Thesis for the Master's degree in Molecular Biosciences
Main field of study in Molecular biology

Kari-Anne Myrum Frikstad

Significance of the metastasis-promoting protein S100A4 in chemotherapy sensitivity in colorectal cancer cell lines

60 study points

Department of Molecular Biosciences
Faculty of mathematics and natural sciences
UNIVERSITY OF OSLO, December 2012

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2012

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Year: 2012

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http://www.duo.uio.no/

Print: Reprosentralen, Universitetet i Oslo

Abstract

Colorectal cancer (CRC) is a common type of cancer, and at present the TNM staging system is the only tool in routine clinical use for predicting patient outcome and making treatment decisions. Nuclear expression of the metastasis associated protein S100A4 is a promising prognostic biomarker in CRC, and has been shown to identify a subset of TNM II patients with poor prognosis. This suggests that patients in TNM II which express nuclear S100A4 might benefit from adjuvant chemotherapy, which is usually given only to TNM III patients.

In this project, we investigated the significance of S100A4 expression on sensitivity towards four commonly used chemotherapeutic drugs in CRC, 5-flourouracil (5FU), irinotecan (IRI), oxaliplatin (OXA) and cetuximab (CET). Two human CRC cell lines (HCT116 and SW620) were experimentally modified to express different levels of S100A4, and were exposed to increasing drug concentrations in 2D and 3D cultures. Cell viability assessed by the MTS assay was used as a screening tool, and some results were followed up using clonogenic survival and spheroid assays.

HCT116 cells were generally more sensitive to the drugs than SW620 cells, while both cell lines were resistant to CET. S100A4 related differences in sensitivity were observed with the MTS assay in HCT116 treated with 5FU and SW620 treated with OXA, while there was no variation with IRI treatment. For OXA, the differences were observed at concentrations that are probably not relevant in cancer treatment in humans, whereas for 5FU the concentration range was probably relevant, but the differences could not be confirmed using other methods.

In conclusion, S100A4 expression did not substantially influence in vitro sensitivity towards the drugs in the two investigated models. However, results from in vitro experiments cannot be directly translated into the clinic, and the relevance of S100A4 expression on drug sensitivity still warrants further investigations. An immunohistochemical study of S100A4 expression is ongoing in primary tumors from patients treated with adjuvant 5FU in a phase III trial. If S100A4 expression is associated with drug efficacy, additional in vitro studies will be necessary and the models systems established in this work may become useful.

Acknowledgements

This work was performed at the Department of Tumor Biology, The Norwegian Radium Hospital, Oslo University Hospital in collaboration with the University of Oslo in the period June 2011 to November 2012.

Thanks to my supervisor at the University of Oslo Ola Myklebost, who have made it possible to work with this master thesis at the Department of Tumor Biology.

My primary supervisors have been senior scientist Kjersti Flatmark and postdoc Kjetil Boye. I want to thank you both for an inspiring and instructive time as a master student. I appreciate all the time you have spent helping me and all the answers you have patiently given. I admire your ability to cooperate and even though both of you periodically have been occupied in the clinic I have never been on my own.

Kjersti, thank you for challenging me and making me think on my own. It has greatly increased my understanding of science and also my interest in research.

Kjetil, thank you for always having time for my questions and for answering them all with a smile. Your positive manner has been helpful in periods of frustration and stress both during the practical work and in the writing process.

Thanks to the S100A4 group led by Gunnhild M. Mælandsmo which has been a great support and set the stage for a good work environment.

I specially want to thank Tove for making the modified cell lines and for her help at the start of this project, Torveig for teaching me how to work safely with the cytotoxic drugs, Solveig for all her help with the spheroids and Eivind for all his help with the western blots. I also want to thank Ingrid for giving me advice along the way and for keeping my spirit up when working late nights.

At last I want to thank my family and my boyfriend for being patient with me during the last months, for believing in me and for keeping my feet on the ground.

Kari-Anne M. Frikstad

Oslo, November 2012

Contents

Abbreviations

(v/v)	Volume/volume
5FU	5-Flourouracil
aa	Amino acid
AUC	Area under the curve
BCA	Bicinchonic acid
BSA	Bovine serum albumin
CET	Cetuximab
CRC	Colorectal cancer
DTT	Dithiothreitol
EDTA	Ethylenediamineteraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FAP	Familiar adenomatous polyposis coli
FBS	Fetal Bovine Serum
FdUMP	Flourodeoxyuracilmonophosphate
FdUTP	Fluorodeoxytriphosphate
FUTP	Flourouraciltriphosphate
g	Gram
G	Gravity
HNPCC	Hereditary non-polyposis colorectal cancer
HRP	Horseradish peroxidase
IC50	Concentration of drug needed to inhibit growth by 50 %
IRI	Irinotecan
kDa	Kilo Dalton
l	Liter
LDS	Lithium dodecyl sulfate
LV	Leucovorin
M	Molar (mol/liter)
m	Milli (10^{-6})
MES	2-(*N*-morpholino)ethanesulfonic acid
Mother strain	Refers to the parental strain of a cell line

mRNA	Messenger ribonucleic acid
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
MTS assay	Refers to the CellTiter 96® AQueous One Solution Cell Proliferation Assay
n	Nano (10^{-12})
OXA	Oxaliplatin
P/S	Penicillin/Streptomycin
PBS	Phosphate buffered saline
p-value	Probability value
shA4-3	Strain of cells transduced with lentiviral shRNA constructs towards S100A4
shNT	Strain of cells transduced with non-target shRNA lentiviral constructs
shRNA	Short hairpin ribonucleic acid
TS	Thymidylat synthase
μ	Micro (10^{-9})

1 Introduction

1.1 General principles of cancer

Cancer is a collective term for disorders of cell growth that results in invasion and destruction of healthy tissue by abnormal cells (Oxford University Press, 2008). It is a common disease especially in the Western industrialized world and in Norway there were 28 271 new cases of cancer in 2010 (Cancer Registry of Norway, 2012).

Cancer usually arises monoclonally when one single cell encounters enough mutations to get an advantage in cell growth and/or avoiding control mechanisms. The mutations that arise are often in tumor suppressor genes or proto-oncogenes (Alberts, 2008). Tumor suppressor genes are genes that suppress tumor formation and typically have gene products that are engaged in control of cell cycle progression, apoptosis and DNA repair mechanisms. Proto-oncogenes are genes that normally function as positive regulators of cell growth and division. These genes typically encode growth factor receptors, signal transduction proteins or transcription factors. The dominant mutant form of a proto-oncogene is called an oncogene and is capable of transforming a normal cell into a cancer cell (Oxford University Press, 2008).

1.2 Metastasis

Cancer progresses by accumulating an increasing number of molecular changes to overcome the control mechanisms and growth barriers (Hanahan and Weinberg, 2000). A tumor cell can thus acquire properties that allow it to invade other tissues and give rise to new tumors in other parts of the body, a process which is termed metastasis. To accomplish this complex task, the cells must detach from the primary tumor, invade neighboring tissue and intravasate into the bloodstream or lymphatic system. After survival in the circulation, cells must be able to extravasate, survive and initiate growth at a distant site (Eccles and Welch, 2007). The ability of tumor cells to metastasize is also considered one of the hallmarks of cancer

(Hanahan and Weinberg, 2000). Importantly, metastatic disease is what usually kills a cancer patient, not the primary tumor itself (Alberts, 2008).

1.3 Anatomy of the colon

The colon and rectum are the last sections of the vertebrate gastrointestinal tract before the anus and are engaged in uptake of water and salts from the indigested remains and storage of feces, respectively. The wall of the colon and rectum is built up of several layers as depicted in figure 1. In the inner layer, facing the gastrointestinal lumen we find an epithelial layer. The epithelial cells are differentiated from stem cells in the crypts between the intestinal villi and are gradually pushed outward towards the lumen. The epithelial cell layer, together with connective tissue, lymphoid and blood capillaries make up the mucosa. Under the mucosa there is a thin muscle layer called the muscularis mucosae and a layer of connective tissue and blood and lymph vessels called the submucosa. Outside the submucosa there are two muscle layers, one transversal and one longitudinal. Together, these two layers make up the muscularis propria. On the outside of the muscularis propria, on colorectal surfaces that face the peritoneal cavity, there is an epithelial layer called the serosa. On the other surfaces, the colorectum is bordered by fatty tissue containing its vasculature and draining lymph nodes.

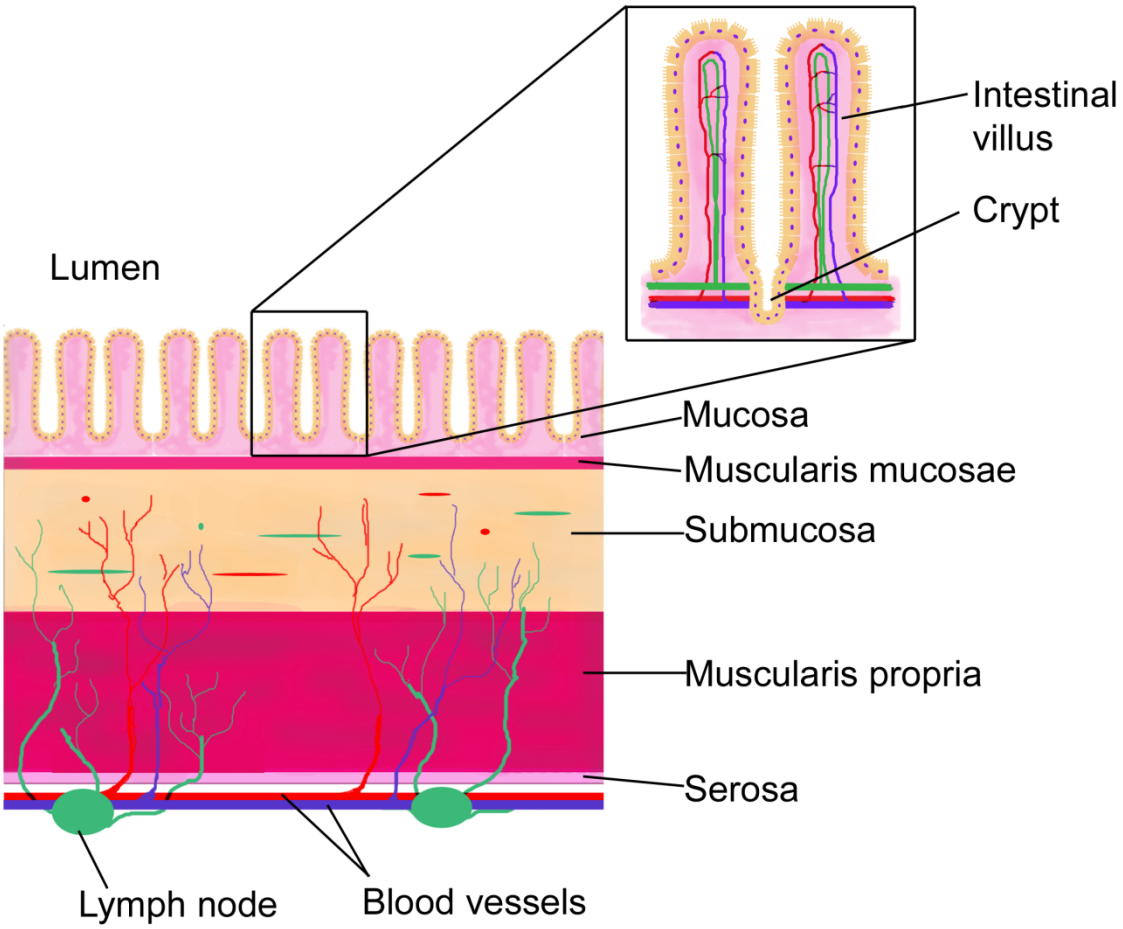

Figure 1: Layers of the colon.

1.4 Colorectal cancer

Colorectal cancers (CRC) are adenocarcinomas that arise from the glandular epithelium of the colon and rectum. CRC is the second most common type of cancer in Nordic countries in both sexes, after prostate cancer in men and breast cancer in women (Figure 2) (Engholm et al.).

The incidence of CRC in Norway was in the period 2005-2009 1767 and 1803 cases per year for men and women respectively (Engholm et al.). The incidence and mortality correlates positively with age (figure 3), and 90% of patients are diagnosed after the age of 55 (Alberts, 2008). This reflects that CRC is a cancer type that usually evolves slowly by acquiring multiple cellular changes.

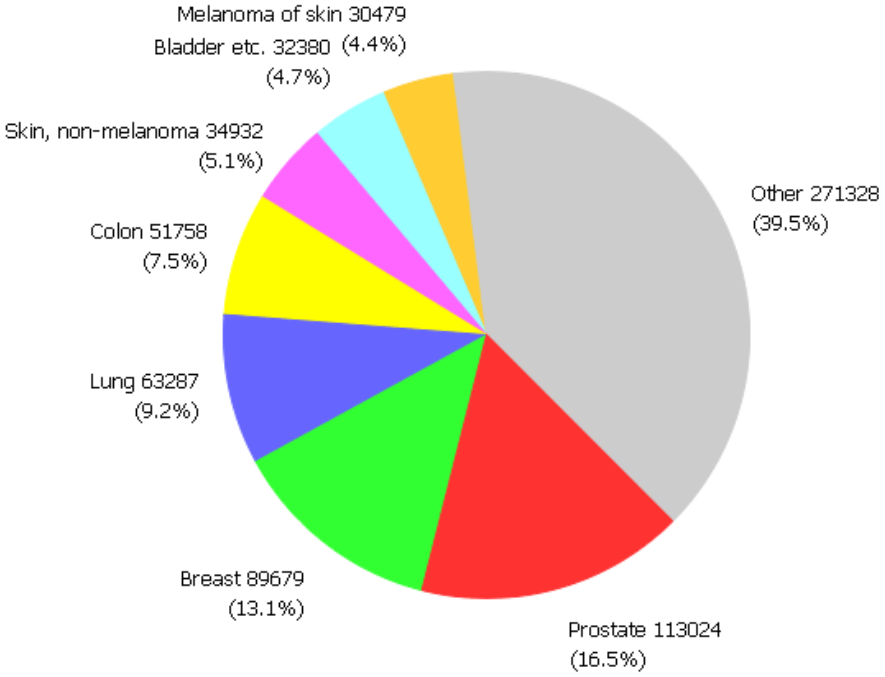

Figure 2: Number of new cases of cancer in Nordic countries in both sexes based on cancer type in the period 2005-2009. Cancer in the colon accounts for 7.5 % of cases whereas cancer in the rectum accounts for 4.3 % of cases (included in the group "other" in this chart). This means that CRC as a whole accounted for 11.8 % of all new cases of cancer in the period. The chart and values were adapted from (Engholm et al.).

