). Due to the high levels of expression of ABCC1 proteins in various solids and hematopoietic cancers such as CRC and chronic lymphocytic leukaemia, ABCC1 is often used as predictive marker for the development of chemoresistance (Kunická and Souček, 2014; Zhao, 2016).

ABCG2 or breast cancer resistance protein (BCRP) is not only highly expressed in breast cancer, but is also expressed in CRC, liver cancer and kidney cancer (Xiong et al., 2015). ABCG2 is reported to efflux a broad spectrum of substrates out of the cells, including various chemotherapeutic drugs such as mitoxantrone and topotecan. The enhanced efflux capacity of ABCG2 is associated with the development of chemoresistance in many cancers (Shigeta et al., 2010; Robey et al., 2010).

Ongoing research on ABC transporters revealed a large group of ABC superfamily members are associated with the development of chemoresistance in various chemotherapeutic drugs as listed in Table 2.2. Due to the adverse effects of ABC transporters in chemotherapy treatment, clinical trials using ABC transporter inhibitors are ongoing. However, identification of ABC transporter inhibitors with high specificity remains a challenge (Falasca and Linton, 2012).

Table 2.2 ABC transporter members and chemotherapeutic drugs efflux from cells

(Adapted and modified from Kunická and Souček 2014)

Gene	Drug Substrates
ABCA2	Estramustine and mitoxantrone
ABCA3	Doxorubicin, daunorubicin and imatinib
ABCB1	Anthracyclines, methotrexate, irinotecan and taxanes
ABCB4	Vinblastine and doxorubicin
ABCB5	5-Fluorouracil and doxorubicin
ABCB11	Paclitaxel
ABCC1	Etoposide, anthracyclines, irinotecan and imatinib
ABCC2	Etoposide, anthracyclines, irinotecan and imatinib
ABCC3	Etoposide
ABCC4	Irinotecan, methotrexate and thiopurines
ABCC5	Cisplatin, methotrexate and thiopurines
ABCC11	5-Fluorouracil
ABCC12	Not identified
ABCG2	Doxorubicin, irinotecan, methotrexate and mitoxantrone

Recent reports linked the chemoresistance properties of CSC population with over-expression of ABC transporter proteins. Melanoma CSC population with enhanced chemoresistance, expressed high levels of the ABCB5 protein. Knockdown of ABCB5 restored the drug sensitivity towards doxorubicin (Frank et al., 2005). ABCC1 is linked with the enhanced survival rate in CD133-expressing glioblastoma CSCs (Jin et al., 2010). ABCG2-expressing hepatocellular carcinoma showed CSC-like phenotype with increased malignancy and chemoresistance (G. Zhang et al., 2013).

However, there is a lack of reports on the expression profile of CrCSC population. The increasing evidence on the role of ABC transporters in one of the major characteristics of CSCs population, chemoresistance, provides an opportunity to use ABC transporters as specific CSC markers. Therefore, more investigations are needed to generate ABC transporter profile on CrCSC population in an attempt to identify potent CrCSC specific markers.

CHAPTER 3

MATERIALS AND METHODS

3.1 Cell Culture

All cell culture activities were performed aseptically in Airstream Class II Biological Safety Cabinets (BSC II, ESCO, Singapore). The cabinets were sterilised by using ultraviolet light and 70% ethanol. Cells were maintained in humidified incubator (ESCO) at 37 °C in an atmosphere of 5% CO_2.

3.1.1 Colorectal Cancer Cell Lines

Human colorectal cancer (CRC) cell lines used in the present study include HCT-15, WiDr and SK-CO-1 were bought from American Type Culture Collection (ATCC, USA). Based on the certificate of analysis of the CRC cell lines, HCT-15 was derived from a male patient with Dukes' Type C colon cancer, WiDr was from a 44 years old female colon cancer patient while SK-CO-1 was from metastatic ascites of colon cancer of 65 years old male patient.

3.1.2 Culture Reagents and Materials Preparation

The reagents and materials used in the present study were prepared as described Table 3.1.

3.1.3 Preparation of Culture Medium

Dulbecco's Modified Eagle Medium (DMEM) high glucose and Minimum Essential Medium (MEM) were used to prepare serum-containing medium (SCM) for the maintenance of 2D monolayer-culture of CRC cell lines. The mediums were prepared according to the manufacturer's instruction (Gibco^{TM}). DMEM high glucose powder was dissolved in 900 mL of deionised water supplemented with 3.7 g of sodium bicarbonate powder (Merck Millipore, USA) using magnetic stirrer (Thermo Fisher Scientific). MEM powder was dissolved in 900 mL of deionised water supplemented with 2.2 g of sodium bicarbonate powder. The pH of the mediums was adjusted to a final range of pH 7.2 to 7.4 using a pH meter (Jenco, California, USA) and the final volume was brought up to 1 L. The mediums were sterilised using 0.22 µm cellulose acetate filter (Sartorius) attached to a vacuum pump system (Gast, Michigan, USA). The complete SCMs were prepared by supplementing 10% (v/v) foetal bovine serum (FBS, Gibco^{TM}) and 1% (v/v) Penicillin-Streptomycin (Penstrep, Gibco^{TM}). The sterilised and complete mediums were stored at 4 °C until further use.

Table 3.1: Culture reagents and materials preparation

Reagent	Preparation method
0.4% (v/v) Bovine Serum Albumin (BSA)	Stock solution of 10% (v/v) of BSA was prepared by dissolving 4 grams of BSA powder (Nacalai Tesque, Japan) in 40 mL of deionised water at 4 °C overnight. The final concentration of 0.4% (v/v) was prepared by mixing 20% (v/v) of the prepared BSA stock with 80% (v/v) of medium.
Epidermal Growth Factor (EGF)	EGF (Miltenyi Biotech, Germany) was prepared according to manufacturer's instructions. The stock solution was prepared by dissolving EGF powder in sterile deionised water to achieve a final concentration of 0.1 mg/mL. The stock solution was stored at –20 °C for long-term storage. The stock was diluted to 1 µg/mL with 0.1% BSA-PBS solution. A final concentration of 20 ng/mL was added into the medium.
Basic Fibroblast Growth Factor (bFGF)	bFGF (Miltenyi Biotech) was prepared the same way as described for EGF. A final concentration of 10 ng/mL was added into the medium.
PolyHEMA-coated culture flasks and plates	Polyhydroxyethylmethacrylate (PolyHEMA) powder (Sigma-Aldrich, USA) was dissolved in

	95% ethanol (Thermo Fisher Scientific, USA) to create a stock concentration of 24 mg/mL. Magnetic stirrer was used to stir the solution overnight. The solution was sterilised using 0.22 µm cellulose acetate filter (Sartorius, Germany) and stored at 4 °C until further use. Sterile T75 non-treated culture flasks (Nest, China) and sterile flat bottom 96-well culture plates (BD Biosciences, USA) were added with 7 mL and 60 µL of polyHEMA solution respectively, in a BSC II and left to air dry overnight. Second coating layer of polyHEMA coating was added to the pre-coated flasks and plates, which were left to dry overnight under sterile conditions in BSC II. The coated flasks and plates were sterilised by UV radiation for 30 minutes in BSC II. The flasks and plates were sealed with parafilm, and stored at 4 °C in dark until further use.
0.24% (v/v) Methylcellulose solution	Methylcellulose stock solution was prepared by autoclaving 6 grams of methylcellulose powder (Sigma-Aldrich) in 500 mL flask containing a magnetic stirrer to create 1.2 % (v/v) solution. The autoclaved methylcellulose powder was dissolved in 250 mL of 60 °C preheated sterile

	DMEM F-12 basal medium for 20 minutes. DMEM-F12 basal room which was warmed to room temperature was added to a final volume of 500 mL. The stock solution was stirred overnight at 4 °C. The stock solution was aliquoted into 50 mL centrifuge tube (BD Biosciences) and centrifuged at 5,000 xg in room temperature for 2 hours (Allegra® X-15R, Beckman Coulter, California, USA). The stock solution was stored at -20 °C until further use. The final concentration 0.24% of methylcellulose was prepared by mixing 20% (v/v) of the methylcellulose stock and 80% (v/v) serum free medium (SFM).
4% Paraformaldehyde	Paraformaldehyde powder (Sigma-Aldrich) was measured and 0.4 g was transferred into 15 mL centrifuge tube (BD Biosciences) containing 10 mL of 1×PBS. The tube was gently agitated to mix the powder. The tube was heated in water bath at 60 °C for 30 minutes. The PFA was prepared freshly for every use.

Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM F-12, Gibco™) was used to prepare serum-free medium (SFM) for the spheroidal culture of CRC cell lines. DMEM F-12 powder was dissolved in 900 mL of deionised water supplemented with 3.024 g of sodium bicarbonate powder. The pH of the mediums was adjusted to a final range of pH 7.2 to 7.4 using a pH meter (Jenco) and the final volume was brought up to 1 L. The medium was sterilised as mentioned above. The complete SFM was prepared by supplementing the sterilised DMEM F-12 with 0.24% (v/v) methylcellulose (Sigma-Aldrich), 1× B27 supplement (Gibco™), 20 ng/µL of epidermal growth factor (Miltenyi Biotec), 10 ng/µL of basic fibroblast growth factor (Miltenyi Biotec), 0.4% (v/v) of bovine serum albumin (Nacalai Tesque) and 1 mg/mL of insulin (Gibco™). The sterilised and complete mediums were stored at 4 °C until further use.

3.1.4 Revival of Cryopreserved Cells

Cryovials frozen in nitrogen tank were thawed immediately in 37 °C water bath (Memmert, Germany). The thawed cells were transferred into sterile 15 mL centrifuge tubes (BD Biosciences) containing 9 mL of pre-warmed complete medium. The cell suspensions were centrifuged at 2,000 rpm for 5 minutes using a benchtop centrifuge (Allegra® X-15R, Beckman Coulter). The supernatants were discarded and the cell pellets were suspended in 1 mL of complete medium and transferred to T75 culture treated flasks (Nunc, USA). The cells were maintained at 37 °C in 5% CO_2 in humidified incubator (ESCO).

3.1.5 2D Monolayer-Culture and Maintenance

The CRC adherent cells which were cultured as 2D monolayer were maintained in SCM. HCT-15 was cultured in DMEM high glucose and, WiDr and SK-CO-1 in MEM. The mediums were changed once every two to three days. The cells were subcultured upon 70% to 80% confluent. The mediums were discarded and the cells were washed with 1x Phosphate Buffered Saline (PBS, Amresco) to remove traces of serum. The PBS was discarded and pre-warmed 0.25% (v/v) Trypsin-EDTA solution (Gibco^{TM}) was added into the culture flask. The flask was incubated at 37 °C in 5% CO_2 in humidified incubator for 3 to 5 minutes to allow enzymatic dissociation of the cells from the flask. The flask was observed under an inverted phase contrast light microscope (Nikon) to check for cellular dissociation. Once the cells were completely detached from the flask, complete medium was added into the flask to stop the enzymatic activity of trypsin. The cell suspension was transferred into centrifuge tubes and centrifuged at 2,000 rpm for 5 minutes. The supernatant was discarded and the cell pellet was suspended using 1x PBS. The total cell concentration was determined using trypan blue exclusion assay. The cells were diluted with 0.4% (v/v) trypan blue solution (MP Biomedicals, USA) and counted using haemocytometer (Hirschmann, Germany). The cell concentration was determined by the formula: cell concentration per mL = (average cells counted) × (dilution factor) × 10^4. The cells were seeded in a ratio of 1:10 into a new culture treated flask for further propagation.

3.1.6 Spheroidal Culture and Maintenance

CRC cells grown in the spheroidal culture were maintained in SFM. Cells grown in 2D monolayer-culture were dissociated into single cell suspension and seeded at a cell density of 5×10^4 cells per mL in 10 mL of SFM on polyhydroxyethylmethacrylate (PolyHEMA, Sigma-Aldrich)-coated T75 non-treated culture flask (Nest). The cells were incubated at 37 °C in 5% CO_2 in humidified incubator for 14 days. On day 7 of the spheroidal culture, 7 mL of SFM were added into the flask. On day 14, the medium containing the spheroids formed were transferred into 50 mL centrifuge tube (BD Biosciences) and centrifuged at 1,800 rpm for 5 minutes. The supernatant was discarded and the cell pellet was suspended in 2 mL of StemPro® Accutase (Gibco™). The centrifuge tube was loosely capped and left to stand in the humidified CO_2 incubator for 5 to 7 minutes. The cells were gently resuspended to form a single cell suspension. The Accutase dissociation activity was stopped by adding 8 mL of 1× PBS into the centrifuge tube. The cells were centrifuged at 1,800 rpm for 5 minutes. The supernatant was discarded and the cell pellet was suspended in 1 mL of 1× PBS. The cell concentration was determined using haemocytometer and the cells were reseeded in the same condition for further propagation. The first passage was labelled as “P0” and subsequent passages were labelled accordingly (P1, P2, P3, P4 and P5).

3.1.7 Cryopreservation of Cells

Following the dissociation of the cells from 2D monolayer-culture and spheroidal culture as described in Section 3.1.5 and 3.1.6, the cells were suspended in 5 mL of 1× PBS and centrifuged at 2,000 rpm for 5 minutes, to remove debris from the cell pellets. The supernatants were discarded and the cell pellets of the monolayer-culture were suspended with 90% (v/v) cold FBS and 10% (v/v) dimethylsulfoxide (DMSO, Sigma-Aldrich), while the cell pellets of the spheroid-cultured cells were suspended with Cell Reservoir One (Nacalai Tesque) serum-free cell culture freezing medium. The cell suspensions of both cultures were aliquoted into cryovials (Corning, USA) and left in -80 °C freezer (ESCO) overnight. The cryovials were transferred into liquid nitrogen container (Chart Industries, USA) for long term-storage.

3.2 Characterisation of Spheroid-Cultured CRC

The spheroid-cultured cells were analysed for colorectal cancer stem cells characteristics. The details of the characterisation analysis are described below.

3.2.1 Analysis of CrCSCs Related Surface Markers

Colorectal cancer stem cell related markers, CD133, CD44 and Aldehyde dehydrogenase 1 (ALDH1) were analysed using flowcytometry. The cells were dissociated using Accutase to produce single cell suspension as described in Section 3.1.5 and 3.1.6. The cells were suspended in PBS and the cell concentration was determined using haemocytometer. Six 5 mL flowcytometry tubes (BD Biosciences), labelled as "A", "B", "C", "D", "E" and "Control" were prepared for each cell type. The cells were seeded at 5×10^5 cells in 1 mL into tube A to E. The ALDH1 marker was analysed using ALDEFLOUR KIT (Stemcell Technologies, Canada) according to the manufacturer's instructions. The tubes A and B were added with 5 µL activated ALDEFLUOR™ Reagent. The control tube which was added with 5 µL of ALDEFLOUR^{TM} DEAD Reagent. From tube A, 0.5 mL of solution containing activated ALDEFLUOR™ Reagent and cell suspension was transferred into the control tube. Tube A to E and the control tube were incubated for 30 minutes in 37 °C incubator. The tubes were centrifuged at 1,800 rpm at 4 °C for 5 minutes. The supernatants were discharged and the pellets were washed with 1 mL of 0.5% BSA – PBS solution. The pellets were resuspended in 100 µL of 0.5% BSA – PBS solution. Tubes A and B were added with 10 µL of CD133-conjugated PE antibody (Miltenyi Biotech), tubes A and C were added with 10 µL CD44-conjugated APC antibody (BD Biosciences). Tube D was added with 10 µL isotype control for CD133-conjugated PE antibody (mouse IgG_{1K}, Miltenyi Biotech) and tube E was added with 10 µL isotype control for CD44-conjugated APC antibody (rat IgG_{2bk}, BD Biosciences). A summary table

of the tubes is shown in Table 3.2. All the tubes were incubated on ice for 20 minutes. The tubes were centrifuged and wash with 0.5% BSA – PBS solution. The pellets were suspended with 300 µL of 0.5% BSA – PBS solution and left on ice until acquisition.

The readings of the flowcytometry analysis were acquired using FACS Canto-II analyser (BD Biosciences). The compensation and acquisition were analysed using (BD FACSDIVA™, BD Biosciences) software. The analyses were done in triplicates.

Table 3.2 Set-up for flowcytometry analysis

Tubes	CONTROL	A	B	C	D	E
Activated ALDEFLUOR™ Reagent	5 µL	5 µL				
ALDEFLOUR^{TM} DEAD Reagent	5 µL					
CD133-conjugated PE antibody		10 µL	10 µL			
CD44-conjugated APC antibody		10 µL		10 µL		
CD133-conjugated PE antibody (mouse $IgG_{1K)}$					10 µL	
CD44-conjugated APC antibody (rat $IgG_{2bk)}$						10 µL

3.2.2 Cell Proliferation Assay

The proliferation rate of the cells was analysed using MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Parental CRC cells and spheroid-cultured cells from P5 were seeded as triplicates in flat bottom 96-well culture plates with their respective SCM. The cells were seeded at 5×10^3 cells in 100 µL per well. The 96-well plates were incubated for 24, 48, 72 and 96 hours. At each time point, 10 µL of 5 mg/mL MTT solution (Sigma-Aldrich) was added and the plates were incubated at 37 °C for 2.5 hours in dark. The supernatant was aspirated. A total of 100 µL of dimethylsulfoxide solution was added to each well and gently mixed. The absorbance of each plate was read at 570 nm using Infinite M200 PRO Microplate Reader (Tecan, Männedorf, Switzerland). The assay was performed in three independent experiments.

3.2.3 Colony Forming Assay

The colony forming ability of the cells were analysed using serum-free methylcellulose-based medium (MethoCult™, Stemcell Technologies). MethoCult™ was prepared and stored as described below. MethoCult™ was thawed overnight at 4 °C. In a sterile BSC II, 2.4 mL of MethoCult™ medium was aliquoted into 15 mL centrifuge tubes (BD Biosciences) using 6 mL Luer lock syringe attached to a 16 gauge blunt-end needle (Terumo, New Jersey, USA). Complete MethoCult™ medium was prepared by adding EGF at

20 ng/mL, bFGF at 10 ng/mL and 1x B27 per mL into the 2.4 mL aliquoted MethoCult™ medium. The final volume was adjusted to 3 mL by adding sterilised DMEM F-12 medium. The complete MethoCult™ medium was stored at -20 °C until further use.

