Targeting cancer stem cells: Modulating apoptosis and stemness
Çolak, S.

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Chapter 9

General discussion and summary
Predicting prognosis and therapy response in CRC patients

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in Europe\(^1\). Treatment decisions are currently largely based on pathological staging of CRC patients\(^2,3\). As discussed in the general introduction (chapter 1) 20% of the stage II patients will inevitably progress upon successful surgery. They will either recur locally or develop distant metastases. However, the benefit of adjuvant chemotherapy on stage II patients is relatively limited and 80% will never develop recurrences making large scale application of adjuvant therapy unethical\(^4,5\). Therefore, patients are selected based on high risk factors and only a subset of the stage II patients are receiving adjuvant chemotherapy\(^3,4\). However, also within the stage II CRC patients that are not classified as high risk, tumor relapse occurs\(^6\). Furthermore, the patients that are classified as high risk and receive therapy do not all respond to therapy\(^6\). Together this implicates that, unfortunately, we are ineffective in predicting which patients will develop a recurrence and also fail at predicting their response to therapy\(^6\). Stratification as well as therapy optimization is therefore of crucial importance to improve therapy in CRC.

In an effort to identify poor-prognosis patients we hypothesized that the enumeration of cancer stem cells (CSCs) in CRC might facilitate selection. CSCs are a minority of cells in a tumor that are defined by their capacity to transplant the human malignancy to immuno-compromised mice and are suggested to fuel tumor growth and cause tumor recurrence and metastasis (chapter 2). There are reports that propose a direct relation between the number of CSCs and patient prognosis in different malignancies including CRC. For instance, Merlos Suarez et al. showed that EphB2 expression marks stem cells in mouse intestine and also in CRC\(^7\). They generated an EphB2 intestinal stem cell (ISC) signature by identifying genes that are highly expressed in EphB2\(^{\text{high}}\) ISCs compared with more differentiated epithelial cells. This signature strongly associated with CRC disease stage and was detected in patients where the tumor recurred and metastasis formed. This has led to the suggestion that an increased number of CSCs is predictive for prognosis.

Previously, we have shown that WNT signaling pathway marks colon-CSCs in primary human CRC\(^8\). Primary isolated spheroid CRC cultures were transduced with a WNT reporter construct and colon-CSCs (cells with high WNT pathway activity) and differentiated progeny (cells with low WNT pathway activity) were sorted to perform gene expression profiling and subsequently to generate a colon-CSC gene expression signature. This colon-CSC signature comprised 187 genes that were most differentially expressed between CSCs and more differentiated cells. Importantly, also this signature was intimately associated with disease recurrence in a set of 90 stage II
CRC patients that underwent intentionally curative surgery at our institute (AMC-AJCCII-90). Therefore, similar to the EphB2 ISC signature, this colon-CSCs gene signature predicts recurrence of CRC (chapter 3). There is a partial overlap between these stem cells signatures, as both signatures were characterized by enrichment in WNT target genes, consistent with the major role of WNT pathway in ISC and colon-CSC biology.

However, to our surprise expression of WNT target genes inversely correlates with prognosis. Correlating CSCs associated WNT target genes with actual CSC numbers revealed no correlation within our AMC-AJCCII-90 tumors, indicating that adherence to the CSC signature does not reflect the number of CSC in CRC. Instead we found that the inverse correlation with the signature pointed to two subgroups in CRC of which the poor prognosis group has low expression of WNT targets due to CpG island methylation of several WNT target genes.

In line with our observations, several WNT target genes have been reported to be methylated in CRC. *DKK1* is one such target gene that binds and inhibits Lrp5/6 receptors required for activation of WNT signaling pathway. Similar to *DKK1*, also *AXIN2* is described to be methylated on CpG islands in a subset of CRC patients. The promoter region of the gene coding for the secreted frizzled related protein 1 (*sFRP1*), that binds to and inhibits WNT ligands is also found to be methylated. Intriguingly, all these target genes are in fact feedback inhibitors explaining why their inactivation would be observed in CRC as they would activate the WNT signaling pathway. In agreement, mutations in either *APC* or β-catenin are found in the majority of CRC patients leading to high WNT pathway activity. However, we observe a relatively high fraction of WNT target methylated cases that do not contain mutations in the *APC* gene, suggesting that these tumors have employed feedback inhibitor inactivation to modulate the WNT pathway activity (unpublished observations). Importantly, treatment of CRC cells *in vitro* with 5-Aza resulted in re-expression of these feedback regulators and inhibited WNT pathway activity (chapter 3). This suggests that epigenetic inactivation of WNT feedback inhibitors is a mechanism to regulate WNT signaling and possible induce positive regulation of growth promoting WNT target genes. This can explain why patients that have methylated WNT feedback inhibitors have poor prognosis.

Currently, we are studying the selectively re-expression of these methylated WNT target genes in CRC cells that display methylation and study the effects on tumor biology.

