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Cell Cycle and Proliferative Activity of Human Colon Cancer Initiating Cells

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Summary

In human colon cancer only a small subfraction of all tumor cells is able to rebuild the tumor in immunodeficient mice. It has been hypothesized that the proliferative activity of these tumor initiating cells (TIC) may differ from the bulk of the tumor cells and that mitotic quiescence of TIC may contribute to chemotherapy resistance or relapse after treatment. By genetic marking, it has previously been shown that a variable proportion of all human TIC contributed to tumor xenograft formation only late after serial transplantation suggesting that these delayed contributing TIC indeed might have been quiescent in primary recipient mice.

In order to investigate the cell cycle and proliferative activity of human colon TIC *in vitro* and *in vivo*, human colon cancer patient samples were dissociated and cultured under serum free conditions favoring the outgrowth of tumor spheres enriched for TIC.

The CFSE label-retaining assay was used to analyze the proliferative activity of human colon TIC *in vitro*. It allowed discrimination of fast (F), slow (S) and rarely dividing (R) cell fractions suggesting that a rarely dividing population of human colon TIC might exist *in vivo* as well. Cell surface markers previously associated with tumor initiating potential (CD133, CD44, EpCAM and CD166) were equally expressed in all proliferative subfractions. A limiting dilution assay confirmed the self-renewal potential of spheroid cells. Furthermore, it revealed that the frequency of sphere forming cells (SFC) was similar in the fast, slow and rarely dividing fraction within individual sphere lines, demonstrating that the vast majority of all SFC were rapidly cycling *in vitro*. To assess the *in vivo* tumor initiating potential and self-renewal ability, equal cell numbers of sorted R, S and F cells were transplanted into immunodeficient mice. All sorted cell fractions of three patients formed tumors, irrespective of their proliferative kinetics *in vitro*. Moreover, the majority of cells within serially transplanted tumors originating from $CFSE^+$ fractions lost fluorescence intensity indicating that they actively cycled after transplantation. Hoechst/Pyronin-staining of dissociated sphere cells allowed investigation of their cell cycle status. Equal numbers of G_1-, $S/G_2/M$- and G_0-cells were transplanted under the kidney capsule of immunodeficient mice. Each cell fraction comprised self-renewing, human colon TIC as shown by a serial transplantation assay. In order to investigate the proliferative activity of human colon TIC within an established tumor *in vivo*, intra-tumoral cell divisions were tracked using a genetic high resolution label-retaining assay. A tetracycline-regulated H2B-GFP expression system was implemented into spheroid cells by lentiviral transduction prior to transplantation. H2B-GFP-expression was suppressed after establishment of the tumor microenvironment. Further cell divisions dilute the GFP-label and thereby enable

analysis of the cell's proliferative activity. FACS analysis of formed tumors revealed fast, slow and rarely dividing cell fractions *in vivo*. All cell fractions harbored self-renewing, human colon TIC as shown by serial transplantation. Interestingly, only quiescent TIC showed a polyclonal contribution to tumor formation in mice. A proportion of quiescent TIC might have been activated to proliferate upon chemotherapeutic treatment.

This study demonstrates that human colon cancer harbours tumor initiating cells with differing cell cycle status and proliferative activity. Self-renewing colon TIC were present in all cell cycle phases demonstrating that the tumor initiating potential is not restricted to a dormant cell cycle status. A rarely dividing population of human colon TIC derived from different patient samples exists *in vitro* and *in vivo*. However, the majority of colon TIC rapidly divided in sphere cultures as well as *in vivo*. Colon TIC were found to be enriched in the quiescent population and were recruited to tumor formation upon chemotherapeutic treatment. Our results provide basis for a better understanding of quiescence and proliferation of human colon TIC. This will hopefully lead to the development of innovative treatment strategies directed against colon cancer initiating cells.

Keywords: colon cancer initiating cell, quiescence, dormancy, chemotherapy, resistance

Zusammenfassung

Nur eine kleine Fraktion der Zellen des humanen kolorektalen Karzinoms ist in der Lage, Tumore in serieller Transplantation in immundefizienten Mäusen zu bilden. Bisher wurde angenommen, dass die proliferative Aktivität solcher Tumor-initiierender Zellen (TIC) sich von der des Großteils der Tumorzellen unterscheidet. Ein mitotischer Ruhezustand, die sog. Quieszenz, trägt möglicherweise zu Therapieresistenz gegenüber Chemotherapeutika und Wiederkehr der Krankheit nach einer Therapie bei. Genetische Markierungsarbeiten haben gezeigt, dass eine humane TIC-Subpopulation erst zu späteren Zeitpunkten zum Tumorwachstum in transplantierten Mäusen beigetragen hat und dass sie anfangs inaktiv waren. Diese verzögert beitragenden TIC (DC-TIC) waren daher möglichweise quieszent und wurden reaktiviert.

Um das Zellzyklus- und Proliferationsverhalten humaner Kolonkarzinom-initiierender Zellen *in vitro* und *in vivo* zu untersuchen, wurden Sphäroidkulturen aus primärem Kolonkarzinom-Patientengewebe etabliert, die mit TIC angereichert sind. Die proliferative Aktivität der TIC wurde *in vitro* mittels CFSE-Färbung untersucht. Es konnten schnell (F), langsam (S) und kaum teilende (R) Kolon-TIC unterschieden werden, was die Existenz ruhender Kolon-TIC *in vivo* impliziert. Außerdem konnte keine Anreicherung von CD133, CD44, EpCAM oder CD166 in einer der proliferativen Fraktionen festgestellt werden. Eine Verdünnungsreihe vereinzelter Sphäroidzellen belegte deren Selbsterneuerungspotential und zeigte weiterhin dass die Frequenz von sphärogenen Zellen (SFC) inter-fraktionär ähnlich ist. Die Mehrheit der SFC proliferierte aktiv *in vitro*. Mittels durchflusszytometrischer Zellsortierung wurden schnell, langsam und kaum teilende TIC aufgetrennt und anschließend in immundefiziente Mäuse transplantiert um Selbsterneuerungsfähigkeit und Tumor-initiierendes Potential in Abhängigkeit von der proliferativen Aktivität *in vivo* zu untersuchen. F-, S- und R-Zellen trugen zum Tumorwachstum in Mäusen bei. Die Mehrheit der angewachsenen Tumorzellen, auch wenn als reine $CFSE^+$-Fraktion transplantiert, zeigte kein CFSE mehr. Demnach hat sich der größte Teil der Zellen nach Transplantation aktiv geteilt. Des Weiteren wurde der Zellzyklusstatus von dissoziierten Späroidzellen mittel Hoechst/Pyronin-Färbung bestimmt. Die serielle Transplantation von G_1-, $S/G_2/M$- und G_0-Zellen zeigte, dass jeder Zellzyklusfraktion selbsterneuerungsfähige humane Kolon-TIC beinhaltete. Das proliferative Verhalten humaner Kolon-TIC innerhalb eines bereits etablierten Tumors wurde an Hand einer genetischen Markierung von Kolon-TIC untersucht. Sphäroidzellen wurden mittels lentiviraler Transduktion mit einem Tetrazyklin-induzierbaren H2B-GFP-Expressionssystem ausgestattet und anschließend in Mäuse transplantiert. Nach Etablierung des Tumormilieus wurde die H2B-GFP-Expression mittels Tetrazyklingabe

über das Trinkwasser der Mäuse inhibiert. Weitere Zellteilungen transduzierter Zellen führen zu einer Verdünnung des GFP-Markers und lassen daher eine Analyse der proliferativen Aktivität solcher Zellen zu. Die durchflusszytometrische Analyse gewachsener Tumoren bestätigte eine schnell, langsam und kaum teilende Zellpopulation innerhalb des Tumors *in vivo*. Die proliferativen Fraktionen zeigten keine Unterschiede hinsichtlich Selbsterneuerungspotentials während serieller Transplantation. Des Weiteren konnte die ruhende Kolon-TIC-Fraktion durch 5-Fluorouracil-Behandlung der Mäuse angereichert werden. Möglichweise wird eine Subfraktion ruhender TIC durch Chemotherapiegabe zur Teilung angeregt.

Diese Studie zeigt, dass das humane Kolonkarzinom TIC mit unterschiedlichem Zellzyklusstatus und Teilungsverhalten beinhaltet. In allen Zellzyklusphasen waren selbst-erneuernde TIC zu finden. Dies beweist, dass das Tumor-initiierende Potential nicht auf eine ruhende Zellzyklusphase beschränkt ist. Eine kaum teilende TIC-Population existiert tatsächlich *in vitro* und *in vivo*. Jedoch war der größte Teil an TIC schnell teilend in Sphäroidkulturen und Mäusen. Die klonalen Analysen implizieren, dass Kolon-TIC in der ruhenden Fraktion *in vivo* angereichert sind und durch 5-Fluorouracil-Behandlung aktiviert wurden um zum Tumorwachstum beizutragen. Unsere Ergebnisse tragen zu einem besseren Verständnis von Quieszenz und Proliferation humaner Kolon-TIC bei. Dies wird hoffentlich zu der Entwicklung von innovativen Behandlungsstrategien führen, die spezifisch gegen Kolonkarzinom-initiierende Zellen gerichtet sind.

Schlüsselwörter: Kolonkarzinom, Quieszenz, Ruhezustand, Chemotherapie, Resistenz

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List of Abbreviations

5-FU	5-Fluorouracil
AML	Acute myeloid leukemia
APC	Adenomatous polyposis coli
ATP	Adenosine-5'-triphosphate
BrdU	5-bromo-2-deoxyuridine
BSA	Bovine serum albumin
CBC	Crypt base columnar cell
cDNA	Complementary DNA
CFDASE	5-(and-6)-carboxyfluorescein diacetate succinimidyl ester
CFSE	5-(and-6)-carboxyfluorescein succinimidyl ester
CML	Chronic myeloid leukemia
COX	Cytochrome c oxidase
CRC	Colorectal Cancer
CSC	Cancer stem cell
DC-TIC	Delayed contributing tumor initiating cell
ddNTP	Dideoxynucleotides
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
dNTP	Deoxynucleosidetriphosphates
Dox	Doxycyclin
ds	Double stranded
dTTP	Deoxythymidine triphosphate
EGF	Human Epidermal Growth Factor
EMT	Epithelial-Mesenchymal Transition
FACS	Fluorescence activated cell sorting or flow cytometry
FAP	Familial adenomateous polyposis
FGF	Human Fibroblast Growth Factor

FOLFOX	Leucovorine + 5-Fluorouracil + Oxaliplatin
H2B-GFP	Histone 2B fused to green fluorescent protein
HIV-1	Human immunodeficiency virus type 1
HNPCC	Hereditary non-polyposis colon cancer
Hoe	Hoechst 33342
HSC	Hematopoietic stem cell
i.p.	Intraperitoneally
ISC	Intestinal stem cell
LIC	Leukemia initiating cell
LRC	Label-retaining cell
LTR	Long terminal repeat
LT-TIC	Long-term tumor initiating cell
LV	Lentivirus
MET	Mesenchymal-Epithelial Transition
MFI	Mean fluorescence intensity
MMR	mismatch repair
MOI	Multiplicity of infection
MRD	Minimal residual disease
MSI	Microsatellite instability
NOD	Non-Obese Diabetic
PCR	Polymerase Chain Reaction
PEI	Polyethylenimine
PIC	Pre-integration complex
PPT	Polypurine tract
PVDF	Polyvinylidene difluoride
PY	Pyronin Y
RNA	Ribonucleic acid
RT	Room temperature
SCID	Severe Combined Immunodeficiency

SFC	Sphere forming cell
SIN	Self-inactivating
ss	Single stranded
TAC	Transit-amplifying cells
TIC	Tumor initiating cell
TS	Thymidilate synthetase
T-TAC	Transient tumor amplifying cell
VSV-G	Vesicular stomatitis virus-glycoprotein g

1 Introduction

1.1 Colon and colonic epithelium

As the physically last part of the digestive system, the colon is mainly responsible for concentration of digestive waste products and their transport. It is densely populated with microorganisms which degrade previously undigested material from the chyme[1]. Water, fatty acids, electrolytes and some vitamins are absorbed by epithelial cells[2]. The colonic epithelium is organized in crypt-like units[3] (Figure 1): at the bottom of each crypt, adult stem cells and mesenchymal cells surrounding the crypt-base can be found making up the stem cell niche. Intestinal stem cells (ISCs) actively proliferate and give rise to transit-amplifying cells (TAC) migrating upwards among the crypt[4]. At the crypt's top they exit the cell cycle and reach a highly differentiated, post-mitotic state fulfilling determined functions: enterocytes are responsible for absorption of nutrients and water whereas enteroendocrine and goblet cells are involved in secretion of hormones and mucus[5-7]. At the end of their life span, they undergo apoptosis and are shed into the gut lumen. The alternation of contraction and relaxation of muscles in the colonic wall is responsible for propelling its content (peristalsis)[8, 9].

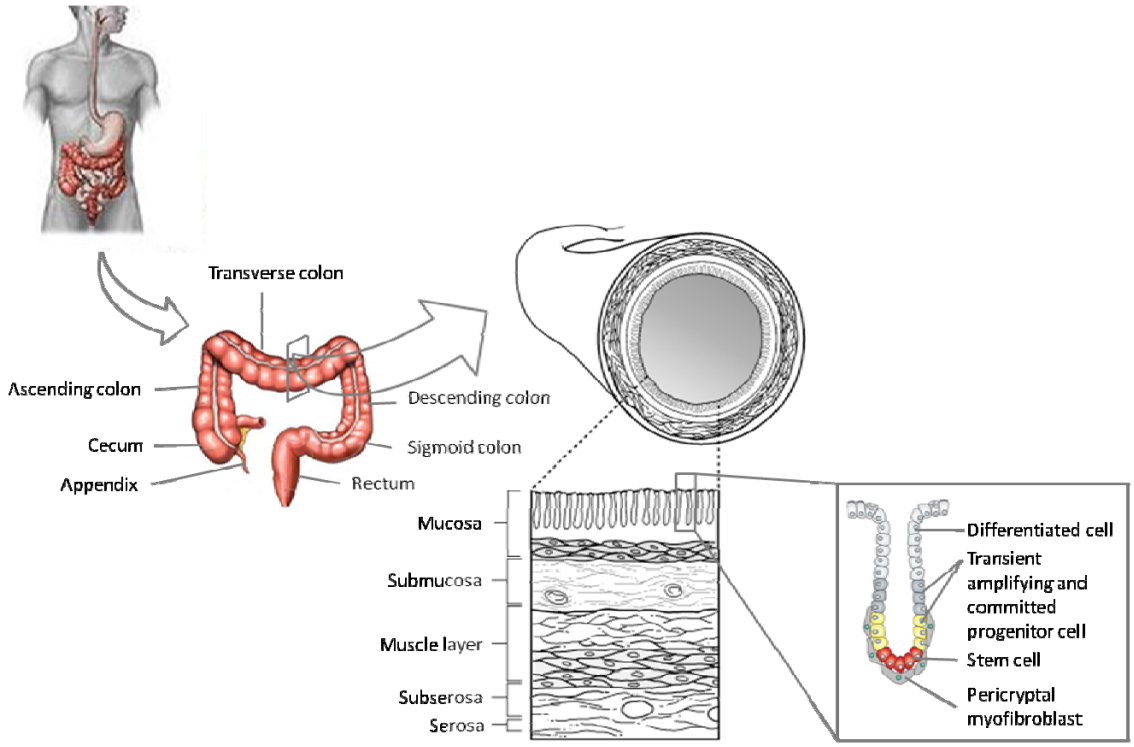
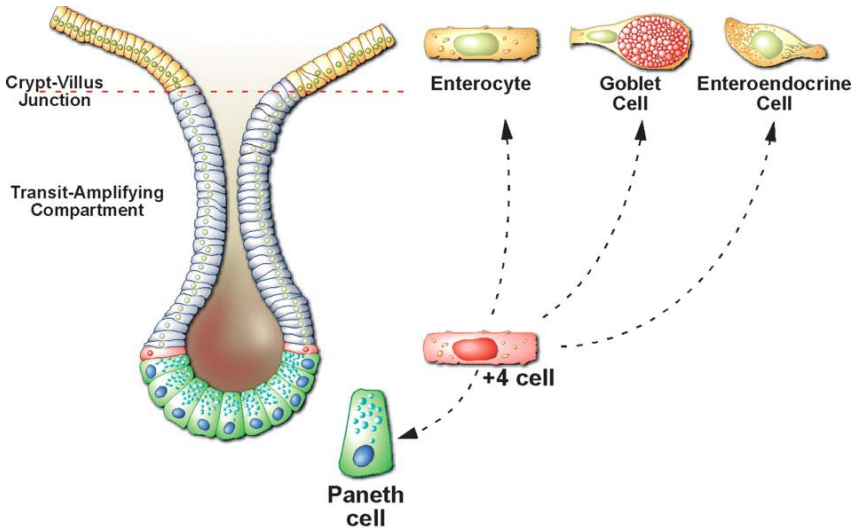


Figure 1: The human colon.

The inner layer of the colonic wall is organized in finger-like invaginations called crypts. Each crypt comprises intestinal stem cells at its bottom giving rise to transient amplifying cells. The differentiated cells at the top fulfill absorbing and secreting functions.

The concept of adult stem cells required for human tissue maintenance and organ homeostasis is well established. Adult stem cells are characterized by two distinct features: they have the ability to self-renew and are multi-potent meaning that they can generate all differentiated cells of the corresponding tissue. Stemness can be experimentally approved either by lineage tracing or transplantation. In the hematopoietic system, the golden proof of stemness is "lifelong reconstitution of hematopoiesis in sublethally irradiated mice"[10, 11]. In the intestine, two stem cell models have been proposed (Figure 2): Potten *et al.* suggested that ISCs can be found at the +4 position, meaning 4 cells upwards from the crypt bottom ("+4 position model")[12]. In the same year, Leblond and Cheng proposed the "stem cell zone model" showing that all differentiated colonic cells originate from multi-potent colon stem cells residing more closely to the crypt bottom (crypt base columnar cells, CBC) with intermingled cells resembling paneth cells from the small intestine[6]. Experimental evidence for the clonal offspring of intestinal crypts has been provided in mice[13-15]. In 1996, Novelli *et al*. then demonstrated that human colonic crypts are clonal populations as well[16].

a) +4 position model



b) Stem cell zone model

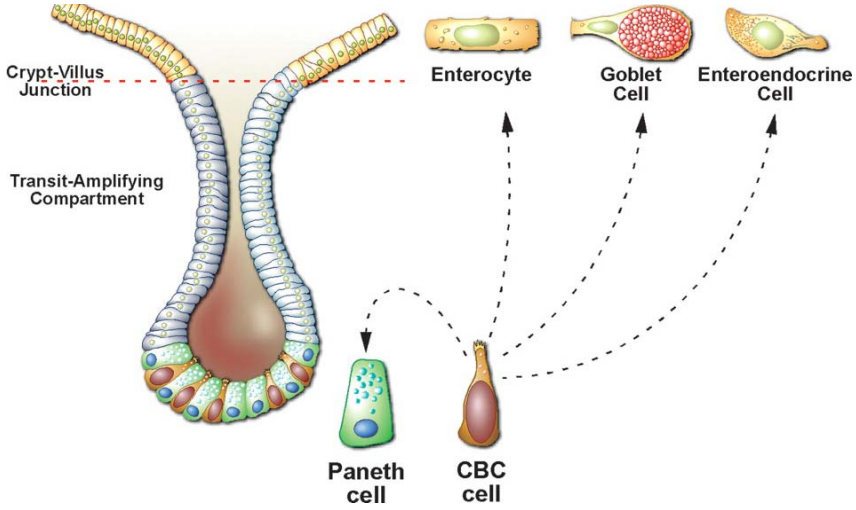


Figure 2: Intestinal stem cell models.

Intestinal stem cells are proposed to reside at the +4 position (a) or to be distributed along the bottom of the crypt (b). Adapted from Barker *et al.*, 2008[17].

Proliferation and differentiation in the crypt are mainly governed by Wnt signaling[18] (Figure 3): Wnt proteins bind to cell surface receptors of the Frizzled and LRP families. The signal is transduced to β-catenin which then accumulates in the nucleus and forms a complex with TCF. This complex acts as a transcriptional activator of target genes which have been identified by microarray-based expression studies[19]. In addition, these experiments showed that only a subset of these genes was expressed in ISC. *In vivo* lineage tracing has shown that Bmi1 expression identifies ISCs in the +4 position[20] whereas Lgr5 has been found on CBC[21]. Recent data indicates that these two stem cell populations might even convert into each other[22, 23]. Expression levels of EphB2, Ascl2, OlfM4, Musashi-1 and Prominin-1 were shown to be associated with intestinal stem

cells[24-28] but the expression of the two latter was additionally found in the TAC compartment[29]. Moreover, transit-amplifying cells showed reduced expression levels of Lgr5 and EphB2[24] indicating that a unique ISC signature remains elusive.

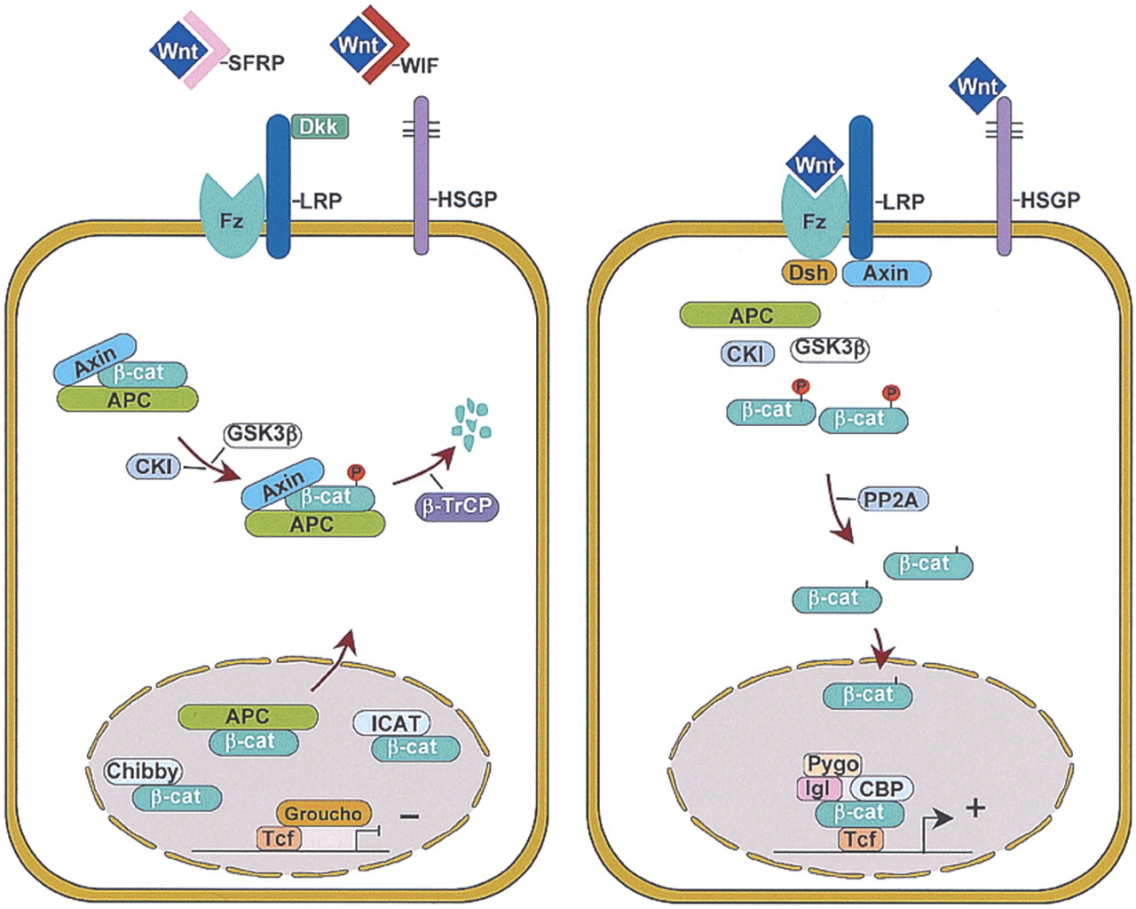


Figure 3: The Wnt canonical pathway.

(*Left*) Without Wnt stimulation, β-catenin is degraded in the cytoplasm by a degradation complex composed of APC, Axin, GSK3β, and CKI. Expression of target genes is blocked by a complex of TCF factors and transcriptional repressors. (*Right*) Wnt stimulation leads to destabilization of the degradation complex and β-catenin accumulates in the nucleus where it activates transcription of target genes. SFRP – secreted frizzled-related protein 1, WIF – *WNT* inhibitory factor, Dkk – Dickkopf, LRP – low-density lipoprotein receptor-related protein, Fz – frizzled receptor, HSGP – heparan sulfate proteoglycan, Axin – Axin 1 , β-cat – β-catenin, APC – adenomatous polyposis coli, CKI – Casein kinase 1, GSK3β – glykogen synthase kinase 3β, β-TrCP – β-transducin repeat containing protein, ICAT – inhibitor of beta-catenin and TCF-4, Tcf – T-cell factor, Dsh – Dishevelled, PP2A – protein phosphatase 2A, Pygo – Pygopus, Lgl – lethal giant larvae, CBP – CREB binding protein. Adapted from Gregorieff *et al.* 2005[30].

Quiescent stem cell pools have been demonstrated in the hematopoietic system[31], brain[32], prostate[33] and pancreas[34] where they are responsible for tissue maintenance and its longevity. In contrast to these adult stem cell systems, at least the vast majority of colonic stem cells are actively-cycling and the epithelial layer is renewed within 4-5 days[35]. TAC divide every 12-16h allowing the fast cell turnover and maintenance of the colonic epithelium[36]. However, quiescence is also supposed to play an important role in

stem cell maintenance via inhibition of differentiation and by limiting the risk of accumulating mutations[37-39]. Pericryptal fibroblasts from the intestinal stem cell niche are also assumed to influence proliferation via secretion of growth factors and cytokines[40]. Expression profiling demonstrated that genes involved in BMP, EPH and MYC signaling pathways are involved in the maturation of colonic crypts[41]. Acquired mutations in such regulatory genes lead to deregulated proliferation or misleaded differentiation which can finally result in colorectal cancer.

1.2 Colorectal Cancer

Colorectal cancer (CRC) is one of the most frequent cancers among men and women worldwide[42]. It is a progressive disease where accumulation of mutations in a cell's genome leads to transformation of normal cells into malignant ones. More than 90% of the cases are diagnosed adenocarcinoma originating from the epithelial mucosa[43]. Lymphoma, squameous cell carcinoma, malignant melanoma, neuroendocrine tumors and sarcomas are rarely accounting for primary colorectal tumors[44-48].

They all share distinct malignant properties: uncontrolled proliferation of cells, invasion into neighbouring tissues and metastasis formation in later stages. Causes of cancer differ widely but they all lead to genetic mutations in the normal cell's genome. A deficient DNA repair machinery then causes accumulation of such mutations in all cell descendants.

1.2.1 Cancerogenesis

The majority of CRC cases is associated with genetic alterations and mutations of genes involved in the Wnt pathway[49]. It is well accepted, that colorectal cancer is driven by multiple mutations accumulating over disease progression and a long period of time. Whole genome sequencing of human colon cancers revealed that only few mutations among many are common in different patient-derived samples[50, 51]. In 1990, Fearon and Vogelstein already published the adenoma-carcinoma model which is the well accepted basis for a genetic model of colon cancerogenesis[10, 52] (Figure 4): inherited or acquired mutations in the tumor suppressor gene *adenomatous polyposis coli* (*APC*) lead to loss of APC protein function. APC is part of an inactivation complex preventing transport of β-catenin into the nucleus where it acts as a transcription factor for different Wnt target genes like *CCND1* encoding cyclin D1[53]. In some cases, the β-catenin-encoding gene *CTNNB1* is mutated itself making the protein insensitive towards degradation[54]. In addition, mutations in genes encoding for components of the degradation complex lead to insufficient β-catenin-degradation[55]. Aberrant degradation of cytoplasmic β-catenin leads to its nuclear accumulation and activates the Wnt signaling pathway (Figure 3). Wnt signaling is usually only active in stem cells at the crypt bottom whereas it

gradually decreases upon migration of cells upwards[30]. Deregulated Wnt signaling drives the cells into proliferation and abnormal cell descendants replace the normal colonic epithelium. A benign cell mass is formed which is called adenoma. According to Fearon and Vogelstein, increasing genomic instability and further oncogenic hits in *KRAS*, *SMAD 4* and *TP53* drive the progression from a benign adenoma to a malignant carcinoma.

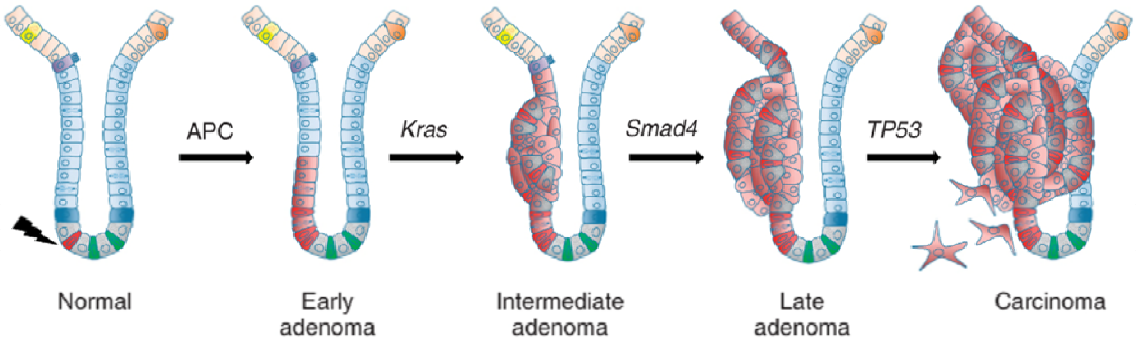


Figure 4: Model of intestinal cancer initiation and progression.

Different mutations drive the progression from a benign adenoma to a malignant carcinoma. Some colon cancer cells can acquire the ability to leave the primary tumor site and form metastasis in distant organs. Adapted from Rizk *et al.* 2012[56].

Widening the Fearon-Vogelstein-model of colorectal cancer initiation and progression, the Cancer Genome Atlas Network very recently published data obtained from a large-scale genomic analysis of colon and rectal cancer patient samples[57]: in addition to the already published colorectal cancer causing mutations, the authors found several other frequently mutated genes, such as *ARID1A*, *SOX9* and *FAM123B,* as potential drivers of the disease. Furthermore, colon and rectal cancers were classified as one cancer since both types showed similar patterns of genomic alterations. *KRAS* was one of the first oncogenes studied in CRC and was found to be mutated in 40-50% of the cases[58, 59]. Its mutations mainly lead to a constantly active KRAS protein which is involved in the translation of growth factor signaling resulting in proliferation[60, 61]. *SMAD4* and *TP53* act as tumor suppressor genes. SMAD4 protein plays an important role in TGF (transforming growth factor)-β signaling where expression of target genes inhibits cell cycle progression. Loss of SMAD4 function confers insensitivity towards growth inhibition via TGF-β signaling[62]. Many colon cancers show a depletion on chromosome 17p which comprises the *TP53* gene[63-65]. Its product, the p53 protein, is a key player in regulation of apoptosis and also in its induction upon DNA damage[65, 66]. Depletion of p53 consequently helps cells to escape from apoptosis and increases genomic instability. ARID1A is part of the chromatin remodelling complex SNF/SWI which plays a role in cell differentiation, development, and tumor suppression[67]. Its functional loss has been suggested to contribute to a variety of different neoplasms[68]. The protein SOX9 is involved in differentiation processes in the ISC niche[69, 70] and FAM123B

negatively regulates Wnt signaling[71] suggesting a tumor suppressive function for both proteins.

The genomic instability described above accounts for 85% of all colorectal cancers. About 15% of all colorectal cancer cases are defined by a genetic condition called microsatellite instability[72, 73]. Microsatellites are DNA regions comprising short sequence repeats. The likelihood that those strands realign incorrectly during replication is increased and a mismatch repair (MMR) system is required to smooth the errors out[74]. Mutations in MMR components encoding genes like MLH1 and MSH2 result in nucleotide insertions and deletions which increases genomic instability (microsatellite instability, MSI). It has also been demonstrated that MMR deficiency plays a major role in carcinogenesis of hereditary non-polyposis colon cancer (HNPCC) [75-77]. Mutations in MLH1 and MSH2 accounted for 80% of the genetic alterations found in HNPCC-families whereas mutations in the other MMR genes MSH6, MSH3, PMS1, PMS2 and MLH3 were rare or absent[78].

Spread of the diseases requires epithelial tumor cells to detach from neighboring cells and migrate through the body until they settle down to form metastasis at distant organ sites. In the 1980s, Greenburg and Hay provided experimental data on a similar transformation process in benign tissue by showing that lens epithelial cells detached from their tissue association in a 3D collagen gel and migrated as individual cells with mesenchymal morpholgy[79-81]. They termed this process Epithelial-Mesenchymal Transition (EMT). However, EMT appeared to be reversible enabling migrating cells to settle down and to form an epithelial layer (Mesenchymal-Epithelial Transition, MET)[82, 83]. E-Cadherin was identified as a gate keeper of the epithelial state: it mediates formation of cell-cell-junctions and its lack of production led to loss of the epithelial cell phenotype *in vitro*[84]. Interestingly, E-Cadherin restorage resulted in suppression of the invasive mesenchymal phenotype. During carcinoma progression, E-Cadherin can be downregulated by several mechanisms: the transcriptional repressors Snail, ZEB1 and ZEB2 bind independently from each other to the E-Cadherin promoter and thereby inhibit E-Cadherin expression in epithelial tumors[85-87]. Gregory *et al.* demonstrated that ZEB1 additionally inhibits the transcription of the microRNA-200 family which in turn regulates expression of ZEB1 and ZEB2 by inhibiting the translation of their RNA[88]. Thus, the microRNA-200 family indirectly controls expression of E-Cadherin as well. Furthermore, transcriptional repression of E-cadherin has been associated with hypermethylation of its promoter[89]. Few cases of gastric carcinomas have been shown to harbor genetic mutations within the E-Cadherin encoding gene leading to non-functional or absent protein[90, 91]. Research from the last years emphasizes the role of EMT and MET in CRC and metastasis initiation: it has been suggested that EMT enables colorectal cancer cells to leave their solid tissue association and, in a reverse way via MET, to settle down and form metastasis at distant organ sites[92, 93]. Moreover, lab

members have shown that the metastastic potential of primary colon sphere cultures in immunodeficient mice was associated with low levels of E-Cadherin. Because of EMT's and MET's transient nature, isolation and investigation of cells in EMT is hardly possible and hampers investigation of the underlying molecular mechanisms[92]. Other studies demonstrate that cancer cells disseminate throughout the body in early stages of cancerogenesis before the primary tumor is surgically resected[94, 95]. This finding additionally implies a hibernating state of tumor cells which would explain the long period of time until appearance of disease relapse. Furthermore, Balic and colleagues suggest the implication of cancer stem cells in metastasis formation[96].