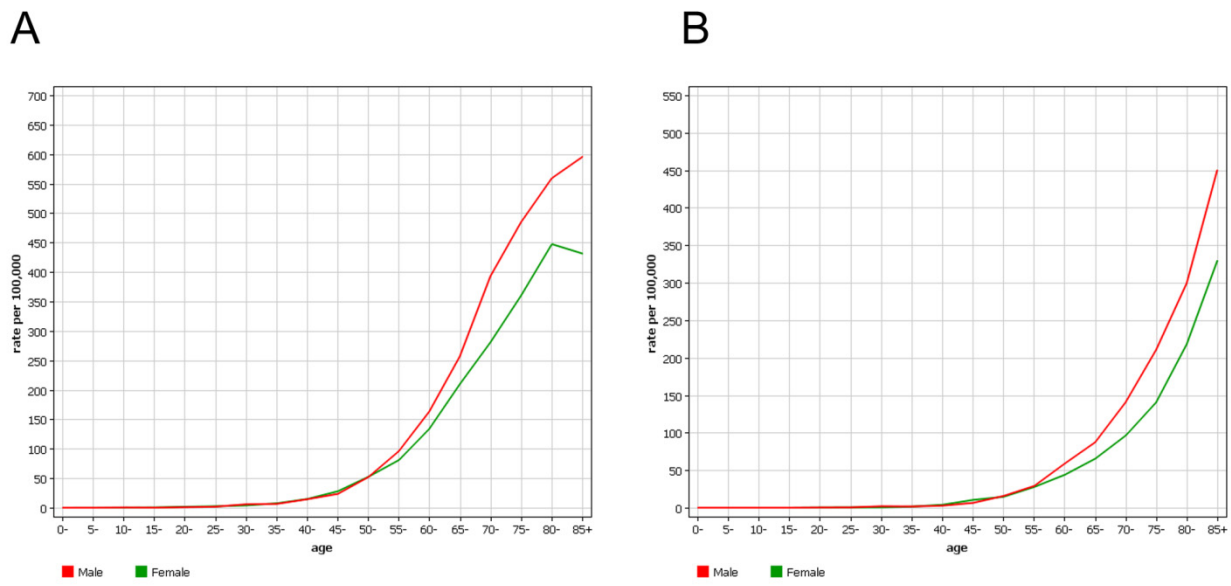

Figure 3: Age specific incidence (A) and mortality (B) of CRC in Nordic countries in the period 2005-2009 given for men (red) and women (green). Both incidence and mortality correlates positively with age as would be expected due to accumulation of molecular changes. The graphs were adapted from (Engholm et al.).

Most cases of CRC are found to be sporadic, but a higher risk of disease can also be related to inheritance. The two most known types of inherited CRC are familiar adenomatous polyposis coli (FAP) which is cause by defective *Apc* gene, and hereditary non-polyposis colorectal cancer (HNPCC, also called Lynch syndrome) which is due to mutations in one of the genes that encode central components of the DNA mismatch repair system (Alberts, 2008). The most commonly mutated genes in CRC are given in table 1.

Gene	Class	Mutation occurrence (% of all CRC cases)
KRAS	oncogene	40
β-catenin	oncogene	5-10
Apc	tumor suppressor gene	>80
p53	tumor suppressor gene	60
TGFβ receptor II	tumor suppressor gene	10
Smad4	tumor suppressor gene	30
MLH1 and other mismatch repair genes	tumor suppressor gene	15

Table 1: The most frequently mutated genes in CRC. The table is adapted from (Alberts, 2008).

1.5 TNM staging of CRC

The TNM staging system classifies tumors according to anatomical extent of disease (American Joint Committee on Cancer et al., 2006):

The T-stage classifies the primary tumors according to growth into the underlying tissue. The stages are: tumors that are not assessable (T_X), no evidence of primary tumor (T_0), intraepithelial tumors or tumors that invade the lamina propria (T_{is}), tumors that invade the submucosa (T_1), tumors that invade the muscularis propria (T_2), tumors that penetrate the

muscularis propria into pericolorectal tissue (T_3), tumors that penetrate to the visceral peritoneum (T_{4a}), and tumors that directly spread or adhere to other organs or structures (T_{4b}).

The N-stage describes whether or not we find cancer cells in regional lymph nodes, and the stages are: regional lymph nodes cannot be assessed (N_X), no metastasis in regional lymph nodes (N_0), metastasis to 1-3 regional lymph nodes (N_1) (subdivided according to number of regional lymph node metastasis or other deposits; 1 lymph node (N_{1a}), 2-3 lymph nodes (N_{1b}) and deposits in the subserosa, mesentery or nonperitonealized pericolic/rectal tissues without regional lymph node metastasis (N_{1c})), metastasis in >4 regional lymph nodes (N_2) (subdivided according to number of regional lymph node metastasis: 4-6 lymph nodes (N_{2a}) or >6 lymph nodes (N_{2b})).

The M-stage tells us whether or not there are secondary tumors (metastases) in distal sites of the body and the stages are: distant metastasis cannot be assessed (M_X), no distant metastasis (M_0) or distant metastasis (M_1), the latter being subdivided into two groups: 1 distal metastasis (M_{1a}) or >1 distal metastasis (M_{1b}). The TNM stages are summarized in table 2.

TNM	T-stage	N-stage	M-stage
0	T_{is}	N_0	M_0
I	T_1 T_2	N_0 N_0	M_0 M_0
II A	T_3	N_0	M_0
II B	T_{4a}	N_0	M_0
II C	T_{4b}	N_0	M_0
III A	T_1-T_2 T_1	N_1/N_{1c} N_{2a}	M_0 M_0
III B	T_3-T_{4a} T_2-T_3 T_1-T_2	N_1/N_{1c} N_{2a} N_{2b}	M_0 M_0 M_0
III C	T_{4a} T_3-T_{4a} T_{4b}	N_{2a} N_{2b} N_1-N_2	M_0 M_0 M_0
IV A	Any stage	Any stage	M_{1a}
IV B	Any stage	Any stage	M_{1b}

Table 2: TNM stages in colorectal cancer. The table was adapted from (American Joint Committee on Cancer et al., 2006).

1.6 Treatment of CRC in Norway

The treatment of CRC depends on the extent of disease, and the TNM staging system is one of the best tools for determining which treatment is most beneficial. At present, primary tumors and regional lymph nodes are treated surgically by removing the affected tissue. Some patients in TNM stage II and most patients in TNM stage III are considered to have a higher risk of recurrence and metastasis. These patients are defined as high-risk patients and will usually get the option of receiving chemotherapy treatment after surgery. This type of chemotherapy is called adjuvant chemotherapy and is given to minimize the risk of relapse and metastasis, in comparison to palliative chemotherapy that is given to alleviate symptoms

of cancer and/or to prolong survival. The baseline chemotherapeutic treatment given is usually 5-fluorouracil and leucovorin alone or given together with oxaliplatin, the latter combination being called FLOX. In TNM stage IV most patients receive chemotherapy, and the four drugs mentioned below are often used, either alone or in combination. The treatment is more individualized, and a simple scheme of treatment for stage IV patients can therefore not be presented. Patients above the age of 75 or with additional health issues are usually not given adjuvant chemotherapy, but each case has to be individually evaluated (Helsedirektoratet, 2010).

1.7 Chemotherapeutic drugs

The most commonly used drugs in treatment of CRC in Norway today is 5-fluorouracil, oxaliplatin, irinotecan, cetuximab or a combination of these drugs.

1.7.1 5-fluorouracil (5FU)

5-Fluorouracil (5FU) is a prodrug that is activated through phosphoribosylation to the nucleotides FdUMP, FdUTP and FUTP. Tymidylate synthase (TS) is an enzyme that normally converts dTMP to dTTP which in turn is incorporated into the DNA. FdUMP inhibits TS and causes less production of dTMP and accumulation of dUMP. The binding to TS require reduced folate and because of this folate is often given together with 5FU in the form of leukovorin (LV). FdUTP and FUTP is incorporated into the DNA and RNA, respectively, and will block further synthesis and repair (Dahl, 2009).

1.7.2 Oxaliplatin (OXA)

Oxaliplatin (OXA) is a platinum compound which is activated by a non-enzymatic process. In its active form OXA binds covalently to the DNA forming DNA adducts which inhibits DNA replication and blocks the cell cycle causing the cell to enter apoptosis (Dahl, 2009).

1.7.3 Irinotecan (IRI)

Irinotecan (IRI) is an analogue of camptothecin which is isolated from the Chinese 'happy tree' *Camtotheca acuminate*. IRI is converted to its 1000 times more active form, SN-38, in the liver and other tissue. Topoisomerase I (topo I) is an enzyme that is engaged in DNA synthesis. It eases the hypertension formed by the replication fork, and works by breaking the parental DNA strand, unwinding the strands and ligating the strands back together. After ligation topo I dissociates from the DNA (Rothenberg, 1997). SN-38 stabilizes the complex between topo I and the DNA strand causing the DNA strand to break when the replication fork collides with the topo I/SN-38/DNA complex. The broken DNA will in turn induce apoptosis through caspase activation mechanisms (Dahl, 2009).

1.7.4 Cetuximab (CET)

Epidermal growth factor receptor (EGFR) is a member of the HER family of receptor tyrosine kinases that when activated is involved in several signaling pathways, including MAPK and PI(3)K/Akt, which affect cell proliferation, angiogenesis, invasion and metastasis. EGFR is often up regulated in CRC, and because of this it was proposed as a target for cancer treatment (Wheeler et al., 2008). Cetuximab (CET) is a monoclonal antibody which binds to the ligand binding site of EGFR with high affinity and prevents EGFR activation (Dahl, 2009). This leads to receptor inactivation and might also lead to internalization and down regulation (Wheeler et al., 2008). A recent study also suggests that the binding of CET to EGFR marks cancer cells for dendritic cell-mediated phagocytosis and that this also activates an antigen mediated cytotoxic T-cell response (Correale et al., 2012).

1.8 S100A4: a metastasis-promoting protein

1.8.1 The S100 protein family

The S100 protein family consists of at least 20 acidic, small (9-13 kDa), calcium binding proteins (Emberley et al., 2004). The first S100 proteins discovered (S100B and S100A1) were isolated from neural tissue, and they were given the name S100 because of their solubility in a 100% saturated ammonium sulfate at a neutral pH (Moore, 1965).

The S100 proteins are known to form homo- and heterodimers intracellularly, and some are also found in multimeric forms extracellularly (Donato, 2003). None of the S100 family members are known to possess enzymatic activity, and they exert their functions by modulating other proteins (Helfman et al., 2005). A common structural characteristic of S100 proteins is the presence of two EF-hand domains which can bind calcium, and this induces a conformational change that allows the protein to interact with its target(s) and exert its biological function (Garrett et al., 2006).

Several of the S100 protein family members have been shown to have functions in progression or suppression of tumors (Donato, 2001). One of the proteins which has been particularly associated with the metastatic potential of cancer cells is S100A4.

1.8.2 S100A4

The *S100A4* gene, also referred to as mts1/pEL98/p9Ka/18A2/42A/calvaskulin/FSP1/CAPL (Marenholz et al., 2006), encodes a 101 aa protein with a molecular weight of 11.5 kDa (Boye and Maelandsmo, 2010). The S100A4 protein has been shown to be located extracellularly, in the cytoplasma and in the nucleus (Flatmark et al., 2003, Boye and Maelandsmo, 2010). This suggests that the protein may have several functions, but at present the functions that S100A4 exerts in the normal cell are largely unknown.

The S100A4 protein has been shown to physically interact with components of the cytoskeleton, such as non-muscular tropomyosin (Takenaga et al., 1994), myosin (Ford and

Zain, 1995) and actin (Watanabe et al., 1993). In addition the S100A4 protein is also shown to be expressed in natural motile cell types such as macrophages and lymphocytes (Takenaga et al., 1997b), and this suggests that the protein is involved in dynamic rearrangements of the cytoskeleton and cell motility.

Davies et al showed in 1996 that S100A4 transgenic mice that were crossed with mice prone to develop mammary tumors due to expression of the MMTV-neu transgene, developed tumors with an increased invasive and metastatic potential (Davies et al., 1996). Another study performed by Takenaga et al in 1997 found that the silencing of S100A4 by antisense RNA reduced the metastatic potential of otherwise highly metastatic Lewis lung carcinoma cells (Takenaga et al., 1997a). These findings also indicate that S100A4 promotes invasive abilities of tumor cells.