In line with an important role for CpG methylation, we observed that treatment of colon-CSCs derived xenografts with a demethylating agent results in suppression of
tumor growth (chapter 3). Previously, it was shown that APC\textsuperscript{min} mice also develop fewer and smaller polyps when treated with a demethylating agent\cite{11}. Together this implies that demethylating agents might provide an exciting therapeutic strategy that deserves further exploration.

In a phase I/II clinical trial metastatic CRC patients with wild-type \textit{KRAS} were treated with a demethylating agent in combination with an antibody targeting \textit{EGFR}\cite{15}. Out of the 20 patients, 2 had a partial response and 10 patients had stable disease. The conclusion of this trial was that demethylating agents are well tolerated and showed clinical response in metastatic CRC patients. Presently, we are conducting a proof of concept clinical trial where CRC patients are pre-operatively treated with the demethylating agent decitabine. By conducting this trial we aim to determine whether decitabine treatment results in demethylation of WNT target genes, re-expression of WNT target genes and if methylation of a set of WNT target genes can be used as a biomarker to predict if a CRC patient will respond to decitabine. For this study tumor specimens will be obtained by endoscopy prior to treatment and compared to tumor resection specimens post decitabine treatment and methylation will be determined.

Besides determining methylation status of a set of WNT target genes there are other possibilities to classify patients in clinically relevant subgroups. One popular approach is gene expression profiling or proteome studies that can be used to identifying signatures associated with prognosis or biological traits. Many such signatures have been used to predict prognosis in CRC\cite{16-46}.

Development of prognostic signatures is normally based on training and validation sets in which the first is used to identify differentially expressed genes between patients that relapse and patients that do not show relapse. Independent validation is subsequently needed to determine the quality of a predictive signature. Other studies rather use gene expression differences of normal mucosa compared to carcinoma. Also this approach has been shown to generate signatures that can classify poor and good prognosis patients.

Based on such signatures several prognostic tests, such as Oncotype DX colon cancer, CoIDX, ColoPrint, ColoGuide Ex and ColoGuide-Pro have been developed and entered the diagnostic market with the aim to identify patients that are at increased risk for recurrence development\cite{47}. Of these the most frequently used are Oncotype DX colon cancer and ColoPrint. Oncotype DX colon cancer is a quantitative reverse transcriptase-PCR (qRT-PCR) based assay that measures the expression level of a subset of 12 genes. Seven of these genes associated with recurrence and 5 genes are reference for standardization. This signature is validated in 1436 stage II CRC
patients from the QUASAR study\textsuperscript{40} and more recently in the NSABP C-07 study\textsuperscript{49}. Importantly, the parallel development of a predictive diagnostic test that could predict response to 5FU-based chemotherapy failed at the validation stage.

Similar to Oncotype DX colon cancer, the 18-gene signature ColoPrint can also identify stage II CRC patients at high risk for recurrence\textsuperscript{41}. Currently, a large phase II clinical trial to validate ColoPrint in stage II CRC (PARSC study, NCT00903565) is under way. This PARSC study is comparing risk assessment using the ColoPrint profile versus a clinical risk assessment based on investigator’s judgment and American Society of Clinical Oncology high-risk recommendations.

Despite the original attempts currently available tests cannot predict whether a patient may benefit from adjuvant chemotherapy. Our laboratory performed research to identify a gene signature that can potentially classify patients relevant for prognosis prediction and therapy response prediction\textsuperscript{50}. We used a set of stage II patients operated at our institute (AMC-AJCCII-90 patients (chapter 3)) and by unsupervised consensus-based clustering, we identified 3 colon cancer subtypes (CCS1,2,3). In contrast to the clinical test like ColoPrint our approach have identified distinct biological subgroups. Interestingly, gene expression profiles of the CCS3 subtype are highly related to those observed in serrated adenomas and, therefore, this is suggested to develop from the serrated pathway. Patient that we classified as CCS3 express high levels of TGF\textbeta target genes, appear mesenchymal and importantly have a poor prognosis. Moreover, CCS3 are shown to be resistant to the EGFR targeting antibody cetuximab\textsuperscript{50}. Many research groups have used similar approaches for CRC patient stratification and identified 3 to 6 subtypes that reflects biological differences in CRC\textsuperscript{51-56}. Many of these stratifications could be in the future translated to clinical use after further validation. However, multiple proposals of classifiers is hampering clinical utility of gene expression based subtyping. To resolve this issue of inconsistencies in subtyping of CRC we teamed up in an international consortium. Six expert groups applied their subtyping classification algorithm to a total of 18 CRC data sets leading to 6 different subtype labels per sample\textsuperscript{57}. Using a network-based approach, four consensus molecular subtypes (CMS1-4) were identified. 15% of the CRC patients are classified in CMS1. These turn out to be more likely right-sided, poorly differentiated, mucinous tumors with a bias to older female patients. Most CMS1 tumors are microsatellite instable and have immune infiltration and activation. Almost half (41\%) of the CRC patients are CMS2, which are predominantly left-sided tumors. These tumors are characterized by high WNT and Myc target gene expression and are characterized as epithelial tumors that may reflect the classical Vogelgram-like cancers\textsuperscript{57, 58}. The third subgroup, CMS3
Discussion