1.3 Tumor initiating cells

In the genetic concept of carcinogenesis, a distinct cell acquires a mutation. A clonal population accumulates mutations over a long period of time which finally leads to uncontrolled proliferation and to a spread of the disease via the lymphatic or venous system. According to the traditional stochastic model, every tumor cell divides uniformly and has the same potential to self-renew and to drive tumor growth[97]. The biological behavior of individual cells within the tumor might be altered by random or stochastic events leading to the tumors functional heterogeneity. In 1997, a study by Bonnet and Dick implied that AML is organized in a different way[98]: serial transplantation of AML patient's bone marrow and blood cells in immunodeficient mice revealed that <0.01% of mononuclear patient-derived cells could launch the disease in those mice. Furthermore, they postulated that these leukemia-initiating cells (LIC) were only found in the $CD34^+CD38^-$ cell population suggesting that malignant transformation took place on the stem cell or early progenitor level. However, later studies questioned the use of these markers for purification of LIC by showing that LIC were also found in the CD34- or CD38+ cell population[99, 100]. These studies indicated that the expression of LIC surface molecules varies between different patients. In 2004, Hope *et al.* demonstrated a hierarchical organization of the leukemic stem cell compartement[101]. In the hierarchical "cancer stem cell" (CSC) model, a small population of cancer stem cells shows self-renewal and pluripotency and gives rise to several more differentiated cell types with limited or no proliferative capacity. Only these CSCs were able to initiate tumor growth in transplanted animals and to maintain the disease[102, 103]. While the term "cancer stem cell" suggests that cancer results from oncogenic transformation of stem cells, it is now well known that stem cell progeny as well might be hit by oncogenic transformation and gain stem cell-like properties[104, 105]. These findings introduced the more appropriate term "tumor initiating cell" (TIC).

Several studies made use of different marker combinations to isolate or enrich for tumor initiating cells in different solid tumor entities like breast[106], brain[107, 108], pancreas[109, 110],

head and neck[111], prostate[112], ovary[113] and colon[114-116]. Whether expression of certain cell surface markers is able to universally distinguish TIC from non-tumorigenic progeny is still under debate[99, 100, 117, 118]. Sphere-forming assays have been established to identify and to study TIC *in vitro*[119-121]. It has been suggested that sphere-forming capacity reflects self-renewal ability of cells, but the relation of stemness and sphere-forming capacity remains discussed[102, 122]. The gold standard for approving the tumor initiating capacity of a given cell population *in vivo* is xenotransplantation into immunodeficient mice: TIC generate xenografted tumors with all the cellular phenotypes and similar properties as the primary, patient-derived tumor. Furthermore, serial transplantation of xenografted cells elucidates their self-renewal ability[101, 123].

1.3.1 Tumor initiating cells in colorectal cancer

The concept of colorectal cancerogenesis resulting from sequential mutations of a single cell is widely-accepted. In the context of the cancer stem cell model, colonic stem cells might accumulate mutations over several years and generate new cancer stem cells and more differentiated progenitor cells loosing the ability to self-renew. Colonic crypts are finally colonized by the mutant stem cell's monoclonal progeny of highly differentiated cancer cells (monoclonal conversion). On the other hand, an initial oncogenic hit might also occur in the more differentiated progeny of colonic stem cells. These cells might then regain stem-like properties. Vermeulen and coworkers provided experimental evidence that stemness of more differentiated colon cancer cells can be induced by growth factor modulation of their microenvironment[124] indicating that oncogenic transformation not necessarily happens on the stem cell level. Upon publication of data on the putative isolation of acute myeloid leukemia (AML) initiating cells, a number of studies followed investigating the possible existence of tumor initiating cells in solid cancers such as CRC. Human colon TIC are defined on a functional basis: transplantation into immunocompromised mice produces xenografted tumors which show similar heterogeneity and properties as the primary, patient-derived tumor. Self-renewal capacity of colon TIC can be confirmed by a serial transplantation assay. Colon TIC research has been performed using several transplantation techniques. Transplantation of human cells under the kidney capsule of immunodeficient NOD/SCID or SCID mice has been favored due to higher tumor take rates as compared to subcutaneous transplantation[114-116, 125, 126]. Additionally, distinct *in vitro* culturing conditions have been described enabling researchers to study human colon TIC in more detail: patient-derived tumor cells are cultured under serum-free, non-adherent conditions where tumor cells form 3-dimensional floating aggregates called spheroids. Dalerba *et al.* described that serum-containing growth medium induces a more differentiated phenotype in tumor cells going along with reduced tumor initiating capacity upon xenotransplantation[115]. Differentiated cells do not survive serum-free

growth conditions enabling an enrichment of cultures for TIC. Furthermore, serum-free spheroid cultures were shown to preserve the "genotype, gene expression patterns and *in vivo* biology of human glioblastomas"[127].

Identification of human colon TIC on a phenotypic level was first published in 2007. CD133 was used as cell surface marker to discriminate a human colon TIC enriched cell population from other cells[114]. Ricci-Vitani *et al.* additionally demonstrated that only $CD133^+$ formed tumors in immunocompromised mice upon subcutaneous transplantation whereas $CD133^-$ cells did not[116]. On the other hand, Shmelkov and colleagues published data demonstrating that CD133 is expressed by differentiated and undifferentiated healthy colon cells[117]. Moreover, the authors show that $CD133^-$ and $CD133^+$ metastatic colon cancer cells contribute to tumor formation in mice. Although CD133 has been shown to be expressed not only on colon TIC but also on more differentiated progenitor cells, expression levels were clinically correlated with relapse-free time intervals and prognosis[128, 129]. In addition, Todaro *et al.* showed that $CD133^+$ colon TIC resist chemotherapeutic treatment by the production of IL-4[126]. IL-4-targeted therapies eradicated tumor bulk cells as well as CD133+ colon cancer cells *in vitro* and *in vivo*. Another study from 2007 has shown that mice transplanted with $EpCAM^{high}/CD44^+$ CRC cells developed tumors whereas $EpCAM^{low}/CD44^-$ CRC cells did not[115]. The authors identified CD166 as an additional marker to further enrich for human colon TIC. Expression of CD166 in CRC was previously already linked to shortened patient survival[130]. Few years ago, Huang *et al.* identified ALDH1 as a colon cancer stem cell marker with respect to tumor formation in mice[131]. The tumor initiating capacity of $EpCAM^+/CD133^-$ cells was also demonstrated in a serial transplantation assay[117]. However, in 2010, Kemper *et al.* published that the AC133 antibody which has generally been used in previous study to enrich for $CD133^+$ colon TIC, does not detect glycosylated forms of CD133 in colon CSCs[132]. The role of cell surface markers in cancer is still not fully understood and needs to be further addressed. Moreover, the controversial findings imply consideration of several TIC phenotypes among different patients and of the existence of several subfractions of colon TIC within individual patients what has recently been demonstrated in different leukemias[133, 134].

1.3.2 Heterogeneity of colon TIC

More recently, the colon cancer initiating cell compartment has for the first time been analyzed surface molecule-independendly[135]: clonal marking revealed the existence of distinct classes of TIC with differing self-renewal and metastasis-initiating capacity (Figure 5). Only self-renewing long-term (LT-) TIC showed tumor- and metastasis-initiating potential in a serial transplantation assay. Tumor transient amplifying cells (T-TAC) had limited or no self-renewal capacity and contributed to tumor formation only

in primary mice whereas rare delayed contributing TIC (DC-TIC) were exclusively active in secondary or tertiary mice.

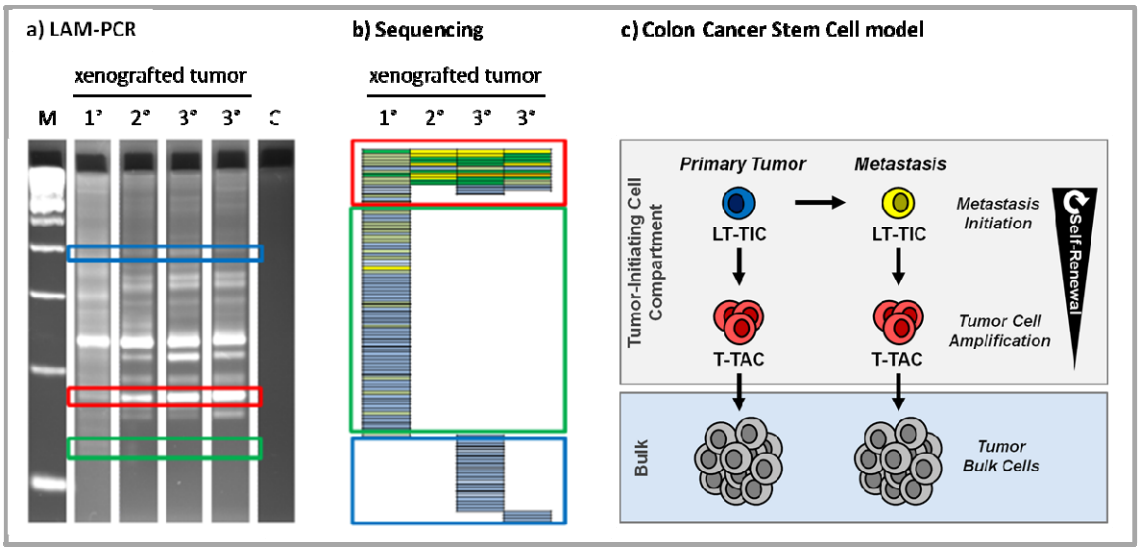


Figure 5: The human colon cancer initiating cell compartment is hierarchically organized.

a) LAM-PCR and b) high throughput sequencing revealed distinct classes of colon TIC and led to a new colon cancer stem cell model (c): Long-term TIC (LT-TIC, red) have self-renewal capacity and can initiate tumor and metastases in a serial transplantation assay. LT-TIC give rise to tumor transient amplifying cells (T-TAC, green). T-TAC show limited or no self-renewal potential and contribute to tumor formation in primary mice only. They form the tumor bulk cells. Delayed contributing TIC (DC-TIC, blue) were rare and became active in later generations of mice. 1-3°: First to third generation of mice; M: 100bp marker; C: control. Adapted from Dieter *et al.*, 2011[135].

DC-TIC did not contribute to primary tumor growth, but were recruited to tumor formation at later time points indicating that they were able to self-renew. In addition, this small cell population was necessarily present in primary tumors although not contributing to its growth. Consequently, DC-TIC might have been present in a mitotically quiescent state. Conversion into a proliferating state might have reactivated them to contribute to tumor formation only in late recipient mice. A possible quiescence of colon TIC in patients might protect them from chemotherapy and would represent a source for chemoresistance and disease relapse after prolonged period of times.

1.4 Tumor dormancy

A substantial number of cancer patients suffer from relapse of their disease after initially successful therapy. This implies that residual cancer cells remained unnoticed in patients (minimal residual disease, MRD) and that they are reactivated after months or years to regrow a tumor[136]. These extended periods of time might be overcome in cellular quiescence or rare cell divisions until cells might be reactivated by genetic or cellular changes in the tumor or by microenvironmental factors[137]. Quiescence also plays an important role under physiological circumstances: it prevents accumulation of

mutations and thus maintains many adult stem cell compartements[138, 139]. Label-retaining assays helped to identify different proliferative fractions in intestinal crypts and suggested that ISCs were less proliferating than other intestinal cells[12, 140]. More recent studies indicated the existence of a quiescent and an actively-proliferating stem cell zone in intestinal crypts building a bridge between the +4 position and stem cell zone model for intestinal stem cells[141, 142]. There might even be a conversion between the ISC's proliferative status which is potentially regulated by the microenvironment[22, 23, 143, 144].

The proliferative biology of many cancer cells and several tissue stem cells like ISCs has been investigated using pulse/chase label-retaining assays[21, 145] (Figure 6): a bulk of cells with a low frequency of stem cells is labeled *in vitro* using a fluorescent dye like CFSE (pulse). Labeling is followed by a prolonged period of time (chase). During this time actively-proliferating cells dilute the label by separating it equally among the daughter cells during cell division until it is not detectable anymore. Slow or rarely dividing cells maintain their label intensities (label-retaining cells, LRC).

A common technique to identify quiescent and slow-dividing cells *in vivo* is labeling by administration of 5-bromo-2-deoxyuridine (BrdU). The nucleoside is incorporated into newly synthesized DNA during S phase of the cell cycle instead of thymidine and distributed among daughter cells during cell division. A subsequent chase period reveals LRCs[146, 147]. However, not all cells might be labeled by BrdU since only a small fraction of a given cell population is actively-cycling at the time of administration. Quiescent cells evade BrdU labeling. Moreover, BrdU incorporation might be associated with DNA damage influencing cell proliferation[148]. Another disadvantage of BrdU is that its detection requires permeabilization of cells which makes further investigations of purified LRCs impossible[149, 150]. To overcome these major impediments, a new pulse-chase-concept was developed making use of the tissue-specific expression of a tetracycline-inducible histone fused to a green fluorescent marker protein (H2B-GFP)[151]. This system was first applied to the hair follicle of mouse skin[152]: H2B–GFP-expressing LRCs were identified at the base of each hair follicle. They were shown to proliferate and to exit their niche upon activation of a new hair cycle or wounding. Their fluorescence was found to be diluted 2-fold with each cell division. Moreover, engraftment in immunodeficient mice revealed multi-lineage reconstitution potential[153]. Thus, the system enabled monitoring of quiescent or slow-cycling stem cells and also of their progeny[154].

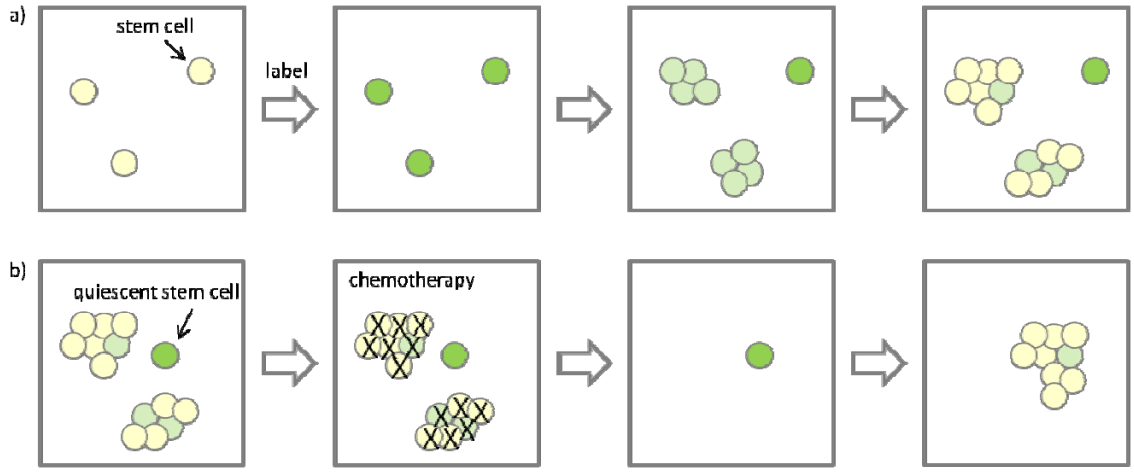


Figure 6: Label-retaining assays to study stem cell behavior and tumor biology.

a) Cell suspensions are labeled and label-retention is investigated over a prolonged chase period. Actively-proliferating cells lose their label whereas less cycling ones maintain label intensities. b) Tumors are supposed to comprise a fraction of quiescent or slowly cycling cells. These cells would survive conventional chemotherapies which only target fast proliferating cells and thus could be the source for disease relapse. Adapted from Moore and Lyle, 2011[155].

It has been suggested that quiescence of tumor cells plays a major role in chemotherapy resistance limiting clinical treatment. Rare quiescent cells are assumed to survive chemotherapeutic treatment which is only targeting actively-proliferating cells. They can be reactivated, enter the cell cycle and contribute to disease relapse. Experimental evidence for chemoresistant cells has been provided in the hair follicle where slowly proliferating cells survived treatment and regenerated the hair follicle[156]. In the context of cancer, Moore *et al.* found small, slow cycling cell populations in spheroid cultures of the colon cancer cell line HCT116 and the breast cancer cell line MDA-MB-231[157]. Both populations were still able to proliferate after chemotherapeutic treatment. Furthermore, Correa and colleagues published data about the activation of previously quiescent ovarian cancer cells[158]. Furthermore, slow-cycling populations of cancer cells have also been demonstrated in cell lines originating from pancreatic cancer[159] and melanoma[160] and in primary patient-derived cancer cells from melanoma[129], breast[133] and ovary[157, 160, 161]. These studies indicate that quiescent and slow cycling cells might be the source for disease relapse and late metastasis formation in patients.

1.5 Therapy of colorectal cancer

Colorectal cancer is diagnosed based on tissue biopsies during colonoscopy[162]. Treatment strategies are initiated according to previous staging[162, 163]: rectal cancers are commonly resected by surgery. Patients receive neoadjuvant or adjuvant radiotherapy in order to reduce the tumor volume. Localized colon cancers are also surgically resected but radiation therapy is less suitable due to the intestines motility. Those patients

receive adjuvant chemotherapy based on 5-Fluorouracil (5-FU) to reduce the risk of disease relapse. The treatment of colon cancer patients with metastatic disease is mainly performed using combinational chemotherapy with 5-FU and oxaliplatin, This treatment scheme was established upon a clinical phase III study by de Gramont and colleagues presenting that the combination of Leucovorine, 5-FU and oxaliplatin (FOLFOX) significantly extended progression free survival of patients with metastatic colorectal cancer[164-166].

5-FU exhibits two mechanisms of action[167, 168]: first, it is a pyrimidine analogue whose metabolites are incorporated into RNA and DNA leading to inhibition of DNA synthesis and apoptosis. Second, 5-FU inhibits the enzyme thymidylate synthetase (TS) which is responsible for generation of deoxythymidine triphosphate (dTTP). dTTP is one of the bases necessary for generation of nucleic acid. Thus, inhibition of TS leads to reduction of dTTP-levels which eventually lets the cell die (thymineless death). Oxaliplatin is a platinum-based agent which produces crosslinks between different DNA molecules and also within one molecule[169]. These links block DNA replication and transcription which finally leads to cell death.

Although many attempts have been made bringing up new agents and treatment options, treatment of advanced colon cancer is still not curative. The 5-year survival rate for colorectal cancer patients in european countries is <60%[170]. Up to 25% of patients suffer from recurrent colon cancer within the first 5 years after initially successful treatment[171]. This suggests that a small fraction of tumor cells is not affected by conventional chemotherapeutic treatment with 5-FU and Oxaliplatin which both require actively-cycling cells to unfold their spectrum of action[169, 172].

2 Scientific Aims

Accumulating studies emphasize that colon cancer is hierarchically organized. Only a small subpopulation of tumor cells is able to self-renew and can thereby maintain the disease. Within this colon cancer initiating compartment, Dieter *et al.* found a small subfraction of TIC contributing to tumor formation only in secondary or tertiary mice in a serial transplantation assay[135]. These delayed contributing TIC (DC-TIC) might have been mitotically quiescent or might have hibernated in a dormant cell cycle state in primary mice. In patients, cellular quiescence of TIC could enable them to evade systemic cancer-therapy and cause cancer relapse or metastasis formation upon reactivation after extended periods of time. Indeed, most colon cancer patients die due to advanced, metastastatic cancer stages after initially successful therapies.

This work aims to characterize the proliferative and cell cycle activity of human colon TIC *in vitro* and *in vivo*. We determined whether a quiescent population of human colon cancer initiating cells exists. Hoechst/Pyronin-staining revealed if colon TIC were arrested in G_0 phase of the cell cycle. Long-term quiescence of human colon TIC was assessed using the CFSE label-retaining assay. In addition, the sphere forming potential of cells with differing proliferative activity was examined by a limiting dilution assay. Xenotransplantation studies elucidated if the tumor initiating potential was associated with mitotic quiescence. A high-resolution label-retaining strategy was used to investigate the proliferative activity of human colon TIC *in vivo.* Unique clonal marking allowed to decipher whether DC-TIC were indeed quiescent *in vivo* and to investigate their role in chemoresistance.

This detailed characterization of the proliferative characteristics and dynamics of human colon TIC will contribute to a better understanding of their biological properties and TIC maintenance. Furthermore, it might extend knowledge about colon cancer disease progression and relapse.

3 Materials and Methods

3.1 Materials

3.1.1 Equipment and Devices

Analytical Balance TE 124S	Sartorius, Göttingen
Biofuge® pico	Heraeus, Hanau
Camera Lumix DMC-FZ50	Panasonic, Hamburg
centrifuge inserts	Kendro, Langenselbold
Centrifuge Multifuge® 3SR	Heraeus, Hanau
Cryobox Nalgene	Thermo Fisher Scientific, Schwerte
Electrophoreses Power Supply 200/2000	Elchrom Scientific, Cham
Flow Cytometer BD™ LSRII	Becton, Dickinson and Company, Heidelberg
Flow Cytometer FACS Aria™ Cell Sorter	Becton, Dickinson and Company, Heidelberg
Fluorescence Microscope Axiovert 200	Zeiss, Oberkochen
Freezer -20°C	Liebherr, Biberach an der Riss
Freezer -80°C	Sanyo (Panasonic), Hamburg
Fridge 4°C	Liebherr, Biberach an der Riss
Gel Documentation	Peqlab, Erlangen
Gel Electrophoreses Chamber	Biometra, Göttingen
Incubator Heracell® 150	Thermo Fisher Scientific, Schwerte
Isoflurane Vaporizer Vapor 19.3	Dräger, Lübeck
Light Microscope	Zeiss, Oberkochen
Magnet MPC-96	Gibco® Invitrogen, Darmstadt
Magnetic Plates for Tissue Culture Dishes	OZ Biosciences, Herford

Microplate Reader	Tecan, Männedorf
Microwave	Bartscher, Salzkotten
Mini Protean® Tetra Cell	BioRad, Munich
Molecular Imager® ChemiDoc™ XRS+	BioRad, Munich
Multipette Plus	Eppendorf, Hamburg
NanoDrop® Spectrophotometer ND-1000	Peqlab, Erlangen
Neubauer counting chamber	Marienfeld, Lauda-Königshofen
Nitrogen System	German-Cryo, Jüchen
PCR-Thermocycler	Landgraf, Langenhagen
Pipetboy acu	Integra Biosciences, Fernwald
Pipettes Research® (10µl; 20µl; 200µl; 1000µl)	Eppendorf, Hamburg
Power Pac™ HC Power Supply	BioRad, Munich
Precision Balance TE3102S	Sartorius, Göttingen
Rotator Reax2	Heidolph, Schwabach
Safety Cabinet Herasafe® KS Thermo Fisher Scientific	Thermo Fisher Scientific, Schwerte
Shaking incubator	Axon, Kaiserslautern
Submerged Gel Electrophoresis Apparatus SEA 2000®	Elchrom Scientific, Cham
TC10™ Automated Cell Counter	BioRad, Munich
Thermo Cycler TPersonal	Biometra, Göttingen
Thermo Mixer comfort	Eppendorf, Hamburg
Tissue-Tek® TEC™ Embedding System	Sakura Finetek, Tokyo
Trans-Blot® semi-dry cell	BioRad, Munich
Trans-Blot® semi-dry cell	BioRad, Munich
Transilluminator	Biotec-Fischer, Reiskirchen
Ultracentrifuge L8-M with Rotor SW27	Beckman Coulter, Krefeld
Vacuum pump	Merck Millipore, Darmstadt
Video documentation system	Peqlab, Erlangen
Vortexer MS1	IKA, Staufen

Water Bath Haake SWB25	Thermo Fisher Scientific, Schwerte
Water treatment plant	TKA, Niederelbert

3.1.2 Plastic and Disposables

Cell Culture Flasks, EasyFlasks™ (25cm^2; 75cm^2; 175cm^2)	Nunc (Thermo Fisher Scientific), Schwerte
Cell Culture Plates (6-; 12-; 24-; 48-; 96-well)	Becton, Dickinson and Company, Heidelberg
cell strainer (0,4 - 1µm pore size)	Becton, Dickinson and Company, Heidelberg
cotton swabs, sterile	Noba Verbandmittel Danz GmbH, Wetter
Cover Glasses Marienfeld	Marienfeld, Lauda-Königshofen
Cryotubes	Corning, Kaiserslautern
Embedding Cassettes	Sanowa Laborprodukte
FACS Tubes, BD™ Falcon™ Round-Bottom Tube (5ml)	Becton, Dickinson and Company, Heidelberg
Filter (0.22µm pore size)	Merck Millipore, Darmstadt
Hollow Needle (23G-26G)	Becton, Dickinson and Company, Heidelberg
Hollow Needle, blunt end 18G	Becton, Dickinson and Company, Heidelberg
Lab Gloves (Nitril)	Microflex, Reno
Microcon®-30	Merck Millipore, Darmstadt
Parafilm	Pechiney Plastic Packaging, Chicago
PCR reaction tube (V=0,2 ml)	Genaxxon, Ulm
Petri dish (d=10 cm)	Genaxxon, Ulm
Petri dish Nunclon™ (d=15 cm)	Thermo Electron, Langenselbold
Pipette Tips (200µl, extended)	Thermo Fisher Scientific, Schwerte

Pipettes (V=2 - 50 ml)	Genaxxon, Ulm
Pipette tips (V=10/20/200/1000 µl)	Starlab, Hamburg
PVDF membrane	BioRad, Munich
Scapel	Feather Safety, Osaka
Stericup vacuum filtration system	Merck Millipore, Darmstadt
Syringe (5/20/50 ml) Omnifix® Solo	B. Braun, Melsungen
Syringe, Omnifix® solo (1ml; 5ml)	B. Braun, Melsungen
Twin.tec PCR plate 96 well	Eppendorf, Hamburg
Ultra Low Attachment Flasks	Corning, Kaiserslautern
Ultracentrifuge tubes	Beranek Laborgeräte, Weinheim
Whatman filter paper Protean®	BioRad, Munich

3.1.3 Molecular Biology Reagents

5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFDASE)	Invitrogen, Darmstadt
Agarose	Serva, Heidelberg
Ammonium chloride (NH_4Cl)	Sigma-Aldrich, Munich
Aqua ad injectabilia (aqua dest)	B. Braun, Melsungen
Baytril®	Bayer, Leverkusen
Bovine Serum Albumin	Sigma-Aldrich, Munich
Bromphenolblue	Sigma-Aldrich, Munich
Dimethylsulfoxide (DMSO)	Sigma-Aldrich, Munich
Disodium-EDTA	Sigma-Aldrich, Munich
DNA ladder 100 bp	Invitrogen, Darmstadt
DNA ladder 1kb	Invitrogen, Darmstadt
DNA, human genomic	Roche, Mannheim
Ethanol	Sigma-Aldrich, Munich
Ethanol denatured	DKFZ, Heidelberg
Ethidiumbromide (0,07%) AppliChem	AppliChem, Darmstadt
Ethylendiaminetetraacedic acid (EDTA) 0.5M,	AppliChem, Darmstadt

pH8	
Fluorogold	Invitrogen, Darmstadt
Formalin solution, neutral buffered (10%)	Sigma-Aldrich, Munich
Glycerol	Serva, Heidelberg
Hoechst 33342	Invitrogen, Darmstadt
Isopropyl alcohol	Sigma-Aldrich, Munich
Lithium chloride	Sigma-Aldrich, Munich
Loading Buffer (5x)	Elchrom Scientific, Cham
Magnesium chloride ($MgCl_2$) 25mM	Invitrogen, Darmstadt
$MgCl_2$ 100mM	Sigma-Aldrich, Munich
Oligonucleotides	MWG Biotech, Ebersberg
Potassium bicarbonate ($KHCO_3$)	Roth, Karlsruhe
Propidiumiodid	Invitrogen, Darmstadt
Pyronin Y	Sigma-Aldrich, Munich
RNase/DNase free water	Ambion, Darmstadt
Sodium azide pure (NaN_3)	AppliChem, Darmstadt
Sodium Chloride (NaCl)	VWR International, Vienna
Sodium hydroxide (NaOH)	Sigma-Aldrich, Munich
Spreadex® Gels, Type EL1200	Elchrom Scientific, Cham
Sputolysin®	Calbiochem (Merck), Darmstadt
Tris HCl pH 7.5 (1M)	USBiological, Swampscott
Trypan Blue Stain 0,04%	Invitrogen, Darmstadt
Viromag R/L Beads OZ Biosciences	OZ Biosciences, Herford
β-Mercaptoethanol	Sigma-Aldrich, Munich

3.1.4 Cell Culture and Bacterial Growth Reagents

Advanced Dulbecco's Modified Eagle (DMEM/F-12)	Gibco Invitrogen, Darmstadt
Dulbecco's phosphate buffered saline (DPBS)	Invitrogen, Darmstadt
Hank's balanced salt solution (HBSS)	Sigma-Aldrich, Munich

Iscove's Modified Dulbecco's Medium (IMDM)	Invitrogen, Darmstadt
Iscove's Modified Dulbecco's Medium (IMDM)	Invitrogen, Darmstadt
Luria Broth (LB) base	Invitrogen, Darmstadt
SOC-Medium	Invitrogen, Darmstadt
Trypsin-EDTA 0,05%	Invitrogen, Darmstadt
Trypsin-EDTA 0,25%	Invitrogen, Darmstadt

3.1.5 Medium Additives and Antibiotics

Ampicillin Sodium Salt	Sigma-Aldrich, Munich
Bovine Serum Albumin (BSA) Solution (7,5%)	Sigma-Aldrich, Munich
Doxycyclin	Clontech, Saint-Germain-en-Laye
Fetal Bovine Serum (FBS)	Biosera, Sussex
Glucose	Invitrogen, Darmstadt
Heparin Sigma-Aldrich	Sigma-Aldrich, Munich
HEPES buffer	Sigma-Aldrich, Munich
Human Epidermal Growth Factor (hEGF)	R&D Systems, Wiesbaden
Human Fibroblast Growth Factor (hFGF basic)	R&D Systems, Wiesbaden
L-Glutamine	Invitrogen, Darmstadt
Penicillin-Streptomycin	Invitrogen, Darmstadt
Polybrene	Millipore (Merck), Darmstadt
Polyethylenimin (PEI)	Sigma-Aldrich, Munich

3.1.6 Media and Buffer Compositions

media	reagent	concentration
Agar	LB	25%
	Agar	12.5%
	H_20_{dest}	
CSC freezing medium	CSC growth medium	
	DMSO	15%
CSC growth medium	D-Glucose	0.6%

	Penicillin/Streptomycin	2mM
	L-glutamine	2mM
	Heparin	4µg/ml
	HEPES	5mM
	BSA	4mg/ml
HF	HBSS	
	FBS	2%
IMDM freezing medium	IMDM growth medium	
	FBS	30%
	DMSO	15%
IMDM growth medium	IMDM	
	Penicillin/Streptomycin	2mM
	L-glutamine	2mM
	FBS	10%
IMDM thawing medium	IMDM growth medium	
	FBS	50%
LB medium	LB	25%
	H_20_{dest}	
LiCl, 3M	Tris-HCl (pH 7.5)	10mM
	EDTA	1mM
	LiCl	3M
	H_20_{dest}	
LiCl, 6M	Tris-HCl (pH 7.5)	10mM
	EDTA	1mM
	LiCl	6M
	H_20_{dest}	
Loading buffer (5x Blue Run)	Tris HCl pH 7.5 (1M)	25mM
	EDTA pH 8.0 (0.5M)	150mM
	Bromphenol blue	0.05%

	Glycerol (100%)	25%
	H_20_{dest}	
staining buffer (intracellular staining)	PBS	
	FBS	1%
	NaN_3	0.09%
storage buffer for DNA ladders	Tris HCl pH 7.5 (1M)	10mM
	EDTA pH 8.0 (0.5M)	1mM
	NaCl	20mM
	H_20_{dest}	

3.1.7 Antibodies

Mouse anti-human CD133/1 (AC133), APC-conjugated monoclonal antibody	Miltenyi-Biotech, Bergisch Gladbach
Mouse anti-human CD133/2 (293C2), APC-conjugated monoclonal antibody	Miltenyi-Biotech, Bergisch Gladbach
Mouse anti-human EpCAM, PerCP-Cy5.5-conjugated monoclonal antibody	Becton, Dickinson and Company, Heidelberg
Mouse anti-human CD166, PE-conjugated monoclonal antibody	Becton, Dickinson and Company, Heidelberg
Mouse anti-human CD44, APC-conjugated monoclonal antibody	Becton, Dickinson and Company, Heidelberg
Mouse IgG1, APC-conjugated Isotype Control	Miltenyi-Biotech, Bergisch Gladbach
Mouse IgG2b, APC-conjugated Isotype Control	Miltenyi-Biotech, Bergisch Gladbach
Mouse IgG1 κ, PerCP-Cy5.5-conjugated Isotype Control	Becton, Dickinson and Company, Heidelberg
Mouse IgG1 κ, PE-conjugated Isotype Control	Becton, Dickinson and Company, Heidelberg
Mouse IgG2b κ, APC-conjugated Isotype Control	Becton, Dickinson and Company, Heidelberg
Mouse anti-human Ki67, Alexa 700-conjugated monoclonal antibody	Becton, Dickinson and Company, Heidelberg

Mouse IgG1 κ, Alexa 700-conuagtes isotype Control	Becton, Dickinson and Company, Heidelberg

Antibody	Corresponding Isotype	Concentration
α-CD133/1-APC	IgG1-APC	0.55µg/ml
α-CD133/2-APC	IgG2b-APC	0.825µg/ml
α-EpCAM-PerCP-Cy5.5	IgG1-PerCp-C5.5	0.2µg/ml
α-CD166-PE	IgG1-PE	3.5µg/ml
α-CD44-APC	IgG2b-APC	0.15µg/ml

3.1.8 PCR-Reagents

PCR Buffer (10x)	Invitrogen, Darmstadt
dNTPs (mM)	Genaxxon, Ulm
Taq DNA Polymerase	Invitrogen, Darmstadt
Dynal M280-Streptavidin Beads	Invitrogen, Darmstadt
Hexanucleotides mix (10x)	Roche, Mannheim
ATP (10mM)	Epicentre Biotechnologies, Madison

3.1.9 Enzymes and Reaction Buffers

Klenow Polymerase	Roche, Mannheim
T4 DNA-Ligase + Puffer	New England Biolabs, Frankfurt/Main
Taq DNA Polymerase	Invitrogen, Darmstadt
Taq-Polymerase	Genaxxon, Ulm
Restriction endonuclease digestion buffers	New England Biolabs, Frankfurt/Main
Restriction endonucleases	New England Biolabs, Frankfurt/Main
Dispase	Becton, Dickinson and Company, Heidelberg

3.1.10 Oligonucleotides

All oligonucleotides were snythesized by Eurofins MWG Operon, Ebersberg.

name	sequence (5' - 3')
SK LTR 1 bio	GAG CTC TCT GGC TAA CTA GG
SK LTR 2 bio	GAA CCC ACT GCT TAA GCC TCA
SK LTR 3 bio	AGC TTG CCT TGA GTG CTT CA
SK LTR 4 bio	AGT AGT GTG TGC CCG TCT GT
SK LTR 5 bio	GTG TGA CTC TGG TAA CTA GAG
LC I	GAC CCG GGA GAT CTG AAT TC
LC II	GAT CTG AAT TCA GTG GCA CAG
LC 1	GAC CCG GGA GAT CTG AAT TCA GTG GCA CAG CAG TTA GG
LC 3	AAT TCC TAA CTG CTG TGC CAC TGA ATT CAG ATC
TitaniumLinker	CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG AGT GGC ACA GCA GTT AGG
Tit3SKLV10-1	ACA GTA TAT ATG TGT GAC TCT GGT AAC TAG
Tit3SKLV10-2	ACG CGA TCG ATG TGT GAC TCT GGT AAC TAG
Tit3SKLV10-3	ACT AGC AGT ATG TGT GAC TCT GGT AAC TAG
Tit3SKLV10-4	AGC TCA CGT ATG TGT GAC TCT GGT AAC TAG
Tit3SKLV10-5	AGT ATA CAT ATG TGT GAC TCT GGT AAC TAG
Tit3SKLV10-6	AGT CGA GAG ATG TGT GAC TCT GGT AAC TAG
Tit3SKLV10-7	AGT GCT ACG ATG TGT GAC TCT GGT AAC TAG
Tit3SKLV10-8	CGA TCG TAT ATG TGT GAC TCT GGT AAC TAG
Tit3SKLV10-9	CGC AGT ACG ATG TGT GAC TCT GGT AAC TAG
Tit3SKLV10-10	CGC GTA TAC ATG TGT GAC TCT GGT AAC TAG
Tit3SKLV10-11	TGT AGT GTG ATG TGT GAC TCT GGT AAC TAG
Tit3SKLV6-3	CAG CAG TGT GTG ACT CTG GTA ACT AG
Tit3SKLV6-4	TGA TCA TGT GTG ACT CTG GTA ACT AG
Tit3SKLV6-5	AGT GTC TGT GTG ACT CTG GTA ACT AG
Tit3SKLV6-6	ACT TGC TGT GTG ACT CTG GTA ACT AG

Tit3SKLV6-7	CCT ATA TGT GTG ACT CTG GTA ACT AG

3.1.11 Plasmids

All plasmids were provided by Andreas Trumpp. Plasmid sequences can be found in supplement A.

