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Investigating a Model Cell Line for Tumour Stem Cells in Colorectal
Cancer

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I wish I had the voice of Homer
To sing of rectal carcinoma,
Which kills a lot more chaps, in fact,
Than were bumped off when Troy was sacked.
J.B.S. Haldane

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Abstract

According to the cancer stem cell theory, only a small subset of cells in a tumour is responsible for tumour initiation, maintenance and growth. The cancer stem cells (CSC) display stem cell characteristics like longevity, self-renewal, a high differentiation potential and protection from apoptosis and are resistant to conventional therapy approaches. For colon cancer this phenomenon can easily be envisioned as the initial APC-mutation observed in most colon cancers leads to a constitutively active Wnt pathway. This signalling cascade is also active in the normal adult stem cells that drive cell turn over in the colonic mucosa and are situated at the bottom of the colonic crypt. Previous studies revealed a subpopulation in LT97 colon adenoma cells positive for the Wnt target protein CD44 that also displayed stem cell characteristics in gene expression (musashi, telomerase) and behaviour (enhanced growth and survival abilities compared to CD44- cells). In the present thesis the underlying mechanisms that lead to enhanced growth and survival of the CD44+ subpopulation were investigated on RNA level by standard and RealTime PCR and on protein level by western blot. The results indicate (1) protection from apoptosis by expression of the Wnt target gene survivin in the CD44+ cells; (2) down modulation of IGF1 survival signalling by IGFBP3 expression in CD44- cells. and (3) up regulation of the FGFR3IIIc splice variant CD44+ cells indicating a FGF18 dependent survival signal. Analysis of downstream signalling by western blot revealed reduced phosphorylation of IRS1, GSK3, ERK1/2 and S6 and decreased protein expression of IRS1, GSK and S6 in CD44- cells. Both inhibition of Wnt signalling by Sulindac and IGF signalling by PPP led to decreased growth and survival of CD44+ cells, indicating the importance of these pathways for colon tumour formation. Expression of a dominant negative FGFR3 construct increased plating efficiency, but led to decreased growth of CD44+ and CD44- cells, while introduction of a FGF18 over expressing construct led to enhanced growth and survival in CD44+ cells.

In a comparative study of candidate stem cell markers a reverse relationship between CD44 and CD133 as well as a direct relationship between CD44 and CD166 was found in both cell lines and primary cultures obtained from colorectal tumours. Musashi and Lgr5 were found more in normal tissue than in tumour sections. In summary, 3 signalling pathways responsible for enhanced growth and survival of CD44+ LT97 cells and promising for therapeutic targeting have been verified; however the stem cell state of CD44+ LT97 has not been appraised.

Zusammenfassung

Laut der Tumorstammzelltheorie ist nur ein Bruchteil der Zellen eines Tumors verantwortlich für Tumorbildung, -erhalt und -wachstum. Die Tumorstammzellen besitzen Eigenschaften von Stammzellen wie Langlebigkeit, Selbsterneuerung, ein hohes Differenzierungspotential, Schutz vor Apoptose und sie sind resistent gegenüber herkömmlichen Therapieansätzen. Im Darmkrebs kann man sich das so vorstellen, dass die initiale Mutation im APC-Gen, welche in den meisten Fällen beobachtet werden kann, zu einem konstitutiv aktiven Wnt Signaltransduktionsweg führt. Diese Signalkaskade ist auch in normalen adulten Stammzellen, die für den hohen Zellumsatz in der Darmschleimhaut verantwortlich sind, aktiv. Vorangegangene Studien zeigten, dass adenome Darmkrebszellen der Zelllinie LT97 eine Unterpopulation besitzen, welche das Wnt- Zielprotein CD44 exprimieren und Stammzeleigenschaften zeigten, sowohl in der Genexpression (musashi, Telomerase), als auch in Kultur (erhöhte Wachstums- und Überlebensfähigkeit verglichen mit den CD44- Zellen). In der vorliegenden Diplomarbeit wurden die grundlegenden Mechanismen, die zu der verstärkten Wachstums- und Überlebensfähigkeit der CD44+ Population führen, auf RNA-Ebene mittels Standard- und RealTime PCR, und auf Protein-Ebene mittels Western blot untersucht. Das Ergebnis zeigt (1) Schutz vor Apoptose durch Expression des Wnt-Zielgens survivin in CD44+ Zellen, (2) Herunterregulierung des IGF1 Überlebenssignals durch IGFBP3 Expression in CD44- Zellen, und (3) Hochregulierung der FGFR3IIIc Splice Variante in CD44+ Zellen, welche ein FGF18 abhängiges Überlebenssignal andeuten. Analyse des downstream signalling durch Western blot zeigte reduzierte Phosphorylierung von IRS1, GSK3, ERK1/2 und S6 und verringerte Expression von IRS1, GSK3 und S6 in CD44- Zellen. Sowohl Hemmung des Wnt Signalwegs mit Sulindac als auch des IGF Signalwegs mit PPP führten zu verringertem Wachstum und Überleben von CD44+ Zellen, was die Bedeutung dieser Signalwege für die Ausbildung von Darmtumoren aufzeigt. Expression eines dominant negativen FGFR3 Konstrukts erhöhte die Effizienz des Ausplattierens, aber führte zu verringertem Wachstum in CD44+ und CD44- Zellen, während die Einführung eines FGF18 überexprimierenden Konstrukts zu erhöhtem Wachstum und Überleben in CD44+ Zellen führte.

In einer vergleichenden Studie von Kandidaten für Stammzellmarker wurde eine umgekehrte Beziehung zwischen CD44 und CD133 sowie eine direkte Beziehung zwischen CD44 und CD166 sowohl in Zelllinien als auch Primärkulturen von kolorektalen Tumoren entdeckt. Musashi und Lgr5 wurden hauptsächlich in Normalgewebe und kaum in Tumorgewebe gefunden. Zusammenfassend kann man sagen, dass drei für verstärktes Wachstum und Überleben von CD44+ Zellen verantwortliche und somit für therapeutische Ansätze vielversprechende Signalwege bestätigt wurden, wohingegen der Stammzell-Zustand der CD44+ LT97 Zellen nicht verifiziert werden konnte.

1 Introduction

1.1 What is Cancer?

The American Cancer Society (ACS 2007) defines cancer as “A group of diseases characterised by uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in death.”

1.1.1 Epidemiology of Cancer

Cancer is one of the leading causes for death in developed countries, it is only second to cardiovascular disease and accounted for 7.9 million deaths (~13% of all deaths) worldwide in 2007.

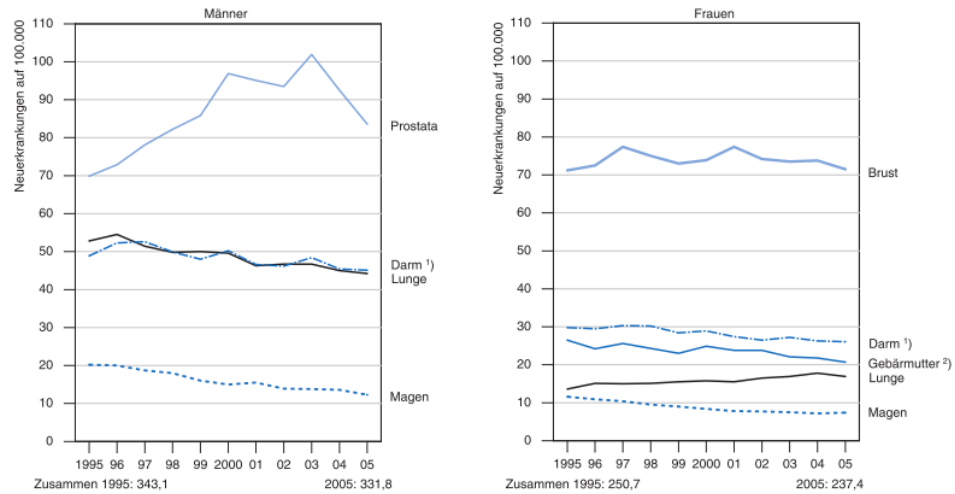
At the age of 70, over 50% of the western population develops a benign colorectal tumour (adenomatous polyp), and in 10% of the cases the tumour progresses towards a malign phenotype (Kinzler et al., 1996).

There are two definitions that can be used to describe the occurrence of cancer in a population: the incidence (number of new cases per 100 000 people per year) and mortality (number of deaths per 100 000 people per year). Incidence and mortality do not necessarily correlate.

The most common cancers regarding incidence world wide, are lung, stomach, liver, breast, prostate and colon cancer (figure 1.1). Deaths from cancer worldwide are estimated to continue rising up to 12 million deaths in 2030. (WHO 2009, <http://www.who.int/cancer/en/>)

Lung and colon cancer have the highest mortality in men, while breast and lung cancer have the highest mortality rate in women world wide (figure 1.2).

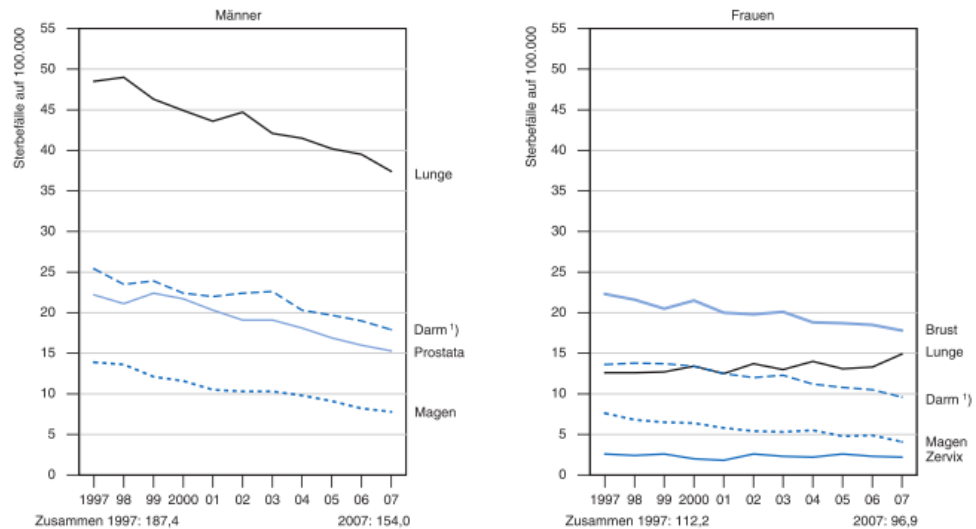
3.01 Krebs-Neuerkrankungen (einschl. DCO-Fälle) 1995 bis 2005 Welt-Standardbevölkerung (WHO/WORLD), altersstandardisiert Cancer incidence (incl. DCO-cases) 1995 to 2005



Q: STATISTIK AUSTRIA. - Für die internationale Anerkennung von Krebsregister-Auswertungen müssen jene Sterbefälle an Krebs, die zuvor nicht dem Krebsregister gemeldet wurden, erfasst und den gemeldeten Inzidenzfällen (Neuerkrankungen) zugezählt werden. Solche Fälle werden DCO-Fälle (Death Certificate Only) genannt, da die Informationen ausschließlich von Totenscheinen stammen. - ¹⁾ Dickdarm und Enddarm. - ²⁾ Ohne Carcinoma in situ (Vorstadien). - Stand 16.09.2008.

Figure 1.1: Cancer incidence from 1992 to 2005 world wide. (Statistisches Jahrbuch 2009, Statistik Austria)

3.03 Krebssterblichkeit 1997 bis 2007 Welt-Standardbevölkerung (WHO/WORLD), altersstandardisiert Cancer mortality 1997 to 2007



Q: STATISTIK AUSTRIA. - ¹⁾ Dickdarm und Enddarm.

Figure 1.2: Cancer mortality from 1992 to 2007 world wide. (Statistisches Jahrbuch 2009, Statistik Austria)

1.1.2 Epidemiology of Colorectal Cancer in Austria

In Austria malignant diseases are with over 25% the second leading cause for deaths (figure 1.3). According to actual WHO statistics, colon and rectum cancer were the second most common new detected cases of cancer both in women and men (see figure 1.4), though men have a 1.6 fold higher risk to come down with colorectal carcinoma than women (Hackl 2004).

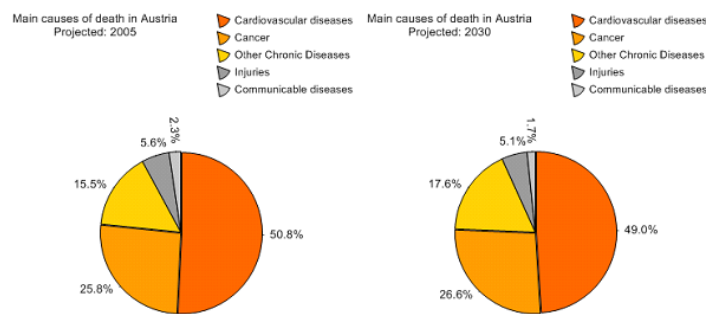


Figure 1.3: Main causes of death in Austria 2005 (WHO global infobase 2009)

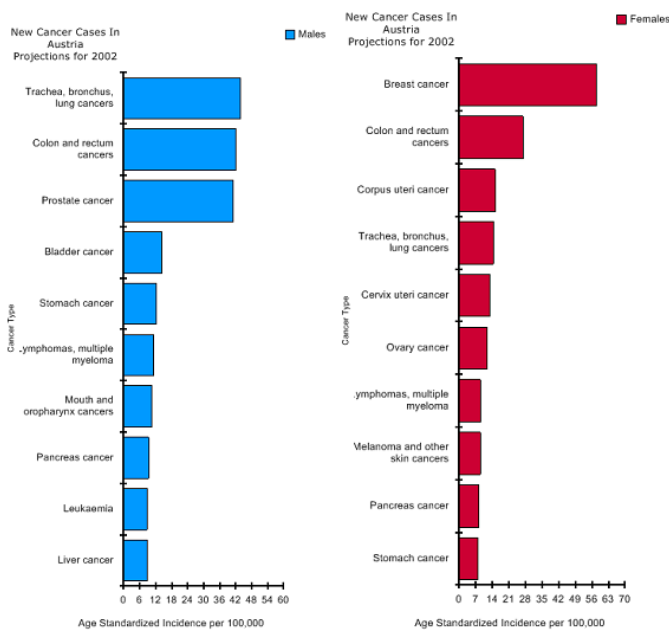


Figure 1.4: Incidence of cancer in men and women in Austria (WHO global infobase 2009)

In general the incidence for colorectal carcinoma is higher in the population over 65 years old than in the population younger than 65 years old in Austria. Though overall mortality from colorectal carcinoma is decreasing, there are about 5000 new cases per year and about 3000 cancer deaths caused by colon cancer (Karner-Hanusch et al., 2006).

1.1.3 Multiple-Stage Hypothesis for Cancerogenesis

Within the body of multicellular, eukaryotic organisms of the kingdom Animalia or Metazoa, the number of living cells is restricted. Complex molecular control circuits make sure that both malfunctioning or excess cells die, and on the other hand dead cells are replaced by new, functional cells. To establish the balance between proliferation and apoptosis, the natural drive of nature to propagate has to be regulated on cellular level. In this case self sacrifice of the cell has to overcome the survival of the fittest. Usually only very few cells, called adult stem cells, maintain the ability to self-renew and proliferate infinitely, although the proliferation rate is very low and regulated. Those cells have a high differentiation potential, giving rise to different cell types.

Cells deriving from such a stem cell lose the capability to self renewal and limitless proliferation, as they start to differentiate. Those differentiated cells do not produce progeny and have in many cases a limited lifespan. Cancer is a group of diseases, where cells evade those control circuits by mutations and start to proliferate offside any regulatory signals.

To prevent a cell from becoming malign, there are many different regulatory pathways that have to be overcome. Hanahan and Weinberg described which capabilities are necessary for a cell to transform into a cancer cell. The cells must be able to evade apoptotic signals, become self sufficient in growth signals, insensitive to anti-growth signals, gain a limitless replication potential, and further on in tumourigenesis also be able to sustain angiogenesis and metastasis (figure 1.5; Hanahan and Weinberg, 2000)

However, the exact order in which those capabilities are acquired is not important, and differs from cancer to cancer.

Most tumours derive from one single aberrant cell. A genetic alteration leads to cancer formation, as carcinogenesis is linked to mutagenesis. Most of the carcinogenic agents, such as chemical carcinogens, ionizing radiation and viruses, are known to also cause genetic mutations.

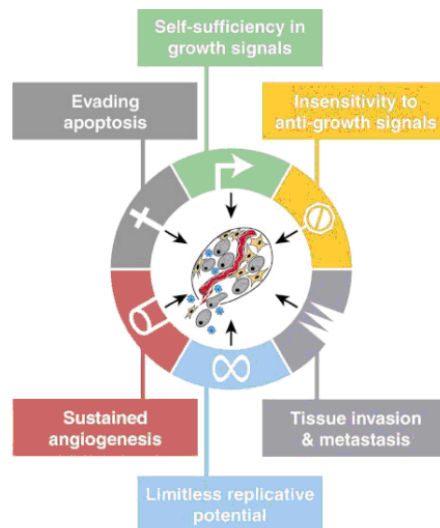


Figure 1.5: The Hallmarks of cancer, Hanahan and Weinberg, 2000

Carcinogenesis is a long term process that requires mostly more than one mutation, and undergoes several cycles of proliferation and mutation, and enhanced by clonal selection, the cancer cell evolves. (figure 1.6)

The genes that cause cancerogenesis are without exception normal genes whose products usually serve a distinct purpose in a healthy organism. Through mutations these genes are altered, leading to a gain or loss of function which destabilizes the balance of metabolic pathways and lead to aberrant characteristics of the cell.

The accumulated mutations occur in two main groups of genes, that have been named oncogenes and tumour-suppressor genes (figure 1.7).

Oncogenes are genes, in which a mutation leads to a gain of function that enhances proliferation of the cell, for example by over expression of growth factors or genes in general that are involved in growth stimulation, like RAS for example. This mutation is dominant, which means, a single mutation event is sufficient to alter the expression of the gene product. Tumour suppressor genes are genes, whose expression normally prevents abnormal proliferation, such as Rb or p53, they mostly cause cell cycle arrest, induce DNA repair or lead to apoptosis. tumour suppressor genes are recessive, which means that the mutation of one gene copy has barely an effect since there is still a second copy of the gene available. Only when both copies are mutated, an effect will take place.

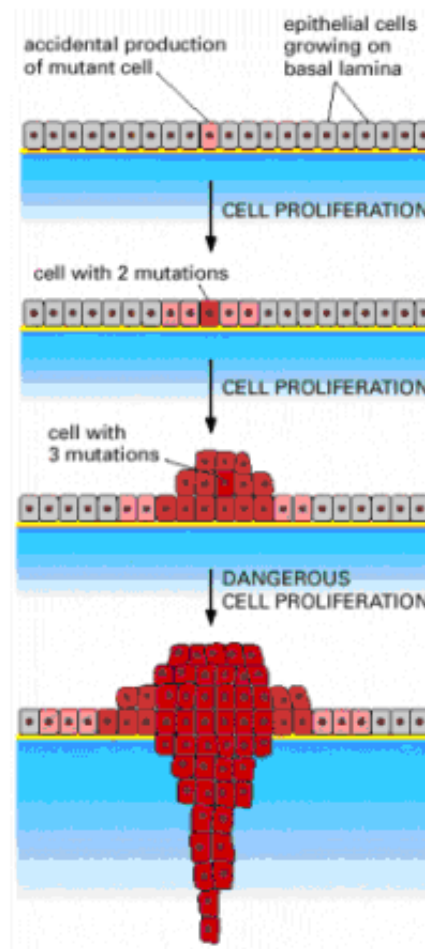


Figure 1.6: Clonal evolution (The Cell, Alberts 4th ed)

However, cancerogenesis is a multiple-stage event, which can be divided into three distinct phases, initiation, promotion and progression.

Initiation

At the beginning, a cell acquires a small growth advantage, mostly caused by a mutation event (DNA damage), which is a rare but irreversible event. Proliferating cells are more susceptible to DNA damage, which then is passed on to the daughter cell. Initiation has many reasons, it can occur spontaneously or due to replication mistakes or endogenous DNA lesions. The frequency for initiation is increased by DNA damaging agents, usually taking place in several tissues at the same time. It is believed that initiation will be successful only if it hits an early progenitor or stem cell, especially in tissues with a high turn over rate and regenerative potential, such as the skin or colon crypts. The terminal

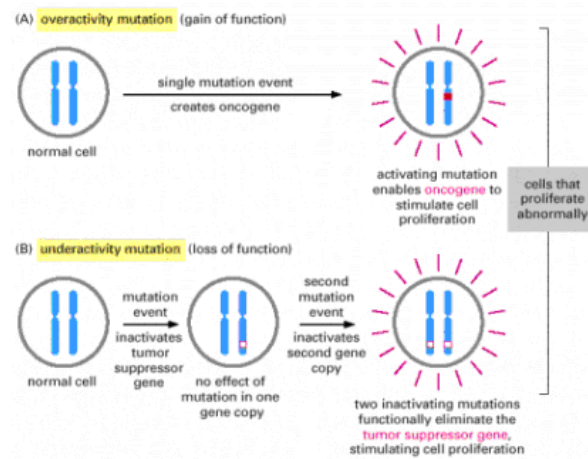


Figure 1.7: Cancer-critical genes fall into two readily distinguishable categories, dominant and recessive (The Cell, Alberts 4th ed)

differentiation will be affected, so the cell will remain in its state of proliferation rather than differentiate and as a consequence go apoptotic. The initiation event itself does not lead to cancer, the next step in cancerogenesis is tumour promotion.

Promotion

Promotion is a process in which tumour growth is accelerated and strengthened after initiation by selection of the cells with the highest growth potential. This process is often due to tissue specific characteristics, as many tumour promoting substances act on tissue specific mechanisms. Promotion takes long periods of time, from weeks to many years, depending on the species. These preneoplastic changes are reversible, and premalignant tumours can regress.

Progression

The transition from a benign to a malignant tumour is called tumour progression. During progression, both genotypic and phenotypic changes occur, leading also to genetic instability. Often care-taker genes, such as DNA damage repair genes or genes that regulate apoptosis and repair, like p53, are lost within this process. This allows further mutations which then give rise to more aggressive tumour cells. Different mutations can occur in this step and create tumour cells with different genotypes named tumour-heterogeneity. Main characteristics of tumour progression are angiogenesis, establishment of unlimited

expansion potential and metastasis (Gotzmann et al., 2004).

1.2 Colon Carcinogenesis

The colon consists of millions of colonic crypts, which have a very distinct compartmentalisation (figure 1.8). At the base of the crypt, adult stem cells can be found; on top of these cells are transient amplifying and committed progenitor cells, and in the upper half of the crypt are differentiated cells. Each crypt is built of about 2000 cells, which are maintained by a very small subset of adult stem cells at the base of the colonic crypt. The cells proliferate, migrate towards the lumen, and differentiate. Then the cells are shed into the medium and die. The epithelial cell turnover is very fast, within a week the differentiated cells of the colonic crypt are replaced.

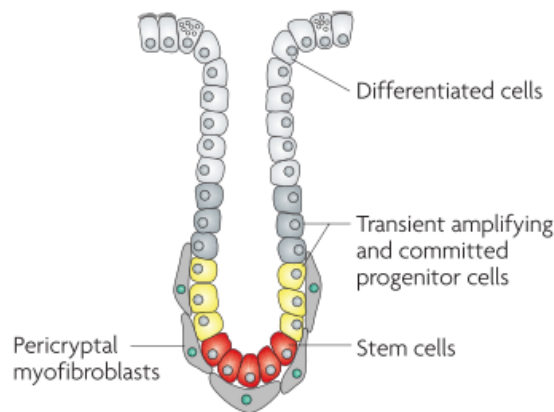


Figure 1.8: The colonic crypt (Humphries and Wrigh, 2008)

1.2.1 Genetic Alterations in Colon Cancer

Colon cancer is the result of a sequence of events, the adeno-carcinoma sequence, in which the morphogenesis of the cells is altered. The earliest lesion is an aberrant crypt focus (ACF) in the colonic epithelium. There are two types of ACF's, (1) the hypercellular type and (2) the dysplastic type which is more relevant for carcinogenesis. The aberrant crypt foci grow by crypt fission and establish micro adenomas.

ACFs grow and build up benign adenomatous polyps, which then are changed during tumour progression into malignant carcinoma. There are two models for the develop-

ment of adenomas. In the top-down morphogenesis model, suggested by Vogelstein and colleagues, mutant cells are found in the intra-cryptal zone, and later on spread down to form new crypts, and form monoclonal lesions (Shih et al., 2001). The bottom-up model suggests, that the basic crypt stem cells are the source for carcinogenesis (Preston et al., 2003). Histopathological evidence has been found for both theories (figure 1.9) and it is conceivable that both mechanisms exist (Wright 2000).

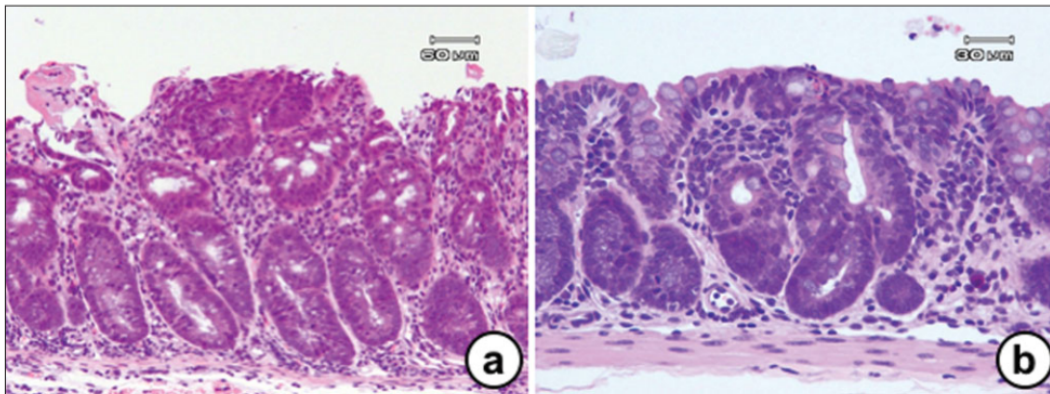


Figure 1.9: Histopathology findings for the top-down (a) and bottom-up (b) models. Atypical dysplastic cells can be found in the upper (a) and lower (b) parts of the crypts. (Tanaka, 2009)

The histological model of colon carcinogenesis is connected to genetic alterations. Aberrant gene expression had been linked to anatomical findings, such as an activation of Ki-Ras and several Loss of Heterozygosis events (LOH) that accumulated during tumour progression (Vogelstein et al., 1989).

It is suggested that a certain preferential sequence of events with about seven mutations is necessary for neoplastic growth (figure 1.10). This multi step process requires a long period of time, and it is well illustrated for colorectal cancer (Kinzler et al., 1996).

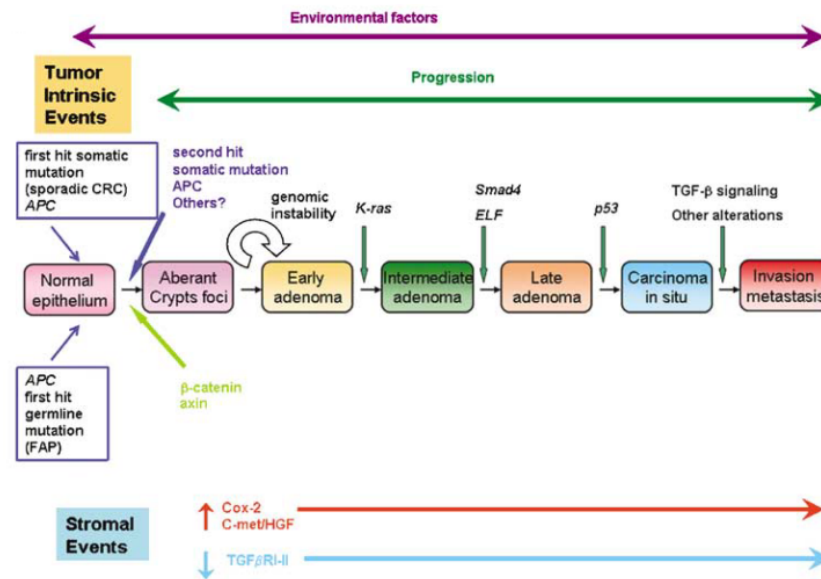


Figure 1.10: Genetic alterations in colon cancer (Mishra et al., 2005)

Well known mutated genes on colorectal cancer are adenomatous polyposis coli (APC), Ki-Ras, p53, deleted in colorectal cancer (DCC), DNA mismatch repair genes and genes of the TGF β -pathway (figure 1.11).

GENE	CLASS	PATHWAY AFFECTED	TUMORS WITH MUTATIONS (%)
<i>K-Ras</i>	oncogene	receptor tyrosine-kinase signaling	40
<i>-catenin</i>	oncogene	Wnt signaling	5–10
<i>p53</i>	tumor suppressor	stress/genetic-damage response	60
<i>APC</i>	tumor suppressor	Wnt signaling	> 60
<i>Smad4</i>	tumor suppressor	TGF β signaling	30
<i>TGF receptor II</i>	tumor suppressor	TGF β signaling	10
<i>MLH1</i> and other DNA mismatch repair genes	tumor suppressor	DNA mismatch repair	15 (often silenced by methylation)

Figure 1.11: Some genetic abnormalities detected in colorectal cancer cells (The Cell, Alberts 4th ed.)

1.3 Cancer Stem Cells (CSC)

Cancer stem cells are thought to be self renewing and to possess a high differentiation potential and mechanisms that protect them from damage and apoptosis, to be able to initiate tumour growth and contribute to tumour therapy resistance (Guo et al., 2006).

1.3.1 Origin of (Cancer) Stem Cells

According to the Unitarian hypothesis, all cell lineages in the intestinal epithelium derive from a single stem cell population (Cheng 1974; Schmidt et al., 1988). The cancer stem cell theory suggests that normal adult colonic stem cells give rise to the cancer stem cells if they undergo certain mutations (Huang and Wicha, 2009).

1.3.2 Stem Cell Attributes

Cancer stem cells and normal stem cells have some characteristics in common. They both exist in small numbers, are quiescent, but capable to proliferate, they have the capacity to self-renew, to regenerate tissue after injury, and to differentiate into all cell lineages found in the tissue/tumour (Potten and Loeffler, 1990; Bach et al., 2000; Guo et al., 2006; Vermeulen et al., 2008). Cancer stem cells and adult stem cells share similar epigenetic profiles (Bloushtain-Quimron et al., 2008), gene expression profiles (Shipitsin et al., 2007) and activated signalling pathways, like Notch, Wnt or Hedgehog (Klonisch et al., 2008).