For colony forming assay, the complete MethoCult™ medium was thawed overnight at 4 °C. Single cell suspensions of the adherent cells and spheroid-cultured cells from P5 were prepared at a density of 1×10^6 cells per mL were prepared in PBS. A final medium volume of 1.1 mL was prepared by mixing 0.1 mL of the cell suspension and 1 mL complete MethoCult™ medium for each cell type. The mediums were vortexed and allowed to stand for 5 minutes. The mediums were dispensed into 35 mm culture dishes (BD Biosciences) at a final volume of 1.1 mL using a 3 cc syringe attached to 16 gauge blunt-end needle. The dishes were gently rotated to distribute the medium evenly. Two dishes containing the medium were placed per 100 mm sterile petri dish (BD Biosciences). A third uncovered 35 mm dish containing 3 mL of sterile water was added to each 100 mm petri dish to maintain humidity. The dishes were incubated at 37 °C in 5% CO_2 in humidified incubator for 10 days. On the 10^{th} day, images of the colonies formed were captured using an inverted phase contrast light microscope (Nikon). The assay was performed in independent biological triplicates.

3.2.4 Cell Cycle Analysis

The cells were dissociated and the cells were washed with 1x PBS. The cells were fixed using 70% cold ethanol (Thermo Fisher Scientific) at -20 °C overnight. The cells were treated with 100 µg/mL RNase A (Thermo Fisher Scientific) and co-stained with 10 µg/mL propidium iodide (Tesque) for 30 minutes at dark on ice. The cells were analysed using FACS Canto-II analyser (BD Biosciences). A total of 10,000 events were recorded and the results were analysed using ModFit LT™ software (BD Biosciences). The experiment was performed in triplicates.

3.2.5 Re-Differentiation Assay

The spheroid-cultured cells from P5 were dissociated into single cell suspension as described in Section 3.1.6. The cells were seeded in SCM, in the same condition as the adherent cells. The medium was changed every 2 days and the cells were allowed to reach confluence before subculturing. The re-differentiated spheroid-cultured cells were grown for up to 10 passages in SCM condition. The images of the cells were captured using an inverted phase contrast light microscope (Nikon). The cells were dissociated as described in Section 3.1.5 and the drug sensitivity of the cells were analysed as described in Section 3.3.

3.3 Drug Sensitivity Assay

3.3.1 Chemotherapeutic Drug Dilutions

The CRC chemotherapeutic drugs used in the present study were 5-fluorouracil (5-FU, Acros Organics, Geel, Belgium) and Oxaliplatin (L-OHP, LKT Laboratories Inc., USA). The stock solutions of 5-FU and L-OHP were prepared by dissolving 5 mg of drug powders in 1 mL of deionised water to give a concentration of 5 mg/mL. The stock solutions were stirred gently overnight at 4 °C in dark. The stock solutions were sterilised using 0.22 µm cellulose acetate filter. Serial dilutions were performed to give concentrations of 100, 200, 400, 600, 800 and 1,000 µg/mL for each drug by diluting the stock solution in sterile deionised water. The stock solutions and dilutions were stored at -20 °C in dark until further use.

3.3.2 Drug Treatment

The cells were dissociated into single cell suspension as described in Section 3.1.5 and 3.1.6. The adherent and spheroid-cultured cells were seeded at 5×10^3 cells/mL in 90 µL of SCM and SFM, respectively. The adherent cells were seeded in flat bottom 96-well culture plates (BD Biosciences), while the spheroid-cultured cells were seeded in polyHEMA coated flat bottom 96-well culture plates (BD Biosciences). The plates were incubated for 24 hours at 37 °C in 5% CO_2 in humidified incubator. The cells were treated with

10 µL from each drug concentration in triplicate wells. The control cells were with treated with 10 µL of sterile deionised water. The plates were incubated further for 48 hours at 37 °C in 5% CO_2 humidified incubator. The drug sensitivity of the cells was analysed using cell proliferation kit (CCK-SK, Dojindo, Japan). All the treated and control wells were added with 10 µL of CCK-SK solution as recommended by the manufacturer. The plates were incubated for 2.5 hours at 37 °C in 5% CO_2 humidified incubator in dark. The absorbance of the wells was read at 450 nm using Infinite M200 PRO Microplate Reader. The viability of the cells was calculated as shown below. The experiments were performed in independent triplicates. The graph of concentrations of the drug versus percentage of viability of the cells was constructed. The IC_{50} was determined using GraphPad Prism software (GraphPad Software Inc., USA).

$$\text{Cell Viability} = \frac{\text{Absorbance of treated well}}{\text{Absorbance of control well}} \times 100\%$$

3.4 Gene Expression Analysis

3.4.1 RNA Extraction

The cell pellets were collected following cell dissociation and centrifugation. Total RNA was isolated using miRNeasy Mini Kit (Qiagen, Germany), according to the manufacturer's instruction. The cell pellets were added with 700 µL of QIAzol lysis reagent. The pellets were homogenised by 2 minutes of vortex. The pellets were incubated for 10 minutes at room

temperature. The pellets were added with 140 µL of chloroform (Amresco) and spun for 2 minutes (450 rpm) in a shaking incubator (IKA, Breisgau, Germany). The homogenates were transferred into 1.5 mL micro-centrifuge tubes and allowed to stand for 3 minutes. The homogenates were separated into three layers by centrifugation at 12,000 xg in 4 °C for 15 minutes. The upper transparent layer containing total RNA was transferred into new micro-centrifuge tubes and mixed with 525 µL of 100 % ethanol (Thermo Fisher Scientific). The mixtures were transferred into RNeasy Mini spin columns and centrifuged at 10,000 xg in room temperature for 15 seconds. The flow through was discarded and 350 µL of buffer RWT was added to the columns. The columns were centrifuged at 10,000 xg in room temperature for 15 seconds and the flow through was discarded. DNase 1 solution was added directly onto the membrane of the columns and allowed to stand for 15 minutes at room temperature. The columns were added with 350 µL of buffer RWT and centrifuged at 10,000 xg in room temperature for 15 seconds. The flow through was discarded and 500 µL of buffer RPE was added. The columns were centrifuged at 10,000 xg in room temperature for 2 minutes and followed by 14,500 rpm in room temperature for 1 minute to dry the membrane. The columns were transferred into new collection tubes and 30 µL of RNase-free water was added on the membrane. The columns were centrifuged at 10,000 xg in room temperature for 1 minute and the flow through was added onto the membrane again. The columns were centrifuged at 10,000 xg in room temperature for 1 minute. The RNA was quantified and stored at -80 °C for future use.

3.4.2 RNA Quantification

The concentration of the extracted RNA was quantified using NanoPhotometer (Implen, Germany). The purity of the RNA was determined by measuring the reading ratio of absorbance 260 and 280 nm (A_{260}/A_{280}). RNA isolates that records a ratio between 1.8 and 2.0 were used for the downstream gene expression study.

3.4.3 cDNA Conversion

The extracted RNAs were reverse transcribed to cDNA using High Capacity RNA-to-cDNA kit (Applied Biosystems, USA) according to the manufacturer's instruction. Two micrograms of RNA were reverse transcribed in a final volume of 20 µL per reaction containing 1× RT buffer, 1× RT enzyme mix and RNase-free water. The reaction tube was spun down and incubated in 96-well Thermal Cycler (Applied Biosystems, USA) at 37 °C for 60 minutes, 95 °C for 5 minutes and 4 °C for infinity. The cDNA was stored at -20 °C for future use.

3.4.4 Real-Time qRT-PCR

The cDNA was subjected to quantitative real-time polymerase chain reaction (qRT-PCR) using SYBR® Select Master Mix (Thermo Fisher Scientific) as described in Table 3.3. The specific mRNA sequences were amplified using specific forward and reverse primers as listed in Table 3.4 and 3.5. The gene primers were screened for specificity using Basic Local Alignment Search Tool (BLAST) of National Center for Biotechnology Information (NCBI) database. The primers were synthesised by 1st BASE Oligos (Singapore). No template control (NTC) was included in each assay by substituting the cDNA with DEPC-treated water. The mixtures were incubated in Rotor Gene Q cycler (Qiagen) under the following conditions; 50 °C for 2 minutes, 95 °C for 2 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minutes. The melt curve analysis was carried out for each assay. The experiments were repeated in triplicates and normalised to housekeeping gene, GAPDH (Glyceraldehyde 3-phosphate dehydrogenase). Relative mRNA expression levels were calculated using the comparative C_T ($\Delta\Delta C_T$) method.

Table 3.3 Quantitative real-time qRT-PCR reaction set-up

Components	Volume per reaction	Final concentration
2X SYBR GreenER qPCR SuperMix	10 µL	1X
Forward primer (10 µM)	0.4 µL	200 nM
Reverse primer (10 µM)	0.4 µL	200 nM
cDNA template (10 ng/µL)	2 µL	20 ng
DEPC-treated water	7.2 µL	-
Total volume	20 µL	

Table 3.4 List of pluripotent gene primers used in qRT-PCR

Genes	Accession Number	Primer Sequence (5' → 3')
OCT4	NM_002701.5	F: GAC AGG GGG AGG GGA GCT AGG R: CTT CC TCC AAC CAG TTG CCC CAA AC
SOX2	NM_003106.3	F: GGG AAA TGG GAG GGG TGC AAA AGA GG R: TTG CGT GAG TGT GGA TGG GAT TGG TG
KLF4	NM_001314052.1	F: TGA TTG TAG TGC TTT CTG GCT GGG CTC C R: ACG ATC GTG GCC CCG GAA AAG GAC C
c-MYC	NM_002467.4	F: GCG TCC TGG GA GGG AGA TCC GGA GC R: TTG AGG GGC ATC GTC GCG GGA GGC TG
NANOG	NM_024865.3	F: AGT CCC AAA GGC AAA CAA CCC ACT TC R: TGC TGG AGG CTG AGG TAT TTC TGT CTC
GAPDH	NM_002046.5	F: CGT GGA AGG ACT CAT GAC CA R: CAG TCT TCT GGG TGG CAG TGA

Table 3.5 List of ABC transporter gene primers used in qRT-PCR

Genes	Accession Number	Primer Sequence (5' → 3')
ABCA2	NM001606	F: AAG CCT GTG GAG GAT GAT GTG R: GGT CAA CGG CCA GGA TAC G
ABCA3	NM001089	F: CAA AAC CCT GGA TCA CGT GTT R: CCT CCG CGT CTC GTA GTT CT
ABCB1	NM000927	F: GTC CCA GGA GCC CAT CCT R: CCC GGC TGT TGT CTC CAT A
ABCB4	NM000443	F: TTT TTA CTT TCT TCC TTC AGG GTT TC R: TAA AAG CCA TTGA CCG CAG TCT
ABCB5	NM178559	F: GCT CTG GCC CCT CAA ACC R: TGG CCT TGA GGT ATG TGC AGT A
ABCB11	NM003742	F: AGG GAG CTA CCA GGA TAG TTT AAG G R: TCG TGC ACC AGG TAA GAA AGC
ABCC1	NM004996	F: GAA GGC CAT CGG ACT CTT CA R: CAG CGC GGA CAC ATG GT
ABCC2	NM000392	F: TGC AGC CTC CAT AAC CAT GAG R: GAT GCC TGC CAT TGG ACC TA
ABCC3	NM003786	F: CAC ACG GAT CTG ACA GAC AAT GA R: ACA GGG CAC TCA GCT GTC TCA
ABCC4	NM005845	F: AAG TGA ACA ACC TCC AGT TCC AG R: GGC TCT CCA GAG CAC CAT CT
ABCC5	NM005688	F: TGA AAG CCA TTC GAG GAG TTG R: CGG AAA AGC TCG TCA TGC A
ABCC11	NM033151	F: AGG GTC TAC CAC CAC TAC ATC CA R: CGA TCA GCA CCA CGA AGA AG
ABCC12	NM033226	F: TTC ATC CAA AGG CCT GTC ATT R: CCG TTC GCA CAC ACA CTT G
ABCG2	NM004915	F: CAG GTC TGT TGG TCA ATC TCA CA R: TCC ATA TCG TGG AAT GCT GAA G

CHAPTER 4

RESULTS

4.1 Study Design

The present study was divided into three major parts as shown in Figure 4.1:

Part 1: Establishment and characterisation of spheroid-cultured colorectal cancer stem cells (CrCSCs)

Part 2: Cellular and molecular profiling of spheroid-cultured CrCSCs

Part 3: Establishment of chemoresistance and ABC gene expression profile of spheroid-cultured CrCSCs

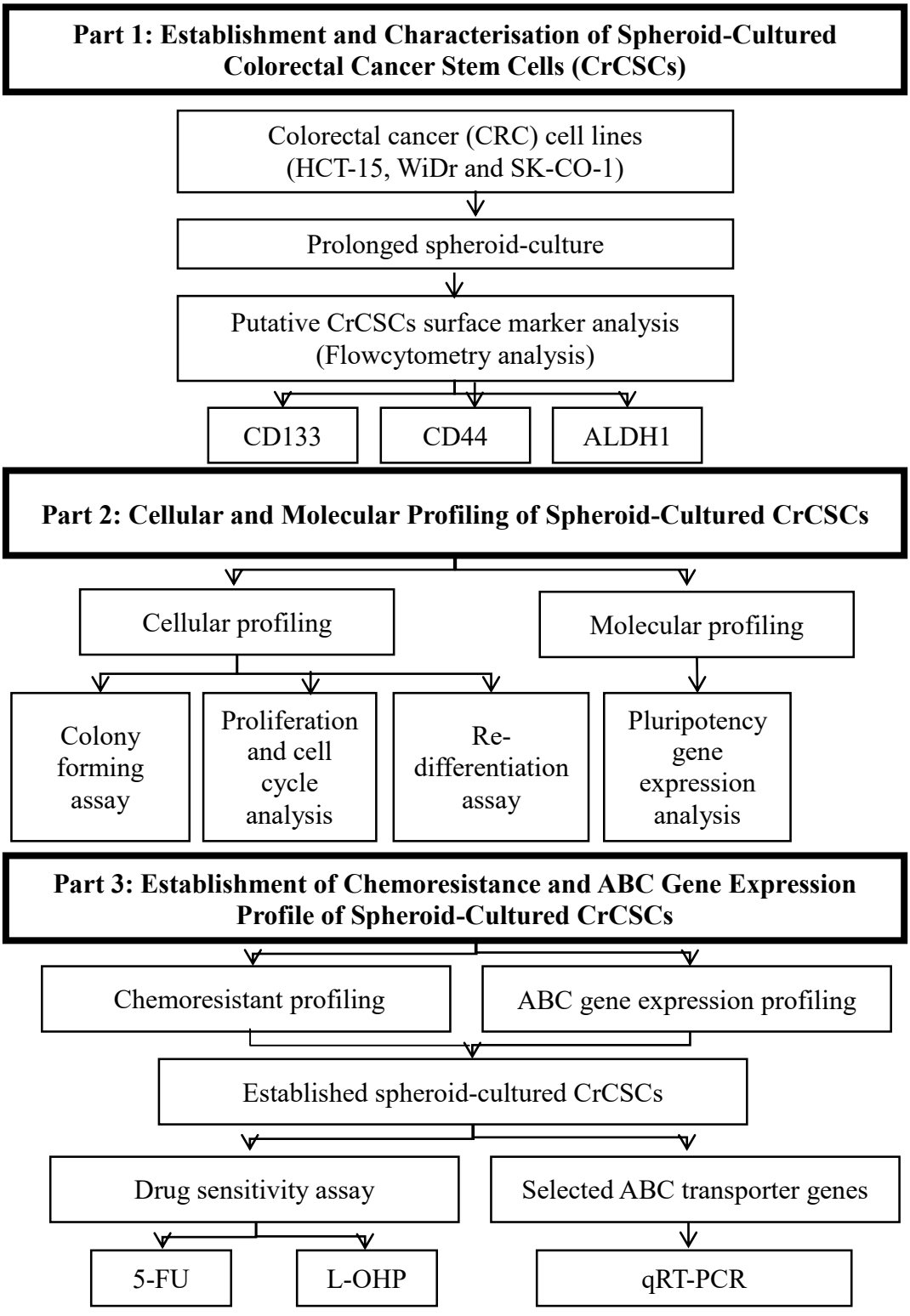


Figure 4.1 Study design. The present study was divided into three main parts. CRC cell lines: HCT-15, WiDr and SK-CO-1 were used in all the three parts of study.

PART I

Establishment and Characterisation of Spheroid-Cultured Colorectal Cancer Stem Cells (CrCSCs)

4.2 CRC Cells Formed Spheroids in Anchorage-Independent Culture

To generate colorectal cancer (CRC) spheroid-cultured cells, CRC cell lines, HCT-15, WiDr and SK-CO-1 were grown in serum-free medium which were supplemented with growth factors, on polyHEMA-coated culture flask as described in Section 3.1.6. The parental CRC cell lines grown in 2D monolayer-culture were in epithelial-like morphology (Figure 4.2 A). In spheroidal culture condition, CRC cells aggregated in small floating clumps of roughly five cells and subsequently enlarged in size through clonal expansion (Figure 4.2 B). The spheroids continued to grow in diameter over 14 days of culture with notable darkening in the centre of the spheroids, suggesting the formation of a necrotic region. The spheroid-cultured cells were able to be propagated from single-cell suspension to form second generation spheroids under the same condition (Figure 4.3). Generally, the spheroids formed were more regular in shape and larger in size at increased passages of the cells in spheroidal culture. However, it was observed that all three CRC cell lines formed spheroids at different efficacy under the suspension culture. HCT-15 and WiDr cells were able to form regular-shaped spheroids consistently from first the spheroidal culture (P0), whereas SK-CO-1 cells were only able to form regular-shaped spheroids in later passages (P3) of spheroidal cultures.