Construct	Promoter	Gene
pMD2.G	CMV	VSV-G
psPAX2	CAG	gag pol rev
pWPXL-TTT-H2B-GFP	EF1α	$tTA2^S$
	P_{Tight}	H2B-GFP

3.1.12 Commercial Kits

Agencourt AMPure XP, 60 mL	Beckman Coulter
BCA Protein Assay	Pierce, Schwerte
Blood and Tissue Kit	Qiagen, Hilden
EndoFree® Plasmid Purification Kit	Qiagen, Hilden
Fast-Link™ Ligation Kit	Epicentre Biotechnologies, Madison
Plasmid Miniprep DNA Purification Kit	GeneMATRIX
QIAquick® Gel Extraction Kit	Qiagen, Hilden
QIAquick® PCR Purification Kit	Qiagen, Hilden
Quant-iT PicoGreen dsDNA Assay kit	Invitrogen
Spreadex 1200	Elchrom Scientific, Cham

3.1.13 Surgical Instruments and Material

Alcohol Pads	B. Braun, Melsungen
B. Braunoderm®	B. Braun, Melsungen
BD Matrigel™ Growth Factor Reduced	Becton, Dickinson and Company, Heidelberg
Bepanthen® eye and nose lotion	Bayer, Leverkusen
Cotton Swabs	Böttger, Bodenmais
Earmarker	Fine Science Tools, Heidelberg

Forceps, Moria Ultrafein	Fine Science Tools, Heidelberg
Forceps, standard anatomical	Fine Science Tools, Heidelberg
Forceps, standard surgical	Fine Science Tools, Heidelberg
Heat Pad	Thermolux, Murrhardt
Insulin Syringe 0.5ml, 27G	Becton, Dickinson and Company, Heidelberg
Isoflurane	Abbott, Ludwigshafen
Reflex Wound Clip System	Fine Science Tools, Heidelberg
Scalpels	Feather Safety, Osaka
Scissors, standard surgical	Fine Science Tools, Heidelberg
Thread PGA Resorba 4-0	Resorba, Nürnberg

3.1.14 Mouse Strains

The *in vivo*-work in this study has been performed using NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice (The Jackson Laboratory, Bar Harbor).

3.1.15 Cell Lines

HEK 293T	ATCC, Wesel
HeLa	ATCC, Wesel

3.1.16 Bacteria Strains

One Shot® TOP10 Chemically Competent *E. coli*	Invitrogen, Darmstadt
One Shot® Stbl3™ Chemically Competent *E. coli*	Invitrogen, Darmstadt

3.1.17 Primary Material

Primary colorectal tumor samples, liver metastases and lung metastasis samples were provided by the surgical department of Heidelberg University Hospital. Respective patients were informed according to the instructions of the universities ethical committee. These instructions have been established in agreement with the declaration of Helsinki[a]. The ethical committee also approved all the experiments performed with the material (Ethikvotum 323-2004).

3.1.18 Histopathological Material

Isopropyl alcohol	Sigma-Aldrich, Munich

Ethanol	Sigma-Aldrich, Munich
Xylol	VWR International, Vienna
Paraplast X-TRA®	Sigma-Aldrich, Munich

3.1.19 Computer Programs

Axiovision Rel. 4.8	Zeiss, Oberkochen
Basic Local Alignment Search Tool (BLAST)	http://www.ncbi.nlm.nih.gov/ blast
FACS Diva Software V6.1.3	Becton, Dickinson and Company, Heidelberg
Lasergene	DNAStar, Madison
L-Calc	Stem Cell Technologies, Grenoble
NanoDrop® ND-1000 V3.2.1	Coleman Technologies, Langley
Microsoft Office 2007	Microsoft, Redmond
Photoshop CS2	Adobe, Dublin
Reference Manager 12	Thomson Reuters, New York

3.2 Methods

3.2.1 Cell-Biological Methods and Flow Cytometry

3.2.1.1 Purification of Primary and Xenografted Colon Cancer and Metastasis Tissue

Primary colorectal tumor samples, liver metastases and lung metastasis samples were provided by the surgical department of Heidelberg University Hospital. Respective patients were informed according to the instructions of the universities ethical committee. These instructions have been established in agreement with the declaration of Helsinki[b]. The ethical committee also approved all the experiments performed with the material (Ethikvotum 323-2004).

The surgically resected tissue sample was fractionated by a scientist of the surgical department laboratory. Tissue samples were kept in PBS on ice and transported to our laboratory as fast as possible after surgery. They were mechanically dissociated in a petri dish with a scapel into small pieces of approximately $1mm^3$ and were spun down in 20ml PBS (900rpm, 5min, 4°C). The pellet was resuspended in a PBS-digestion mix (20ml/3g sample) containing 0.08U dispase per ml and 50µM $MgCl_2$. Enzymatic digestion was carried out at 37°C in a rotating incubator for 1.5hours. Subsequently, the

solution was amended with PBS to a final volume of 50ml. An initial filtering step (100µm pore size) was performed to remove remaining tissue and mucous. After centrifugation at 900rpm, 5min, 4°C, the pellet was dissolved in 20ml PBS and filtered through a 40µm-filter. In case of excessive erythrocytes, the pellet was lysed with 5ml red blood cell lysis buffer, washed with 10ml PBS and subsequently resuspended in 5ml PBS and filtered (40µm). Cells were counted and stored on ice until they were further processed.

3.2.1.2 Determination of Cell Numbers

Scientific Background

Cells were counted using the dye exclusion test. The membrane of intact cells cannot be overcome by dyes like trypan blue. Dead cells lose their property to maintain their impermeable cell membrane. Thus, dead cells are stained and appear blue under the light microscope. Cell viability can be easily assessed.

Methods

Singularized cells were mixed with trypan blue in specific dilution (1:2 – 1:100). A cover slip was placed on a Neubauer chamber and 10µl of the cell mixture was loaded into the counting chamber. Light, viable cells were counted in 4 quadrants under a light microscope. The number of cells per ml original cells suspension was calculated according to this formula:

$$\frac{\#\ of\ cells}{ml} = \frac{\#\ of\ cells}{4\ quadrants}\ x\ dilution\ factor\ x\ 10^4$$

1 quadrant holds 0.1µl (= 10^{-4}ml). The average number of cells per quadrant multiplied with the dilution factor gives the average number of cells per 0.1µl. In order to calculate the average number of cells per ml, the average number of cells per 0.1µl has to be multiplied with 10^4.

3.2.1.3 Culturing of Purified Tissue Samples and Sphere Cultures

Scientific Background

Primary human colon cancer cells can be cultured under serum-free conditions that favor the growth of 3-dimensional floating cell aggregates, so-called tumor spheres. This culture assay allows for the enrichment of TIC and prevents cancer cells from differentiation[116, 125, 126]. It has first been published for normal and neoplastic stem cells from neural and epithelial organs[107, 108, 173, 174]. Additionally, non-neoplastic cells present in the primary tumor specimens do not survive in spheroid cultures.

Methods

After purification of clinical or xenografted cancer samples and depending on their further experimental processing, $1x10^4$ – $1x10^7$cells were spun down (900rpm, 5min, 4°C), resuspended in 5-15ml CSC-medium and seeded in an ultra low attachment flask (passage 0). The culture medium was supplemented with the cytokines hFGF-basic (10ng/ml) and hEGF (20ng/ml). hFGF-basic and hEGF were added twice a week. Medium was changed at least once a month. When spheres were formed and medium was used, indicated by a yellow color, the sphere culture was split and entered a new passage number. Therefore, cells were spun down at 900rpm for 5min at 4°C and the pellet was resolved in 1-10ml fresh CSC-medium. The spheres were manually dissociated by pipetting. Depending on the growth kinetics of each individual sphere line, 1/10 to 1/3 of the cells were than seeded in a new ULA flask containing up to 50ml medium supplemented with hFGF-basic and hEGF. Cells were kept in a humified atmosphere at 37°C and 5% CO_2.

The experiments in this work were performed with sphere cultures in passage numbers 3 to 13 closely resembling the original patient sample without a selective process undergone during culturing. All centrifugation steps with primary, xenografted or sphere cells were carried out at 900rpm for 5min at 4°C unless otherwise stated. CSC-medium, was always supplemented freshly with hFGF-basic and hEGF at a final concentration of 10ng/ml and 20ng/ml respectively.

3.2.1.4 Culturing of Adherent Cell Lines

Scientific Background

In this work, HEK-293T- and HeLa-cells were used. HEK-293T-cells are human embryonic kidney cells which stably express the SV40 T-antigen. HeLa cells are derived from human cervical carcinoma. Both are commercially available.

Methods

Culturing of adherent cell lines was performed in cell culture flasks in an incubator at humidified atmosphere (37°C, 5% CO_2). At 80% confluency, medium was aspirated and the cell layer was washed with 10ml PBS. 5ml 0.05% Trypsin was added and cells were incubated at 37°C for 5min. The reaction was stopped by the addition of 20ml stopping medium. The cell suspension was centrifuged (1200rpm, 5min, 4°C) and resuspended in 5ml growth medium. 1/100 – 1/10 of the cell suspension was transferred into a new flask containing 15ml growth medium and culturing was continued at 37°C.

3.2.1.5 Freezing and Thawing of Purified Tissue Cells, Sphere Lines and Adherent Cell Lines

Scientific Background

For preparation of back up stocks of sphere and cell lines or simply for preserving cells, cells can be frozen and kept viable at very low temperatures for many years. Therefore, cells should be frozen in complete growth medium in the presence of dimethylsulfoxide (DMSO). DMSO acts as a cryoprotective agent: it lowers the freezing point and allows slower cooling rates reducing ice crystal formation which can damage the cells[c].

Methods

If not all purified cells were required for further experimental processing or culturing, a part of the cells was viably frozen. Same was performed for sphere cultures and adherent cultures when expanded and when the cells were still in log phase of growth or at about 80% confluency. Respective cell suspensions were centrifuged or adherent cells were harvested (3.2.1.4) and resolved in 700µl growth-medium. The cell suspension was transferred into a cryotube and mixed with 700µl of the respective freezing-medium reducing the amount of DMSO to 7.5%. Cells were placed in a cryobox filled with 250ml isopropyl alcohol providing a cooling rate of 1°C/min which is required for successful cryopreservation of cells[d]. The box was than kept at -80°C for 24-48hours. Frozen cells were moved to the vapor phase of liquid nitrogen for long-term storage.

For thawing of cells, the respective cryotube was placed in a 37°C water bath in order to thaw the cells until only a small ice piece was left. Cells were transferred in a 50ml falcon tube and 1ml growth-medium or in case of adherent cells conditioned thawing medium was added drop wise in the time span of 1 minute to thaw the remaining cells completely. In order to further dilute the DMSO from the freezing medium, another 5ml of medium were slowly added during the next minute. Finally, 20ml medium were added to the cell suspension which was then centrifuged. Depending on the amount of thawed cells, the pellet was resuspended in 5-15ml fresh growth-medium. Adherent cells were seeded into a cell culture flask. Sphere cells were seeded in a ULA flask and medium was supplemented with hFGF-basic and hEGF as mentioned in chapter 3.2.1.3. Cells were then kept at 37°C, 5% CO_2.

3.2.1.6 Flow Cytometry

Flow Cytometry (fluorescence activated cell sorting, FACS) is a technology that measures cells or particles in liquid suspension. A flow cytometer, like the BD™ LSRII, is composed of three subsystems: fluidics, optics and electronics. Samples are kept in suspension and loaded into the cytometer's fluidic system which brings the sample to a point of interrogation where it is hit by a laser beam of a certain wavelength. Because of

the samples fluorescent properties or previous treatment of the sample with fluorescent dyes and the like, the fluorescent components are excited by the laser light and emit fluorescent light which is subsequently modified by mirrors, a photo multiplier tube system and filters. The modified light is then detected by collection components of the optics subsystem. The electronic subsystem converts the light signal into an electronic one and digitizes the data which is then displayed by a computer using software. The experimenter can measure fluorescence intensity, counts, the relative complexity and the relative size of particles or cells.

Additionally to the BD™ LSR II, the BD FACS Aria™ II Cell Sorter was used in this work. A cell sorter works similar to a common cytometer. The optics system contains additional features for sorting the samples according to different characteristics of choice: the sample drop is charged depending on the emitted fluorescence signal after excitation and deflected in a subsequent electrical field. Tubes at respective positions collect the separated samples which can then be further used.

For flow cytometric measurement or sorting of viable cells, specific fluorescent dyes can be used to exclude dead cells which allows enrichment of a population of interest. Membrane integrity of viable cells guarantees that these dyes cannot invade a cell. Cell death results in loss of membrane integrity. Thus, the stain enters a cell and dead cells can be identified.

For the flowcytometric experiments in this work, Fluorogold or ToTo®-3 Iodide was used. ToTo®-3 Iodide exhibits far-red fluorescence which makes it an excellent stain in combination with fluorochromes excited by the UV- (355nm) or green laser (488nm). Fluorogold was only used in combination with stains occupying the red laser (635nm).

3.2.1.7 Preparation of Cells for Flow Cytometry

Cells were centrifuged and the pellet was washed once with 1ml PBS. Subsequently, cells were resuspended in 500µl ToTo®-3 Iodide and spun down. Samples were resuspended in 300µl PBS and stored on ice until FACS analysis.

3.2.1.8 Hoechst/Pyronin-Staining of Colon Cancer Sphere Cells

Scientific Background

The DNA content of a cell changes with increasing cell age: in G_1-phase, the cell is diploid (2n). The genome is transcribed and DNA is synthesized during S-phase until it is duplicated in G_2-phase. The chromosomes are then separated during mitosis (M-phase). Cytokinesis completes the cell cycle. Each daughter cell is diploid and can reenter G_1-phase. However, some cells that only divide rarely can leave G_1 and enter a stage called G_0. It is supposed to be a quiescent state and is characterized by diploid DNA and low RNA content whereas the RNA content in actively-cycling cells remains

relatively stable[175, 176]. Using the fluorescent DNA-intercalating dye Hoechst 33342 (Hoe) and the RNA-specific dye Pyronin Y (PY) the DNA or RNA contents can be correlated with the fluorescence intensity of the stained cells[176, 177].

Methods

Spheres in medium were pelleted and resuspended in 5ml PBS. After manual dissociation of spheres, single-cell suspensions were prepared by filtering through a cell strainer (40µm pore size). Cells were counted and $5x10^5$ cells were transferred into a sterile FACS tube. They were spun down and resolved in 2ml staining solution containing 1µM Hoechst, PY, a combination of Hoe and PY in the mentioned concentrations or PBS only for an unstained control. Samples were incubated in a waterbath at 37°C for 45min. Addition of PY to a final concentration of 0.3175µg/ml was followed by further 45min incubation. All tubes were gently vortexed every 15min during incubation. Cells were centrifuged and washed once in 2ml PBS or the respective washing buffer (0.66µM Hoe, 0.21µg/ml PY in PBS). Cell pellets were washed once more in PBS or the respective washing buffer with 0.2µM ToTo®-3 Iodide. Finally, cells were resuspended in washing buffer and stored on ice until FACS analysis. Cells were sorted according to their cell cycle status and collected in 0.5ml CSC-medium. The walls of the collection tubes were rinced with PBS. Samples were then centrifuged (1200rpm, 5min, 4°C) and washed once with PBS until they were resuspended in CSC-medium supplemented with hFGF-basic and hEGF.

3.2.1.9 Intracellular staining of colon cancer sphere cells against Ki-67

Scientific Background

Ki-67 is an intracellular, nuclear protein which is only expressed in G_1, S, G_2 and M phase of actively-cycling cells[178]. Its absence from resting cells in G_0 makes it an outstanding marker to discriminate cells in G_0 from proliferating ones. Intracellular proteins can be detected by antibodies. If respective antibodies are conjugated to a fluorochrome, they can be visualized and quantified via flow cytometry. Since antibodies are not membrane permeable, cells have to be fixed and permeabilized in advance.

Methods

$5x10^2$ – $3x10^4$ sorted cells were transferred into a FACS tubes and centrifuged (G_0-, S/G_2/M-cells, 3-fold amount of cells in G_1) All centrifugation steps in this experiment were performed at 1200rpm, 5min, 4°C. The supernatant was discarded and 4ml ice-cold Ethanol was added while vortexing. Cells were then incubated in Ethanol at -20°C for 2h.

They were spun down and washed twice with 4ml staining buffer. Before the last centrifugation step, G_1-cells were separated among three different FACS tubes. Cell

pellets were resuspended in100µl staining buffer (unstained control), 100µl isotype control (0.01µg/µl staining buffer) or 100µl Ki-67-antibody (0.01µg/µl staining buffer). Incubation was performed at room temperature (RT) for 20-30min in the dark. Finally, 2ml staining buffer were added and all samples were centrifuged and resuspended in 200µl staining buffer. Cells were stored on ice until FACS analysis.

3.2.1.10 5-(and-6)-carboxyfluorescein succinimidyl ester (CFSE)-Staining of Colon Cancer Sphere Cells

Scientific Background

The proliferative activity of cells can be investigated through staining cells with the originally non-fluorescent dye 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFDASE). It is highly membrane permeable and is cleaved by intracellular esterases yielding the fluorescent dye 5-(and-6)-carboxyfluorescein succinimidyl ester (CFSE). CFSE is highly reactive and can covalently bind to intracellular aminogroups. Its high lipophilicity also allows it to freely exit from cells. After equilibration, stable fluorescent labeling of cells is achieved[179]. During each cell division CFSE fluorescence is equally distributed among the two daughter cells allowing tracking of cells according to their proliferative history[180].

Methods

The sphere cell suspension was spun down and the pellet was resuspended in 5ml PBS. Spheres were manually dissociated by thorough pipetting and filtering through a 40µm cell strainer. Cells were counted and $1x10^5$ cells were transferred to a 50ml falcon. After centrifugation, cells were resuspended in 4ml CFSE-solution (1µM in PBS) or in PBS only (unstained control). BSA was added to every tube at a final concentration 0.1%. Cells were then stained for 10min at 37°C in a waterbath. After another centrifugation step, cells were washed twice with 50ml 0.1% BSA/PBS before they were resuspended in 3ml CSC-medium and kept at 37°C, 5% CO_2 for 18 hours. Medium was replaced after spinning the cells down and hFGF-basic and hEGF were added. Culturing was continued for further 8 days at 37°C, 5% CO_2. Medium and cytokines were refreshed every 72hours.

3.2.1.11 Determination of CFSE-Staining Efficiency and 10-Days-Kinetics

Before medium was replaced 18hours after staining, an aliquot of cells was withdrawn to determine the initial CFSE intensity (day 0). 500µl of cells were transferred into a FACS tube and centrifuged. They were washed once with 1ml PBS and once with 0.2µM ToTo®-3 Iodide/PBS. Samples were stored on ice until FACS analysis. Fluorescence intensity of CFSE was determined in the FITC-channel in relation to an unstained control.

In order to investigate the label-loss-kinetics in detail, CFSE-stained cells were centrifuged 18hours after staining and resuspended in CSC-medium. Cells were counted and $5x10^4$ stained cells were seeded into 11 wells of a 24-well-plate in 500µl total volume. Every 24hours, one well was emptied and prepared for FACS analysis as mentioned above.

3.2.1.12 Detection of Cell Surface Markers CD133, EpCAM, CD44 and CD166 on Colon Cancer Sphere Cells

Scientific Background

Previous studies suggest that colon TIC can be identified using the cell surface marker CD133 or a combination CD44/EpCAM and CD166[114-116]. Later studies questioned the use of these marker, especially of CD133[117, 181]. In order to investigate whether these markers are enriched in one of the proliferative subfractions, CFSE-stained singularized sphere cells were stained with different antibodies against the respective cell surface molecules.

Methods

Before CFSE-stained samples were analyzed 8 days after culturing via FACS, cells were also stained with different antibodies specific for the surface molecules CD133, CD44, EpCAM and CD166. Therefore, CFSE-stained cells were transferred to a falcon and centrifuged. The pellet was resuspended in 5ml PBS and spheres were manually dissociated and filtered through a 40µm-filter in order to singularize them. Cells were counted and $5x10^4$ cells were kept in a FACS tube and spun down at 1200rpm, 5min, 4°C. Samples were then incubated with the different antibody-mixes in HF as indicated above. One sample was incubated in HF only as an unstained control. Incubation was performed at 4°C for 30min in the dark. 100µl HF were added and samples were centrifuged (1200rpm, 5min, 4°C). Cells were washed with 500µl 0.02µM Fluorogold/HF and the pellet was resuspended in 300µl PBS. Samples were stored on ice until FACS analysis.

3.2.1.13 Determination of the Frequency of Sphere Forming Cells

To investigate the frequency of sphere forming cells in cell fractions with differing proliferative activity, singularized sphere cells were stained with CFSE (3.2.1.10), cultured for subsequent 8 days and analyzed using flow cytometry. Fast, slow and non-dividing sphere cells were sorted in wells of a 96-well-plate in limiting dilution. The number of sorted cells per well varied depending on the growth kinetics of the different sphere lines (1 cell/well, 5 cells/well, 10 cells/well, 50 cells/well, 100 cells/well, 1000 cells/well). To determine the sorting efficiency, one plate was always prepared with 1 cell/well and checked manually under the microscope. Only wells with 1 cell were counted as a successful event.

Sorted cells were cultured in 200µl CSC medium per well supplemented with hFGF-basic and EGF. Every 3 to 4 days, the 96-well-plates were centrifuged at 1000rpm, 7min, 4°C and 100µl medium was carefully withdrawn by a multichannel-pipette. 100µl medium containing the 2-fold concentration of hFGF-basic and EGF were added for maintenance of the cells. Plates were inspected for sphere formation weekly. Wells where spheres developed were counted and compared to number of wells successfully sorted. Frequencies were calculated using the software L-Calc (Stem Cell Technologies).

3.2.1.14 Serial Plating Assay

8 to 12 weeks after the initial sort, single spheres were picked, transferred to a FACS tube and carefully dissociated in 100µl PBS. Cells were spun down at 1200rpm, 5min, 4°C and washed with 100µl 0.2µM ToTo®-3 Iodide/PBS. Living cells were sorted into in wells of a 96-well-plate in limiting dilution. The sorting efficiency was determined by manual observation and cells were cultured as described in 3.2.1.6. The frequencies of sphere forming cells were calculated and compared to the previously analyzed generation of cells.

3.2.1.15 Cytotoxicity Tests of Hoechst 33342, Pyronin Y, CFSE and Cytostatic Drugs

In this work, different sphere lines were used for analyzing the proliferative and cell cycle activity of colon TIC *in vitro* using the dyes CFSE, Hoechst 33342 and Pyronin Y. Since sphere cells were sorted and needed to be kept viable for further culturing or transplantation into immunodeficient mice, the long-term toxicity of these dyes needed to be determined.

CFSE-, Hoechst-, Pyronin Y- or Hoechst/Pyronin Y-stained and unstained cells were counted and $1x10^4$cells were seeded into 12 wells of a 96-well-plate into 200µl CSC growth medium. Every 24h, 100µl were withdrawn from one well, pipette carefully up and down for 10 times to singularize the cells and mixed 1:1 with trypan blue. Cells were counted with an automated cell counter and the number of stained cells was normalized to the number of unstained cells to assess an effect of the staining on cell viability.

3.2.1.16 Production of Lentiviral Particles

Scientific Background

In order to stably express H2B-GFP and the corresponding repressor $tTA2^S$ in sphere lines, lentiviral vectors were produced which encode the respective expression cassette. These vectors were used as a gene shuttle delivering the genetic material of interest into the target cells. The lentiviral genome encodes for three essential viral proteins: gag is

required for the formation of structural proteins to protect the viral core and genome, pol is involved in protein processing, reverse transcription of the viral genome and integration into the host genome and the envelope-glycoprotein env is necessary for the attachment to and fusion with the target cell. The products of the regulatory genes *tat* and *rev* and of the accessory genes *vif, vpu, vpr* and *nef* play a role in regulation of gene expression, replication and host recognition. Furthermore, gene expression is controlled by two long terminal repeats (LTR), one at each end of the viral genome (5'- and 3'-LTR). LTRs contain the Psi-sequence which is required for packaging of the viral RNA into the virus capsid to continue the infection of HIV in its host and the primer binding site where tRNA binds and acts as a primer to initiate reverse transcription. The polypurine tract (PPT) enables DNA synthesis[182].

In order to improve the safety in molecular biology and gene therapy trials, the viral wildtype genome was modified by deleting a short region within the 3'LTR which contains transcription factor binding sites[183]. This modification led to the development of self-inactivating (SIN) lentiviral vectors which are not able to replicate anymore (replication incompetent). As a result, new expression cassettes composed of promoter and a corresponding gene of interest were introduced facilitating development of genetically engineered lentiviral vectors for research and for clinical applications[184-187]. In this context, the envelope-protein env of HIV-1 was replaced by the glycoprotein G of the vesicular stomatitis virus envelope protein (VSV-G). In contrast to HIV-1-env, VSV-G pseudotypes allow transduction of cells independent of CD4 and co-receptor and thus show extended host tropism[184]. In addition, HIV-based lentiviral vectors allow efficient transduction of cells in G_1 or G_0 phase of the cell cycle[188].

In this work, 293T-cells were co-transfected with plasmids encoding all viral components required for the formation of SIN-HIV-1-derived lentiviral vectors. All viral components were separated among three plasmids in order to minimize the risk of replication competent virus formation.

Methods

$5x10^6$ 293T-cells were seeded into 15cm-dishes. Cells were grown in 15ml IMDM growth medium for 24hours at 37°C and 5% CO_2. Medium was replaced with 13ml fresh growth medium. Polyethyleneimine (PEI, 179.25µg/500µl blank IMDM) was used as a transfection agent. PEI packs the negatively charged DNA into positively charged particles facilitating cellular uptake by endocytosis. 500µl plasmid-mix was prepared with blank IMDM. The solution was filter sterilized (0.22µm pore diameter) and mixed with an equal volume of PEI/IMDM. After 20min incubation at RT, the mixture was added onto the cells and the dish was carefully shaken in order to distribute the DNA among the cells.

Plasmid	Function	Amount per dish
psPAX2	Packaging vector	18.75µg
pMD2.G	Envelope vector	9µg
pWPXL-TTT-H2B-GFP	Transfer vector	32µg

Medium was refreshed after 12h incubation (15ml). After further 48hours of incubation, medium containing lentiviral particles was harvested and filter sterilized (stericup vacuum filtration system). It was seperated among ultracentrifugation tubes and lentiviral particles were pelleted at 20.000rpm, 2h, 20°C. The supernatant was discarded and tubes were put upside down on an ethanol-wetted tissue. After 10min incubation at RT, residual medium was removed by a sterile cotton-tip. 30µl PBS were added and incubation was performed at RT for 30min. Then, the pellet was washed 25 times with the PBS. The supernatants from all centrifuge tubes were collected in a 1.5ml-reaction tube and shaken at RT for further 20min. The concentrated viral supernatant was aliquotted and stored at -80°C for at least 16h.

3.2.1.17 Determination of Functional Lentiviral Titers

Scientific Background

The efficiency of lentiviral infection depends on the amount of functional and infectious viral particles present at the time of infection. This amount might differ depending on production and harvest conditions. It is therefore helpful to know the amount of infectious particles in a given solution (functional titer). The titer can be quantified using a serial limiting dilution transduction assay under standardized conditions.

Methods

HeLa cells were grown in a cell culture flask in IMDM-growth medium. At about 80% confluency, cells were harvested as described in 3.2.1.4. Cells were counted (3.2.1.2) and $5x10^4$ cells were seeded into each well of a 6-well-plate in 3ml growth medium. After 24hours of incubation (37°C, 5% CO_2) one well was trypsinized and counted to determine the numbers of cells at the timepoint of infection (3.2.1.2).

Growth medium was replaced by 500µl conditioned growth medium containing 16µg Polybrene per ml. Virus was diluted in a 24-well-plate: 2µl virus stock were added into 1ml growth medium and mixed properly. Five serial 1:10-dilutions were prepared by transferring 100µl of the previous well into 900µl fresh growth medium (dilution factor 10^{-3} – 10^{-7}). Subsequently, 500µl of each viral dilution were added into the wells containing the cell layer in 500µl conditioned medium. Transduction was allowed for 72hours at 37°C, 5% CO_2. Then, cells were trypsinized and prepared for flow Cytometry (3.2.1.7).

The functional titer of the lentiviral stock solution was calculated with the following formula from the sample where 1% to 25% (z%) of all living cells were transduced:

$$\#\ of\ TU/_{ml} = \#\ of\ cells\ (time\ of\ infection) x\ dilution\ factor\ x\ \frac{z\%\ GFP^{+} cells}{100}$$

3.2.1.18 Transduction of Primary Colon Cancer Cells with Lentiviral Vectors

Scientific Background

Lentiviruses are frequently used to genetically modify a cell. The functional basis is integration of the viral DNA into a host cells genome followed by stable expression of the newly introduced genetic material. Infection of a cells starts with recognition of its receptor by a viral particle (Figure 7). Once a particle is attached, it fuses with the targets cell's membrane and deposits its genomic RNA into the cytoplasm. Viral proteins, host proteins and the viral RNA form the pre-integration complex (PIC). Single-stranded (ss) RNA is reversely transcribed into ss cDNA by the viral reverse transcriptase. A host cell's DNA polymerase transcribes it into double-stranded (ds) cDNA. Processed DNA is then actively transported into the nucleus where integration of the viral DNA into the host cell's genome is promoted by a viral integrase. The newly generated DNA ("provirus") is transcribed and subsequently translated in the cytoplasm by the host cell machinery. Viral proteases modify the synthesized polypeptides and finally proteins assemble to form the new lentiviral particle which is released into the surrounding medium and ready to infect new target cells.

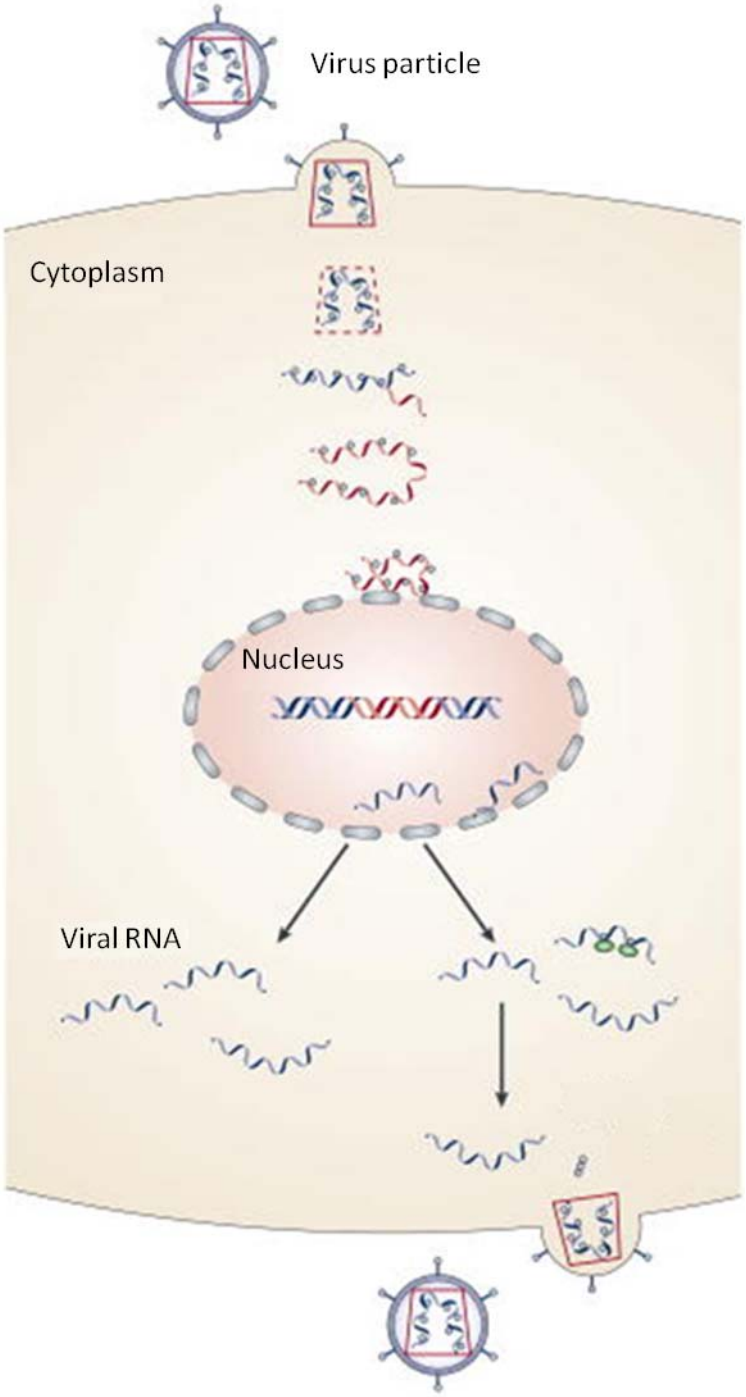


Figure 7: Life Cycle of HIV-1-derived lentiviruses.