is relatively small and contains a relatively high percentage (75%) of mutant RAS. Often these RAS mutations are combined with PI3KCA mutations. In contrast to CMS2, WNT pathway activity is not high in CMS3 patients, but a metabolic activation profile is evident, suggesting that these tumors are distinct from the classical Vogelgram pathway. Finally CMS4 cancers have a dismal prognosis and show a mesenchymal gene expression profile with expression of EMT genes and high TGFβ signaling activation. Similar to CCS3, the CMS4 patients respond relatively limited to EGFR targeting therapy50, 57. Although the mesenchymal subtype is identified in all subtyping studies its existence as a tumor-specific trait was challenged as it was suggested to emanate from a larger fraction of stromal cells present in these tumors. In agreement, the laboratory of Medico further explored this mesenchymal subtype59 and showed that patient-derived xenografts, which contain mouse stromal cells surrounding and supporting human cancer cells, lost the typical mesenchymal appearance, suggesting that this feature is derived from the high expression of cancer associated fibroblast (CAF). Calon et al. further substantiated this idea by isolation of the various cell types present in CRC samples and showed that CAFs express high levels of genes that are dictating the mesenchymal subtype60. If fibroblast genes were taken out of the gene signature, the patients could no longer be classified as mesenchymal. The authors therefore proposed that TGFβ activates stroma and this activated stroma including fibroblasts promote tumor initiation and metastasis. Both studies highlighted the importance of the stromal contribution to the molecular signature in comparison with the cancer epithelium itself and conclude that mesenchymal subtype is a reflection of the amount and activation of the stroma rather than a reflection of an EMT program in the tumor cells 59, 60.

As discussed above in several CRC patients stratification studies the TGFβ pathway is shown to be highly active in poor prognosis patients. This pathway is activated when TGFβ ligands (like TGFβ 1, 2 or 3) bind to TGFβ type 2 receptor (TGFβR2) that subsequently dimerizes and phosphorylates TGFβ type I receptor (TGFβR1). Activated TGFβR1 can phosphorylate SMAD2 and SMAD3, which then bind to SMAD4 and this complex translocates to the nucleus and act as a transcription factor to stimulate transcription of TGFβ target genes61. Inactivating mutations in TGFβR2 and in SMAD4 are found in many CRC patients62-64. However, even in the presence of specific mutations in TGFβR2, this receptor can still be responsive to TGFβ ligands65. Furthermore, when cells lack functional SMAD4, TGFβ can activate so called non-canonical TGFβ signaling that involves activation of many signaling pathways including Pi3K, p38, and NFκβ signaling66, 67.
TGFβ is an EMT inducer and is implicated in therapy resistance in many cancers. In a shRNA screen to identify genes that can cause resistance to BRAF inhibitor in BRAFV600E melanoma, SOX10 was identified. Downregulation of SOX10 was associated with increased TGFβ pathway activation and resistance to BRAF inhibitor. MED12 was also picked up in a shRNA screen. Loss of this protein was associated with resistance to receptor tyrosine kinase (RTK) inhibitors and chemotherapy. MED12 is a negative regulator of TGFβR2, meaning that suppression of MED12 is followed by TGFβ pathway activation. Inhibition of TGFβ with the inhibitor LY2157299 is sufficient to restore sensitivity to RTK inhibitors in MED12 knockdown cells. Combined this suggests that TGFβ can mediate resistance to therapy. This concept was substantiated by Arteaga and colleagues who analyzed RNA expression of matched pairs of primary breast cancer biopsies prior and post chemotherapy. This analysis showed increased RNA expression of CSCs and TGFβ signaling. Treatments of triple negative breast cancer xenografts with LY2157299 blocked CSC expansion and sensitized tumors to paclitaxel treatment showing the importance of TGFβ signaling pathway in therapy resistance.

In conclusion, recent studies have identified that TGFβ signalling is highly active in mesenchymal CRC subtypes. Further study is required to understand whether targeting TGFβ signalling is beneficial in these poor prognosis CRC patients. Moreover, in chapter 3 we discussed that methylation of a set of WNT target genes predict prognosis and targeting methylation by agents inducing demethylation is clinically relevant. Further studies will reveal if patients with high WNT target gene methylation also show high TGFβ signaling. A link between methylation and the mesenchymal subgroup was substantiated by the observation that mir200 family CpG methylation is pivotal in CMS4 mesenchymal CRC subtype. As TGFβ signaling cross talk with mir200 target genes is well known, it is worth pursuing the link with methylation further.

**Apoptotic threshold in colon-CSCs**

In this thesis, we describe our effort to predict prognosis of CRC patients using CSC signatures (chapter 3). In addition, we were also highly interested in studying the role of colon-CSCs in CRC treatment response (chapter 4-8).