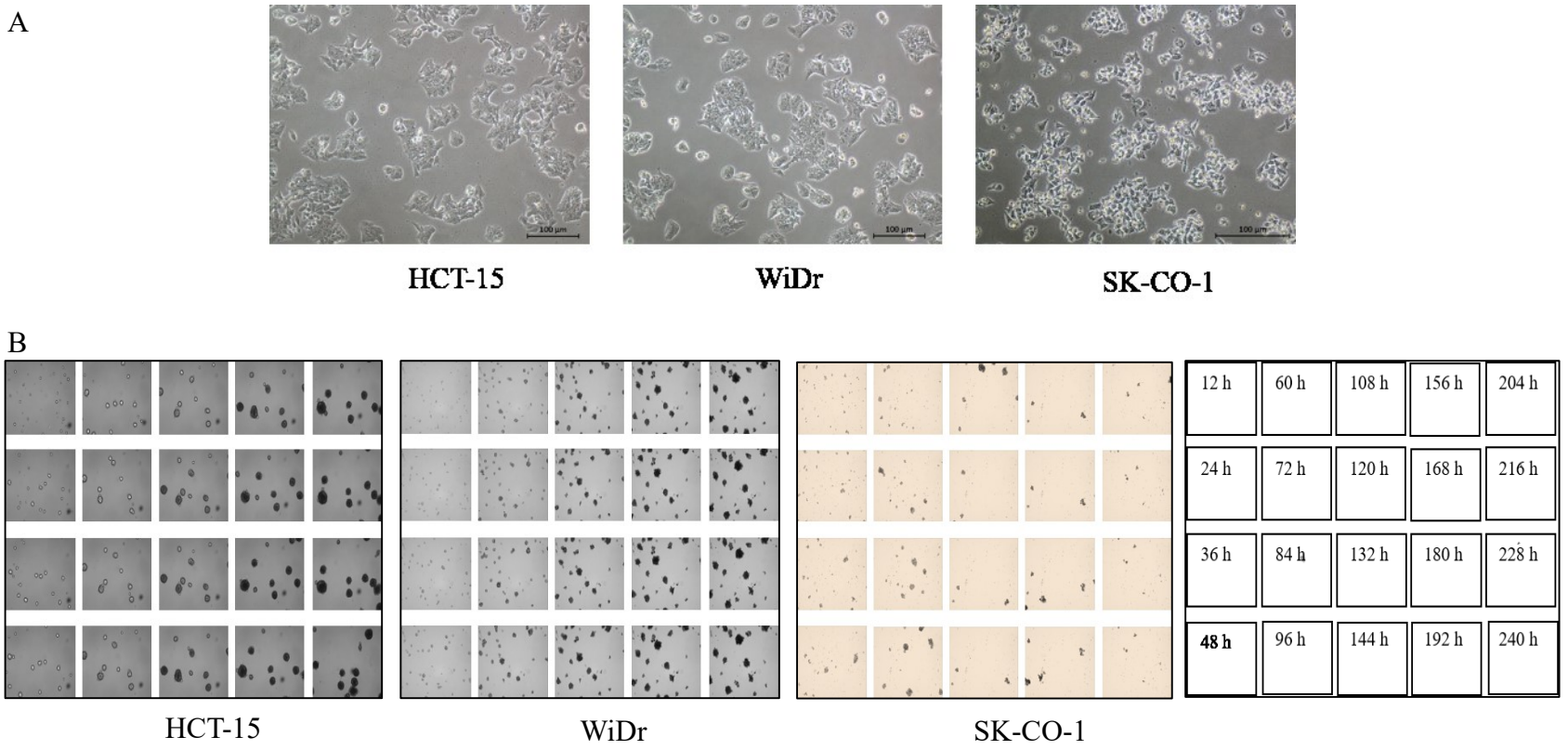


Figure 4.2 Cellular morphology and formation of CRC spheroids. (A) Morphology of parental CRC cell lines, HCT-15, WiDr and SK-CO-1. (B) Progression of spheroids formation over 10 days of culture in serum-free anchorage-independent culture of CRC cells. Bottom-left image is a schematic representation of the time-lapse image arrangement of spheroid formation.

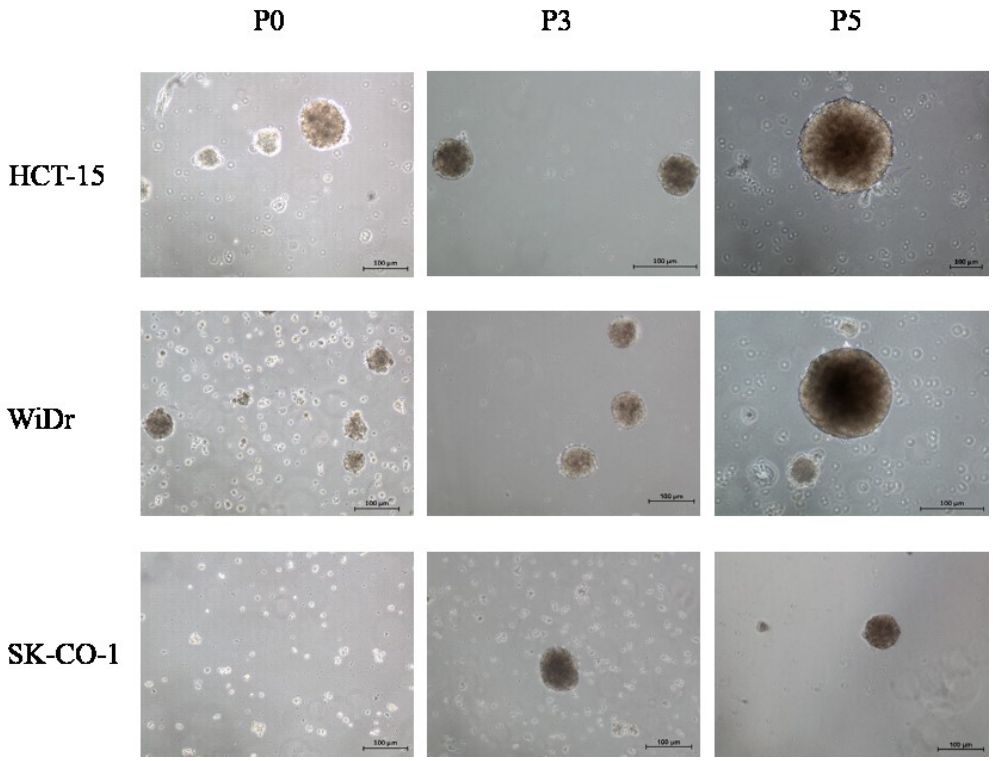


Figure 4.3 Representative images of spheroids formed at different passages (P) in spheroidal culture. Bright field microscopy images of spheroids formed on day 14 in spheroidal culture of CRC cell lines with 10X magnification. (Bars = 100 µm).

4.3 Enhanced Expression Levels of CrCSCs-Related Surface Markers in Spheroid-Cultured CRC Cells

CD133, CD44 and aldehyde dehydrogenase 1 (ALDH1) are cellular surface markers associated with colorectal cancer stem cells (CrCSC) population (Langan et al., 2013). Hence, the expression of the putative surface markers of the CRC parental and P5 spheroid-cultured cells was analysed using flowcytometry (Figure 4.4 and Figure 4.5). Due to the lack of specificity of single markers for CrCSCs population, co-expression levels of the markers were also analysed (Skoda et al., 2016). Based on the results obtained, the parental CRC cells, HCT-15, WiDr and SK-CO-1 expressed high levels of CD133, CD44 and ALDH1. On the contrary, the expression levels of CD133 and ALDH1 were markedly down-regulated in the spheroid-cultured cells. In addition, the co-expression of levels of triple markers, ALDH1-CD44-CD133 and double markers, ALDH1-CD133 were also down-regulated compared to their parental cell expression levels. Conversely, the expression levels of CD44 were consistently up-regulated in all three CRC spheroid-cultured cells. The co-expression levels of ALDH1-CDD44 population were also up-regulated in the spheroid-cultured cells compared to their parental cells. Collectively, the spheroid-cultured cells showed increased population of cells expressing CD44 and ALDH1-CD44 markers, indicating CrCSC-like phenotype.

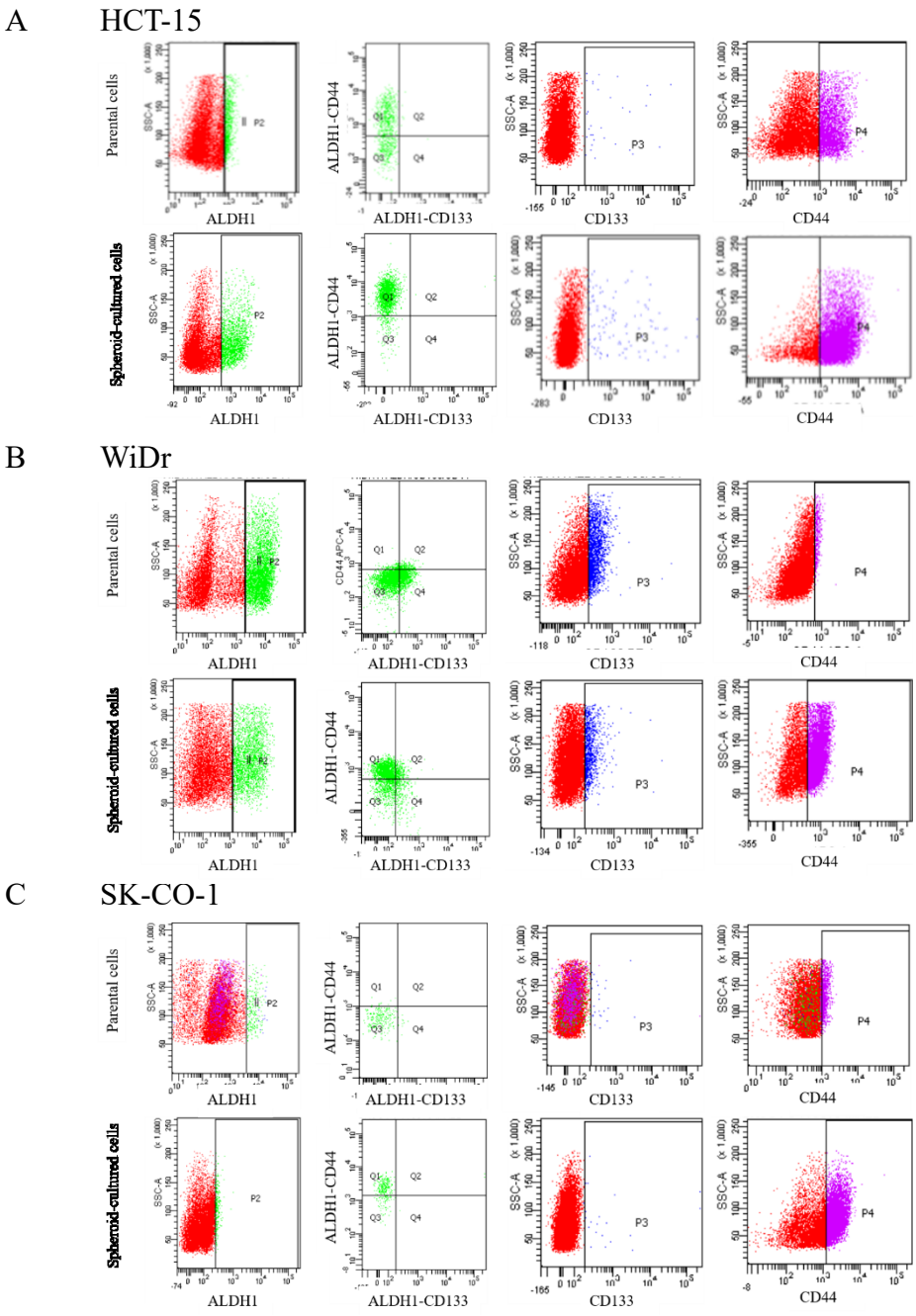
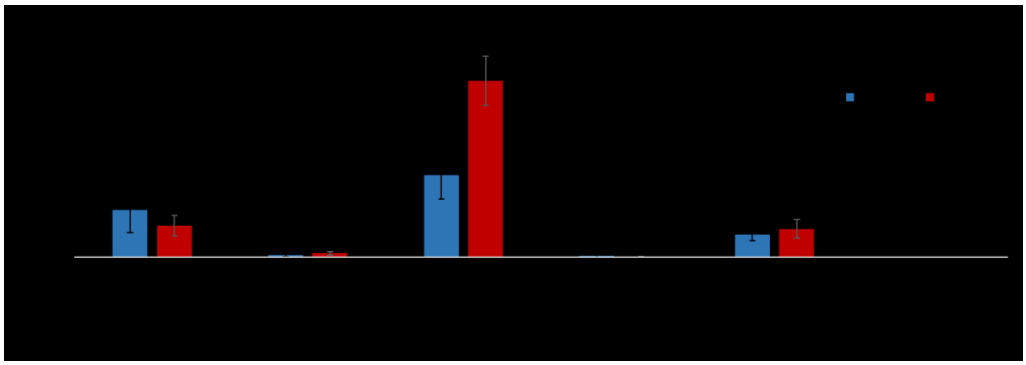
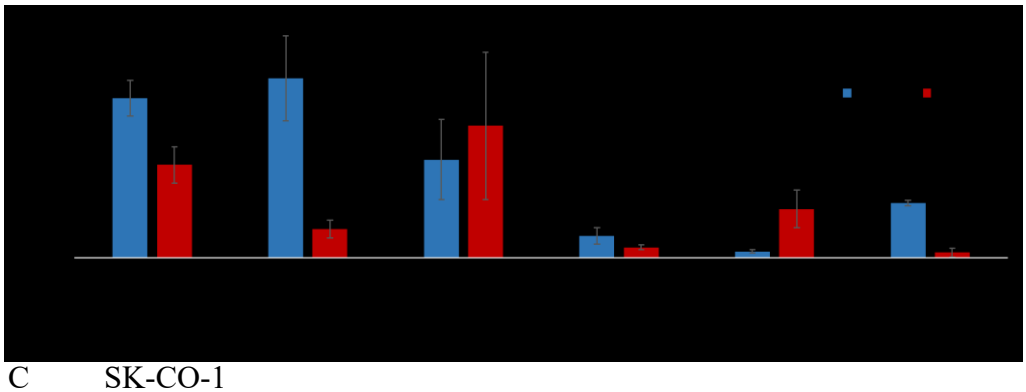


Figure 4.4 Flowcytometry analysis of putative CrCSC surface markers. Representative images of (A) HCT-15, (B) WiDr and (C) SK-CO-1 parental and spheroid-cultured cells analysed for CD133, CD44 and ALDH1 expression levels using flowcytometry. The dot plot graphs for each independent experiment are shown in Appendices A-F.

A HCT-15



B WiDr



C SK-CO-1

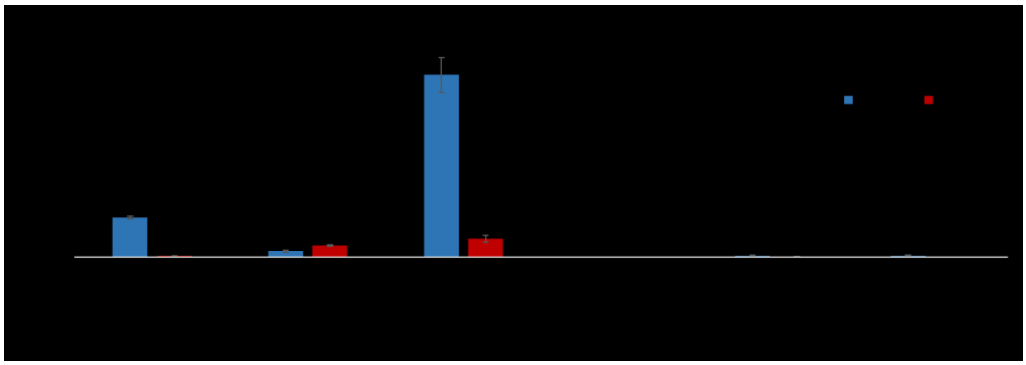


Figure 4.5 Expression levels of CrCSCs-related surface markers by flowcytometry analysis. (A) HCT-15, (B) WiDr and (C) SK-CO-1 parental and spheroid-cultured cells analysed for CD133, CD44 and ALDH1 expression levels using flowcytometry. Data from three independent experiments. *p<0.05 and **p<0.01 were obtained by comparing to the parental CRC cells

PART II

Cellular and Molecular Profiling of CrCSCs

4.4 Spheroid-Cultured Cells Formed Colonies in Semi-Solid Medium

To assess the clonogenic ability of CRC cells, colony-forming assay was performed. CRC parental and spheroid-cultured cells were seeded as single cell suspension in semi-solid medium to allow colony formation over 10 days. It was observed that all the CRC cells were able to survive and proliferate in semi-solid medium (Figure 4.6A). However, only the spheroid-cultured cells were able to form large colonies (>100 μm). The parental CRC cells were unable to form large colonies while spheroid-cultured cells formed higher numbers of colonies ranging from 5- to 40-folds (Figure 4.6B). Although the parental CRC cells, HCT-15 and WiDr, were able to form small colonies, these colonies were not able to grow in size and meet the criteria set for the colonies. Only colonies with minimum diameter of 100 μm were counted. In conclusion, the colony forming assay suggested that CRC spheroid-cultured cells had higher clonogenic ability than the parental cells.