Viral particles recognize the host cell's receptor. Virus-host-fusion is followed by release of the viral genome into the cytoplasm. ssRNA is reversely transcribed into ds cDNA which is transported into the nucleus. Integration of the viral DNA into the host cell's genome leads to efficient production of viral proteins and finally to formation of new lentiviral particles ready to infect new target cells. Adapted from Ozato *et al.* 2008[189].

The viral DNA semi-randomly integrates into the host cell's genome. All cell descendants also carry the viral DNA at exactly this site and thus a unique clonal mark is established in each successfully transduced cell.

The transduction efficiency of target cells depends not only on the functional titer of a viral stock solution but also on the actual number of viruses entering a cell. One cell can be entered by more than one virus while another cell may not absorb any virus particle. The transduction efficiency therefore directly depends on the ratio of functional virus particles to target cells. This ratio is named *multiplicity of infection* (MOI). In order to increase infection efficiencies, MagnetoFection™ technology was used (Figure 8): virus particles were associated with magnetic nanoparticles. Application of a magnetic field targets the virus-nanoparticles to the cells and increases infection rates.

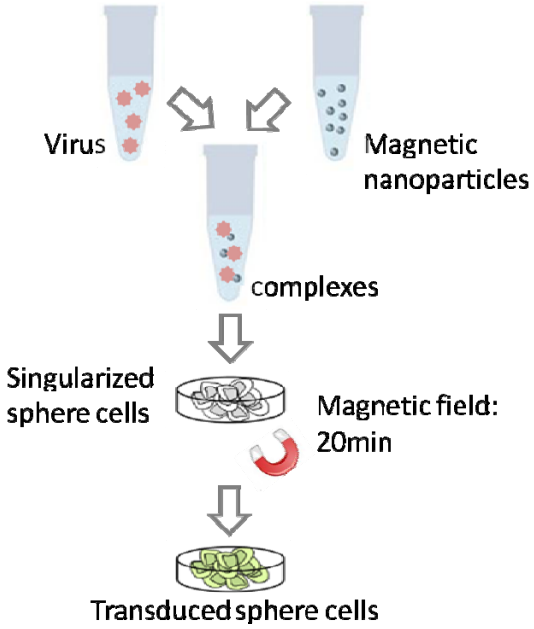


Figure 8: Transduction using MagnetoFection™ technology.

Virus-nanoparticle-complexes are targeted to spheroid cells by application of a magnetic field which increases infection rates.

Methods

For lentiviral transduction of TIC, sphere cultures were centrifuged and resuspended in 1ml fresh CSC medium. Spheres were manually dissociated and singularized via pipetting with a 200µl tip. Cells were counted and $5x10^4$cells were transferred into the well of a 24-well-plate and medium was brought to a total volume of 400µl. To achieve an MOI of 100, $5x10^6$ viral particles were mixed with 1.5µl Viromag magnetic beads and H_2O in a final volume of 100µl. After 20min incubation at RT, the virus-mix was added into the wells and mixed with the cells. Infection was allowed for 16h in an incubator at 37°C; 5% CO_2. Sphere cells were harvested, centrifuged and medium was replaced with fresh CSC medium containing FGF and EGF. Depending on their intentional use, cells were kept on ice or culturing was continued in an incubator.

3.2.1.19 Determination of Transduction Efficiencies

The transduction efficiency was determined 72h after transduction: for low cell numbers, cells carrying the reporter gene GFP were observed under the fluorescence microscope, positive events were counted manually and the percentage of GFP-positive cells was calculated. For higher cell numbers and for a more accurate determination, an aliquot of cells was prepared for flow cytometry (3.2.1.7) and the percentage of GFP-positive cells in a given population of viable cells was determined.

3.2.1.20 Validation of the Tet-Off-regulated expression of H2B-GFP in HeLa cells and sphere cultures

After determining the transduction efficiency, $5x10^4$ transduced HeLa cells were kept in culture in a 6-well-plate. The same number of untransduced cells was also kept in parallel as a negative control. Cells were cultured in 3ml IMDM growth medium until 80% confluency, harvested as described in 3.2.1.4 and culturing was continued with 1/10 to 1/100 of the cells. Remaining cells were prepared for FACS analysis (3.2.1.7). The percentage of GFP-positive cells was determined everytime when splitting the cells. After 4 to 6 weeks in culture, untransduced and transduced HeLa cells were counted and $5x10^4$cells were seeded. An equal number of transduced cells were seeded into an additional well and treated with 10ng doxycyclin per ml medium. The percentage of GFP^+ cells under doxycyclin-treatment was compared to that of untreated transduced cells. The same experiment was performed with TSC-01 and TSC-08. Sphere cells were maintained in 5ml medium in an ULA T25 flask as described in 3.2.1.3. GFP precentages were measured frequently over 5 months mimicking the time period sphere cells spent in mice.

3.2.1.21 Testing the Sensitivity of sphere lines towards 5-Fluorouracil and Oxaliplatin

Scientific Background

Since transplanted mice were planned to be treated with chemotherapy, the effect of 5-Fluorouracil (5-FU) and Oxaliplatin on respective sphere lines was estimated *in vitro* as a preliminary test. These drugs are commonly used in colon cancer chemotherapy[165, 166, 190].

Methods

Sphere cells were harvested and centrifuged. The pellet was resuspended in 1ml CSC medium and spheres were manually dissociated and singularized via pipetting and filtering (40µm pore size). Cells were then counted and $1x10^4$cells were seeded into 12 wells of a 96-well-plate into 100µl CSC growth medium containing the 2-fold concentration of FGF and EGF. Then, 100µl of 2-fold concentrated drug were added

into the wells. Final concentrations of 5-FU and Oxaliplatin were 0, 0.001, 0.01, 0.1, 1, 10, 100 and 1000µg/ml. Every 48h, 100µl were withdrawn from one well, pipette carefully up and down for 10 times to singularize the cells and mixed 1:1 with trypan blue. Cells were counted with an automated cell counter and the number of treated cells was normalized to the number of untreated cells to assess sensitivity of the cells for the chemotherapeutic drug.

3.2.1.22 Preparation of cells for Xenotransplantation

For transplantation, tumor sphere cells were dissociated, counted and 500 to $1x10^6$ cells were transferred into a 1.5ml reaction tube. Cells were pelleted at 2000rpm, 5min, RT and resuspended in 25µl CSC medium including FGF and EGF. The cell suspension was mixed with an equal volume of matrigel and transferred into an insulin syringe. The syringe was kept on ice until transplantation.

3.2.2 Animal Experiments

3.2.2.1 Mouse Strain and housing

Scientific Background

In this work, only NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice, also known as Il2rg$^{-/-}$, Il2RG^{null} or NOD SCID gamma (NSG), were used. This mouse strain lacks B-cells, T-cells and functional NK-cells. Consequently, it displays a severe immune deficiency facilitating the engraftment of human cancer cells[191-193]. Colon sphere cells were injected into the kidney capsule of these mice. Injection of colon cancer cells into the murine kidney capsule has been associated with the best tumor take rate, generation of tumors that closely resembled the patient material and high cell numbers could be transplanted which minimizes the risk of losing cell clones in genetic marking experiments[114, 125].

Methods

NSG mice were kept at the animal facility in individually ventilated cages (IVC) and isolators. These housing conditions are pathogen free and provide a clean atmosphere for immunodeficient animals. Mice received sterilized feed and bedding. All supplies and equipment was disinfected prior to animal contact. Gloves, coat, mask and cap were worn in respective housing rooms. Experiments were performed under sterile conditions in a laminar flow hood. Animals were sacrificed by cervical dislocation when a tumor reached a size of $1cm^3$ or when the mice suffered.

3.2.2.2 Transplantation of Cells under the Kidney Capsule of Immune Compromised Mice and Serial Transplantation Assay

Cells were prepared for transplantation according to 3.2.1.22. At the animal facility, a mouse was anesthetized using 1.8% isoflurane (inhalation narcosis) and narcosis was maintained on a 37°C heat pad. Eyes of the mouse were covered with Bepanthen (eyes and nose lotion) to keep them moist. The left flank was shaved using a scapel and the skin was disinfected with sterile alcohol pads. On the upper right of the spleen, a skin incision of about 1cm was made. Using blunt scissors, the skin was mobilized from the peritoneum. The abdominal cavity was opened by a small cut above the kidney. Light manual pressure was applied to expose the kidney. Peritoneum and kidney were kept moist using sterile cotton tips and PBS. The syringe containing cells in matrigel was inserted under the kidney capsule and the viscous mixture was injected before the syringe was carefully removed. PBS-soaked cotton tips facilitated the repatriation of the kidney into the abdominal cavity. The peritoneal cut was stitched using resorbable material and the skin was closed using wound clips. Iodide solution was applied liberally and the mouse was uniquely ear marked. Narcosis was ended and the mouse was kept on the heat pad until reaching its consciousness. From then on, animals were continuously treated with 50µg Baytril® per ml drinking water. Transplanted mice were checked every day during the first week after surgery and every second day in subsequent weeks. The wound clips were removed 10 days after transplantation.

3.2.2.3 Excision of organs and xenografted tumors from transplanted mice

Mice were sacrificed by cervical dislocation and fixed backwards on a solid underground. The abdomen was opened along the median ventral line and the skin was also fixed on the underground. Tumor and corresponding kidney were carefully separated from the surrounding tissue and excised. Liver, spleen and lungs were also carefully removed and all was kept in PBS on ice until further processing.

3.2.2.4 Administration of Tetracycline to NSG mice

In order to suppress H2B-GFP-experssion in transduced and transplanted cells, doxycyclin was applied to the drinking water of mice (2g/L) from three weeks on after transplantation. The dox-water was additionally supplemented with 1.5% sucrose to mask the bitter taste of doxycyclin.

3.2.2.5 Administration of 5-FU on NSG mice

5-FU was administrated intraperitoneally (i.p.). A stock solution was prepared at a concentration of 10mg/ml PBS. The mouse was hold in a safe and tight grasp with one hand, it was turned around to expose the abdominal site and the head was tilted downwards to move inner organs upwards. Using a 26G needle, a syringe loaded with

5-FU in PBS was inserted into the lower right quadrant of the abdomen and 5-FU was injected. The needle was removed carefully and the mouse was returned to its cage.

Starting three weeks after transplantation, 85 mg 5-FU/kg body weight was administered weekly for three weeks in a row. Mice were then sacrificed and tumors and organs were harvested. 150 mg 5-FU/kg body weight was injected in another experiment 3 days prior to scarification. The total volume injected did not exceed 350µl.

3.2.3 Molecular Biological Methods

3.2.3.1 Transformation of Competent *Escherichia coli*

Scientific Background

Bacteria exhibit distinct features which make them ideal organisms to amplify DNA: Various methods have been described to introduce exogenous DNA into bacteria (transformation)[194-202], always provided that they were in DNA-uptake-enabled state (competence). For this work, One Shot® TOP10 chemically competent *E. coli* (genpotype F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G λ^-) and One Shot® Stbl3™ chemically competent *E. coli* (genotype F^-*mcr*B *mrrhsd*S20(r_B^-, m_B^-) *rec*A13 *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Str^R) *xyl*-5 λ^-*leumtl*-1) were purchased from Invitrogen. The competent state was achieved by Ca^{2+}-treatment leading to masking of the negatively charged outer bacterial membrane which consists of lipopolysaccharides and phospholipids. The negatively charged DNA molecules are attracted and can enter the cell upon heat shock which weakens the membrane structure and leads to pore formation[203].

Methods

Chemically competent *E.coli* were stored at -80°C and thawed on ice for 30min. 1µg plasmid was added and incubated on ice for further 30min. Bacteria were heat shocked for 30s on 42°C (Stabl3 45s on 42°C). The mix was cooled on ice for 2min and 250µl SOC-medium was added. Incubation was performed for 60min at 37°C to help bacteria recover from the heat shock.

3.2.3.2 Propagation of Transformed Bacteria

Scientific Background

If absorbed plasmids harbor a gene conferring antibiotic resistance, transformed bacteria can be enriched by antibiotic selection: the bacterial cell suspension is grown on agar plates containing the respective antibiotic and only plasmid containing bacteria will

have the ability to metabolize the drug and to form colonies. Single colonies can then be picked and amplified in liquid cultures.

All plasmids used in this study harbored an ampicillin-resistance-gene.

Methods

50-100µl of the bacteria suspension was plated onto ampicillin-containing agar plates (100µg/ml). Plates were incubated upside down at 37°C for 12-16h. Formed colonies were picked by an autoclaved toothpick and transferred into 5ml LB medium containing 100µg ampicillin/ml. Bacteria were allowed to grow for 8-14h at 37°C, 190rpm. Depending on the desired amount of DNA, liquid cultures were either used for DNA isolation (Mini Prep) or used for inoculation of 250ml ampicillin-LB medium which was then kept on the shaking incubator for further 12-14h and finally used for DNA isolation (Maxi Prep).

3.2.3.3 Isolation of Plasmids

Scientific Background

Plasmids can be isolated by alkaline lysis: addition of an alkaline solution containing a detergent like sodium hydroxide (NaOH)/Sodium dodecyl sulfate (SDS) disrupts bacterial cell membranes, the chemical can enter the cell and nucleic acid is denatured. A neutralizing solution like sodium acetate allows the renaturing of plasmid DNA, but precipitates chromosomal DNA and the detergent. The lysate can be filtrated to separate precipitated components and debris. The plasmid can then be purified using an ion-exchange polymer column. DNA is bound to the column, washed and finally eluted with a saline solution. Purified DNA is then pelleted by isopropanol-percipitation and centrifugation and dissolved in H_2O.

Methods

Bacterial cultures were centrifuges at 8000rpm, 5min, 4°C (5ml Mini culture) or at 4600rpm, 30min, 4°C (250ml Maxi culture) and either frozen and stored at -80°C or directly submitted to alkaline lysis according to the kit-manufacturers protocol. The following kits were used:

- Mini Prep: GeneMATRIX Plasmid Miniprep DNA Purification Kit
- Maxi Prep: Qiagen EndoFree® Plasmid Purification Kit.

The concentrations of DNA were determined using the spectrophotometer NanoDrop® ND-1000. Purified DNA was stored at -20°C.

3.2.3.4 Restriction Digest

Scientific Background

DNA can be digested enzymatically using restriction endonucleases. These enzymes recognize particular and unique sequences of DNA and act as molecular scissors producing DNA fragments of distinct sizes with 5'-, 3'- or no overhang (blunt end). Visualization of these DNA fragments using agarose gel electrophoresis and ethidium bromide as an intercalating agent helps to determine fragment sizes which can then be compared to theoretically expected ones. Fragments can be extracted from the gel and be used in further cloning experiments or the gel is simply used to determine integrity of isolated DNA samples like in this work.

Methods

2µl of the purified DNA sample were mixed with 18µl digestion buffer (see Table 1) and incubated at 37°C for 1h. In some cases, bovine serum albumin (BSA) had to be added (final concentration 100µg/ml) to stabilize enzymes and to prevent their adhesion to plastic surfaces during incubation. The plasmids pMD2.G, pSPAX2 and pWPXL-TTT-H2B-GFP were digested with the enzyme Kpn1 and its corresponding NEB buffer 1 under presence of BSA.

Table 1: Buffer composition for restriction digestion.

10x NEB buffer	2µl
10x BSA (if necessary)	2µl
NEB enzyme	5 units
H_2O	ad 20µl

3.2.3.5 Agarose Gel Electrophoresis

Scientific Background

DNA molecules are negatively charged due to their phosphate groups. They can thereby move in an electrical field. The velocity of their movement depends on their size (correlating with the molecules total charge and mass) and on the constitution of the medium. One can take advantage of these facts using agarose gel electrophoresis: DNA is loaded onto an agarose gel, an electrical field is applied and the molecules start to move through the electrical field. Agarose is a natural polysaccharide found in red algae. As a gel, agarose medium is porous. Its pore size depends on the concentration of agarose (0.8 - 2%). Smaller molecules migrate faster than bigger molecules because they move much easier through the pore mesh. Thus, DNA fragments can be separated according to their size. A DNA intercalating dye helps to visualize the fragments and comparison to a marker of known size lets one determine the size of these fragments.

Fragments can be extracted from the gel and be used in further cloning experiments or the gel is simply used to determine integrity of isolated DNA samples like in this work.

Methods

Depending on the intended concentration, 0.8 – 2g of purified agarose were dissolved in 100ml 1x TBE buffer under heating in a microwave. After a short cool down, 2 drops of ethidiumbromide (1mg/ml) were added and the mixture was pivoted carefully and filled into a gel carrier. A comb was inserted for the formation of chambers and the gel was solidified and cooled for 20min at RT. The carrier containing the gel was placed into a chamber with 1x TBE buffer. 8µl of the digested DNA samples were mixed with 2µl loading buffer 5x Blue Run and loaded into the gel chambers. Gel electrophoresis was perfomed for 45min at 150V. Fragment sizes were estimated by visualization under UV light and comparison of signals to a DNA ladder loading control.

3.2.3.6 Sequencing of plasmids

Scientific Background

Plasmids had to be checked for their integrity before amplification and usage in further experiments. Sequencing was performed according to Sanger *et al.*[204]: DNA is replicated *in vitro* using a primer, a DNA polymerase, a mix of normal deoxynucleosidetriphosphates (dNTP) and only one type of radio-labeled dideoxynucleotides (ddNTP) lacking a 3'-OH group. If ddNTPs are incorporated into the newly synthesized DNA, replication is terminated since a new phosphodiester bond cannot be formed. These fragments are subsequently separated via electrophoresis and the sequence can directly be read from the gel picture.

Methods

2.5µg of the DNA sample were diluted in 30µl H_2O in a 1.5ml reaction tube. Sequencing was performed at GATC Biotech in Konstanz. Primers were chosen based on the following criteria: 17-28 bases in length, G/C-content of about 50-60%, location about 50bp up- or downstream of region to be sequenced, melting temperature preferentially 52 – 58°C and a G- or C-3'end was favored. If not available, primers were also synthesized by GATC Biotech.

3.2.3.7 Isolation of Genomic DNA from Xenografted Tumors

After purification of xenografted tumors, at least 1/3 of the cells were pelleted at 2000rpm for 5min. The supernatant was discarded and samples were frozen and kept at -20°C.

Pellets were thawed on ice for 30min and DNA was isolated according to Qiagens DNeasy Blood & Tissue Kit protocol. Cells were lysed, DNA bound to silica surfaces and was thereby separated from debris and contaminants. DNA was finally eluted in 50-

100µl H_2O and concentrations were determined using the spectrophotometer NanoDrop® ND-1000. Purified DNA was stored at -20°C.

3.2.3.8 Linear Amplification Mediated PCR

Scientific Background

Efficient transduction of cells is based on integration of viral DNA into the host cell's genome. These integration sites (IS) are unique for each cell and its descendants. Tracking of IS by linear amplification mediated PCR (LAM-PCR) and subsequent sequencing allows to determine the clonal composition of xenografted tumors in serial transplantation assays. Schmidt *et al.* established this method in order to detect viral IS in clinical gene therapy samples[205] harboring the possibility of insertional mutagenesis and subsequent malignant transformation[206-208].

Virus-specific, biotinylated primers were used to amplify the genome-virus junctions. Single-stranded DNA fragments composed of the known primer-vector-sequence and the unknown adjacent part of the host cells genome were immobilized on streptavidin-coated magnetic beads. Double-stranded DNA was enzymatically synthesized and subsequently digested. A restriction-site-specific linker of a known sequence was coupled to the remaining dsDNA. The DNA fragments were denatured and removed from the solid phase. ssDNA was further amplified using linker-specific and nested primers. Finally, amplified DNA was purified, concentrated and submitted for high resolution gel electrophoresis.

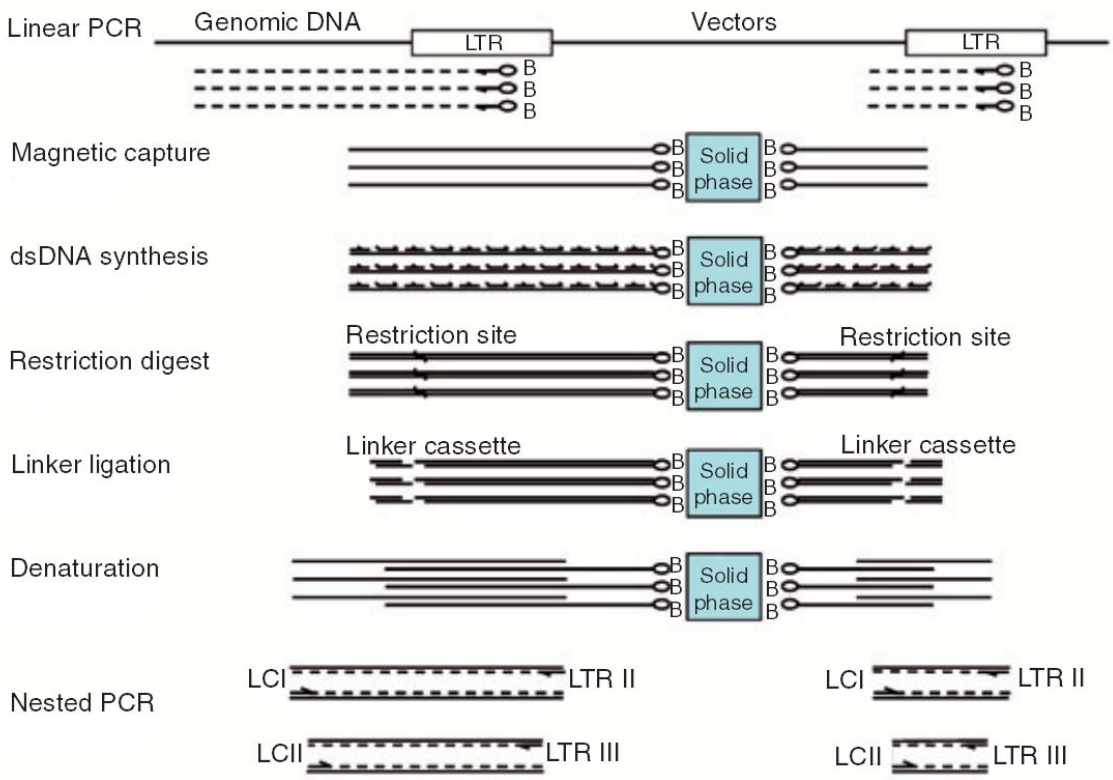


Figure 9: Schematic outline of linear amplification mediated PCR (LAM-PCR).

Genome-virus junctions were amplified using virus-specific, biotinylated primers. ssDNA products were immobilized on streptavidin-coated magnetic beads and dsDNA was synthesized and subsequently digested. Coupling of restriction-site-specific linkers to immobilized dsDNA and subsequent denaturation yielded ssDNA which was further amplified using nested primers. Adapted from Schmidt *et al.* 2007[205].

Methods

Generation of linker

The linker was generated by ligation of two oligonucleotides: a universal long one (LC 1) and a short one which had to be chosen according to the overhanging sequence produced by the enzyme used for restriction digestion. In this study, the enzyme Tsp509I was used for restriction digestion yielding an AATT-overhang. Thus, LC 3 was mixed with LC 1 according to the following protocol:

LC 1 (100µM)	40µl
LC 3 (100µM)	40µl
$MgCl_2$ (100mM)	10µl
Tris (250mM)	110µl

The mix was incubated for 5min at 95°C in a thermo mixer which was subsequently switched off and the reaction mix was cooled down over night. 300µl H_2O were added and transferred into a Microcon-30 column. Centrifugation was performed at 12600rpm, 12min, RT and the flow through was discarded. The column was turned upside down

and placed into a new collection tube. DNA was harvested by centrifugation (3600rpm, 3min, RT) and filled up to 80µl with H_2O. The linker was aliquotted and stored at -20°C.

Linear PCR

A 96-well-PCR-plate was prepared containing 50µl DNA-reaction mix per well:

DNA	100-500ng
10x PCR-buffer	5µl
dNTP (10mM)	1µl
Taq DNA Polymerase (5U/µl)	0.5µl
Primer SK LTR 1 bio (0.167µM)	0.25 µl
Primer SK LTR 2 bio (0.167µM)	0.25 µl
H_2O	ad 50µl

DNA was amplified using the following program

step	Temperature	Time
Initial Denaturation	95 °C	2min
Denaturation	95 °C	45sec
Annealing	58 °C	45sec
Elongation	72 °C	1min
Final Elongation	72 °C	5min

After 50 cycles of denaturation, annealing and elongation, 2.5 U of fresh Taq DNA Polymerase were added. DNA was then amplified for further 50-cycles using the same program.

Magnetic Capture

The amplified, biotin-tagged ssDNA fragments were then captured by streptavidin-coated magnetic beads. Therefore, 200µg beads per sample were collected by a magnet at the wall of a reaction tube. The storage buffer was removed and beads were washed twice with PBS/0.1% BSA (40µl/200µg beads) and once with 3M LiCl (20µl/200µg). Finally, beads were resuspended in 6M LiCl (50µl/200µg) and added to 50µl PCR product (ratio 1:1). Capturing of biotinylated DNA by the streptavidin-coated beads was performed at 300rpm, over night, RT.

dsDNA synthesis

Complementary DNA was synthesized using klenow-enzyme: the PCR-pate was exposed to a magnetic field and the supernatant was withdrawn. Beads/DNA-complexes were washed with 100µl H_2O and resuspended in 10µl reaction mix.

H_2O	8.25µl
10x Hexa-Nucleotide-Mix	1µl
dNTPs (200µM)	0.25µl
Klenow (2U/µl)	0.5µl

Samples were incubated at 37°C, 1h and then stored on ice.

Restriction digest

90µl H_2O were added to each sample and a magnetic field was applied. The supernatant was discarded and beads/DNA-complexes were washed once more with 100µl H_2O. Finally, they were resuspended in 10µl reaction mix.

H_2O	8.8µl
10x NEB-buffer 1	1µl
Enzyme Tsp509I (5U/µl)	0.2µl

Samples were incubated at 65°C for 1h.

Linker ligation

Digested, dsDNA fragments were ligated with the restriction site-specific linker using "Fast-Link™ Ligation Kit": 90µl H_2O were added to each sample which was then exposed to a magnetic field. The supernatant was discarded and samples were washed one more time with 100µl H_2O. The beads/DNA/linker-complexes were resuspended in 10µl ligation mix.

10x ligation buffer	1µl
ATP	1µl
Ligase	1µl
Linkerkassette	2µl
H_2O	5µl

They were incubated for 5min at RT and subsequently stored on ice.

Denaturation

DNA was removed from the beads and denatured by addition of an alkaline solution: first, 90µl H_2O were added to each sample and the supernatant was removed after

exposure to a magnetic field. Samples were again washed with 100µl H_2O and then resuspended in 5µl 0.1M NaOH. Denaturation was allowed for 10min at 300rpm, RT. Beads were attracted by a magnet and the supernatant containing DNA fragments was carefully transferred into 0.5ml reaction tubes which were finally stored on ice.

Nested PCRs

The denatured DNA was submitted to a first round of nested PCR: a 96-well-PCR-plate was prepared containing 50µl DNA/reaction mix per well:

DNA (denaturation product)	2µl
H_2O	40.5µl
10x buffer (Qiagen)	5µl
dNTP (10mM)	1µl
Taq DNA Polymerase (5U/µl)	0.5µl
Primer LC I (16.7µM)	0.5 µl
Primer SK LTR 4 bio (16.7µM)	0.5 µl

DNA was amplified using the following program:

step	Temperature	Time
Initial Denaturation	95 °C	2min
Denaturation	95 °C	45sec
Annealing	60 °C	45sec
Elongation	72 °C	1min
Final Elongation	72 °C	5min

35 cycles of denaturation, annealing and elongation were carried out.

The first round of nested PCR was followed by second round using two other, more nested primers:

DNA (product from 1st nested PCR)	2µl
H_2O	40.5µl
10x buffer (Qiagen)	5µl
dNTP (10mM)	1µl
Taq DNA Polymerase (5U/µl)	0.5µl
Primer LC II (16.7µM)	0.5 µl
Primer SK LTR 5 bio (16.7µM)	0.5 µl

DNA was amplified using the same program. 8µl of each sample were submitted to high resolution gel electrophoresis whereas the remaining part was frozen and stored at -20°C.

3.2.3.9 High resolution gel electrophoresis

DNA fragments were seperated using spreadex EL 1200 gels which are made of a synthetic polymer. Samples were mixed with 5x Elchrom loading buffer (ratio 1:5) and 10µl were loaded into the chambers of the gel. Gel electrophoresis was performed in a tank containing Elchrom buffer (mixed 1:40 with H_2O) for 90min at 130V. Gels were incubated in 100ml H_2O containing 2 drops of ethidiumbromide (15min, RT). Fragment sizes were estimated by visualization under UV light and comparison of signals to a DNA ladder loading control.

3.2.4 Histopathological Methods

3.2.4.1 Fixation and Processing of Tissue Samples

In order to obtain tissue slices (primary and xenografted tumor or organs), tissue samples had to be embedded into a solid media like paraffin or paraplast (a mixture of paraffin and synthetic polymers).

First, tissue samples in embedding cassettes were incubated in 10% formalin for 24hours, 4°C. They were then stored in PBS at 4°C for up to 3 weeks. Since paraffin is not water-miscible, tissue had to be drained in an ascending ethanol series (2h 70% EthOH, 2h 85% EthOH, 2h 95% EthOH, 3x 2h Isopropanol). Incubation with Xylol (2x 2h) removed remaining alcohol. Finally, samples were kept in melted paraffin for 2-8h until they were transferred into metal forms and covered by melted paraffin. Solid tissue blocks were produced by letting the paraffin harden at 4°C. Blocks were then stored at room temperature until usage.

3.2.4.2 Pathology and Generation of Tissue Slices

Pathological evaluation of primary patient tumors, spheroid cultures, xenografts and respective organs was performed in collaboration with PD Dr. Karsten Brand and Prof. Dr. Wilko Weichert. Prof. Weichert also provided tissue slices and HE staining of submitted samples.

3.2.4.3 Immunohistochemistry

Tissue slices of H2BGFP-transduced tumors were stained with an anti-GFP-antibody by the Weichert lab. The distribution of GFP-label-retaining cells was assessed and evaluated by Prof. Dr. Wilko Weichert.

4 Results

Our lab has shown that the human colon cancer TIC compartment is composed of distinct types of TICs differing in their ability to contribute to tumor formation in a serial transplantation assay[135]. DC-TIC were only active in secondary or tertiary mice suggesting an initial mitotic quiescence in primary generations of mice. The proliferative and cell cycle activity of human colon TIC has not been investigated so far. In order to characterize human colon TIC in more detail, this study thoroughly investigates their proliferative activity and dynamics *in vitro* and *in vivo*.

4.1 Primary Sphere Cultures are enriched for Human Colon Cancer initiating Cells

Colon cancer cells lines have widely been used to study the disease *in vitro*. Stable cell lines are usually cultured over several months where cells accumulate phenotypic and genetic aberrations diverging from the original patient tumor. To overcome this limitation, only primary patient-derived tumor material was used in this study. Patient tumor or metastasis samples were purified and isolated cells were expanded using two different strategies to enlarge engraftment efficiency: cells were grown *in vitro* under serum-free, growth factor supplemented and low attachment conditions. Cells grew in 3-dimensional, non-adherent structures called spheroids. Sphere formation in freshly purified samples started 2 weeks to 5 month after purification. However, not all purified samples directly engrafted *in vitro*. This is why freshly purified patient-derived tumor cells were additionally transplanted into immunodeficient mice. Patient material was expanded *in vivo* and xenografted tumors were subsequently purified and cultured allowing spheroid formation.

In total, 194 samples were purified in our lab. 16 sphere cultures could have been established from purified patient's or xenograft's material (success rate 8.2%) of which 4 have been used in this study (TSC-01, TSC-03, TSC-04 and TSC-08, Figure 10).

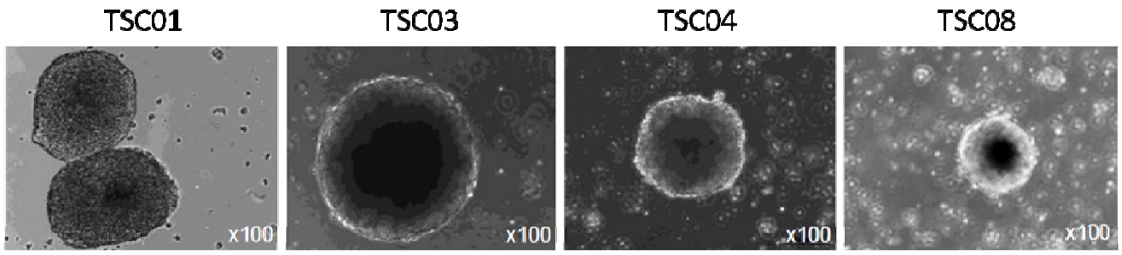


Figure 10: Primary sphere cultures derived from patient tumors and metastasis.

TSC-01 was derived from a colon cancer patient's liver metastasis, TSC-03 was established out of a patient's lung metastasis, TSC-04 was developed from a xenografted tumor which grew after transplantation of purified primary colon cancer tumor tissue and TSC-08 originated from xenografted patient's liver metastasis tissue.

Enrichment of spheroid cultures for human colon TIC has been demonstrated by a limiting dilution experiment where $1x10^6$ freshly purified TSC-02 cells did not form a tumor in mice but $1x10^4$ primary sphere culture cells formed tumors. Histological analysis revealed that both, spheroid cultures and xenografted tumors resembled the corresponding patient tissue. Moreover, all samples showed similar intra-individual marker expression (Figure 11).