Previously, to study chemotherapy response of colon-CSCs and differentiated cells, colon CSCs were forced to undergo differentiation in vitro by growing spheroid cultures adherently in medium containing Foetal Calf Serum (FCS). Chemotherapy induced cell death was compared between these sphere-derived adherent
cultures (SDAC) and CSCs enriched cultures, i.e. spheroid cultures grown in suspension in medium supplemented with growth factors. This approach therefore compares cells maintained under different culture conditions and therefore observed differences may not necessarily relate to the intrinsic resistance of CSCs.

In chapter 5 we introduced a FACS-based assay that allows treatment and cell death measurement of colon-CSCs and their differentiated progeny under the exact same conditions. We used WNT pathway activity to segregate colon-CSCs and differentiated cells and measured caspase 3 activity at the single cell level after chemotherapy treatment. In contrast to differentiated cells, little caspase 3 activity was measured in colon-CSCs after chemotherapy treatment.

A recent study describes that this low efficacy of chemotherapy could even be detrimental as it may drive the induction of genomic instability. As we see only little caspase activity in our colon-CSCs when we expose them to chemotherapeutic insults it is possible that these cells are not undergoing apoptosis but are becoming more aggressive because of the low caspase activity-induced genomic instability. Therefore it is interesting to study if colon-CSCs that survive chemotherapy treatment are more tumorigenic and metastatic.

To undergo apoptosis a cell needs to surpass a so called apoptotic threshold. For example, chemotherapy can induce BH3 proteins that affect this threshold and induces apoptosis. When a cell is primed to undergo apoptosis it is considered to be close to this threshold and requires less chemotherapy to die. Using the newly developed assay we showed that colon-CSCs have a higher apoptotic threshold when compared to differentiated progeny and therefore colon-CSCs resist chemotherapeutic insults (chapter 5).

The underlying mechanism of the difference in apoptotic threshold between colon-CSCs and differentiated cells is still not clear. High apoptotic threshold in colon-CSCs can be caused by high expression of anti-apoptotic molecules (e.g. BCLXL) or decreased expression of pro-apoptotic molecules (e.g. BAX). However, in gene expression profiling studies and Multiplex Ligation-dependent Probe Amplification assays on colon-CSCs and their differentiated progeny, only little difference in expression of pro- and anti-apoptotic genes were observed, suggesting that expression differences are probably not underlying the higher apoptotic threshold in colon-CSCs.

We cannot rule out that proteins levels of apoptotic proteins, which are strongly regulated by post-translational modifications, can be differentially expressed between colon-CSCs and their differentiated progeny leading to a differential apoptotic threshold. Other studies have shown differences in the expression of apoptotic molecules between colon-CSCs and differentiated cells (e.g. BIRC6).
However, as mentioned before we defined CSCs based on WNT pathway activity and the other studies compared colon-CSCs with SDACs\textsuperscript{73}. Apoptotic proteins can be regulated by phosphorylation\textsuperscript{79, 80}. To illustrate, the pro-apoptotic BAD protein can be phosphorylated by AKT leading to its inactivation\textsuperscript{81}. Phosphorylation induced inhibition of BAD can result in BCLXL activation. In contrast to BAD, phosphorylation of BID can result in activation of this pro-apoptotic protein\textsuperscript{82}. During the cell cycle BID gets phosphorylated as a cell enters mitosis and BID phosphorylation is lost during metaphase to anaphase transition. This phosphorylation induced activation of BID makes a cell more dependent on anti-apoptotic proteins during mitosis. This suggests that a potential difference in cell cycle between colon-CSC and differentiated cells can result in differential BID activation and thereby differential apoptotic threshold between colon-CSCs and differentiated cells. However, when we performed cell cycle analysis, we did not see a difference in cell cycle profiles of colon-CSCs and differentiated cells making this cell cycle dependent BID phosphorylation less likely to explain the difference in apoptotic threshold between colon-CSCs and differentiated cells.

Exposure of cells to chemotherapy induces expression of pro-apoptotic molecules\textsuperscript{83}. Chemotherapy induces DNA damage and DNA double strands breaks. DNA double strand breaks are detected by ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3 related) proteins, which signal downstream to CHK1, CHK2 and p53\textsuperscript{84}. Activation of p53 can result in cell death. The p53 targets considered most important in this respect are the pro-apoptotic BAX and the BH3-only proteins NOXA and PUMA\textsuperscript{85-87}. Chemotherapy induced DNA damage can also lead to activation of CHK2 that phosphorylates and activates E2F1 transcription factor. One of E2F1 target genes is p73. Upregulation of p73 in turns induces transcription of BAX, PUMA and NOXA. Combined these data indicate that chemotherapy decreases the apoptotic threshold by increasing expression of pro-apoptotic molecules\textsuperscript{88}. It would be interesting to determine how CSCs and differentiated cells respond to chemotherapy in context of apoptotic proteins and whether this may explain their differential response to therapy.