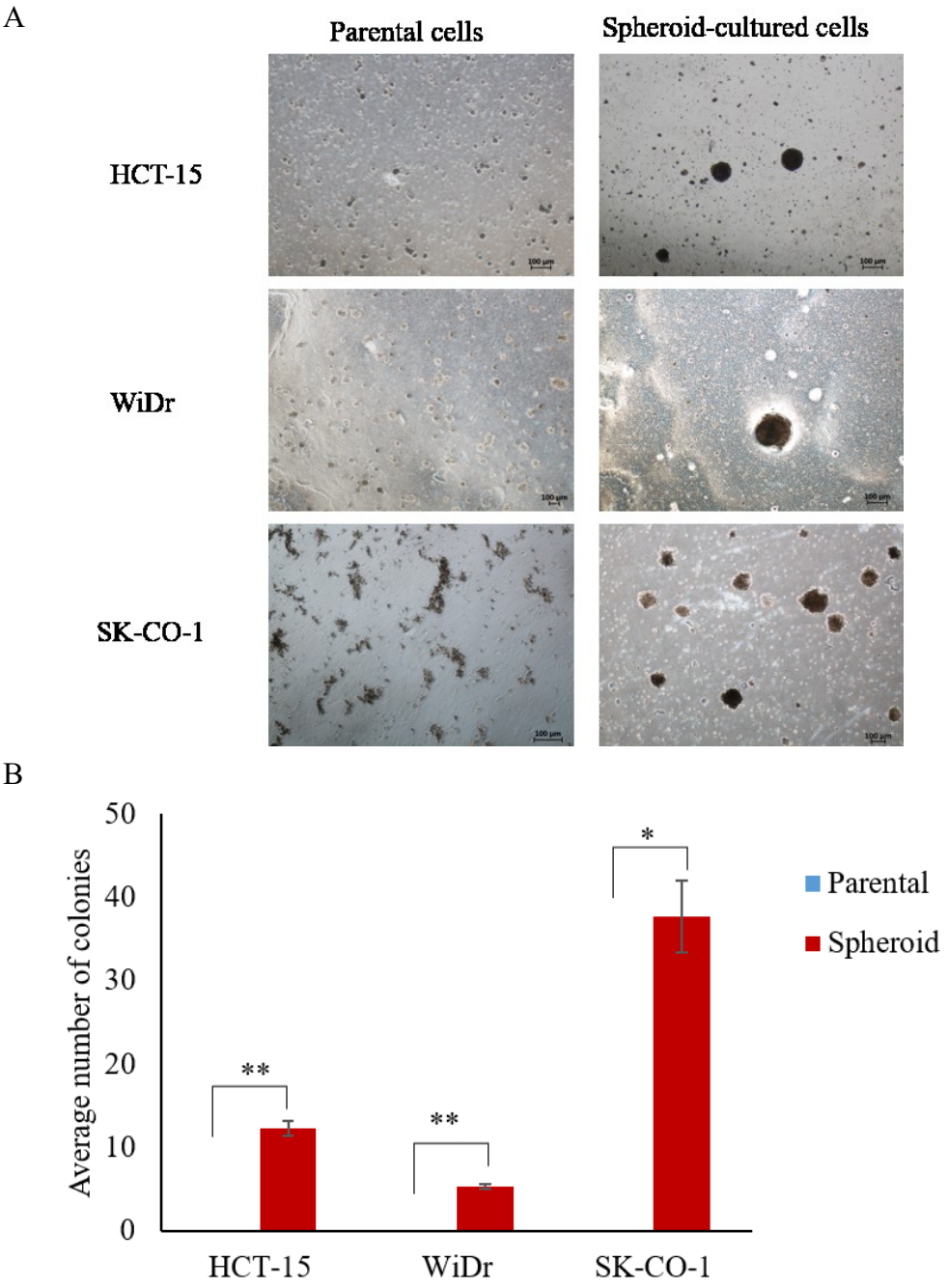


Figure 4.6 Clonogenic ability of parental and spheroid-cultured CRC cells. (A) Representative images of cells in semi-solid medium after 10 days of culture, at 10X magnification (Bar = 100 µm). (B) Average numbers of colonies formed. Data from three independent experiments. *p<0.05 and **p<0.01 were obtained by comparing to the parental CRC cells.

4.5 Spheroidal Culture Generated Quiescent Population of CRC

Cancer stem cells population has been reported to be in quiescent state of cell division (N. Wang et al., 2015; Lin et al., 2015). Hence, to investigate the proliferative state of the parental and spheroid-cultured CRC cells, cell cycle analysis using flowcytometry and proliferation analysis using MTT assay were performed. The cell cycle analysis was performed by staining the CRC cells with propidium iodide (PI). Histograms generated by flowcytometry were used to determine the state of cell cycle of spheroid-cultured cells relative to their parental cells (Figure 4.7A). All the parental CRC cells showed active dividing cell cycle profile; with low G_1 phase and high S and G_2 phase. Whereas, the spheroid-cultured cells showed slow dividing cell cycle profile, evident by higher G_1 phase and lower S and G_2 phase. Figure 4.7 B shows the proliferative curves of parental CRC and their counterpart spheroid-cultured cells constructed from MTT assay. The proliferation rate of the all CRC spheroid-cultured cells was lower than their parental counterpart which corroborated the results obtained from the cell cycle analysis. The results collectively suggest that the CRC population derived from spheroidal culture were relatively dormant compared to their parental cells, which is a characteristic of CSC population.

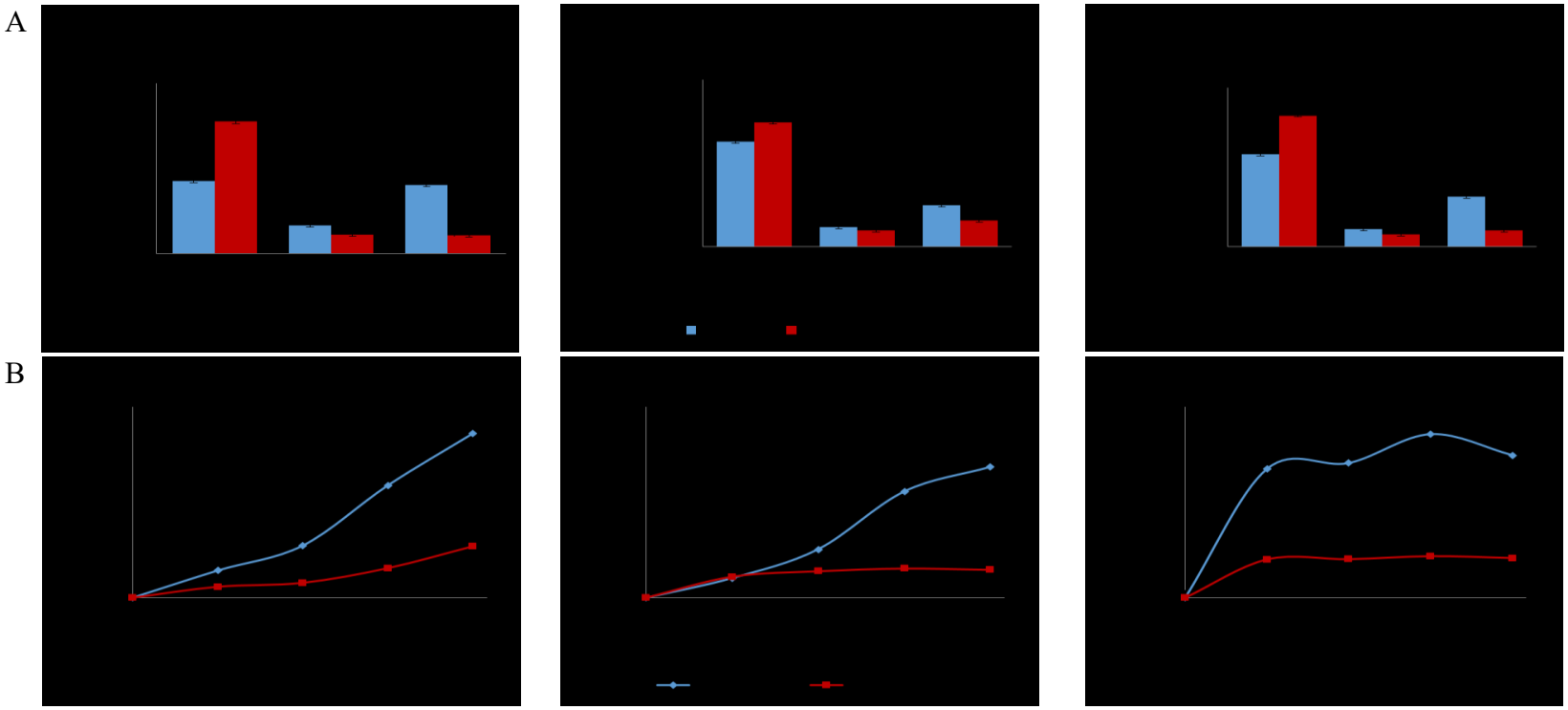


Figure 4.7 Cell cycle and proliferation analysis CRC parental and spheroid-cultured cells. (A) Cycle cell analysis using flowcytometry and (B) proliferation curve of parental and spheroid-cultured CRC cells. Results were obtained from three independent experiments. *p<0.05 and **p<0.01 were values obtained in comparison to their parental cells. Cell cycle histograms for each independent experiment are shown in Appendices G-I.

4.6 Re-Differentiated Spheroid-Cultured Cells Exhibited Similar Morphology as the Parental Cells

Spheroidal culture was reported to be able to maintain the "stemness" of cell population (Li et al., 2015). To evaluate the morphology changes upon re-differentiation, the P5 spheroid-cultured cells were dissociated and seeded in parental condition with serum containing medium. Serum has been reported to act as differentiating agent in stem cells (Nestor et al., 2013). The re-differentiated spheroid-cultured cells were able to survive and proliferate in the parental culture condition up to 10 passages. The re-differentiated spheroid-cultured cells showed close resemblance to their parental counterpart cells (Figure 4.8). HCT-15 and WiDr spheroid-cultured re-differentiated cells were able to form large flatten colonies while SK-CO-1 formed small clusters of cells, which were similar to their parental morphology. However, the re-differentiated spheroid-cultured cells showed increased subpopulation of small-sized cells in all three CRC cells. Collectively, the re-differentiation of the spheroid-cultured cells exhibited similar morphology as their parental cells.

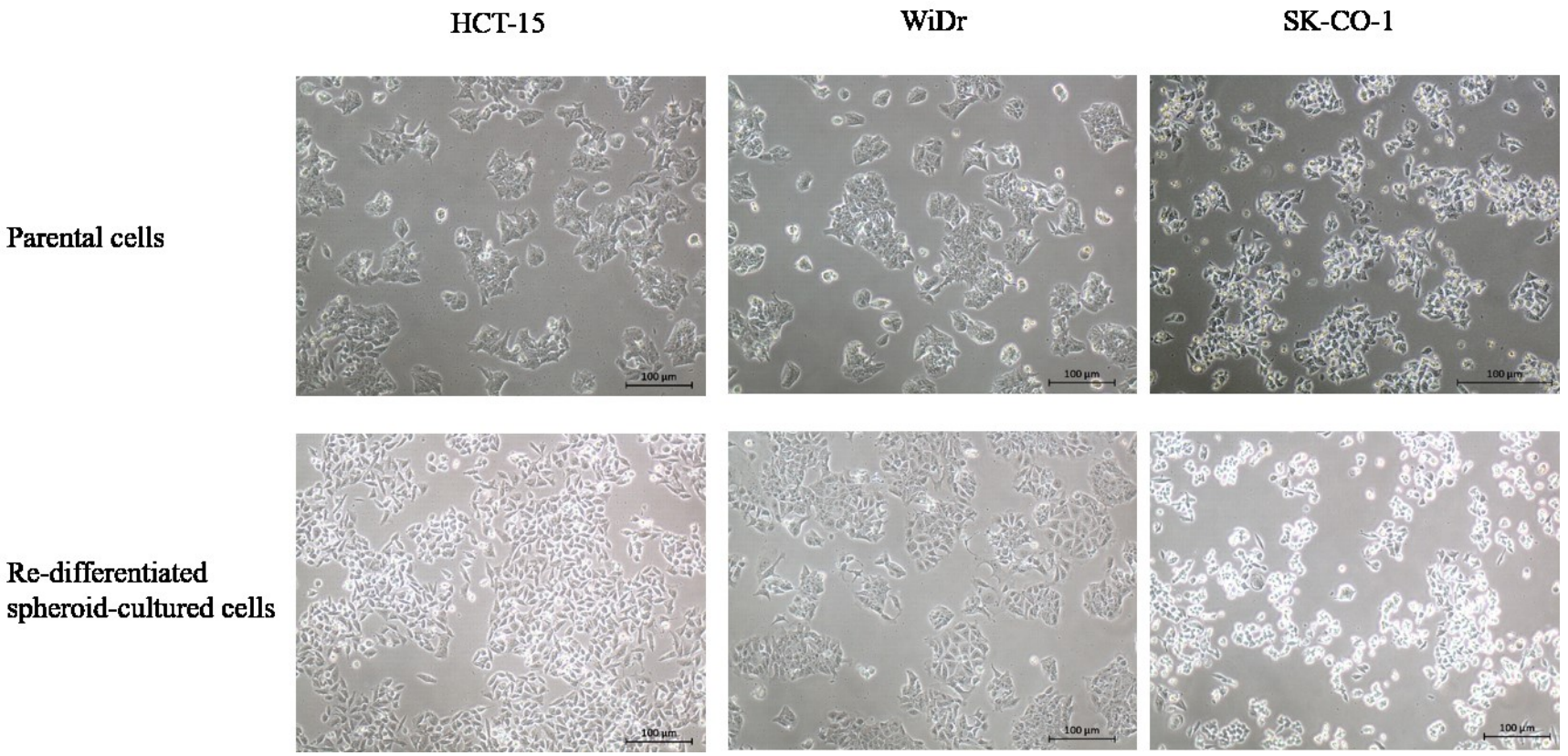


Figure 4.8 Morphology of re-differentiated spheroid-cultured cells. Morphology of the parental and re-differentiated spheroid-cultured CRC cells**.** Images were taken in 10x magnification (Bar = 100 µm).

4.7 Up-Regulated Expression Levels of Pluripotency and Tumourigenicity Genes at Increased Passages of Spheroidal Culture

To evaluate the "stemness" enrichment of spheroidal culture, mRNA expression levels of pluripotency and tumourigenicity associated genes, *OCT4*, *SOX2*, *KLF, c-MYC* and *NANOG* (OSKMN) were analysed using real-time qRT-PCR at different passages of CRC cells in spheroidal culture. Expression levels were analysed at P0, P3 and P5 passages of the spheroidal culture, and the log fold change graphs were constructed relative to the parental CRC expression levels (Figure 4.9). Generally, the OSKMN mRNA expression levels of all three CRC cell lines were noted to be up-regulated at increasing passages in spheroidal culture. *OCT4* and *c-MYC* genes were steadily up-regulated at increasing passages of spheroidal culture although WiDr showed down-regulation of *OCT4* expression level at P0. Expression of *SOX2* and *NANOG* genes were inconsistent in all the CRC spheroid-cultured cells, however the overall expression level of spheroid-cultured cells at P5 was higher than that their parental cells. The expression levels of *KLF4* were uniformly up-regulated at increasing passages of spheroidal culture with an exception for HCT-15. Despite the unique expression regulation of OKSMN at different passages of CRC cells in spheroidal culture, predominantly the expression level of all genes at P5 were greater than their parental. In summary, the genes associated with pluripotency and tumourigenicity associated genes were markedly up-regulated at increasing passages of spheroidal culture suggesting enhanced stemness and tumourigenicity.

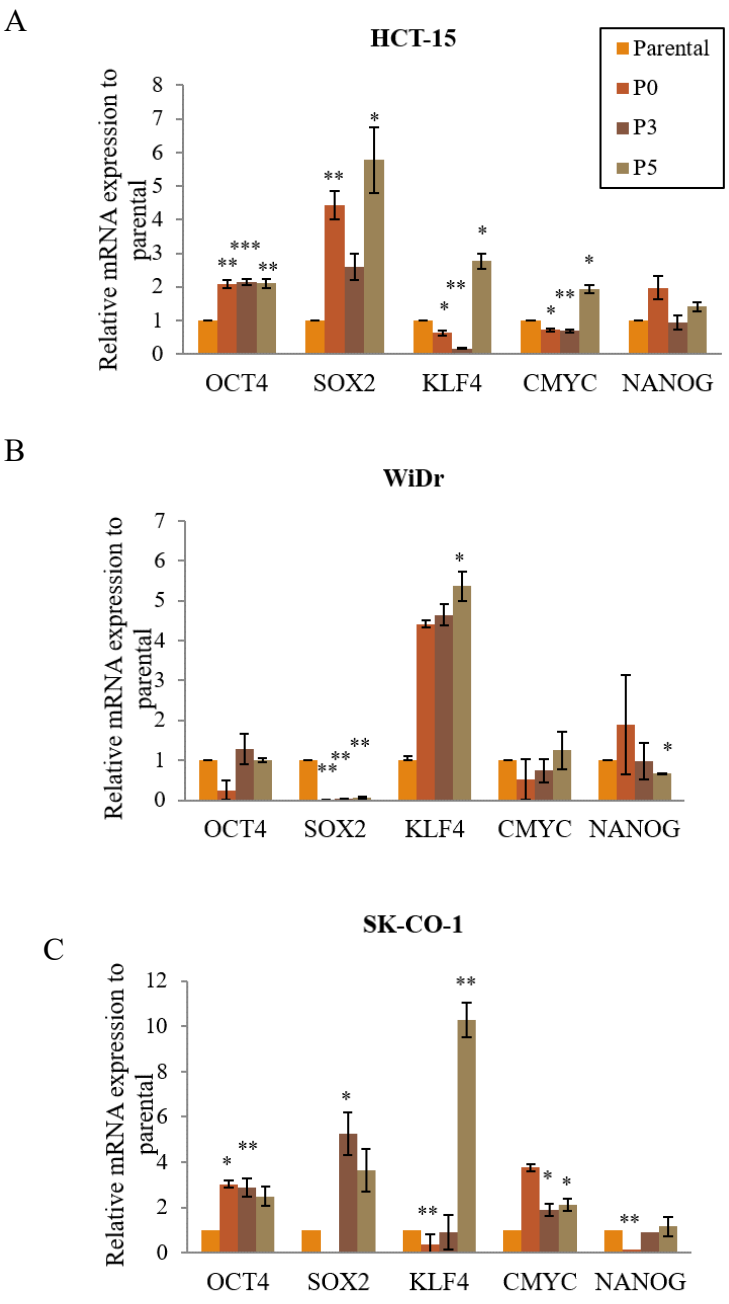


Figure 4.9 mRNA expression levels of pluripotency and tumourigenicity genes of CRC spheroid-cultured cells. (A) HCT-15, (B) WiDr and (C) SK-CO-1. Results obtained from three independent experiments. *p<0.05, **p<0.01, ***p<0.001 were values obtained in comparison to their parental cells.

4.8 Reduced Expression Levels of Pluripotency and Tumourigenicity Genes in Re-Differentiated Spheroid-Cultured Cells

To investigate the effect of re-differentiation of spheroid-cultured cells, mRNA expression levels of OSKMN were analysed on the re-differentiated CRC spheroid-cultured cells using real-time qRT-PCR. The gene expression levels of the re-differentiated cells were evaluated at passage 4 (denoted as "Dif-S P4") and passage 10 (denoted as "Dif-S P10"), and the relative expression levels were compared to their parental cells (Figure 4.10). Across all the CRC cells, the OSKMN gene expression levels were markedly down-regulated upon re-differentiation at passage P4 as compared to their respective P5 spheroid-cultured cells. The expression levels were further down-regulated at passage P10. Generally, the gene expression levels of OSKMN at P10 re-differentiated spheroid-cultured cells were evidently down-regulated compared to their parental cells. Collectively, upon re-differentiation, the spheroid-cultured cells lost the enriched "stemness" properties observed in Figure 4.8.