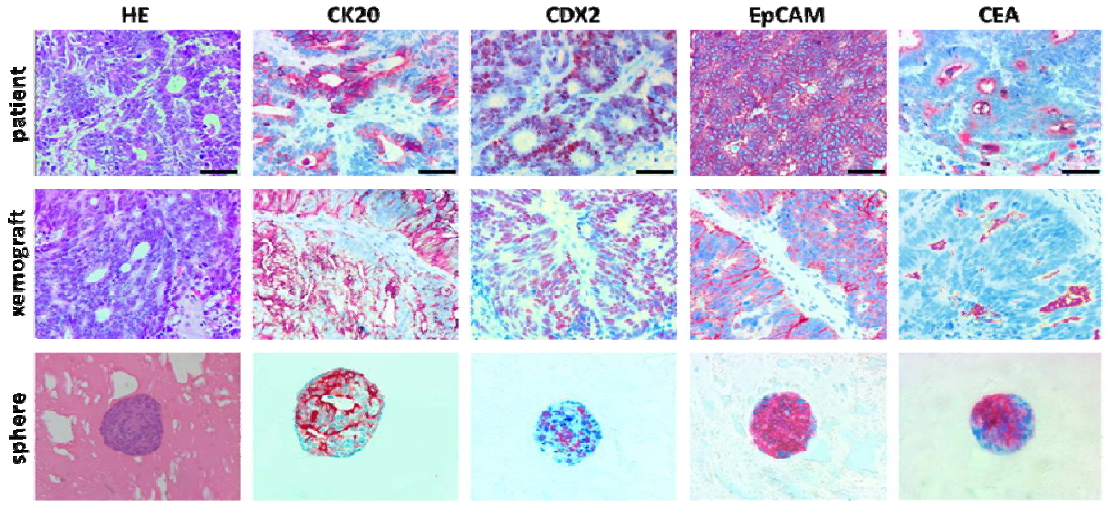


Figure 11: Spheroid cultures and xenografted tumors resemble the original primary patient material.

The expression of diagnostically relevant colon cancer antigens was similar in patient tissue and corresponding xenografted tumors and spheroid cultures. TSC-02 is exemplarily displayed. Scale bar 50µm. HE – Hematoxylin and eosin stain, CK20 – Cytokeratin 20, CDX2 - caudal type homeobox 2, EpCAM - Epithelial cell adhesion molecule, CEA - Carcinoembryonic antigen. Data produced by Dr. Christopher Hoffmann and Dr. Sebastian Dieter.

Once spheres were formed and the medium was used, sphere cultures were harvested and pellets were resuspended in fresh medium. Everytime, sphere cultures were splitted or frozen, spheres were manually dissociated and cells entered a new passage number.

Only low to medium passage numbers were used in this work (P<13). Aliquots of passage numbers were routinely investigated in the lab and proved no change of morphology or expression of cell surface markers (CD133, EpCAM, CD44 and CD166) up to P30. Patient characteristics are depicted in Table 2.

Table 2: Patient characteristics of distinct primary sphere cultures.

Primary sphere culture	age	sex	Tumor site	Tumor stage (UICC)
TSC-01	54	m	Liver metastasis	IV
TSC-03	67	m	Lung metastasis	IV
TSC-04	53	m	Colon	IV
TSC-08	49	f	Liver metastasis	IV

4.2 The majority of human colon TIC is actively-cycling *in vitro* and *in vivo*

To address whether a highly quiescent population of colon cancer initiating cells persists in G_0 phase of the cell cycle, sphere cells were stained with the DNA-intercalating dye Hoechst 33342 (Hoe) and the RNA-intercalating dye Pyronin Y (PY). During progression through the cell cycle, a cell changes its DNA- and RNA-content. The intercalating fluorescent dyes were used to visualize DNA and RNA via flow cytometry in order to discriminate cells in G_0-phase of the cell cycle from actively-cycling ones (G_1-, S-, G_2- and M-phase). G_2- and M-phase are characterized by a doubled amount of DNA (correlated to the Hoechst fluorescent intensity) as compared to the signal of cells in G_0- and G_1-phase. Cells in S-phase of the cell cycle show an intermediate Hoechst-intensity. G_0-cells, which have a low RNA content, can be distinguished from G_1-cells by their reduced PY-staining. Different concentrations and combinations of Hoe and PY were tested to minimize the cytotoxic effect of each compound. Cytotoxicity tests helped to avoid a selective process and overgrowth of a subsequently transplanted population by one resistant subfraction. Suitable concentrations of Hoe and PY were used to stain different sphere cultures. Subsequent FACS analysis revealed sphere cells in the different cell cycle phases G_1, G_0, S, G_2 and M (Figure 12). Cells were sorted according to their cell cycle status. The gating strategy was verified using Ki-67 as a marker for actively-cycling cells. Cells from each cell cycle phase were then transplanted into immunodeficient mice. The tumor forming capacity of cells from each fraction was investigated in order to see whether the tumorigenic potential depended on the cell cycle status of transplanted cells. Formed tumors were harvested and the *in vivo* cell cycle status of xenografted cells was evaluated. A serial transplantation assay was carried out to decipher which cell cycle phase comprised self-renewing colon TIC.

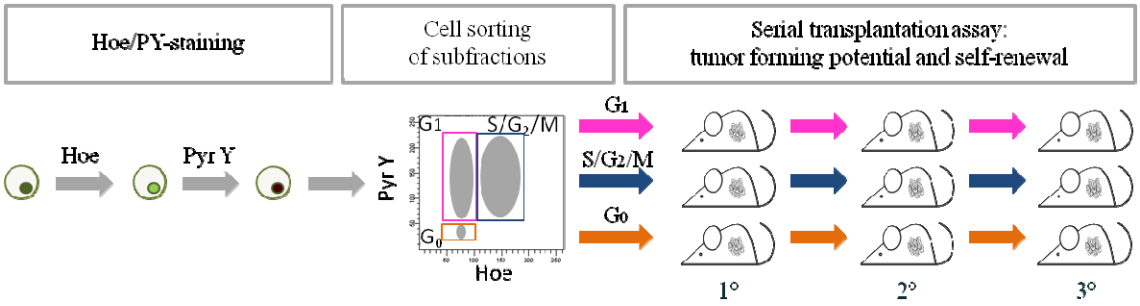


Figure 12: Cell cycle status of colon cancer initiating cells.

Tumor sphere cells were stained with Hoechst 33342 and Pyronin Y. Cells were sorted according to their cell cycle status (G_1, $S/G_2/M$, G_0) and transplanted into immunodeficient mice. A serial transplantation assay was carried out in order to evaluate the tumor forming potential and self-renewal ability of cells with differing cell cycle status.

4.2.1 Hoechst/Pyronin-staining allows discrimination of primary sphere culture cells in G_0-, G_1- and $S/G_2/M$-phase of the cell cycle

Primary sphere culture cells were stained with Hoechst and PY in order to discriminate cells in different phases of the cell cycle. TSC-03 and TSC-04 were chosen for assessment of the spheroid cell's cell cycle status due to their homogenous morphological appearance regarding size and shape. Other sphere cultures tested were relatively heterogeneous causing an inconsistent sample flow in the sorting device's fluidic system which is required for high resolution stable sorting experiments. Consequently, these spheroid cultures were inappropriate for cell cycle analysis and subsequent cell sorting. Different concentrations of the fluorescent dyes were tested for their staining efficiencies and long term toxicities. PY did not have an impact on cell viability up to 1µM (Figure 13a). Spheroid cells tolerated low Hoechst concentrations (<0.5µM) for 48h and showed slightly reduced viability afterwards (Figure 13b). 1µM Hoechst showed a cytotoxic effect already in the first 24hours after staining but then viability stayed stable over further 3 days *in vitro*. When comparing the staining efficiencies of several Hoechst-concentrations, only 1µM Hoechst revealed cell cycle profiles with a sufficient resolution to discriminate G_0/G_1 and $S/G_2/M$ phase (Figure 13c).

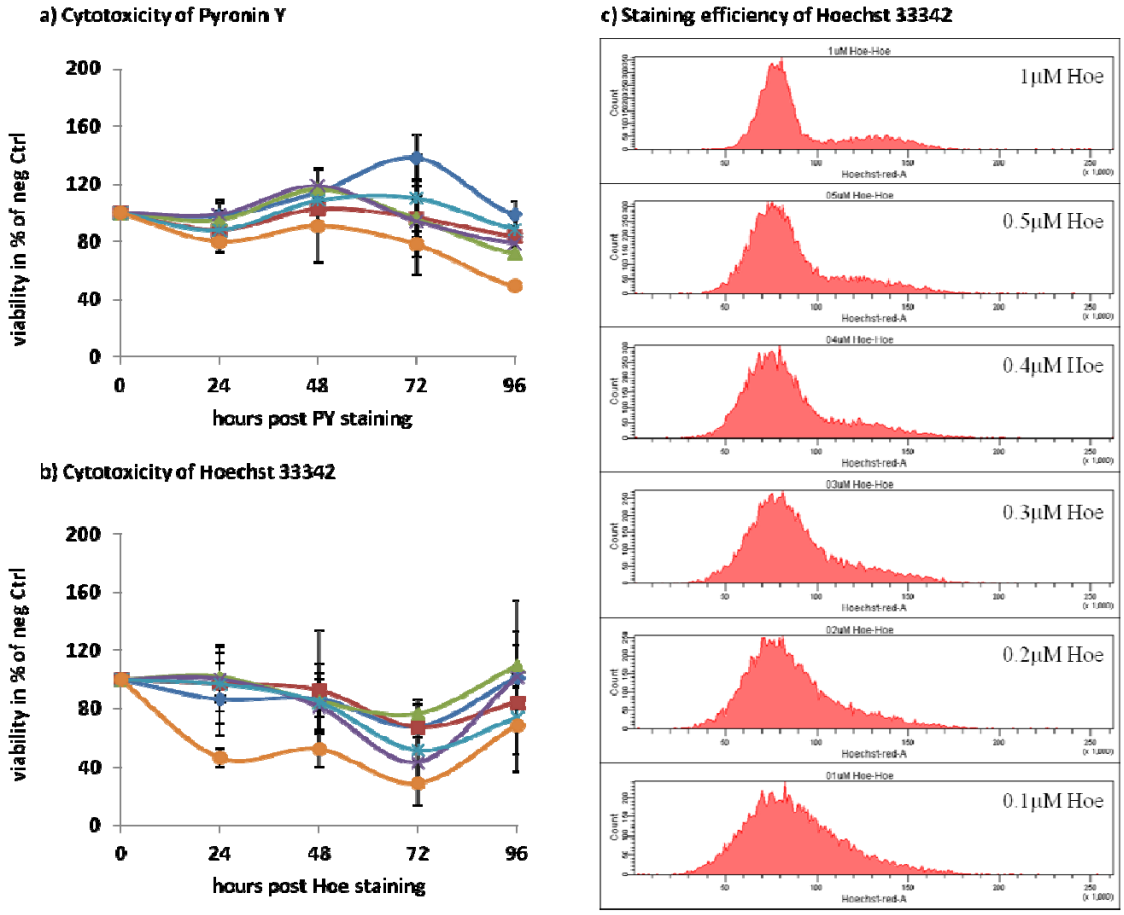


Figure 13: Spheroid cells can be efficiently stained using 1µM Hoechst 33342 and 1µM Pyronin Y.

Cytotoxicity of Pyronin Y (a; 0.2µM, 0.4µM, 0.6µM, 0.8µM, 1µM, 2µM), Hoechst 33342 (b; 0.1µM, 0.2µM, 0.3µM, 0.4µM, 0.5µM, 1µM) and Hoechst staining efficiencies of different concentrations (c) on TSC-04. Pyronin Y had only a small impact on cell viability whereas Hoechst showed a cytotoxic effect at concentrations of 0.4µM or higher. Only 1µM Hoechst allowed discrimination of cells with differing cell cycle status.

Consequently, TSC-03 and TSC-04 spheroid cells were stained using 1µM Hoe and 1µM PY (Figure 14). The majority of cells were actively-cycling: TSC-03 showed 72.3% in G_1-phase and 27.4% in S/G_2/M-phase of the cell cycle. For TSC-04, 83.2% of spheroid cells were present in G_1-phase and 16.5% in S/G_2/M-phase. Both sphere cultures exhibited 0.3% of cells in G_0.

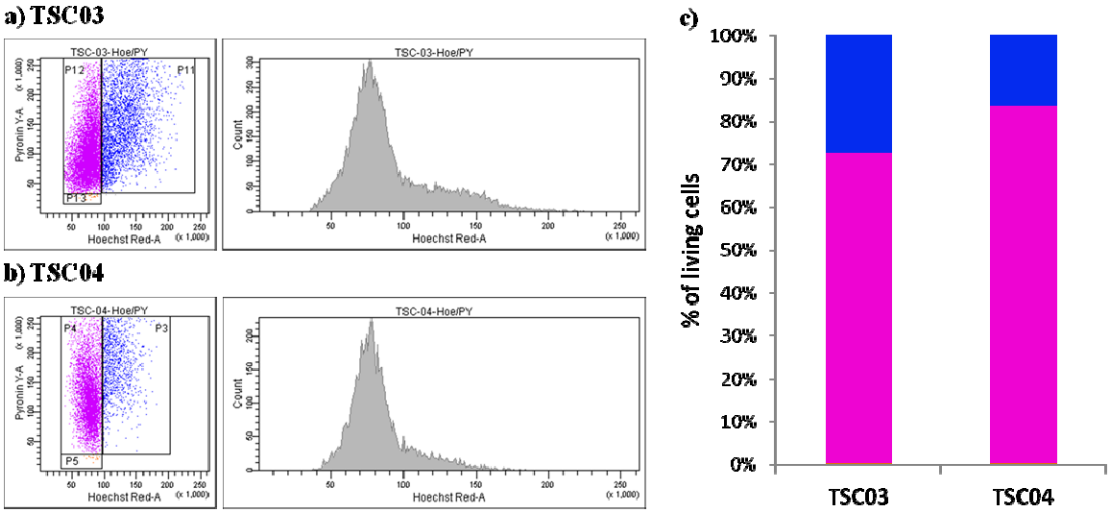


Figure 14: Cell Cycle Profiles of spheroid cultures using Hoechst/Pyronin staining.

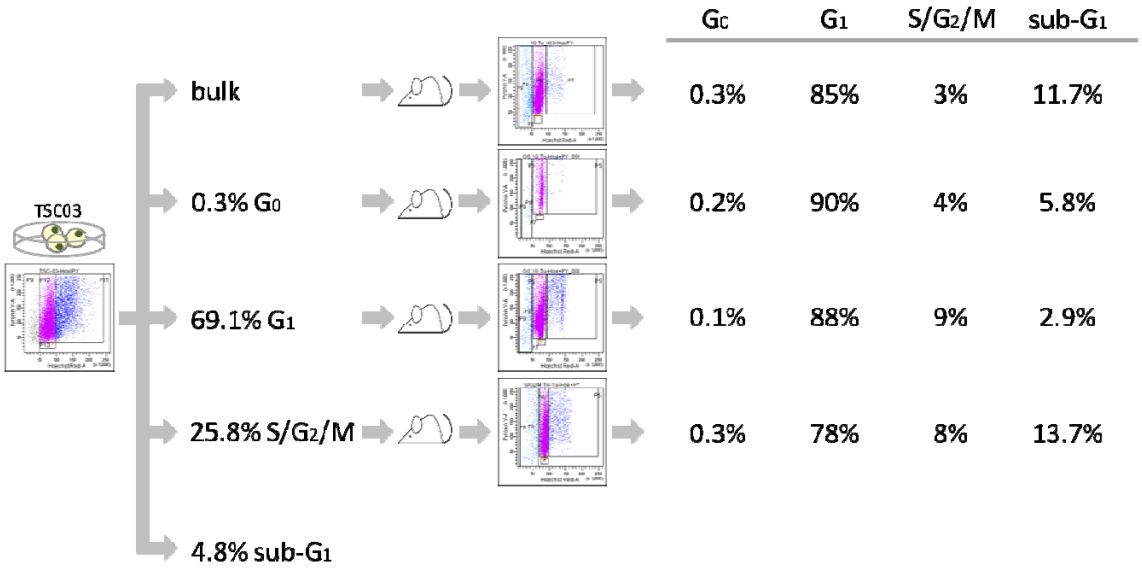
a) TSC-03 and b) TSC-04 spheroid cells were stained using 1µM Hoe and 1µM PY. The majority of cells were in G_1- (■) and S/G_2/M-phase (■) and only 0.3% cells were hibernating in G_0 (■) as shown in the cell cycle phase distribution (c).

4.2.2 Sphere cells from G_0-, G_1- and S/G_2/M-fractions form tumors with a similar cell cycle profile

To address whether cell cycle quiescence is a property of human colon TIC and whether the tumor initiating and self-renewal potential are restricted to cells in G_0 phase of the cell cycle, primary patient-derived spheroid culture cells were stained with Hoechst and Pyronin, sorted according to their cell cycle status and serially transplanted into immunodeficient mice. In TSC-03, 0.3% of living cells were present in G_0 whereas the majority of cells were actively cycling (69.1% G_1 and 25.8% S/G_2/M, Figure 15a). 4.8% of cells were in sub-G_1-phase. Cells in sub-G_1 are apoptotic and characterized by degradation of DNA resulting in a decreased Hoechst signal as compared to G_1-phase. TSC-04 exhibited a similar pattern: 0.5% G_0, 80.7% G_1, 16.3% S/G_2/M and 2.5% sub-G_1 (Figure 15b). Cells were sorted according to their cell cycle status and equal cell numbers of bulk or sorted fractions were transplanted into immunodeficient mice to assess the tumor initiating capacity of cells with differing cell cycle status. All fractions of TSC-03 and TSC-04 contributed to tumor formation irrespective of their cell cycle status in spheroid cultures. In addition, purified cells from xenografted tumors were analyzed for their cell cycle status to determine the cell cycle activity of human colon TIC *in vivo* (Figure 15). TSC-03 tumors showed a remarkably similar distribution of cells in the different cell cycle phases as compared to the *in vitro* situation of sphere cultures: in the tumor grown out of bulk transplanted spheroid cells, 0.3% of viable cells were in G_0, 85% in G_1 and 3% in S/G_2/M-phase of the cell cycle, the G_0-tumor exhibited 0.2% in G_0, 90% in G_1 and 4% in S/G_2/M, G_1-tumor 0.1% G_0, 88% G_1, 9% S/G_2/M and the S/G_2/M-tumor revealed 0.3% of cells in G_0, 78% in G_1 and 8% in

S/G_2/M-phase. TSC-04 sphere culture yielded 0.4% of viable cells in G_0, 77% in G_1 and 13% in S/G_2/M-phase of the cell cycle. Xenografted tumors showed the following cell cycle phase distribution: bulk tumor 0.3% G_0, 61% G_1 and 12% S/G_2/M-phase; the; G_1-tumor 0.9% G_0, 7% G_1 and 4% S/G_2/M-phase and S/G_2/M-tumor 0.5% G_0, 64% G_1 and 12% S/G_2/M-phase. The G_1-tumor additionally exhibited a huge proportion of apoptotic cells in sub-G_1-phase. The cell cycle status of TSC-04's G_0-tumor could not be determined due to a very small amount of viable cells.

a)



b)

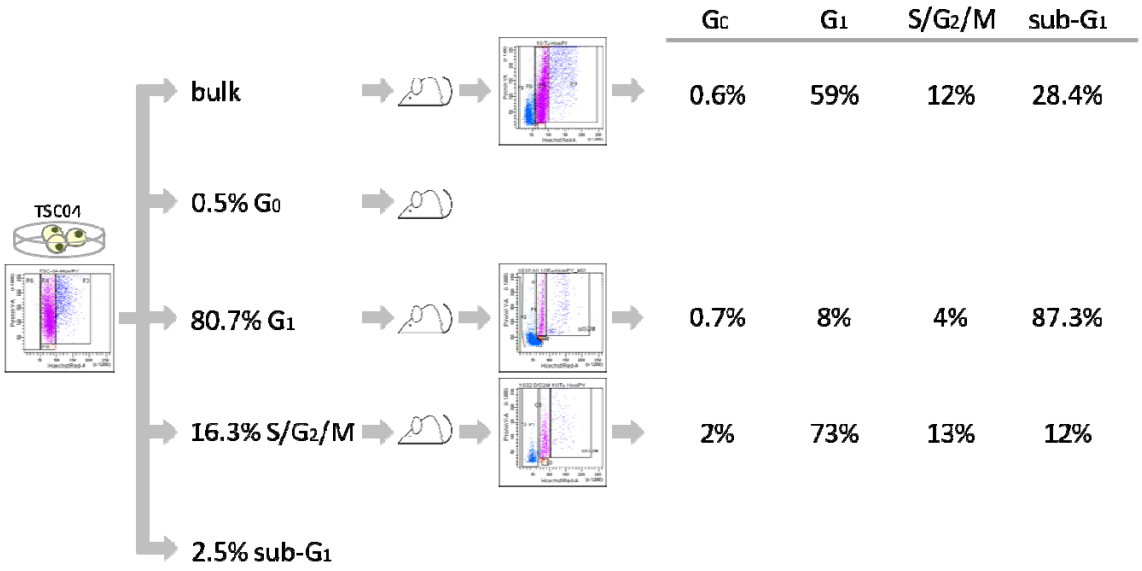


Figure 15: Cell cycle status of colon spheroid cells and xenografted tumors.

Singularized TSC-03 (a) and TSC-04 (b) sphere cells were stained with Hoechst and Pyronin. Bulk and sorted cells originating from G_0 (■), G_1 (■) and S/G_2/M phase (■) were transplanted into immunodeficient mice. Xenografted tumor cells were again analyzed for their cell cycle status and serial transplantation was performed. Cells in sub-G_1 (■) showed a decreased Hoechst staining due to DNA-degradation.

4.2.3 Ki-67 is a nuclear marker to discriminate cells in G_0-phase of the cell cycle from actively-cycling cells

In order to verify the sorting gates and to approve the dormant cell cycle state of transplanted G_0-cells, aliquots of sorted cells were stained with an antibody against Ki-67. Sorted TSC-03 and TSC-04 could be discriminated according to their Ki-67 expression levels determined by the mean fluorescence intensity (MFI) of fixed and stained cells (Figure 16): G_0-cells show similar fluorescent intensities as unstained and isotype-stained control cells indicating the level of autofluorescence. Contrarily, G_1- and S/G_2/M-fractions exhibited 2- to 5-fold increased expression levels of Ki-67.

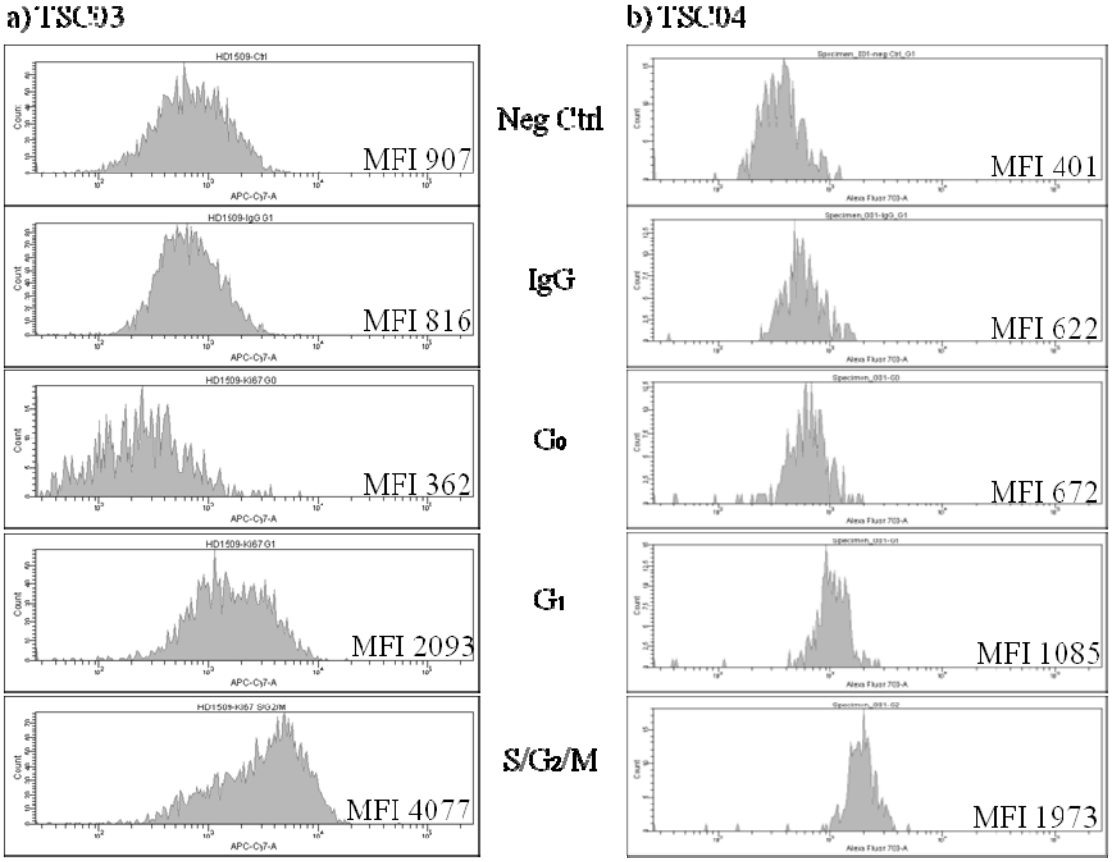


Figure 16: Ki-67 is only expressed by actively-cycling cells.

Aliquots of Hoe/PY-sorted cells from a) TSC-03 and b) TSC-04 were stained with an antibody against Ki-67. Mean fluorescence intensities (x1000 fluorescent units) increased with cell cycle progression. Neg Ctrl – negative control, IgG – isotype control, MFI – mean fluorescence intensity.

4.2.4 G_0-, G_1- and S/G_2/M-fractions comprise self-renewing human colon TIC

Long-term tumor initiating capacity and self-renewal-potential of primary sphere cells were assessed in a serial transplantation assay: singularized spheroid cells were transplanted into immunodeficient mice as an unsorted and unstained bulk or as sorted fractions defined by their cell cycle status (G_0, G_1 and S/G_2/M). Mice transplanted with

TSC-03 and TSC-04 cells originating from the different cell cycle phases all formed tumors (Figure 15). Xenografted tumors were harvested, digested and equal numbers of tumor cells were transplanted into new recipients. Serial transplantation was carried out until 3 generations of mice indicating that G_0-, G_1- and $S/G_2/M$-fractions from spheroid cultures comprised self-renewing colon TIC.

Aliquots of tumor cells from primary recipients were restained with Hoe and PY and sorted according to their cell cycle status *in vivo*. Sorted cells were subsequently transplanted into a second generation of mice. Mice transplanted with 500 to $1x10^4$ cells did not develop tumors. However, 2nd generation mice transplanted with 500 unstained and unsorted cells from the same primary recipient showed tumor formation.

Bulk transplantation of unstained and unsorted TSC-03 and TSC-04 spheroid cells yielded tumors in primary recipients. 2nd and 3rd generation tumors developed in mice serially transplanted with unstained bulk cells. Aliquots of primary tumor cells were additionally stained and sorted according to their cell cycle status. Secondary mice transplanted with 500 to $1x10^4$ cells of the different cell cycle fractions did not develop tumors for TSC-03. For TSC-04, 500 G_1- and $1x10^4$ $S/G_2/M$-phase-cells from primary xenografted bulk tumors led to tumor formation in mice.

Summarizing, all initially sorted cell cycle fractions yielded long-term tumor forming potential in three generations of mice indicating that G_0-, G_1- and $S/G_2/M$-fractions harbored self-renewing colon TIC.

4.3 The tumor initiating potential is not tightly linked to mitotic quiescence *in vitro* and *in vivo*

It has been hypothesized that mitotic quiescence and resulting chemoresistance are functional characteristics of human colon TIC but experimental evidence has not been provided yet. We aimed to investigate whether colon cancer comprises cell fractions with differing proliferative activity. Analysis of the cell cycle status via Hoe/PY-staining is a snapshot at the time of analysis. However, the momentous cell cycle status does not equally indicate quiescence *per se*. In order to detect cells which are long-term quiescent, label-retaining assays have been developed based on DNA-intercalation or binding of cellular components[147, 179]. In this study, the CFSE label-retaining assay was used to investigate prolonged quiescence of primary, human colon TIC enriched spheroid cultures. Furthermore, the H2BGFP label-retaining assay was applied to identify quiescent human colon TIC *in vivo*.

Singularized sphere cells were stained with CFSE. The fluorescent dye is highly reactive and can covalently bind to intracellular aminogroups resulting in stable fluorescent labeling of cells[179]. During each cell division half of the CFSE fluorescence intensity is distributed into each of the two daughter cells allowing tracking of cells according to their proliferative history[180]. Systematic titration experiments were performed to test different dye-concentrations for their cytotoxic effect, their staining efficiency and 10-days-kinetics of label loss. A suitable concentration showing no cytotoxicity, maximum staining efficiency after equilibration and a small fraction of label-retaining cells after 8 days in culture was used to stain different sphere cultures. Subsequent FACS analysis revealed spheroid cells with differing proliferative activity. Fast, slow and rarely dividing sphere cells were separated by FACS. Cells from each proliferative fraction were sorted in limiting dilution and cultured over a prolonged period of time. The frequency of sphere forming cells in each proliferative subfraction was determined *in vitro* to test whether the sphereogenic potential correlated with the original proliferative velocity of sorted cells. Formed spheres were picked and replated to elucidate the self-renewal ability of colon sphere cells. Furthermore, the expression of cell surface markers previously associated with tumor initiating potential (CD133, EpCAM, CD44 and CD166) was analyzed among the sorted proliferative fractions. In addition, sorted cells with differing proliferative activity were transplanted into immunodeficient mice. The tumor forming capacity of cells from each fraction was investigated in order to see whether the tumorigenic potential depended on the original proliferative velocity of transplanted cells. Formed tumors were harvested and a serial transplantation assay was carried out to investigate the self-renewal ability of colon cancer spheroid cells with differing proliferative activity *in vivo*.

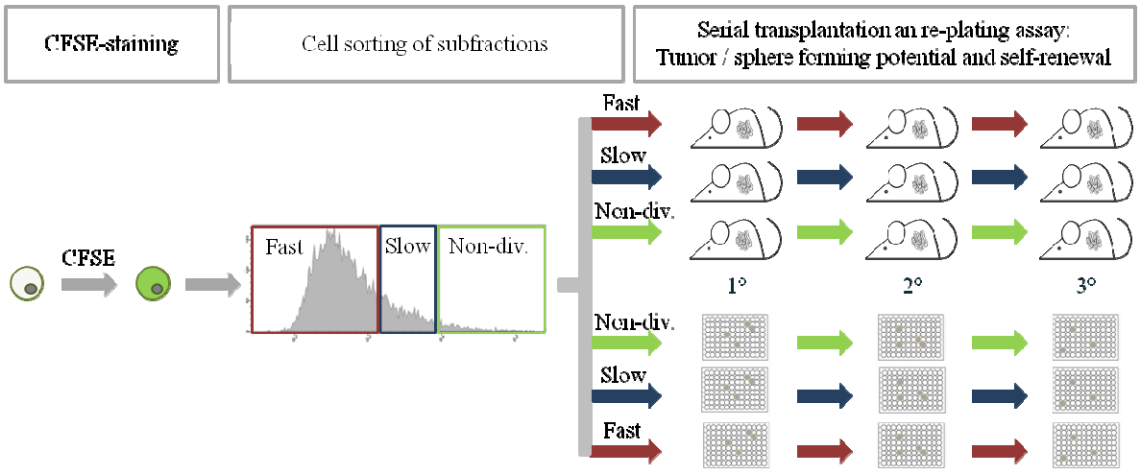


Figure 17: Proliferative capacity of colon cancer initiating cells.

Tumor sphere cells were stained with CFSE and cells were sorted according to their proliferative history at day 8 after staining. Fast, slow and rarely dividing cells were transplanted into immunodeficient mice or sorted in limiting dilution into 96-well-plates. A serial transplantation and replating assay were carried out in order to evaluate the tumor and sphere forming potential and self-renewal ability of cells with initially differing proliferative activity.

4.3.1 CFSE-staining allows discrimination of primary sphere culture cells with differing proliferative activity and reveals a rarely dividing subpopulation

TSC-01, TSC-03 and TSC-04 cells were stained with different concentrations of CFSE in order to test for their initial staining efficiencies and for their long-term cytotoxic effect. The CFSE-intensity was determined after equilibration to assure successful staining. Cells were cultured for 10 days and cytotoxicity of CFSE was evaluated by cell counting. Labeling of spheroid cells with 1µM – 25µM CFSE showed 100% staining efficiency. 1µM CFSE showed no cytotoxic effect over 10 days for all primary sphere cultures tested. CFSE concentrations higher than 2.5µM inhibited cell growth and had a negative impact on cell viability after 2-3 days in culture (Figure 18).

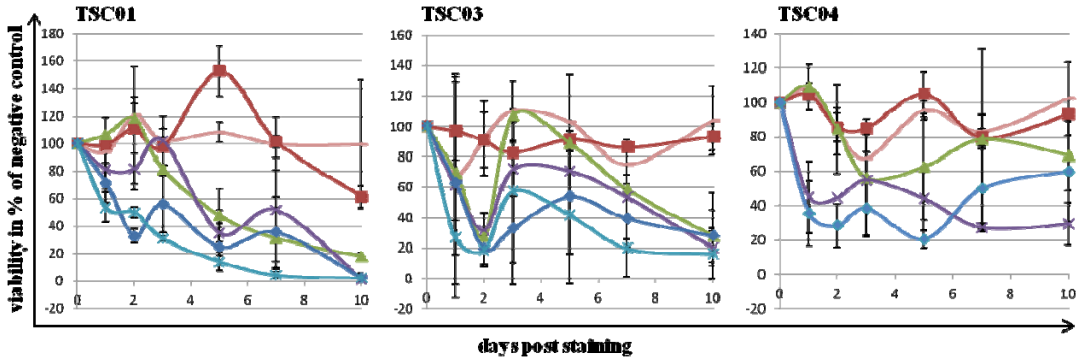


Figure 18: Cytotoxicity of CFSE.

TSC-01, TSC-03 and TSC-04 sphere culture cells were stained with 1µM (—■—), 2.5µM (—▲—), 5µM (—✳—), 10µM (—◆—) or 25µM (—✳—) CFSE or treated with 0.2% DMSO (—) as a control. Cell viability was determined by cell counting and observed over 10 days.

Spheroid cells, which were previously stained with 1µM CFSE, were cultured for 10 days and FACS measurements were performed daily in order to investigate the label-loss-kinetics for three sphere cultures (Figure 19): all cells were efficiently labeled with 1µM CFSE. When determining initial CFSE intensities at about 16h after staining (day 0), corresponding histograms revealed a gaussian distribution. In the next 24 hours, mitotic profiles slightly changed. On day 2, a decrease of CFSE intensities was observable in all sphere cultures tested. During the following days *in vitro*, the majority of cells lost their CFSE until only a small subpopulation still maintained the label. After day 8, the proliferative profile was not altered anymore.