Although it is unclear what determines the difference in sensitivity our data indicates that the resistance is dictated by BCLXL. Compounds that inhibit anti-apoptotic BCL2 family members can decrease the apoptotic threshold. ABT-737 and the highly related ABT-263 are small molecule inhibitors, so called BH3 mimetics, which both inhibit BCL2, BCLXL and BCLW\textsuperscript{89}. More selective inhibitors have also been developed including ABT-199 and WEHI-539\textsuperscript{90, 91}. ABT-199 is a compound that inhibits BCL2
only, while WEHI-539 is specifically inhibiting BCLXL. Our data reveal that inhibition of anti-apoptotic molecules with either ABT-737 or WEHI-539 by-passes chemotherapy resistance showing that colon-CSCs rely on BCLXL for their chemotherapy resistance (chapter 5).

Our data on BCLXL dependent CSC resistance (chapter 5) was recently confirmed in a separate study on lung-CSCs92. However, a study performed by Prehn and colleagues revealed an important role for BCL293. In this study the authors extracted proteins from 26 CRC patients and by Western blotting quantified expression of pro- and anti-apoptotic molecules. Experimental findings were combined with systems modelling to develop a tool called DR_MOMP (dose response medicinal outcome model predictor). The authors concluded that DR_MOMP is able to predict chemotherapy response in CRC patients and this was dependent on BCL2 expression93.

Stromal cells, like immune cells express BCL2 and it is possible that BCL2 expression in patients is not derived from cancer cells but stromal cells, which potentially explains the difference between our and Prehn laboratory findings94. Previously it was published that BCL2 expression is restricted to stem cells in normal healthy colon95. In a recent study, we showed that crossing APC\textsuperscript{min} mice with BCL2-deficient mice results in decreased polyp formation, suggesting that BCL2 is important for CRC initiation (Van der Heijden et al. Nature Communications 2016). Furthermore, expression of BCL2 and BCLXL in adenomatous polyps and primary colorectal adenocarcinomas was studied by John Reed and his colleagues96. They demonstrated that expression of BCL2 is high in adenomas and low in carcinomas. However, in contrast to BCL2, BCLXL expression is high in CRC96. This is in line with our findings that colon-CSCs derived from primary CRC are dependent on BCLXL for chemotherapy resistance.

In summary, colon-CSCs have a high apoptotic threshold, which makes these cells resistant to chemotherapy. Inhibition of BCLXL decreases the apoptotic threshold and thereby sensitizes colon-CSCs towards chemotherapy. Therefore BCLXL is an interesting candidate to target in CRC and potentially in other solid tumors.

However, inhibition of BCLXL can lead to thrombocytopenia, which relates to a dependence of platelets on BCLXL for survival97-99. Further studies need to be performed to unravel if it may be feasible to spare thrombocytes while effectively targeting CRC.

**Differentiation therapy decreases apoptotic threshold**

Next to directly targeting the threshold can also be decreased in colon-CSCs by forcing them to differentiate. In chapter 7 and 8 we discussed two potential means to differen...
entiate colon-CSCs. First in chapter 7, HDAC inhibitors were used to differentiate colon-CSCs and thereby sensitized them to chemotherapy. Interestingly, sensitization induced by HDAC inhibition can be blocked by ectopic overexpression of BCLXL. In addition, HDAC inhibition sensitized colon-CSCs to ABT-737. This suggests that HDAC inhibitors lower the apoptotic threshold by differentiating colon-CSCs. However, we cannot rule out that HDAC inhibitors, in addition to kick starting the differentiation program, also simultaneously regulate apoptotic protein expression and activity. In agreement with this idea is the observation that HDAC inhibition was previously shown to induce expression of pro-apoptotic molecules BMF and BIM and decrease expression of anti-apoptotic protein BCLXL. Gene expression profiling on colon-CSCs treated with HDAC inhibitors confirmed the regulation of many pro- and anti-apoptotic proteins, including upregulation of BMF and BAX and downregulation of Aven and BCLXL. Aven is a poorly studied apoptotic regulator that was identified in a screen for BCLXL binding proteins. Aven was reported to stabilize BCLXL and decreasing Aven expression can result in decreased BCLXL protein levels, thereby facilitating apoptosis. This indicates that HDAC inhibitors can lower BCLXL protein levels and thereby increase sensitivity of CSCs toward chemotherapy.

Acetylation is a posttranslational modification that affects histone proteins and non-histone proteins. In tumors where p53 is not mutated acetylation of p53 protein stabilizes the protein and enhance transcriptional activity. Some of the pro-apoptotic p53 target genes are BAX, PUMA and NOXA. β-catenin can also be acetylated, which increases its binding to TCF4 and enhances WNT signaling. There are various transcription factors that can be acetylated including Forkhead box O (FOXO) transcription factors. Acetylation of FOXO proteins has different functions including triggering apoptosis by inducing expression of pro-apoptotic molecule BIM.