Cell motility, invasion and metastasis are all processes involved in cancer progression, and this implies that S100A4 might have functions in the progression of cancer. The *S100A4* gene is located in a gene cluster with several other S100 genes on chromosome 1q21, and this gene cluster have been shown to be frequently rearranged in cancer (Ridinger et al., 1998). A positive correlation between tumor progression and expression of S100A4 had also been reported by Ebralidze et al in 1989 (Ebralidze et al., 1989), and this indicates that S100A4 in addition to the functions mentioned above also has a function in tumor progression and the metastatic process.

Because S100A4 has been shown to play a role in tumor progression and metastasis, the protein could possibly be used as a prognostic marker of cancer to supplement the TNM staging used for predicting patient outcome.

In 2000 Rudland et al performed a large study involving 349 patients with breast cancer (Rudland et al., 2000). In this study the expression of S100A4 was investigated in tumor tissue from patients and compared to the survival statistics. The study showed a strong correlation between expression of S100A4 and poor prognosis. Eighty percent of patients with S100A4 negative tumors were still alive 19 years after surgery, while the survival of patients with S100A4 positive tumors were only 11%. Similar studies have been done to investigate the significance of S100A4 in CRC.

1.8.3 S100A4 in CRC

In a study performed by Takenaga et al in 1997 (Takenaga et al., 1997b), the amount of S100A4 mRNA was analyzed by Northern blotting in normal and CRC tissue from patients in different TNM stages. The results showed that benign polyps and normal colonic mucosa had a relatively low expression of S100A4 mRNA, while malignant tumors had a significantly higher expression. Immunohistochemical staining showed that S100A4 expression was higher in the deep, invading parts of the tumors, and that the expression levels were highest in the metastases.

In 2002 Gongoll et al investigated the significance of S100A4 as a prognostic marker in CRC (Gongoll et al., 2002). The immunohistochemical expression of S100A4 was compared with clinicopathological parameters and outcome from 709 CRC patients. The expression was divided in high, low and no expression of S100A4, and a correlation was found between high expression of S100A4 and women, tumor stage T3-4 and relapse. A correlation was also found between S100A4 expression and long term survival of the patients. The median survival of patients in the S100A4 negative group was more than 120 months, while for the groups with low and high expression of S100A4 the median survival was 106 and 58 months, respectively.

In 2003 Flatmark et al performed a study of the expression of S100A4 in resection specimens from 277 CRC patients (Flatmark et al., 2003). Immunohistochemical expression of S100A4 was observed in the nucleus, and this had not been previously described. Nuclear expression was confirmed by Western blot analysis of nuclear fractions. The expression of S100A4 was graded from 0 to 5 according to the percentage of stained tumor cells, and nuclear expression was significantly associated with TNM stage, suggesting that nuclear S100A4 could be a negative prognostic factor. In a subsequent study, Boye et al indeed demonstrated that expression of S100A4 in the nucleus was associated with a poor prognosis, whereas expression of S100A4 in the cytoplasm did not show any prognostic significance (Boye et al., 2010). Furthermore, the prognostic impact of expression of S100A4 within the separate disease stages was investigated, showing that the prognostic significance of S100A4 was restricted to patients in TNM stage II. Interestingly, patients in TNM stage II which had expression of nuclear S100A4 had a similar prognosis as patients in TNM stage III.

In conclusion, S100A4 seems to be a promising prognostic biomarker in CRC. The prognostic significance of S100A4 is especially interesting to pursue further because of the ability of nuclear S100A4 to predict which patients in TNM stage II that have the worst prognosis. These patients might benefit from adjuvant chemotherapy, which is usually offered mainly to patients in TNM stage III. A better prediction of which patients that might not benefit from adjuvant chemotherapy could also prevent unnecessary treatment with chemotherapeutic drugs that are known to have many toxic side effects.

2 Aims of the study

In CRC, tumor stage at diagnosis is still the most important factor used for identification of patients with high risk of disease recurrence and selection for adjuvant chemotherapy. S100A4 expression is a promising prognostic biomarker that may be used to supplement TNM stage, particularly in TNM II patients who normally do not receive adjuvant chemotherapy. If S100A4 can be used to identify high-risk TNM II patients, it would be interesting to know if S100A4 expression is associated with response or resistance towards particular chemotherapeutic drugs. Chemotherapeutic treatment has a lot of unpleasant side effects and if S100A4 not only can predict which patients will benefit from treatment but also which chemotherapeutic drug that would be most efficient the patients would be saved from unnecessary suffering from side effects.

In this work, we aimed to:

- determine if the established cell lines are suitable models of high and low S100A4 expression
- analyze the relevance of S100A4 expression on drug sensitivity towards four commonly used drugs in CRC treatment
- establish and compare different methods for screening of in vitro drug sensitivity

3 Materials and methods

3.1 Cell lines

The human colon cancer cell lines HCT116, SW620 and CaCo2 were originally obtained from American Type Cell Collection (ATCC) (Mannassas, VA, USA). S100A4 knockdown variants of HCT116 and SW620 had already been made at The Department of Tumor Biology, The Norwegian Radium Hospital, when starting this project. Briefly, HCT116 and SW620 cells were transduced with lentiviral constructs expressing short hairpin RNA (shRNA) directed against S100A4 or non-target shRNA as a control, and shRNA-expressing clones were selected using puromycin resistance. The resulting strains were named shA4-1 – shA4-5 and shNT, respectively. Of the shA4 strains (containing different lentiviral constructs) shA4-3 showed the lowest expression of S100A4 in both cell lines and was chosen for further studies.

3.2 Cell culture and treatment

The cells were grown in monolayers in T-75 Nunc cell culturing flasks (Thermo Fisher Scientific Waltham, MA, USA) with suitable medium for each cell line. The standard medium used for HCT116 and SW620 was RPMI 1640 (Lonza, Basel, Switzerland) added 8% (v/v) fetal bovine serum (FBS)(Life Technologies, Carlsbad, CA, USA), 1 % (v/v) Hepes buffer (Lonza) and 1% (v/v) Glutamax (Life Technologies). Medium used for CaCo2 was Dulbecco's modified eagle's medium (DMEM) (Lonza) added 5 % FBS, 1 % Hepes buffer and 1 % (v/v) Glutamax.

The cells were all grown in a humid environment with 5% CO_2 and without antibiotics, and all work with the cells was performed under sterile conditions in a LAF bench. For all experiments, 1 % (v/v) 5000 U/ml penicillin/streptomycin (P/S) (Lonza) was added to the medium to minimize the risk of bacterial infections.

For maintenance or when seeding out cells for experiments, the cells were detached from the growth surface using Trypsin/EDTA (170 000 U/l Trypsin and 200 mg/l EDTA) (Lonza) and diluted in the fresh medium according to cell density. One fraction of cell solution was retained in the cell culturing flask and fresh growth medium was added to a total of 15 ml for continued culturing. The other fraction was transferred to 50 ml tubes (Sarstedt, Nümbrecht, Germany) and used for seeding out experiments or discarded. Maintenance was required approximately twice a week to ensure that the cells did not grow too dense and became less viable. When seeding out for experiments the cells were counted either manually using a Bürcher chamber or automatically using the Countess Automatic Cell Counter (Life Technologies).

For manual counting we used 10 µl of cell suspension between a Bürcher chamber and a cover slip. The cells inside three or more of the small squares were counted and the mean value was found. This number equals the number of cells per ml x 10^4.

For automatic counting we followed the manufacturer's recommendations (LifeTechnologies, 2009). Briefly, we used 10 µl of cell suspension mixed with 10 µl of 0.4% tryphan blue stain (Life Technologies) applied to a Countess® Cell Counter Chamber Slide (Life Technologies). Viable cells transport the blue dye out of the cells whereas dead cells will remain dark blue. The Countess Automatic Cell Counter uses an algorithm to designate cells according to roundness and size, and counts the total number of cells in an area of the slide corresponding to 0.4 µl of cell suspension. Live cells will transport the tryphan blue stain out of the cell and appear white with a blue ring around, while dead cells will be colored dark blue. The instrument uses this difference in staining pattern to distinguish live cells from dead cells and calculates the total number of cells, the number of live cells and the number of dead cells per ml cell suspension (LifeTechnologies, 2009). The counting of cells is necessary to be able to seed out the correct number of cells required for the individual experiments. This number must be estimated for each cell line and each method.

For storing cells over shorter periods of time cells were frozen at -70°C. For storage over longer periods of time cells were frozen and kept in liquid nitrogen. Before freezing the cells were detached using Trypsin/EDTA and dissolved in fresh medium containing 20% FCS and 10% dimethyl sulfoxide (DMSO) (Sigma Aldrich). DMSO was used as a cryoprotectant (Day et al., 2007).

For the protein analysis that is described in 3.4, dry pellets were made. The harvesting of cells for protein isolation was performed in parallel with seeding of cells for other experiments. The cells were detached from the growth surface using Trypsin/EDTA and then dissolved in growth medium. The cells were transferred to a 50 ml tube and centrifuged at 235 x G for 7 min. The supernatant was removed and the pellet was resolved in 10 ml cold 6.7 mM (PO_4) phosphate buffered saline without Ca and Mg (PBS) (Lonza) and transferred to a 15 ml tube (Sarstedt) before spinning at 235 x G for 7 min. This washing procedure was done twice before resolving the pellet in 1 ml cold PBS, transferring it to an eppendorf tube (Eppendorf, Hamburg, Germany) and spinning at 235 x G for 7 min. The supernatant was removed and the dry pellet was stored in the freezer at -70°C.

3.3 Mycoplasma testing

All cell cultures that are grown in the lab over time should be tested for mycoplasma infection every 4-6 weeks. Mycoplasma is a small, pathogenic organism that can infect the cell cultures and make them behave in abnormal ways. Signs of infection are yellow colored growth medium and cells with abnormal behavior, but infection might also be unrecognized.

The method that is currently in use in the lab is a VenorGem Mycoplasma detection kit (Minerva Biolabs, Berlin, Germany). This method is PCR based and detects mycoplasma DNA in medium from the cell culture.

All cells used in experiments performed in this thesis have been tested mycoplasma free before freezing, and the cells have only been grown in the lab for some weeks before they were discarded and a new vial of cells was thawed.

3.4 Protein analysis by polyacrylamide gel electrophoresis and Western blotting

3.4.1 Protein isolation

Cells frozen as dry pellets as described in 3.2 were thawed slowly on ice. The lysis buffer used was made at the Department of Tumor Biology, The Norwegian Radium Hospital, and the formula is given in the appendix. The protease and phosphatase inhibitors used were Complete Mini Protease Inhibitor Cocktail tablets provided in EASYpack (Roche, Basel, Switzerland) and phosphoSTOP phosphatase inhibitor cocktail tablets provided in EASYpack (Roche). One tablet with protease inhibitors and one tablet of phosphatase inhibitors were solved in 1.5 ml ddH_2O to yield a 10x stock solution which was frozen in aliquots of 250 µl at -20°C. An aliquot was thawed and diluted to 1x concentration in lysis buffer. Two-hundred µl of lysis buffer containing protease and phosphatase inhibitors were added to the dry pellet. The sample was mixed well using a vortex, and kept on ice at all times. After vortexing, the samples were sonicated three times for five seconds. The sonicator tip was washed before and between each sample to avoid contamination of the samples. The samples were then incubated on ice for 15 min before spinning down at 16 000 x G at 4°C for 15 min, and the supernatant was transferred to an eppendorf tube.

3.4.2 BCA measurement of protein concentration

The BCA protein assay kit from Pierce, Thermo Fisher Scientific (Waltham, MA, USA) was used to measure the protein concentration in each lysate. This kit contains a 2 mg/ml stock solution of bovine serum albumin (BSA), Reagent A containing bicinchonic acid (BCA) and tartrate in an alkaline carbonate buffer, and reagent B containing 4% copper sulfate pentahydrate solution. Briefly, lysates with an expected concentration of 1.5-13 µg/µl were diluted 10x in ddH_2O and 25 µl of each sample were added in 3 wells of a 96-well plate. BSA was diluted in ddH_2O to concentrations between 125 µg/ml and 1500 µg/ml, and 25 µl were added to two wells per concentration. We also added 25 µl ddH_2O to two wells to use as a

blank. Reagents A and B were mixed 50:1 and 200 µl were added to each well containing sample, standard or blank, and the plate was incubated at 37°C for 30 min before measuring the absorbance at 540 nm in a 96-well plate reader (Wallac 1420 VICTOR^{2TM} (PerkinElmer, Waltham, MA, USA)).

In an alkaline environment containing sodium potassium tartrate, proteins reduce the copper ions from Cu^{2+} to Cu^{1+} and form a light blue complex (known as the Biuret reaction). BCA reacts with the Cu^{1+} ions that were formed in the first reaction to form a complex consisting of two molecules of BCA and one Cu^{1+} ion. This complex has a purple color and can be detected by measuring absorbance at 540 nm (Thermo Fisher Scientific). The BSA standards are used to make a standard curve from which we can calculate the concentration of the other lysates.

3.4.3 Polyacrylamide gel electrophoresis

Depending on the protein concentrations measured with the BCA method described in 3.4.1, the samples were diluted with ddH_2O to a final amount of 20 µg in a volume of 30 µl. NuPAGE sample reducing agent (Life Technologies) and NuPAGE lithium dodecyl sulfate (LDS) sample buffer (Life Technologies) were added to each sample before denaturation at 95°C for 5 min. The sample reducing agent was purchased as a 10x stock solution containing 500 mM dithiothreitol (DTT) and reduces disulfide bonds and stabilizes the resulting SH groups of the protein (Cleland, 1964). The lithium dodecyl sulfate (LDS) sample buffer is purchased as a 4x stock solution containing LDS in a Tris/glycerol buffer, pH 8.5. Dodecyl sulfate is a detergent with a hydrophobic hydrocarbon tail which can bind to proteins through hydrophobic interactions and a negatively charged sulfate head. The negative charge of dodecyl sulfate is much greater than the charges of the amino acids of the protein, and therefore the amino acid charge does not need to be taken in consideration in the electrophoresis separation.