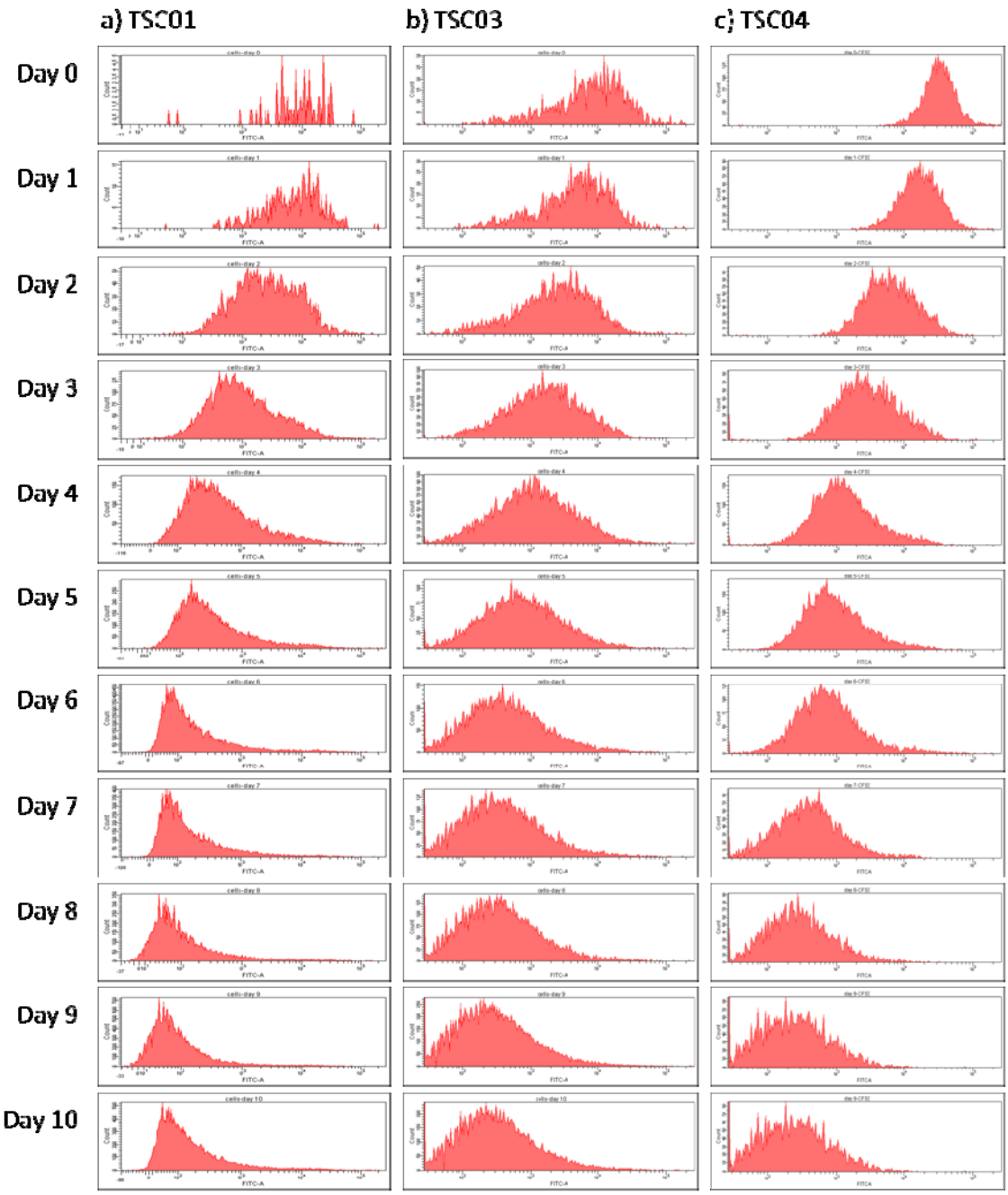


Figure 19: CFSE label loss kinetics.

Singularized spheroid cells from a) TSC-01, b) TSC-03 and c) TSC-04 were stained using 1µM CFSE and cultured for 10 days. FACS measurements were performed daily in order to study the label loss of each individual primary sphere culture.

After 8 days of culture, three subfractions with differing proliferative activity could be distinguished: fast dividing cells lost all the label dye and exhibited the same CFSE intensity as compared to a negative, unstained control (autofluorescence), cells maintaining at least 90% of the initial CFSE-intensity represented a rarely dividing or quiescent population and slow-dividing cells showed an intermediate CFSE fluorescence. On day 8, less than 5% of cells in all sphere cultures tested could be defined as rarely dividing (Figure 20).

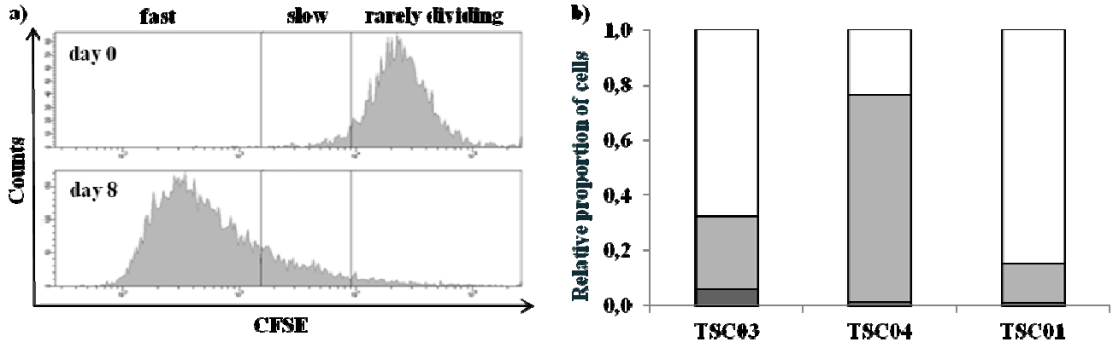


Figure 20: CFSE staining of primary sphere culture cells reveals different proliferative subfractions.

a) Sorting gates used to separate cells in culture are indicated (fast, slow and rarely dividing). b) The distribution of fast (□), slow (▣) and rarely dividing (■) cells among different sphere cultures varies, but each spheroid culture comprised a small fraction of rarely dividing cells at day 8 after staining.

4.3.2 Cancer stem cell markers are not enriched in fast, slow or rarely dividing cell fractions

To further analyze whether published markers, which have previously been associated with colon cancer initiating cells, are enriched in individual proliferative fractions, we analyzed sphere cells which have been prepared for sorting after CFSE culture for the expression of CD133, CD166, CD44 and EpCAM by flow cytometry. Interestingly, no enrichment of any marker could be identified among fast, slow and rarely dividing spheroid cells (Table 3).

Table 3: Expression of cell surface markers.

Fast, slow and rarely dividing TSC-01, TSC-03 and TSC-04 sphere cells were stained with antibodies against CD133, CD166, CD44 and EpCAM. The cell surface markers were not enriched in any proliferative subfractions.

		CD133	CD166	CD44	EpCAM
	Fast	0%	99.3%	64.7%	0%
TSC-01	Slow	0%	99.4%	69.1%	0%
	Rarely div.	0%	93.3%	57%	0%

	Fast	47.3%	96.3%	72.2%	0.6%
TSC-03	Slow	51.3%	98.4%	85.2%	0.6%
	Rarely div.	39.5%	91.1%	75%	0.4%
	Fast	0%	98.2%	89.1%	3.9%
TSC-04	Slow	0%	95.7%	91.4%	17.9%
	Rarely div.	0%	48.5%	71.1%	19.1%

4.3.3 The frequency of sphere forming cells is similar among fast, slow and rarely dividing cells

To test the hypothesis that colon cancer initiating cells are quiescent, the frequency of sphere forming cells in rarely, slow and fast dividing subfractions of three primary colon sphere cultures was determined *in vitro*. Therefore, cells from each subfraction were sorted in limiting dilution series into the wells of a 96-well-plate. 1 cell/well, 10 cells/well and 100 cells/well were seeded and sphere formation was observed weekly. Formed spheres were picked, singularized and replated twice in order to test the self-renewal ability of spheroid cells with differing proliferative activity *in vitro*. The frequency of sphere forming cells (SFC) was calculated for every proliferative subfraction and each plating-generation (Table 4). In TSC-01, every second to every 11^{th} cells was sphereogenic, independent from its original proliferative activity and plating generation. The SFC-frequency for TSC-03 ranged from 1 in 20 cells to 1 in 97. For TSC-04, frequencies were only determined for two spheroid generations (1 SFC in 34 to 1 SFC in 160 cells). They did not grow from single-cell level. TSC-04 spheroids regrew when plated at a density of at least 500 cells/ml, in rare cases also at 50 cells/ml.

Comparison revealed that the frequencies of sphere forming cells (SFC) were similar among the different proliferative fractions but varied among different sphere cultures, each representing one patient.

Table 4: Frequencies of sphere forming cells.

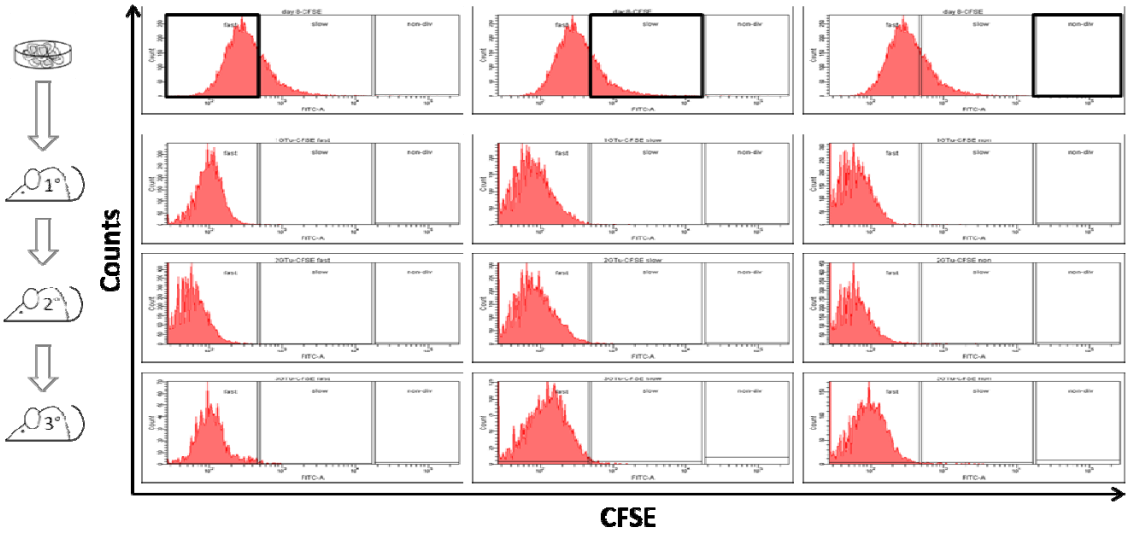
Fast, slow and rarely dividing TSC-01, TSC-03 and TSC-04 sphere cells were sorted in 96-well plates in limiting dilution and the frequencies of sphere forming cells (SFC) were calculated using the software L-Calc. SFC frequencies were similar among fast, slow and rarely dividing colon cancer sphere cells.

		1st generation	2nd generation	3rd generation
TSC-01	Fast	1 SFC in 2	1 SFC in 4	1 SFC in 6
	Slow	1 SFC in 8	1 SFC in 5	1 SFC in 4
	Rarely div.	1 SFC in 10	1 SFC in 11	1 SFC in 7
TSC-03	Fast	1 SFC in 23	1 SFC in 97	1 SFC in 20
	Slow	1 SFC in 29	1 SFC in 62	1 SFC in 63
	Rarely div.	1 SFC in 61	1 SFC in 94	1 SFC in 45
TSC-04	Fast	1 SFC in 76	1 SFC in 34	n.d.
	Slow	1 SFC in 160	1 SFC in 68	n.d.
	Rarely div.	1 SFC in 81	1 SFC in 75	n.d.

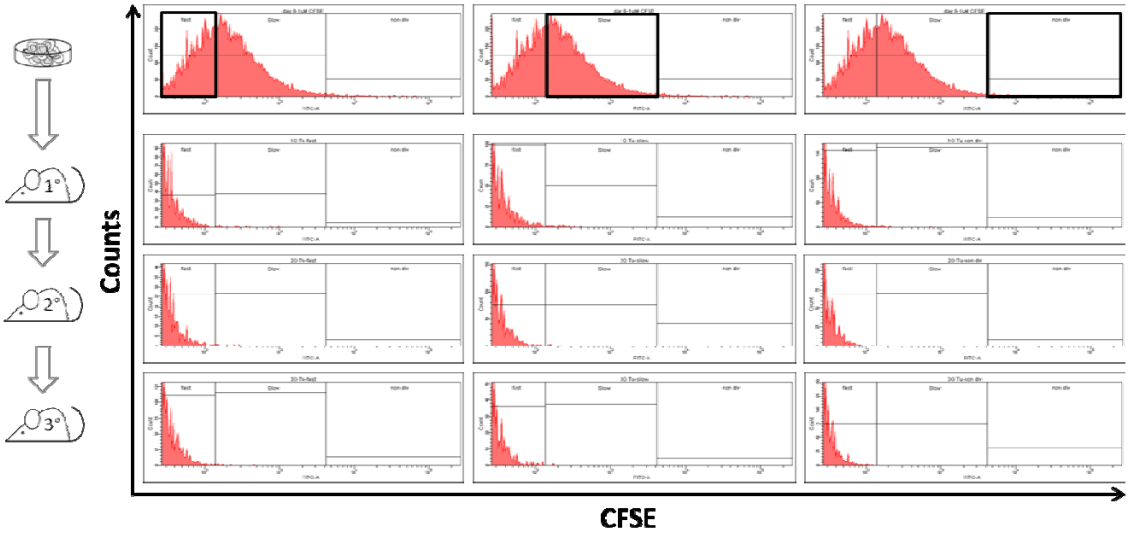
4.3.4 Fast, slow and rarely dividing cell fractions comprise self-renewing human colon TIC

In order to assess the tumor initiating capacity of human colon TIC with differing proliferative activity, equal cell numbers from each subfraction of rarely, slow and fast dividing cells were transplanted under the kidney capsule of immunodeficient mice. Sorted cell fractions of all three patients formed tumors, irrespective of their proliferative kinetics *in vitro*. Tumors formed in the first generation (1°) of mice were digested, analyzed for their CFSE intensities and serially transplanted (2° and 3°) with equal numbers of bulk cells. TSC-01, TSC-03 and TSC-04 initially sorted rarely, slow and fast dividing cells were able to form tumors in 3 generations of mice confirming the self-renewal ability of cells in all proliferative subfractions. Respecive tumors within one generation of mice exhibited a similar weight. In all cases, first generation tumor cells from originally fast ($CFSE^{neg}$), slow ($CFSE^{low}$) and rarely dividing ($CFSE^{high}$) cells were $CFSE^{neg}$, indicating that all cells within this fraction actively proliferated upon transplantation (Figure 21).

a) TSC-01



b) TSC-03



c) TSC-04

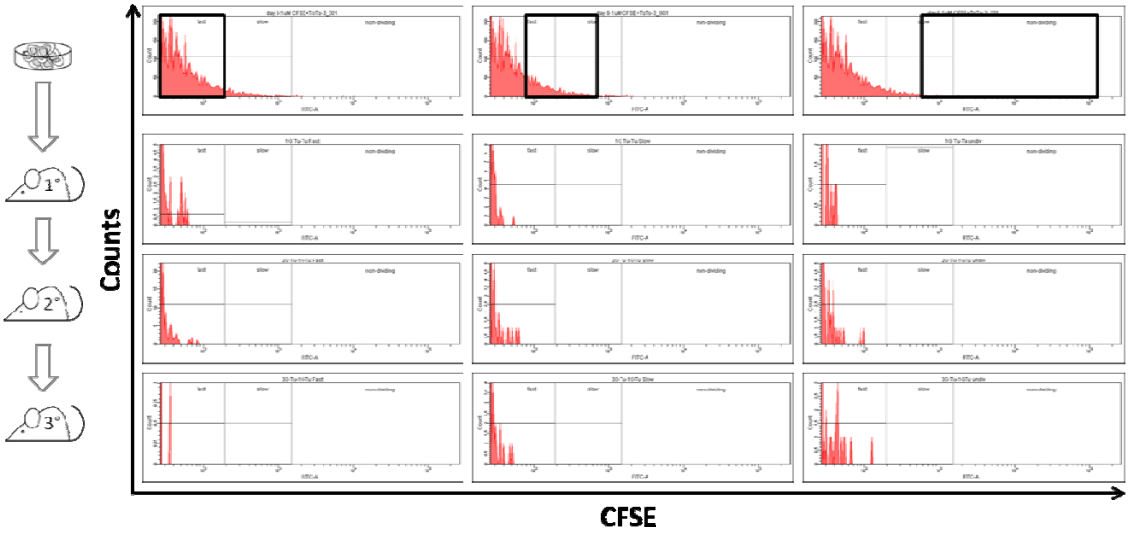


Figure 21: Fast, slow and rarely dividing human colon TIC form tumors in immunodeficient mice.

CFSE-stained TSC-01 (a), TSC-03 (b) and TSC-04 (c) cells were sorted as indicated by the gates (fast – left, slow – middle, and rarely dividing – right) and serially transplanted over three generations.

4.4 A quiescent population of human colon cancer TIC exists *in vivo* and is enriched by chemotherapeutic treatment

Dieter *et al.* described delayed contributing tumor-initiating cells in the TIC compartment of human colorectal cancer[135]. These DC-TIC only contributed to tumor formation in a serial transplantation assay in secondary or tertiary mice suggesting an initial mitotic quiescence *in vivo*. To understand whether DC-TIC are indeed quiescent *in vivo* and whether quiescent TIC can be recruited to tumor formation after chemotherapeutic treatment, a high resolution genetic label-retaining strategy was used: tumor sphere cells were genetically marked by transduction with a lentiviral vector encoding a tetracycline-regulated gene expression system[151]. This expression construct comprised a transcriptional transactivator protein ($tTA2^S$) and a transactivator-controlled human histone 2B (H2B) fused to a green fluorescent marker protein GFP (H2B-GFP). Transduced cells constitutively express $tTA2^S$ (EF1α promoter) whereas H2B-GFP is under control of the tetracycline-responsive promoter P_{Tight}. In the absence of tetracycline, the transactivator binds to P_{Tight} and activates transcription of H2B-GFP. Proliferating cells continuously incorporate the H2B-GFP fusion protein into their nucleosomes. Addition of tetracycline leads to conformational changes in the transactivator which cannot bind to P_{Tight} anymore and thereby expression of H2B-GFP is stopped ("Tet-Off regulated gene expression system"). During each cell division half of the GFP is distributed among the two daughter cells allowing tracking of cells according to their proliferative history.

Singularized sphere cells were transduced with the H2B-GFP lentiviral vector (H2B-GFP-LV). Different MOIs were tested for their transduction efficiencies (MOI 0.1-500). MOI 100 was used to transduce different primary sphere cultures. We validated the Tet-Off-regulated expression of H2B-GFP by long-term-culturing of transduced cells with or without tetracycline and frequent FACS measurements. Transplantation of infected cells into immunodeficient mice yielded green fluorescent tumors. After establishment of the tumor microenvironment, tetracycline was applied to the drinking water of mice. Consequently, H2B-GFP expression was repressed and tumor cells lost half of their GFP expression with each cell division. Formed tumors were harvested and purified. FACS analysis revealed tumor cells with differing proliferative activity. Cells were sorted according to their proliferative velocity and a serial transplantation assay demonstrated whether the self-renewal ability of colon TIC was correlated to their proliferative activity. In addition, we determined whether quiescent TIC can be reactivated to regrow the tumor after chemotherapy. Therefore, we tested the *in vitro*-sensitivity of different sphere lines towards 5-Fluorouracil (5-FU) and Oxaliplatin, two

commonly used drugs for the clinical treatment of colorectal cancer patients[165, 166, 190]. Two different concentrations of 5-FU were tested for its long-term-toxicity on immunodeficient mice (85mg/kg, 150mg/kg). Cohorts of mice transplanted with H2B-GFP expressing sphere cells were treated with 85mg 5-FU per kg bodyweight. The proportion of label-retaining cells was quantified in order to determine whether mitotically quiescent TIC within established tumors survived chemotherapeutic treatment. Serial transplantation of sorted cells with differing proliferative activity originating from 5-FU-treated tumors allowed us to determine whether quiescent TIC can be reactivated to regrow the tumor after chemotherapy. The clonal composition of tumors engrafted from sorted cells with differing proliferative activity was analyzed via LAM-PCR. We determined whether quiescent TIC can be recruited to regrow the tumor after drug treatment. Furthermore, identification of DC-TIC in 5-FU treated tumors reflected their role in chemoresistance.

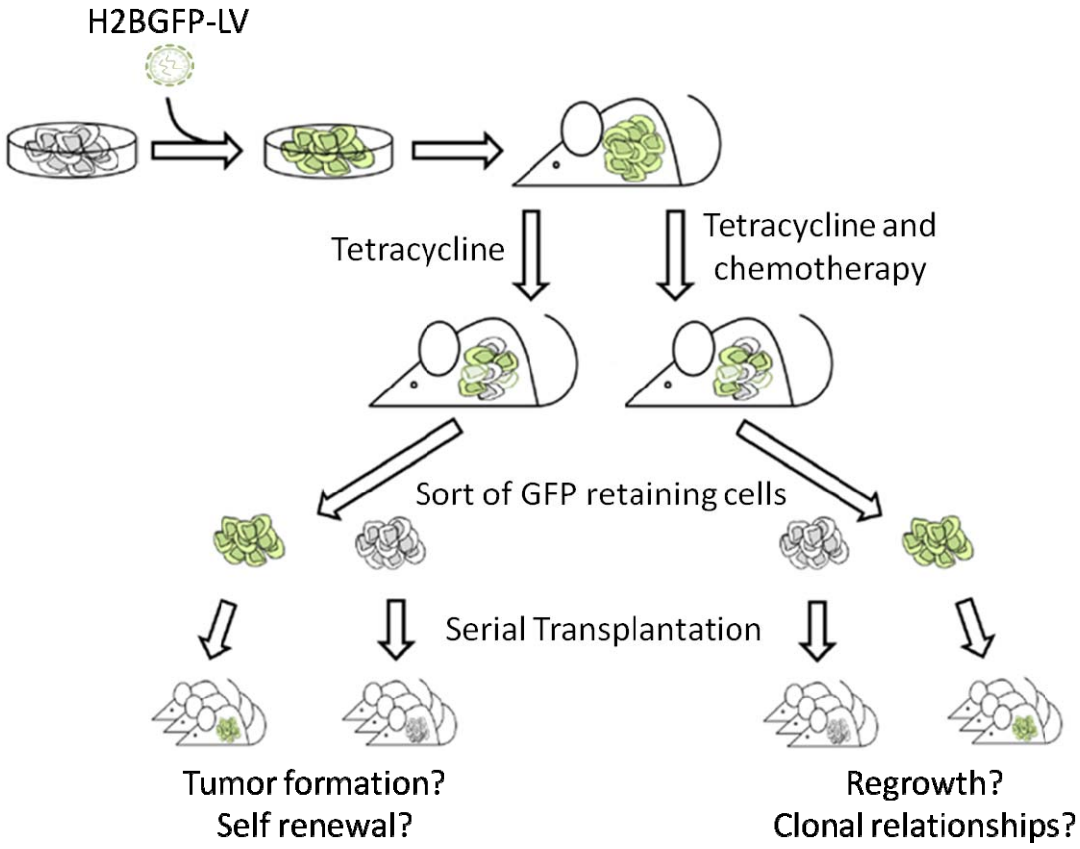


Figure 22: *In vivo* quiescence and chemotherapy resistance of delayed contributing TIC.

Tumor sphere cells were dissociated and transduced with an H2B-GFP-lentiviral vector resulting in green fluorescent tumors following xenotransplantation under the kidney capsule of immunodeficient mice. Upon treatment with tetracycline, H2B-GFP expression was shut off. Consequently, dividing tumor cells loose half of their GFP expression with each cell division. Tumors were harvested and label-retaining cells were sorted. Subsequent serial transplantations into immunodeficient mice assessed repopulation ability and self-renewal potential of different cell clones. Chemotherapeutic treatment of mice bearing H2B-GFP expressing tumors allowed evaluation of the recruitment of previously quiescent clones to tumor formation upon drug treatment.

4.4.1 Efficient production of lentiviral particles encoding the human-derived histone H2B-GFP

The production of lentiviral particles encoding the tet-regulated H2B-GFP expression system required bacterial plasmids encoding the viral components. These plasmids were amplified in *E.coli* and subsequently checked for their integrity by restriction digestion. Figure 23 shows representative results. Digestion of pMD2.G, psPAX2 and pWPXL-TTT-H2B-GFP were performed using NEB enzyme Kpn1.

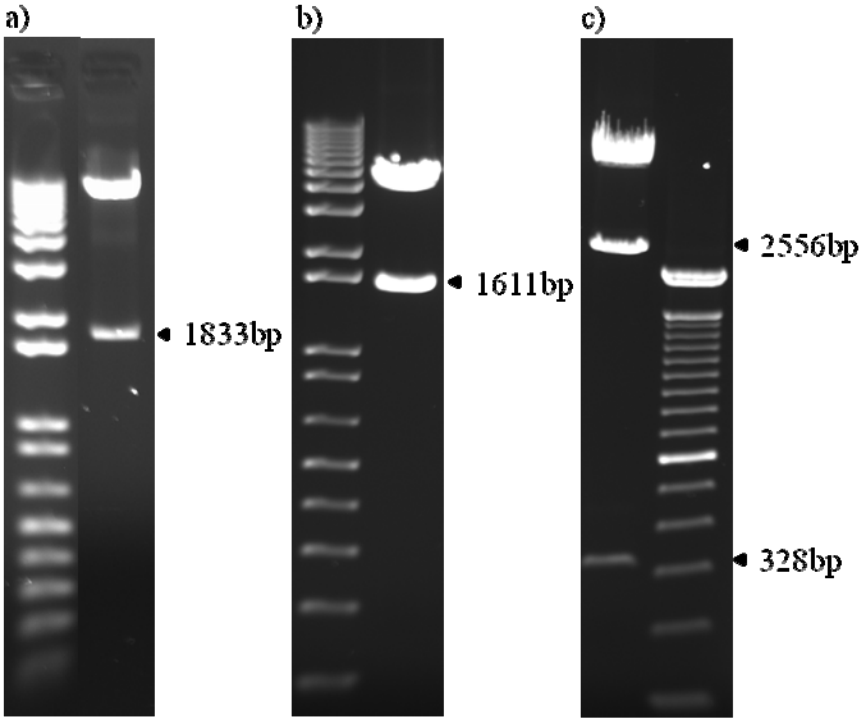


Figure 23: Agarose gel displaying digested DNA.

The plasmids pWPXL-TTT-H2B-GFP (a), pMD2.G (b) and psPAX2 (c) were digested using the restriction enzyme Kpn1 (37°C, 1h). Gel electrophoresis was performed in a 2% agarose gel (150V, 45min).

Isolated plasmid DNA was used for the production of lentiviral particles. Titer assays were performed on HeLa cells. The percentage of GFP-positive cells was determined via flow cytometry and the functional titer was calculated according to the formula

$$\#\ of\ TU/_{ml} = \#\ of\ cells\ (time\ of\ infection) x\ dilution\ factor\ x\ \frac{z\%\ GFP^{+}\ cells}{100}$$

(Figure 24). 2.8% of 9x104 cells were successfully transduced by a viral stock solution (10^4-fold dilution) giving a functional titer of $2.52x10^7$ TU/ml.

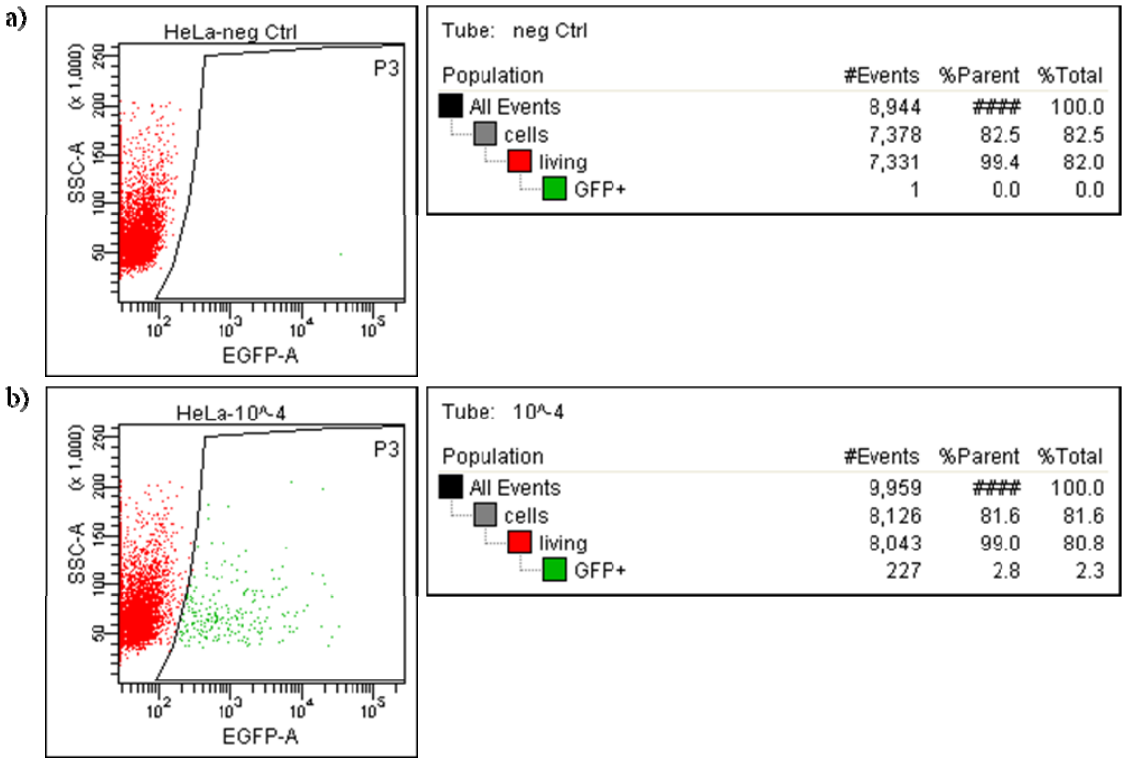


Figure 24: Determination of functional virus titers.

FACS plot and corresponding statistics from a) untransduced HeLa cells and b) HeLa cells transduced with a lentivirus encoding the tet-regulated H2B-GFP expression system. The percentage of GFP^+ cells was determined and functional titers were calculated.

4.4.2 Expression of H2B-GFP in infected cells is regulated by tetracycline *in vitro* and *in vivo*

In order to validate the tet-regulated H2B-GFP expression system *in vitro*, HeLa cells were transduced with the H2B-GFP-LV and cultured. Transduced HeLa cells showed a distinct nuclear GFP signal (Figure 25a). Stable integration of viral DNA into the host cells genome was verified by measuring GFP-intensity of transduced HeLa cells at frequent timepoints over 8 months. Additionally, a part of those cells was cultured in the presence of doxycyclin to approve the functionality of the tet-regulated system. First, the system was tested in HeLa cells (Figure 25): transduced cells (approximately 25%) maintained their GFP intensity over 3 months. Then, the proportion of GFP expressing cells slightly increased and remained stable for further 4 months. In the following two weeks, the amount of GFP expressing cells was tripled. Doxycyclin was added to the medium after 4 weeks of culturing and GFP-expression was completely suppressed. Removal of doxycyclin after further 4 weeks restored GFP-expression. Same hold true for a later removal of doxycyclin from transduced cells. GFP-expression was further suppressable by renewed doxycyclin-addition. After 7 months of culture, GFP-expression of the untreated, GFP-expressing cells started to increase 3-fold.

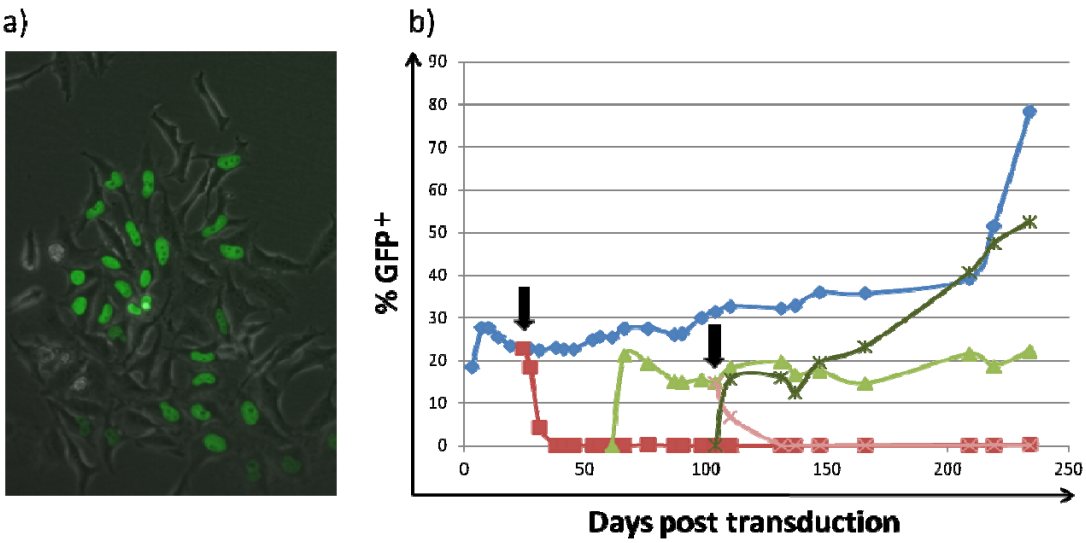


Figure 25: Tet-regulated H2B-GFP expression system in HeLa cells.

a) Transduced HeLa cells show nuclear localization of GFP expression. b) Cells were cultured for 8 months (—◆—). 10ng/ml Doxycyclin was added as indicated by black arrows and GFP-expression was completely abolished (—■—). Removal of Doxycyclin restored GFP-expression at two different timepoints (—▲— and —✳—) which remained reversible (—✳—).

Regulation of H2B-GFP expression in mice harbored the risk that doxycyclin was only available for distinct regions of the xenografted tumor. Thus, label-retention would have been due to insufficient doxycyclin-supply and not due to mitotic quiescence. Therefore, the distribution of GFP within a tumor was assessed using tissue slices which were immunohistochemically stained with an antibody against GFP (Figure 26). Doxycyclin-untreated control tumors showed homogeneous GFP-expression for TSC-01 and TSC-08. Single clones showed no GFP-expression in both cases. Treatment of mice with doxycyclin via the drinking water led to almost complete abundance of GFP-expression in TSC-01- and TSC-08-derived tumors. Single GFP^+ signals were equally distributed over the tumor and showed no regional enrichment.