As discussed in chapter 7, HDAC inhibitor induced differentiation requires FOXO transcription factors. It is therefore possible that HDAC inhibitors activate transcription or induces acetylation of FOXO proteins and these proteins increase expression of pro-apoptotic proteins or decrease expression of anti-apoptotic proteins. In chapter 8 we show that activation of UPR pathway by inducing ER-stress also differentiates colon-CSCs. Similar to HDAC inhibitor treatments, ER-stress induction can lower the apoptotic threshold by differentiating colon-CSCs. However, ER-stress can simultaneously to differentiation induction also regulate apoptotic protein expression and activity. In agreement with this idea is the observation that CHOP, which is one of the
main downstream activators upon UPR activation, induce expression of pro-apoptotic molecules like BIM\textsuperscript{114}.

Furthermore, one of the features of ER-stress induced UPR is inhibition of translation\textsuperscript{115, 116}. Some apoptotic molecules have a short half-life, including for instance MCL\textsuperscript{117}, allowing apoptosis to be induced. However, it is unlikely that translation inhibition is the mechanism of ER-stress induced sensitization because translation as well transcription inhibitors failed to sensitize colon-CSCs (chapter 7), suggesting that ER-stress sensitizes colon-CSC in a different fashion.

Both differentiation inducing agents, HDAC inhibitors and ER-stress inducing compounds, can decrease the apoptotic threshold by directly regulating pro- and anti-apoptotic proteins or by differentiating colon-CSCs. We think that inducing differentiation plays an important role in sensitization of colon-CSCs. First, chemotherapy sensitization by HDAC inhibitors and ER-stress induction is more specifically in colon-CSCs and much less in differentiated cells. If HDAC inhibitors and ER-stress inducing agents solely regulate apoptotic molecules, we would not expect this colon-CSCs specific sensitization of these compounds.

Second, caspase-3 activity measured in colon-CSCs treated with HDAC inhibitor followed by ABT-737 treatment is similar to the ABT-737 only treatment of differentiated cells. This suggests as well that HDAC inhibition sensitizes colon-CSCs and thereby sensitizes cells to different agents to the same extent as differentiated cells. Although we cannot rule out a possible role of direct apoptotic protein regulation by HDAC inhibitors and ER-stress inducing agents, we think that differentiation induction is playing a crucial role in sensitization of colon-CSCs towards chemotherapy.

**Therapeutic window for differentiation inducing therapies**

Current adjuvant therapies in cancer including CRC fail in part due to selective resistance of CSCs (reviewed in chapter 2). We show that an effective means to circumvent this resistance can be achieved by pushing CSCs to undergo differentiation. Both inhibition of HDACs and activation of UPR will induce differentiation of colon-CSCs (chapter 7 and chapter 8). Although this would argue for the use of such combination therapies in cancer patients, one would have to consider the effects of these treatments on normal stem cells first as both have previously been shown to regulate intestinal homeostasis in mice. In our laboratory we performed knock-out studies and demonstrated that HDAC1 and HDAC2 are required for stem cell maintenance in mouse intestine\textsuperscript{118}. Combined removal of HDAC1 and HDAC2 or treatment with HDAC inhibitor leads to loss of stem cells in mice intestine\textsuperscript{118}. 
Next to a role for HDAC1 and HDAC2 in ISC differentiation, we have shown that ER-stress inducing agents force normal stem cells differentiation as well. Normal healthy ISCs in mouse have low ER-stress response compared to their progeny, the transit amplifying cells and differentiated cells. More importantly, as is the case for colon CSCs, induction of ER-stress forces ISCs to differentiate. This ER-stress induced differentiation is also observed in other tissues including the hematopoietic system and is suggested to serve as a mechanism that protects the integrity of the stem cell compartment, driving differentiation under unwanted stress conditions. In conclusion, inhibition of HDACs and enhancing ER-stress differentiate healthy stem cells and colon-CSCs.

Increasing evidence suggests that normal stem cells and CSCs use analogues morphogenic pathways to regulate self-renewal and differentiation. Likewise to HDAC inhibition and ER-stress induction, targeting WNT, Notch, and BMP pathways can also lead to loss of stemness. To illustrate, WNT signalling is crucial for maintenance of ISC and colon-CSCs. High pathway activity is observed in normal colon stem cells and in colon-CSCs. Next to WNT pathway, Notch signaling is shown to be essential for stem cell maintenance and inhibition of this pathway results in loss of ISC as well as colon-CSCs. In contrast to WNT and Notch signaling, not inhibition but activation of BMP signaling pathway results in loss of normal healthy stem cells and colon-CSCs.