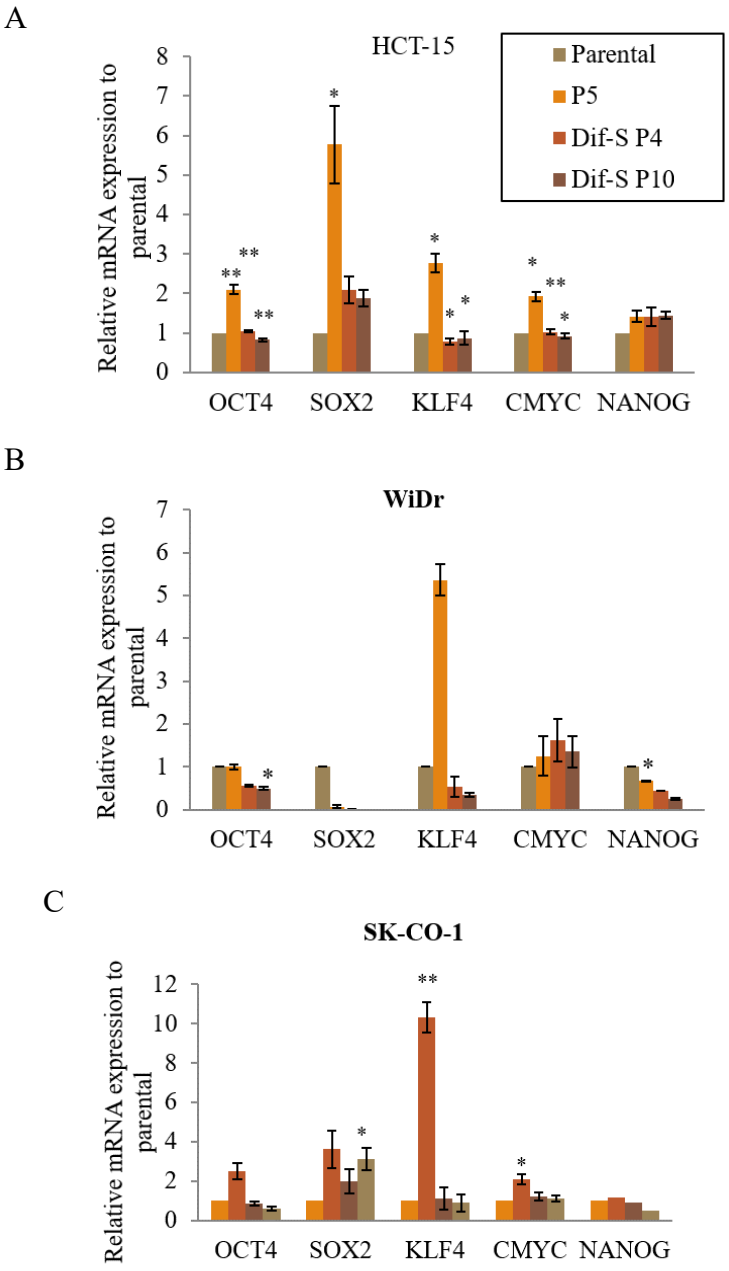


Figure 4.10 mRNA expression levels of pluripotency and tumourigenicity genes of re-differentiated spheroid-cultured CRC cells. (A) HCT-15, (B) WiDr, (C) SK-CO-1. Results obtained from three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 were values obtained in comparison to their parental cells. Dif-S refers to the re-differentiated spheroids at passage 4 (Dif-S P4) and at passage 10 (Dif-S P10).

PART III

Establishment of Chemoresistance and ABC Gene Expression Profile of Spheroid-Cultured CrCSCs

4.9 Spheroid-Cultured Cells Displayed Enhanced Chemoresistant Properties in Passage-Dependent Manner

The hallmark of cancer stem cells (CSCs) is the chemoresistance ability (Dallas et al., 2009; Onaitis and Hanna, 2013). Hence, chemoresistance properties of CRC cells and the spheroid-cultured counterpart cells were evaluated using common CRC chemotherapeutic drugs; 5-fluorouracil (5-FU) and oxaliplatin (L-OHP). The chemoresistant ability was assessed by measuring the viability of the cells after treating the single cell suspension at different dosages of the drug. The viability graphs and IC_{50} values derived from GraphPad Prism were constructed in Figure 4.11 and Figure 4.12, respectively. All spheroid-cultured cells showed higher viability after treatment with 5-FU and L-OHP compared to the parental. It was also observed that the spheroid-cultured cells showed higher viability at increasing passages in spheroid-culture. This was evident by the uniform increment of IC_{50} values from P0, P3 and P5 in all three CRC cell lines; HCT-15, WiDr and SK-CO-1. Collectively, the results suggest the spheroid-cultured cells showed higher chemoresistance at increasing passages in spheroid-culture.

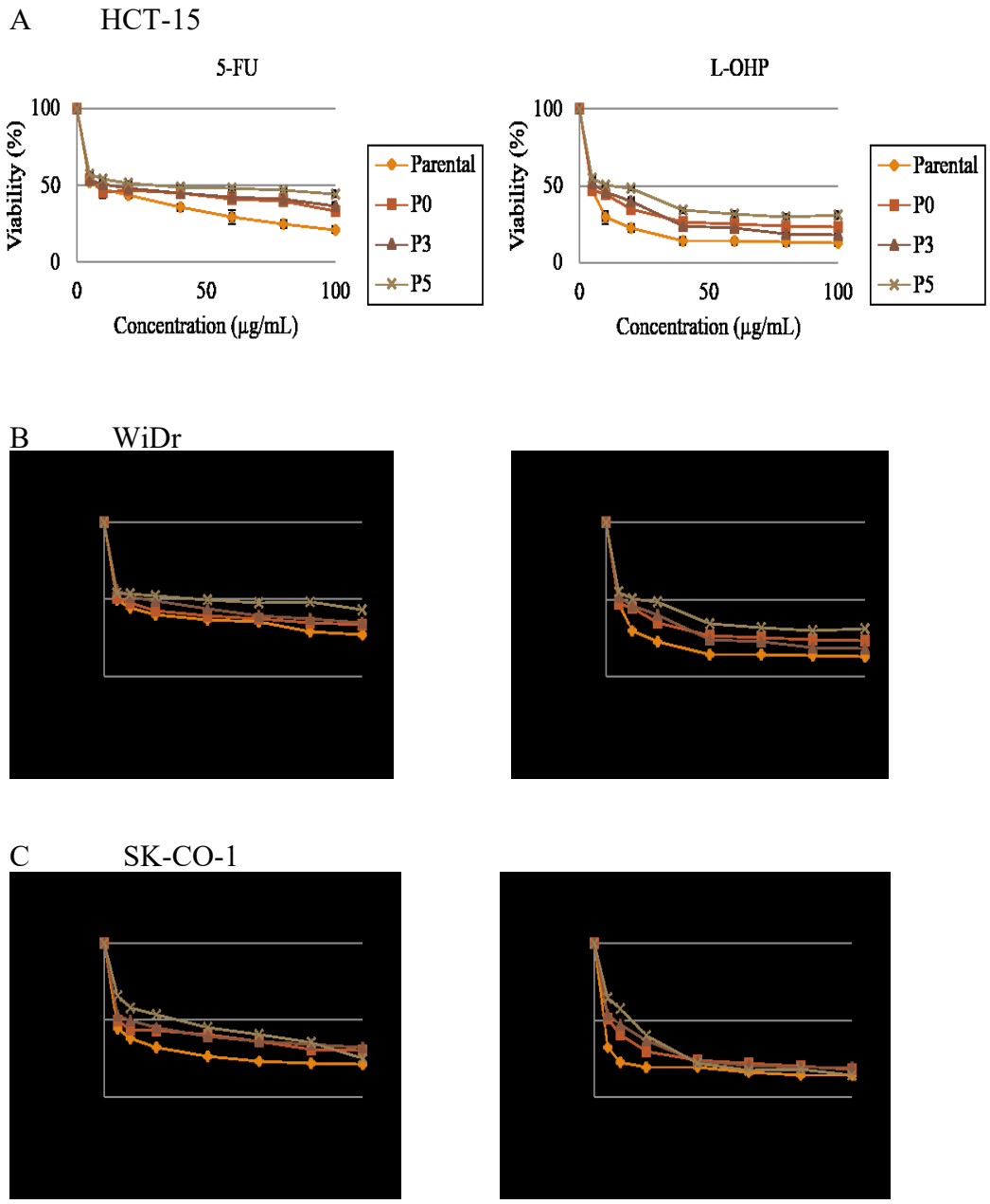


Figure 4.11 Chemoresistance properties of CRC parental and spheroid-cultured cells. Viability of (A) HCT-15, (B) WiDr and (C) SK-CO-1 parental, P0, P3 and P5 spheroid-cultured cells upon treatment with 5-FU and L-OHP drugs. Results were obtained from three independent experiments.

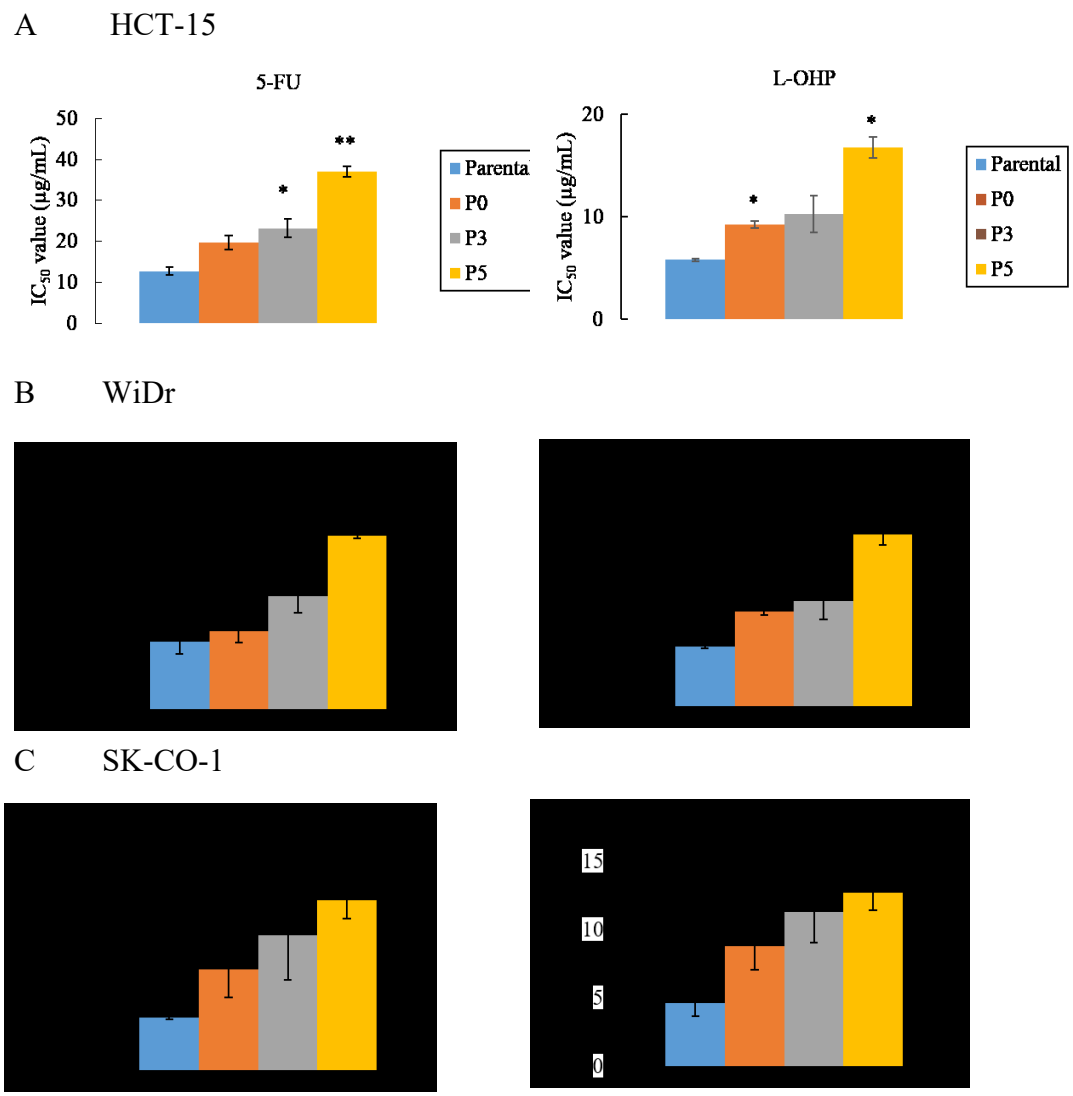


Figure 4.12 IC_{50} values of CRC parental and spheroid-cultured cells. Bar graphs represent IC_{50} values of (A) HCT-15, (B) WiDr and (C) SK-CO-1 parental, P0, P3 and P5 spheroid-cultured cells upon treatment with 5-FU and L-OHP drugs. Results were obtained from three independent experiments. *p<0.05 and **p<0.01 were values obtained in comparison to their parental cells.

4.10 3D Structure of the Spheroid Had Limited Contributions Towards Chemoresistance Properties of Spheroid-Cultured Cells

The 3D structure of spheroid-cultured cells may confer chemoresistance by limiting the entry of drugs into the core centre of the spheroids (Sgouros et al., 2003). Therefore, the drug sensitivity assay was also performed on a monolayer-culture (2D) derived from the spheroidal culture (denoted as mono-spheroid). CRC spheroid-cultured cells at P5 were dissociated into single cell suspension and grown in their parental condition as monolayer 24 hours prior to the drug treatment. Based on the results obtained, the CRC mono-spheroids showed similar level chemoresistance as compared to their spheroid-cultured cells (Figure 4.13 and Figure 4.14). The mono-spheroids of HCT-15 and WiDr showed consistent results of chemoresistance in both 5-FU and L-OHP drug treatment, compared to their spheroid-cultured cells. However, SK-CO-1 mono-spheroids showed lower chemoresistance level compared to their spheroid-cultured cells. Nevertheless, all of the mono-spheroids showed higher chemoresistance level relative to their parental cells. This suggests that the chemoresistance observed in Figure 4.11 and Figure 4.12 were not contributed by the structural properties of the spheroid-cultured but was due to the enrichment of spheroidal culture.

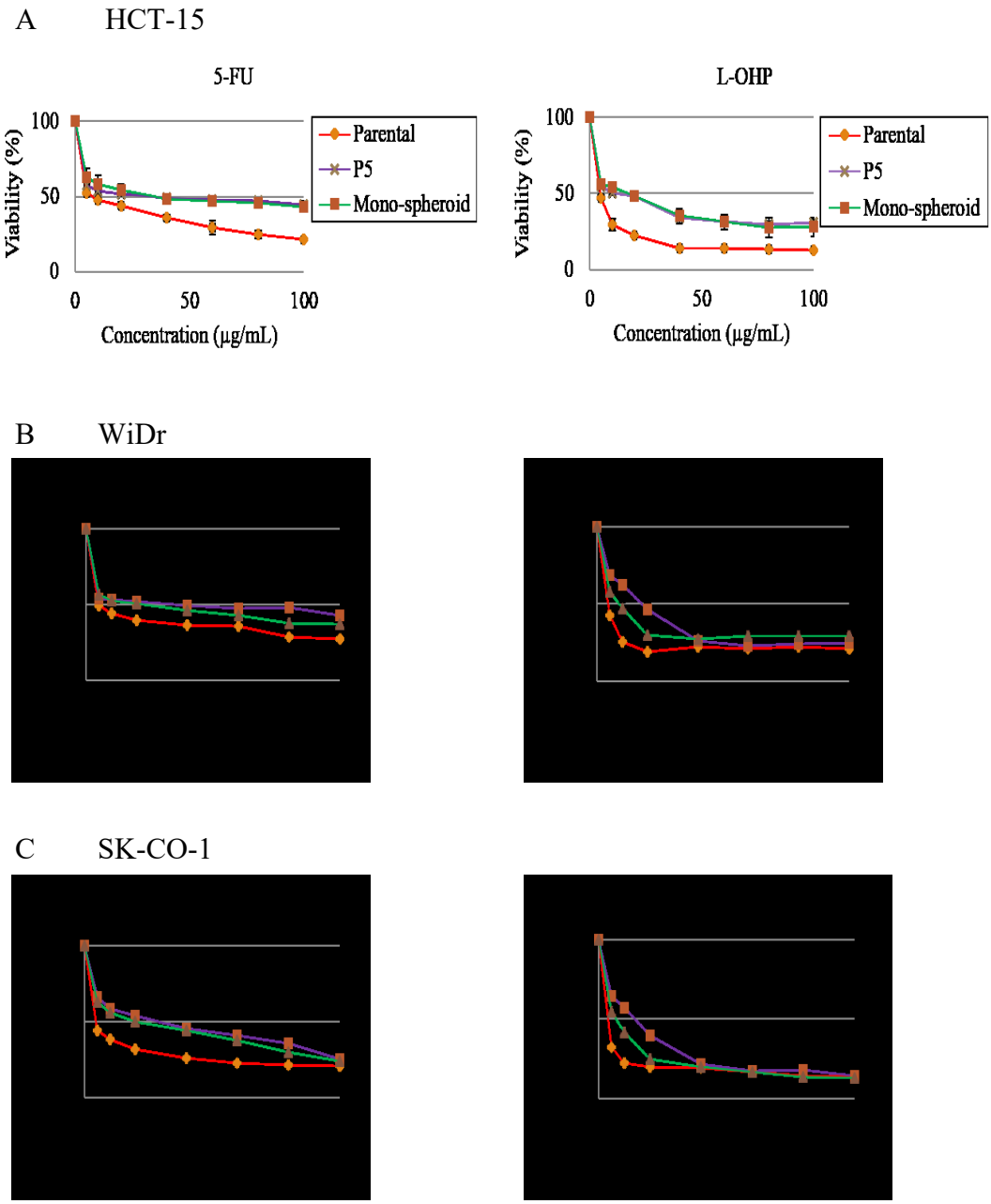


Figure 4.13 Chemoresistance properties of CRC mono-spheroids. The viability of (A) HCT-15, (B) WiDr and (C) SK-CO-1 parental, P5 spheroid-cultured cells and P5 monolayer culture-derived from spheroid culture (mono-spheroid) after treatment with 5-FU and L-OHP drugs. Results obtained from three independent experiments.