1.3.3 Stem Cell Location and Number

The colonic crypt base contains the adult stem cells (Qiu et al., 1994), approximately 4-6 cells per crypt, the precise number being still a matter of discussion (Potten et al., 2003). However, at least in the mouse, it has been found that approximately 6 long lived stem cells can be found in each colonic crypt base (Barker and Clevers, 2007).

Actual stem cells divide more slowly compared to early progenitor cells higher up in the crypt, perhaps to grant error free DNA synthesis and more time for repair (Potten, 1986; Potten et al., 1997). These cells can be regarded as lineage ancestor cells. Upon asymmetrical cell division the stem cell gives rise to another stem cell which will remain in the stem cell niche, and another cell that has been described as transit-amplifying cell (TA-cell), or multipotent/committed progenitor cell in haematopoietic terminology (Miller et al., 2005). The TA-cells can still be seen as stem cells that can dedifferentiate

if necessary, but further cell division creates more TA-cells (Potten et al., 1990). The TA-cells give rise to about 20-30 clonogenic progenitor cells, that still could dedifferentiate if necessary (Potten et al., 1990), though these cells are usually destined to rapidly proliferate, until the proliferative potential is exhausted, and then to terminally differentiate (Renahan et al., 2002).

Cancer stem cells are thought to derive from all of these cell types (figure 1.12, Marotta and Polyak, 2009)

1.3.4 The Stem Cell Niche

The microenvironment of the stem cells, which consists both of cells and extracellular matrix, regulates all aspects of stem cell behaviour (Alison et al., 2002), and has been defined as the stem cell niche (Spradling et al., 2001). It creates the specific location in a tissue where stem cells can reside for an infinite period of time and produce progeny cells while self-renewing (Ohlstein et al., 2004).

1.3.5 Theories about Heterogeneity and tumour Growth

It has been observed in experiments with human acute myeloid leukemia stem cells (Bonnet et al., 1997) and other solid tumours, that only a small subset of cells within any tumour is capable of forming colonies in *in vitro* clonogenicity assays (Heppner 1984, Weisenthal et al., 1985). Cancer stem cells are proposed to be involved in tumour growth, but the details are still unclear (Marotta and Polyak, 2009). Because cancer cells are constantly evolving by developing new genetic and epigenetic changes, any of them can eventually become a cancer stem cell (Campbell et al., 2007). In addition, micro environment can also influence cancer stem cell status. This makes it likely that more than one population of cancer stem cells can be present in one tumour. Cancer stem cells are not necessarily derived from normal stem cells, the name reflecting more stem-cell-like characteristics than the origin. Adult stem cells, progenitor cells and more differentiated cells may become cancer stem cells (figure 1.12)(Marotta and Polyak, 2009).

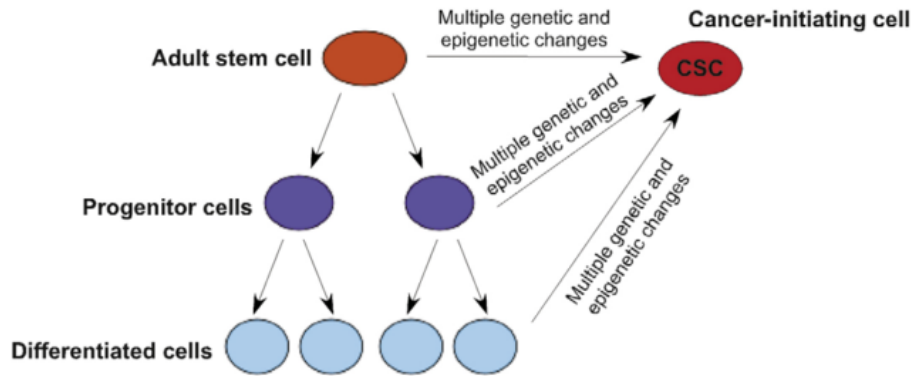


Figure 1.12: A current view of the cancer stem cell model: adult stem cells, progenitor cells, or differentiated cells may acquire the multiple genetic and epigenetic alterations required to become the cancer stem cell (CSC) involved in oncogenesis. (Marotta and Polyak, 2009)

1.3.6 Conventional Therapy vs. Targeting of CSC

Most existing therapeutic approaches have targeted the main tumour cell mass without distinction between cancer stem cells and normal cancer cells. These therapies usually fail to cure solid cancers, as cancer stem cells can survive and are then able to re-establish the original tumour. Therefore it has been assumed that most cancer cells do have a limited proliferation potential, and only a subset of cancer cells has the capacity to maintain and initiate new tumours (Reya et al., 2001). Cancer stem cells show a heightened resistance to chemotherapeutics, as they express high levels of anti-apoptotic proteins (Feuerhake et al., 2000; Peters et al., 1998) and ABC transporters like the multidrug resistance gene (MDR) (Terskhik et al., 2001; Zhou et al., 2001).

As cancer stem cells differ from their progeny in gene expression and growth regulation, the identification and isolation of cancer stem cell specific (marker) proteins is the first step towards a new therapeutic approach (Golub, 2001). This might include the inhibition of cancer stem cell specific signalling pathways, or differentiation therapy (Sell 2004) to approach CSC induced cancer by influencing the microenvironment to develop back into normal tissue.

1.3.7 Putative Surface Markers of the Cancer Stem Cell

Unlike in other cancers, there is no established colonic cancer stem cell marker yet. It has been proposed that the exact cell-of-origin, acquired genetic and epigenetic changes and the micro environmental influence combine to produce a cancer stem cell, and most likely also determine what kind of tumour will develop from it and which markers the cells will express on their surface (Marotta and Polyak, 2009). So far there exist several candidates to mark the colon cancer stem cell, like musashi and phosphor-Pten (He et al, 2007), Lgr5/Gpr49 (Barker et al, 2007), Bmi-1 (Sangiori and Capecchi, 2008), CD133 (O'Brien et al, 2007; Ricci-Vitiani et al, 2007), CD166 (Dalerba et al., 2007) and CD44 (Schulenburg et al, 2007; Dalerba et al., 2007).

CD44 belongs to a family of transmembrane glycoproteins that are generated by alternative splicing and glycosylation. CD44 has the ability to bind and interact with hyaluronic acid (Aruffo et al., 1990), which enables the regulation of different aspects of cell behavior (Naor et al., 1997) such as cell proliferation, cell motility, and cell survival for both malignant and nonmalignant cells (Goebeler et al., 1996; Naor et al., 1997; Morrison et al., 2001; Ahrens et al., 2001a,b). It is widely expressed in different tissues, and its expression level is increased in many different malignant tumours (Dietrich et al., 1997; Naor et al., 1997).

1.4 Molecular Regulation of Intestinal Stem Cell Function

1.4.1 The Wnt-Pathway

Signaling of the canonical Wnt-Pathway is important for cell proliferation, cell polarity and fate decision (Wodarz et al., 1998). The Wnt pathway belongs to a group of signalling cascades which are involved in stem cell homeostatic self-renewal and tissue regeneration. In tissues, where the Wnt-pathway controls stem cells, cancer develops, if the signalling becomes dysregulated, which suggests that hijacking of physiological regulators of the stem cell leads to cancer (Reya and Clevers, 2005).

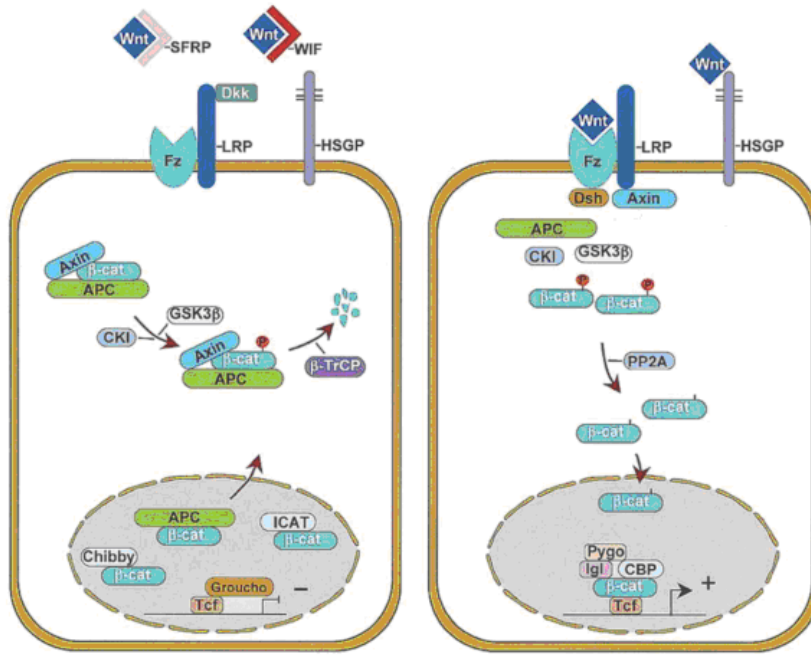


Figure 1.13: The Wnt canonical pathway. (Gregorieff and Clevers, 2005)

Wnt signalling is initiated when Wnt ligands bind to their cognate receptor complex, which consists of a serpentine receptor of the Frizzled family and a member of the LDL receptor family, Lrp5/6 (figure 1.13). The cytoplasmic protein β -catenin is usually regulated by a destruction complex, consisting of the scaffolding proteins adenomatous polyposis coli (APC) and axin, which bind β -catenin, and the kinases CKI and GSK3, which phosphorylated a set of conserved Ser and Thr residues in the amino terminus of β -catenin. This phosphorylation pattern recruits a β -TrCP-containing E3 ubiquitin ligase, which targets β -catenin for proteasomal degradation.

If Wnt is bound to the receptor complex, the kinase activity of the destruction complex is inhibited. The exact mechanism is not yet fully understood, but it involves direct interaction between axin and Lrp5/6, and actions by the axin binding protein Dishevelled. β -catenin is no longer degraded, accumulates and travels to the nucleus, where it binds to the amino terminus of DNA binding proteins of the Tcf/Lef family (Eastman et al., 1999). This interaction converts the Tcf/Lef factors from repressors to transcriptional activators and induces transcription of specific Tcf/Lef target genes (Giles et al., 2003). In the colon epithelium, Wnt signalling appears to be the most dominant force in controlling cell fate (Reya and Clevers, 2005).

Loss of APC is not only the main reason for familial adenomatous polyposis, a heredi-

tary cancer syndrome of the colon, but also occurs in most sporadic colorectal cancers (Kinzler and Vogelstein, 1996). This implies that the absence of functional APC transforms epithelial cells by constitute activation of the Wnt cascade, which has been shown by Korinek and colleagues with a reporter plasmid assay (Korinek et al., 1997). The candidate gene approach reveals several essential compounds of the Tcf4 target genes, such as c-myc (He et al., 1998) and cyclin D1 (Tetsu and McCormick, 1999). A constitutive active β -catenin-Tcf4 complex indeed drives a genetic programme in colorectal cancer cells which is also expressed in crypt stem and progenitor cells (Van de Wetering et al., 2002).

1.4.2 The MAP-Kinase Pathway

Mitogen activated protein kinases (MAPKs) are highly conserved serin-threonin kinases that build up a signalling cascade from the cell surface to the nucleus, which is important in regulating cell differentiation, cell proliferation and apoptosis (Fang and Richardson, 2005). The activation of the players of the MAPK cascade is usually transferred by phosphorylation. There are three major branches of MAP kinases, the extracellular regulated kinases (ERK), the c-jun N-terminal kinase (JNK) and p38 (figure 1.14). The MAPK pathways are located downstream of several growth factor receptors, such as the Fibroblast growth factor receptor family, Insulin and Insulin-like growth factor receptors, and several key-growth factor and proto-oncogene signals meet at the MAPK cascade (Fang and Richardson, 2005). The signals are transmitted through Ras or protein kinase C (PKC), activating Raf1 and a cascade of MEKs, until ERK1/2 is phosphorylated and active and travels to the nucleus, where transcription factors such as cFos, c-Myc and Elk1 are phosphorylated and activated (Khokhlatchev et al., 1998).

The signalling via the MAPK pathways can lead either to proliferation or to differentiation, depending on signal intensity and duration (Fang and Richardson, 2005).

In 36% of all colorectal cancers, Ras is mutated and leads to increased ERK activity. Mutations of B-RAF can be found in about 11% of colorectal cancers. Also increased JNK activity was found in many human tumours (Licato and Brenner, 1998). Most importantly, upregulation of growth factor receptors like FGF receptors lead to an increased ERK signalling and mitogenesis (Fang and Richardson, 2005).

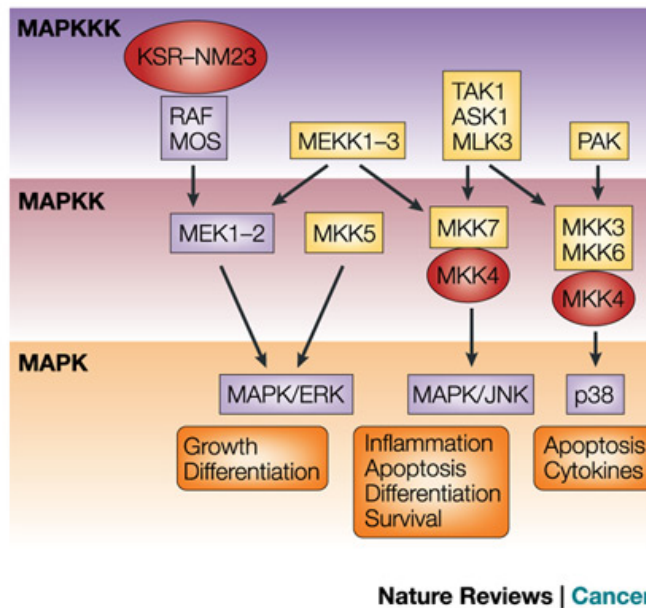


Figure 1.14: The three arms of the mitogen-activated protein kinase (MAPK) signalling pathway - ERK, JNK and p38 - are presented schematically (Steeg, 2003)

1.4.3 The Phosphoinositol 3-Kinase signalling Pathway

A different pathway which is activated upon growth factor stimuli is the phosphoinositol 3-kinase (PI3K) signalling pathway. PI3 kinases phosphorylate the 3'-hydroxyl-group of phosphatidylinositol and phosphoinositides, which leads to the activation of many different intracellular signalling cascades, which regulate diverse functions, such as cell metabolism, survival and polarity (Engelman et al., 2006). It has been found that PI3K is most likely a key component in insulin and growth factor responses that regulate cell growth and metabolism. Further, the PI3K signalling pathway is one of the most highly mutated systems in human cancers, which underlines its importance in human carcinogenesis (Engelman et al., 2006). There are three classes of PI3K, according to the substrate preference and sequence homology (Cantley, 2002), though we only focus on signalling by class I PI3 kinase as it is best understood.

PI3K generates phosphatidylinositol-3,4,5-trisphosphate (PIP3), which is a lipid second messenger within the cell membrane. Molecules with a pleckstrin homology domain can bind to PIP3 and are activated by this interaction (DiNitto et al., 2003). Akt/PKB, which is a serine/threonine kinase, is a principal target of PIP3 (Franke et al., 1997; Klippel et al., 1997), which is recruited to the membrane and further phosphorylated and activated by PDK1 (Mora et al., 2004) and mTOR-ricor kinase complex (Sarbasov et

al., 2005). The activated Akt/PKB activates and inhibits many key signalling molecules downstream, such as the mTOR-raptor kinase complex, which mediates phosphorylation of the eukaryotic translation initiation factor 4E-binding protein (4E-BP1) and p70S6-kinase (whose target is S6), leading to increased protein synthesis (Richardson et al., 2004). Akt also promotes glucose uptake by stimulating translocation of GLUT4 to the cell membrane (Thong, 2005) and inhibits glycogen synthase kinase 3 β (GSK3 β) which activates glycogen synthase (Cohen and Frame, 2001) and also influences the Wnt signalling. Akt has many more target molecules, which stimulate growth, translation, glucose metabolism, and which are anti-apoptotic (figure 1.15).

PI3K-signalling has been found to be involved in deregulated translation, which could

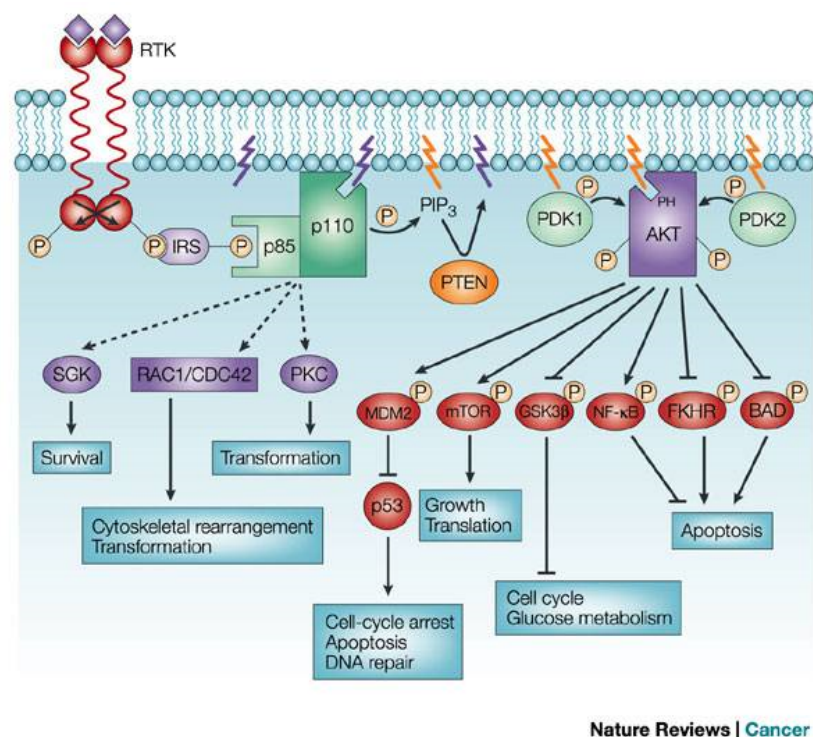


Figure 1.15: Akt signalling (Vivanco and Sawyers, 2002)

be caused by mutations of the PI3K-pathway (Parsons et al., 2005) and lead to tumour development in general (Seger and Krebs, 1995) and specifically progression of colon adenocarcinoma (Hommes, Peppelenbosch and van Deventer, 2003).

The PI3K pathway is an interesting target for cancer therapies, and with rapamycin a promising therapeutic has been introduced for clinical trials (Mita, Mita and Rowinsky, 2003).

1.4.4 Cross-talk between Several Signalling Pathways

All signalling pathways that have been mentioned so far are connected with each other as depicted in figure 1.16.

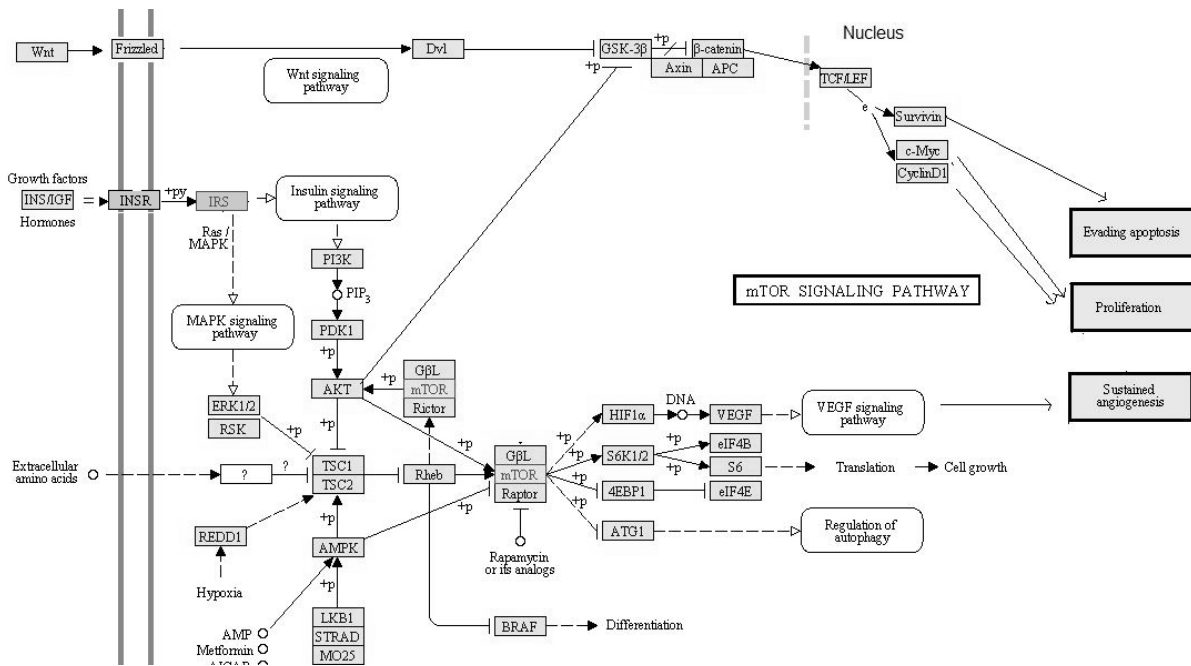


Figure 1.16: Interaction of several signalling pathways that have been investigated in CD44 positive and negative LT97 cells. (Adapted from “Insulin signalling Pathways”, “Pathways in cancer”, “mTOR signalling pathway” (©Kanehisa Laboratories), published in Kyoto Encyclopedia of Genes and Genomes (KEGG))

An IGF mediated stimulating signal leads to activation of both the PI3K pathway and the ERK/MAPK pathway via IRS phosphorylation. Akt, the downstream target of PI3 kinase, activates mTOR which activates p70S6 kinase and S6. Akt also inhibits GSK3 β , hence interfering with the Wnt pathway, leading to accumulated β -catenin in the nucleus and Tcf/Lef mediated gene transcription, which has proliferative and anti-apoptotic effects, for example mediated by survivin.

1.4.5 Fibroblast Growth Factors and Fibroblast Growth Factor Receptors

The first fibroblast growth factor (FGF) had been discovered as a mitogen for fibroblasts (Gospodarowicz, 1974). About 23 distinct FGFs have been identified so far (reviewed by L'Hôte and Knowles, 2005). FGFs are important for morphogenesis in embryonic development, as they regulate proliferation, differentiation, and cell migration. In the adult, FGFs play a role in controlling the nervous system, in tissue repair, wound healing and also in tumour angiogenesis (reviewed by Givol et al., 2003). FGFs bind and activate one of the four FGF receptors (FGFR1-4), which are high affinity receptor tyrosine kinases (Lee et al., 1989; Givol et al., 1992; Schlessinger et al., 1992). Additionally to the four known FGF receptors, a fifth FGFR (FGFR-5) has also been found (Kim et al., 2001). The FGF receptors consist of an extracellular ligand binding domain, a trans-membrane domain and a cytoplasmic domain with a catalytic protein tyrosine kinase core and regulatory sequences (Hunter, 2000; Schlessinger, 2000).

There is a variety of FGFR isoforms, produced by alternative splicing of FGFR transcripts. One region for alternative splicing lies in the third Ig-like domain (D3), which alters the ligand binding specificity (Bottaro et al., 1992; Yayon et al., 1992). Isoforms with alternative splicing in the D3 region have been found in FGFR1,-2 and -3, but not in FGFR4, and alternative splicing of the D3 region is also the main difference between FGFR3IIIb and FGFR3IIIc. FGFR3IIIb and IIIc are both activated by FGF1 and FGF9, though FGF9 is a much weaker ligand for IIIb than for IIIc and remains weaker than FGF1 (Hecht et al., 1995; Santos-Ocampo et al., 1996). Additionally, FGF 2,4,6 (Kanai et al., 1997), 8 (Santos-Ocampo et al., 1996) and FGF18 (Liu et al., 2002; Ohbayashi et al., 2002) are ligands for FGFR3IIIc. FGF18 $-/-$ mice have a similar phenotype as FGFR3 $-/-$ mice, though with more severe consequences due to the reduced signalling by FGFR 1 and 2 (Liu et al., 2002). An interesting autoinhibitory mechanism has been found in FGFR3IIIc as well as in FGFR1 and 2, in which ligand binding is regulated, which involves the first Ig domain and the following linker region (Olsen et al., 2004).

The function of FGFR3, FGFR4 and FGF18

FGFR3 is expressed in kidney, lung and brain (Chellaiah et al., 1994), cartilage (proliferating and hypertrophic chondrocytes), in the intestine (Patstone et al., 1993; Vidrich

et al., 2004), pancreas and testis (see gene expression atlas: <http://symatlas.gnf.org/SymAtlas>).

The FGFR3IIIc isoform is expressed in chondrocytes, while FGFR3IIIb is expressed in epithelial cells (reviewed by L'Hôte and Knowles, 2005). A switch from IIIb to IIIc can be induced by FGF1, probably correlating with a loss of epithelial phenotype (Scotet et al., 1998). Cytokines or growth factors such as EGF, TGF β or IL-2 up regulate IIIb expression in the intestinal epithelial cell line Caco2 (Kanai et al., 1997).

FGF receptors in general play a very important role at most stages of mouse development and organogenesis (Ornitz and Itoh, 2001). In mice in vivo, FGFR3 is expressed in undifferentiated crypt epithelial cells, which suggests a role for intestinal cell proliferation (Vidrich et al., 2004). The targeted disruption of FGFs and FGFRs in mice showed that disruption of FGFR3 led to bone dysplasia and bone overgrowth, associated with an increased number of proliferating chondrocytes during embryogenesis (Deng et al., 1996) while a disruption of FGFR4 did not have an obvious phenotype, but there was a growth retardation and lung defects in FGFR3 null background (Weinstein et al., 1998). FGF18 $-/-$ mice were also embryonic lethal at P1, they showed delayed ossification and increased chondrocyte proliferation, and decreased alveolar spaces in the lung (Liu et al., 2002; Ohbayashi et al., 2002; Usui et al., 2004).

FGF18 also has been shown to enhance cell migration in response to mechanical damage in human cardiovascular tissues (Antoine et al., 2006).

Signalling via FGF-Receptor

Fibroblast growth factors bind the tyrosine auto-phosphorylation sites on the activated FGF receptor. Directly linked docking proteins become tyrosine phosphorylated, and form a complex with additional complements of signalling proteins (reviewed by Eswarakumar et al., 2005). The cytoplasmic domain of FGFR also contains regulatory sequences; the juxtamembrane domain of FGF-receptors is longer than in other receptor tyrosine kinases (RTKs), it contains a binding site for phosphotyrosine binding domains (PTB) of the FRS2 family of lipid anchored multi-docking proteins FRS2 α and FRS2 β (Ong et al., 2000; Dhalluin et al., 2000). The recruitment of these proteins is followed by binding of multiple Grb/SOS complexes which activates the RAS/MAPK pathway (Kouhara et al., 1997) and PI3 kinase (reviewed by L'Hôte and Knowles, 2005).

Autophosphorylation on Tyr766 in the c-term of FGFR1 creates a binding site for phospholipase C γ (PLC γ) (Mohammadi et al., 1991), which leads to PLC γ activation, and as a consequence, generation of the second messengers diacylglycerol (DAG) and IP3.

Also the PI3K pathway can be activated over Grb2 interaction with Gab1 (reviewed by Eswarakumar et al., 2005).

FGF Signalling in Human Disease

Genetic alterations of FGF and FGFR genes play mostly a role in human skeletal dysplasias (review Webster et al., 1997; Wilkie, 1997), but they have also been found to play a role in several cancers. FGFRs associate with proteins involved in cell-cell interaction and may contribute by this mechanism to cancer metastasis (Cavallaro et al., 2004). It appears that cell-cell interaction can either attenuate FGFR signalling and repress cell transformation (Small et al., 2003), or maintain or even increase it, and therefore lead to transformation, e.g. by stimulating invasion (Suyama et al., 2002).

Although it has been shown that FGFR3 is a negative regulator of bone growth, it is unlikely that activation of FGFR3 in cancer leads to growth inhibition. Its oncogenic effects are mediated by Ras-MAPK and STAT signalling (reviewed by L'Hôte and Knowles, 2005). It has been found that alternative splicing of FGFR3 plays a role in multiple myeloma patients who are over expressing FGFR3 (Soverini et al., 2002), and it also plays a role in bladder cancer (Tomlinson, L'Hote, Kennedy, Pitt and Knowles, 2005), and in colon cancer (Sonvilla et al., 2007). In colorectal cancer, aberrant splicing and activation of cryptic splice sequences in FGFR3 have been found (Jang et al., 2000; Jang et al., 2001). FGF18 is upregulated in colon cancers as direct downstream target of the Wnt pathway (Shimokawa et al., 2003), and it has been suggested to exert autocrine and paracrine oncogenic functions in colorectal cancer (Sonvilla et al., 2008). FGFR3IIIc has been shown to mediate FGF18 effects in colon cancer cells (Sonvilla et al., submitted 2009).

1.4.6 The IGF-1/ IGF-1 Receptor Pathway

Both the insulin receptor (IR) and the insulin like receptor (IGF-1R) derive from an ancestral receptor involved in regulation of metabolism, organismal size and longevity. They share a relatively conserved structure, consisting of two half receptors, each having an extracellular α -subunit and a transmembrane β -subunit with tyrosin kinase activity (Ullrich et al., 1986; Adams et al., 2000). The IR is activated by insulin, which derives from the pancreas, while the IGF-1R is activated by IGF I and IGF II, which derives from the liver, but is also produced in stromal fibroblasts and tumour cells. IGF I and IGF II have about 50% homology to insulin, have a mass of about 7 kDa, and they

have multiple paracrine and autocrine functions (Steward and Rotwein,1996). IGF I is mainly produced by the liver and has an important role in growth and development.

IGF Signalling

When the ligand binds to the IGF-1R, autophosphorylation of the tyrosines in the kinase domain takes place, and also juxtamembrane tyrosines and c-term serines are phosphorylated, creating binding sites for docking proteins like IR substrate1-4 (IRS 1-4), Src homology and collagen domain protein (Shc). Recruitment of these proteins activates signalling by the PI3K-Akt and Ras/Raf/MAPK pathways (Baserga et al., 2003; Manning et al., 2007; Johnson et al., 2002).