Interestingly, regulation of stemness by these morphogenic pathways can be used to target CSCs. WNT pathway can for example be inhibited with salinomycin, which results in differentiation of breast-CSCs. Notch signalling pathway can be inhibited with a -secretase inhibitor DBZ and in APC mice this inhibition leads to conversion of proliferative stem cells into non-proliferative goblet cells. In addition, human CRC xenografts treated with anti-DLL4 displayed enhanced differentiation and sensitization of colon-CSCs towards chemotherapy. Finally, differentiation and sensitization of colon-CSCs can be achieved by activating BMP signalling. Together these data indicate that differentiation of colon-CSC can be induced by different means utilizing their normal homeostatic regulation or forcing differentiation pathways. Because differentiated colon-CSCs lose their tumorigenic capacity and therapy resistance, such therapies can be an interesting tool to treat CRC patients. However, differentiation inducing agents will have to regard unwanted side effects that are in fact on-target as they affect the normal stem cell compartment as well as the CSCs. The efficacy of such therapies will thus dependent on the existence of a therapeutic window.
Interestingly, single knock out of only HDAC1 or HDAC2 is not sufficient to induce mouse ISC differentiation\(^\text{118}\). This appears to be distinct in tumors where often inactivation of a single HDAC is sufficient to obtain anti-tumor activity\(^\text{130}\). This suggests that specific targeting of HDACs can have low toxicity and may still show clinical benefit. In colon-CSCs MS275 treatment differentiates these cells, suggesting that HDAC1, HDAC2, and / or HDAC3 are important for stemness in these cells (chapter 7). Genome editing technologies like CRISPR/Cas9 can be used to generate knock-outs of a single or multiple of these HDACs. These experiments will tell us which HDAC is required for colon-CSCs and if it sufficient to specifically target HDACs.

Alternatively, the colon CSCs may rely more heavily on the activity of HDACs and as such lowering the activity to 50% may be sufficient to drive differentiation. However, in mice when HDAC2 is partially deleted in combination with complete HDAC1 deletion, proliferation was increased, suggesting that complete deletion of HDAC1 and HDAC2 is required in mouse intestinal stem cells to differentiate these cells\(^\text{118}\). The increased dependency of tumors cells on HDACs may determine the width of this therapeutic window. In colorectal lesions, a therapeutic window may be present as HDAC1 and HDAC2 have been found to be up-regulated in CRC cells in patients and HDAC2 is increased expressed in APC\(^\text{min}\) mouse\(^\text{130, 131}\). When APC\(^\text{min}\) mice were crossed with HDAC2 deleted mice this resulted in decreased amount of polyp formations, suggesting an important role for HDAC2 in CRC\(^\text{132}\). As mentioned above, ER-stress differentiates normal ISCs as well as colon-CSCs\(^\text{119}\) (Chapter 8). For both, it is shown that there is regeneration. In normal healthy intestinal cells, Lgr5 has been identified as a stem cell marker\(^\text{133}\). However, depletion of Lgr5 expressing cells in the intestinal epithelium does not disturb homeostasis as would be expected after killing a stem cell population\(^\text{134}\). This suggests that there are stem cells in the intestine that do not express LGR5. Such back-up stem cells may be encoded by the BMI1 expressing cells which appear to be able to regenerate the LGR5\(^+\) population\(^\text{134, 135}\). Alternatively non-cycling cells, also called label retaining cells, were also shown identified in the mouse intestine that express LGR5 but also Paneth and enteroendocrine cell markers\(^\text{136}\). Similar to BMI expressing cells, there are many other stem cell populations identified in mouse intestine that are able to repopulate damaged crypts\(^\text{135, 136, 137, 138, 139, 140}\).

Such stem cell plasticity as well the existence of various pools of stem cells in normal healthy tissue can potentially make a tissue resist differentiation induced therapies without causing toxicity for the patients. It is suggested that also in CRC there can be different stem cell populations. To illustrate, Dieter and colleagues lenti-virally
marked tumor cell populations in human CRC samples and revealed that there are distinct types of CSCs existing in CRC. Serial transplantation experiments in mice showed that some clones only appeared in primary recipients while so called delayed contributing CSCs are only found in the secondary and tertiary recipients. This suggests the existence of quiescent CSCs in CRC. Similarly, Kreso et al marked 150 cells from 10 CRC samples and also performed serial transplantation experiments. 34 of the 150 marked clones were below detection limit (approximately 10^4 cells/tumor) in the first recipient but could be identified in the later transplants. This suggest that these clones were quiescent or slow-proliferating but became activated in later transplants. In addition, chemotherapy treatment of xenografts enriched in quiescent clones indicating that these quiescent cells can survive chemotherapy and reinitiate tumor growth. Hence, likewise to the normal tissue, also in CRC there are quiescent stem cells that can get activated. How the existence of quiescent CSCs is related to therapeutic window needs to be determined. A difference in regeneration between normal and tumor cells will generate a therapeutic window. Based on the finding that HDAC inhibitors and ER-stress induction differentiate colon-CSCs and normal healthy stem cells, would argue that it would give toxicity. However, in our xenograft studies we did not see any toxicity. One possible explanation for this is that the concentration we used was not sufficient to completely block HDACs or induce sufficient ER-stress and therefore to differentiate normal stem cells. Tumors did not disappear in our xenograft studies supporting the idea that a higher dose may be required to fully eradicate the tumor. On the other hand, as described above there are various (reserve) stem cells in the normal healthy intestine. It is possible that we do not differentiate all stem cells and therefore in a case of damage reserve or quiescent stem cells can repopulate normal tissue. To test these possible explanations we need to treat mice with different doses of HDAC inhibitors and ER-stress inducing compounds and study at different time points normal stem cells numbers and intestinal histology. In conclusion, differentiation therapy sensitizes tumor cells to chemotherapy in vitro and in vivo, without a clear sign of toxicity. We therefore believe that HDAC inhibitor treatment and/or induction of ER-stress can be clinically relevant for the treatment of patients with CRC.