Precasted NuPAGE 10-well 4-12% BisTris gels (Life Technologies) were used. The wells were washed with MES buffer (Life Technologies) containing 50 mM 2-(N-morpholino)ethanesulfonic acid (MES), 50 mM Tris Base, 0.1% SDS and 1 mM EDTA. The MES buffer was purchased as a 20x stock solution and diluted to 1x in ddH2O. After washing the wells, the gel was installed in the electrophoresis chamber with the wells pointing

inwards. The inner chamber that was formed between two gels or between the gel and a gel dummy was filled with MES buffer and the outer chamber was filled half full. Seven µl of SeeBlue prestained protein standard (Life Technologies) was applied to at least one of the wells per gel, and the samples were applied to the other wells. The electrophoresis was run on 130V and 125mA for approximately 1 hour and 10 min. The proteins migrate during electrophoresis towards the positive electrode and separate according to size. The gel was taken out of the electrophoresis chamber, the plastic cover surrounding the gel was forced open using a suitable tool, and the gel was placed in Bjerrum-Schafer-Nielsen (BSN) buffer so that it does not dry out. The BSN buffer was made at the Department of Tumor Biology and the formula is given in the appendix.

3.4.4 Western blotting

The membrane used for blotting was an Immobilon-P polyvinylidenfluoride (PVDF) membrane with a pore size of 0.45 nm (EMD Millipore, Billerica, MA, USA). This membrane is highly hydrophobic and must be activated in methanol before soaking in BSN buffer. Whatman paper (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and sponge pads for blotting were also soaked in BSN buffer. The blotting equipment was assembled in the following order: sponge pad, 2 Whatman papers, gel, PVDF membrane, 2 Whatman papers and sponge pad. Air bubbles were removed by rolling a pipette over the stack, and the equipment was closed and installed into the blotting chamber. A cooling element and a stirring pin were inserted and the chamber was filled with BSN buffer. The blotting equipment was placed on a magnetic stirrer at 4°C and the blotting was done at 30V constant voltage overnight. After blotting, the bands of the protein standard were marked on the PVDF membrane using a pen, and the membrane was stained with amidoblack (Department of Tumor Biology, formula found in the appendix) for 5 min, followed by 3x5 min destaining (Department of Tumor Biology, formula found in the appendix). The membrane was then washed in TBS buffer containing 0.1 % Tween (Department of Tumor Biology, formula found in the appendix) and scanned for documentation.

3.4.5 Immunostaining

The membrane was blocked with 10% non-fat dry milk in TBS buffer containing 0.1 % Tween for 1 hour. The blocking and all staining and washing steps were performed on a rotation device. The membrane was cut in two according to the protein standard, and the upper part of the membrane was stained with anti α-tubulin (T9026, Sigma-Aldrich, St. Louis, MO, USA), diluted 1:5000 in 5% non-fat dry milk in TBS buffer containing 0.1 % Tween. The lower part of the membrane was stained with anti-S100A4 (22.3, monoclonal antibody made at Department of Tumor Biology, the Norwegian Radium Hospital (Paus et al.)), diluted 1:1000 in 5% non-fat dry milk in TBS buffer containing 0.1 % Tween. Staining with both primary antibodies was performed overnight at 4°C. Thereafter, the membranes were washed 3 x 10 min in TBS buffer containing 0.1 % Tween before incubation with horseradish peroxidase (HRP)-labelled rabbit anti-mouse antibody (Dako, Glostrup Denmark), diluted 1:5000 in 5% non-fat dry milk in TBS buffer containing 0.1 % Tween for 1 hour at room temperature. The membrane was washed 3 x 10 min in TBS buffer containing 0.1 % Tween before signal detection.

3.4.6 Signal detection

For signal detection the SuperSignal® West Dura Extended Duration Substrate (Thermo Fisher Scientific) was used according to manufacturer's instructions. The kit contains Luminol/Enhancer and Stable Peroxide Buffer which was mixed 1:1 to produce the enhanced chemiluminiscent substrate solution for detecting HRP. The membrane was covered with the solution and incubated for 1 min before detection on the G:BOX iChemi (SynGene, Cambridge, UK) using the software GeneSnap (SynGene). The resulting pictures were used in densitometry measurements using the software GeneTools (Syngene). Densitometry measures the light intensity from each band on the membrane minus the background intensity to gain a raw volume value. The raw volume value from the S100A4 band was divided by the raw volume value of the α-tubulin band from the same sample to get a raw volume ratio. These ratios were used to compare S100A4 expression between different samples.

3.5 Growth curve determination

SW620 cells were counted manually using a Bürcher chamber and seeded out in two Nunc 6-well plates (Thermo Fisher Scientific) per strain, each well containing 3 ml of growth medium and $2x10^5$ cells. The cells were then placed in a humid incubator at 37°C and 5% CO_2. The cells were harvested after 24, 48, 72 and 96 hours, 3 wells at the time. At the time of harvest the growth medium was removed from the wells and the cells were washed once with PBS before detaching them from the well using Trypsin/EDTA. The cells were then diluted in 4 ml growth medium and this suspension was then diluted 40 times in PBS. For counting the cells we used Z1™ Coulter Counter®, Dual Treshold Analyzer (Beckman Coulter, Brea, CA, USA). The settings that were used for counting and calculating the number of cells in each well is shown in table 3. The apparatus was flushed with Coulter Isoton® Diluent (Beckman Coulter) before and between samples to avoid contamination, and placed in Coulter Clenz® Cleaning Agent (Beckman Coulter) after use.

Parameter	Settings
Dilution factor	40
Upper/Lower size	8 μm/24 μm
Count mode	Between
Result type	Concentration

Table 3: Settings used for counting the cells on the cell Z1 Coulter Counter

The doubling time was calculated using the formula $DT = (t - t_0)\log 2/(\log N - \log N_0)$, where t_0 and t is the time point of the initial and the last count of cells respectively, and N_0 and N is the initial and last number of cells counted.

The same experimental setup was also used for measuring growth rate of HCT116 cells. Because HCT116 cells spontaneously detached from the surface of 6-well plates, T-25 cell flasks were used instead.

3.6 Chemotherapy

The work with cytotoxic substances requires a foam hood which is specialized for work with hazardous substances. It is important that the hood does not circulate the air from the bench back into the workspace outside. There should also be a bench coat in the hood to protect the hood from spills. It is also important to wear protective clothing while working with cytotoxic substances. A disposable lab coat, one pair of nitrile gloves and one pair of latex gloves is recommended.

We have tested the three most common chemotherapeutic drugs currently used in treatment of colorectal cancer in Norway on our cell lines HCT116 and SW620. These drugs are: 5-fluorouracil (5FU) (Hospira, Lake Fores, IL, USA), irinotecan (IRI) (Fresenius Kabi, Bad Homburg, Germany) and oxaliplatin (OXA) (Teva, Petah Tikva, Israel). In addition we have tested the monoclonal antibody cetuximab (Merck, Darmstadt, Germany).

Cells were seeded out as described in sections 3.7-3.9. The cell culture medium was then removed and replaced with fresh culture medium with or without chemotherapeutic drug as indicated. The cells were incubated for 4 hours before the drug was removed and fresh medium was added to each well. The cells were then incubated, before final analysis as described in sections 3.7-3.9.

For CET, cells were incubated in the presence of drug for 72 hours, but the protocol was otherwise the same as for the other drugs.

3.7 CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS assay)

The cells were counted on the Countess® Automated Cell Counter and 5000 cells/well (HCT116) or 7500 cells/well (SW620) were seeded in a volume of 200 µl/well in a Nunc 96-well plate (Thermo Fisher Scientific). The cells were allowed to attach to the wells in a humidified incubator at 37°C and 5% CO_2 for 24 hours before treatment with OXA, IRI, 5FU or CET for four hours as described in section 3.6. After removing the drug and adding 200 µl fresh medium the cells were incubated for 72 hours before final analysis with the CellTiter

96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Fitchburg, WI, USA) (also called MTS assay) according to the manufacturer's instructions (Promega Corporation, 2009).

The MTS assay is a colorimetric method to determine the number of viable, proliferating cells. The method contains a single reagent solution which consists of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) and phenazine ethosulfate (PES). PES is an electron coupling reagent that is stabile in solution with MTS.

Following the manufacturer's instructions, 10 µl of reagent solution was added directly to each well where the cells had been grown. The 96-well plate was placed back in the incubator at 37°C for 1-4 hours. The cells bioreduce the yellow colored MTS to a brown colored formazan product, which is soluble in culture medium. After incubation the absorption at 490 nm is recorded with a Wallac 1420 VICTOR2™ (PerkinElmer, Waltham, MA, USA) 96-well plate reader.

3.8 Clonogenic survival assay

The clonogenic survival assay determines a cell's ability of to proliferate indefinitely, thereby retaining its capacity to form a clone, defined as a cell colony containing at least 50 cells. It has been used extensively to evaluate efficacy of cytotoxic treatment in vitro, particularly in radiobiological research. HCT116 shNT, HCT116 shA4-1 and HCT116 shA4-3 were used for the clonogenic survival assay. Cells were detached using Trypsin/EDTA, dissolved in fresh medium, and 100 cells/well were seeded in a volume of 2 ml in three 6-well plates for each cell line. The cells were incubated for 24 hours. The medium was then removed and fresh medium with or without 5FU was added. The plates were incubated for 4 hours before the medium was removed and fresh medium was added to each well. The plates were incubated for about one week until colonies of appropriate size were detectable by visual inspection. The medium was removed and the colonies were fixed using ice cold methanol for 1 min at room temperature. The plates were placed upside down on a bench paper to dry. The colonies were stained by incubating with 1 ml of 0.05 % crystal violet dye (Apotekproduksjon, Oslo, Norway) for 1 min at room temperature. The dye was removed and the plates were placed

upside down on a bench paper to dry. Colonies containing ≥ 50 tumor cells were then counted manually at a Gerber Counter (Gerber Scientific Products, Tolland, CT, USA).

3.9 Spheroid assays

3.9.1 Spheroid volume

The protocols for forming spheroids and measuring cell viability by calculated spheroid volume were adapted from Vinci and co-workers (Vinci et al., 2012). Briefly, HCT116 cells were seeded in 96-well, ultra-low attachment plates (Costar, Washington, DC, USA), 1000 cells/well, and incubated in a humidified atmosphere at 37°C and 5% CO_2 for 72 hours. The spheroids were then treated with the indicated concentrations of 5FU for 4 hours. Since the spheroids were not attached to the wells, we removed 100 µl of medium and added 100 µl of 5FU in 2x concentration. The treatment was ended by removing 100 µl of medium and adding 100 µl of fresh medium without 5FU. This process was repeated 5 times to dilute the concentration of 5FU to an insignificant level. The spheroids were photographed using the IX81 motorized inverted microscope (Olympus, Shinjuku, Tokyo, Japan) 96 hours after treatment, and the software Cell^P (Olympus) was used to calculate the spheroid volume. This was primarily done by using the magic wand function which marks the darker colored spheroid. The output was visually inspected and corrected manually if needed. The software calculated the average radius of the spheroids, and from this value we calculated the spheroid volumes with the formula $V=4/3\pi r^3$, based on the assumption that the spheroids were approximately spherical.

3.9.2 CellTiter-Glo

The viability of cells in the spheroid was investigated by using CellTiter-Glo® Luminescent Cell Viability Assay (Promega) according to the manufacturer's instructions (Promega Corporation, 2011). CellTiter-Glo is a luciferase based assay that measures the number of viable cells based on quantification of the ATP present. The kit was thawed and placed in

room temperature to equilibrate. The CellTiter-Glo reagent was made by mixing the buffer and lyophilized substrate components. The spheroids were harvested 96 hours after treatment with 5FU and transferred in a volume of 50 µl to a 96-well black plate with clear bottom (Costar), one spheroid per well. For reference purposes, 50 µl of fresh medium were added to two wells per plate. Fifty µl of CellTiter-Glo reagent was added to each well using a multichannel pipette and mixed well using an orbital shaker to induce cell lysis. The plate was incubated at room temperature for 10 min before mixing again with a pipette. Luminescence was recorded at a Modulus Microplatereader (Turner Biosystems, Sunnyvale, CA, USA). The average value recorded in the medium-only wells was used as a blank and subtracted from the other values recorded. All results recorded were divided by the relevant vehicle treated control to take variation in seeding of cells into account.

3.10 Statistical analysis

To analyze the results we obtained from the different assays we used the software SigmaPlot 11.0 (Systat Software, Inc., Chicago, IL, USA) to calculate the area under the curve (AUC) for each strain of each cell line and in every experiment. The AUC is a commonly used measure to compare whole curves instead of comparing all measured points singularly. The calculated AUC values were compared using Student's t-test, which is a parametric test that requires a dataset with a normal distribution. The Shapiro-Wilk Normality Test was used to ensure that each dataset did not violate this assumption. However, since normality assessment may be uncertain when each data set contains relatively few observations, results were also compared using the non-parametric Mann-Whitney U test. The tests gave the same conclusions.

The inhibitory concentration where 50% of cells die (IC50) was calculated for each drug on each strain. This was done by finding the two measured points closest to 0.5 and calculating the IC50 value linear between these points. In cases where the IC50 value was out of our concentration range we define the IC50 as higher than the last measured concentration.