Regulation of IGF Signalling

The signalling via the IGF-1R pathway is tightly regulated on many levels. The ligand availability is regulated, IGF II expression is subject to genomic imprinting, and IGF-2R competes for IGF II, and both IGF I and IGF II are bound by high affinity IGF binding proteins (IGFBP), which inhibit IGF bioactivity (Firth et al, 2002; Pollak et al., 2004). Inside the cell, the IGF-1R kinase activity is regulated (O'Connor, 2003), and IGF-1R effectors, like mTOR, p70S6 kinase, ERK or JNK establish a feedback suppression of the IRS-PI3K-Akt signalling (Manning et al., 2007; O'Connor, 2003; Wan et al., 2007).

The IGF Binding Proteins

So far six different IGF binding proteins (IGFBP 1-6) have been identified (Walker et al., 2004). Hepatic IGF I is bound to >99% to IGFBPs, mostly to IGFBP3 (75-90%). It builds a ternary complex consisting of IGFBP3, IGF and an acid-labile subunit (ALS) that stabilizes the complex and prolongs its half-life (Domene et al., 2005). The IGFBPs have an intrinsic biologic activity that are independent of IGF signalling. In addition to their IGF/IGF-1R dependent action to bind IGF and thereby reduce the IGF biologic activity (Martin et al., 1995), IGFBP3 has some distinct characteristics, like phosphorylation on serin residues, heparin binding motifs and a nuclear localization sequence (Walker et al., 2004).

IGF/IGF-1R Independent Pro-apoptotic Effects of IGFBP3

Additionally to the IGF/IGF-1R dependent effects, the IGF binding proteins serve as pro-apoptotic signalling molecules with a lot of different target molecules. IGFBP3

increases the ratio of pro-apoptotic (BAX, Bad) to anti-apoptotic (Bcl-2, Bcl-xL) in apoptotic breast cancer cells (Butt et al., 2000). Some studies suggest that IGFBP3 inactivates Bcl-2 through serine phosphorylation (Rajah et al., 2002). Furthermore, IGFBP3 has a growth inhibitory effect as it induces apoptosis by activation of caspase-8 and -7 (Kim et al., 2004).

It has also been shown that there is an interaction between IGFBP3 and the TGF- β signalling pathway, the IGFBP3 inhibitory signal requires active TGF- β signalling and the presence of smad 2 and 3 (Fanayan et al., 2000). IGFBP3 inhibits TNF- β induced NF κ B activity in human colonic carcinoma cells (Zadeh et al., 2006) and significantly enhances TNF-related apoptosis-inducing ligand (TRAIL)-induced cell death in colonic carcinoma cells by inhibition of NF κ B (Williams et al., 2007). Constitutive nuclear activation of NF κ B leads to a resistance to chemotherapeutic agents and radiotherapy (Nakshatri et al., 1997; Bo et al., 2005), and as IGFBP3 interferes with NF κ B signalling, it might have therapeutic potential for chemodrug- and radiotherapy-resistant cancer. IGFBP3 can translocate to the nucleus in human breast cancer cells, even if bound to IGF (Schedlich et al., 1998). It is a binding partner for the nuclear retinoid x receptor α RXR- α (Liu et al., 2000; Schedlich et al., 2004), a nuclear receptor, which, upon binding of a ligand such as a steroid hormone, serves as transcription factor with target genes that are important for embryonic development, growth, differentiation, apoptosis or homeostasis. RXRs form also heterodimers with the orphan receptor Nur 77 (Kastner et al., 1995; Mangelsdorf and Evans, 1995). This enhances its DNA binding capability and transcriptional regulation. Nur 77 is an important regulator of apoptosis (Hazel et al., 1988), as apoptosis is induced, it translocates from the nucleus to mitochondria to induce Cytochrom C release and apoptosis in several cancer cells, also in colonic cancer (Wilson et al., 2003). IGFBP3 modifies the RXR/Nur 77 complex from a DNA binding state to one that targets the mitochondrion and induces apoptosis (Lee et al., 2005).

Function of IGF-Signalling

IGF-1R signalling is important for prenatal and postnatal growth (Liu et al., 1993), in muscle, cartilage and bone. It leads to differentiation, and it is also important for maintenance of the myocardium and brain (Laustsen et al., 2007; Russo et al., 2005). IGF Signalling has shown to regulate the life span, as attenuation of IGF signalling relieves the inhibition of the FOXO family of transcription Factors. Interestingly, some FOXO targets are involved in suppression of tumourigenesis (Pinkston-Gosse et al.,

2007).

IGF-Signalling and Cancer

Many tumours show an altered expression of IGF-1R, its ligands and the IGFBPs. It has been shown that tumour growth was influenced by the circulating IGF levels of the host organism in different *in vivo* model organisms with mutations associated with low IGF1 levels or genetic manipulations to influence ligand levels (reviewed by Pollak et al., 2004). IGF-1R is required for cellular transformation by most oncogenes; an altered expression of IGF-1R follows usually a loss of function of a tumour suppressor, or a gain of function mutation in p53 (Sarfstien et al., 2006; Yuen et al., 2007). IGF-1R mediates a proliferation and survival signal, which enables anchorage independent growth. This allows the transformed cells to build macroscopic tumours, and survive the detachment that is required for metastasis (Baserga et al., 2003; Sell et al., 1994). Preclinical studies suggest, that IGF-1R overexpression leads to tumour formation and metastasis (Lopez and Hanahan, 2002; Jones et al., 2007).

IGF signalling as Target in Cancer Therapy

It has been found in many models that interruption of IGF signalling inhibits tumour growth, blocks metastasis, and enhances the efficiency of other cancer treatment (Baserga et al., 2003; Yuen et al., 2008; Samani et al., 2007). A big concern are the activating mutations downstream of the IGF-1R, which might negate a receptor blockade, but in colorectal cancer, the inhibition of IGF signalling still led to a block in growth and survival of tumour cells in which PI3K-Akt or ERK were activated (Belfiore et al., 2007; Yeh et al., 2006; Plymate et al., 2007). It has been shown that IGF-1R protects tumour cells from apoptosis induced by cytotoxic drugs, and it also influences DNA damage repair (Dunn et al., 1997; Trojanek et al., 2003). It has also been suggested that IGF-1R inhibition alters sensitivity to chemotherapy (Yuen et al., 2008; Samani et al., 2007).

1.5 Aims of the Thesis

Previous experiments by Schulenburg et al. have identified a CD44-positive subpopulation in LT97 human colorectal adenoma cells that display enhanced growth and survival capacity and express genes that are also observed in the lower crypt compartments that

also contain crypt stem cells (Schulenburg et al., 2008). Our working hypothesis is that this cell population contains the adenomatous tumour stem cells from which colon cancer stem cells and consequently colorectal carcinomas eventually arise. A whole genome micro-array analysis of gene expression has pointed towards 3 signalling pathways that might be responsible for the enhanced growth and survival capacity in this subpopulation. Specifically, the Wnt-pathway, the IGF1 pathway and FGF pathways were implicated. Based on these observations, the first objective of this thesis was to investigate the impact these 3 signalling pathways have on the control of growth and survival of colon cancer stem cells. To achieve this objective, the following aspects of tumour cell growth control had to be addressed: The expression of growth and survival related genes on the RNA (RT-PCR) and protein (western Blot) level had to be analyzed with a specific focus on those genes that were up-regulated in the micro-array - IGFBP, FGFRs -, and/or are Wnt target genes like survivin and FGF18.

The impact of pathway stimulation and inhibition on the attachment, growth and survival of CD44+ and CD44- cells had to be assessed. For this purpose, the Wnt-pathway was to be inhibited by analogues of the NSAID Sulindac, and the IGF pathway was to be stimulated by IGF1 and inhibited by PPP. The FGFR3 pathway was to be stimulated by FGF18 - either by addition of the recombinant factor to the culture medium or by over-expression of the FGF18 gene from an adenoviral vector. Inhibition of FGFR3 signalling was attempted using a dominant negative receptor construct expressed from an adenoviral vector. The pathway activity was to be investigated by western blot for phosphorylated signalling molecules. The second objective of this thesis was the examination of a colorectal carcinoma cell line and primary colon cancer cells for the presence of a subpopulation with similar longevity.

The expression of putative stem cell markers in LT97 cells had to be analyzed on the RNA (RT-PCR) and/or protein (FACS, immunocytochemistry) level. This had to be done for CD44 and further putative stem cell markers described by other groups for CSC - Lgr5, CD133, CD166 and musashi. Then the presence of these markers had to be assessed in carcinoma cells obtained from early passage cell lines or directly isolated from surgical specimen of colorectal tumours, as well as in tumour tissue by immunohistochemistry, followed by comparative investigation.

2 Materials and Methods

2.1 List of Materials

2.1.1 Cell Culture

FCS: fetal calf serum (Vitromex, D)

BSA: bovine serumalbumine (Sigma, USA)

HAM F-12: medium with 25mM Hepes, 1.8g/L D-Glucose, 0.15g/L L-Glutamine, essential amino acids, anorganic salts and vitamins (Sigma, USA) supplemented with 2 μ g/mL penicillin-streptomycin and 0.088g/L CaCl (Sigma, USA)

L-15 Medium: Medium with 0.9g/L D-Galactose, amino acids, anorganic salts and vitamins (Sigma, USA) and freshly added 0.3g/L L-Glutamin (Sigma, USA)

MEM: Minimal essential Medium with 1g/L D-Glucose, 0.3g/L L-Glutamine, 25mM HEPES, essential amino acids, anorganic salts and vitamins (Gibco Life Technologies, USA), supplemented with 20 μ g/mL Penicillin/Streptomycin

EGF: Epidermal Growth factor (Preprotec, UK)

Hydrocortisone: (Sigma, USA)

Insulin: (Sigma, USA)

Sodiumselenit: (Sigma, USA)

Transferrin: (Sigma, USA)

Triiodotyronin: (Sigma, USA)

Putrescine: (Sigma, USA)

FGF-2: PeproTech EC LTD

Amphotericin B: 0.25 mg/mL in water, PAA

Penicillin/Streptomycin: 10000 U/mL Penicillin, 10mg/mL Streptomycin, PAA

Gentamycin: 10mg/mL, Biochrom AG

PBS: Phosphate buffered saline: 0.2g/L KCl, 0.2g/L KH₂PO₄, 0.049g/L MgCl₂ (anhyd.), 8g/L NaCl, 1.15g/L Na₂HPO₄ (Sigma, USA)

PBS/EDTA: 10mM EDTA in PBS

Trypsin in PBS: stock solution: 20mM Trypsin, 2mM EDTA pH7.4 (Gibco Life Technologies, USA), dilution 1:10 in PBS for use.

Cell culture dishes: Petri dishes with a diameter of 6cm and 10cm, 24-well-plates (Falcon, USA)

LT97 Medium

HAM-F12 medium

20% L-15 medium

2%FCS

10 μ g/mL Insulin

2 μ g/mL Transferrin

2x10⁻¹⁰ M Triiodothyronine

1 μ g/mL Hydrocortisone (V10)

5x10⁻⁹ M Sodiumselenite

30ng/mL EGF

0.8mg Gentamycin

LT97 Minimal Medium

HAM-F12 medium

20% L-15 medium

2 μ g/mL Transferrin

2x10⁻¹⁰ M Triiodothyronine

1 μ g/mL Hydrocortisone (V10)

5x10⁻⁹ M Sodiumselenite

30ng/mL EGF

0.8mg Gentamycin

Primary Culture Medium

HAM-F12 medium

20% L-15 medium

2 μ g/mL Transferrin

1 μ g/mL Hydrocortisone (V10)

5x10⁻⁹ M Sodiumselenite

30ng/mL EGF

10 ng/mL FGF-2

9.6 microg/ml putrescine (Sigma)

25 U/ml of penicillin

25mg/mL Streptomycin

10 mg/mL Amphotericin B

ACL Medium (Brower et al., 1986)

ACL-4 medium

5% heat inactivated fetal bovine serum (AR5)

Serum Free Medium

500mL MEM

2mL Pen/Strep

MEM 10% FCS

500mL MEM

50mL FCS

2mL Pen/Strep

2.1.2 Cell Lines

LT97

This human colon microadenoma cell line was established by Richter et al., 2002 from small colorectal polyps, the cells lost both APC alleles, and have a ki-Ras mutation. They build 3D plateau forming colonies, and single cells are no longer viable, therefore they need to be passaged as cell aggregates with PBS/EDTA instead of Trypsin. They grow at standard cultivation conditions at 37°C, 5% CO₂ in an incubator. They are fed twice a week with HAM-F12 medium supplemented with 20% L-15 medium, 2%FCS, 10µg/mL insulin, 2µg/mL transferrin, 1µg/mL hypocortisone (V10), 5x10⁻⁹ M sodium selenit and 30ng/mL EGF. We observed a shift in this cell line, which altered characteristics such as doubling time, gene expression, including CD44 marker expression, attachment time and survival in dense cultures.

Therefore I will refer to the cultures before the shift as LT97-2, and the cell line which developed from this cell line as LT97-3, the suffix 2 and 3 referring to stages after passing. Additionally the early passage LT97-1 has been used as well.

LT97-1

This early passage LT97 strain has a doubling time of around 96 hours. The cells grow in 3D plateau forming colonies, and in the centre of these plateaus long, tail shaped structures grow out. The attachment time is 48-72 hours after splitting. A density of 90-100% is lethal for the cells, they detach from the culture dish and die. The growth potential is very low, therefore they can only be passaged 1:2.

LT97-2

The cells of the later passage, referred to as LT97-2, have a doubling time of 72-96 hours, the attachment usually takes 48 hours. They are passaged usually 1:2 or 1:3 at maximum. The 3D plateaus no longer show the tail like structures in their centre, instead the formation found on top of the plateaus resembles a dumpling. Density of 90-100% is lethal for these cells as well. The expression of CD44 surface marker is between 10 and 70%.

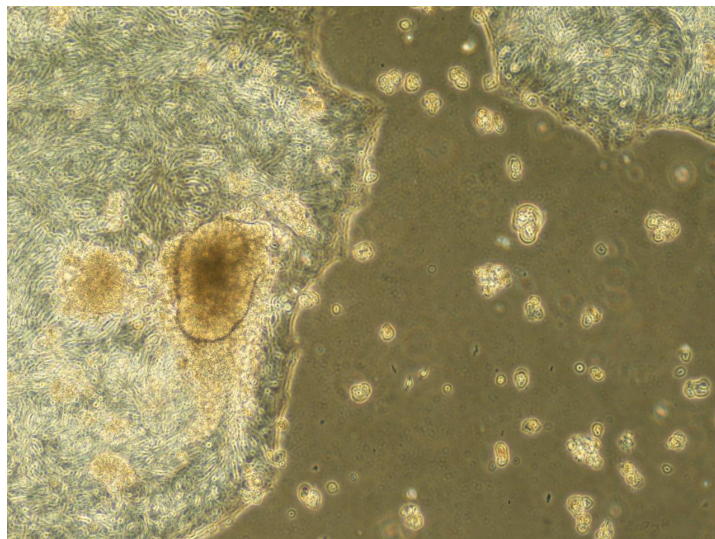


Figure 2.1: LT97-2 at 20x magnifications

LT97-3

The cell line, that developed from the later passage LT97-2 and adapted differing characteristics, has a doubling time of around 48-72 hours. Attachment takes 24 hours. A density of 100% does not seem to affect the cells viability, and they can be passaged up to 1:10. They are 90-100% CD44 positive and show an altered gene expression profile,

which will be described later on. Although they differ immensely from the LT97-2 cells, the optical appearance does not differ from these cells.

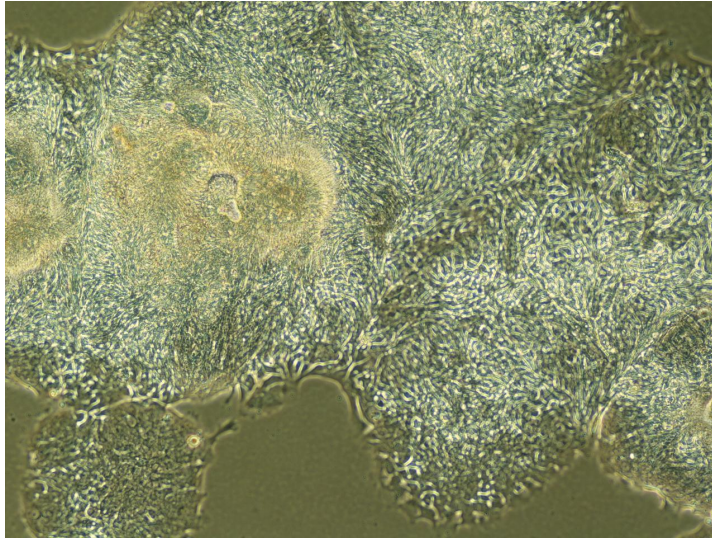


Figure 2.2: LT97-3 at 20x magnifications

AKH4

AKH4 cells were established at the institute for cancer research, MUW, by Grasl, Eisenbauer and Wrba.

The cells derived from a liver metastasis of a colon carcinoma from a Caucasian male patient. The cells contain a mutated p53 and a normal ki-Ras; they are well differentiated and they produce spheroblasts. Another characteristic of this cell line is the observation that at high density they differentiate into enterocytes and start to pump water underneath the cell monolayer to form domes. The AKH4 cells were fed with ACL + 1% Transferrin medium and split once a month 1:2 with Trypsin-EDTA.

AKH14

This cell line was obtained from the same tumour as the AKH4 cell line, though they differ in the cell line passing rhythm and are less differentiated. Neither domes nor spheroblast like formations are observed. They are passaged 1:2 each 14 days.

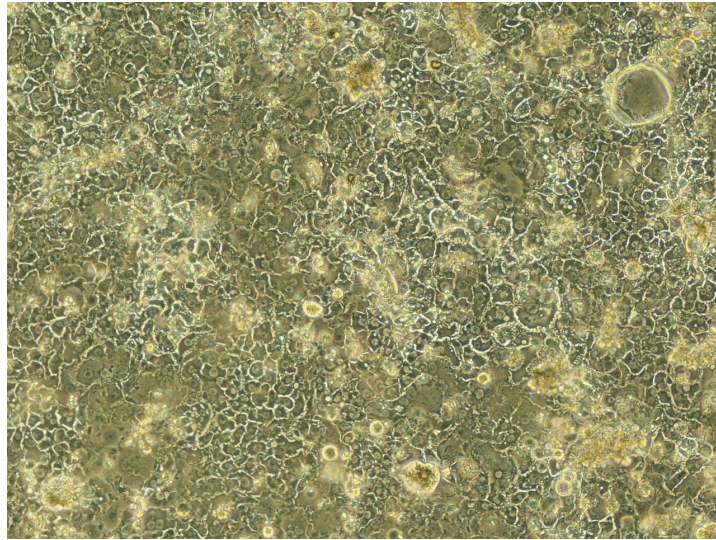


Figure 2.3: AKH4 at 20x magnifications

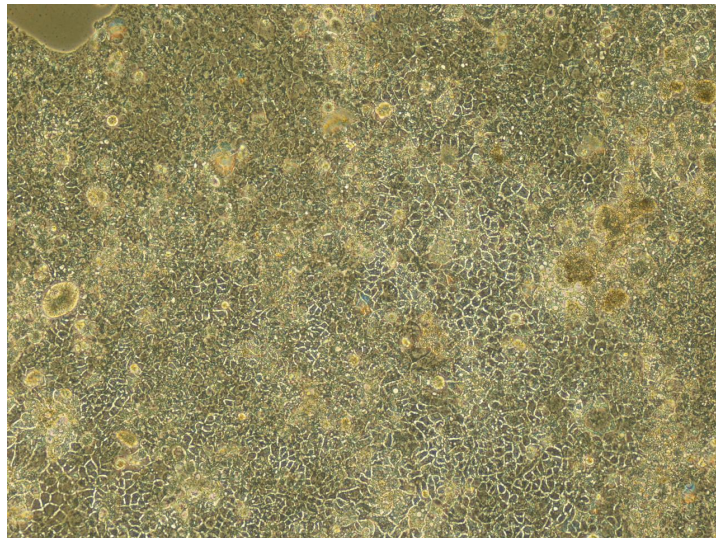


Figure 2.4: AKH14 at 20x magnifications

2.2 Production of Primary Cultures by Isolation from Adenoma Tissue

Materials

Dispase I: 0.5U/mL PBS, Roche

Collagenase Type 1: 50U/mL PBS, Worthington Biochemical Corporation

Desoxyribonuclease I: 1000 U/mL PBS, Sigma

N-Acetylcysteine: 2mg/mL serum free medium, pH 7.4, sterile filtrated, Sigma

Primary cultures were established from patient colon cancer samples to obtain cancer stem cells and support their survival prior to other cell subtypes. The epithelial cell fraction was isolated with scalpels. The cells were washed with 5mL MEM medium supplemented with 5 mM N-acetylcysteine and 10mg/mL Amphotericin B. After centrifugation 5 minutes at 1100 rpm, the pellet was resuspended with MEM medium supplemented with 5mM N-acetylcysteine, 1.5mg/mL collagenase I, dispase, 40 U/mL DNase , Gentamycin and Amphotericin B and incubated for approximately 90 minutes at 37°C in a water bath. The volumes of the substances and the incubation time were varied depending on the size and appearance of the tissue sample.

After incubation, the cell suspension was smeared through a cell strainer (120 μ m pore size) in order to obtain single cells and small aggregates of cells. Then the cells were washed 5 times with MEM, then centrifuged 5 minutes at 1100 rpm. The cell pellet was resuspended in primary culture medium especially designed to support survival of cells with stem cell like features (HAM-F12 medium supplemented with 20% L-15 medium, 2 μ g/mL transferrin, 1 μ g/mL hypocortisone (V10), 5x10⁻⁹ M sodium selenit , 30ng/mL EGF, 10 ng/mL FGF-2 , 9.6 μ g/mL putrescine (Sigma) 25 U/mL of penicillin, 25mg/mL Streptomycin and 10mg/mL Amphotericin B)

The primary cultures were fed twice a week. Non adherent cells were digested if necessary with 40U/mL DNase 15 minutes at 37°C in water bath to get rid of debris and adherent dead cells.

2.3 Neutral Red Uptake

The principle of this assay is to determine the amount of living cells. The living cells take up neutral red into the lysosomes. After incubation the dye is washed out and measured. The amount of washed out dye correlates with the number of living cells.

The cells were incubated with 0.05mg/mL neutral red (Merck, Darmstadt, G) in MEM medium 2 hours at 37°C and 5% CO₂ in the incubator. Then the cells were washed with PBS and incubated 5 minutes with a neutral red fixation solution consisting of 70% Ethanol and 1% glacial acetic acid to wash out the neutral red. The optical density was measured photometrically at 562nm (620nm as reference). The optical density correlates

with living cell density.

2.4 Crystal Violet Assay

In this assay, the ability of crystal violet to bind DNA is used to stain adherent cells and then to determine the cell number by either measuring the optical density photometrically or by determining the percentage of the ground of the Petri dish that is dyed.

The cells were washed with PBS, then incubated with 0.1% crystal violet in PBS 10 minutes, then washed with PBS again. The cells were dried and then either measured photometrically at 620nm, or alternatively by photographing and analysis with Lucia (Nikon).

2.5 Transient Infection with Adenovirus

Adenoviruses are large, icosahedral, non-enveloped viruses with a dsDNA genome who replicate in the nucleus of the host cell. Different Adenovirus constructs (table 1) were used to infect LT97 cells and to transiently alter the gene expression of these cells.

Table 2.1: Adenovirus constructs used for transient infection of LT97 cells

Virus construct	Function	Concentration (ifu/ μ L)
Dominant negative FGFR3-IIIc Virus KD3	Blockade of FGFR3 IIIc	$1 \cdot 10^8$
FGF18 Virus	Overexpression of FGF18	$8 \cdot 10^7$
Shuttle Virus	control	$5 \cdot 10^6$
GFP-virus	Expression of GFP, control	10^8
Cox-2 antisense	Antisense mRNA of Cox-2, control	$5.5 \cdot 10^6$

LT97 cells were infected with a MOI of 10. The medium was changed, then the virus was added and incubated 24 hours. The supernatant was removed and fresh medium

was added. Further analysis and experimentation were performed from now on.

2.6 FACS Analysis

2.6.1 The Principle of FACS Analysis

Fluorescent associated cell sorting (FACS) is a method to determine the percentage of cells carrying a specific surface marker molecule, and to separate those cells that have the marker from those who do not.

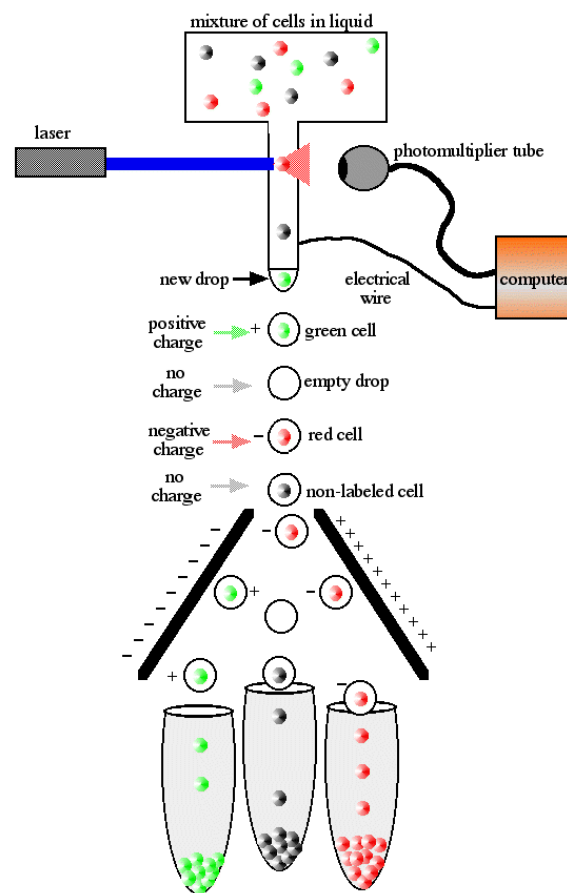


Figure 2.5: The principle of fluorescent associated cell sorting (FACS).(<http://www.bio.davidson.edu/COURSES/GENOMICS/method/FACS.html>)

The cells are first brought into a single cell solution. Then they are incubated with a specific antibody against the surface molecule of interest, which is also conjugated to a fluorescent dye, such as phycoerythrin (PE) or fluorescein isothiocyanate (FITC). Then the cells are analyzed and if necessary sorted by a Flow cytometer. Each single cell is exposed to a laser which excites the fluorochrome bound to the surface molecule on the cell. As a consequence, the cell emits light of a different, specific wavelength. The light scattering is measured by a photomultiplier tube, or light detector, so the number of cells carrying the specific fluorochrome can be counted. To sort the cells, an electric charge is applied to each drop containing a cell. By deflecting the drop according to its charge by charged electrodes, the cells can be collected in different sample tubes.

The Flow cytometer used for analysis was a FACScalibur (Becton and Dickinson, Sunnyvale, CA, USA) with a 15mW Argon Laser for 488nm with the software CELLQUEST (Becton and Dickinson) and MOD-FIT.

2.6.2 Cell Staining for Analysis

The soluble primary cell cultures were centrifuged, and the cell pellet was digested 5 minutes with Trypsin at 37°C. Then 5mL medium containing FCS was added, the cells were centrifuged 5 minutes at 1100rpm at RT. The cell pellet was resuspended in 3mL serum free medium containing 30μL DNase and incubated 15 minutes at 37°C. The cells were washed with serum free medium to get rid of the enzymes.

Then the cells were centrifuged 5 min at 1100 rpm at RT. The cell pellet was resuspended in 100μL PBS, and 30μL horse serum was added and incubated 15 minutes at RT (blocking). Then 10μg/μL antibody against the molecule of interest (table 2) were added and incubated 45 minutes at RT. The cells were washed in 1mL PBS, centrifuged 5 minutes at 1100 rpm at RT, and the cell pellet was resuspended in 1mL serum free medium.

2.6.3 Cell Staining for Sorting

A 10cm PD with cells was washed with PBS/EDTA. The cells were detached with trypsin 10 minutes at 37°C and 5% CO₂ (Incubator) and separated further by mechanical means (pipetting up and down 10-20 times). Then the cells were centrifuged 5 minutes at 1100 rpm at RT. The cell pellet was resuspended in 300μL PBS containing 90μL horse serum,

and incubated 15 minutes at RT (blocking). Then 30 μ L specific monoclonal antibody against CD44 directly coupled to PE (CD44-PE, Becton and Dickinson) were added and incubated 20 minutes at RT. The cells were washed in 1mL PBS, centrifuged 5 minutes at 1100 rpm at RT, and the cell pellet was resuspended in 1mL serum free medium.

Table 2.2: Antibodies used for FACS

Antibody target	Specificity	Label	Company	Concentration (μg/mL)
carcino embryonic antigen CEA	Mouse	FITC	Abcam	10
human CD44	Mouse	PE	BD Pharmin-gen	10
hALCAM (CD166)	Mouse	PE	R&D systems	10
CD133	Mouse	PE	Miltenyi	10
Mouse IgG1	Rat	PE	Becton Dickinson	10
Rabbit IgG (whole molecule)	Goat	FITC	Sigma Aldrich	10

2.7 Plating Efficiency / Survival Assay

3000 or 30000 cells per well were seeded in a collagen coated 24-well plate with serum and insulin free LT97 medium (LT minimal medium). Then different substances were added to the cells. After 24 hours (plating efficiency) or 7 days (survival) the cells were analyzed by Neutral red uptake assay or Crystal violet assay.

Table 2.3: Substances used in the Plating Efficiency and Viability Assay

Substance	Function	Concentration
PPP (cyclo lignan picropodophyllin, Calbiochem)	IGF-1 Receptor inhibitor	100 / 1000 μ M
Sulindac sulfid (BIOMOL Research Laboratories)	Wnt inhibitor	5/20 μ M
IGF I (Sigma)	IGF-1 receptor activator	10/50ng/mL
FGF18 (Strathmann Biotec)	FGFR3 activator	20ng/mL
DMSO (Dimethylsulfoxid)	Control for Sulindac(dissolved in DMSO)	Same volume as Sulindac 20 μ M

2.8 RNA Analysis

2.8.1 RNA-Isolation

Cells were harvested by addition of Trizol per well and 5 minutes incubation. The cells were scraped off with RNase free spatulas followed by homogenization of the cells by forcing them through a 0.6mm-in-diameter needle with a 2mL syringe. Then 1/5 Volume (of Trizol Volume) chloroform was added for RNA extraction, the lysate was centrifuged 10 minutes at 15.000 rpm and 4°C. The upper phase was transferred into a fresh eppendorf tube and $\frac{1}{2}$ volume (of Trizol Volume) Isopropanol was added. After 10 minutes incubation at RT the lysate was centrifuged again 10 minutes at 12.200 rpm and 4°C. The pelleted RNA was washed with 70% ethanol in DEPC-water, and 15 minutes centrifuged at 15.000 rpm and 4°C. The Pellet was dried and resolved in 30-50 μ L DEPC-water. The sample was heated 5 minutes at 65°C to denature the RNA, and the RNA was stored on -80°C.