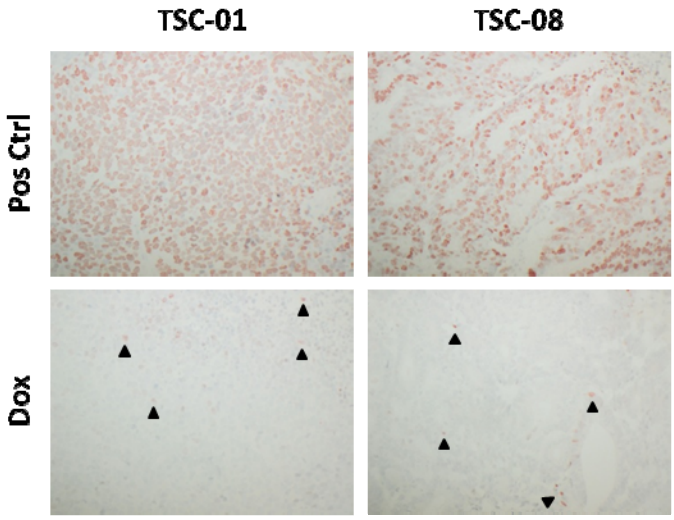


Figure 26: Immunohistochemical staining of GFP within H2B-GFP-transduced tumors.

Tumor tissue slices were stained using an antibody against GFP. Positive control tumors showed a homogeneous distribution of GFP (pos Ctrl). Doxycyclin inhibited GFP expression (Dox). Only rarely dividing cells maintained the label.

4.4.3 Xenografted tumors comprise different proliferative subfractions of self-renewing human colon TIC *in vivo*

Lentivirally marked tumor sphere culture cells were transplanted under the kidney capsule of immunodeficient mice and gave rise to green fluorescent tumors. Doxycyclin was applied to the drinking water of mice starting 3 weeks after transplantation. GFP expression was suppressed from then on resulting in dilution of GFP expression in rapidly dividing cells. Only slow or rarely dividing cells maintained their GFP. After further 3 weeks of tumor growth, mice were sacrificed and tumors were harvested and analyzed for their GFP intensities. Doxycyclin-treated tumors were compared to untreated control tumors. Subfractions with differing proliferative activity could be discriminated (Figure 27): fast dividing cells (78.8% of TSC-01, 99.6% of TSC-08) lost all their GFP and showed the same fluorescent intensity as untransduced tumor cells. Rarely dividing tumor cells (TSC-01 0.3%, TSC-08 0%) were defined by the same GFP-intensities as the highly GFP^+ population in the doxycyclin-untreated tumor. Slow dividing cells (TSC-01 21.1%, TSC-08 0.4%) exhibited an intermediate GFP-fluorescence. The untreated H2B-GFP expressing control tumor showed 60% GFP^+ cells in first generation recipients of TSC-01. First generation mice were sacrificed and tumors were analyzed for their GFP expression. Untransduced and transduced but doxycyclin-untreated tumor cells were serially transplanted as control groups. Untreated, H2B-GFP expressing 2^{nd} generation TSC-01 tumors contained 64% GFP^+ cells. The GFP^+-proportion remained stable in 3^{rd} generation mice (70% GFP^+). Doxycyclin-treated tumor cells were sorted according to their proliferative history and each subfraction was transplanted into a second generation of mice. Tumors were

formed by control bulk cells and originally fast and rarely dividing TSC-01 cells. In TSC-01, 4.5% of the transplanted rarely dividing cell population still maintained their GFP-intensities upon tumor formation. A third generation of mice was transplanted with xenografted 2nd generation tumor bulk cells. Originally fast, rarely dividing and unfractionated cells maintained their tumor forming capacity confirming the presence of self-renewing TIC. The size of the rarely dividing population decreased to 2.5% in the 3rd generation tumor originating from rarely dividing cells. For TSC-08, transduced control cells formed tumors over two generations whereas untransduced TSC-08 contributed to tumor formation over three generations of mice (Figure 27b). Upon sorting of proliferative subfractions, only fast dividing cells contributed to tumor formation in a 2nd generation of mice.

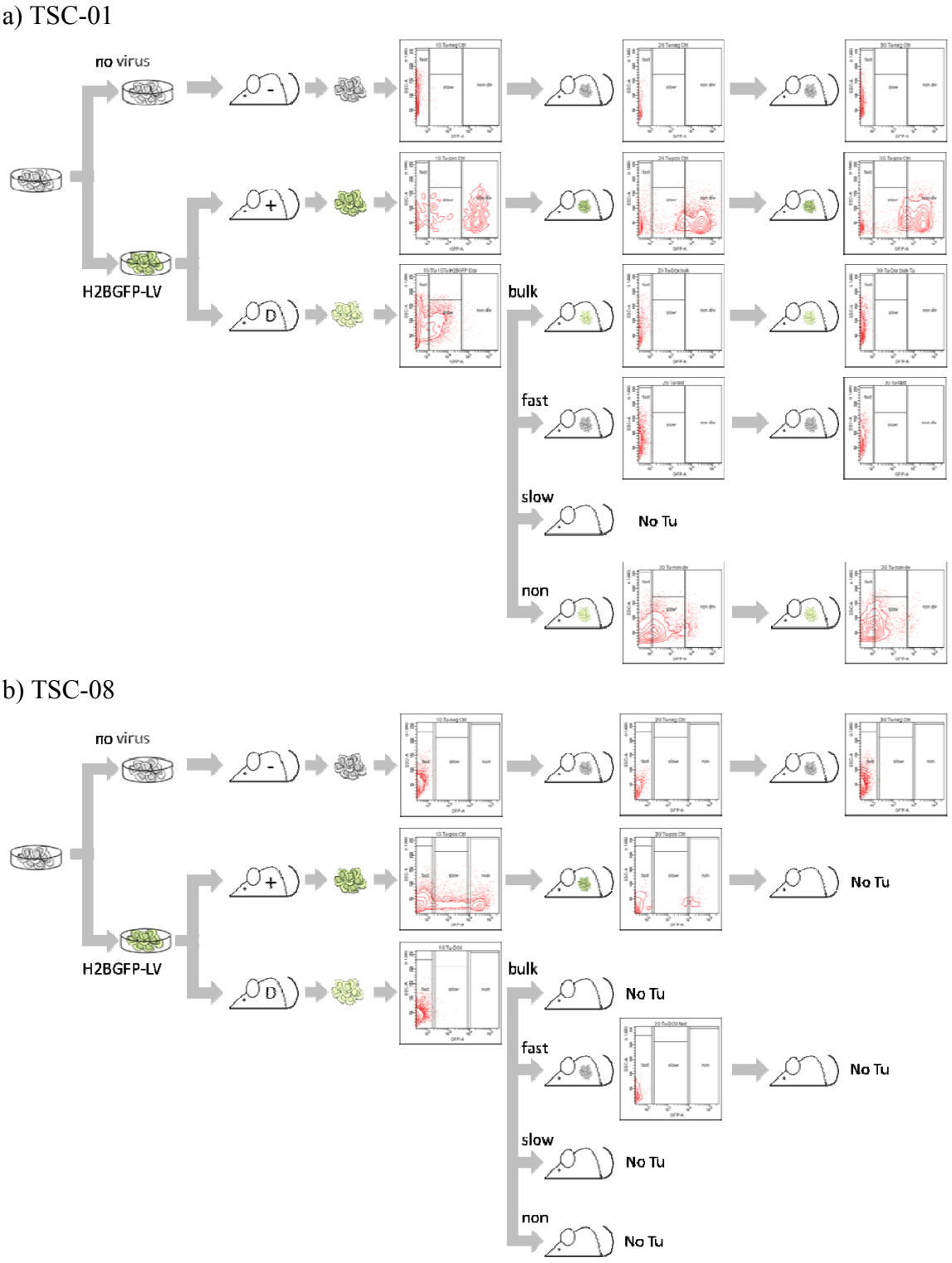


Figure 27: Xenografted tumors comprise fast, slow and rarely dividing human colon TIC.

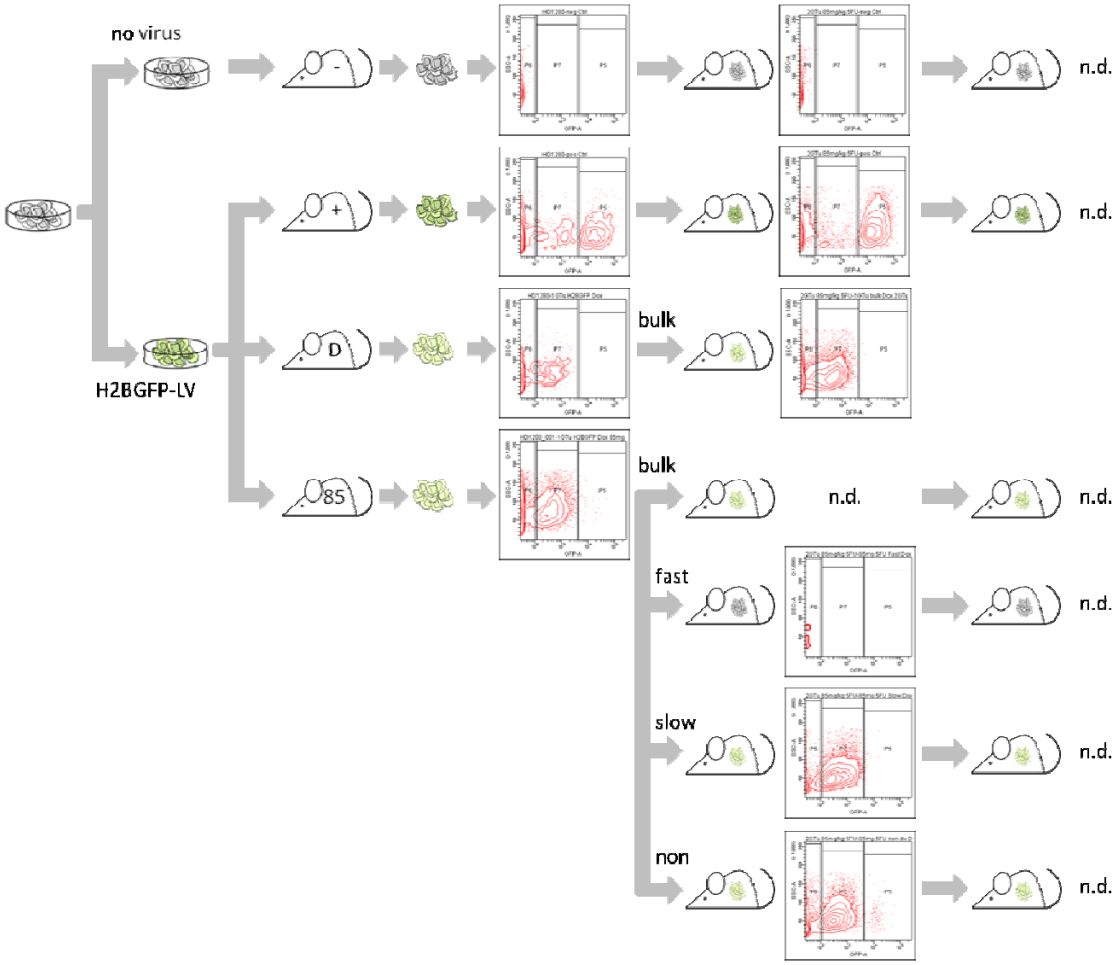
Untransduced (-) and transduced (+ and D) cells were transplanted into immunodeficient mice. After three weeks of tumor growth, a cohort of mice was treated with doxycyclin in the drinking water (D), tumors were harvested after further three weeks. Purified tumor cells were sorted according to their proliferative activity or further assessed as unfractioned bulk. a) TSC-01 fast and rarely dividing tumor cells maintained their tumor initiating potential over three generations of mice; the size of the originally quiescent population decreased to 2.5%. b) Fast dividing TSC-08 contributed to tumor formation in a 2nd generation of mice.

4.4.4 A quiescent population of human self-renewing colon TIC is enriched by chemotherapeutic treatment

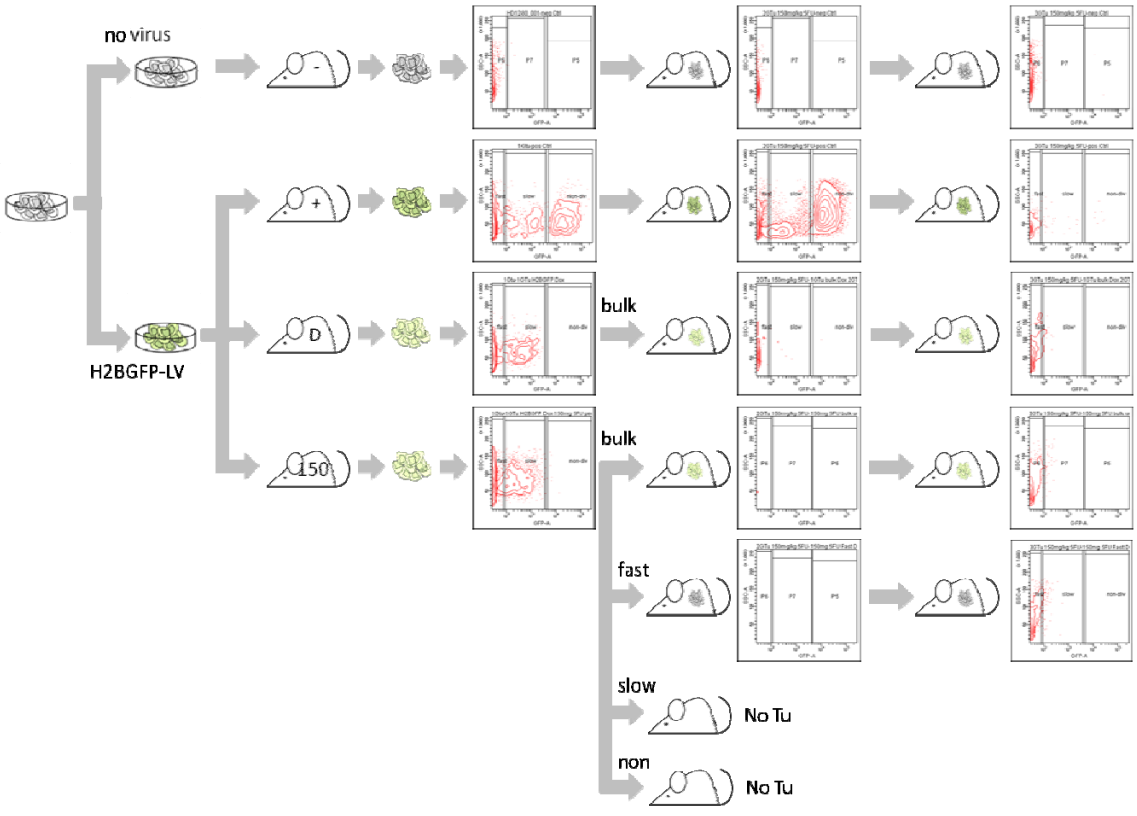
In order to investigate whether quiescent TIC can be recruited to tumor formation after chemotherapeutic treatment, cohorts of mice were treated with 5-FU, a chemotherapeutic drug applied in standard colon cancer therapies.

Prior to these experiments, 5-FU *in vivo* toxicity tests were performed in agreement with the DKFZ veterinarian: weekly intraperitoneal injection of 85mg 5-FU per kg body weight on 3 consecutive weeks and bolus injection of 150mg/kg 3 days prior to sacrification did not show a viability-reducing effect in treated animals. 2 out of 2 mice for each group survived. Consequently, 85mg 5FU / kg were injected once a week for the final three weeks of tumor growth in parallel to application of doxycyclin whereas 150mg 5-FU / kg were only applied once, 3 days prior to sacrification (Figure 28). In this experiment, the size of a quiescent population in 5-FU-treated mice was compared to 5-FU non-treated mice. Interestingly, 5-FU-treatment increased the size of the quiescent population (TSC-01: 0.1% in non-treated mice vs. 0.3% with 1x 150mg/kg and 0.5% with 3x 85mg/kg; TSC-08: 0% in non-treated mice vs. 0% with 1x 150mg/kg and 0.1% with 3x 85mg/kg). Sorting and subsequent serial transplantation of fast, slow and rarely dividing cells revealed that all proliferative subfractions of TSC-01 and TSC-08 comprised self-renewing TIC. The quiescent fraction of TSC-01 cells was even more enriched in the 2nd generation tumor originating from those cells (1.7% vs. 0.5% in the 1st generation tumor), indicating that these cells admittedly proliferated but, however, comprise a small fraction which is still not contributing to tumor formation. Slow and rarely dividing cells from 150mg 5-FU-treated tumors (Figure 28b) did not engraft in transplanted mice. Treatment of TSC-08-tumor-bearing mice with 5-FU led to an enrichment of label-retaining cells in the first generation. All cells in 2nd generation tumors, no matter from which cell fraction they were derived, actively proliferated and lost all their GFP. Rarely dividing cells from 85mg-treated mice and slow-dividing and unsorted cells from 150mg-treated mice did not form a tumor in the second generation.

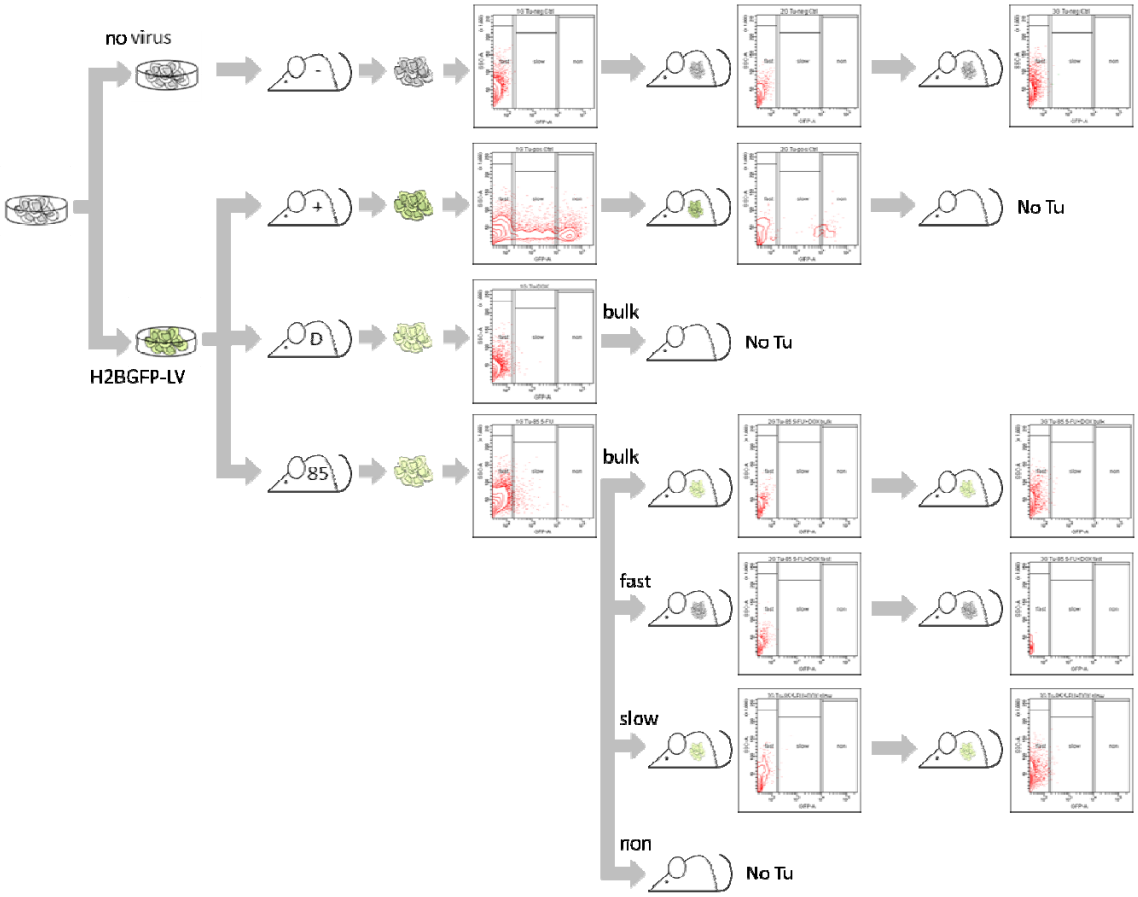
a) TSC-01, 85mg 5-FU/kg



b) TSC-01, 150mg 5-FU/kg



c) TSC-08, 85mg 5-FU/kg



d) TSC-08, 150mg 5-FU/kg

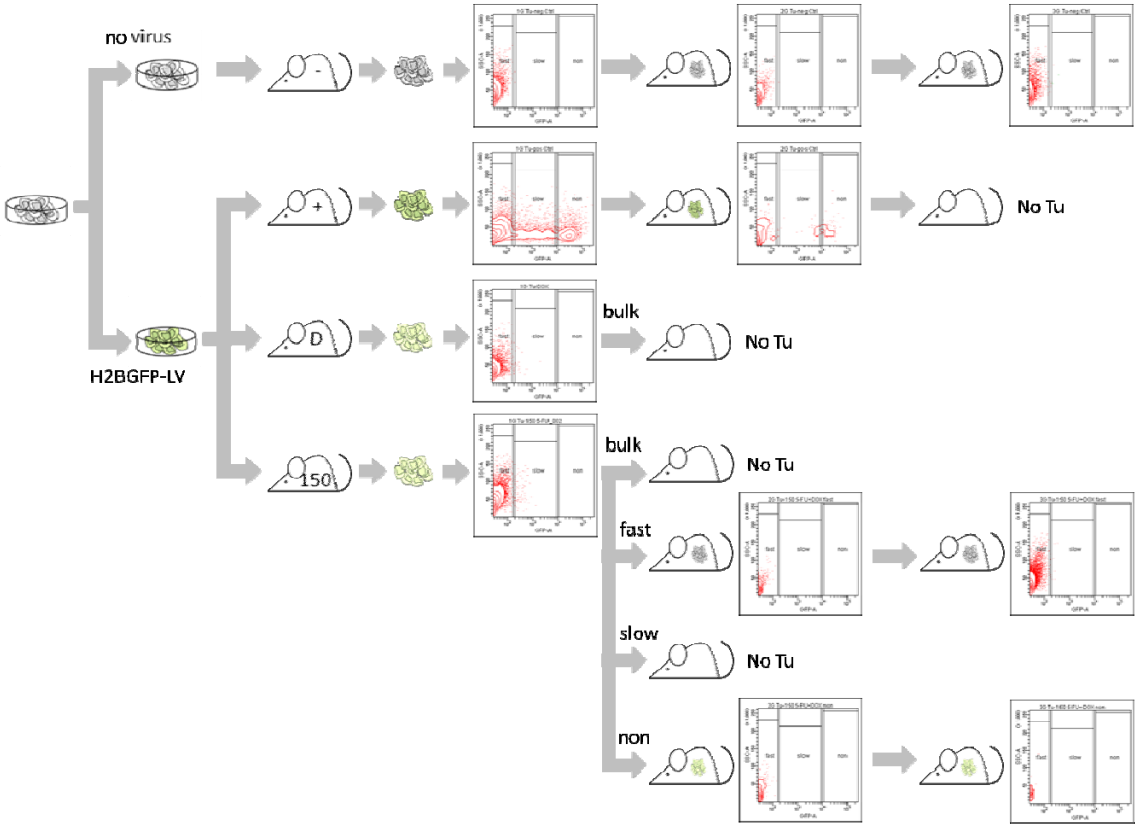


Figure 28: A quiescent population of human colon TIC can be enriched using chemotherapeutic treatment.

Fast, slow and rarely dividing cell fractions of TSC-01 (a and b) and TSC-08 (c and d) tumor cells comprised self-renewing TIC. The quiescent fraction of TSC-01 and TSC-08 was enriched in 85mg 5-FU/kg-treated mice. TSC-01-rarely dividing tumor cells were even more concentrated in the 2nd generation tumor originating from those cells. Fast, slow and rarely dividing TSC-08 tumor cells highly proliferated and lost all their GFP.

4.4.5 Clonal Composition of xenografted tumors formed by colon TIC with differing proliferative activity

Our group has recently shown that human colon cancers comprise a small subfraction of delayed contributing TIC (DC-TIC) which becomes activated upon serial transplantation. These cells might have rested in a mitotically inactive state. We hypothesized that cellular quiescence might enable them to survive chemotherapeutic intervention. To track the contribution of individual cell clones, we analyzed the clonal composition of xenografted tumors. In addition, we assessed the effect of 5-FU treatment on the clonal contribution of distinct TIC clones.

H2B-GFP expression in human colon TIC was driven by lentiviral integrating vectors. The viral DNA carrying the H2BGFP expression cassette semi-randomly integrates into the host cell's genome. All cell descendants inherit the viral DNA at exactly the same site, and thus a unique clonal mark is established in each successfully transduced cell. Lentiviral integration sites in the host cell's genome were tracked using LAM-PCR. Amplification and restrictive digestion of vector-genome-junctions during LAM-PCR yielded DNA-fragments of different length which were finally displayed by high resolution gel electrophoresis (Figure 29). Assuming that each clone harbors one integration, every band on the gel represents one distinct cell clone contributing to tumor formation in respective samples. Primary recipient mice developed tumors with polyclonal offspring (Figure 29a). Several clones contributed to tumor formation in three generations of mice whereas other cell clones were only active in 1st generation mice. A small fraction of TIC only proliferated in the third generation, its clonal contribution to first or second generation tumors was not detectable via gel electrophoresis. Moreover, the number of clones contributing to tumor formation decreased during serial transplantation.

First generation tumors comprised fast, slow and rarely-dividing cells. Cells with differing proliferative activity were separately transplanted into NSG mice and the clonal composition of xenografted tumors was analyzed (Figure 29b): tumors derived from initially fast dividing cells were formed by few cell clones with a relatively small band size of 100-150bp. In contrast, tumors formed by originally rarely-dividing cells showed a polyclonal pattern with numerous additional cell clones with band sizes between 200 and 500bp. Chemotherapeutic treatment led to a different clonal pattern as compared to non-treated tumors (Figure 29c). Some clones were eliminated and did not

contribute to tumor formation in chemo-treated mice. Few clones survived and were still detectable after chemotherapy. In addition, new cell clones which were previously not detectable in chemo-free mice ("no 5-FU 1°") proliferated and contributed to the tumor ("5-FU bulk 1°"). Follow-up of chemo-exposed tumor cells over three generations of mice showed that one cell clone was detectable in primary and tertiary recipients of chemo-treated, unsorted bulk cells. If at all contributing, it was below the detection limit in secondary tumor samples. The polyclonal pattern from 1st generation tumors was reduced to an oligoclonal level within three generations. One very weak clone contributed to the tertiary tumor only. Fast, slow and rarely-dividing cells could be discriminated in 1st generation chemo-treated tumors. They were sorted and the clonal pattern of formed tumors was analyzed and compared to each other and to the 1st generation bulk tumor from which they were derived. The gel revealed that the primary tumor's polyclonal pattern ("5-FU bulk 1°") was only maintained by the rarely-dividing cell fraction in a second generation of mice ("5-FU rarely div. 2°"). The third generation tumor derived from initially rarely-dividing cells showed an oligoclonal composition as seen before. Moreover, the gel picture suggests that another cell clone which was not detectable in primary or secondary recipients actively contributed to tumor formation in the tertiary mouse. Secondary recipients transplanted with fast or slow dividing tumor cells both developed tumors showing only few of the initially detected cell clones. Tertiary tumors of both proliferative subfractions exhibited a reduced number of contributing cell clones but showed activity of an additional, previously undetected cell clone.

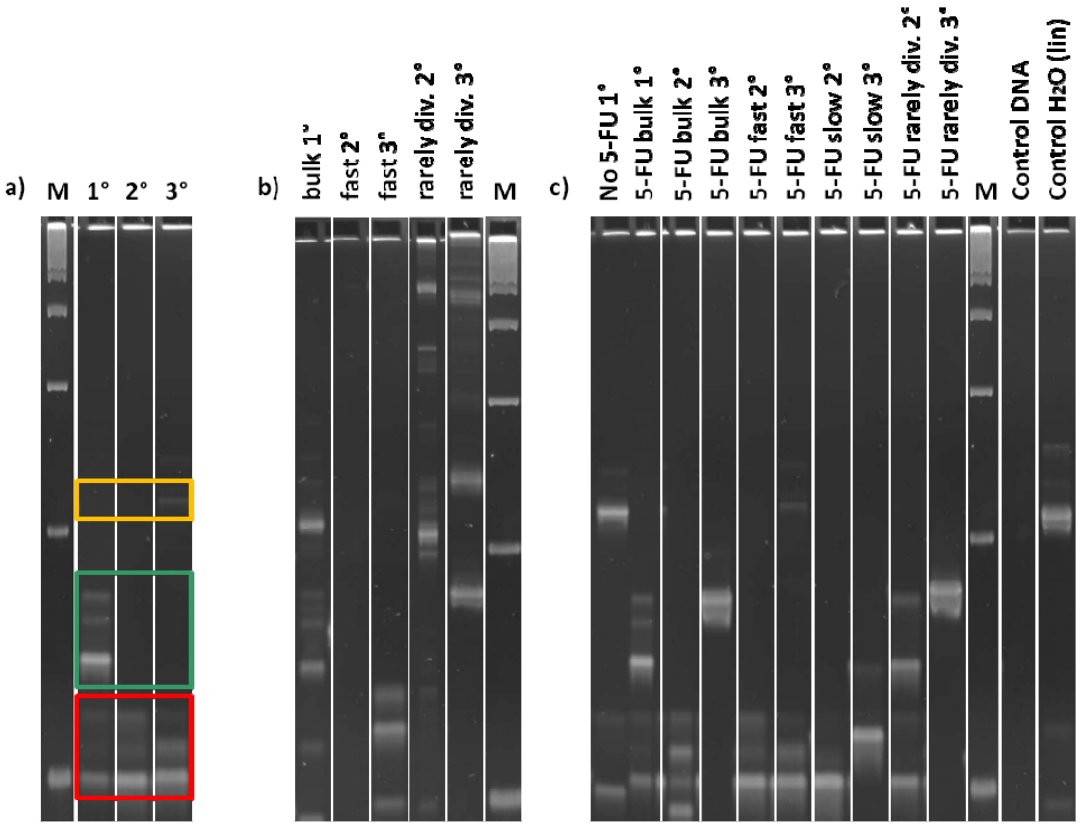


Figure 29: Clonal contribution of individual cell clones with differing proliferative activity under chemotherapeutic treatment determined by LAM-PCR.

H2B-GFP-LV-transduced TSC-01 spheroid cells were serially transplanted over three generations of NSG mice (1°-3°). a) Three classes of TIC could be discriminated. Several clones contributed to tumor formation in three generations of mice (*red*) whereas other cell clones were only active in 1st generation mice (*green*). A small fraction of TIC only proliferated in the third generation (*yellow)*. Tumor cells from non-treated (b) or 5-FU-treated mice (c) were analyzed using flow cytometry and sorted according to their proliferative activity. Fast, slow and rarely-dividing cell fractions were serially transplanted. The clonal contribution of individual cell clones, defined by their unique integration site of the viral genome, was analyzed via LAM-PCR and high resolution gel electrophoresis.

4.5 Summary

The intestinal stem cell compartment is becoming more and more characterized: it has been shown that the majority of colonic stem cells are actively-cycling enabling fast cell turnover and renewal of the epithelial layer[35, 36]. A protective role is ascribed to rare quiescent intestinal stem cells[37-39]. An equivalent counterpart in the malignant stem cell compartment has not been demonstrated. We aimed to elucidate whether quiescent TIC exist in human colorectal cancer and if they play a major role in chemoresistance. They could potentially be either hibernating in G_0 phase of the cell cycle or they could be long-term inactive. In this study we now investigated the link between cell cycle status, proliferative activity and stem-like properties in human colon cancer. Hoe/PY- and CFSE staining of primary human colon cancer spheroid cultures enriched for human

colon TIC allowed discrimination of TIC subfractions with differing cell cycle status and differing proliferative activity *in vitro*. A small subfraction of TIC indeed hibernated in G_0 phase of the cell cycle but self-renewing colon TIC could be found in all cell cycle phases. Moreover, the majority of all colon TIC from different colon cancer patient samples was actively-proliferating *in vitro* and *in vivo* as shown by lentiviral marking experiments. Nevertheless, a quiescent population of human colon TIC exists *in vitro* and *in vivo*. Clonal analysis revealed that colon TIC were enriched in the quiescent population and indicated that quiescent TIC were recruited to tumor formation upon chemotherapeutic treatment.

5 Discussion

5.1 Maintenance of primary human colon TIC in spheroid cultures and xenografts

5.1.1 *In vitro* maintenance of human colon TIC in spheroid cultures

In this study, human colon cancer specimen from patients who underwent surgery were enzymatically purified and maintained *in vitro* as non-adherent, 3-dimensional spheroid cultures. To date, human colorectal cancer has been studied in different model systems starting with patient-derived cancer cell lines growing as 2-dimensional monolayers in serum-containing growth medium. In an attempt to simulate the clinical diversity of colon cancers, low-passage, serum-containing cell culture models were developed reflecting the individual combinations of oncogenes and mutations found in the parental primary patient tumor[209]. To better approximate the 3-dimensional *in vivo* situation of a tumor, spheroid culture systems were established using non-adherent culture-conditions: cells form 3-dimensional, freely floating cell aggregates. Their superior role in mimicking the *in vivo*-situation was demonstrated by Kobayashi *et al*.[210]. The authors generated 2- and 3-dimensional cell cultures out of murine mammary tumors which showed *in vivo*-resistance to the alkylating agents Thiotepa, Cyclophosphamid and Cisplatin. They found that 2-dimensional monolayer cultures did not recapitulate the chemoresistant phenotype whereas multicellular spheroids under serum-free conditions did. In addition, serum-free culture conditions have been described for neuronal stem cells[211], mammary stem cells[174], mesenchymal stem cells[212] and hematopoietic stem cells[213]. In the context of human cancer, the sphere culture technique has been widely used to enrich putative cancer stem cells from breast[106], brain[108] and colon[116, 126]. Lee *et al.* showed that serum-treatment of isolated human glioblastoma cells leads to genotypic and biological changes whereas cells in serum-depleted growth medium maintained the primary patient tumor characteristics[127]. Additionally, it has been shown that serum-depletion prevents colon cancer initiating cells from differentiation[125, 126] and Vermeulen *et al.* demonstrated multi-lineage differentiation capacity of single colon cancer stem cells from serum-free cultures[214]. Well in line with already published data, our study provides experimental evidence for the clonal origin of cells within one sphere by showing that single sphere cells generated new spheroids. DNA analysis of all cells comprised in one spheroid might approve the clonal conditions on a molecular level. The clonality of these cultures might be questioned in high density sphere cultures where fusion of spheroids has already been observed in a neurosphere assay[215]. The

analogy between sphere-forming capacity and stemness of human primary tissue-derived cells remains under debate. Within this study, dissociation and serial replating of formed spheres demonstrated that at least a subfraction of spheroid cells shows long-term self-renewal ability which links spheroid-formation to stem-like properties. Furthermore, xenotransplantation of singularized, spheroid cells resulted in long-term tumor formation throughout a serial transplantation assay demonstrating the self-renewal ability of spheroid cells *in vivo*. Pastrana *et al.* summarized that the sphere-forming assay is a functional assay which evaluates "the potential of a cell to behave as a stem cell when removed from its *in vivo* niche"[122]. Indeed, in the context of colon spheres, one should not speak about cultivating stem cells uniquely. Although serial passaging of spheroid cultures progressively enriches for long-term self-renewing tumor initiating cells, self-renewing tumor transient amplifying cells (T-TAC) from the colon cancer initiating compartment are also maintained and contribute to spheroid formation *in vitro*[135]. This has also been shown in the mammary stem cell compartment. Progenitor enriched populations of mammary cells generated "pure luminal colonies" and "multi-lineage colonies"[216]. Moreover, murine hematopoietic stem cell progeny gained stem cell-like properties via malignant transformation by the leukemic oncogene MOZ-TIF2[104]. These studies indicate that not only stem cells but also more differentiated stem cell progeny is able to self-renew and potentially maintained in spheroid cultures. Our lab has shown that the majority of several colon sphere cultures and corresponding xenografted tumors resembled the original patient material, demonstrating the multi-lineage reconstitution potential of TIC maintained in spheroid cultures. TSC-01 and TSC-04 showed a reduced differentiation level than respective patient tissues. Genetic or epigenetic changes might have led to a dedifferentiated phenotype with growth advantage, and thus clonal selection might have happened during establishment of the spheroid culture. The efficacy of establishing spheroid cultures out of primary patient tissue was 8%. Major limitations were given by the mass of received tissue samples and their tumor cell content, the time between surgery and purification, the treatment patients received before undergoing surgery and *E. coli* contaminations.