4 Results

4.1 Stability of S100A4 expression

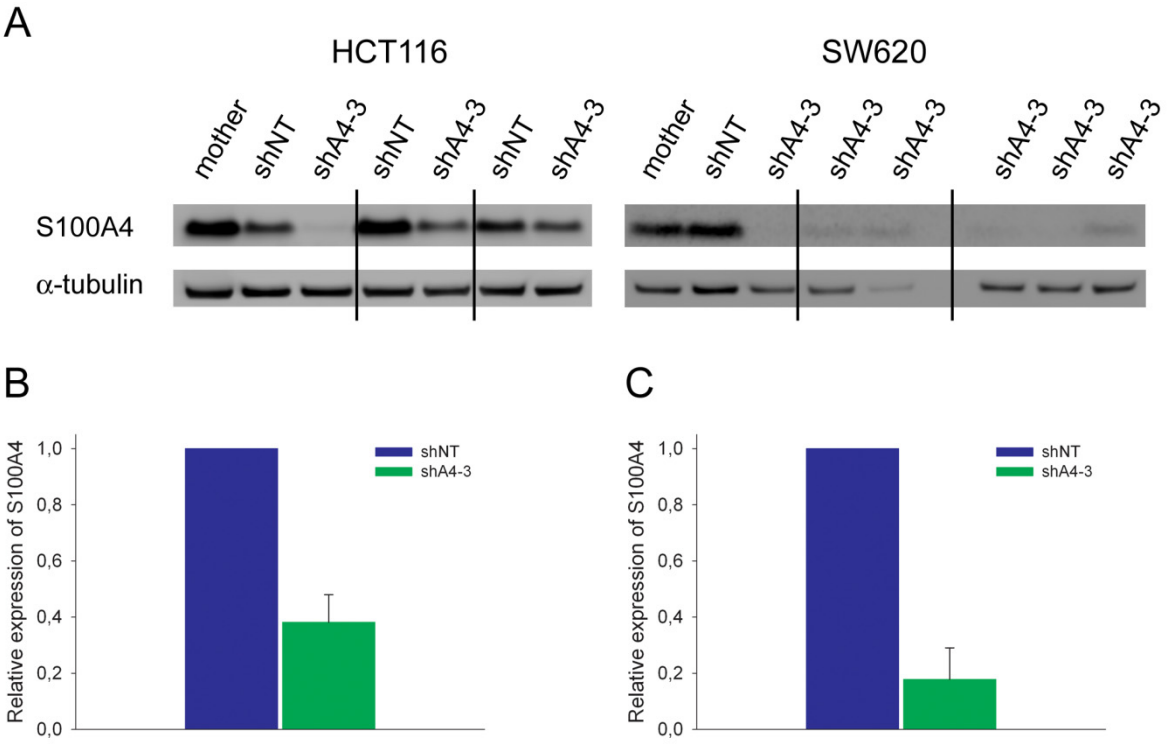

Figure 4: Expression of S100A4 shown by Western blots (A). The three first samples in both panels were harvested right after transduction. The vertical lines in the HCT116 membrane separate samples harvested at different time points. The vertical lines in the SW620 membrane separate different batches of cells. The lower panel shows densitometry measurements for HCT116 (B) and SW620 (C).

To verify that the generation of stable S100A4 knockdown cell lines had been successful, protein samples were collected repeatedly over time and analyzed by Western blotting (figure 4). The results demonstrate that the expression of S100A4 in shA4-3 was lower than in the parental ("mother") and shNT variants of both cell lines, and the Western blots in figure 4A

was chosen as a representative picture of this. The expression of S100A4 in mother and shNT strains has been shown to be relatively stable (figure 4 and previously obtained data from the Department of Tumor Biology). The difference in layout for the two membranes was due to the first sample of HCT116 shNT, which show lower expression of S100A4 than later samples. In the other HCT116 shNT samples we typically see higher expression of S100A4 and to prove this, samples taken at the same time point as HCT116 shA4-3 were also included.

Expression of S100A4 was stably low in the SW620 shA4-3 cell line, while for HCT116, the down-regulation was less consistent over time when grown in the laboratory. This can also be seen in figure 4A. To keep the expression on a low level cells were grown for only 4-5 weeks before they were discarded and a new aliquot were taken from the -70°C freezer. Cells were harvested for Western blot analysis every other week to ensure low expression, and densitometry was performed on these Western blots. Densitometry showed a mean expression of S100A4 in shA4-3 that was 62 % lower than shNT in HCT116, and 82 % lower than shNT in SW620 (figure 4B and 4C).

4.2 Growth rate of HCT116 and SW620

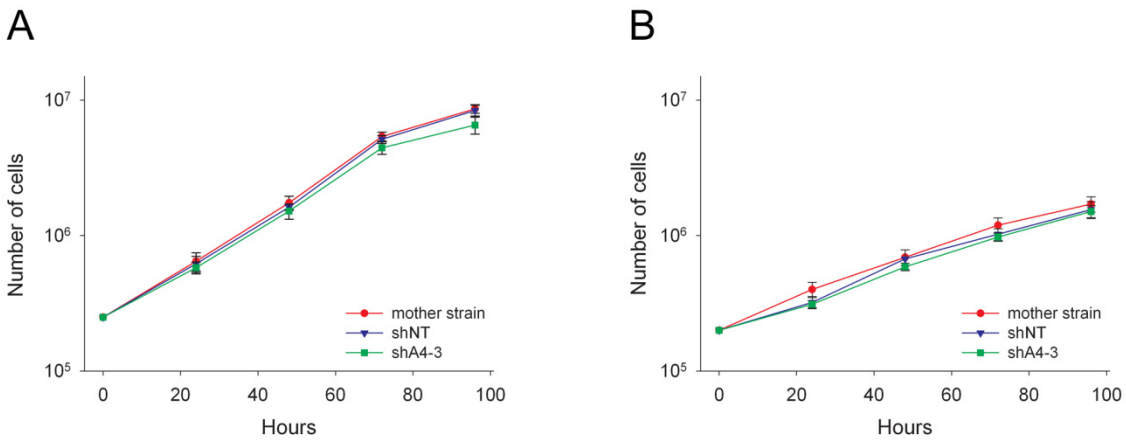

Figure 5: Growth curves for HCT116 (A) and SW620 (B). Standard error of the mean (SEM) is shown for each time point.

Strain/Cell line	HCT116	SW620
mother vs shNT	p = 0.69	p = 0.53
mother vs shA4-3	p = 0.17	p = 0.28
shNT vs shA4-3	p = 0.25	p = 0.67

Table 4: Probability values (p-values) from AUC comparisons by the Student's t-test.

Growth rates for the three strains were measured to investigate if the lentiviral vector with or without the shRNA construct gave the cells any advantages or disadvantages in growth compared to the mother strain. The growth curves are depicted in figure 5. The strains from the same cell line seemed to have approximately the same growth rate. For HCT116, the shA4-3 strain seemed to exhibit a somewhat reduced growth over time in comparison to the mother and shNT strain, but the difference was not statistically significant. The probability values (p-values) were calculated by comparing the AUC values from each experiment and can be found in table 4. A summary of the average AUC values for all curves presented in this thesis can be found in table 13 on page 43.

We observed a difference in growth rate between the two cell lines, and by calculating the doubling time for each cell line we can confirm this. The calculated doubling time of HCT116 and SW620 is given in table 5.

Strain/Cell line	HCT116	SW620
Mother strain	18.8 h	31.0 h
shNT	18.9 h	32.4 h
shA4-3	20.4 h	32.9 h

Table 5: Doubling time for HCT116 and SW620 strains.

4.3 Effect of OXA, IRI, 5FU and CET on HCT116 and SW620 2D cultures measured by MTS

4.3.1 OXA

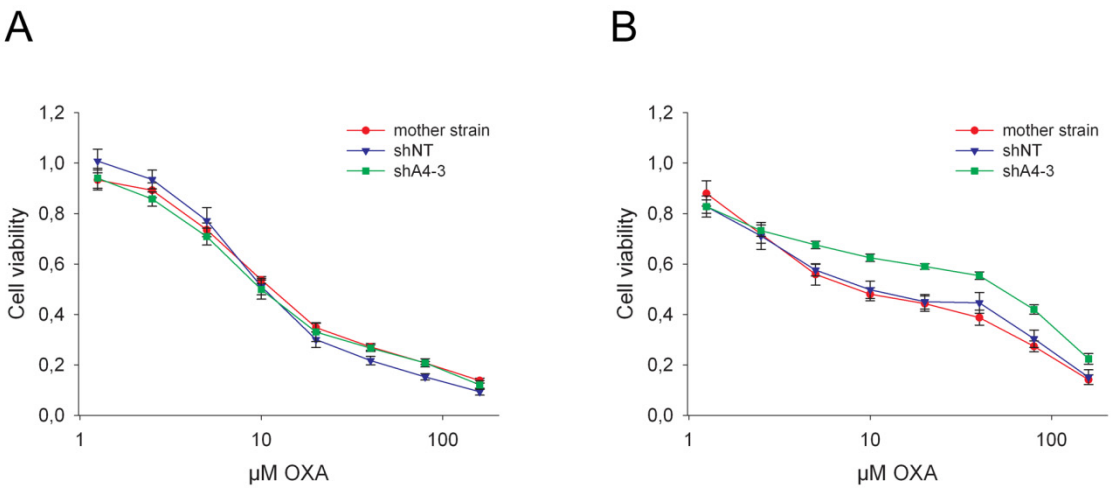

Figure 6: Viability of HCT116 (A) and SW620 (B) cells after treatment with OXA. Standard error of the mean (SEM) is shown for each concentration.

The cells were treated with OXA to investigate the sensitivity towards this drug. The viability of the treated cells relative to control was plotted as a function of the concentration of OXA and depicted in figure 6. We observed no major differences between the strains of HCT116, and there were no notable differences between the HCT116 strains and the SW620 mother and shNT strain. However, SW620 shA4-3 was observed to be less sensitive towards OXA than SW620 mother and shNT.

Strain/Cell line	HCT116	SW620
mother vs shNT	p = 0.03	p = 0.70
mother vs shA4-3	p = 0.62	p < 0.001
shNT vs shA4-3	p = 0.16	p = 0.005

Table 6: Probability values from AUC calculations from OXA curves compared by the Student's t-test.

The low p-values (table 6) support that there is a difference between SW620 mother and shNT, and shA4-3. The t-test also gave a low p-value to the comparison of AUC from HCT116 mother and shNT, but the curves for these cell line variants cross each other, so it seems doubtful that there is an actual difference between these cell lines.

Strain/Cell line	HCT116	SW620
Mother	12 μM	10 μM
shNT	10 μM	10 μM
shA4-3	10 μM	55 μM

Table 7: IC50 estimates for the OXA curves

IC50 estimated for the curves (table 7) gave roughly the same value for the HCT116 strains and the SW620 mother and shNT strains, whereas a difference of 45 μM between these strains and SW620 shA4-3 was observed. This finding also supports that there is a difference between SW620 shA4-3 and the other SW620 variants.

Having observed the similarities in both growth and viability between the mother and the shNT strain in both cell lines, we assumed that these strains have the same properties and decided to focus on the differences between shNT and shA4-3 in the further work.

4.3.2 IRI

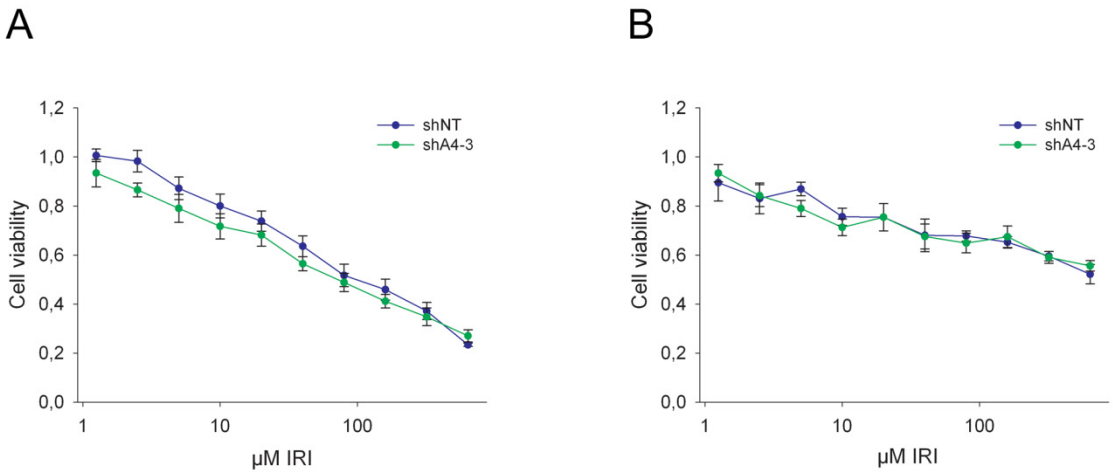

Figure 7: Viability of HCT116 (A) and SW620 (B) cells after treatment with irinotecan. Standard error of the mean (SEM) is shown for each concentration.

Figure 7 shows the viability of cells relative to control after treatment with IRI. We observed no major differences between shNT and shA4-3 from the same cell line, and the high p-value of the AUC comparison by the Student's t-test of p = 0.68 (HCT116) and p = 0.80 (SW620) support this observation.

Strain/Cell line	HCT116	SW620
shNT	108 µM	>640 µM
shA4-3	72 µM	>640 µM

Table 8: IC50 estimates for the IRI curves

When comparing the IC50 estimates (table 8) we observed a difference of 32 µM between the HCT116 shNT and shA4-3. For SW620 shNT and A4-3, IC50 was not reached in our span of drug concentrations and a good estimation could not be performed.