2.8.2 RNA Quantification

Measuring of RNA concentration has been performed with a PEQLAB spectrometer and ND1000 software.

2.8.3 cDNA Synthesis

Random Hexamer Primer Mix

1 μ L 100 μ M Hexamer primer solution

1.5 μ L DEPC-water

Mastermix

4 μ L 5x first strand buffer (M-MLV-buffer, Fermentas)

2 μ L dNTP mix (10 μ L of each nucleotide in 60 μ L DEPC-water)

0.5 μ L RNase inhibitor (20 U)

Taq Polymerase

1 μ L Polymerase (RevertAid M-MuLV RT (Fermentas))

2 μ g RNA were diluted with DEPC-water to a total volume of 10 μ L and transferred into a PCR tube, then 1 μ L Hexamer Primer Master Mix diluted in 1.5 μ L DEPC-water was added. The sample was heated 5 minutes to 70°C, then the tubes were put on ice and 6.5 μ L Master Mix (4 μ L 5x first strand buffer (M-MLV-buffer, Fermentas), 2 μ L dNTP- Mix (10 μ L dGTP, dATP, dTTP and dCTP each +60 μ L DEPC-water) and 0.5 μ L RNase inhibitor) were added. The samples were heated 5 minutes to 25°C, then 1 μ L M-MuLV reverse transcriptase (RevertAid M-MuLV RT (Fermentas)) was added. The tubes were incubated 10 minutes at 25°C, 60 minutes at 42°C and then 10 minutes at 70°C. The tubes were put back on ice, 80 μ L DEPC-water was added. The cDNA was stored on -20°C, the final concentration of cDNA was approximately 2 μ g/100 μ L.

2.8.4 Polymerase Chain Reaction

The Principle of Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a method for qualitative and to a certain extent quantitative analysis of expression of a certain gene. To determine if the gene of interest is expressed, the RNA of a certain cell population is harvested and transcribed into cDNA, representing the expressed genes of this subpopulation in a stable, double stranded DNA form although without any introns or regulatory sequences. PCR permits amplification of specific genes by a heat stable DNA polymerase, usually Taq polymerase, which derives from the bacterium *Thermus aquaticus*.

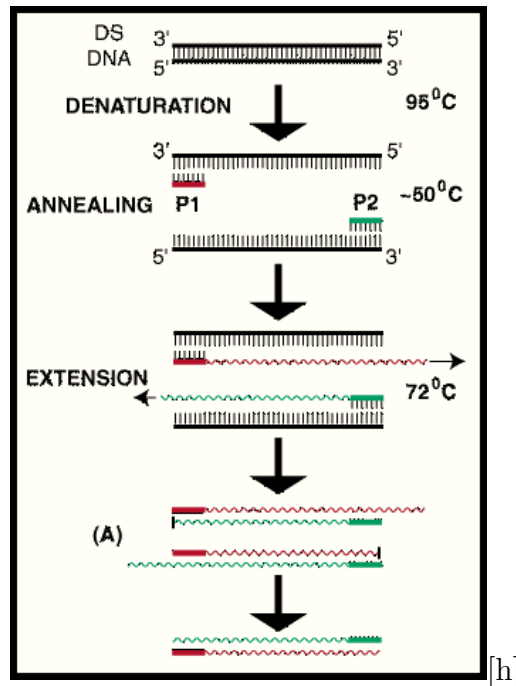


Figure 2.6: The principle of Polymerase Chain Reaction (PCR). Image taken from <http://www.flmnh.ufl.edu/cowries/PCR.gif>

As it is shown in figure 2.6, the double strands are melted at 95°C , a step that is called denaturation, granting access to the single strands. Now the temperature is lowered to a temperature which allows annealing of two complementary gene sequences to anneal to the single strand DNA. Those are called primers, and flank a sequence of defined length in the gene of interest. The exact annealing temperature varies, depending on length and GC percentage of the primers. Then, at a temperature of 72°C , the Taq polymerase binds to the primers and extends them in 5'-3' direction. This step is called extension. Upon repetition of this cycle, primers specific for the opposite strand anneal to the newly synthesized DNA fragment, only amplifying the defined fragment within the gene of interest. After 20-40 cycles, the amount of this fragment surpasses the amount of all other DNA molecules. The PCR product is now applied to a polyacrylamid gel, and the nucleotid fragments are separated by size via electrophoresis. Ethidium bromide is a fluorescent dye, excited by ultraviolet light, that intercalates between base stocks in the DNA and is used to dye the bands of DNA fragments within the gel. Upon comparison to a DNA marker with fragments of defined size it is possible to determine the size of the bands. The gene expression of the gene of interest is compared to the gene expression of a housekeeping gene (GAPDH, glycerinaldehyd-3-phosphat-dehydrogenase) as a control.

Table 2.4: The Primer Sequences used for Standard Polymerase Chain Reaction Gel electrophoresis

Gene	Primer	Annealing Temperature (°C)	Cycles	PCR Product Size(bp)
GAPDH	5'-GGC-AGT-GAT-GGC-ATG-GAC-TG-3' 5'-CGG-GAA-GCT-TGT-GAT-CAA-TGG-3'	56	23	356
GAPDH	5'-GGG-TGT-CGC-TGT-TGA-AGT-CAG-AGG-3' 5'-GGC-TCT-CCA-GAA-CAT-CAT-CCC-TGC-3'	68	23	369
BAX	5'-CAC-CCA-ACC-ACC-CTG-GTC-TTG-GAT-C-3' 5'-TGC-AGA-GGA-TGA-TTG-CCG-TCG-TGG-3'	68	35	4727
CD44	5'-TTT-GTT-AGA-AGC-CAT-CCA-TAG-CAC-ACC-C-3' 5'-AAC-CAT-TAC-AGG-GAG-CTG-GGA-CAC-TTA-A-3'	67	32	255
survivin	5'-GAA-ACA-CTG-GGC-CAA-GTC-TG-3' 5'-AGA-ACT-GGC-CCT-TCT-TGG-AG-3'	55.5	35	107
IGFBP3	5'-CGT-CAA-CGT-TAG-TGC-CGT-CAG-CCG-3' 5'-GAC-CAT-ATT-CTG-TCT-CCC-GCT-TGG-ACT-3'	67	32	293
FGF18	5'-ACT-TGC-CTG-TGT-TTA-CAC-TTC-C-3' 5'-CCA-GAA-CCT-TCT-CGA-TGA-AC-3'	53	35	379
FGFR4	5'-GAT-CCA-CAG-GCC-TTT-CAT-GGG-3' 5'-TGC-TGC-GGT-CCA-TGT-GGG-GTC-CTC-3'	56	43	525

Table 2.5: Ingredients of 6x Loading Dye

Substance	Amount
Urea	0.24 g
Saccharose	0.4 g
Bromphenol blue	0.001 g
Xylenol blue	0.001 g
1 x TAE	to 1 mL

Standard PCR

Components

1μL 3' primer

1μL 5' Primer

2μL cDNA

12.5μL 2xPCR Master Mix (Fermentas)

The polymerase chain reaction was performed with primer dilutions of 1:50 (nuclease free water), the primer mix contained 1μL of pre-diluted 3' primer and 1μL of pre-diluted 5'-primer, diluted with 4μL DEPC-water. For each sample 2μL cDNA were diluted in 4.5μL DEPC-water. Then 6μL cDNA sample, 6.5μL primer mix and 12.5μL 2xPCR Master mix were transferred into Eppendorf tube strips and spun down. The PCR was performed using an iCycler (Bio-Rad, Hercules, CA, USA).

Table 2.6: Gel Pipetting Scheme for a 6% Acryl Amid Gel

Substance	Volume/Gel
TAE 50%	150 μ L
Acrylamid (40%)	1.125 mL
A.bidest	6.175 mL
TEMED	5 μ L
APS (10%)	50 μ L

Table 2.7: Ingredients of the 50x TAE buffer

Substance	Amount
TRIS	242 g
Glacial acetic acid	57.1 mL
0.5M EDTA pH 8.0	100 mL
A. Bidest	to 1L

The PCR products were applied on a 6% Acrylamid gel for analysis. 10 μ L PCR product was mixed with 2 μ L 6x loading buffer. 1 μ L of a 100bp marker (Fermentas) was used for standardisation. The gel was run at 120V in 1x TAE buffer for 1h, then stained with ethidium bromide and the resulting fluorescence was measured at the Fluorolmager595.

Real time PCR

The Principle of Real time-PCR

The general principle of real time PCR is basically the same as for standard PCR, as cDNA is denaturated, annealed with primers and a defined fragment within the gene of interest is amplified. In addition, it is a method to exactly quantify the amount of PCR product in real time, simultaneously to the amplification. This is made possible by addition of a short nucleotide probe labelled with fluorescent dye, called reporter, as well as a quencher molecule. The quencher absorbs the fluorescence of the probe as reporter and primer are in close proximity. As the primers bind and are extended, the probe is hydrolyzed by the exonuclease activity of the Taq polymerase and the fluorescent dye is released (figure 2.7). Hence the fluorescent dye is able to emit fluorescence, correlating

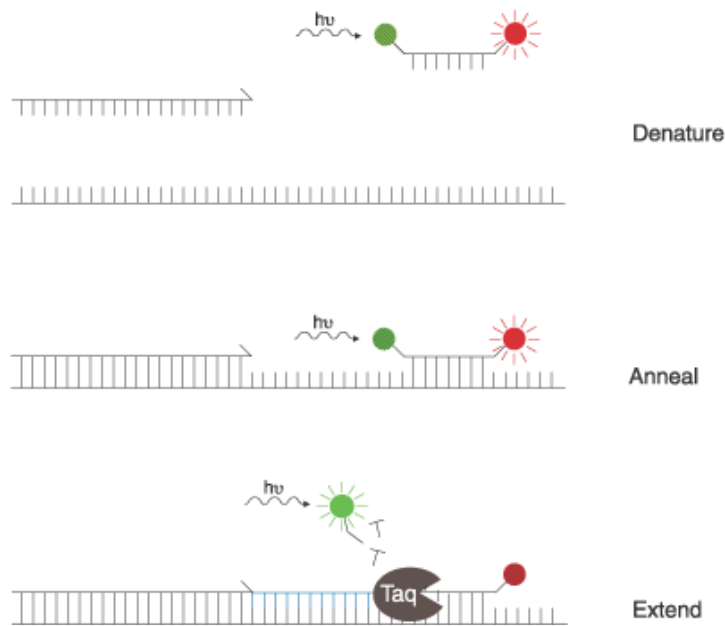


Figure 2.7: The Principle of Real Time Polymerase Chain Reaction. (<http://www.bio.davidson.edu/Courses/Molbio/MolStudents/spring2003/Pierce/threestep.gif>)

to the amount of PCR product.

As a consequence, the total amount of PCR product can be measured at every point of time during the process. Upon comparison with a housekeeping gene (here GAPDH, glycerinaldehyd-3-phosphad-dehydrogenase), the exact amount of the gene of interest expressed in the cell sub population can be determined.

Real time PCR with Taq man® kits cDNA mix:

1 μ L cDNA

8 μ L nuclease free water

Taq man® Mix:

1 μ L Taq man® probes (FAM labelled, AppliedBiosystems)

10 μ L Taq man® Master Mix

9 μ L cDNA mix and 11 μ L Taq man® mix were applied to a microtiter plate. The plate was centrifuged and then inserted into a ABI PRISM 7000 thermocycler. The conditions

were 50 cycles, 2-step real time PCR.

Real time PCR with Quiagen QuantiTect Primer Assay cDNA mix:

2 μ L cDNA

4.5 μ L nuclease free water

Primer mix:

12.5 μ L SYBR® Green RT-PCR Reagents

1 μ L Quiagen QuantiTect Primer

6.5 μ L cDNA mix and 13.5 μ L Primer mix were applied to a microtiter plate. The plate was centrifuged and then inserted into an ABI PRISM 7000 thermocycler. The conditions were 50 cycles, 2-step real time PCR.

Table 2.8: List of RT-PCR Primers

Manufacturer	Target Gene	Product Number
Taq man	CD44	Hs00153304.m1
Taq man	FGF18	Hs00818572.m1
Taq man	FGFR4	Hs00242558.m1
Taq man	GAPDH	Hs99999905.m1
Taq man	FGFR3IIc	Hs00997397.m1
Taq man	FGFR3IIb	Lot529030
Qiagen	BAX	Hs_BAX_1_SG
Qiagen	Survivin	Hs_BIRC5_2_SG
Qiagen	IGFBP3	Hs_IGFBP3_1_SG
Qiagen	Lgr5	Hs_Lgr5_1_SG
Qiagen	Bcl-2	Hs_Bcl2_1_SG
Qiagen	GAPDH	Hs_GAPDH_2_SG

2.9 Western Blot

2.9.1 The Principle of Western Blotting

The western blot is a standard method for detection of specific proteins. Protein lysates of a defined total protein concentration are first separated for the protein sizes via gel electrophoresis. The polyacrylamid gel contains sodiumdodecylsulfate (SDS), an ionic detergent which forces proteins into their monomeric form, and also denaturates the proteins secondary and non-disulfide-linked tertiary structures. SDS attaches to the proteins and adds a negative charge to the protein relative to the protein mass, which makes it possible to separate proteins via electrophoresis.

The proteins which are now separated for size in the gel are now transmitted onto a polyvinylidene fluoride membrane in the main blotting process, which uses electric current to pull the proteins from the gel onto the surface of the membrane, conserving the band pattern. Now the proteins are fixed on the membrane surface, and it is possible to detect specific proteins by antibodies. The membrane is incubated with primary antibodies specific for the protein of interest, followed by a secondary antibody with an enzymatic label, such as horse radish peroxidase. After addition of a chemiluminescent substrate for this enzyme onto the membrane, the substrate is oxidized and hence gives off chemiluminescence, which can be detected by a photosensitive film.

Production of Protein Lysates (RIPA-Lysate)

The cells were washed with cold PBS plus phosphatase inhibitors in case of phosphorylated proteins to prevent dephosphorylation. 200 μ L RIPA lysis-buffer was added to a 10cm PD and incubated 5 minutes in order to lyse the cells. Afterwards, the cells were scraped off and homogenised by forcing the lysate through a 0.6mm-in-diameter needle with a 2mL syringe. The sample was incubated 20 minutes on ice. The DNA was broken by three 1 second pulses of ultrasound. Finally the sample was centrifuged 5 minutes, 15.000 rpm at 4°C. The supernatant, which contains the proteins, was stored at -20°C.

Table 2.9: Ingredients of the RIPA Buffer

Final Concentration	Amount	Stock Solutions
50mM Tris/HCl pH7.4	1.5 mL	1M Tris/HCl pH 7.5
500mM NaCl	7.5 mL	2M NaCl
1% NP40	300 μ L	NP-40
0.5% Na-DOC	150 mg	Na-DOC
0.1% SDS	300 μ L	10% SDS
0.05% NaN ₃	1.5 mL	1% NaN ₃ -Lsg
A bidest	18.9 mL	

Table 2.10: Ingredients of the RIPA solution

Amount	Stock Solutions
10 μ L/mL	1M NaF (Phosphatase Inhibitor)
1 μ L/mL	Na ₃ VO ₄ (Phosphatase Inhibitor)
20 μ L	Complete (Protease Inhibitor)
969 μ L	RIPA buffer

Determination of the Protein Concentration

A standard curve with bovine serumalbumine (BSA) was prepared in a 96-well-plate after the following pipetting scheme:

Table 2.11: Pipetting Scheme for the BSA Standard

BSA concentration (μ g/ μ l)	0	1	2	3	4	5	6	7	8	9
μ L Abid	9	8	7	6	5	4	3	2	1	0
μ L Lysis-Buffer	1	1	1	1	1	1	1	1	1	1
μ L BSA (1 μ g/ μ L)	0	1	2	3	4	5	6	7	8	9
Total volume (μ L)	10	10	10	10	10	10	10	10	10	10

For the samples, each 1 μ L protein lysate and 9 μ L A. bidest were pipetted into separate wells.

Then 150 μ L of a fresh 1:5 dilution of Bio-Rad Coomassie's Protein Assay Dye Reagent

Concentrate in A. bidest were added to each well. Absorbance was measured at 590nm with an Immunoreader (no reference filter).

Acryl Amid Gel Electrophoresis

Materials

Table 2.12: Ingredients of the 2x Sample Buffer

Substance	Concentration	Amount
SDS	4%	0.2 g
Glycerol	20%	1 g
2-Mercaptoethanol	10%	0.5 mL
0.125M Tris/HCl pH 6.8	0.125M pH 6.8	0.4 mL
Bromphenolblau		

Table 2.13: Ingredients of the Electrophoresis Buffer

Substance	Concentration	Amount
Glycin	1.44%	7.2 g
Tris	0.3%	1.5 g
SDS	0.1%	0.5 g
A. bidest		500mL

Table 2.14: Pipetting Scheme for the SDS Separating Gel

Substance	7%	12%
40% Acrylamid	0.875 mL	1.5 mL
1.5M Tris pH 8.8	1.25 mL	1.75
A. bidest	2.8 mL	2.15 mL
10% SDS	0.05 mL	50 μ L
10% APS	0.025 mL	25 μ L
TEMED	0.0025 mL	2.5 μ L

Table 2.15: Pipetting Scheme for the SDS Collecting Gel

Substance	Volume
40% Acrylamid	0.25 mL
1.0M Tris pH 6.8	0.313 mL
A. bidest	1.9 mL
10% SDS	25 μ L
10% APS	12.5 μ L
TEMED	2.5 μ L

The separating gel was overlaid with 70% ethanol, and after 1h polymerisation the collecting gel was poured. Polymerisation of the collecting gel took 2h.

20 μ g of protein in 2x sample buffer were applied per lane. Also 5 μ L of a protein marker (PageRulerTM Prestained Protein Ladder, Fermentas, Burlington, Canada) was loaded onto the gel. Electrophoresis was performed 90 minutes at 125V.

Blotting

Materials

Table 2.16: Ingredients of the 10x Blotting Buffer

Substance	Concentration	Amount
Glycin	14.4%	72 g
Tris	3%	15 g
SDS	1%	5 g
A. bidest		To 500mL

Table 2.17: Ingredients of the 1x Blotting Buffer

Substance	Concentration
10x blotting buffer	10%
Methanol	20%
A. bidest	70%

For the transfer blot, a PVDF membrane was activated in methanol and placed on top of the gel. The transfer of the proteins to the membrane is performed by electric current, the negative charge is on the side of the gel, whereas the positive charge is on the side of the membrane, so that the negatively charged proteins are pulled onto the membrane. A Mini Protean 3 was used for this, the transfer was performed over night at 4°C at 25V.

After blotting, the membrane was washed with A. bidest, after this the membrane was swayed in methanol, dried and stored at 4°C.

Ponceau S Staining

Ponceau S stains proteins, so it is used to verify whether the transfer has been successful. The membrane was incubated with Ponceau S and then washed with A. bidest until bands became visible, photographed for documentation and destained via washing in A. bidest.

Immunological Detection of Proteins

Materials

Table 2.18: Ingredients of the Washing Buffer

Substance	Concentration	Amount
PBS	10x	100mL
Tween 20	0.05%	0.5 mL
A.Bidest		To 1L

Table 2.19: Ingredients of the first Antibody Solution

Substance	Concentration	Amount
Wash buffer		10mL
Milk Powder	1%	0.1g
0.1% NaN ₃	0.02%	200μL
Antibody	See list below	

Table 2.20: Primary Antibodies used for Immunostaining of the Western Blot

Antibody target	Specificity	Dilution	Company	Size of Detected Bands (kDa)
Bax	Rabbit polyclonal	1:5000	SC-493	28
p-IRS-1/2 (Tyr 612)-R	Rabbit	1:100	SC-17195-R	165-185
panIRS	Rabbit	1:1000	C.S.2382	180
FGFR3	Rabbit polyclonal	1:1000	SC-123	135
FGFR4	rabbit polyclonal	1:500	SC-124	125
Survivin	rabbit polyclonal	1:5000	Novus	16.5
β -actin	mouse	1:5000	Sigma	42

Table 2.21: Secondary Antibodies used for Immunostaining of the Western Blot

Antibody target	Specificity	Dilution	Company	label
Rabbit IgG	Goat	1:1000	PIERCE Nr. 1858415	HRP
Mouse IgG	Goat	1:10000	PIERCE Nr. 1858413	HRP

For the immunologic detection of proteins, the blot was washed in wash buffer and incubated with the first antibody over night. Then the blot was washed 30 minutes in wash buffer, incubated 60 minutes in the secondary antibody solution (the secondary antibodies are conjugated to HRP), washed again 30 minutes in wash buffer, incubated 5 minutes with the detection reagent (Super Signal West Dura Extended Duration) and detected with x-ray film for different periods of time in the dark room. As a loading control the blots were immunostained afterwards with antibodies against the housekeeping gene β -actin.

2.10 Immunohistochemistry and Immunocytochemistry

2.10.1 The Principle of Immunohistochemistry and Immunocytochemistry

Immunohistochemistry and Immunocytochemistry are methods to detect specific molecules in fixed cells or tissue slides. These Methods share the same principle.

Tissue slices are sometimes fixed with formaldehyde and embedded in paraffin, which has to be removed to make the proteins within the tissue accessible for the antibody. This is usually achieved by washing with xylol followed by a series of alcohol dilutions of a decreasing percentage.

The following steps are common for both immunohistochemistry and immunocytochemistry. Specimens are cooked with citrate buffer to demask the antigens. Further, if the detecting reagent is horseradish peroxidase, the endogenous peroxidases have to be blocked by incubation with H_2O_2 to prevent interference; unspecific protein binding sites have to be blocked by incubation with normal horse serum. Now as it is shown in figure 2.8 an antibody specific for the molecule of interest is added and binds it. A secondary antibody against the first antibody conjugated to biotin is added and binds the first antibody. Finally streptavidin coupled to horseradish peroxidase is added and binds biotin. Several HRP molecules are bound to one streptavidin, and several streptavidin molecules are bound to one biotin molecule, which is an additional amplification of the signal. Now the substrate for the HRP, usually 3,3'-Diaminobenzidine (DAB) is added and oxidize to produce brown precipitate, which can be detected via light microscope.

2.10.2 Immunohistochemistry

The tissue sections were dried 30 minutes at 55°C and incubated 2x5 minutes in Xylol followed by 2x5 minutes in 96% ethanol, and short immersion in 80%, 70% and 60% ethanol. Then the tissue slices were incubated 15 minutes in 3% H_2O_2 in methanol to block the endogenous peroxidases. The slides were washed 20x in tap water and cooked at 900 W 5x5 minutes (CD44: 3x5 minutes) in citrate buffer in a microwave oven. Then 2% horse serum in 1% Triton X supplemented PBS was added and incubated for 20 minutes for blocking, and after draining the blocking solution the primary antibody diluted in 2% horse serum in 1% Triton X supplemented PBS was added and incubated 1h. The slices were washed 2x10 minutes in 1% Triton X supplemented PBS. Then the

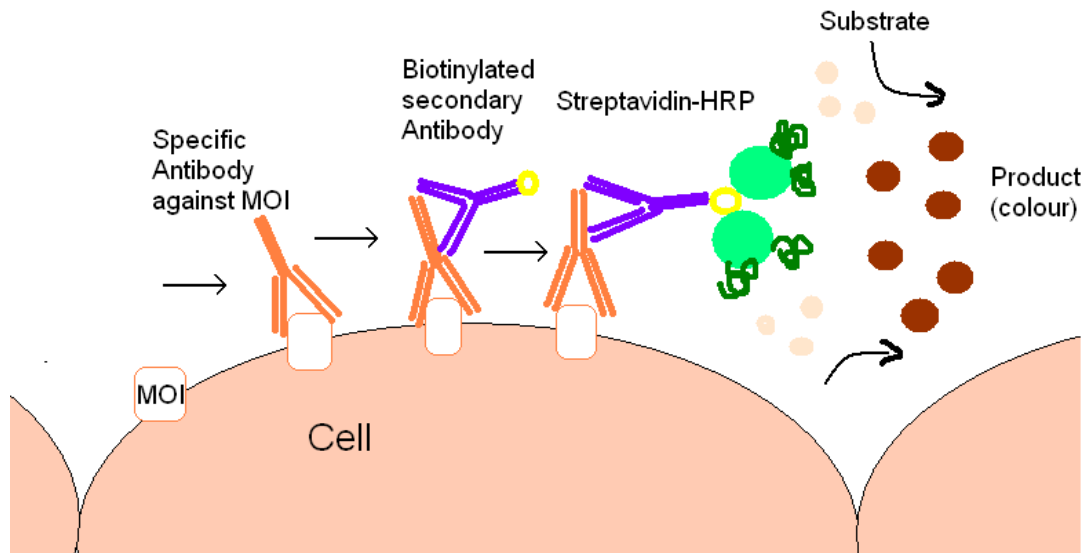


Figure 2.8: The Mechanism of Immunohistochemistry

secondary antibody anti-mouse/anti-rabbit 1:200 dilution in 1% Triton X in PBS (Vectastain(R) Elite ABC KIT PK-6200) was added and incubated 30 minutes, followed by 2x10 minutes washing with 1% Triton X in PBS. The streptavidin-HRP solution (20 μ L A, 20 μ L B from the Vectastain(R) Elite ABC KIT PK-6200 in 1mL PBS) was added and incubated 30 minutes. The slices were washed 2x10 minutes in 1% Triton X in PBS, then the chromogen (DakoCytomation Liquid DAB Substrate Chromogen system, Code K3466) was added and incubated 2-60 minutes until a specific staining was detectable under the light microscope. The slides were washed 20x with water. Then the slides were incubated 50 seconds in Hematoxylin and washed 20x with water, and then covered with a drop of Mowiol and a cover slip.

2.10.3 Immunocytochemistry

Cells were grown on sonic seal slides. The medium was sucked away and the cells were washed with PBS. Then the cells were incubated 30 minutes with Methanol:Aceton 1:1 at -20°C for permeabilisation and fixation of the cells. The cells were dried for about 30 minutes at RT, then rehydrated 10 minutes with PBS. Then the cells were incubated 30 minutes in 3% H_2O_2 in methanol to block the endogenous peroxidases. The cells were washed 2x10 minutes in PBS and cooked 30 minutes at 60°C in citrate buffer. Then 10% horse serum in 1% Triton X supplemented PBS was added and incubated 15 minutes

Table 2.22: Antibodies used for Immunohistochemistry and Immunocytochemistry

Antibody target	Specificity	Company	Concentration ($\mu\text{g/mL}$)
Lgr5/Gpr49	Rabbit	Abcam	20
Anti-Human CD44	Mouse	Dako	15
musashi 1/Msi1(EP1302)	Mouse	Abcam	10

for blocking, and after draining the blocking solution the primary antibody diluted in 2% horse serum in 1% Triton X supplemented PBS was added and incubated over night at 4°C. The cells were washed 10 minutes in 1% Triton X supplemented PBS. Then the secondary antibody anti-mouse/anti-rabbit 1:200 dilution in 1% Triton X in PBS (Vectastain(R) Elite ABC KIT PK-6200) was added and incubated 30 minutes, followed by 2x10 minutes washing with 1% Triton X in PBS. The streptavidin-HRP solution (20 μL A, 20 μL B from the Vectastain(R) Elite ABC KIT PK-6200 in 1mL PBS) was added and incubated 30 minutes. The cells were washed 2x10 minutes in 1% Triton X in PBS, then the chromogen (DakoCytomation Liquid DAB Substrate Chromogen system, Code K3466) was added and incubated 2-60 minutes until a specific staining was detectable under the light microscope. The cells were washed 20x with water. Then the slices were incubated 50 seconds in Hematoxilin and washed 20x with water, and then covered with a drop of Geltol and a cover slip.

2.11 ELISA

2.11.1 The Principle of ELISA

Enzyme-linked immunosorbent assay (ELISA) is a method to detect and quantify a specific protein in a sample. An antibody specific for the protein of interest is affixed onto a surface in a well of a polystyrene microtiter plate . The sample is incubated in the well so the protein can bind to the antibody. After washing away the non bound proteins, a secondary antibody conjugated to an enzyme is added and incubated, which binds to the protein of interest on top. This is the reason why it is also called a "sandwich ELISA". Then a substrate for the enzyme is added, which forms a detectable signal after processing. The signal is measured and compared to the signal of a protein standard.

ELISA

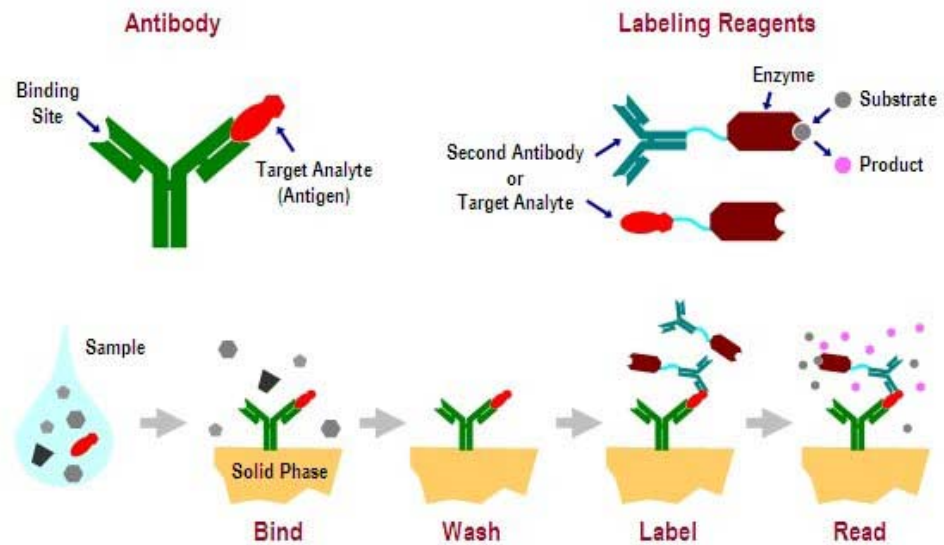


Figure 2.9: The principle of Sandwich ELISA. (<http://64.202.120.86/upload/image/articles/2006/biopen/biopen-elisa-schematic.jpg>)

2.11.2 ELISA

ELISA was performed using the human Bcl-2 ELISA kit (BenderMed Systems).