In summary, the serum-free, non-adherent, growth factor supplemented culture conditions maintained a sub-population of primary human colon cancer cells with tumor-forming potential and self-renewal ability, defined as colon cancer initiating cells. To date, spheroid cultures represent the assay of choice for maintenance of cells *in vitro* in best possible similarity to the patient material which is clinically most important. Furthermore, they allow a detailed molecular characterization and genetic engineering via lentiviruses.

5.1.2 *In vivo* maintenance of human colon TIC in NSG mice

Although *in vitro* studies facilitated molecular characterization of colorectal cancer and colon TIC, translational research requires detailed investigation of their biological function *in vivo*. Several transgenic animal models were developed on the basis of the most prevalent mutations in CRC: various *APC* mutant mouse strains were generated, starting with the most prominent Apc^{Min} mouse[217-220]. All of them form polyp adenomas preferentially in the small intestine differing only in number and exact localization. $Apc^{\Delta 716}$ mice with an additional mutation of the *Cdx2* gene exhibited more polyps in the colon[221]. Reduction of polyp numbers was observed by equipping *Apc* mutant mice with further mutations in *Smad4*[222], in the nitric oxide synthase gene *iNOS*[223], in the DNA methyltransferase gene *Dnmt1*[224], in the transcription regulating *Mbd2* gene[225] and in the ABC transporter gene *Mdr1*[226]. Mouse models harboring several other gene mutations were developed including modifications of the TGF-β1 encoding gene *Tgfb1*[227], of *Kras*[228] and of the β-catenin gene *Ctnnb1*[229, 230]. In order to address specific questions of inflammation-related colon cancer, *in vivo* models have been developed where adenocarcinomas grew in interleukin-10 knockout mice[231] or in mice deficient for interleukin-2 and β_2-microglobulin expression[232]. All these genetically engineered CRC models are driven by oncogenic mutations, and only few showed metastasis formation. Thus, these models only reflect a part of CRC biology and hamper investigation of advanced cancer stages. Furthermore, they only mirror the murine *in vivo* situation and might underestimate the huge mutational diversity of human colon cancers[50, 51]. Transplantable colon cancer models were introduced facilitating investigations of metastasis[233, 234] and of tumor invasion into neighboring tissue[235, 236]. Many of these models are still based on murine cancer cells, making them less appropriate to study the human disease. However, each of these colon cancer animal models shows distinct characteristics to investigate specific aspects of CRC. The choice of animal model depends upon the aim of a study. Consequently, in order to study the human colon cancer initiating compartment, xenogenic transplantation is the gold standard. Its major limitation is given by inefficient engraftment of human cells in respective animals due to the host's immune response. To overcome this impediment, immunodeficient mice were developed. The NOD.Cg-$Prkdc^{scid}$ $Il2rg^{tm1Wjl}$/SzJ ($Il2rg^{-/-}$, NSG) mouse strain was generated by Shultz *et al.* and is one of the most immunodeficient strains available to date[237, 238]. It lacks functional activity of mature B cells, T cells and NK cells which are responsible for recognition and elimination of foreign contaminants. Evading recognition and rejection of transplanted material, NSG mice are thus most eligible for xenotransplantation studies on human colorectal cancer. Several studies have been performed using subcutaneous or kidney capsule injections of human cells whereas the latter was reported to yield higher tumor take rates[114-116, 125, 126]. Moreover, subcutaneous tumor formation might hinder metastasis formation due to the

tissue barrier[239]. Consequently, in this work, primary patient-derived colon cancer cells were transplanted under the kidney capsule of NSG mice, facilitating tumor formation and subsequent investigation of the proliferative behavior of colorectal cancer cells *in vivo*.

5.2 Quiescence of colon cancer initiating cells

Adult stem cells are functionally defined by their ability to self-renew and by their capacity to generate all organ-specific differentiated cell types. Murine hematopoietic stem cells (HSC) represent the best-studied stem cell compartment to date. They reestablish normal hematopoiesis in lethally irradiated mice and thereby lead to hematopoietic recovery and survival. Several groups have directly linked quiescence of long-term HSC to most efficient reconstitution potential[147, 240, 241] suggesting that stem cell quiescence plays a protective role in HSC maintenance[31, 242, 243]. The finding that some leukemia and solid tumor cells also show a heterogeneous potential to self-renew suggested the existence of a stem cell-like population in the malignant compartment[244-247]. It is now a widely-accepted concept that in many cancer entities tumor maintenance is driven by a subset of stem-like cells. Since HSC properties like self-renewal and multi-lineage-reconstitution potential have also been demonstrated for TIC of different solid cancers, it appears reasonable to assume that TIC also exhibit other HSC characteristics like quiescence. Indeed, dormant TIC have been demonstrated in AML[248, 249] and CML[250]. Moreover, a correlation between low mitotic activity and tumor forming ability has been demonstrated for several cancers including brain[251], breast[252], skin[160] and pancreas[159] but has not been found in colorectal cancer. However, proliferative studies of human colon cancer initiating cells are highly relevant for clinical treatment of the disease. Quiescent cells could evade conventional 5-FU-based chemotherapy which is only affecting actively-cycling cells[172]. They might be reactivated at later time points and contribute to recurrent disease.

This work characterizes the proliferative activity and dynamics of human colon TIC independent of any cell surface marker.

5.2.1 The cell cycle status of human colon TIC

It has been demonstrated that several solid cancers harbor a subfraction of slow-cycling cells[159, 160, 252, 253]. In terms of the cell cycle, TIC might persist in G_0 in which cells do not divide. To address whether cell cycle quiescence is a property of human colon TIC and whether the tumor initiating potential is restricted to cells in G_0 phase of the cell cycle, primary patient-derived spheroid culture cells were stained with Hoechst and Pyronin. These intercalating fluorescent dyes enabled simultaneous analysis of cellular RNA and DNA content, and thereby discrimination and subsequent cell sorting of colon

TIC in G_0, G_1 and $S/G_2/M$ phase of the cell cycle. In order to exclude contamination of the G_0 cell fraction with actively-cycling cells, the expression of Ki-67 was analyzed in aliquots of all sorted fractions. Ki-67 is a nuclear protein only expressed by actively-cycling cells whereas exit from the active cell cycle leads to a rapid decrease of mRNA and protein expression[254, 255]. A more recent study indicates that Ki-67 can nevertheless be detected in quiescent cells, namely at the site of ribosomal RNA synthesis[256]. However, the authors also showed that proliferating and quiescent cells could still be clearly distinguished by differences in intensity and spatial distribution of $Ki67^+$ events. Consequently, Ki-67 can still be used as a proliferation marker.

Cell sorting of spheroid cells with differing cell cycle status and subsequent xenotransplantation of equal cell numbers revealed tumor-forming potential in every cell cycle phase. Interestingly, cell cycle profiles of first generation xenografted tumors exhibited a similar cell cycle distribution of tumor cells as compared to spheroid culture cells. This implies that cells with differing cell cycle status equally contributed to tumor formation. It additionally indicates that colon TIC from spheroid cultures did not change their proliferative behavior when removed from *in vitro* conditions and transplanted into mice. Moreover, tumors formed by unstained and unsorted spheroid cells showed a similar cell cycle profile as previously stained and sorted spheroid cells. Thus, staining and sorting procedures did not have any impact on the biological behavior of cells as well. Bulk transplantation of 1^{st} generation tumor cells revealed long-term tumor forming potential in three generations, no matter if transplanted cells originated from cell cycle phase tumors or from the bulk tumor. Cells which have been stained and sorted twice (the first time from spheroid cultures and the second time from first generation tumors) did not form tumors even from high cell numbers (500 to $1x10^4$ cells). In contrast, mice transplanted with 500 TSC-04 cells from once stained and sorted first generation bulk tumor cells developed a tumor. A strongly decreased viability after a second Hoe/PY staining and sorting procedure probably led to TIC frequencies below the threshold necessary for tumor formation. Nevertheless, self-renewing colon TIC were present in G_0, G_1 and $S/G_2/M$ fractions, showing long-term tumor-forming potential in a serial transplantation assay. The proportion of G_0-cells from spheroid cultures was maintained in mice. A switch between a dormant and active cell cycle state appears likely since at least a proportion of G_0-cells must have been activated to leave their dormant state and to initiate tumor growth. Interestingly, a few years ago, it has been demonstrated in the murine hematopoietic system that mouse HSC are activated to self-renew upon hematopoietic stress and return to a quiescent state after reestablishment of homeostasis[257]. A similar role might be ascribed to quiescent human colon TIC enabling maintenance of the TIC pool upon cellular stress.

Here, we demonstrate that the majority of human colon TIC is actively-cycling. A small proportion of TIC in G_0 exists. However, quiescent and actively-cycling TIC populations equally contributed to tumor formation in immunocompromised mice.

5.2.2 The proliferative activity of human colon TIC

The cell cycle analysis performed above showed that a small fraction of human colon TIC was in an inactive state at the time of analysis. However, cells might transiently adopt the G_0 state and begin to cycle upon activation. In order to detect cells which are long-term quiescent, label-retaining assays have been developed based on DNA-intercalation or binding of cellular components[147, 179]. In this study, the CFSE label-retaining assay was used to investigate prolonged quiescence of primary, human colon TIC enriched spheroid cultures. Furthermore, the H2BGFP label-retaining assay was applied to identify quiescent human colon TIC *in vivo*.

5.2.2.1 The proliferative activity of human colon TIC in spheroid cultures

CFSE has been originally developed to track lymphocytes in animals[258]. It has been demonstrated that CFSE is equally distributed among daughter cells during each cell division[259], initiating its use in proliferative studies. Several dyes have been used to study cellular proliferation[179]. The membrane inserting dye PKH 26 additionally allowed long-term observation of CFSE labeled cells *in vivo*[260]. However, it is much more expensive and has been shown to give less uniform staining than CFSE[179] leading to low resolution profiles during flowcytometric analysis of the cell's proliferative history. 1µM CFSE was identified to yield optimum staining conditions for our experiments. Initial fluorescence intensities of labeled cells enabled follow-up of around 8 cell divisions demonstrating the efficient labeling of spheroid cells. Blood cells were commonly studied upon staining with 5µM to 10mM CFSE[180, 258, 259] indicating that colon TIC were more susceptible to CFSE staining. A reduced concentration of CFSE was also used to study a mammary adenocarcinoma-derived cell line and glioma sphere cultures reflecting the higher CFSE sensitivity of such tumor cells[253, 261]. The more efficient staining of tumor cells by lower CFSE concentrations might have been due to their increased metabolic activity, as compared to their healthy counterpart, leading to increased transcriptional and translational activity. The cell's protein content directly correlates with CFSE-intensity due to the formation of stable conjugates[179]. Nevertheless, a cytotoxic effect of CFSE could be excluded by thorough titration experiments. Colon TIC from spheroid cultures showed a broad range of initial CFSE intensities. Cell sorting of a stained subfraction would have yielded a distinct intensity-peak, allowing determination of exact numbers of divisions after prolonged culturing. In order to maintain the diversity present in human colon cancers and corresponding spheroid cultures, cells were not sorted and proliferation was allowed during culturing

of the unselected bulk population. The wide range of initial CFSE intensities can be explained by cell aggregation during staining and by a heterogeneous appearance of spheroid cells in terms of size, morphology and protein content. This is supported by the finding that TSC-04 exhibited the narrowest "peak" after staining. TSC-04 was the spheroid culture which was easiest to singularize and most homogeneous in terms of size and cellular shape when viewed under the microscope. Fast, slow and rarely dividing cells could be discriminated on day 8, after initially determining CFSE intensities. The proliferative status was maintained afterwards. Thus, day 8 was chosen to sort cells according to their proliferative activity *in vitro*.

All proliferative subfractions harbored self-renewing spheroid cells. Moreover, the frequencies of sphere forming cells (SFC) were similar among the different proliferative subfractions, indicating that the sphere-forming potential is not correlated to the proliferative activity of spheroid cells. This finding is supported by very recent data obtained with glioma spheres: slow and fast dividing cells derived from glioma sphere cultures formed new spheres after separation by CFSE-dependent FACS sorting[253]. Singularization and serial replating of formed spheres showed that the frequency of SFC was retained over 3 spheroid generations. This indicates that a cellular hierarchy present in individual patient-derived colon spheres is maintained. In addition, inter-individual differences reflected by varying SFC frequencies in different spheroid cultures were also preserved. TSC-04 cells did not regrow spheres from single-cell level but at higher cell densities, implying that growth stimulation was dependent on co-culture of several spheroid cells. This finding is supported by a study using a co-culture system of intestinal stem cells and Paneth cells. The authors demonstrated the relevance of growth-stimulating factors secreted by surrounding cells for regulating stem cell proliferation[262].

Upon transplantation of equal cell numbers from each proliferative subfraction, all mice developed tumors. Serial transplantation revealed long-term tumor forming potential of all proliferative subfractions. Thus, rarely, slow and fast dividing cell fractions comprised self-renewing human colon TIC and mitotic quiescence could not be approved as a functional criterion of human colon TIC from spheroid cultures. Moreover, the separation of quiescent cells from more actively-proliferating ones did not have an impact on long-term tumor formation whereas the long-term repopulating activity of HSC was lost when disrupting maintenance of a quiescent HSC pool[263]. Mice from each generation were sacrificed on the same day and the CFSE intensities of xenografted tumor cells were analyzed. All tumor cells were $CFSE^-$, indicating that all cells including originally rarely dividing and slow-dividing cells actively proliferated upon transplantation and lost their CFSE due to divisions. First generation mice were sacrificed 5 to 13 weeks post-injection which still allows monitoring of CFSE-stained

cells *in vivo*[258, 264]. It has also been postulated that up to 8 divisions can be tracked[264] which is well in line with our data.

5.2.2.2 Relationship of proliferative activity and cancer stem cell markers in spheroid cultures

An aliquot of CFSE stained cells was also analyzed for the expression of cell surface markers which have previously been associated with tumor initiating potential. Expression of CD133, CD44, CD166 and EpCAM were, if at all detected, not found to be enriched in any of the proliferative subfractions. Since Kemper *et al.* published that the AC133 antibody does not detect glycosylated forms of CD133 in colon CSCs[132], a combination of two CD133-antibodies was used with differing epitopes (AC133 and 293C3). Thus, CD133 was indeed not expressed by TSC-01 spheroid cells. Investigation of the corresponding primary patient material might have shown whether CD133 was not expressed by this patient, or if its expression was lost upon *in vitro* cultivation. Consequently, expression of any of these markers was not correlated to the proliferative activity of spheroid cells. Recently and consistent with our finding, two independent groups could not link $CD133^+$-expression to the proliferative activity of colon TIC[265, 266]. Furthermore, Huang and coworkers demonstrated for several other cancer types that cell fractions defined by putative cancer stem cell markers, e.g. $CD44^+CD24^-$ for breast cancer, showed a similar proliferative potential as the CSC-marker negative fraction[266]. The authors also showed that primary, patient-derived $CD133^-$ colon cancer cells could generate $CD133^+$ progeny and *vice versa*, demonstrating the phenotypic plasticity exhibited by colon cancer cells. Investigations from the last decade have already led to controversial findings and have questioned the use of distinct marker combinations to identify human colon TIC[115-117]. Taken together, these very recent studies emphasize limitations of using cell surface markers alone to distinguish CSCs from non-tumorigenic cells.

5.2.2.3 The proliferative activity of human colon TIC within established tumors

To gain a thorough insight into the proliferative activity of human colon TIC within an established tumor, a TetOff-H2BGFP expression system was introduced into TIC-enriched spheroid culture cells. A study by Pfizer demonstrated that doxycyclin, a member of the tetracycline antibiotics group, forms stable calcium complexes and is stored in bone, teeth, kidney and liver of tested animals[e]. Induction of H2BGFP-expression and a subsequent doxycyclin-free chase period (TetOn-H2BGFP expression system) are thus inappropriate to study label-dilution since basal activity of the transactivator would lead to falsely GFP^+ cells. Therefore, the TetOff-H2BGFP expression system was chosen where presence of tetracycline stops H2B-GFP expression and dividing cells dilute their GFP label, allowing tracking of cells according to their proliferative activity. To stably implement the H2BGFP-expression system into

human colon TIC, we transduced spheroid cells with a TetOff-H2B-GFP-encoding lentivirus. Lentiviruses are frequently used as gene delivery vectors. In comparison to other viruses they show the unique ability of infecting fast dividing and rarely dividing cells[188]. Stable integration of the viral genome leads to tissue-specific life-long expression of the transactivator protein and, if no doxycyclin is present, H2B-GFP. Moreover, it allows studying viable cells after purification based on their label-retaining ability.

Long-term stable expression of H2B-GFP was approved in HeLa cells. After 3 months of culture, the proportion of transduced untreated control cells imcreased. When culturing cells over extended periods of time, a clonal selection process might happen. A transduced clone probably developed a growth advantage and overgrew the culture, explaining the increasing proportion of GFP^+ cells. Nevertheless, addition of doxycyclin completely abolished GFP-expression *in vitro*. Withdrawl of doxycyclin led to incomplete restorage of original GFP expression levels. Possible reasons include (1) a reduced activity of the transactivator protein after its conformational change and (2) epigenetic silencing of H2B-GFP expression. For the TetOn system, where presence of doxycyclin induces transgene expression under control of the reverse transactivator protein rtTA, it has been demonstrated that rtTA shows weak binding to the transgene-controlling promoter P_{Tight} even without doxycyclin being present, leading to a basal expression of the transgene[267]. As the TetOff-tansactivator $tTA2^S$ induces H2B-GFP expression only in the absence of doxycyclin, and doxycyclin itself induces a conformational change of this protein hampering binding to P_{Tight}, doxycyclin withdrawl from once-treated cells might not restore the original $tTA2^S$ conformation completely. This might lead to inefficient binding and thereby to reduced expression levels of H2B-GFP. On the other hand, Kues *et al.* reported that reduced transgene expression was correlated with the methylation status of P_{Tight} in pigs[268]. The authors concluded that promoter methylation led to epigenetic silencing of their transgene. In our hands, methylation of P_{Tight} might lead to binding of methyl-CpG-binding domain proteins. These proteins attract histone deacetylase complexes (HDAC) and several factors which modify histones leading to dense chromatin structure (heterochromatin)[269]. The compressed state of the DNA region might result in inefficient binding of the transactivator protein and thereby in H2B-GFP silencing. Bisulfite sequencing might help to determine the methylation status of P_{Tight} in HeLa cells. In addition, direct histone modifications such as methylation might lead to dense chromatin structure and epigenetic H2B-GFP silencing. Nevertheless, we could show that H2B-GFP expression in transduced cells is regulated by doxycyclin *in vitro*. In addition, when transplanting transduced cells into immunodeficient mice, a stable proportion of GFP^+ cells was detected in the control group which was not treated with doxycyclin over three generations of mice.

In order to approve that label-retention was not due to insufficient supply of doxycyclin, the GFP-distribution in tissue slices of doxycyclin-treated tumors was investigated. Local enrichment of GFP^+ cells would have indicated that doxycyclin was not equally available throughout the tumor. In our study, an unequal spread of doxycyclin appears unlikely because single GFP^+ events were equally distributed over the tumor slice. It can be concluded that label-retaining cells were indeed less-dividing than other cells. GFP^- regions in doxycyclin-untreated control tumors can be explained by the contribution of untransduced cell clones.

The tumor from a 1st generation recipient mouse treated with doxycyclin revealed cell fractions with differing proliferative activity including a small fraction of label-retaining cells. Fast and rarely dividing tumor cells formed secondary and tertiary tumors. Slow-dividing tumor cells contributed to long-term tumor formation in chemotherapeutically-treated mice. Consequently, the absence of tumor-formation in 2nd generation recipients of slow-dividing tumor cells in chemotherapy-untreated mice might have been due to a technical mistake performed during transplantation. In doxycyclin-treated primary mice, all cells actively proliferated and lost their label due to divisions. In contrast, a second generation tumor xenografted from the same number of purified LRC from primary recipients still contained a small fraction of rarely dividing cells. First, it is obvious that cell sorting prior to secondary transplantation enriched for label-retaining cells. Flow cytometric detection of rare LRCs in bulk transplanted 2nd generation mice was limited by the number of recorded events. Additionally, populations smaller than 0.1% are below the detection limit of the FACS Diva software. Second, this finding indicates that a small fraction of initially label-retaining cells is still inactive in secondary and tertiary recipients reflecting a long-term quiescent subfraction of human colon TIC *in vivo*. But third and more important, the majority of initially label-retaining cells was activated upon transplantation and contributed to tumor-formation in every generation. In the benign intestinal stem cell compartment, a quiescent nature has been ascribed to label-retaining +4 ISCs[12, 140] whereas $Lgr5^+$ CBC cells are assumed to be more actively-cycling[270]. It might be interesting to find out whether Lgr5 is uniquely expressed by actively-cycling cells with decreased to no H2B-GFP expression. On the other hand, two groups have independently suggested that the label-retaining +4 ISCs are able to generate $Lgr5^+$ cells and *vice versa*[22, 23]. Moreover, the authors show, that +4 ISCs are activated upon irradiation, indicating that these cells function as a quiescent stem pool responsible for stem cell maintenance under very distinct circumstances. In line with this, we here show that quiescent and actively-cycling colon TIC co-exist in human colon cancer.

Summarizing, our data goes along with observations made for healthy intestinal stem cells where a quiescent and an actively-proliferating stem cell population have been demonstrated[141, 142]. A quiescent TIC subpopulation indeed exists *in vivo*. It might be

responsible for TIC maintenance upon injury or stress such as chemotherapeutic treatment. Reactivation of quiescent TIC by chemotherapeutic drugs might lead to tumor regrowth and recurrent disease in patients.

5.2.3 Quiescent TIC and their role in chemoresistance

The behavior of quiescent TIC upon chemotherapeutic treatment was thoroughly investigated. A cohort of H2B-GFP-transplanted mice was additionally treated with 5-Fluorouracil after xenografted tumors and their microenvironment were already established. Quiescent TIC were enriched by single bolus treatment and to an even higher extent by frequent treatment with a lower dose. 5-FU was injected intraperitoneally, mainly because of the ease of administration. In addition, it has been demonstrated that intraperitoneal injection of 5-FU offers high local- and systemically-effective concentrations of the drug[271, 272]. But the relatively short half-life of 5-FU[273] might explain the lower effect of the single bolus-injection, although it was administered in higher concentrations. Frequent treatment leads to a more constant bioavailability over a longer time. This is well in line with the finding, that prolonged continuous 5-FU low-dose infusion is superior to bolus treatment of colorectal cancer patients in terms of response rates and side effects[274].

In contrast to TSC-01, TSC-08 showed little to no sensitivity towards 5-FU. 85mg 5-FU/kg increased the proportion of quiescent cells to 0.1%, 150mg/kg-bolus treatment had no detectable effect. Moreover, although the 5-FU-treated xenografted TSC-01 tumors showed an enrichment of quiescent cells, indicating the effectiveness of the chemotherapeutic drug, the majority of tumor cells present at the time of analysis were fast dividing. Thus, 5-FU did not have an effect on the majority of fast dividing cells as well. Given the fact that 5-FU shows a low therapeutic index[275], studies have been performed to increase 5-FU efficacy. Well in line with our finding, Francescangeli *et al.* recently demonstrated that chemotherapy-treated colon cancer xenografts contained actively-proliferating cells[265]. Additional inhibition of the mitotic regulator polo-like kinase1 (Plk1) eradicated all proliferating cells. Moreover, several mechanisms including imbalanced drug-uptake and –efflux[276, 277], increased 5-FU-metabolism[278] and alteration of the drug target thymidilate synthetase[279], have been identified leading to 5-FU-resistance. As 5-FU requires actively-cycling cells, specifically in S-phase of the cell cycle, cellular quiescence is a major impediment for 5-FU treatment. Contrarily, TSC-01 and TSC-08 tumor-bearing mice mainly harbored fast dividing tumor cells even after 5-FU treatment. For the moment, this finding implies that cellular quiescence is no major factor being responsible for 5-FU resistance of TSC-08 and TSC-01 in these experiments. Effectiveness of 5-FU is also influenced by DNA repair mechanisms like the mismatch repair (MMR) machinery. MMR corrects replication errors, including base mismatches and insertions and deletions due to polymerase slippage at nucleotide

repeats[280]. It also plays a role in apoptotic signaling induced by DNA damage[281]. Deficiency of the MMR machinery, mainly caused by inherited mutations in MMR genes or by their silencing[282], leads to tolerance of DNA damage and apoptosis, and is consequently another reason for 5-FU resistance. This has been experimentally shown by Meyers *et al*., who demonstrated that restoration of MLH1 activity in an MMR-deficient colon cancer cell line increased sensitivity to 5-FU[283]. Moreover, MMR-defects cause increasing genomic instability and the formation of microsatellites, repeated sequences of few nucleotides. These microsatellites become instable during replication when a deficient MMR system does not properly repair newly developed errors (microsatellite instability, MSI). Thus, the MSI status correlates with sensitivity towards 5-FU. Indeed, investigation of TSC-01's and TSC-08's MSI status by another lab member revealed that both spheroid cultures were MSI^{+}. Interestingly, the MSI phenotype has been associated with increased patient-survival[284]. This can be explained by the fact, that most MSI^{+} patients exhibit a p53-wildtpe status whereas mutated p53 was associated with MSI^{-} patients. The p53 protein functions as a transcriptional activator of cell cycle arrest inducing genes like *CDKN1*[285]. Arrested cells are not able to incorporate metabolites of 5-FU and consequently, 5-FU treatment yields only reduced effects by TS inhibition. Furthermore, depending on the type of DNA damage, p53 triggers induction of apoptosis via activation of pro-apoptotic genes like *FAS* and down-regulation of anti-apoptotic genes such as *BCL2*[286]. Thus, $p53^{mut}$ patients show loss of p53 function resulting in 5-FU resistance and dysfunctional apoptosis. TSC-01 and TSC-08 were both classified as MSI^{high}, and additionally harbored mutated p53. It is worth testing combinational chemotherapy on transplanted mice to increase therapy efficiency. Clinical therapies are mainly based on the combination of 5-FU and Oxaliplatin[164-166]. Thus, combination of 5-FU and Oxaliplatin which has a different spectrum of action producing DNA crosslinks[169], is a promising strategy to improve treatment response in mice.

The unique clonal mark established by lentiviral transduction of spheroid cells was utilized to determine the contribution of distinct cell clones with differing proliferative activity to tumor formation in mice. Within a serial transplantation assay, three TIC classes could be discriminated which is well in line with data obtained by Dieter *et al.*[135]: (1) long-term tumor initiating cells (LT-TIC) showed long-term self-renewal ability contributing to tumor formation in 3 generations of mice, (2) transient tumor amplifying cells (T-TAC) which were only detectable in primary mice and showed no self-renewal ability and (3) delayed contributing tumor initiating cells (DC-TIC) which proliferated only in late generations and were not detectable in primary recipients. The contribution of fast, slow and rarely-dividing cell clones was investigated. Tumors formed by initially fast and rarely-dividing cells exhibited a different clonal pattern. The rarely-dividing cell fraction from 1^{st} generation bulk tumors might have comprised

numerous quiescent cell clones which potentially became activated when separated from the bulk and contributed to tumor formation.

According to the LAM-PCR gel, chemotherapeutic treatment of tumor-bearing mice eliminated numerous cell clones. On the other hand, several previously undetected cell clones quantitatively contributed to tumor formation in 5-FU-treated mice indicating that they were mitotically inactive before. They might have been activated upon chemotherapeutic treatment. Activation of previously quiescent cells by 5-FU treatment has been extensively studied in the murine hematopoietic system. Treatment with 5-FU is commonly used to force quiescent murine HSC into proliferation replenishing the impaired hematopoietic system[288]. This furthermore demonstrates the important role of a quiescent HSC pool for maintenance of the hematopoietic system. Our work already suggested a similar role for quiescent colon TIC. Now it appears even more likely, since chemotherapeutic stress might activate mitotically quiescent colon TIC to cycle and replace the previously eliminated TIC and thereby the tumor bulk cells. The LAM-PCR-gel suggests that tumors from primary and tertiary chemo-treated, bulk-transplanted mice harbored the same cell clone. If it was really the same clone, it must have been inactive in secondary recipients indicating a switch between an actively cycling and quiescent state. Few clones were only detectable in third generation tumors formed by initially fast, slow and rarely-dividing cells. In order to validate the existence of different TIC clones, high-throughput sequencing needs to be performed elucidating the contribution of individual cell clones to tumor formation in respective mice. Independently from 454 sequencing, primers might be designed binding to viral LTR and flanking host genome region. These integration site-specific “Tracking PCRs” will elucidate whether cell clones which were only rarely detected were indeed in an inactive state in remaining mice.

6 Conclusion and Perspective

It is a widely-accepted concept that colon cancer is hierarchically organized with long-term tumor initiating cells at the top being able to self-renew and to maintain the disease. It has been hypothesized that mitotic quiescence and resulting chemoresistance are functional characteristics of human colon TIC being responsible for late disease relapse and metastasis formation. However, experimental evidence has not been provided yet. Within the colon cancer initiating compartment, our lab determined a small subfraction of delayed contributing TIC (DC-TIC) which contributed to tumor formation only in secondary or tertiary mice in a serial transplantation assay[135]. This was the first experimental result indicating the existence of a quiescent population of human colon TIC *in vivo*.

Here we provide a marker-independent characterization of the proliferative activity and dynamics of human colon TIC *in vitro* and *in vivo*. We have shown that a quiescent population of human colon TIC indeed exists, both *in vitro* and *in vivo*, but that the majority of colon TIC is actively-proliferating. Quiescent and actively-cycling colon TIC equally contributed to tumor formation in immunocompromised mice. It is likely that cells selectively switch between a dormant and an actively-cycling state. Interestingly, only quiescent TIC showed a polyclonal contribution to tumor formation *in vivo*. 5-FU-treatment might activate previously quiescent TIC to drive tumor growth.

We conclude, that quiescent and actively-proliferating colon TIC co-exist in human colorectal cancer. A quiescent TIC subpopulation might be responsible for TIC maintenance. Furthermore, our data implies that the biological properties of colon TIC are similar to those of benign colonic stem cells which have been shown to be mainly actively-cycling. Our *in vivo*-data demonstrated that colon TIC were enriched in the quiescent population. It additionally indicated that quiescent TIC were recruited to tumor formation upon chemotherapeutic treatment. Future studies are essential to understand mechanisms that control quiescence and proliferation of human colon TIC. Further downstream analysis of quiescent colon TIC might unravel new therapeutic targets, in order to eliminate actively-proliferating as well as quiescent TIC. This will hopefully provide basis for the development of innovative treatment strategies for colon cancer, leading to improved therapy response rates and prolonged survival.

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[c] http://de-de.invitrogen.com/site/de/de/home/References/gibco-cell-culture-basics/cell-culture-protocols/freezing-cells.html

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Supplement A: Plasmid sequences

A.1 pMD2.G (5.824bp)

5’-

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A.3 pWPXL-TTT-H2B-GFP (12.127bp)

5’-

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Publications and Conferences

Cell cycle activity of transplantable colon cancer initiating cells

Hartinger, EM; Ball, CR; Dieter, S; Hoffmann, C; Weitz, J; Koch, M; von Kalle, C and Glimm, H

DKFZ PhD Retreat, July 2011, Weil der Stadt

Proliferative Activity of Human Colon TIC in Sphere Cultures

Hartinger, EM; Ball, CR; Dieter, SM; Hoffmann, CM; Weitz, J; Koch, M; Schmidt, M; von Kalle, C and Glimm, H

DKFZ PhD Student Poster Presentation, November 2011, Heidelberg, Germany

Proliferative Activity of Human Colon TIC in Sphere Cultures

Hartinger, EM; Ball, CR; Dieter, SM; Hoffmann, CM; Weitz, J; Koch, M; Schmidt, M; von Kalle, C and Glimm, H

AACR 103rd Annual Meeting 2012, April 2012, Chicago, USA

Proliferative Activity of Human Colon TIC in Sphere Cultures

Hartinger E.-M., Ball C., Dieter S., Hoffmann C., Weitz J., Koch M., Schmidt M., von Kalle C., Glimm H.

Jahrestagung der Deutschen, Österreichischen und Schweizerischen Gesellschaften für Hämatologie und Onkologie 2012, October 2012, Stuttgart, Germany

Declaration

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Es wurden nur die in der Arbeit ausdrücklich benannten Quellen und Hilfsmittel benutzt. Wörtlich oder sinngemäß übernommenes Gedankengut habe ich als solches kenntlich gemacht.

Ich erkläre außerdem, dass diese Arbeit weder in dieser noch in einer anderen Form anderweitig als Dissertation oder Prüfungsarbeit verwendet oder einer anderen Fakultät als Dissertation vorgelegt wurde.

Heidelberg, _____ _____

Ort, Datum Unterschrift

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