4.3.3 5FU

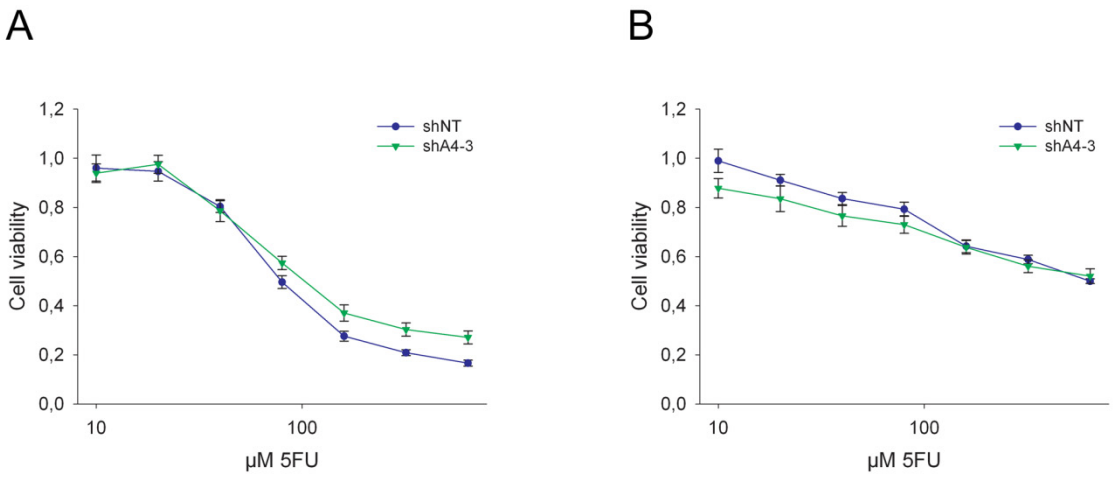

Figure 8: Viability of HCT116 (A) and SW620 (B) cells after treatment with 5FU. Standard error of the mean (SEM) is shown for each concentration.

Figure 8 shows the viability of cells relative to control after treatment with 5FU. HCT116 shNT and shA4-3 seemed to be equally sensitive to 5FU at low concentrations whereas shA4-3 was less sensitive at the higher concentrations ($p = 0.02$). In the case of SW620, the curves had a more linear shape, and there was no apparent difference between SW620 shNT and shA4-3 at the low concentrations ($p = 0.65$). At the higher 5FU concentrations, there were no noticeable differences between the two strains.

Strain/Cell line	HCT116	SW620
shNT	80 μM	640 μM
shA4-3	107 μM	>640 μM

Table 9: IC50 estimates for the 5FU curves

The estimated IC50 (table 9) show a difference of 27 μM between the HCT116 strains. In the case of SW620 the shA4-3 did not reach IC50 in our span of concentrations and a good estimation could not be done. The IC50 for SW620 shNT was estimated to be at the highest concentration used.

4.3.4 Cell line specific chemotherapy sensitivity

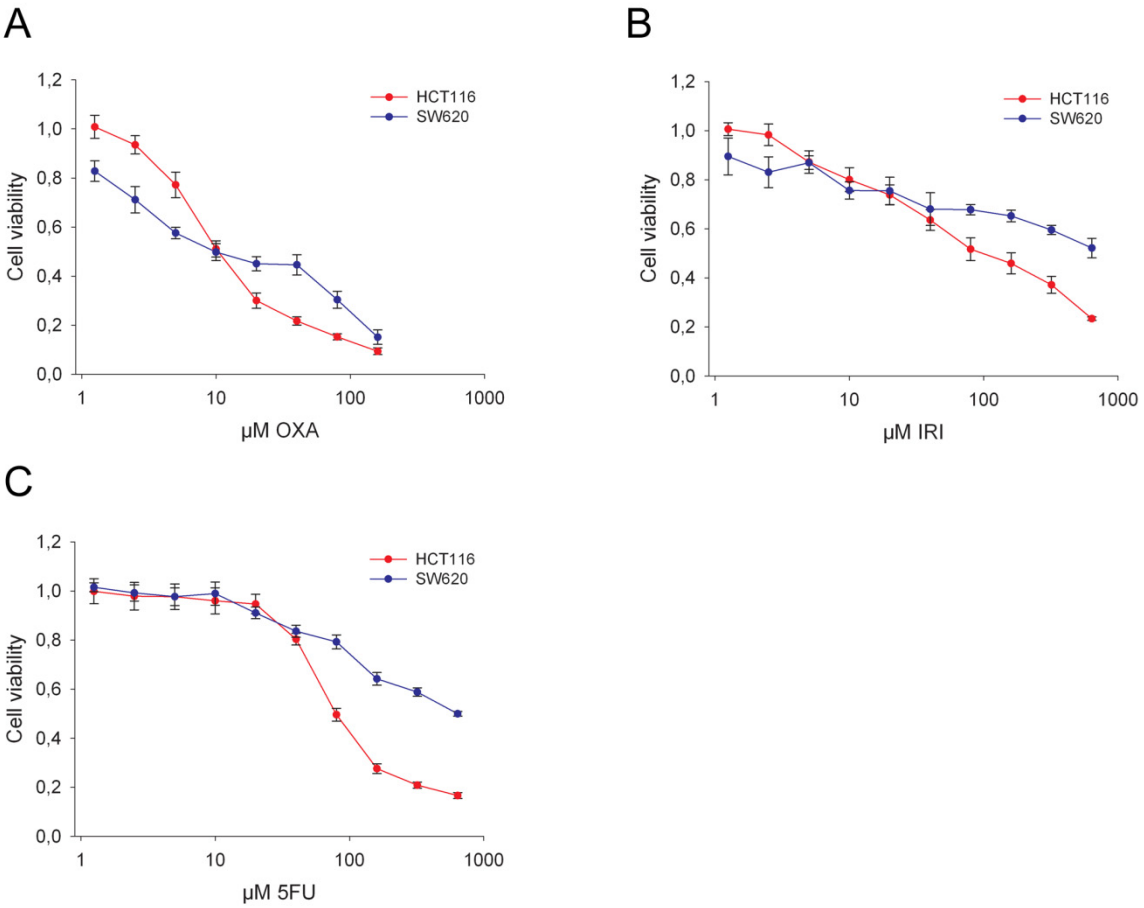

Figure 9: Viability of HCT116 shNT and SW620 shNT after treatment with OXA (A), IRI (B) and 5FU (C). Standard error of the mean (SEM) is shown for each concentration.

The shNT strain of each cell line was compared to investigate the difference in drug sensitivity between the cell lines, and results are shown in figure 9. In the case of OXA, HCT116 seemed to be less sensitive at low concentrations but more sensitive than SW620 in the higher concentration range. The AUC calculations returned higher values for SW620 than for HCT116 ($p = 0.008$), but the curves crossed at about 10 µM and this makes the results difficult to interpret.

HCT116 was also less sensitive to IRI at low concentrations and more sensitive at high concentrations than SW620. The low p-value ($p < 0.001$) supported the observation that there

was a difference between the two cell lines, the AUC calculations from SW620 being higher than AUC from HCT116, but the results are similarly difficult to interpret.

For 5FU, the cell lines was shown not to be sensitive to concentrations below 10 μM and at the higher concentrations, HCT116 was more sensitive than SW620 ($p < 0.001$).

Strain/Cell line	HCT116 shNT	SW620 shNT
OXA	12 μM	10 μM
IRI	108 μM	>640 μM
5FU	80 μM	640 μM

Table 10: IC50 estimates for the HCT116 shNT and SW620 shNT curves when treated with OXA, IRI and 5FU.

The estimated IC50 for the shNT curves are summarized in table 10. We observed that there was no difference between IC50 in HCT116 and SW620 treated with OXA. However, we observed relatively large differences between the cell lines when treated with IRI and 5FU, HCT116 being more sensitive than SW620.

4.3.5 CET

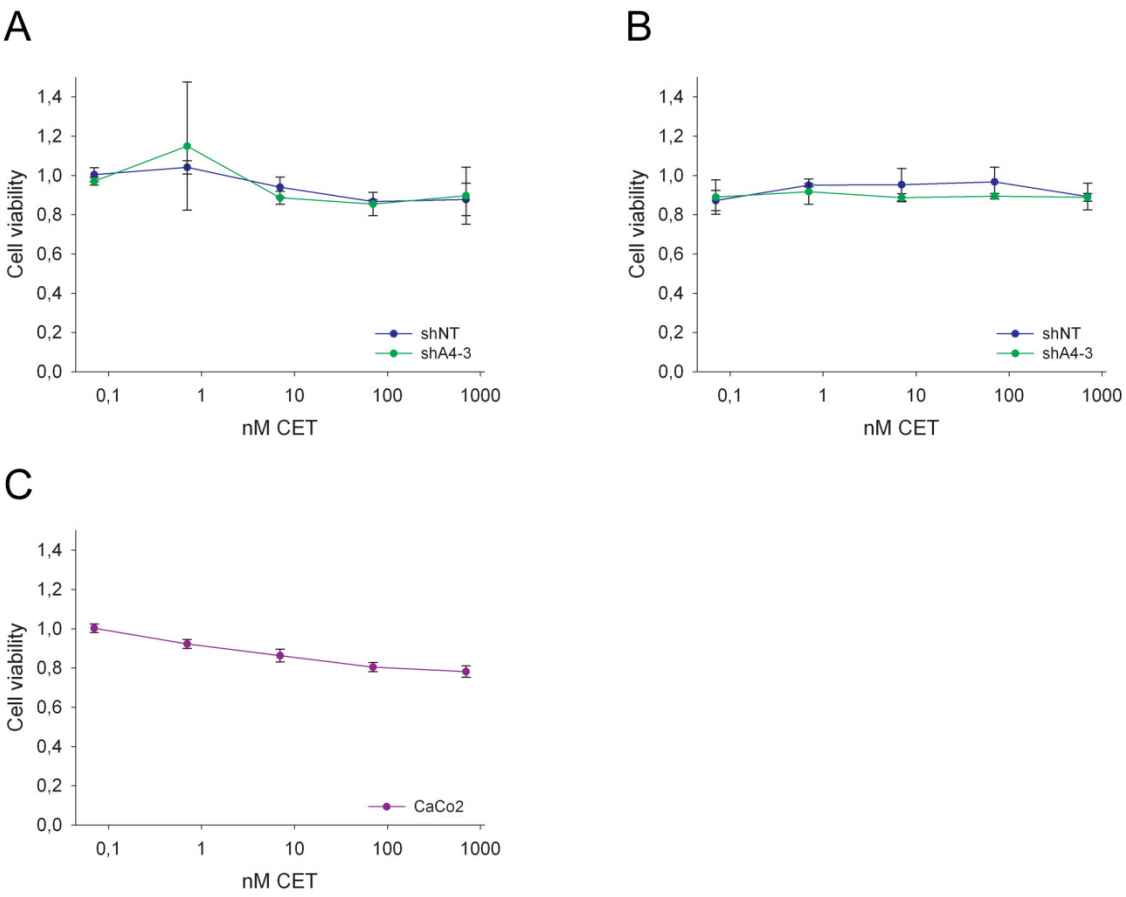

Figure 10: Viability of HCT116 (A), SW620 (B) and CaCo2 (C) cells after treatment with cetuximab. Standard error of the mean (SEM) is shown for each concentration.

Figure 10 shows the viability of cells relative to control after treatment with cetuximab (CET). CaCo2 cells were used as a positive control for CET treatment and showed a slight reduction in viability. Neither HCT116 nor SW620 exhibited noticeable reduction in viability upon treatment with CET, and the shNT and shA4-3 strains seem to be equally resistant towards this treatment.

4.4 Effect of 5FU on HCT116 2D cultures measured by clonogenic survival assay

After obtaining significant differences between HCT116 shNT and shA4-3 upon treatment with 5FU in the MTS assay, clonogenic survival assay was run to investigate if the differences also could be observed by a different method.

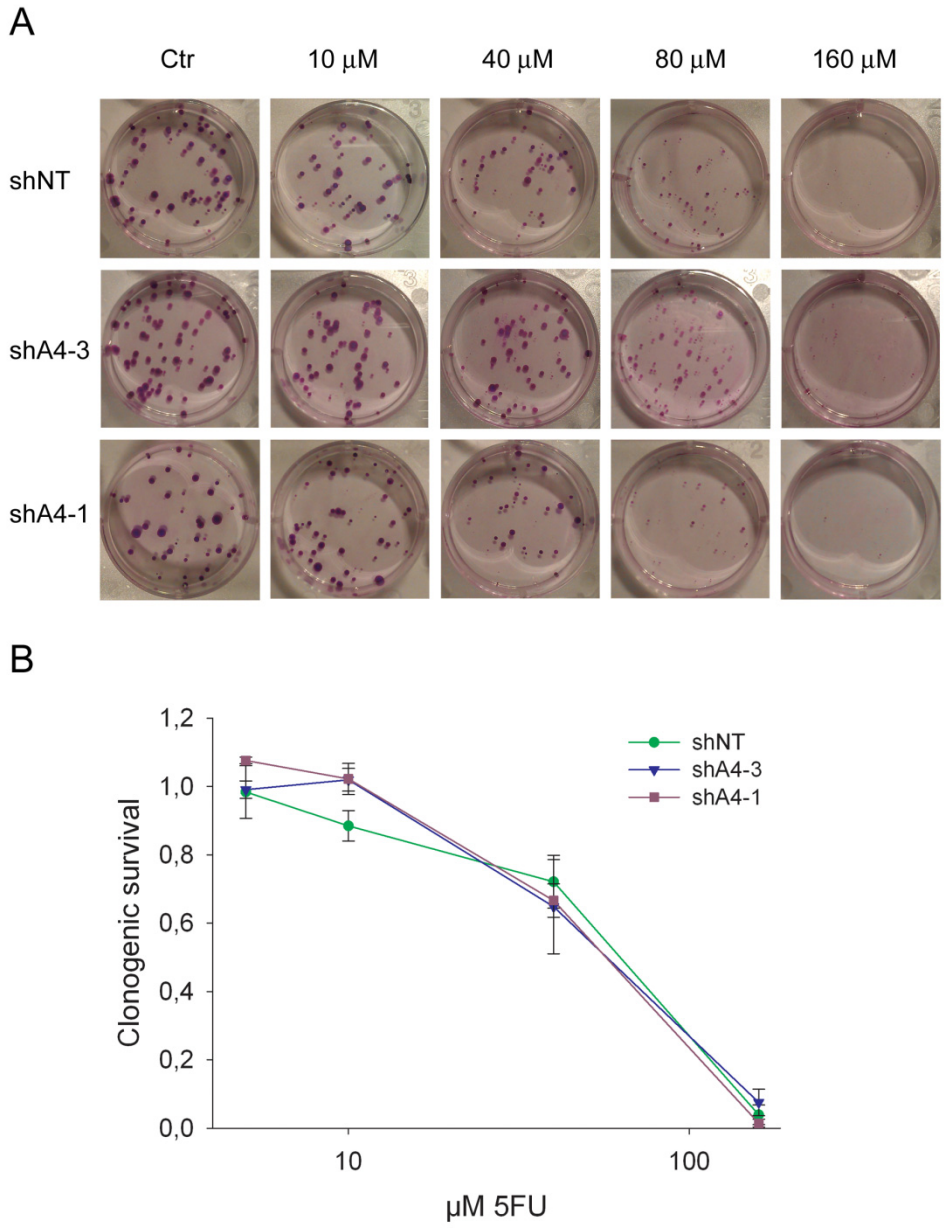

Figure 11: Survival of HCT116 in clonogenic survival assay after treatment with 5FU. Standard error of the mean (SEM) is shown for each concentration.