Wash Buffer

25mL wash buffer concentrate

475mL A. bidest

Assay Buffer:

2.5mL Assay buffer concentrate

47.5mL A. bidest

Biotin-conjugate:

30 μ L biotin-conjugate concentrate

2.97mL Assay-buffer

Streptavidin-HRP:

60 μ L streptavidin - HRP concentrate

5.94mL Assay buffer

BCL-2 standard:

Stock= 64ng/mL

Dilutions of 32,16,8,4,2,1 and 0.5 ng/mL in each 225 μ L sample diluent were prepared.

The microstrips were washed with wash buffer, then each 100 μ L of the standard dilutions and blanks (sample diluent) were applied to the wells. For the samples, each 80 μ L sample diluent and 20 μ L sample were added to a well. Then 50 μ L of the biotin conjugate were added to each well, the wells were covered with a film and incubated 2h at RT on a shaker (100rpm). The wells were 3x washed with wash buffer, then 100 μ L streptavidine-HRP were added to each well, the wells were covered with a film and incubated 1h at RT on a shaker (100rpm). The wells were washed 3x with wash buffer and incubated 10 minutes at RT. The substrate product has a blue colour. Then 100 μ L of the stop solution were added to each well, causing a colour change to yellow. Then the wells were measured at 450nm, 620 nm as reference. The result was calculated by linear regression from the standard.

2.12 Statistical Analysis

Standard deviation, two-way ANOVA and t-test were calculated with GraphPad Prism 4 and GraphPad Prism 5. Linear regression was calculated with Microsoft Excel 2003.

3 Results

3.1 Investigating LT97 as Model Cell Line for Premalignant Colon Cancer Stem Cells

In order to determine characteristics of the proposed tumour stem cells in colorectal adenoma, the model cell line LT97 has been investigated. This colon adenoma cell line contains a subpopulation that is positive for the proposed tumour stem cell marker CD44. It is suggested that the CD44 positive subpopulation might serve as a model for tumour stem cells, as its growth potential and plating efficiency is significantly higher as the CD44 negative subpopulation.

This leads to the question, which pathways might be involved to grant the CD44 positive cells its remarkable survival qualities in comparison to the CD44 negative cells?

The first necessary step was to separate the two subpopulations of the cell line LT97 by fluorescence activated cell sorting (FACS). The cells were stained with Phycoerythrin (PE) labeled antibodies against CD44 and sorted with a flow cytometer. In figure 3.1 a typical result of this experiment is shown. The CD44 positive cells are depicted in blue, both in figure 3.1A, which shows the counts per fluorescence intensity, and in figure 3.1B which shows the gates that have been used. There were about 43% CD44+ cells. The CD44 negative cells are depicted in pink, both in figure 3.1A and figure 3.1B. About 30% of the cells were CD44 negative. Also an intermediate population is shown in figure 3.1B, depicted in green, which contains both weak CD44 positive and negative cells, this fraction of cells was discarded.

From the two subpopulations that have been obtained by FACS, RNA and proteins were isolated for further examinations.

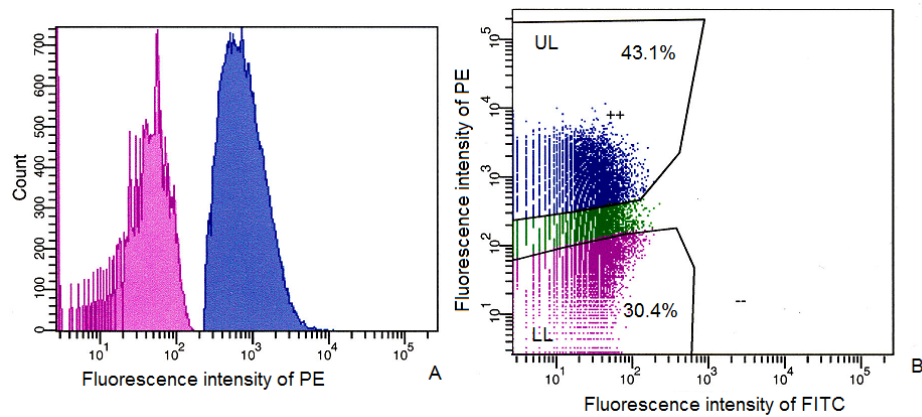


Figure 3.1: FACS for CD44-PE in LT97 cells. A=counts per fluorescence intensity level, B=the gates used for sorting. Y-axis: PE-fluorescence intensity. UL: gate for CD44+ LT97 cells, LL: gate for CD44- LT97 cells. The experiment has been conducted 7 times.

3.2 Do Apoptosis Associated Proteins Play a Role in Reduced Survival of CD44- LT97 Cells?

To determine why the CD44 negative cells have such a low growth and plating potential, several apoptosis associated proteins were investigated. First the gene expression of survivin and BAX was examined by standard PCR. The results are shown in figure 3.2A for BAX and figure 3.2B for survivin. The bands for BAX seem to be of the same thickness, hence there seems to be no regulation of this gene on RNA level. The bands for survivin in CD44 negative cells seems to be a bit weaker than the band for survivin in CD44 positive cells. It was not possible to amplify Bcl-2 by standard PCR. For a quantitative analysis of gene expression on RNA level, Real time PCR has been performed. In figure 2C the results of the Real time PCR for survivin, BAX and Bcl-2 are shown. The gene expression level of CD44 negative LT97 cells has been set to 100% and the gene expression level of the CD44 positive cells has been calculated accordingly. The result for BAX shows that this gene is expressed at the same level in CD44 positive and negative LT97 cells. It verifies the outcome of the standard PCR and suggests that this gene is not differently regulated in the CD44 positive and negative subpopulations.

The result for Bcl-2 shows that this gene is expressed at over 150% in the CD44 positive cells and hence seems to be significantly up regulated in this subpopulation at RNA level.

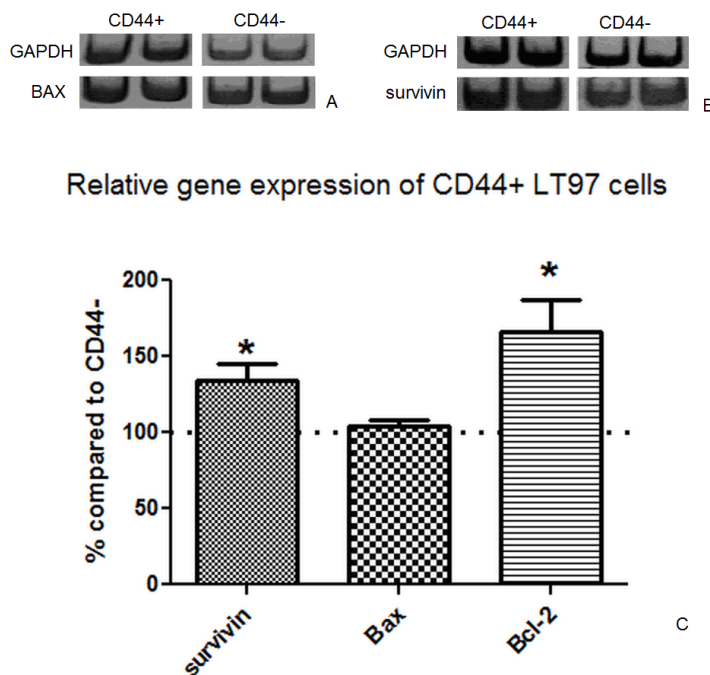


Figure 3.2: Gene expression on RNA level of survivin, BAX and Bcl-2 in CD44+ and CD44- LT97 cells. A= standard PCR result for BAX, B= standard PCR result for surviving, C= Real time PCR result for survivin, BAX and Bcl-2, the gene expression level in CD44- cells has been set to 100%, expression in CD44+ cells has been calculated as % compared to the CD44- expression level. Significances: survivin: $p = 0.0257$, Bcl-2: $p = 0.0316$. The experiments have been conducted 3 times (standard PCR) or 6 times (real time PCR) respectively.

Survivin is also expressed significantly higher in CD44 positive cells with about 140%. Following the RNA analysis, the gene expression level at protein level was examined. Survivin and BAX protein expression have been investigated by Western blot analysis, (figure 3.3A and B), and Bcl-2 has been analyzed by ELISA (figure 3.3C), as it was not possible to detect this protein by Western blot analysis. The Western blot results confirm the findings of the RNA analysis, survivin is expressed at a higher level in CD44 positive cells, and at a very low or non detectable level in CD44 negative cells (figure

3.3A). BAX is expressed at the same level, both in CD44 positive and negative cells. The result of the ELISA shows that Bcl-2 protein seems to be expressed at a higher level in CD44 negative cells with about 0.6ng Bcl-2 per μg total protein, while it is only expressed at about 0.2ng Bcl-2 per μg total protein in CD44 positive cells. This result contradicts the result of the RNA analysis where Bcl-2 was significantly higher expressed in the CD44 positive cells.

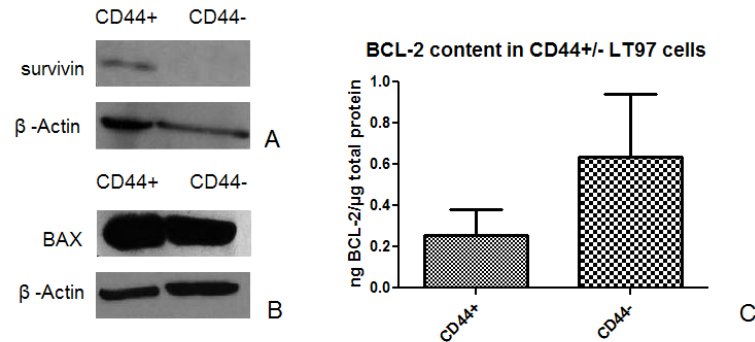


Figure 3.3: Protein expression of survivin, BAX and Bcl-2 in CD44+ and CD44- LT97 cells. A= Western blot result for survivin, β -actin as housekeeping protein, B= Western blot result for BAX, β -actin as housekeeping protein, C= ELISA result for Bcl-2, result is depicted as ng Bcl-2 per μg total protein. The experiments have been conducted 3 times.

3.3 The Role of Wnt Pathway Activation in CD44+/- LT97 Cells

As we found survivin to be significantly higher expressed both on RNA and protein level in CD44 positive cells, and as survivin is a β -catenin target gene, the question arose, whether the modulation of the Wnt-pathway might play a role in the enhanced plating efficiency and growth potential of CD44 positive cells.

Therefore a plating efficiency and viability experiment with CD44 positive LT97 cells was performed. The cells were plated with and without the Wnt inhibitor sulindac (SRI) to determine if blocking of the Wnt pathway reduces the plating efficiency and growth potential of the CD44 positive cells. For the plating efficiency 3×10^4 CD44 positive cells were plated with or without $20 \mu\text{M}$ sulindac per well in a 24 well plate and analyzed 24

hours after plating by Crystal violet staining. The result is shown in figure 3.4, which shows the CD44 positive cells plated with 20 μ M sulindac (SRI) after 24 hours. Most of the cells are not attached to the ground and seem to be dead. Figure 3.4B shows the untreated control, the CD44 positive cells shown in this picture are all attached to the ground and vital.

To determine the growth and survival potential of the CD44 positive cells, 3x10³ CD44 positive cells were plated with or without 5 or 20 μ M sulindac per well in a 24 well plate and analyzed 7 days after plating by a Neutral red uptake assay (figure 3.4C).

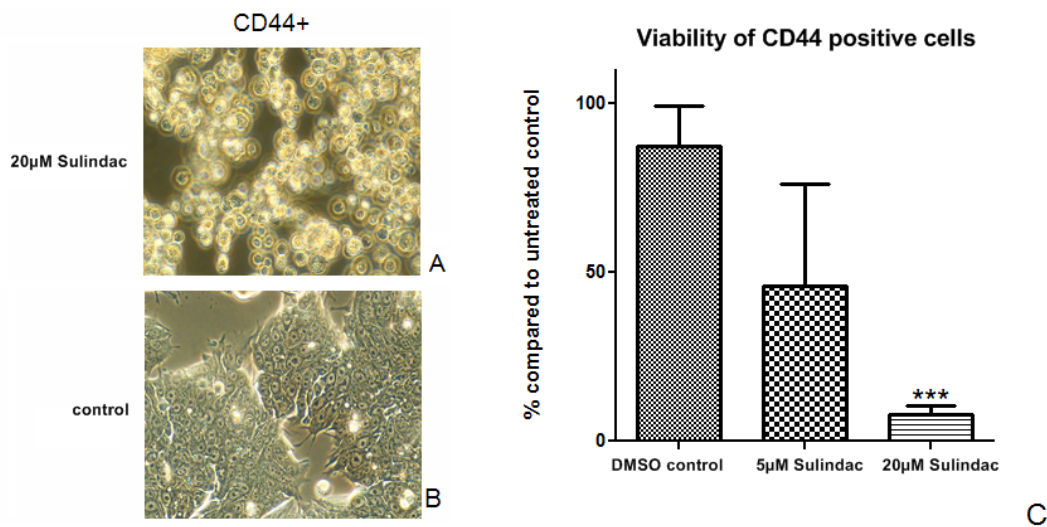


Figure 3.4: Plating efficiency and Viability of CD44+ LT97 cells treated with and without sulindac. A,B= Plating efficiency, 3x10⁴ cells per well, picture taken after 24 hours at 40x magnifications A= treated with 20 μ M sulindac, B= untreated control, C= 3x10³ cells per well, viability: result of Neutral red uptake assay after 7 days. significance: Sulindac 20 μ M: p=0.0005. Experiments have been conducted 4 times.

The results are depicted as percent of the untreated control. As sulindac is dissolved in Dimethylsulfoxid (DMSO), an additional control treated with DMSO was analyzed to exclude a possible interference of this cytotoxic substance. The viability of CD44 positive cells plated with DMSO is not significantly affected. In cultures plated in the presence of 5 μ M sulindac (SRI) a not significant reduction of about 50% in the number of viable cells could already be observed. Cultures treated with 20 μ M sulindac were re-

duced to less than 10% of the viable cells found in the CD44 positive controls ($p=0.0005$).

3.4 Is Survival of CD44+ LT97 Cells Conferred by IGF-1 Pathway Activation?

Preceding RNA microarray experiments suggest the IGF pathway as a target for further investigations. IGFBP3, a protein which binds IGF-1 and hence prevents binding to and activation of the IGF-1 receptor, has shown to be upregulated in CD44 negative cells. Maybe a modulation of the exogenous IGF pathway is essential for the higher growth and plating efficiency of CD44 positive cells.

First the gene expression of IGFBP3 in CD44 positive and negative LT97 cells was examined by standard PCR (figure 3.5A). The result shows a thicker band for IGFBP3 in CD44 negative cells than in CD44 positive cells, therefore it seems that IGFBP3 is indeed higher expressed at RNA level in CD44 negative cells. For comparison the expression of CD44 at RNA level is shown in the same figure. IGFBP3 seems to be conversely expressed to CD44. To quantify these findings, real time PCR was performed for IGFBP3 and CD44 (figure 3.5B). While CD44 was expressed significantly higher in CD44 positive cells, IGFBP3 was found to be expressed at a lower level in CD44 negative cells. As it proved to be impossible to determine the expression of this soluble protein, which is furthermore also excreted by the cell, after a cell sorting by FACS, other components of the IGF-1 pathway were investigated on protein level instead.

The protein IRS becomes phosphorylated when the IGF-1 pathway is activated; hence phosphorylated IRS was examined by Western blot analysis, and also the pan IRS protein. The result is shown in figure 3.5C. Phosphorylated IRS (pIRS) seems to be expressed at a slightly higher level in CD44 positive cells, although this might also be due to the fact that the pan IRS is already expressed at a higher level in CD44 positive cells. The β -actin bands show that a comparable amount of total protein had been applied for both CD44 positive and negative samples.

One of the downstream targets of an activated IGF-1 pathway is ERK1/2. It becomes phosphorylated upon activation, hence the phosphorylated form of this protein (pERK) has been investigated by Western blot, along with the pan protein form (ERK1/2). As it is depicted in figure 3.5D, there is much more pERK visible for CD44 positive cells than for CD44 negative cells, while ERK1/2 expression seems to be at the same level

in both samples. The β -actin bands are of comparable size as well. This indicates a phosphorylation and activation of ERK1/2 in CD44 positive specimen.

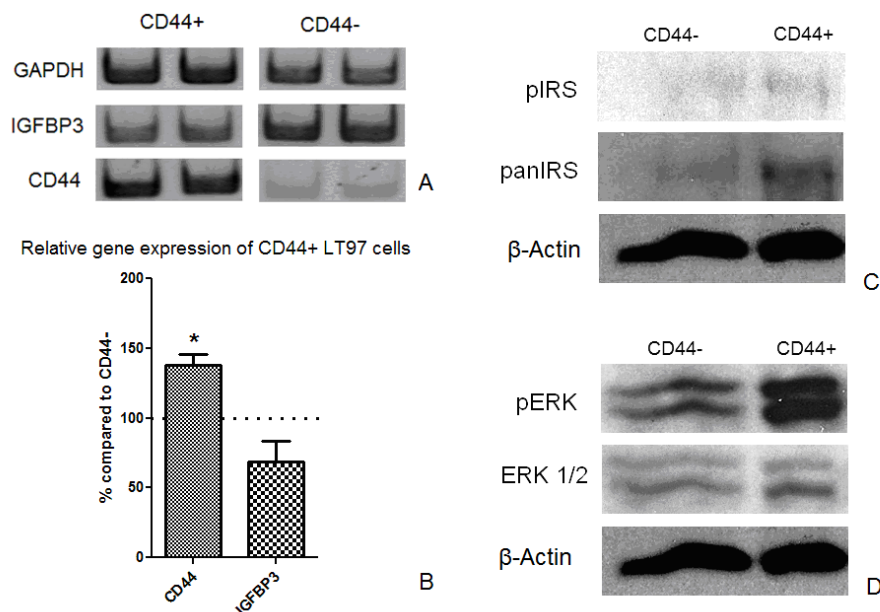


Figure 3.5: RNA and Protein expression of several components of the IGF pathway in CD44+ and CD44- LT97 cells. A= standard PCR result for IGFBP3 and CD44, GAPDH as housekeeping control gene. The experiment has been conducted 2 times. B= real time PCR result for IGFBP3 and CD44, gene expression of CD44- cells has been set to 100% and expression in CD44+ cells is shown as % of CD44- LT97 gene expression. The experiment has been conducted 6 times. Significances: CD44: $p=0.0391$. C= Western blot result for phosphorylated IRS (pIRS) and pan-IRS, β -actin as housekeeping control protein. The experiment has been conducted 3 times. D= Western blot result for phosphorylated ERK1/2 (pERK), ERK1/2 and β -actin as housekeeping control protein. The experiment has been conducted once.

Furthermore, the pan expression and the phosphorylation of GSK3 β and S6 have been analyzed on protein level by Western blot (figure 3.6). GSK3 β does not seem to be expressed differentially on protein level, though it seems to be slightly more phosphorylated in CD44 positive LT97 cells (figure 3.6A). In contrast, the expression of pan S6 on protein level seems to be regulated differentially, as there is a much stronger band for S6 already shown for CD44 positive cells than for CD44 negative cells. The phosphorylation

does not seem to be significantly different in CD44 positive and negative cells, the CD44 positive cells have a much stronger band for pS6 than the CD44 negative cells, however, the pan expression of S6 is already much higher in CD44 positive cells (figure 3.6B).

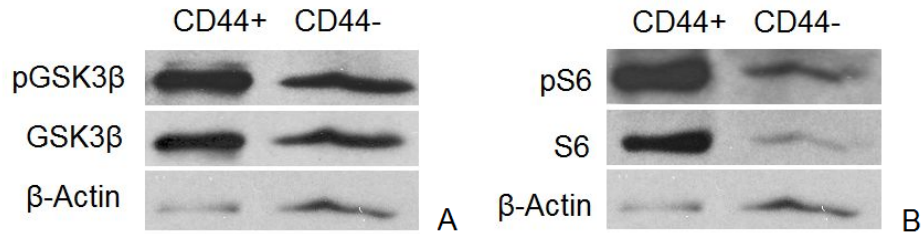


Figure 3.6: Protein expression in CD44 positive and negative LT97 cells. A= Western blot result for pGSK3β and GSK3β, B= Western blot results for pS6 and S6. β-actin has been used as housekeeping control protein. The experiments have been conducted once.

As these findings further support the theory of an IGF-1 pathway activation in CD44 positive cells, plating efficiency experiments were set up to further examine the role of this pathway.

For this plating efficiency and viability assay, 3×10^3 CD44 positive or negative cells were plated per well in a 24 well plate with serum free medium. The CD44 negative cells were treated with 10 or 50ng/mL IGF-1 respectively to find out whether the survival in CD44 positive cells is transmitted by an IGF-1 survival signal and if a stimulation of the IGF-1 pathway might rescue the CD44 negative cells by enhancing their plating efficiency and growth potential. Photos were taken 24 hours after plating at 20-fold magnifications (figure 3.7). Figure 3.7A shows the CD44 positive cells treated with DMSO as positive control, 3.7B shows the untreated CD44 negative cells, and in 3.7C the CD44 negative cells treated with 50ng/mL IGF-1 are depicted.

There is no significant improvement in plating efficiency visible for the CD44 negative cells treated with IGF1, if compared to the untreated control. About the same conclusion derives from the viability assay (figure 3.7D). The results of the Neutral red uptake assay after 7 days are depicted as percentage compared to the untreated control. There is only a minimal increase of viability for the cells treated with 50ng/mL, but it is not significant.

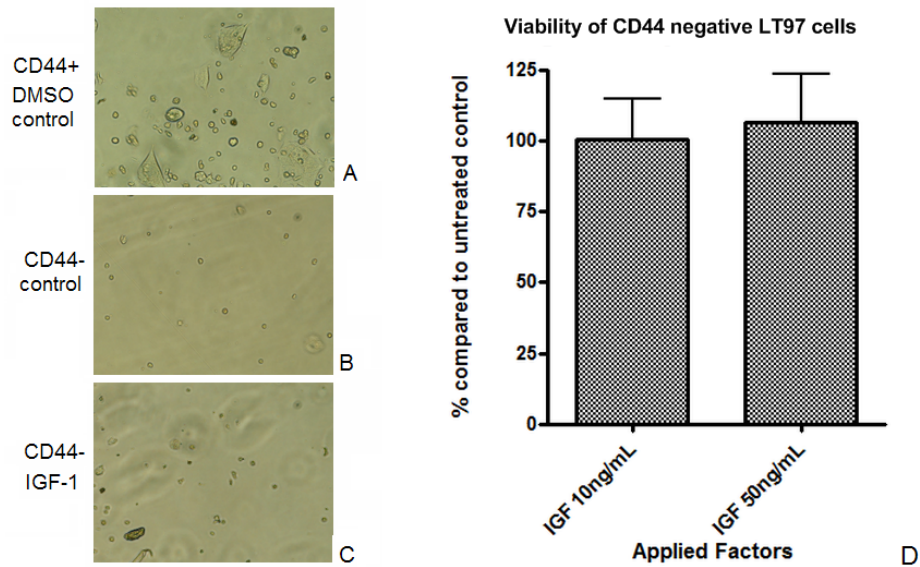


Figure 3.7: Plating efficiency and viability of CD44- LT97 cells treated with and without 10 or 50ng/mL IGF-1 respectively. A-C= Plating efficiency after 24 hours, pictures taken at 20x magnifications. A= CD44+ LT97 cells treated with DMSO, B= CD44- LT97 cells untreated, C= CD44- LT97 cells treated with 50ng/mL IGF-1. D= Viability after 7 days, result of Neutral red uptake assay relative to untreated control. The experiment was conducted 4 times.

To investigate the IGF-1 pathway by inhibition of the IGF-1 receptor in the CD44 positive cells, another viability and plating efficiency experiment was set up. 3×10^4 CD44 positive cells were plated per well in a 24 well respectively. The cells were treated with 100 or 1000nM of the IGF-1 receptor inhibitor cyclolignan picropodophyllin (PPP). After 24 hours pictures were taken at 40x magnifications. In figure 3.8A the cells treated with 1000nM PPP are shown. The cells look detached from the ground and apoptotic, while the untreated control cells in 3.8B look healthy and are all attached to the ground. After one week a Neutral red uptake assay had been performed to determine the viability, the result is shown as percentage of the untreated control. Upon application of 100nM PPP there is not much difference to the untreated control cells, but after adding 1000nM PPP, there is a significant reduction of viability of over 50%.

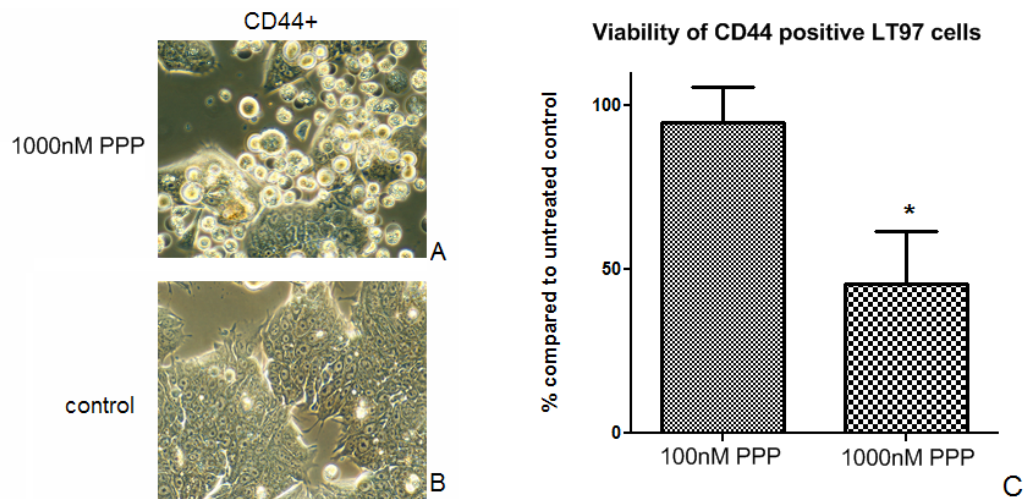


Figure 3.8: Plating efficiency and viability of CD44- LT97 cells treated with and without 100/ 1000 μ M PPP. A,B= Plating efficiency after 24 hours, picture taken at 40x magnifications. A= CD44+ LT97 cells treated with 1000 μ M PPP, B= CD44- LT97 cells untreated, C= Viability after 7 days, result of Neutral red uptake assay relative to untreated control. The experiment was conducted 4 times.

During the course of the plating efficiency experiment, another fact stood out. While after 24 hours, CD44 positive cells treated with sulindac were mostly apoptotic, the cells treated with PPP looked still rather fine, but after 7 days both the cells treated with PPP and the cells treated with sulindac were mostly dead. This observation is shown in figure 3.9. Here the results of the plating efficiency and the viability assay are depicted. The plating efficiency was determined by crystal violet staining, photographing and then calculating the percentage of the ground covered with cells, while the Viability was determined by Neutral red uptake assay. Both results are displayed as percentage compared to the untreated control. It is shown here that the cells treated with 1000nM PPP show about 75% of the plating efficiency of the untreated control, but after 7 days the survival rate is only about 25% of the untreated control. By comparison cells treated with sulindac were reduced to about 25% plating efficiency after 24 hours already, and after 7 days the survival is almost zero.

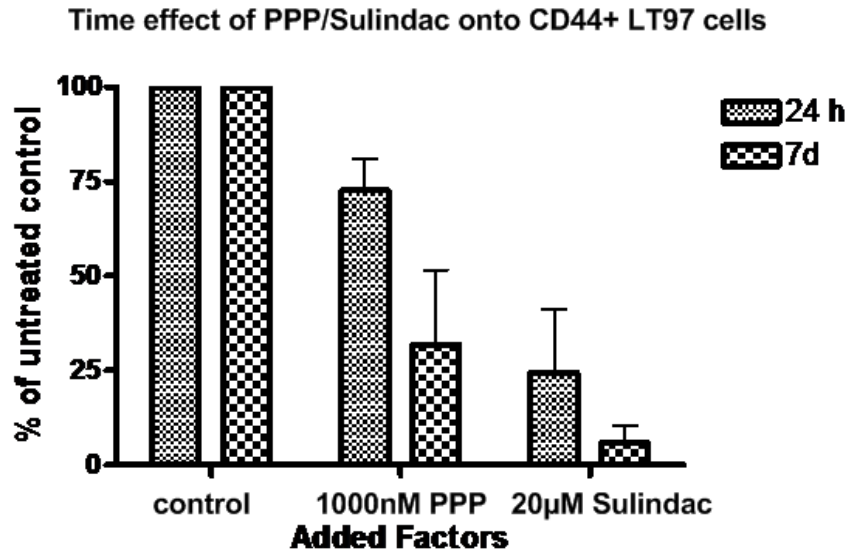


Figure 3.9: Time effect of PPP/sulindac onto CD44+ LT97 cells. Plating efficiency and viability for CD44+ LT97 cells treated with Wnt-Inhibitor sulindac or IGF-1 receptor inhibitor PPP 24h (plating efficiency) and 7 days (viability) after plating. 3×10^3 cells per well in a 24 well plate.

3.5 The Role of FGF Receptor Signalling in CD44+/- LT97 Cells

Another possible pathway target involved in growth and survival of the CD44 positive cells was the fibroblast growth factor (FGF) receptor pathway. In a preceding RNA microarray experiment, FGFR3 and FGFR4 seemed to be upregulated in CD44 positive LT97 cells, while FGFR2 was downregulated. This makes FGFR4 and FGFR3 candidates for the transmission of survival signals in the CD44 positive cells. Additionally to those receptors also the FGFR3 agonist FGF18, which is also a β -catenin target gene, was investigated.