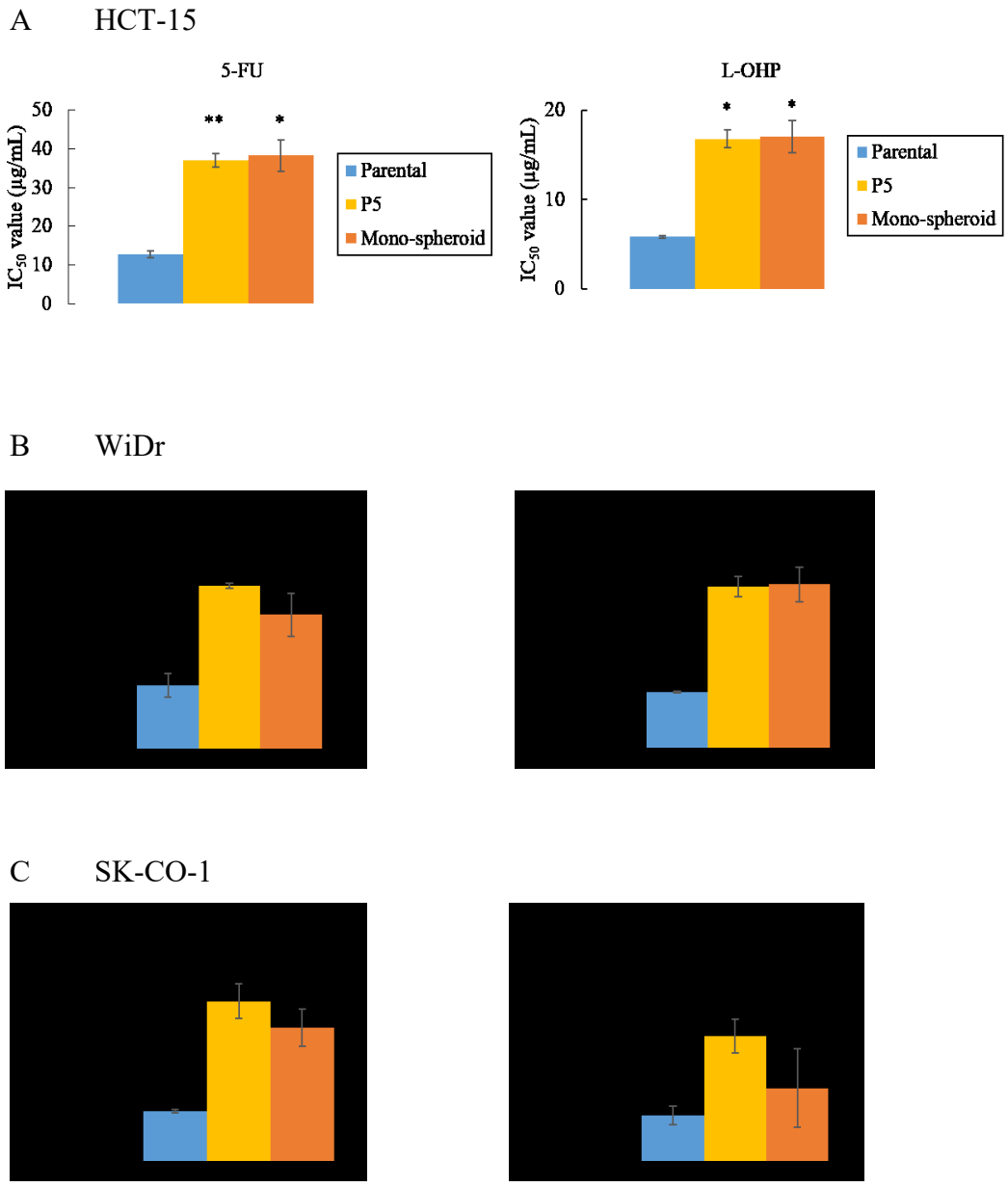


Figure 4.14 IC_{50} values of CRC mono-spheroids. Bar graphs represent IC_{50} values of (A) HCT-15, (B) WiDr and (C) SK-CO-1 parental, P5 spheroid-cultured cells and P5 monolayer-culture-derived from spheroid culture (mono-spheroid) after treatment with 5-FU and L-OHP drugs. Results obtained from three independent experiments. *p<0.05 and **p<0.01 were values obtained in comparison to their parental cells.

4.11 ABC Transporter Genes Displayed Distinctive Expression Profiles in Spheroid-Cultured Cells

ATP-binding cassette transporters have been reported to be linked with the development of chemoresistance in cancer and cancer stem cells (Rebucci and Michiels, 2013). To investigate the possible roles of ABC transporters in the enhanced chemoresistance properties observed in Figure 4.11, the gene expression profile of selected ABC transporters in the CRC parental and spheroid-cultured cells was established. Out of the known 49 members in ABC transporters family, 14 ABC transporter genes were selected based on literature reviews and mRNA levels were evaluated using real-time qRT-PCR. The selection criteria set was based on reports of involvement of the ABC transporters in the development of chemoresistance in any type of cancers. The expression levels of CRC P5 spheroid-cultured cells were compared relative to their parental cells (Figure 4.15). All 14 ABC transporter genes were uniquely regulated in CRC spheroid-cultured cells with regards to their parental expression level. In at least 2 of the CRC spheroid-cultured cells, four genes were up-regulated including *ABCA2*, *ABCB1*, *ABCC5* and *ABCC12*, while 5 genes were down-regulated including *ABCB4*, *ABCB11*, *ABCC1*, *ABCC3* and *ABCC11*. On the other hand, *ABCA3* and *ABCC2* were consistently and significantly up-regulated while *ABCB5*, *ABCC4* and *ABCG2* were consistently down-regulated in all 3 CRC spheroid-cultured cells, compared to their parental cells (Table 4.1).

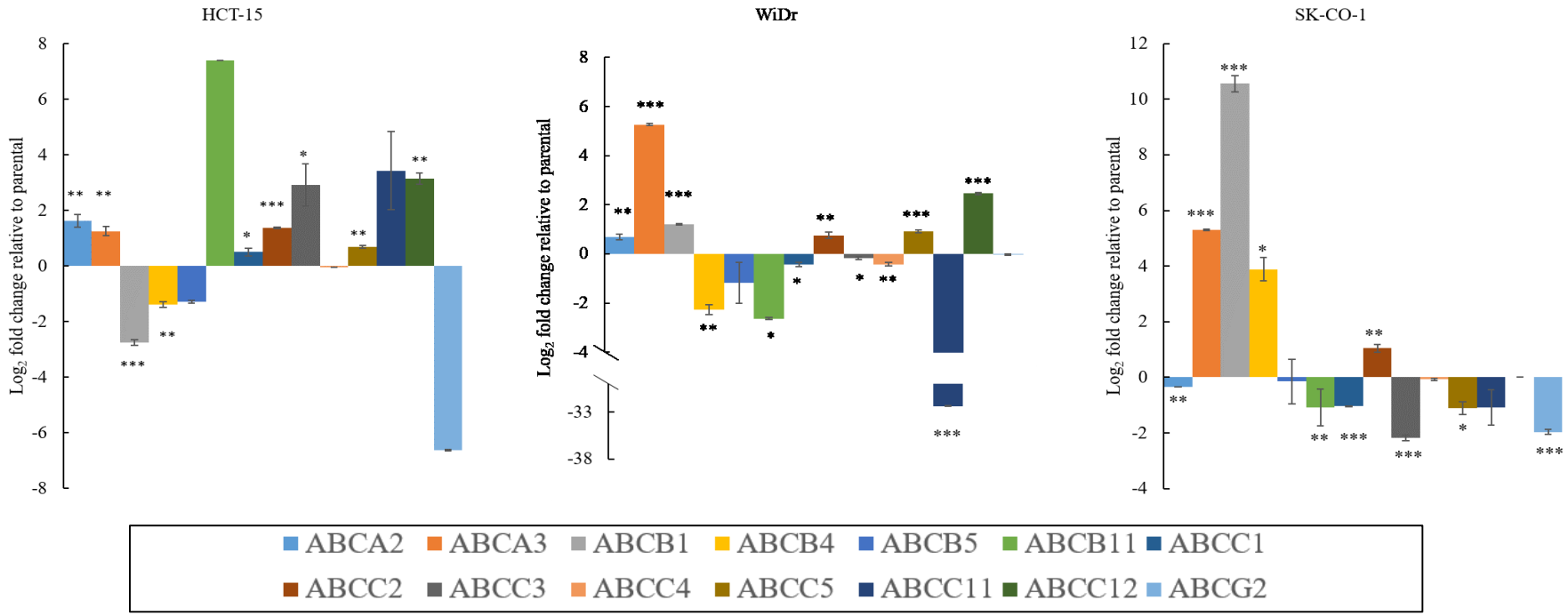


Figure 4.15 Selected ABC transporter gene analysis via real-time qRT-PCR. The bar graphs represent the $\log_2$ fold change of selected ABC transporter genes of HCT-15, WiDr and SK-CO-1 P5 spheroid-cultured cells relative to their parental cells. Results obtained from three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 were values obtained in comparison to their parental cells.

Table 4.1 Expression profile of selected ABC transporter genes in CRC spheroid-cultured cells

ABC Transport Genes	Colorectal Cancer Cell		
	HCT-15	WiDr	SK-CO-1
ABCA2	+	+	-
ABCA3	+	+	+
ABCB1	-	+	+
ABCB4	-	-	+
ABCB5	-	-	-
ABCB11	+	-	-
ABCC1	+	-	-
ABCC2	+	+	+
ABCC3	+	-	-
ABCC4	-	-	-
ABCC5	+	+	-
ABCC11	+	-	-
ABCC12	+	+	/
ABCG2	-	-	-

(+) indicates up-regulated, (-) indicates down-regulated, (/) indicates no difference in the expression of ABC genes in CRC spheroid-cultured cells relative to their parental expression levels.

CHAPTER 5

DISCUSSION

5.1 Properties of CrCSCs Enriched Via Spheroidal Culture

5.1.1 Enhanced Self-Renewal Ability and Cellular Dormancy of CrCSCs

Evidence on the theory of cancer stem cells (CSCs) suggest the need to revise the current understanding of cancer and conventional chemotherapeutic approaches (Liu et al., 2015). Due to the rarity of CSC population, various characteristics of CSC population remains to be investigated (Clevers, 2011; Yang et al., 2015). The present study showed that spheroidal culture was able to enrich CRC population with CSC-like properties, indicating that spheroidal culture may be used as a working model to elucidate the characteristics of CSC population. Prolonged spheroid-cultured cells showed increased expression levels of some CrCSC-associated markers such as CD44 and ALDH1, enhanced clonogenic ability and slower proliferation rate compared to their parental cells (Figure 4.4 – 4.7). The observation on CrCSC-associated surface markers will be discussed in the next section (Discussion Section 5.2).

The spheroid-cultured CRC cells showed enhanced clonogenic ability. Similar findings were also reported by Dallas et al. (2009), Wei et al. (2012) and Leng et al. (2013). The increased clonogenic ability reflects the enhanced

self-renewal capacity, an important hallmark of stem cells (SCs) and CSCs (Nakahata et al., 2015). Although serial xenotransplantation is the golden standard to date for functional assay of self-renewal capacity, however colony forming assay can also be used to access short-term self-renewal capacity of the spheroid-cultured cells (Steffen et al., 2011; Facompre et al., 2012).

SCs and CSCs with enhanced self-renewal abilities undergo cell division, which produce daughter cells with similar proliferation and differentiation potential, resulting in maintenance of cell pool (Garza-Treviño et al., 2015). This unique feature that was once thought to be exclusive to only SC population has now been linked to carcinogenesis, specifically with initiation and maintenance of cancer growth (Wu, 2008). Numerous signalling pathways have been proposed for the maintenance of self-renewal ability in various CSC populations. In CrCSC population, Wnt, Notch and Hedgehog pathways have been reported to be the main key players in maintaining self-renewal ability of CSCs (Borah et al., 2015). Collectively, the spheroid-cultured cells derived from CRC presented in the present study showed enhanced self-renewal ability, suggesting possible roles in CRC tumour maintenance.

The cellular dormancy observed in CRC spheroid-cultured cells has been widely reported as a trait of CSC population (Lyle and Moore, 2011; Kleffel and Schatton, 2013; Enderling, 2013). Zhao et al. (2013), Nyga et al. (2013) and Luca et al. (2013) reported that the proliferation rates in CRC cells cultured in 3D spheroidal culture were significantly lower compared to cells

grown in 2D monolayer-culture. However, Wei et al. (2012) and Li et al. (2012) reported that CRC spheroid-cultured cells showed higher proliferation rate than the parental cells. These differences were likely due to the use of different CRC cell lines and duration of culture. The present study focused on CRC cell lines, HCT-15, WiDr and SK-CO-1, grown in spheroidal culture for a prolonged duration of 3 months. On the other hand, the previously mentioned studies used different CRC-derived cell lines which were cultured for a shorter duration in the spheroidal culture. The phenotypic and genotypic characteristics of the resulting population of spheroidal culture is dependent on the parental properties of the cells and niche created, accounting for the dissimilarities in the observation (Edmondson et al., 2014).

Quiescent or cellular dormancy is a protective mechanism engaged by stem cells that arrest the cell cycle progression during encounters of futile environments, hence ensuring the survival of stem cells (Glauche et al., 2009). This unique property has been associated with CSC population, evident by the high expression level of anti-apoptotic proteins, enhanced capacity of DNA repair mechanism and reduced proliferation and cell cycle arrest (Patel and Chen, 2012). Quiescent is often linked with the development of chemoresistance among CSCs, which will be further be discussed in Section 5.3.

5.1.2 Maintenance of Stemness by OSKMN

In the present study, the CRC population derived from spheroidal culture showed large similarities with reported properties of CrCSC population. Further analysis revealed that spheroidal culture was also able to enrich and maintain the CSC population in a passage-dependent manner. At increasing passages of spheroidal culture, the expression levels of OSKMN were generally up-regulated. Enhanced expression of OSKMN indicates the enrichment of "stemness" in the spheroid-cultured cells. Conversely, the expression levels of OKSMN were down-regulated upon re-differentiation, suggesting the loss of "stemness" when the cells were grown in non-spheroidal culture condition.

The discovery Yamanaka factors allowed systemic reprogramming of cells to a pluripotent state using a set of defined factors, OSKM. This leads to the elucidation of the functional roles of *OCT4*, *SOX2* and *NANOG* as pluripotency regulator and differentiation repressor. Subsequently, OSKMN have been individually and collectively associated with CSC genotypic characteristics (Takahashi and Yamanaka, 2006; Wang et al., 2012).

The enhanced expression of *OCT4* in cells of higher level of cellular hierarchy and decreased expression upon differentiation, has been linked with poor cancer prognosis (Gazouli et al., 2012; Kumar et al., 2012). *SOX2* plays a pivotal role in determining cellular fate. Aberrant regulation of *SOX2* has been linked with poor prognosis of cancers (Lundberg et al., 2016). *KLF4* is

responsible for the maintenance of the self-renewal ability of stem cells and regulates biological processes such as proliferation, differentiation and apoptosis (McConnell and Yang, 2010). *c-MYC,* a known oncogene that plays role in the development cancers, regulates various aspects of cells, including cell growth, cell apoptosis and cell cycle (Wang et al., 2008; Kress et al., 2015). *NANOG,* which regulates the embryonic development and cancer progression, has been correlated in the maintenance of the "stemness" in CSC population (J. Zhang et al., 2013; Jeter et al., 2015).

The OSKMN machinery is reported by regulated by an interdependent mechanism (Figure 5.1). *KLF4*, the master up-stream regulator in OKSMN interactions, activates *OCT4* and *SOX2*, forming a protein complex. The protein complex in return regulates the downstream effector, *NANOG*, which determines the cellular fate and differentiation state of the cells. *KLF4* also regulates *c-MYC,* which controls genes associated with progression in cell cycle (Niwa, 2007; Kim et al., 2008; Zhang et al., 2010). Collectively, the enhanced expression of OSKMN in the spheroid-cultured cells indicates higher population of undifferentiated proliferating cells, which is a common characteristic of CSCs.

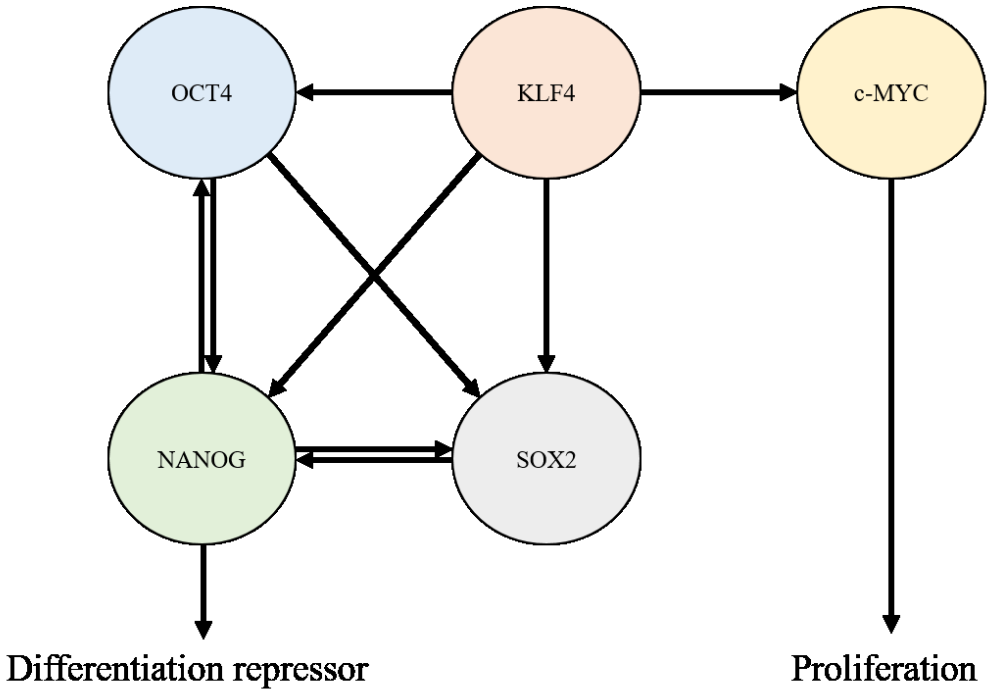


Figure 5.1 Schematic diagram of OSKMN gene interactions in "stemness" maintenance in CSC population. The coloured circles indicate *OCT4*, *SOX2*, *KLF4*, *c-MYC* and *NANOG*, respectively. The arrow indicates the direction of gene regulation. Figure adapted and modified from McConnell and Yang (2010).