Strain/Cell line	HCT116
shNT vs shA4-3	p = 0.95
shNT vs shA4-1	p = 0.79
shA4-3 vs shA4-1	p = 0.90

Table 11: Probability values from AUC calculations from clonogenic survival curves compared by the Student's t-test.

HCT116 was shown to be well suited for assessment of clonogenic survival, generating large, round colonies after just one week. Figure 11 shows relative survival of HCT116 shNT, shA4-3 and shA4-1 after treatment with 5FU. The strain shA4-1 was included to ensure that the properties of the shA4-3 cells were due to reduced expression of S100A4, and not as a result of clonal variation. The results from both the Student's t-test (table 11) and the IC50 calculations (table 12) show no significant difference between either of the HCT116 strains.

Strain/Cell line	HCT116
shNT	79 μM
shA4-3	71 μM
shA4-1	71 μM

Table 12: IC50 estimates for the curves of HCT116 strains in clonogenic survival assay

We also tried to perform clonogenic survival assays using SW620 cells, but they formed small colonies with several satellite colonies, making these cells unsuitable for this assay.

4.5 Effect of 5FU on HCT116 3D cultures measured by spheroid volume and CellTiter-Glo

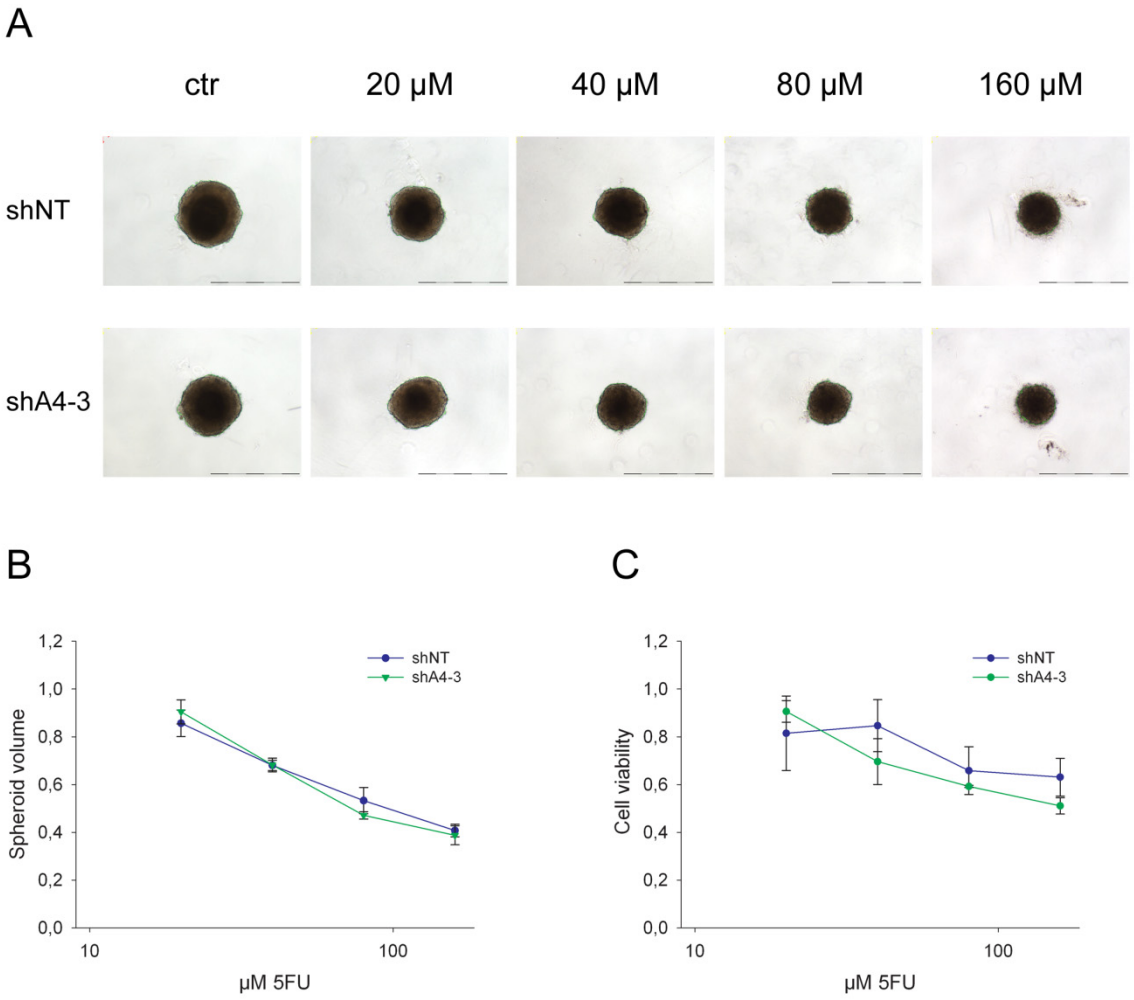

Figure 12: HCT116 spheroids treated with 5FU and photographed 96 hours after treatment (A). Scale bar 1000 µm in all pictures. The estimated volume of spheroids was compared to a vehicle treated control (B) and the viability was measured by CellTiter-Glo (C).

HCT116 spheroids were treated with 5FU to investigate if the differences shown in 2D cultures also were visible in 3D cultures. The spheroids were shown to shrink after treatment with 5FU (figure 12A), and the reduction in calculated spheroid volume was shown to correlate negatively with increasing concentrations of 5FU (figure 12B). HCT116 shNT and

shA4-3 cells grown in spheroids seemed to be equally sensitive towards 5FU and was supported by the t-test which gave $p = 0.59$ for the spheroid volume curves. The estimated IC50 values were found to be 99 μM for shNT and 74 μM for shA4-3.

When looking at the spheroids in the microscope we only see the cells on the surface of the spheroid, and cell viability was therefore also measured to investigate the whole spheroid. In the CellTiter-Glo graph (figure 12C) there seemed to be a difference in mean value for the curves, shNT being less sensitive towards 5FU, but the SEM were high in comparison to the SEM seen for the spheroid volume assay. The shape of the curves were similar and the p-value predicted by the Student's t-test were $p = 0.42$. The IC50 could not be estimated because the curves did not reach this point in our span of concentrations.

We also tried to grow spheroids from SW620 cells, but these cells only formed loose aggregates instead of firm spheroids with a defined edge. Some effort was made to optimize the assay by growing the spheroids in Matrigel (BD Bioscience, Franklin Lakes, NJ, USA) and centrifugation straight after seeding (Vinci et al., 2012), but the cells then formed numerous small spheroids and could not be used in drug sensitivity experiments.

4.6 Comparison of HCT116 sensitivity to 5FU between assays

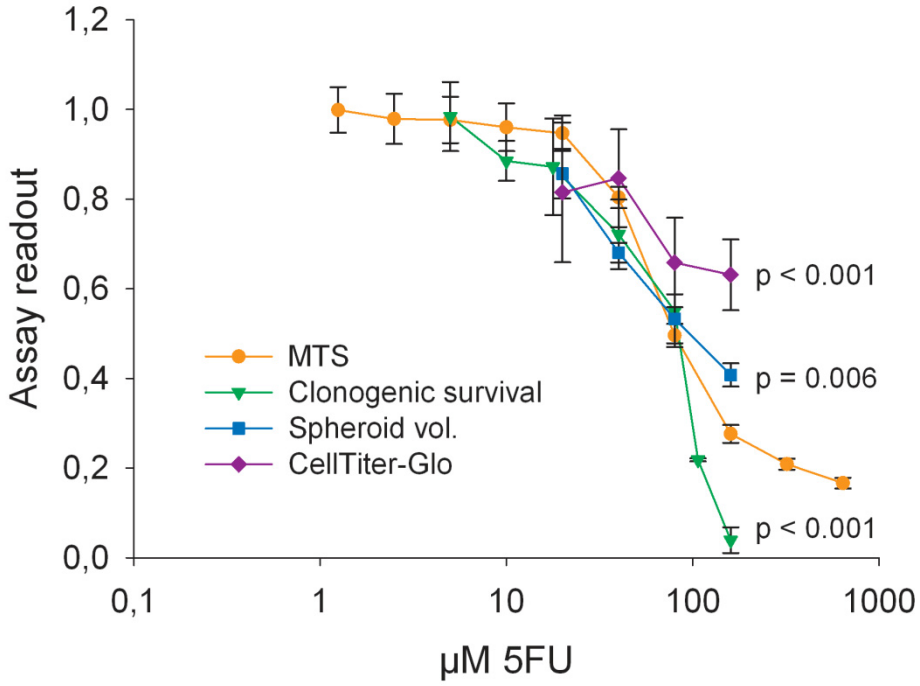

Figure 13: Sensitivity of HCT116 shNT towards 5FU in four different assays (MTS, clonogenic survival, spheroid volume and CellTiter-Glo). P-values (Student's t-test) from comparisons of assays to the MTS assay for the concentration 160 µM 5FU are shown.

We observed that the different assays gave similar results in the intermediate concentration range (figure 13). The highest concentration of 5FU tested in all assays (160 µM), almost completely inhibited clonogenic regrowth, whereas, in the spheroid assays, HCT116 cells seemed to be more resistant towards 5FU at this concentration. The values measured at the single point of 160 µM were compared for all assays against the MTS assay in the Student's t-test and the p-values are shown in figure 13. All three assays (clonogenic survival assay, spheroid volume assay and CellTiter-Glo assay) are found to differ from the MTS assay at this point.

4.7 Average AUC values

Method	Drug	Range	Average AUC								Designation
			HCT116				SW620			CaCo2	
			mother	shNT	shA4-3	shA4-1	mother	shNT	shA4-3		
Growth rate	-	0-96 hours	$2.9x10^8$	$2.8x10^8$	$2.38x10^8$	-	$7.78x10^7$	$6.96x10^7$	$6.55x10^7$	-	No. of cells x hours
MTS assay	OXA	0-160 µM	42	34	40	-	51	53	70	-	Cell viabillity x [OXA]
	IRI	0-640 µM	-	256	277	-	-	389	394	-	Cell viabillity x [IRI]
	5FU	0-640 µM	-	192	248	-	-	399	387	-	Cell viabillity x [5FU]
	5FU	10-640 µM	-	183	238	-	-	384	402	-	Cell viabillity x [5FU]
	CET	0-700 nM	-	613	614	-	-	653	624	558	Cell viabillity x [CET]
Clonogenic survival assay	5FU	0-160 µM	-	79	78	76	-	-	-	-	Cell viabillity x [5FU]
Spheroid volume assay	5FU	0-160 µM	-	96	92	-	-	-	-	-	Cell viabillity x [5FU]
CellTiter-Glo assay	5FU	0-160 µM	-	116	105	-	-	-	-	-	Cell viabillity x [5FU]

Table 13: Average AUC values calculated for all curves shown in sections 4.2-4.6.

AUC values were calculated for all curves shown in sections 4.2-4.6 and a summary of the average AUC values is found in table 13. The average AUC values should only be used to compare experiments within the same range and method.

5 Discussion

5.1 Cell lines

The present project was based on cell lines stably transduced with shRNA against S100A4, and it was therefore of great importance to thoroughly validate the expression of S100A4 at multiple time points. The knockdown of the protein was shown to be successful, and even though the expression seemed to increase slightly over time for the HCT116 cell line, the knockdown was relatively stable. The expression of S100A4 was more decreased in SW620 than in HCT116 and this reflects that the efficiency of the lentiviral shRNA might be cell line specific.

To be certain that the transduction procedure itself did not affect the cells to a large extent, cells were also transduced with a non-target shRNA vector control (shNT). In theory, the mother strain and the shNT strain should have the same properties, and the comparison made in both growth rates and sensitivity to OXA showed that the mother and shNT strains of both HCT116 and SW620 seemed to have the same properties. This also implies that any differences seen between the mother and shNT strains and the shA4-3 were probably due to knockdown of S100A4 and not the lentiviral vector or the transduction procedure.