At first the gene expression of FGF18 and FGFR4 was examined by standard PCR in CD44 positive and CD44 negative LT97 cells. The results are shown in figure 3.10. In figure 3.10A the result for FGF18 is depicted, but there is no significant difference in the two subpopulations. The result for FGFR4 is shown in figure 3.10B, but in this experiment also no difference in gene expression can be seen between CD44 positive

and negative cells. To additionally verify these findings and to investigate the splice variants of the FGFR3, real time PCR has been performed. In figure 3.10C the result of this experiment is depicted with expression in CD44 negative cells calculated as percentage of gene expression in CD44 positive cells. This experiment verifies the finding that FGFR4 is not differentially regulated in CD44 positive and negative cells. FGF18 gene expression has been found to be slightly, but significantly reduced in CD44 positive cells. Of FGFR3 the IIIb subunit does not seem to be differentially regulated, but mRNA of the FGFR3IIIc subunit is increased about 350% in CD44 positive cells (<0.05).

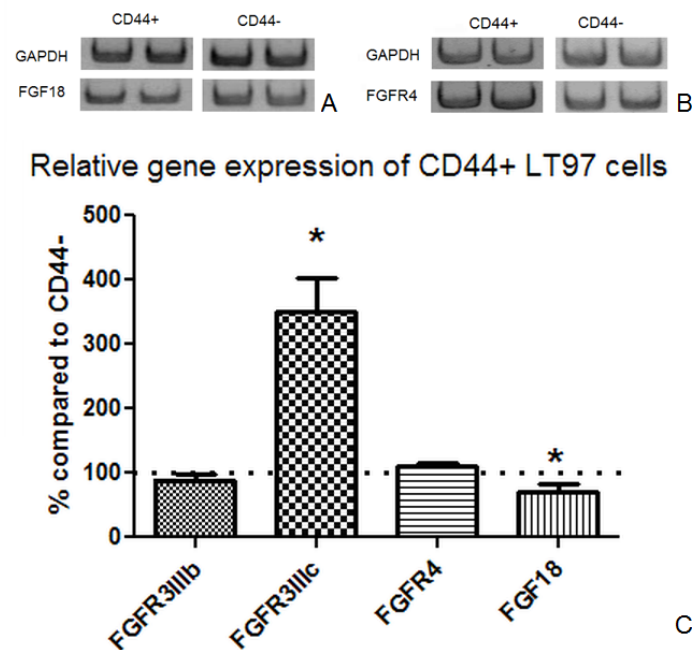


Figure 3.10: Gene expression of FGF receptors and FGF18 in CD44+ and CD44- LT97 cells on RNA level. A= Standard PCR for FGF18, the experiment has been conducted twice, B= Standard PCR for FGFR4, the experiment has been conducted once, C= Real time PCR gene expression of FGFR3IIIb, FGFR3IIIc, FGFR4 and FGF18 in CD44 positive LT97 cells relative to the gene expression of CD44 negative LT97 cells. The significances of gene expression in CD44+ cells compared to the gene expression in CD44- cells were calculated by a t-test. FGFR3IIIc: $p=0,0181$, FGF18: $p=0,0480$, the experiment has been conducted 6 times.

Protein expression of FGFR3 and FGFR4 has been examined by Western blot analysis, though, as it is shown in figure 3.11, neither the FGFR3 (figure 3.11A) nor the FGFR4 (figure 3.11B) seem to be expressed differently in CD44 positive and negative cells on protein level.

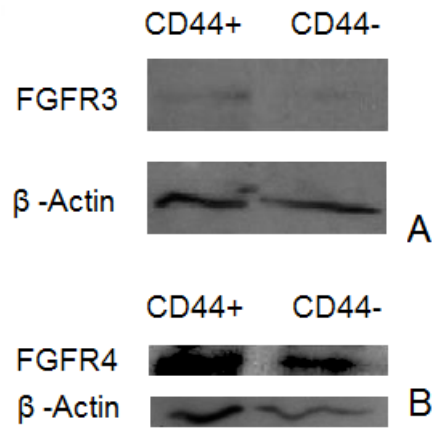


Figure 3.11: Protein expression of FGFR3 and FGFR4 in CD44+ and CD44- LT97 cells.

A= result of Western blot for FGFR3, B= result of Western blot for FGFR4.

The experiments have been conducted 3 times.

For a functional characterisation plating efficiency and viability experiments were set up to determine whether FGFR3 stimulation by FGF18 might rescue the CD44 negative LT97 cells and improve their growth and survival abilities.

For this experiment 3×10^3 CD44 positive and negative cells per well were plated in a 24 well plate respectively. The CD44 negative cells were treated with 10ng/mL FGF18, and analyzed after 24 hours for plating efficiency by taking photos (figure 3.12A-C) and after 7 days for viability by Neutral red uptake assay (figure 3.12D).

After 24 hours, the CD44 negative cells treated with 10ng/mL FGF18 (figure 3.12C) seem to be growing slightly better than the untreated CD44 negative cell control (figure 3.12B), although the CD44 positive DMSO control (figure 3.12A) shows many more viable cells than both of the CD44 negative subpopulations. The result of the Neutral red uptake assay after 7 days in culture (figure 3.12D) is depicted as percent compared to the untreated control. The viability seems to be slightly enhanced, though this result is not significant.

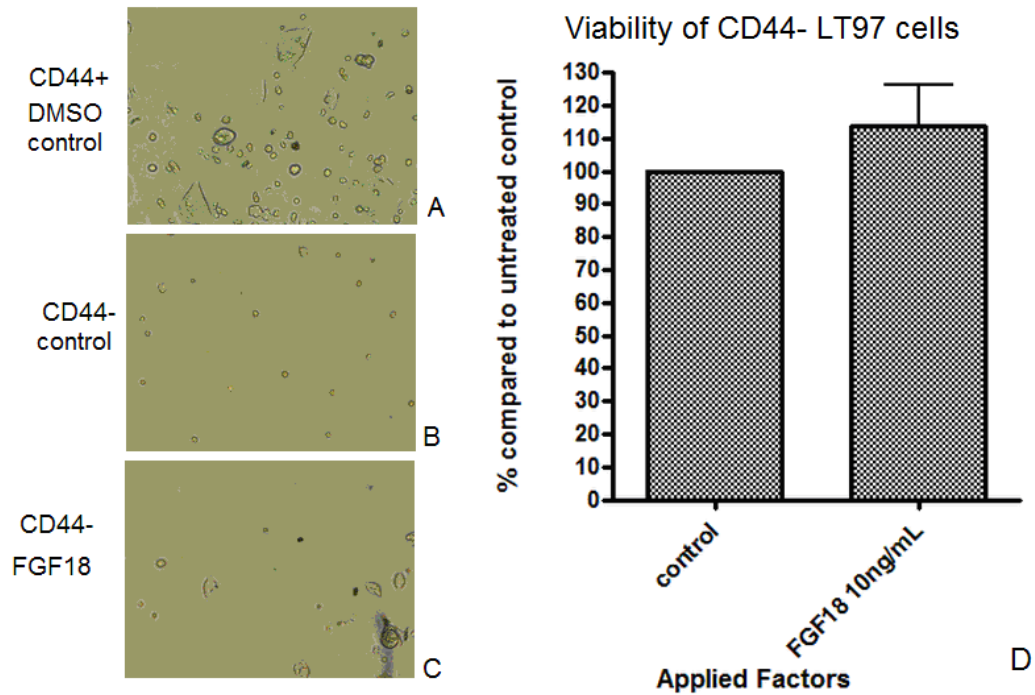


Figure 3.12: Plating efficiency and viability of CD44- LT97 cells treated with and without 10ng/mL FGF18. A-C= Plating efficiency after 24 hours, picture taken at 20x magnifications. A= CD44+ LT97 cells treated with DMSO, B= CD44- LT97 cells untreated, C= CD44- LT97 cells treated with 10ng/mL FGF18. D= Viability after 7 days, result of Neutral red uptake assay relative to untreated control. The experiment was conducted 4 times.

As the findings of the investigations of the FGF receptors were not really explicit, but nevertheless hint to a possible role of the FGFR3 activation in the survival of CD44 positive cells, additional experiments were performed using adenoviral constructs expressing either FGF18, or a dominant negative form of FGFR3 (KD3). As control a GFP encoding adenovirus was used. 24 hours after infection, the cells were sorted for CD44 by FACS, and each 3×10^4 cells were plated for a plating efficiency experiment, or 3×10^3 cells were plated for a viability assay. After 2 days the plating efficiency was determined by a Neutral red uptake assay (figure 3.13A). We can see that the CD44 negative cells of all virus infected cells are strongly reduced in their growth potential without any significant difference between them.

CD44 positive cells that had been infected with the FGF18 virus show a higher plating

efficiency than the CD44 positive cells infected with the GFP control virus. The CD44 positive cells infected with the dominant negative FGFR3 virus (KD3) show a better plating efficiency than those infected with the control virus, though the plating efficiency is not as high as of the CD44 positive cells infected with the FGF18 virus.

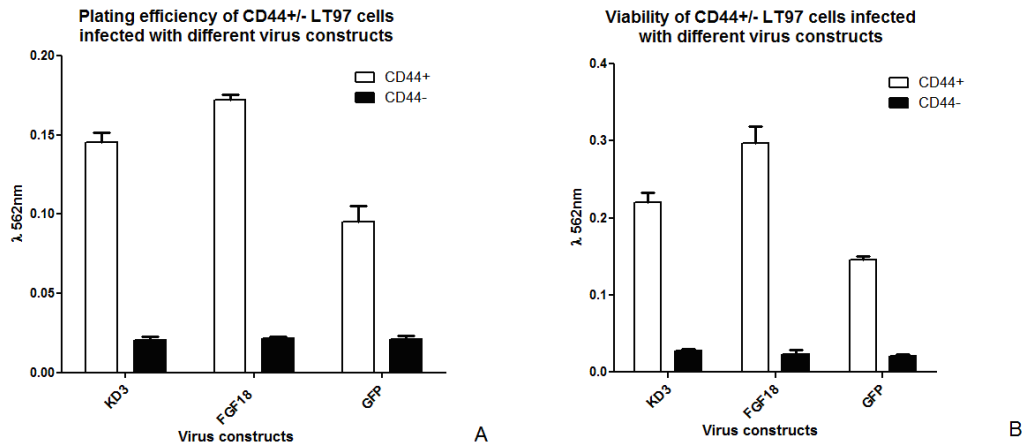


Figure 3.13: Result of the plating efficiency and viability assay with CD44 positive and CD44 negative LT97 cells that had been infected with different virus constructs the day prior to FACS sorting. KD3: cells infected with a virus who knocks down the FGFR3IIIc subunit, FGF18: cells infected with a virus who over expresses FGF18, GFP: control virus expressing GFP. The experiment has been conducted twice. A= plating efficiency, Neutral red uptake assay 2 days after plating 3×10^4 cells per well in a 24 well plate. B= Viability, Neutral red uptake assay 7 days after plating 3×10^3 cells per well in a 24 well plate.

After 7 days the viability was measured by a Neutral red uptake assay (figure 3.13B). All CD44 negative cell fractions show a very low viability of about the same level. Both FGF18 virus and the DNFGFR3 virus increased viability of the CD44 positive cells as compared to the GFP control virus.

To investigate the interaction of the FGFR3 signalling with the Wnt and the IGF-1 pathway, a viability assay with CD44 positive LT97 cells that had been infected with different adenoviral constructs the day prior to sorting was performed. 3×10^3 CD44 positive cells per well were plated in a 24 well plate and treated with 1000nM PPP, 20 μ M sulindac or DMSO as control.

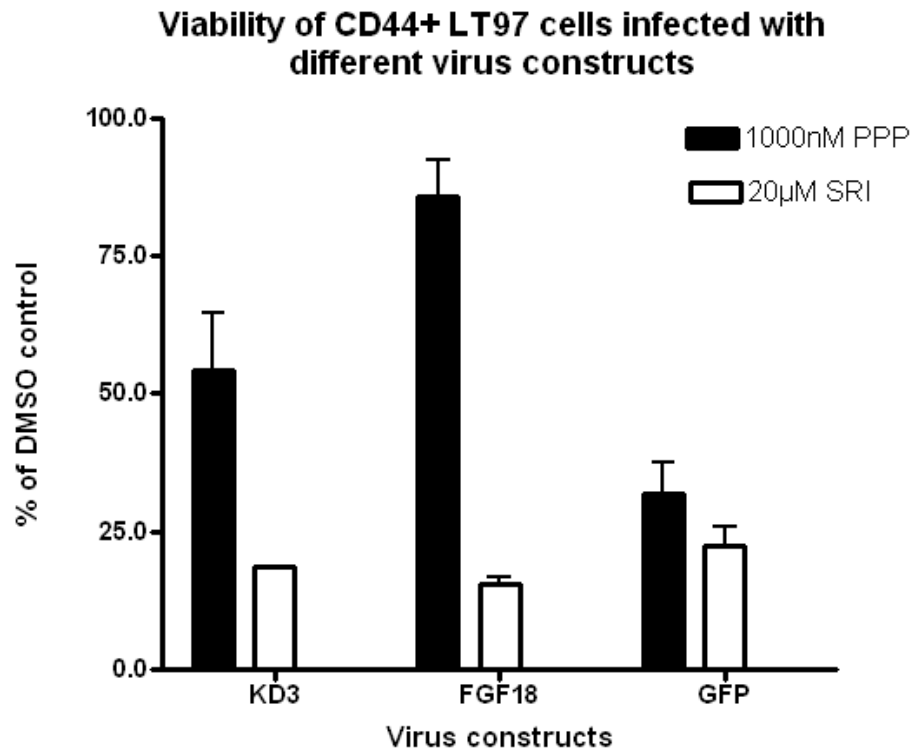


Figure 3.14: Result of the Viability assay with CD44 positive (CD44+) LT97 cells who had been infected with different Virus constructs the day prior to FACS sorting and who have been plated with and without 1000nM PPP or 20μM Sulindac. Viability is expressed as percent of viability of the DMSO control. KD3: cells infected with a Virus who knocks down the FGFR3IIIc subunit, FGF18: cells infected with a virus who over expresses FGF18, GFP: control virus expressing GFP. Neutral red uptake assay 7 days after plating 3×10^3 cells per well in a 24 well plate. The experiment has been conducted 2 times.

After 7 days a Neutral red assay was performed (figure 3.14).

Cultures treated with 20μM Sulindac show only minimal viability, independent of the virus construct used. Inhibitory effects of PPP were counteracted by FGF18 virus so that viability was almost as high as in FGF18 controls. The dnFGFR3 construct also protected from PPP-induced cells loss but less than the FGF18 construct.

3.6 Progression of the LT97-2 Cell Line to the LT97-3 Cell Line

In the course of experiments performed with the LT97-2 cell line, it became obvious that this cell line had started to change its characteristics over time. The first observation was the altered behaviour of the cells in culture. While LT97-2 cells usually need about 24 to 48 hours after passaging to attach to the ground of the dish with a plating efficiency of about 50-80%, and had to be passaged 1:2 once a week, the cells attached now within 24 hours with a 100% plating efficiency and had to be passaged 1:3 to 1:4 once a week. Not only the growth and plating efficiency potential of this cell line improved, also it became obvious that the CD44 positive subpopulation increased in size. Therefore the properties of this cell line at different points of time were investigated.

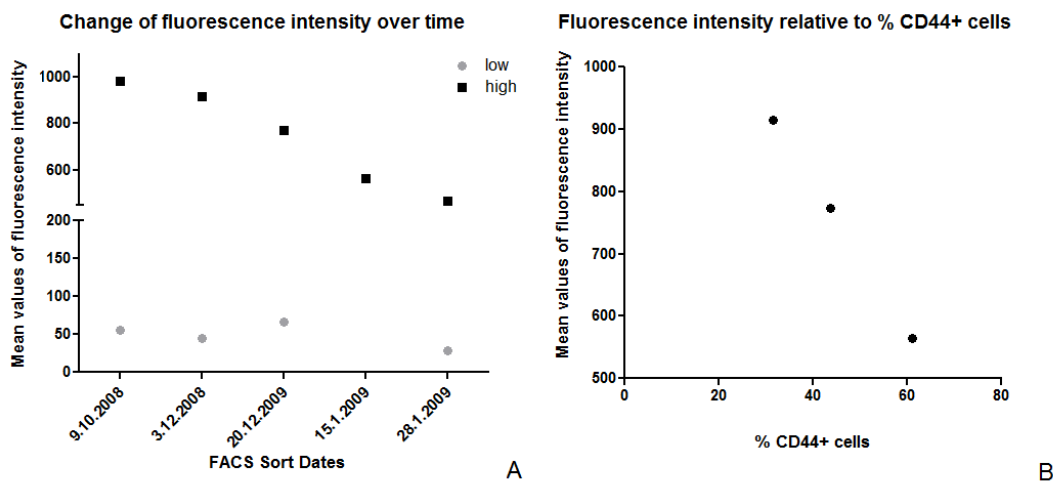


Figure 3.15: The change of fluorescence intensity over time and relative to the size of the CD44+ subpopulation in FACS for CD44 in LT97. A= change of the mean value of fluorescence intensity in LT97 cells over time in FACS. B= Change of the mean value of fluorescence intensity relative to the size of the CD44+ subpopulation.

As the size of the CD44 positive subpopulation seemed to grow, a point of interest was if the CD44 positive cells still expressed the same amount of CD44 molecules on their surface or if the expression level of this protein changed as well. Determination of the mean value of fluorescence intensity relative to the size of the CD44 positive subpop-

ulation demonstrated that the mean value for the CD44 positive cells decreased with each FACS experiment and was inversely correlated to the size of the CD44 positive population indicating that the CD44 positive cells changed towards a phenotype which expresses less CD44 per cell (figure 3.15A). This is further demonstrated in figure 3.15B. Here the mean value of fluorescence intensity level was set into correlation to the size of the CD44 positive subpopulation. It is shown that as the percentage of CD44 positive cells increases, the mean value of fluorescence intensity level decreases. The correlation between CD44 RNA expression and CD44 protein expression is shown in figure 3.16.

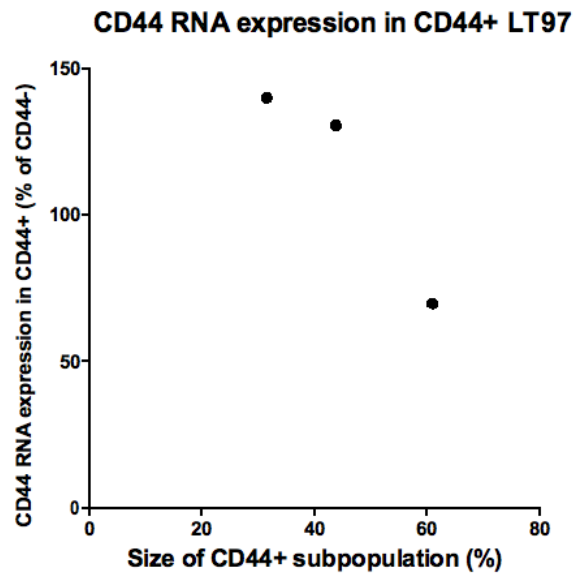


Figure 3.16: CD44 gene expression level relative to the size of the CD44 positive subpopulation in LT97 cells

To find out, whether this was caused by the amount of antibody becoming a limiting factor or a regulatory shift in the advanced population, we determined CD44 expression at the RNA-level. In figure 3.16 CD44 RNA expression in CD44 positive cells is plotted against the percentage of CD44 positive cells determined by FACS demonstrating a transcriptional down-regulation of CD44 as the population expands.

Then the gene expression level of CD44, IGFBP3 and Lgr5 in CD44 positive cells compared to the gene expression level in CD44 negative cells over time was analyzed (figure 3.17). While the gene expression level of CD44 decreases over time (figure 3.17A), IGFBP3 gene expression increases. In contrast, the gene expression of Lgr5 in CD44

positive cells decreases over time parallel to CD44 gene expression (figure 3.17B).

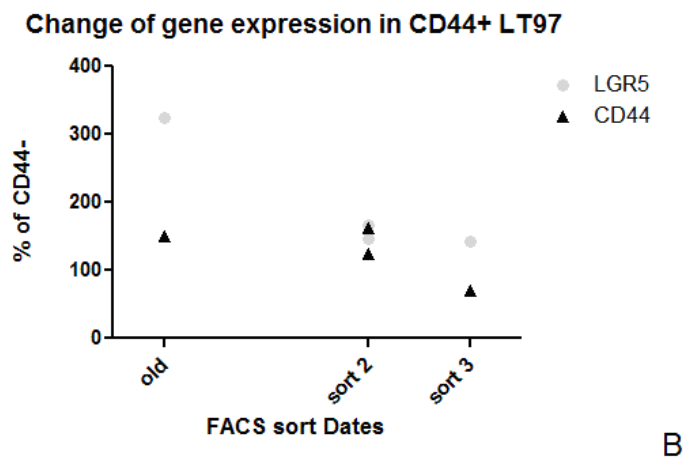
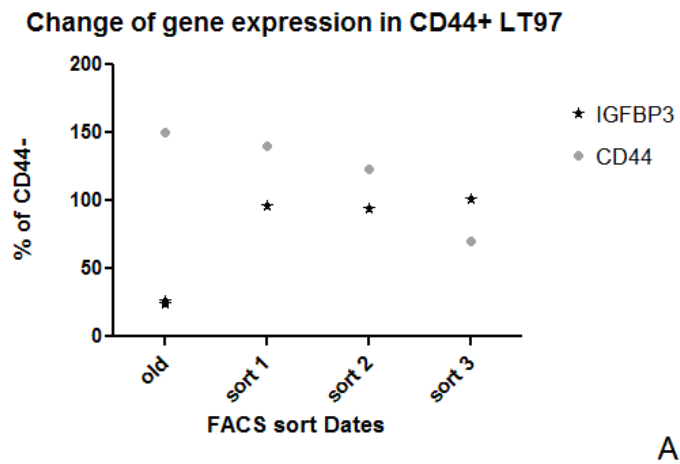


Figure 3.17: Change of gene expression in CD44+ LT97 cells . A= Change of CD44 and IGFBP3 expression. Y-axis: gene expression of CD44+ cells as percentage of gene expression of CD44- cells. X-axis: points of time (different FACS sort dates). Significances: 2-way-ANOVA, column factor: 23.11%, $p=0.0166$, Interaction: 44.67%, $p=0.0239$. B= Change of CD44 and LGR5 expression. Y-axis: gene expression of CD44+ cells as percentage of gene expression of CD44- cells. X-axis: points of time (different FACS sort dates). Significances: 2-way-ANOVA, column factor: $p=0.0324$,Row-factor: $p=0.0481$

As the alterations in gene expression and growth behaviour were substantial the cell line was considered a new line and named to LT97-3 to distinguish it from earlier passages LT97-1 and LT97-2.

To determine the difference in surface marker expression between the three passage lines LT97-1, LT97-2 and LT97-3, FACS analysis was performed for the markers CD44, CD133 and CD166 (figure 3.18A). As observed before, the size of the CD44 positive subpopulation increased with passage number. In LT97-1 less than 60% CD44 positive cells can be found, in LT97-2 about 60% CD44 positive cells can be found, and the size of the CD44 positive subpopulation is the highest with over 80% in LT97-3. Expression of the marker CD166 increases in parallel to CD44 while CD133 decreased reaching only about 10% in LT97-3.

The mean values of fluorescence are depicted in figure 3.18B.

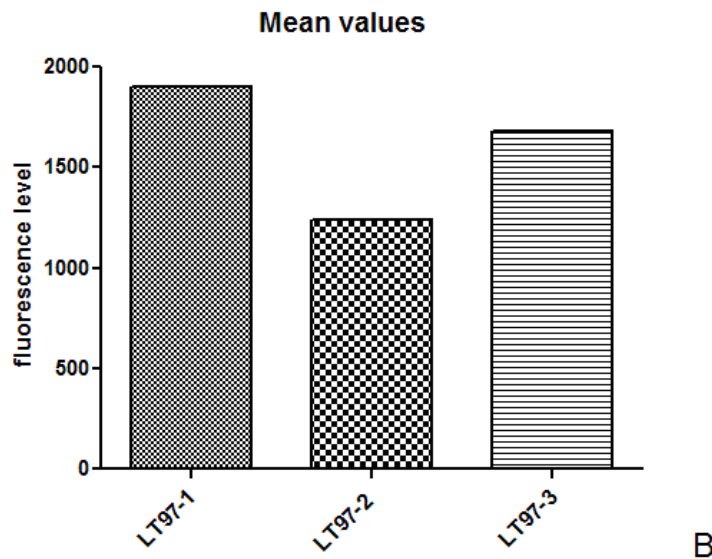
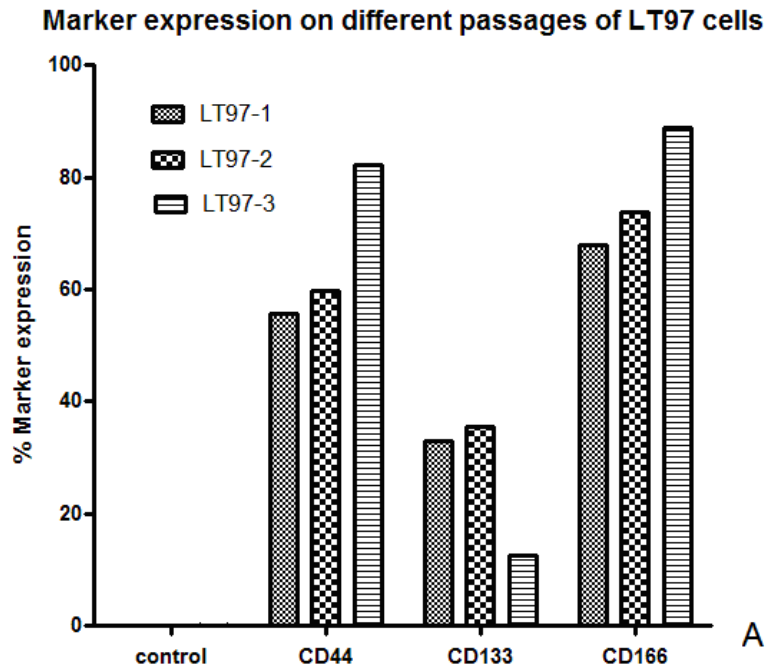


Figure 3.18: A: Marker expression on the LT97 passages LT97-1 (early passage), LT97-2 (later passage) and LT97- 3 (late passage). FACS analysis with cells who were stained for CD44, CD133 and CD166 (each PE labeled). Control: isotype control anti-rabbit PE. B: Mean values of the CD44 FACS analysis of the LT97 passages LT97-1 (early passage), LT97-2 (later passage) and LT97- 3 (late passage). The experiment has been conducted once.

3.7 Are AKH4/AKH14 Cells Suitable as Model Cell Line for CSCs in Colon Carcinoma?

As it would be convenient to obtain, in addition to the adenoma cell line LT97 also a carcinoma cell line as a model for tumour stem cells in colorectal carcinoma, the cell line AKH4/AKH14 was examined.

AKH4/AKH14 cells derive from a liver metastasis of a colon carcinoma, they derive from the same cells, but have a different passaging rhythm. AKH4 cells grow more slowly, only need to be passaged once a month, and show a more differentiated phenotype, while the AKH14 are passaged every other week and are more a model for self renewal.

The first question was: Can a tumour stem cell like phenotype also be found within this carcinoma cell line?

To determine this, gene expression on both RNA and protein level was investigated.

AKH4 and AKH14 cells were analysed shortly after plating (4 days after plating for the AKH4 cells, 2 days after plating for the AKH14 cells) and after 21 days in culture for gene expression of CD44, the suggested tumour stem cell marker. Further, the cells were analysed for gene expression of IGFBP3, as this gene seems to be conversely expressed to CD44, and Lgr5 as adult colon stem cell marker.

It can be observed that CD44 is expressed both in AKH4 (figure 3.19A) and AKH14 (figure 3.19B), and that the expression level increases over time in culture for both cell lines. The expression level in AKH14 cells is much higher than in AKH4 cells. IGFBP3 is also expressed in both cell lines, though as the expression level of IGFBP3 decreases over time in AKH4 cells, it increases over time in AKH14 cells. Lgr5 has been found to be expressed only at a very low level both in AKH4 and AKH14 cells, though the expression level increases over time in culture.

To determine the protein expression of CD44 in AKH4 cells in comparison to LT97 cells, an immunocytochemistry experiment was performed with antibodies against CD44 for both LT97 and AKH4 cells around 3 days after plating in sonic seal slides. As it is depicted in figure 3.20, CD44 is expressed in distinct foci both in LT97 cells (figure 3.20A) and in AKH4 cells (figure 3.20B) and, according to the previous results, the protein expression level of CD44 in AKH4 cells is lower than in LT97 cells.

In an additional experiment, only AKH4 cells were plated in sonic seal slides. After 3

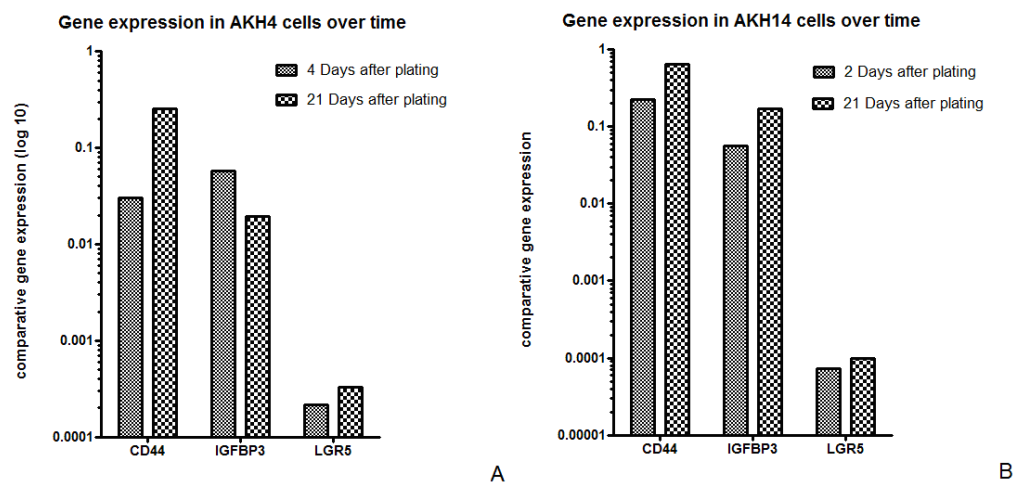


Figure 3.19: Gene expression in AKH4 and AKH14 cells over time. A= gene expression of CD44, IGFBP3 and Lgr5 in AKH4 cells 4 and 21 days after plating. B= gene expression of CD44, IGFBP3 and Lgr5 in AKH14 cells 2 and 21 days after plating. The experiment has been conducted once.