5.1.3 CrCSC Enrichment by Spheroidal Culture is Cell Line-Dependent

The use of spheroidal culture to study CSC population has been in a constant debate with contradicting reports on the characteristics of population derived from spheroidal culture. Reports by Kai et al. (2009), Muraro et al. (2012) and Wu et al. (2014) suggested that spheroid-cultured CRC cells showed reduced levels of CSC characteristics as compared to the parental cells. On the other hand, Fan et al. (2011), Shaheen et al. (2016) and Liu et al. (2013) reported that spheroidal culture resulted in enrichment of CrCSC population. The difference in the observations can be accounted for by a few factors, such as culture technique and media composition (Schatton and Frank, 2010). Besides that, the population derived from spheroidal culture is dependent on the type of cell lines used (Miki and Rhim, 2008; Calvet et al., 2014).

The CRC cell lines used in the present study which were HCT-15, WiDr and SK-CO-1, showed different levels of CSC-associated characteristics. HCT-15 and WiDr were derived from colon cancer adenocarcinoma while SK-CO-1 was derived from malignant metastatic ascites. Based on the results, HCT-15 and WiDr showed a uniform pattern of increment in the expression levels of CSC-related phenotypes and gene regulation in passage-dependent manner, while SK-CO-1 showed a dysregulated pattern. For instance, the OSKMN expression in SK-CO-1-derived spheroid cells showed no clear pattern at increasing passages of spheroidal culture as compared to HCT-15 and WiDr. Poorly differentiated cancer cells have been reported to show more dysregulated

cellular control, correlating with the data observed in SK-CO-1 (Stigliano et al., 2014).

Based on the results, cells derived from prolonged spheroidal culture (P5-derived CRC cells) matches the CSC profile. Most of the published reports on the use of spheroidal culture utilise a short-term duration of culture. Our results suggested that the prolonged spheroidal culture maximises the enrichment effect on cancer cells, producing less differentiated cells with higher chemoresistance and self-renewal ability. These phenotypes resemble CSC population (Garza-Treviño et al., 2015). Collectively, the results suggested that prolonged spheroidal culture was able to enrich the CRC population with CSC-like population.

5.2 Lack of Specific CrCSC-Related Surface Markers

Identification and validation of CrCSC population are often performed by analysing the putative CSC surface markers (Dou and Gu, 2010; Kantara et al., 2015; Sun et al., 2016). The present study analysed the expression of three common surface markers, CD133, CD44 and ALDH1, which have been associated with CrCSC population. Based on the results, the parental CRC cells showed high expression levels of all three surface markers, while only some of the surface markers were over-expressed in spheroid-cultured cells.

The current observation is in line with other reports. $CD133^+$- and $CD133^-$-expressing CRC cells showed no significant difference in the tumourigenic ability *in vivo* (Ieta et al., 2008; Shmelkov et al., 2008; Muraro et al., 2012). Both, $CD44^+$- and $CD44^-$-expressing CRC cells were able to form *in vivo* tumours (Du et al., 2008; Kai et al., 2009). Down-regulation of CD44 in CRC cells showed increased tumourigenicity in CRC cells (Dallas et al., 2012). This, the contradicting reports on CD44 further question the validity of CD44 as a putative CrCSC marker. Moreover, Khorrami et al., (2015) corroborated with the result obtained in the present study, in which CRC spheroid-cultured cells showed reduction in ALDH1 expression, corresponding to no significant difference in the tumourigenicity compared to the high expression in parental cells. This collectively suggests that CD133, CD44 and ALDH1 lack correlation with CSC population.

CD133, CD44 and ALDH1 are reported to be non-specific cancer markers (Medema, 2013). The ubiquitous expression of the acclaimed CSC markers in various other cancers could suggest a need to review the usage of CD133, CD44 and ALDH1 as CrCSC-specific biomarkers. CD133, CD44 and ALDH1 have been reported to be expressed in breast, liver, lung, melanoma, pancreatic and prostate cancers (Klonisch et al., 2008; Keysar and Jimeno, 2010; Medema, 2013). Although CSC populations derived from various cancers showed some overlapping similarities, the generalisation that all CSC population derived from different cancers share the same genotypic and phenotypic characteristics (including surface marker antigens) should be validated further (Tirino et al., 2013). Moreover, the expression of CSC markers

are not limited to only cancerous population but some are highly expressed in non-cancerous population too (Karsten and Goletz, 2013).

Collectively, the lack of exclusive biomarkers in discriminating CSC and non-CSC population prompts a need to find novel biomarkers of CSC that are highly preserved in specific cancer cell population. The growing numbers of reports on CSC research with contradicting observations of the biomarker expression levels further suggest the need for a set of meticulous criteria for defining possible CSCs biomarkers.

5.3 Development of CrCSC-Mediated Chemoresistance

Chemoresistance is often regarded as a hallmark of CSCs (Onaitis and Hanna, 2013). The enhanced chemoresistant ability observed in the CRC spheroid-cultured cells is a common observation reported by many groups (Fan et al., 2011; Colak and Medema, 2016). Besides that, the results from the present study are agreement with Boo et al., (2016) that suggest the chemoresistance observed in the spheroid-cultured cells was contributed by the true nature of the cells and not imposed by the physical barrier of the 3D spheroid structure. Moreover, the up-regulated expression of *ABCA3* and *ABCC2* genes in all CRC spheroid-cultured cells further suggest the possible role of these genes in the development of chemoresistance in CrCSCs (to be discussed further in Section 5.4).

The development of chemoresistance in CSC population has been reported to be mediated by various mechanisms (Zhao, 2016). Besides ABC transporter proteins, the slow-proliferation of spheroid-cultured cells might have contributed to the chemoresistance observed. The quiescent state in CSCs is reported to be regulated by PI3K-AKt signalling, which controls the G_0-G_1 cell cycle transition (Chen et al., 2016). As the conventional cancer treatment targets rapidly dividing cells, a hallmark of cancer, the quiescent spheroid-cultured cells are less likely to be affected by the chemotherapeutic treatment (Pece et al., 2010).

The enhanced expression of ALDH1 in the spheroid-cultured cells may also be a contributing factor. ALDH1, a detoxifying enzyme, has also been reported to confer chemoresistance by metabolising aldehyde, a common intermediate metabolite of chemotherapeutic drugs such as cisplatin (Keysar and Jimeno, 2010). The oxidation of the intermediate metabolite reduces the bioavailability of chemotherapeutic drugs in biological system, hence rendering the treatment ineffective (Marcato et al., 2011). Other mechanisms such as altered DNA repair response and loss of balance in pro- and anti-apoptotic proteins have been proposed to confer chemoresistance in CSC population (Abdullah and Chow, 2013). All things considered, the chemoresistance mechanisms acquired by CSC population imposes great limitation to the current treatment approach, prompting novel therapies targeting different aspect of the cancerous population. CSC-targeted therapy would significantly reduce the cancer recurrence and provide an effective tool in eradicating cancer (Dragu et al., 2015).

5.4 Proposed Use of ABC Transporters as Specific CrCSCs Biomarkers

Gene expression analysis of 14 selected ABC transporters showed distinct regulation of ABC transporters in CRC spheroid-cultured cells as compared to their parental cells. *ABCA3* and *ABCC2* were consistently up-regulated in all three CRC spheroid-cultured cells, while *ABCB5*, *ABCC4* and *ABCG2* were consistently down-regulated. The overexpression of *ABCC2* has been previously linked with CRC chemoresistance phenotype. However, there is a lack of association *ABCA3* with chemoresistance in CRC (Fujita et al., 2008; Salphati et al., 2009; Xu et al., 2012; Vinette et al., 2015). Overexpression of *ABCA3* has been commonly associated with the development of chemoresistance in acute myeloid leukaemia and neuroblastoma (Marzac et al., 2011; Jeong et al., 2015; Khalil et al., 2012).

ABCA3, a member of ABCA family, functions as intracellular vesicular transport while *ABCC2* also known as multiple drug resistance protein 2 (MRP2) is an apical membrane commonly expressed in polarised cells of liver, intestine and kidney (Hirschmann-Jax et al., 2004; Steinbach et al., 2006; Yamasaki et al., 2011; Song et al., 2015). *ABCA3* and *ABCC2* have been reported to efflux a wide range of substrates including chemotherapeutic drugs (Andersen et al., 2015). Despite ABC transporters have been strongly associated with the development of chemoresistance, only few ABC transporters have been associated with chemoresistant CSC population as reviewed by Fletcher et al.

(2010) (Table 5.1). To the best of our knowledge, this is the first study to correlate *ABCA3* and *ABCC2* with CrCSC population.

Over-expression of ABC transporter proteins in cells is conventionally taken to indicate high drug efflux activity that results in high chemoresistant ability (Zinzi et al., 2014). Therefore, the over-expression of *ABCA3* and *ABCC2* suggests a common factor that could have contributed the enhanced chemoresistance observed in the all the spheroid-cultured CRC cells. Despite the lack of reports on the role of ABC transporters in CrCSC-mediated chemoresistance, further investigations would be able to elucidate the roles of *ABCA3* and *ABCC2* in the development of chemoresistance in CrCSC population.

On the other hand, the consistently down-regulated *ABCB5* and *ABCC4* are not in agreement with previous investigations. CRC cell lines and tissues which exhibited high chemoresistance were reported to express high levels of *ABCC4* and *ABCB5* (Kool et al., 1997; Maubon et al., 2007; Gradilone et al., 2008; Wilson et al., 2011; Hu et al., 2016). As CrCSC population could be genotypic-phenotypically distinct compared to the normal CRC population, the expression of *ABCB5* and *ABCC4* in CrCSC population could be different (Akunuru et al., 2012). This may account for the differences in the findings. The down-regulation of *ABCG2* was consistent with other findings (Gupta et al., 2006; Andersen et al., 2015). The down-regulation of ABCG2 hampers the physiological role of ABCG2 in protecting normal colon cells from diet-derived carcinogen, thus resulting in CRC oncogenesis (Andersen et al., 2015).

Table 5.1 Expression of selected ABC transporters in CSC-like populations

(Adapted and modified from Fletcher et al., 2010)

ABC subfamily	ABC subunits	Expression in CSC-like populations
ABCA	ABCA1	ND
	ABCA2	Lung cancer cell lines and acute myeloid leukaemia
	ABCA3	Neuroblastoma
ABCB	ABCB1	Acute myeloid leukaemia and lung cancer cell lines
	ABCB4	ND
	ABCB5	Melanoma
	ABCB11	ND
ABCC	ABCC1	Squamous cell carcinoma lines, glioma, lung cancer cell lines, and acute myeloid leukaemia
	ABCC2	ND
	ABCC3	ND
	ABCC4	ND
	ABCC5	ND
	ABCC6	ND
	ABCC10	ND
	ABCC11	ND
ABCG	ABCG2	Acute myeloid leukaemia, lung cancer, oesophageal carcinoma, neuroblastoma, squamous cell carcinoma cell lines, ovarian cancer, melanoma, glioma and nasopharyngeal carcinoma cell lines

ND: Not determined

The current surface markers used in the identification of CrCSC population, CD133, CD44 and ALDH1, shows a lack of correlation to CSC population as shown in the present study. The co-expression of CSC-associated surface markers, such as CD133, CD44 and ALDH1 could be used to further refine the population, in an attempt to get the true population of CSCs. However, the distinct regulation of ABC transporter genes in the CrCSC population derived from spheroidal culture could be exploited as prospective biomarkers for CrCSC population (Zinzi et al., 2014). The present study proposes the use of $ABCA3^{high}/ABCC2^{high}/ABCB5^{low}/ABCC4^{low}/ABCG2^{low}$ phenotype as an expression profile to isolate true CrCSC population with enhanced chemoresistant properties. Further investigation of this population could potentially aid in the understanding of phenotypic and genotypic regulation of CrCSC population.

Taking all into consideration, spheroidal culture is an effective model in enriching the population with cancer stem cells. The chemoresistance observed in the CSC population could highly be mediated by the ABC transporter genes; *ABCA3* and *ABCC2*. These genes could facilitate further understanding the properties and mechanism of chemoresistance in CrCSC population.

CHAPTER 6

CONCLUSIONS

6.1 Conclusions

In the present study, spheroidal culture demonstrated a systematic enrichment of CSC population in passage-dependent manner. The characterisation of the prolonged CRC spheroid-cultured cells revealed a close resemblance to CrCSC population as compared to single spheroidal culture. However, the prolonged spheroidal culture derived CrCSC population in the present study showed a lack of association with the putative markers of CrCSC. Besides the gradual up-regulation of pluripotent and tumourigenic gene expression levels at increasing passages of spheroidal culture, similar observation was recorded for the chemoresistant properties of the CRC spheroid-cultured cells. The present study identified two consistently up-regulated ABC transporter genes, *ABCA3* and *ABCC2*, and three down-regulated, *ABCB5*, *ABCC4* and *ABCG2*, in all three CRC cell-lines derived CrCSC population. The unique gene regulation profile of selected ABC transporters suggests the possible role ABC transporters in the development of chemoresistance in CrCSC population. In conclusion, the present study showed prolonged spheroidal culture is an effective method in enriching CrCSC population with enhanced CSC-associated characteristics, including chemoresistance. The unique profile of ABC transporter genes, especially the

up-regulated ABC transporters, ABCA3 and ABCC2 can be used as novel biomarkers for CrCSC identification.

6.2 Limitations of Present Study and Future Direction of Research

The present study generated spheroid-cultured CrCSC population from CRC cell lines, HCT-15, WiDr and SK-CO-1. The limitations with the use of cell lines in CSCs research are well-documented and include the fact that the stable fraction of CSC population in cell lines are significantly smaller as compared to patient-derived tissues. Long-term *in vitro* culture of cell lines would have accumulated additional mutations to cause genetic and epigenetic changes.

The present study only assessed the expression profile of common putative CrCSCs markers CD133, CD44 and ALDH. Other markers such as CD166 and CD24, which have been associated with CrCSC population were not assessed. Other than that, some of the ABC transporters, including ABCC6 and ABCC10, that have been recently associated with chemoresistance in cancer were also not screened in the present study.

The limitations of the present study will be addressed in the next phase of further study. Western blot analysis of ABC transporters will be performed to validate the protein expression of the up-regulation and down-regulated ABC transporter genes. Furthermore, the effect of the prolonged spheroidal culture

will be accessed using primary CRC tissue obtained from patients. The spheroid-cultured primary tissues will be subjected for extensive characterisation, including more putative CrCSC markers. Subsequently, *in vitro* limiting dilution assay will be performed to investigate the capacity of the spheroid-cultured cells to form colonies at lower cell density. Isolation and characterisation of ABCA3 and ABCC2 population from the spheroidal culture will be performed to evaluate the potential use of ABC transporters as CrCSC biomarkers.

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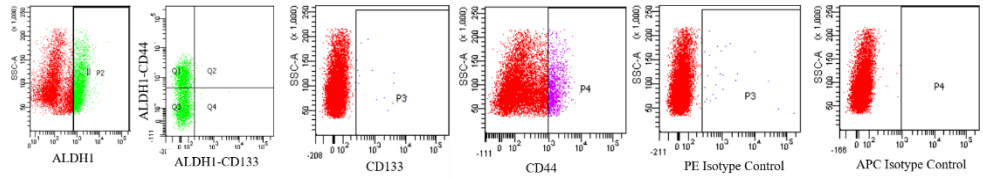
APPENDICES

APPENDIX A

Flowcytometry dot plot graphs of HCT-15 parental cells analysed by BD FACSDIVA™ software (BD Biosciences)

1st replicate



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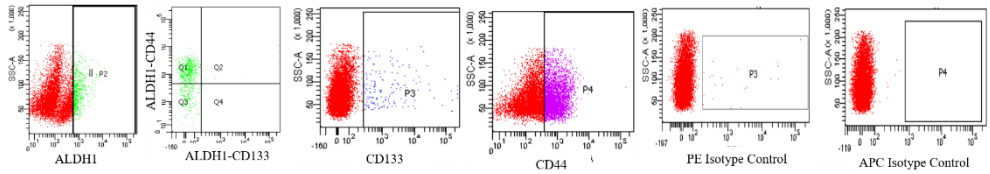

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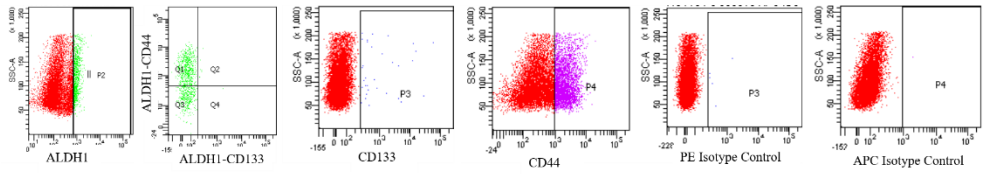
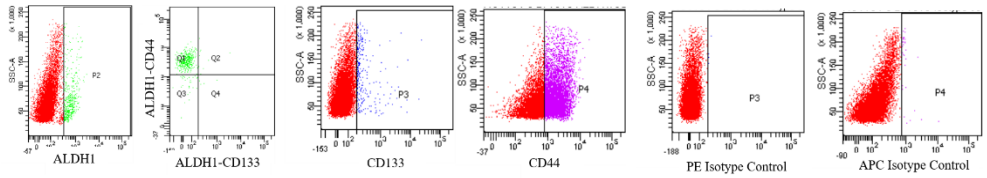


Three independent experiments were carried out (equivalent to Figure 4.4 and Figure 4.5).