The HCT116 and SW620 strains were all multiclonal cell cultures. This is considered an advantage compared to picking and expanding single cell colonies after transduction, because the risk of clonal variation is substantially smaller. Since considerable intratumor heterogeneity is present in human cancer, multiclonal cell lines might also better reflect the actual tumors in patients (Marusyk et al.).

One limitation of this thesis is that only one S100A4 knockdown variant of each cell line was used for the majority of the experiments conducted. Even though the cell lines generated were multiclonal, it is a possibility that the differences observed could be due to clonal variation. Analyzing more than one shS100A4 cell line would to a large extent overcome this problem, but we chose not to include several variants because the experimental set-ups then would have become relatively large. However, for the clonogenic survival assays, two shS100A4 knockdown strains were used and the results were similar for both these cell lines.

Furthermore, presence or absence of S100A4 did not seem to influence cell growth, as there were no differences when we compared growth rate between the strains. Based on this finding, we were able to compare the shRNA-transduced cells directly with the shNT and mother strains, without taking into account the effect of S100A4 on cell growth.

In conclusion, based on continuous routine controls of the S100A4 expression, our cell system seems to be quite robust and could be used to investigate whether S100A4 is involved in chemosensitivity in colorectal cancer.

5.2 Impact of S100A4 expression on the sensitivity to chemotherapeutic drugs

The drugs OXA, IRI, 5-FU and CET were all chosen because they are currently the most commonly used in Norway for chemotherapy in CRC.

5.2.1 OXA

When investigating the sensitivity of OXA in the MTS-assay we found that the expression of S100A4 did not appear to influence the sensitivity of HCT116, whereas low expression of S100A4 in SW620 seemed to decrease the sensitivity towards OXA in the intermediate and high concentrations (5-160μM). In a study of OXA pharmacokinetics in humans, the average max concentration (C_{max}) of OXA in blood was measured after infusion of 85 mg/m^2 (over 2 hours every second week) or 130 mg/m^2 (over 2 hours every third week). The C_{max} for the two therapy regimens were 0.8 μg/ml (2.1 μM) and 1.2 μg/ml (3.1 μM), respectively (Norwegian Medicines Agency, 2007). A dose of 85 mg/m^2 is the highest recommended dose used in treatment of CRC in Norway. It is important to remember that the C_{max} in blood not necessarily corresponds to the concentration that the tumor cells are exposed to, but it might give us a hint regarding the concentration span where we can find differences that can be relevant in treatment of patients. At a concentration of 2-3 μM we did not observed any notable difference in sensitivity in the SW620 strains. The dose limiting factor of OXA is neurological toxicity due to peripheral sensory neuropathy (Norwegian Medicines Agency,

2007), and to give patients high doses of the drug may be harmful or even lethal. Thus, it is questionable whether the differences we observed for the relatively high concentrations of OXA are clinically relevant.

5.2.2 IRI

Cells treated with IRI were in the MTS-assay shown to be sensitive towards the drug in both HCT116 and SW620, but the expression of S100A4 did not seem to affect the cells' sensitivity towards IRI in either of the cell lines. In the cell viability results from treatment with IRI we observed a larger variation between experiments than for the other drugs, visualized by the larger SEM-bars in figure 7 on page 32.

5.2.3 5FU

Cells treated with 5FU in the MTS-assay showed to be sensitive towards the drug in both HCT116 and SW620. In SW620, the expression of S100A4 did not seem to affect the sensitivity of the cells, whereas low expression of S100A4 in HCT116 appeared to be associated with decreased sensitivity towards high concentrations of 5FU (80-640 μM). In a pharmacokinetic study (Bocci et al., 2000), the patients were given an infusion of 250 or 370 mg/m^2 and the C_{max} in blood were 18.2 (140 μM) and 48.4 μg/ml (372 μM), respectively. This is within the range that we observed a difference between HCT116 cells with high and low levels of S100A4, and means that any observed differences could be biologically relevant. In the treatment of CRC patients in Norway 500 mg/m^2 of 5FU for two subsequent days is usually administered (Sorbye and Dahl, 2003), further suggesting that the highest concentrations in our study might be clinically relevant. However, the differences were not present when the cells were studied using the clonogenic survival assay or in the spheroid assays. Thus, although the results from the MTS assay indicated that the presence of S100A4 was associated with a 5FU sensitive phenotype, the fact that the difference was relatively small and could not be confirmed in the other assays argue against its biological importance.

5.2.4 CET

Treatment of shNT and shA4-3 with CET did not decrease cell viability of HCT116 and SW620 in MTS-assay, but a slight effect was observed in CaCo2 cells. CaCo2 has previously been shown to be sensitive towards CET (Dunn et al., 2011), and was included in the experimental setup as a positive control. Since there was a negative correlation between cell viability and concentration of CET in this cell line, it is reasonable to believe that the HCT116 and SW620 cells were actually more resistant to CET. In addition, we tested two different batches of CET, which gave similar results, demonstrating that our results were not due to an inactive batch of drug.

HCT116 and SW620 have both been reported to have mutations in the *KRAS* gene (Dunn et al., 2011). Mutations in *KRAS* have been shown to be associated with resistance to CET treatment (De Roock et al.), and this may explain why HCT116 and SW620 were shown to be resistant towards CET treatment. Because the mechanism by which CET exerts its functions *in vivo* might include lymphocyte mediated cell death (Correale et al., 2012), the lack of response might also be because of the simplified microenvironment in *in vitro* cell cultures.

5.3 Cell line specific chemotherapy sensitivity

A difference in chemotherapy sensitivity was observed between HCT116 and SW620 for OXA, IRI and 5FU, as HCT116 appeared to be more sensitive than SW620 within the high concentration range for all three drugs. The AUC values calculated were higher for SW620 than HCT116 (table 13 on page 43), and the Student's t-test concluded that the HCT116 and SW620 curves were significantly different from each other for the three cases compared. However, the curves for OXA and IRI cross in the mid-concentration range, and this may question the use of this type of statistics, and may make interpretation of results difficult.

HCT116 and SW620 are established from tumor tissue from two different colon cancer patients (Brattain et al., 1981, Leibovitz et al., 1976) and it is therefore not surprising that the sensitivity to chemotherapeutic drugs varies between these cell lines.

5.4 Methodological considerations

In this thesis, cell viability analysis using the MTS assay was chosen as the principal method to investigate the effects of chemotherapeutic drugs. This method was chosen because it is robust, relatively high-throughput, technically simple to conduct, and because the output (cell viability) is a relevant measure in studies of cytotoxic and cytostatic drugs. Based on the results from the MTS assays, we chose to focus on the effect of 5FU in HCT116 cells, for further studies in clonogenic survival and spheroid assays.

The methods used to analyze drug sensitivity reflect different aspects of how the drugs influence cell survival. The MTS- and CellTiter-Glo assays measure the number of viable cells indirectly through a quantitative analysis of metabolic components. A limitation associated with these assays is that the methods only give an indirect measurement of cell viability. The assays return a total measurement of the whole well of cells and cannot alone distinguish whether a decrease in cell viability is due to cell death or an overall reduction in metabolism. These two parameters are usually linked, but can be of great interest when studying sensitivity to drugs that aim to kill cells. Measuring spheroid volume, on the other hand, analyzes the number of cells indirectly according to the space they occupy, but this assay does not necessarily give a precise indication of the relative fraction of live and dead cells.

In comparison, the clonogenic survival assay measures the number of cells that survived the treatment directly and this makes the clonogenic survival assay especially relevant in relation to risk of cancer recurrence (relapse) in patients. The cells that cause a relapse are the ones that survived the initial treatment and can give rise to a new tumor. Each colony in this assay represents a single cell that survived the treatment and which could possibly cause a relapse in a patient. The number of colonies in this assay reflects the risk of relapse in a patient.

All results were calculated relative to a vehicle treated control cells, and because of this we could compare the readout from each of the assays. For the intermediate concentration range the differences seen were only minor, whereas for 160 μM, which was the highest concentration that was investigated with all methods, cells were more sensitive in clonogenic survival and less sensitive in the spheroid assays. Epithelial cells normally grow in cell layers and depend on contact with its neighboring cells and the basal lamina to avoid going into apoptosis (Grossmann, 2002). The Nunclone delta 6-well plates, that were used for the

clonogenic survival assay, have surfaces that are modified to assist adhesion to the growth surface (Granchelli, 2009), but the low number of cells seeded in the clonogenic survival may represent a more challenging growth environment, which might explain why the cells were more sensitive in this setting.

The CellTiter-Glo and Spheroid volume assays will be discussed further in the section 2D versus 3D cultures.

5.5 2D versus 3D cultures

In a 2D culture growing as a monolayer on plastic, the cells will have equal access to nutrients and oxygen anywhere in the culture, whereas there in a 3D culture with a large enough size will be gradients of nutrients and oxygen. The HCT116 spheroids used in this project were typically at a size of 400 μm at the time of treatment and the vehicle treated control spheroids grew to a size of 600-700 μm. Tumor spheroids have been shown to have hypoxic regions and necrotic centers when grown to sizes larger than 500 μm in diameter, and since this feature has also been observed in tumor microregions, this type of assay has been suggested to be more clinically relevant than 2D assays (Sutherland et al., 1986). One of the possible drawbacks of measuring cell viability by spheroid volume is that we do not know if the centre of the spheroid is necrotic and if so to what extent. When performing parallel analysis of spheroid volume and CellTiter-Glo assay, we obtained similar results, and this can indicate that the spheroid volume correlates with the actual number of viable cells.

Another aspect to think about is that the drug might also make gradients in a spheroid. It has been shown that the delivery of many anticancer drugs has been incomplete in tumors because the distance the drug must diffuse from the blood vessels to the cells is greater than is usually seen in normal tissue (Minchinton and Tannock, 2006). If the drug does not readily penetrate the spheroid, the cells in the centre will experience a lower concentration than cells in the periphery of the spheroid. The concentration in the periphery of the spheroid will probably reflect the concentration in the medium and also the concentration in 2D cultures treated with the same drug solution. This means that one could expect 3D cultures to be less sensitive to chemotherapy than 2D cultures. This could be one explanation for the difference observed

between results from the spheroid assays compared to MTS and clonogenic survival for the highest concentration of 5FU (160 μM) in HCT116 cells (figure 13 on page 42).

5.6 Chemotherapy schedule

In this project, we chose to treat cells with chemotherapeutic drugs for 4 hours and ended the treatment by removing the drug solution and adding new fresh medium. In patients, the exposure time and concentration that cancer cells are exposed to is not only dependent on the total dose administered, but also on the rates of absorption, distribution, metabolism and excretion, collectively called the pharmacokinetics of the drug. In infusional chemotherapy, the drug concentration peaks shortly after administration to the patient, and the concentration will then decrease gradually over time. The four-hour schedule used in this work adequately mimics high initial drug exposure and a subsequent drug free interval, and is and effective experimental setup, but does not take into account gradual elimination of the drugs. To imitate this we could have decreased drug concentration in a stepwise fashion, by for example changing part of the medium at regular intervals. However, since the actual time-concentration exposure in tumors is essentially not known for the investigated drugs, the ideal in vitro experimental setting cannot be determined.

6 Conclusion and future perspectives

The background for this project was the discovery of nuclear S100A4 as a possible biomarker for poor prognosis in patients with TNM stage II CRC (Boye et al., 2010). Since TNM stage II patients do not regularly receive adjuvant chemotherapy, nuclear S100A4 could function as a biomarker to decide which patients should be offered such treatment. In relation to this it would be interesting to know if S100A4 expression is associated with sensitivity to relevant chemotherapeutic drugs.

To investigate this question, we exposed cell lines experimentally modified to express different levels of S100A4 to four drugs commonly used in CRC treatment. Expression levels were shown to be relatively stable within each experimental series, suggesting that the cell lines were suitable tools for this purpose. In general, HCT116 cells were more sensitive to the investigated drugs than SW620 cells, while both cell lines were resistant to CET. Some S100A4 related differences were observed in sensitivity in HCT116 treated with 5FU and SW620 treated with OXA. For OXA, the differences were observed at concentrations that are probably not relevant in cancer treatment in humans, whereas for 5FU the concentration range was probably relevant, but the differences could not be confirmed using other methods. In addition to assessment of cell viability by MTS, clonogenic survival and 3D cultures were established for HCT116 cells. With the exception of one high concentration, sensitivity to 5FU was relatively similar when assessed with these methods.

Based on the present work S100A4 does not seem to influence in vitro sensitivity to the investigated drugs to a large extent in the two investigated models. However, results from two in vitro models cannot be directly translated to the clinical situation. The observed association between S100A4 expression and prognosis still makes the initial question highly relevant. Our group is currently pursuing this issue by performing immunohistochemical analysis of primary tumors from patients in a randomized Norwegian phase III study where 5FU was given as adjuvant treatment (Dahl et al., 2009). By analyzing associations between S100A4 expression and outcome, we hope to be able to answer this question. If this is the case, further in vitro elucidation of effects and mechanisms will be even more interesting, and some of the models systems established in this work may become very useful.

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Appendix

The formula of solutions used in this project are listed below.

Amidoblack

1 g naphtol Blue Black
450 ml methanol
100 ml acetic acid
450 ml ddH_2O

Amidoblack destaining solution

900 ml methanol
20 ml acetic acid
80 ml ddH_2O

BSN buffer (20x)

116 g Tris
58 g glycin
ddH_2O to 1L

TBS buffer containing 0.1% Tween

20 ml 1M Tris-HCl pH: 7.5
30 ml 5M NaCl
1 ml Tween 20
949 ml ddH_2O

Lysis buffer

150 mM NaCl
50 mM Tris-H
0.1 % Nonidet-P40 (v/v) Cl, pH 7.5