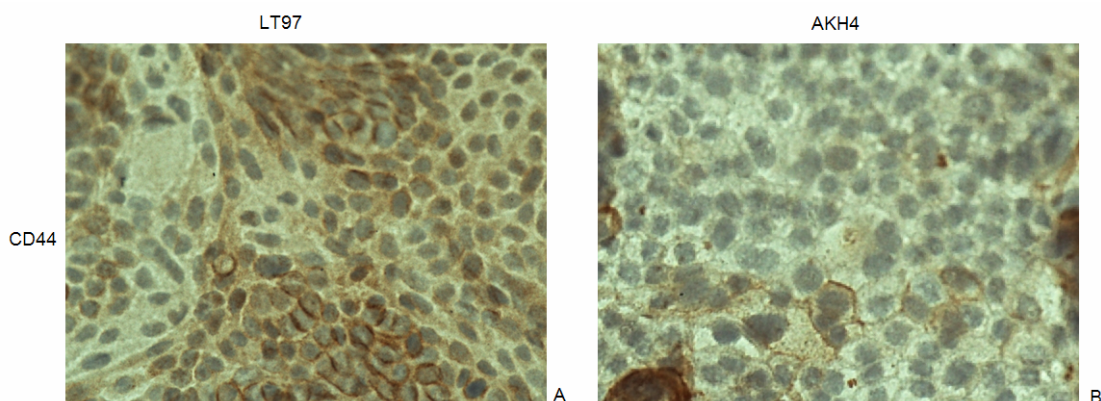


Figure 3.20: Immunocytochemistry for CD44 in LT97 and AKH4 cells 3 days after plating. 40-fold magnifications. A= LT97 cells stained for CD44, B= AKH4 cells stained for CD44. The experiment has been conducted twice.

days, not all cells had attached to the surface of the sonic seal slide, and were plated as “late adherent AKH4” cells into new sonic seal slides. It was observed that a few days later these cells had adhered and after 21 days in culture an immunocytochemistry was performed for CD44 and Lgr5 (figure 3.21).

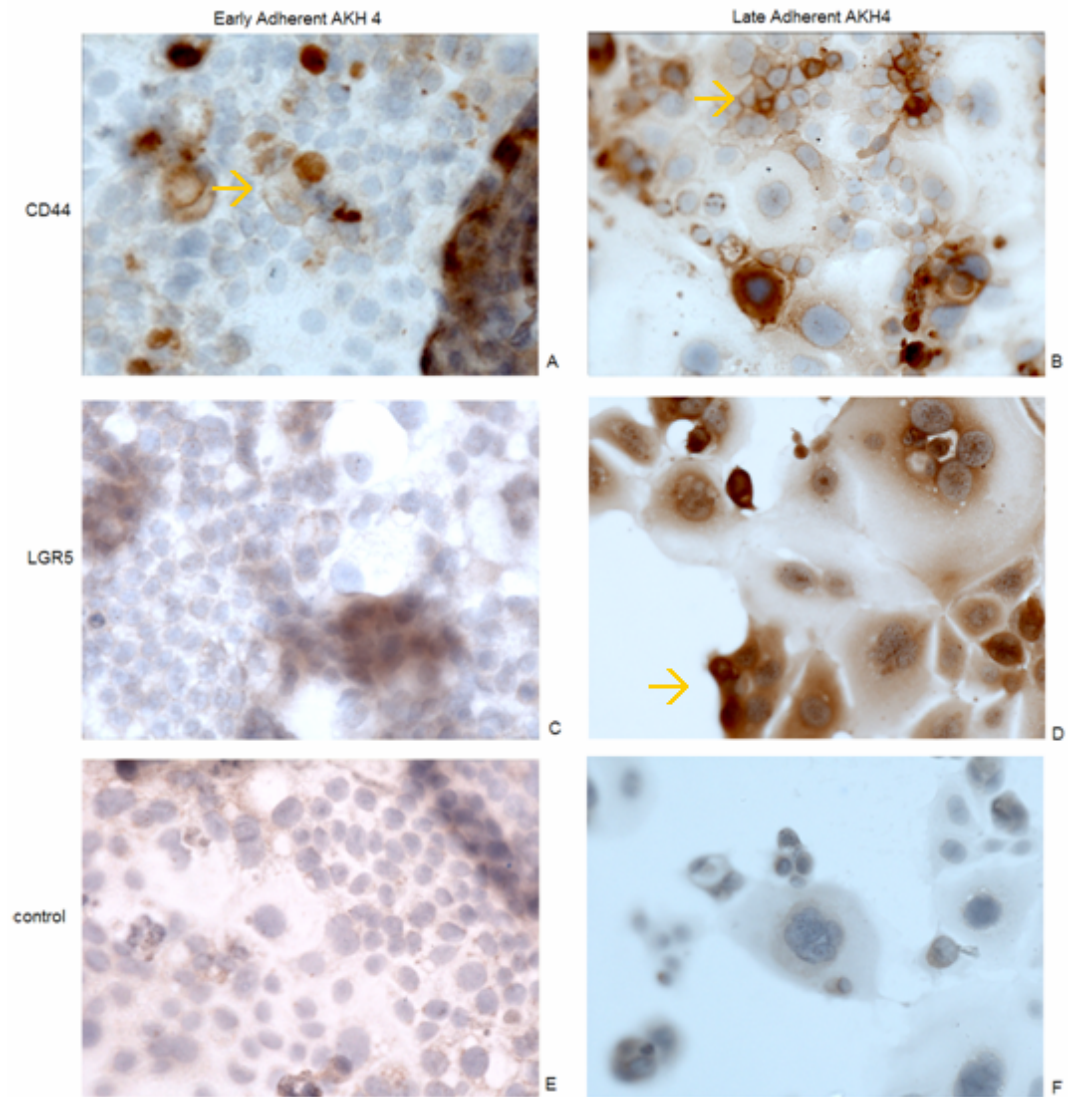


Figure 3.21: Immunocytochemistry for CD44 and Lgr5 in AKH4 early adherent and late adherent cells. Late adherent AKH4 cells were gained by transferring the supernatant of AKH4 cells 3 days after plating into a new sonic seal slide. Staining 21 days after plating the early adherent, photos were taken at 40-fold magnification. A= early adherent AKH4 cells stained for CD44, B= late adherent AKH4 stained for CD44, C= early adherent AKH4 stained for Lgr5, D= late adherent AKH4 stained for Lgr5, E= early adherent control (no first antibody), F= late adherent control (no first antibody)

The first notable observation made was that the late adherent AKH4 cells (figure 3.21B,D,F) look distorted, if compared to the early adherent AKH4 cells (figure

3.21A,C,E). All pictures were taken at 40-fold magnifications. The size of the nuclei varies, as does the overall cell size. Also cells with multiple nuclei can be found (figure 3.21 D). As it is depicted in figure 22A, the early adherent AKH4 cells show a few CD44 positive cells in distinct foci (see arrow). In comparison to this, the late adherent AKH4 cells stained for CD44 (figure 3.21B) show many more CD44 positive cells, which also grow in distinct foci. Furthermore, it seems that mainly cells of normal size are CD44 positive while the blown up cells seem to be more CD44 negative.

The cells were also stained for Lgr5. In figure 3.21C we can see that early adherent AKH4 cells do not show any Lgr5 positive cells, but the late adherent AKH4 cells (figure 3.21D) show Lgr5 positive cells (see arrow).

Now as the AKH4/AKH14 cells had been proven to have a CD44 positive subpopulation, they were sorted for CD44 by FACS. About 70% of the cells were CD44 positive, as it is shown in the upper left (UL) gate (figure 3.22B), and about 21% of the cells were CD44 negative, as it is shown in the lower left (LL) gate. The cells depicted in green are an intermediate subpopulation containing both CD44 positive and CD44 negative cells (figure 3.22B), those cells have not been used.

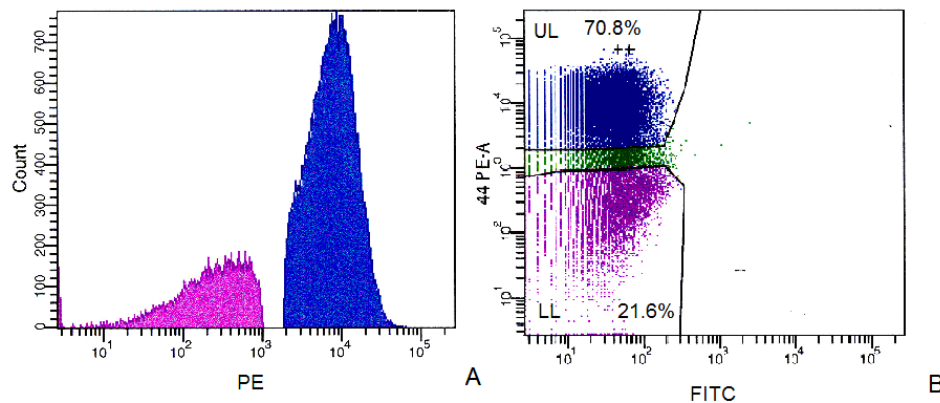


Figure 3.22: FACS for CD44-PE in AKH14 cells. A=counts per fluorescence intensity level , B= the gates used for sorting. Y-axis: PE-fluorescence intensity. UL: gate for CD44+ AKH14 cells, LL: gate for CD44- AKH14 cells. The experiment has been conducted twice.

Now the question was: Do the two distinct subpopulation show the same viability characteristics as the CD44 positive and negative LT97 cells? To determine this, a viability assay was set up. 3×10^3 CD44 positive and CD44 negative cells per well were

plated onto a 24 well plate, and after 3 weeks in culture, a Neutral red uptake assay was performed (figure 3.23). As we can see, the CD44 positive AKH14 cells show a significantly higher viability than the CD44 negative AKH14 cells.

The subpopulations were isolated to analyse expression of those genes that had been differentially regulated in LT97 subpopulations.

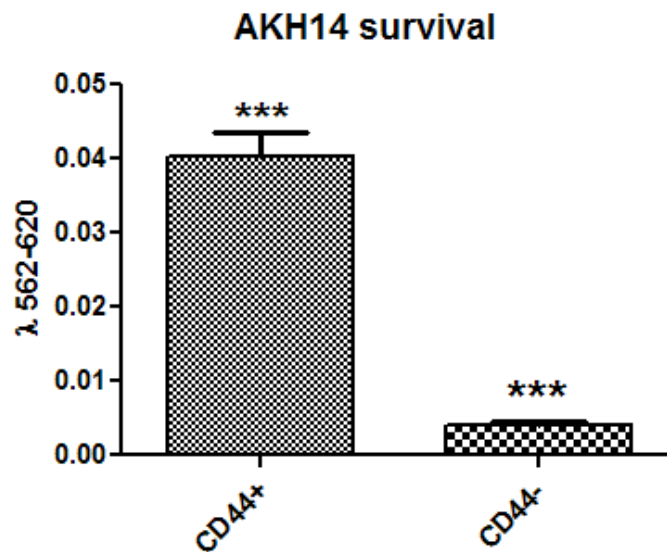


Figure 3.23: Long time survival of AKH14 cells sorted for CD44. Neutral red uptake assay was performed 14 days after plating 3×10^3 sorted cells. The experiment has been conducted twice. Unpaired t-test: $p=0.0004$

A real time PCR for the same genes that were investigated in LT97 cells was performed (figure 3.24).

However, the results show that almost all of the genes were expressed at about the same level in both CD44 positive and CD44 negative AKH14 cells and did not seem to be regulated on RNA level. The only exception found was the expression of Bcl-2, which shows to be higher in CD44 positive AKH14 cells.

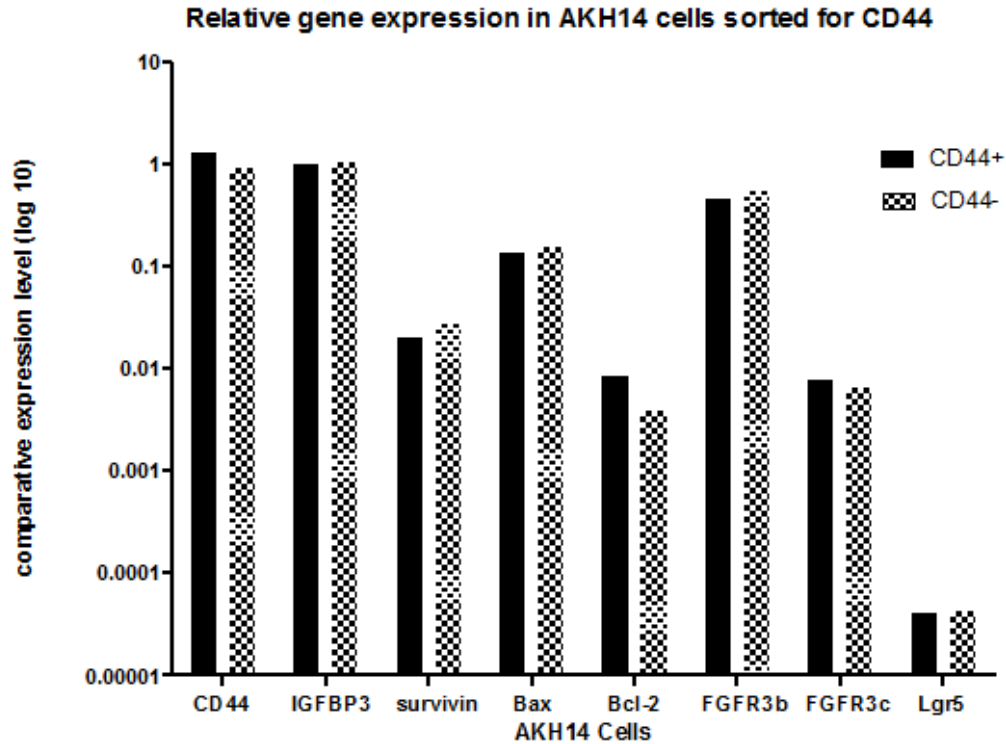


Figure 3.24: Gene expression in CD44 positive (CD44+) and CD44 negative (CD44-) AKH14 cells for CD44, IGFBP3, survivin, BAX, Bcl-2, FGFR3IIIb, Lgr5 and FGFR3IIIc. The experiment has been conducted once.

3.8 Investigating Musashi and Lgr5/Gpr49 as Possible Additional tumour Stem Cell Marker

Another topic of interest within the course of my work was to determine if there might be other possible markers aside from CD44 for the proposed tumour stem cells in colon cancer.

Therefore, additionally to CD44, the protein expression of Lgr5 and musashi had been investigated in tissue of normal mucosa, adenomas and carcinomas sections by immunohistochemistry. We can see a distinct stain of adult colon stem cells in normal tissue (figure 3.25A), but no Lgr5 positive cells have been found in adenomatous tissue at all (figure 3.25B).

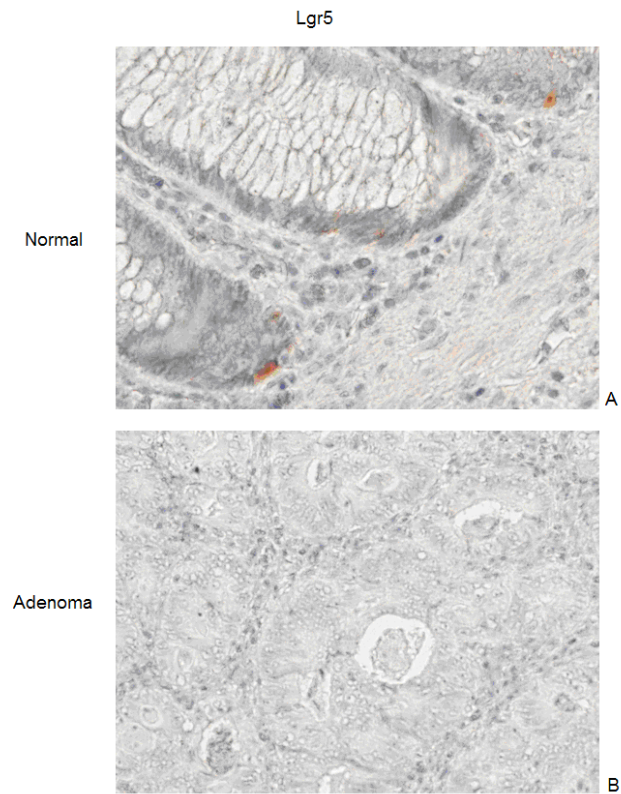


Figure 3.25: Lgr5/Gpr49 expression in normal and adenoma colon tissue. 60x magnification. A= normal tissue, B= adenoma tissue.

Additionally an immunocytochemistry experiment was performed for LT97 and AKH4 cells that did not detect any staining for LGR5 in either cell line (figure 3.26).

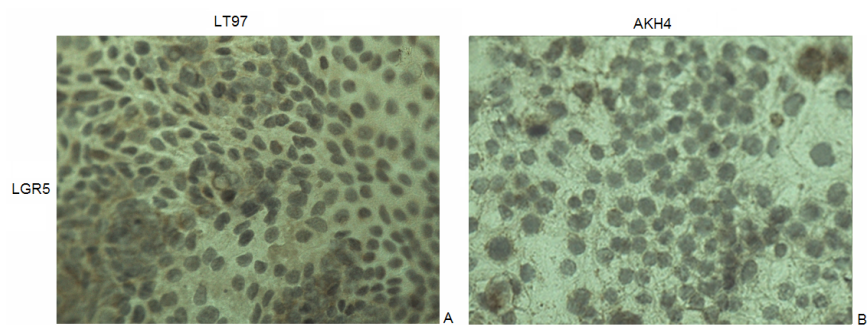


Figure 3.26: Expression of Lgr5 in LT97 and AKH4 cells 3 days after plating. A= Immunocytochemistry for Lgr5 in LT97 at 40x magnifications, B= Immunocytochemistry for Lgr5 in AKH4 at 40x magnifications.

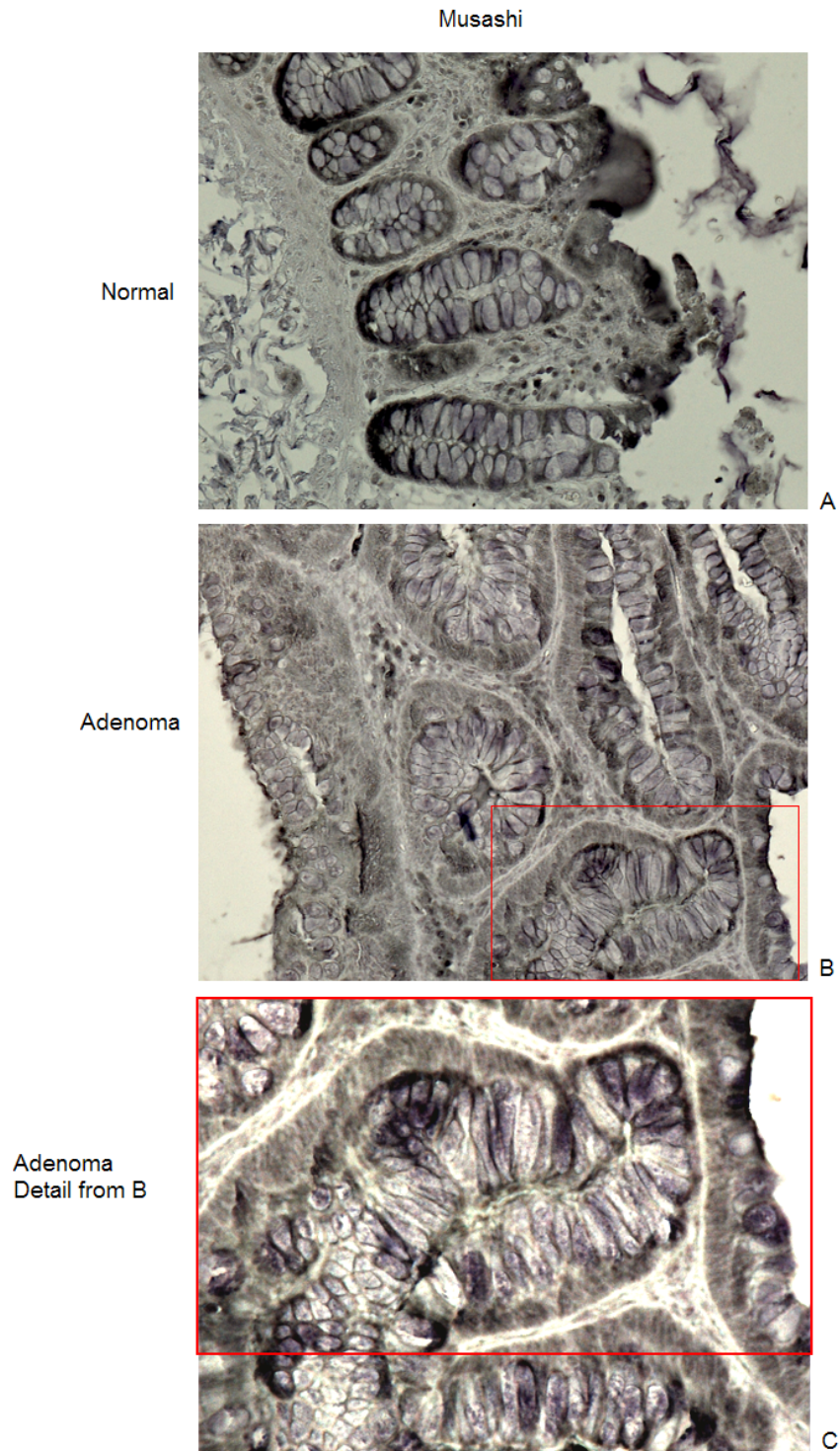


Figure 3.27: Expression of Musashi in normal and adenoma colon tissue at 40x magnification. A= normal tissue, B= adenoma tissue, C= detail from B (red box) at 60x magnification.

To determine the expression of musashi in colon adenoma tissue, an immunohistochemistry experiment was performed (figure 3.27). Musashi can be detected to be expressed at the bottom of the colonic crypts in normal tissue (figure 3.27A), and in adenoma tissue (figure 3.27B,C), we can find musashi in regions of the crypt that are not at the bottom, though only in very few cells.

3.9 Establishing Primary tumour Cell Cultures from Colon Cancer Tissue

Another aim of this study was to establish primary cell cultures from colon cancer tissue and to investigate, if a tumour stem cell like subpopulation could be isolated.

The tissue samples were mechanically minced with scalpels and enzymatically digested to obtain single cells and small aggregates of cells. Then the cells were taken into culture for at least one day, the looks of the primary cultures were observed and the expression of several proposed tumour stem cell markers - CD44, CD133 and CD166- was examined by FACS analysis.

The primary cell culture T0302 shows the characteristics of an average primary cell culture that had been investigated within the course of experiments and shall serve as an example for these.

The T0302 cells consist of two distinct subpopulations (figure 3.28). While a part of the cells attached to the ground, and grew in 3D plateaus that slightly resembled LT97 cell colonies with a dumpling or spheroid shaped aggregate of cells growing on top of these plateaus (figure 3.28A), the rest of the cells did not attach to the ground and showed a spheroid form (figure 3.28B). Pictures had been taken at different points of time. As it is depicted in figure 3.28, the culture did not grow, but the cells died slowly over time. After one month in culture (figure 3.28C), the adherent cells showed reduced size of the plateaus, and they also appeared to have a darker colour. After 6 weeks in culture, all adherent cells had died, and only very few of the spheroid cells could be seen (figure 3.28E), who had also taken on a darker colour. Only a week after the picture was taken, the spheroid cells also had died.

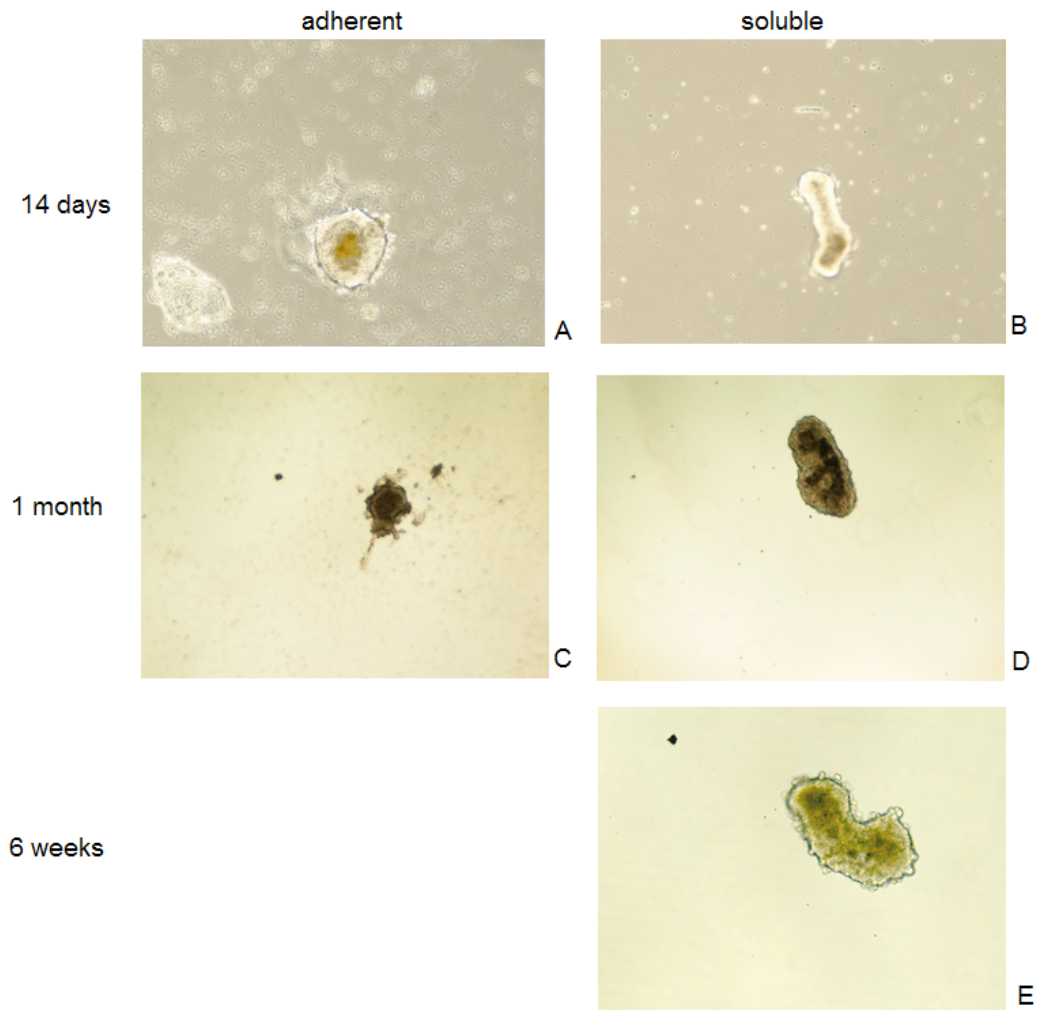


Figure 3.28: Primary cell culture from the colon tumour T0302 over time. Pictures taken at 20-fold magnification. A= adherent cells after 14 days, B= soluble cells after 14 days, C= adherent cells after 1 month, D= soluble cells after 1 month, E= soluble cells after 6 weeks.

Expression of several proposed tumour stem cell markers were analysed by FACS analysis on two different points of time (figure 3.29). Two days and two weeks after establishing the culture, the cells were analysed for the expression of CD44, CD133 and CD166 as proposed tumour stem cell markers, and for carcinoembryonic antigen (CEA) as established cancer cell marker. CD44 was only expressed at a low level, and the expression level decreased over time. CD133 was expressed at a slightly higher level, and the expression increased over time, suggesting a slight enrichment of CD133 posi-

tive cells. CD166 was expressed at a low level and the expression level did not change over time. Almost all cells were CEA positive, suggesting that the population consisted mainly of cancer cells.

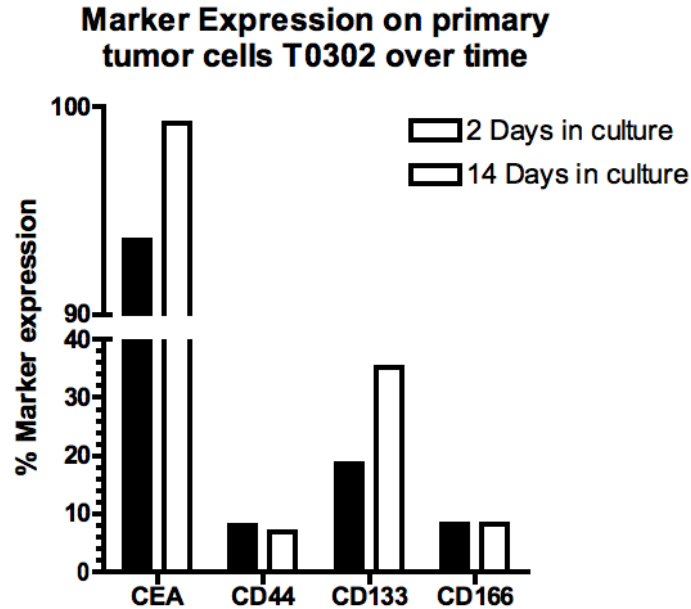


Figure 3.29: Marker expression on primary tumour cells T0302 over time. FACS analysis for the proposed tumour stem cell markers CD44, CD133 and CD166 and the cancer cell marker carcinoembryogenic antigen (CEA) 2 days and 14 days after establishing a primary tumour cell culture of the colon tumour T0302.

In the course of these experiments, two primary cell cultures stood out as they had remarkably different characteristics than the average primary cell cultures. The primary cell culture T2312 was one of these (figure 3.30).

The most remarkable feature of this cell culture was the longevity, as those cells are still in culture after more than 6 months in culture at the current point of time.

But also its morphological characteristics are remarkable. T2312 consists of two distinct subpopulations. One subpopulation grows adherently in 3D plateaus that highly resemble LT97 cell cultures (figure 3.30A) with spheroid shaped structures growing on top of the plateaus. The other subpopulation consists of spheroid shaped aggregates of cells that constantly could be harvested from the adherent cell culture.