APPENDIX B

Flowcytometry dot plot graphs of HCT-15 spheroid-cultured cells analysed by BD FACSDIVA™ software (BD Biosciences)

1st replicate



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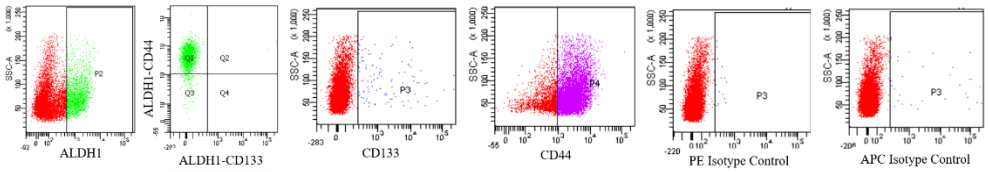

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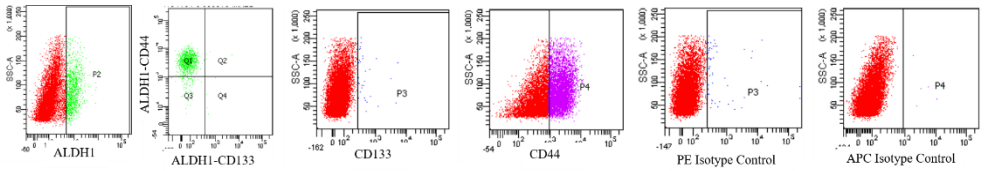
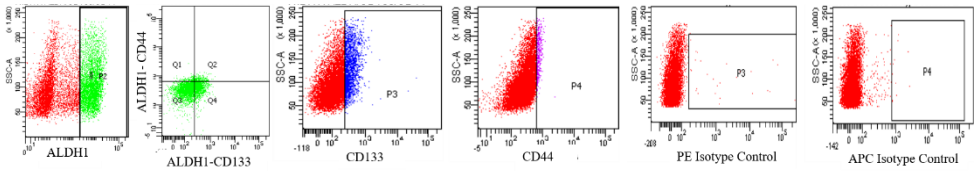


Three independent experiments were carried out (equivalent to Figure 4.4 and Figure 4.5).

APPENDIX C

Flowcytometry dot plot graphs of WiDr parental cells analysed by BD FACSDIVA™ software (BD Biosciences)

1st replicate



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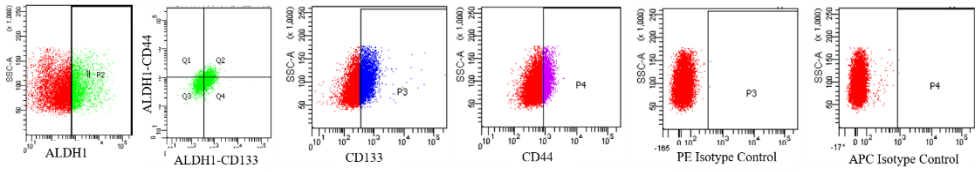

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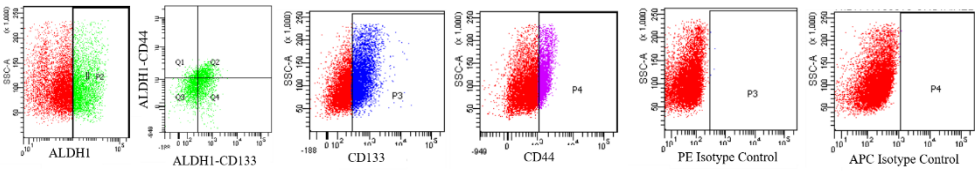
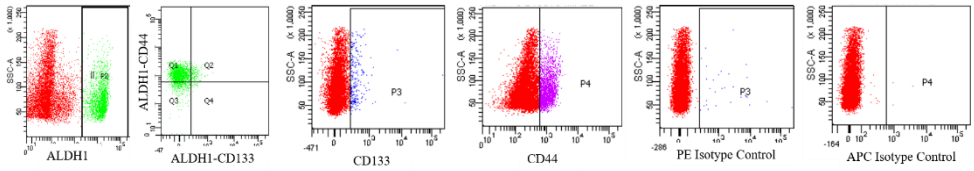


Three independent experiments were carried out (equivalent to Figure 4.4 and Figure 4.5).

APPENDIX D

Flowcytometry dot plot graphs of WiDr spheroid-cultured cells analysed by BD FACSDIVA™ software (BD Biosciences)

1st replicate



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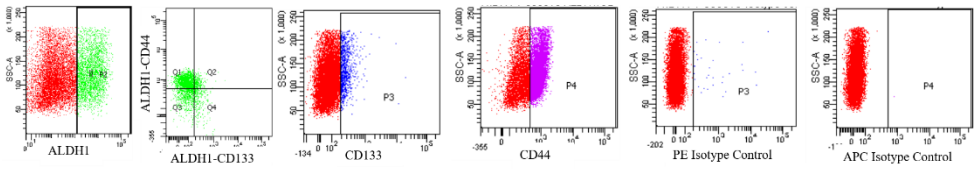

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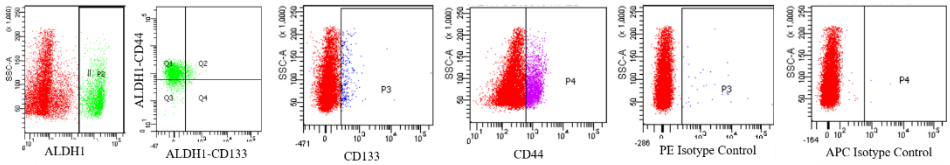
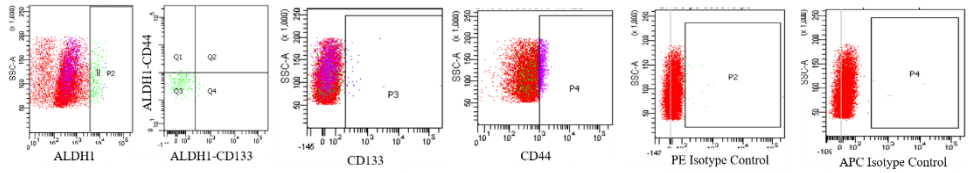


Three independent experiments were carried out (equivalent to Figure 4.4 and Figure 4.5)

APPENDIX E

Flowcytometry dot plot graphs of SK-CO-1 parental cells analysed by BD FACSDIVA™ software (BD Biosciences)

1st replicate



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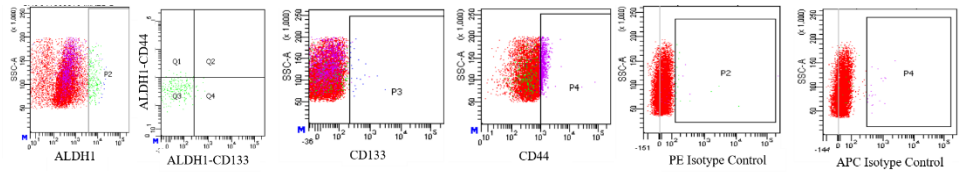

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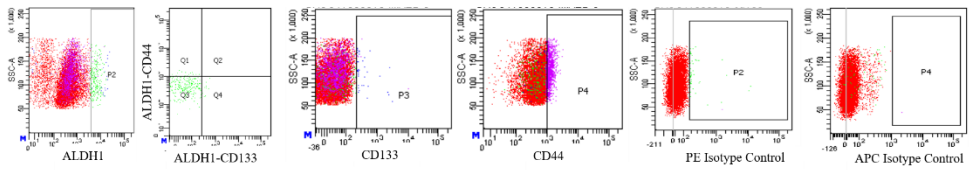
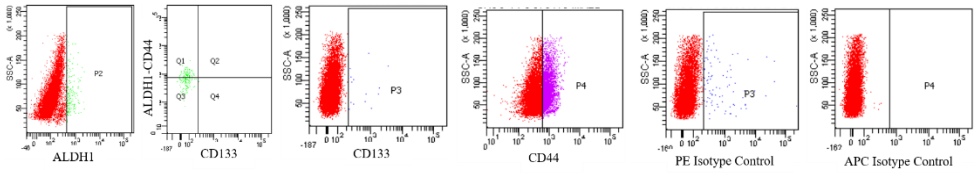


Three independent experiments were carried out (equivalent to Figure 4.4 and Figure 4.5).

APPENDIX F

Flowcytometry dot plot graphs of SK-CO-1 spheroid-cultured cells analysed by BD FACSDIVA™ software (BD Biosciences)

1st replicate



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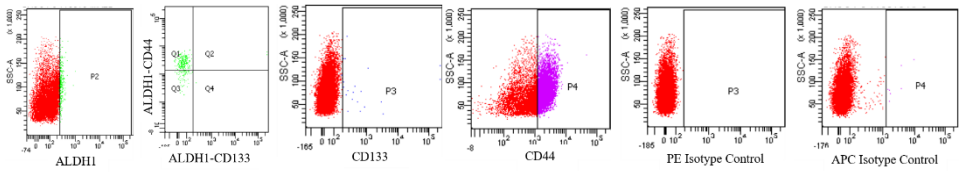

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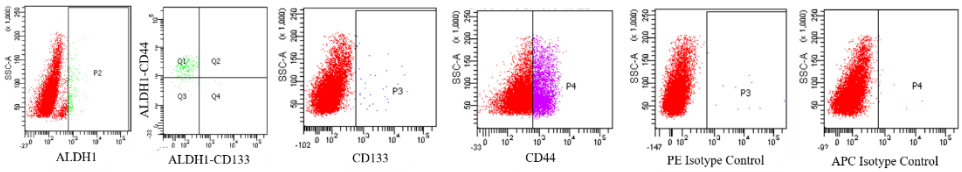
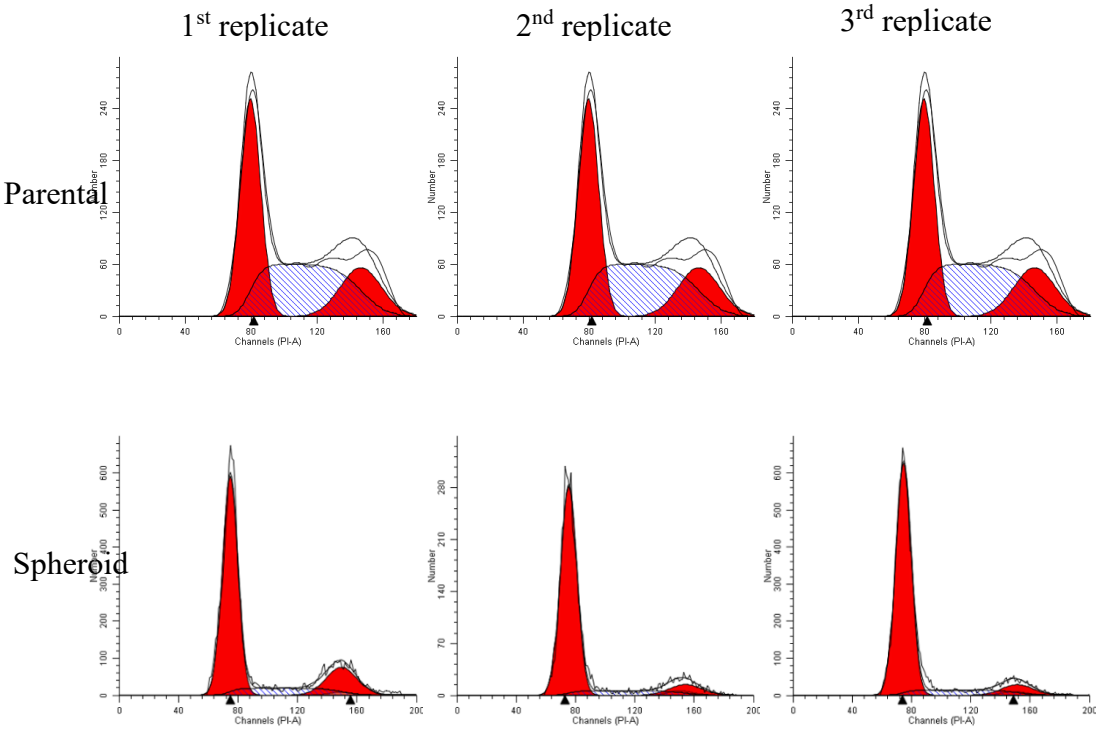


Three independent experiments were carried out (equivalent to Figure 4.4 and Figure 4.5).

APPENDIX G

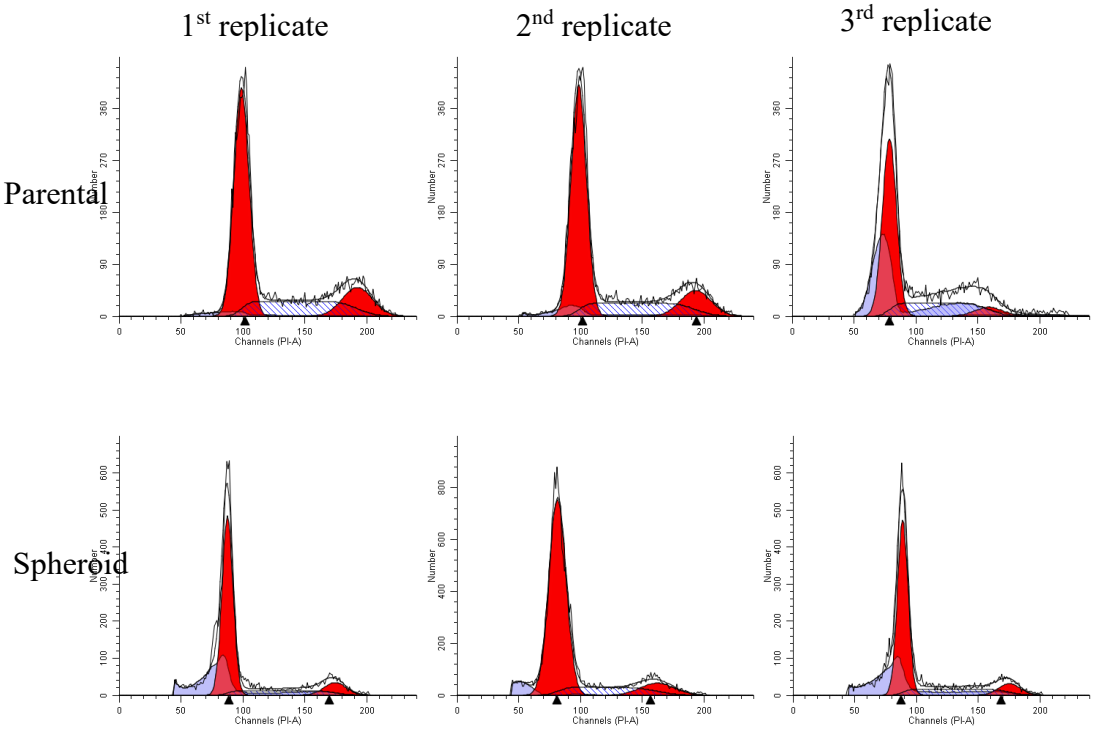
Cell cycle histograms of HCT-15 parental and spheroid-cultured cells analysed by ModFit LT™ software (BD Biosciences)



Three independent experiments were carried out (equivalent to Figure 4.7).

APPENDIX H

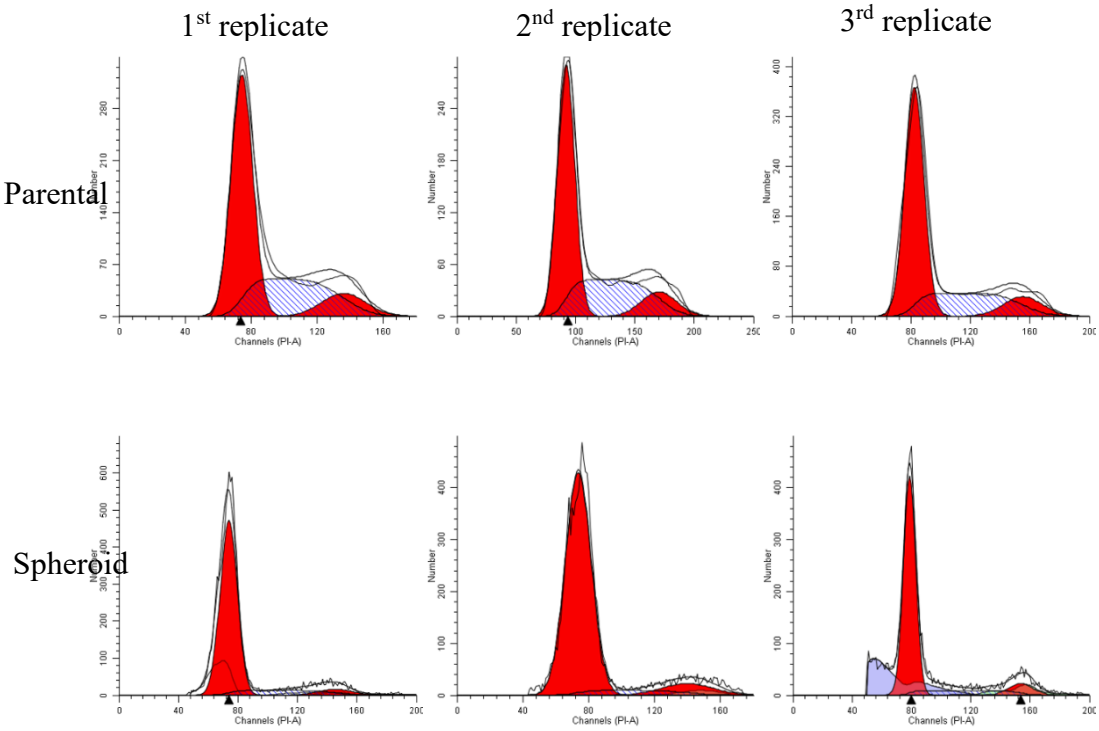
Cell cycle histograms of WiDr parental and spheroid-cultured cells analysed by ModFit LT™ software (BD Biosciences)



Three independent experiments were carried out (equivalent to Figure 4.7).

APPENDIX I

Cell cycle histograms of SK-CO-1 parental and spheroid-cultured cells analysed by ModFit LT™ software (BD Biosciences)



Three independent experiments were carried out (equivalent to Figure 4.7)