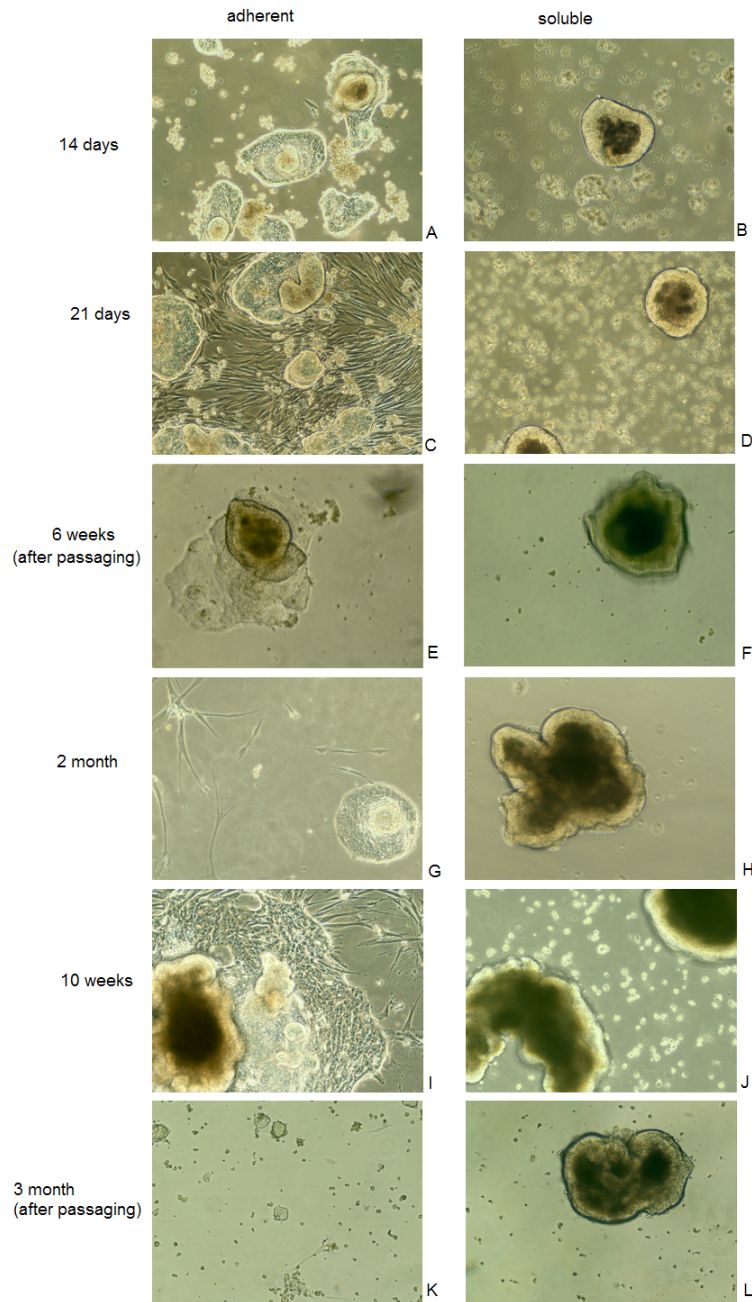


Figure 3.30: Primary cell culture from colon tumour T2312. Pictures taken at 20x magnification. A= adherent cells after 14 days, B= soluble cells after 14 days, C= adherent cells after 21 days, D= soluble cells after 21 days, E= adherent cells after 6 weeks, 1 week after 1:2 passage, F= soluble cells after 6 weeks, G= adherent cells after 2 month, H= soluble cells after 2 month, I= adherent cells after 10 weeks, J= soluble cells after 10 weeks, K= adherent cells after 3 month, 1 week after 1:2 passage, L= soluble cells after 3 months.

It could be observed that these spheroids are shed into the medium from the spheroid like structures on top of the plateaus. At each medium change for the adherent cell culture spheroids were found in the cell supernatant that were isolated and collected in a separate culture flask (figure 3.30B). Also the detachment of the spheroid like structures from the 3D plateaus could be observed under the light microscope several times. The spheroids were round in shape, and slightly resembled blastocysts.

After 21 days in culture, it has been observed that fibroblasts started to grow out of the adherent cell colonies (figure 3.30C). It has been verified that these cells indeed were fibroblasts by a cytokeratine/vimentin staining performed by Brigitte Marian. The adherent colonies were proliferating, not only by producing spheroid cells, but also increasing the size of their 3D plateaus. After 22 days in culture, the adherent cell culture was passaged. The cells were attached very tightly to the ground, so that incubation with Trypsin/EDTA was necessary.

After the first passage, the adherent cells needed several days to attach to the ground, and only grew slowly. The fibroblasts had disappeared and did not reappear for about a month, as it is shown in figure 3.30E (after 6 weeks in culture, 3 weeks after passaging). However after about 2 months in culture (figure 3.30G), fibroblasts could again be found in the adherent culture, though the proliferation and growth ability of the adherent cells was still reduced compared to any point of time before the passage.

Meanwhile, it could be observed that the spheroids in the separate culture flask had not only grown in numbers, as constantly new spheroids were harvested from the adherent subpopulation, but also the single spheroids had grown in size (figure 3.30 B,D,F,H). After 2 months in culture (figure 3.30H), the spheroids were about twice the size they had after 21 days in culture (figure 3.30B), and their shape also started to change. The shape of the spheroids was not homogenous (figure 3.30H,J,L), though most of the spheroids developed short, round excrescences as it is depicted in figure 3.30H.

After 10 weeks in culture, the number of the fibroblasts had increased (figure 3.30I). At the same time, the proliferation rate of the adherent cells seemed to have increased as the colonies were increasing in size at a higher rate than before. One week later, after 11 weeks in culture, the adherent cells had proliferated enough for another passage. This time one week after passaging the cells, after 3 month in culture (figure 3.30K), very few fibroblasts could be found already aside to the reattached adherent cells, though the colonies were still small in size.

To further analyse the primary cell culture T2312, with special attention on the two distinct subpopulations, the expression of the proposed tumour stem cell markers CD44,

CD133 and CD166 as well as the expression of the established cancer cell marker CEA was examined by FACS analysis after 21 days in culture (figure 3.31).

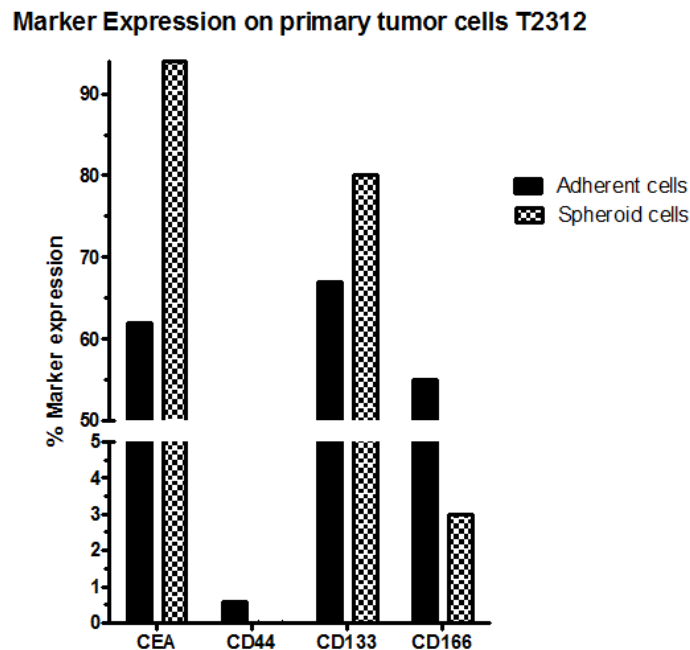


Figure 3.31: Marker expression on adherent and spheroid soluble primary tumour cells from the colon tumour T2312. FACS analysis for the proposed tumour stem cell markers CD44, CD133 and CD166 and the cancer cell marker carcinoembryonic antigen (CEA).

The FACS analysis revealed that the two subpopulations have different characteristics. First of all, only about 60% of the adherent cells expressed the cancer cell marker CEA, while the spheroid cells are almost all CEA positive. Further the suggested tumour stem cell marker CD44 is only expressed at a very low level in adherent cells, and not at all expressed in spheroid cells. CD133 is expressed at a moderate level with about 65% in the adherent cells, and with 80% at a high level in the spheroids. CD166 is expressed in about 50% of the adherent cells, but it is barely expressed in the spheroids with less than 3%.

4 Discussion

4.1 Investigating LT97 as Model Cell Line for Premalignant Colon Cancer Stem Cells

According to the tumour stem cell theory (Zajicek, 1980), adult stem cells and early progenitor cells are very likely the origin of a range of different cancers. As the mucosa of the colon and rectum constitutes a tissue which is constantly renewing, adult colon stem cell play a significant role in normal tissue homeostasis and their activity is essential for the high turn over rate of the epithelial cells. The colon and colorectal adult stem cells are located at the bottom of the colonic crypts, and although it is thought that there are only a few adult stem cells per crypt (Potten, 1995), those cells produce all necessary cell types of the colonic mucosa.

Adult colon stem cells and early progenitor cells express the hyaluronic acid receptor CD44 (Dalerba et al., 2007), which is a Wnt-target gene and indicates Wnt pathway activation (Battle et al., 2002; Van de Wetering et al., 2002). In a vast majority of colon cancers the initial step for tumourigenesis is a deletion or mutation of APC, which leads to a constitutively active Wnt pathway (Fearon et al., 1990). Furthermore, CD44 has been found to be a marker for breast (Waterworth, 2004) and prostate (Collins et al., 2005) tumour stem cells. Due to these findings, CD44 has been suggested to be a possible marker for colon tumour stem cells (Dalerba et al., 2007; Schulenburg, 2006).

In preceding experiments the human colorectal adenoma model cell line LT97, which has an APC deletion, a mutated Ki-Ras, but a still functional p53, proved to possess a distinct CD44 positive subpopulation of about 60%, that had better growth and plating efficiency characteristics than the CD44 negative subpopulation (Schulenburg et al., 2007). The CD44⁺ cells did not only have a higher survival rate, they also produced CD44⁻ progeny over time, re-establishing the original composition of this cell line. The CD44 positive cells showed a high expression of nuclear β -Catenin, the CD44 negative cells on the other hand did not have nuclear β -Catenin and rapidly underwent apoptosis

(Schulenburg et al., 2007). Hence the CD44 positive subpopulation has been used as model for the proposed colon tumour stem cells.

4.2 Do Apoptosis Associated Proteins play a Role in Reduced Survival of CD44- LT97 Cells?

Adult colon cells express anti-apoptotic proteins like Bcl-2 and survivin. However, Bcl-2 has been found to be up regulated in the CD44 positive cells only on RNA level, but not on protein level. The Bcl-2 associated Protein BAX was not expressed differently in the CD44 positive and negative cells both on protein and RNA level. This suggests that these proteins do not account for the enhanced survival abilities of the CD44 positive subpopulation.

In contrast, the anti apoptotic protein survivin, which is also a β -Catenin target gene, was clearly up regulated both on RNA and protein level in CD44 positive LT97 cells. The high expression level of survivin may be due to activation of the Wnt-pathway in CD44 positive LT97 cells as described by Schulenburg et al., 2007.

signalling through the Wnt-pathway has been shown to be important for development, cellular homeostasis, and disease (Rizvi et al., 2005). Constitutive activation of the Wnt-pathway has been found in a multitude of cancers. Additionally, blocking of the Wnt pathway with the inhibitor sulindac, a small molecule antagonist of β -Catenin (Clapper et al., 2004), led to a strongly reduced plating efficiency and viability of the CD44 positive cells. Sulindac can modulate the sub cellular localization of β -Catenin in vivo, decreasing the amount of nuclear β -Catenin and leading to accumulation of β -Catenin in the plasma membrane (Boursi et al., 2007). The Wnt pathway activation seems to be important for both the survival and plating efficiency, which might also be connected to the increased expression of anti-apoptotic Wnt- target proteins like survivin.

4.3 Is Survival of CD44+ LT97 Cells transmitted by IGF-1 Pathway Activation?

Deregulated tyrosine kinase receptor signalling has been found in many colon cancers (reviewed by Kumar, 2005). This involves EGFR (Barnard et al., 1995), FGF receptors

(Sonvilla et al., 2008, Shimokawa et al. 2003), as well as IGFR (Calle and Kaaks, 2004). In CD44 positive LT97 cells IGFBP3 was found to be down regulated in a preceding RNA microarray experiment. IGFBP3 is a protein which binds IGF-1 and IGF-2 and therefore prevents binding and activation of the IGF-1 receptor (Durai et al., 2005; Polak 2004), and its serum levels have been related to colon cancer risk in several reports (Zhang et al., 1997). Its secretion by tumour cells might locally inhibit IGF mediated survival signals. Hence the role of IGF-1 signalling modulation as exogenous factor has been investigated in CD44 positive and negative LT97 cells. IGFBP3 was down regulated in the CD44 positive cells on RNA level, which should permit IGF-1 mediated receptor activation and increased growth potential of the CD44 positive cells. Actually, insulin receptor substrate 1/2 (IRS), the primary substrate phosphorylated upon IGF-1R activation, was found to be higher phosphorylated in CD44 positive than in CD44 negative cells. In addition, IRS was found to be up regulated in the CD44 positive cells on the protein level. Further, downstream ERK1/2 has a higher phosphorylation status in the CD44 positive cells.

In the Akt-pathway both GSK3 β and S6 were both down regulated on the protein level and less phosphorylated in the CD44 negative cells. The down regulation of key molecules of the Akt and IGF pathways on protein level in CD44 negative cells suggest that these pathways are per se shut down within the CD44 negative cells.

The cyclolignan picropodophyllin (PPP) blocks the IGF-1 Receptor and down regulates IGF-1R mediated signalling (Girnita et al., 2004), and has been used to further examine the IGF-1 signalling pathway. Blocking of the IGF-1 Receptor with the compound PPP in approved concentrations (100nM) proved to reduce the growth and viability, but had less impact on the plating efficiency of CD44 positive LT97 cells. Only at concentrations that exceeded IGF-1R specificity (1 μ M) plating efficiency was reduced.

In view of the extensive shut down of IGF1 signalling in CD44 negative cells it is not surprising that it was not possible to enhance plating efficiency and viability by stimulation with IGF-1 in this cell population. From the collected data, it can be concluded that the IGF-1 signalling seems to be important in providing the CD44 positive cells with growth and survival signals. However, this seems to be due to an up regulation of endogenous players of this pathway, which enables a better response to IGF-1 stimulation in CD44 positive cells.

This seems to indicate that other receptor tyrosine kinases such as FGFR, EGFR or PDGFR, activate similar pathways as the IGF-1R upon stimulation ("Pathways in cancer - Homo sapiens (human)" © Kanehisa Laboratories, published in Kyoto En-

cyclopaedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg>) and might compensate for the lack of IGF-1R signalling to a certain degree.

4.4 The Role of FGF Receptor Signalling in CD44+/- LT97 Cells

In a RNA microarray experiment, FGFR3 and FGFR4 were up regulated in CD44 positive LT97 cells, indicating a possible role for FGF receptor signalling in the enhanced growth and survival abilities of these cells. FGF receptors undergo ligand-dependant dimerisation and autophosphorylation and induce several signalling pathways such as the MAPK pathway, the STAT pathway and the Akt pathway (Pawson, 1995). Akt signalling is implicated in several processes including cell proliferation, differentiation and survival (Neri et al., 2002), and constitutively active STAT has been connected to malignant transformation (Turkson, 2004), tumour angiogenesis and modulation of immune responses in favor of tumour immune invasion (Chen and Han, 2007).

The FGFR3 splice variants FGFR3IIIb and FGFR3IIIc have been investigated in several tumour types. Alternative splicing of FGFR3 has been detected to play a role in multiple myeloma patients over expressing FGFR3 (Soverini et al., 2002) and bladder cancer (Tomlinson, L'Hote, Kennedy, Pitt and Knowles, 2005), and furthermore it has suggested to also play a role in colon cancer (Sonvilla et al., 2009). Additionally, a binding partner of FGFR3IIIc, FGF18, is up regulated in colon tumourigenesis (Shimokawa et al., 2003) and has been suggested to exert autocrine and endocrine oncogenic functions in colorectal cancer (Sonvilla et al., 2008). However, we did not find a significant difference in gene expression of FGFR4 and the FGFR3IIIb splice variant and protein expression of both total FGFR3 and FGFR4. Only the receptor splice variant FGFR3IIIc was proven to be significantly up regulated on RNA level in the CD44 positive subpopulation. The FGFR3 agonist FGF18 has been investigated as well, though its expression on RNA level has not been increased in CD44 positive cells. Treatment of CD44 negative LT97 cells with FGF18 did not increase the plating efficiency and viability characteristics of this subpopulation. However, infections with an adenoviral construct which over expressed FGF18 increased both plating efficiency and viability of the CD44 positive subpopulation, but not of the CD44 negative subpopulation.

A stimulating signal can only be established if the growth factor can bind to a functional

receptor. FGF18 stimulates FGFR3IIIc but not FGFR3IIIb (Sonvilla et al., submitted 2009). The CD44 positive but not the CD44 negative LT97 cells showed an up regulation of FGFR3IIIc. Consequently, CD44 negative cells were insensitive to FGF18 stimulation, while CD44 positive cells were responsive. This conclusion was also supported by the effect of over expression of FGF18 from an adenoviral vector in CD44 positive and negative cells. Plating and viability was increased in CD44 positive cells, which suggests that FGFR3IIIc plays a role in the enhanced growth and survival features of this sub-population.

The infection with a dominant negative FGFR3IIIc virus construct led to enhanced plating efficiency and viability of CD44 positive cells if compared to the control cells infected with a GFP expressing virus construct. But the stimulation was still lower than with the FGF18 virus infected cells, which could indicate to a non adequate control virus. There is also the possibility that the dominant negative FGFR3IIIc dimerizes with a growth inhibiting receptor, since this receptor can also heterodimerize with other FGF receptors. In addition, there was a decrease in cell survival induced by dominant negative FGFR3IIIc. Viability after seven days was lower for the FGFR3IIIc infected cells, compared to plating efficiency that had been assessed after 2 days. This indicates that FGFR3 signalling may not be as important for plating, but more for growth and viability.

Another interesting observation was that FGF18 over expression in CD44 positive cells protected the cells from PPP mediated apoptosis. It might indicate that both FGFR3 and IGF-1 signalling share an equal responsibility in providing the cell with a survival signal, since both signalling pathways modulate the MAPK and Akt pathway. Hence the overly stimulated FGFR3 pathway could balance the loss of IGF-1 signalling.

4.5 The Development of the LT97-2 Cell Line to the LT97-3 Cell Line

In tumourigenesis, cells obtain a state of genetical change, which is called mutator phenotype (Martin, 1991). A deletion of APC as it is found in LT97 cells (Richter et al., 2002) may destabilize the karyotype of the cell and cause this phenomenon. Usually carcinoma model cell lines have reached an equilibrium in which further genetic alterations cause more damage than advantage for the cell so that it remains stable, unless conditions are drastically altered (eg. presence of a chemotherapeutic compound exerts selective pressure). However, LT97 cells are still at an early passage number and more

rapidly growing subpopulations are still emerging and can easily be selected by variations of culture conditions. In the course of experiments an enhanced passage rhythm selected for the cells with the best attachment and self renewal characteristics. The end product of this process was named LT97-3. We observed that the size of the CD44 positive subpopulation increased, while the expression level of CD44 both on RNA and protein level decreased.

In plating efficiency experiments with LT97-3 cells (data not shown) the CD44 negative cells showed comparable plating efficiency as the CD44 positive cells. As the CD44 expression decreased, IGFBP3 expression increased. Those data suggest that the CD44 positive and CD44 negative subpopulations started to become more alike to each other to form an intermediate population. At the same time, the overall plating, growth and survival capabilities of this cell line was increased, which is in line with tumour progression towards a more carcinoma cell type. Changes in the gene expression of IGFBP3 and CD44 were inversely related, indicating some sort of connection between those pathways in LT97 cells. IGFBP3 was one of the genes with the highest regulation factor in CD44 negative cells in the microarray experiment. Therefore the question is whether CD44 and IGFBP3 share common regulatory elements or whether they control one another. Looking at potential stem cell markers, it was observed that Lgr5 RNA expression decreased parallel to CD44 RNA expression, indicating that Lgr5 and CD44 might be within the same cluster of gene regulation. The expression level of CD44 went up in the LT97-3 cells, the expression level of CD133 decreased, indicating an inverse regulation.

4.6 Can AKH4/14 Cells be used as Model Cell Line for tumour Stem Cells in Colon Carcinoma?

AKH4 and AKH14 are two cell lines that were derived from the same liver metastasis of a colon carcinoma by different culturing schemes producing different growth characteristics. Both contained a CD44 positive subpopulation which had enhanced growth and survival characteristics compared to the CD44 negative subpopulation. The CD44 positive cells grew in distinct foci, as it is also the case in LT97 cells. However, with the only exception of Bcl-2 gene expression, analysis demonstrated that most of the genes differentially regulated in LT97 subpopulations were not affected. A future RNA microarray experiment should be able to detect possible other pathways involved in the

enhanced survival features of the proposed cancer stem cells in colon carcinoma.

4.7 Establishing Primary tumour Cell Cultures from Colon Cancer Tissue

In order to investigate a tumour stem cell subpopulation in colon cancer it is necessary to examine cells obtained directly from solid colon tumour specimen. For this purpose primary cell cultures were established from patient colon tissue by mechanical separation and enzymatic digestion. Most primary cultures died within a matter of weeks, which suggests that either no tumour stem cells were present within those cultures, or that the culture conditions were not sufficient for the survival of the tumour stem cells. As only a small fraction of the whole cancer specimen was used to establish a primary cell culture, and it is not yet known, where the proposed tumour stem cells in colon cancer might be located, it is possible that no tumour stem cells were contained in the tissue to begin with. However two primary cultures showed extended growth characteristics suggesting that indeed tumour stem cells were present. It is remarkable that fibroblasts were found only in these cultures, and enhanced growth and proliferation was found when fibroblasts were present in the culture. As also embryonic stem cells are usually cultured on a feeder layer of fibroblasts (Thomson et al., 1995), this suggests that a co-culture with fibroblasts may be necessary to provide the cancer stem cells with necessary factors that inhibit differentiation and maintain the stem cell state, which has been suggested before (Koopman et al., 1984). Those long living primary cultures also showed to grow in two distinct subpopulations. One subpopulation attached to the culture dish and formed 3D platforms. The other subpopulations were spheroids that grew in suspension and seemed to arise from the attached cells.

Both subpopulations showed growth and proliferation and longevity unlike the average primary cultures that have been established, indicating that tumour stem cells might be present and viable within this culture.

The two subpopulations differed in their expression of marker molecules. Spheroid cultures of colon cancer cells have been shown to express marker molecules that have been suggested for colon cancer stem cells, such as CD44, CD133, CD166, Lgr5 and nuclear β -Catenin and showed tumour initiating capacity (Vermeulen et al., 2008).

Attached cells contained a 63% CEA-positive fraction, but the spheroids were almost

completely CEA positive, which means that those cells probably consisted of cancer cells only. While the adherent cells expressed CD133 on a medium level, the spheroids expressed those markers at a high level. CD133 positive cells from colon cancer cells growing in spheroids have been shown to have a heightened resistance to cancer therapeutics (Todaro et al., 2007), and to be highly tumourigenic (Todaro et al., 2007, Vermeulen et al., 2008, Ricci-Vitiani et al., 2007). CD44 was barely expressed in the adherent cells and not at all in the spheroids. CD166 showed to be expressed on a medium level in the adherent cells but not in the spheroids.

4.8 Investigating Musashi and Lgr5/Gpr49 as Possible Additional tumour Stem Cell Marker

Several molecules have been suggested as markers for colon cancer stem cells, such as musashi and phosphor-Pten (He et al., 2007), Lgr5/Gpr49 (Barker et al., 2007), Bmi-1 (Sangiori and Capecchi, 2008), CD133 (O'Brien et al., 2007; Ricci-Vitiani et al., 2007), CD166 (Dalerba et al., 2007) and CD44 (Dalerba et al., 2007; Schulenburg et al., 2007). In our immunocytochemistry experiments with colon adenoma tissue slides we could not detect Lgr5 to be expressed in colon adenoma tissue. Also Lgr5 could not be detected by immunocytochemistry; expression of this molecule on RNA level was detectable. This questions Lgr5 as possible marker molecule for colonic cancer stem cells.

Musashi, which is expressed at the bottom of colonic crypts and is said to play a role in asymmetric cell division of stem cells (Potten et al., 2003), was found to be expressed in colon adenoma tissue, though the expression level was very low.

In the course of experiments the assumption came up that CD44 might not be sufficient as a single colonic cancer stem cell marker. Although CD44 seems to be a valuable marker within the context of the colon adenoma stem cell model line LT97, CD44 expression was barely detectable on some of the primary tumour cells. CD133 positive cells growing in spheroids derived from colon cancer tissue have shown better long time survival than CD133 negative cells (Ricci-Vitiani et al., 2007), and they have found to be more resistant to cancer therapeutics and to be highly tumourigenous (Todaro et al., 2007).

In our experiments, CD133 expression was stable in the primary cell cultures, and its expression on protein level increased over time, indicating that due to the enrichment of CD133 cells in culture over time these cells might have increased longevity.

Also CD133 positive cells were found more in spheroids than in adherent cells. It had been found before that CD133 positive colon cancer cells grew *in vitro* as undifferentiated tumour spheres, unlike CD133 negative colon cancer cells (Ricci-Vitiani et al., 2007), that they have higher resistance to cancer therapeutics, and were highly tumourigenic in immune suppressed mice (Todaro et al., 2007). CD133 positive cells have been used to enrich for cancer stem cells in the brain (Singh et al., 2004) and the colon (O'Brien et al., 2007, Ricci-Vitiani et al., 2007).

It was also observed in the LT97-2 cells that developed towards the LT97-3 cell line, that while the expression level of CD44 went up in the LT97-3 cells, the expression level of CD133 decreased.

Possibly each marker that has been suggested so far stands for a specific feature of tumour stem cells, which might also be shared with non cancer stem cells. A cancer stem cell subpopulation might be rather detectable by an overlap of a subset of different markers than by one general marker, which is also the case in acute myeloid leukemia (Bonnet and Dick, 1997).

4.9 Summary

The results compiled in this thesis support the hypothesis that Wnt-, IGF1- and FGFR3-IIIc signalling all contribute to the enhanced growth and survival capacity of CD44+ LT97 colorectal adenoma cells. It was not possible to demonstrate that the CD44+ cells actually are colon adenoma stem cells from which carcinoma stem cells arise by additional mutation. However, the up-regulation of 3 cancer specific signalling pathways in this subpopulation as well as the observation that selection of a more rapidly growing culture from LT97 cells was accompanied by an expansion of the CD44+ population indicate that CD44 expression is connected to tumour growth and expression. For the definition of actual CSC additional markers will be needed. Among the candidates described in the literature CD133, CD166 was found on the surface of cell subpopulations. LGR5 and musashi were identified in CD44+ LT97 cells as well as in normal tissue sections, but could not be detected in tumour sections so that their value as CSC markers have to be reassessed.

5 Appendix

5.1 Abbreviations

APC	Adenomatous Polyposis Coli
APS	Ammoniumpersulfate)
BAX	Bcl-2-associated X protein
Bcl-2	B-cell CLL/lymphoma 2
bp	base pair(s)
B-RAF	V-raf murine sarcoma viral oncogene homolog B1
BSA	Bovines serum albumin
(C)CSC	(Colon) Cancer stem cell
CEA	Carcinoembryogenic antigen
Cox-2	Cyclooxygenase 2
DAB	3,3'-Diaminobenzidine
DCC	Deleted in Colorectal Carcinoma
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide
dsDNA	double-strand DNA
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
FACS	Fluorescence activated cell sorting
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
FOXO	Forkhead box O
FRS2	Fibroblast growth factor receptor substrate 2

GAPDH	Glycerinaldehyd-3-phosphat-dehydrogenase
GFP	Green fluorescent protein
GSK3(β)	Glycogen synthase kinase 3 beta
HRP	Horseradish peroxidase
ifu	Infectious units
IGF	Insulin-like growth factor
IGF-1R	Insulin-like growth factor 1 Receptor
IGFBP	IGF binding protein
IRS	Insulin receptor substrate
JNK	c-Jun N-terminal kinase
Ki-Ras	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
Lgr5	Leucine-rich repeat-containing G protein-coupled receptor 5
MAPK	Mitogen-activated protein kinase
MOI	Multiplicity of infection
Msi(1)	Musashi
mTOR	Mammalian target of rapamycin
MUW	Medical University of Vienna
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSAID	Non-steroidal anti-inflammatory drugs
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PI3K	Phosphoinositide 3-kinases
PLCγ	Phospholipase C gamma
PPP	Cyclolignan picropodophyllin
PVDF	Polyvinylidene Fluoride
RT	Room Temperature
RT-PCR	Reverse transcription polymerase chain reaction
RXR	Retinoid X receptor
SDS	Sodiumdodecylsulfate
Shc	Src homology
STAT	Signal Transducers and Activator of Transcription
TAE	Tris-acetate-EDTA
TCF/Lef	T cell factor/lymphoid enhancer factor
TEMED	Tetramethylethylenediamine

TGF-β	Transforming growth factor beta
TNF-β	tumour necrosis factor beta
TRAIL	TNF-related apoptosis-inducing ligand
TRIS	Tris(hydroxymethyl)aminomethane

5.2 Literature

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Curriculum Vitae

Personal Data

Date of Birth	• December 10, 1981
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Nationality	• German

Education

2003 - 2009	• Diploma study of Molecular Biology at the University of Vienna, Austria
2001 - 2003	• Diploma study of Molecular Biotechnology at the University of Bielefeld, Germany
1992 - 2001	• Aldegrevier Gymnasium Soest, Germany, Graduation: Abitur (Diploma from German secondary school qualifying for university admission or matriculation) passed with honours
1990 - 1992	• Freie Waldorfschule Hamm, Germany
1988 - 1990	• Patroklischule Soest, Germany

Lab Experience

May 2008 - March 2009	• Diploma work "Investigating a model cell line for colorectal adenoma stem cells", Medical University of Vienna
April 2008	• Lab rotation "Optimization of Methods for Characterisation of tumour stem cells in colon carcinoma", Medical University of Vienna
October 2007	• Lab rotation "The Role of Anti-MuSK Antibodies in AChR seronegative Myasthenia gravis", Center for Brain Research, Vienna
February - March 2007	• Lab rotation "The differentiation of microglia cells in vivo and in vitro", Center for Brain Research, Vienna
November 2006	• Lab rotation "Basics of Neuroscience", Center for Brain research, Vienna
2001 - 2006	• Lab rotations within the scope of the undergraduate school programmes in chemistry, organic chemistry, molecular biology, cell biology, biochemistry, structural biology and biotechnology, University of Vienna, University of Bielefeld