**Concluding remarks**

Despite the fact that knowledge about cancer is continuously increasing a lot of research is needed still to increase understanding and to enhance cure rates for patients. Administration of chemotherapy is limited by its toxic side-effects, which is especially
relevant for stage II patients where the majority is cured by surgery alone. More importantly, current therapy does not benefit all patients. Therefore patient stratification is important to predict patients prognosis and therapy response. In this thesis, we put forward that methylation status of a small set of genes is prognostic in CRC. Ongoing clinical investigations will show whether demethylating agents will revert CpG island methylation of CRC patients and potentially affect the outcome for these patients.

Moreover, patients that receive chemotherapy can show relapse many years after therapy. Here, we studied therapy resistance in CRCs. We showed that colon-CSCs are more resistant to conventional chemotherapy, suggesting that colon-CSCs survive therapy and reform a tumor in CRC patients many years after initial chemotherapy treatment. Therefore, elimination of colon-CSCs is required to completely cure CRC patients. We describe in this thesis two means to target these therapy resistance colon-CSCs. The first is by targeting the apoptotic machinery, which shows a higher apoptotic threshold in colon-CSCs compared to differentiated progeny. Decreasing the apoptotic threshold with BH3 mimetics like ABT-737 or a BCLXL specific inhibitor WEHI-539 is sufficient to sensitize colon-CSCs towards chemotherapy. The second means is to target colon-CSCs by forcing them to differentiate and thereby lose their stemness associated chemotherapy resistance. HDAC inhibitors and agents that induce ER-stress can both induce differentiation and sensitize colon-CSCs to chemotherapy. Currently approved HDAC inhibitors including panobinostat were extensively used in our studies in chapter 7. Ongoing clinical studies will show if patients with CRC will benefit from HDAC inhibitor treatment in combination with chemotherapy and if differentiation inducing therapy will help us to cure more CRC patients.
References


Discussion


Summary

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers worldwide and the second leading cause of cancer mortality in Europe. Stage of disease at initial presentation determines treatment strategy. Different types of treatment are available for patients with CRC. To illustrate, treatment for stage I CRC patients is resection only. CRC stage III patients receive FOLFOX or CAPOX chemotherapy regimens after surgery. In FOLFOX and CAPOX regimens oxaliplatin treatment is combined with either 5 Fluorouracil (5FU) and leucovorin or capecitabine, respectively.

For stage II the situation is more complex. The benefit of chemotherapy is limited for stage II CRC patients and therefore only high risk stage II CRC patients are considered for receiving adjuvant chemotherapy treatment, in which high risk is defined by clinicopathological characteristics, such as a poorly differentiated tumor, presence of bowel obstruction or perforation, less than 12 lymph nodes evaluated (in the Netherlands less than 10), tumor penetration into visceral peritoneum or vascular, lymphatic or perineural invasion of tumor cells. This stratification is far from perfect though as some patients diagnosed with stage I or low risk stage II can progress to more advanced stage of disease. In addition, adjuvant chemotherapy is only curative in part of the patients. It is therefore crucial to better identify patients at risk of recurrence and to optimize therapy for these patients before the disease progresses and will be lethal. This heterogeneity in tumor progression can be explained by inter-tumor heterogeneity (difference between patients) and possibly by intra-tumor heterogeneity (difference within a tumor). Better insight into inter-tumor heterogeneity may allow us to predict outcome of CRC patients and identify patients that have a poor prognosis. Inter-tumor heterogeneity may also provide clues to the benefit from chemotherapy and insight in the potential mechanisms of chemotherapy failure. Hence, better stratification of patients will allow for selection of patients that need treatment and potentially the assignment of more effective treatments.

Next to patient-to-patient variation it is vital to understand the intra-tumor heterogeneity as it will allow us to understand why tumors recur and potentially how we can prevent this tumor regrowth. Intra-tumor heterogeneity is in part caused by the existence of a small population of so called cancer stem cells (CSCs) that drive tumor growth and resist therapy in contrast to their more differentiated progeny that lack these properties. Therefore, it is suggested that therapies can kill differentiated tumor cells, but spare CSCs. Consequently, upon cessation of therapy, CSCs can re-initiate proliferation and lead to tumor regrowth. This theory provides an explanation for recurrence of a tumor many years after initial treatment. The current view therefore
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proposes that successful therapy requires effective targeting of CSCs as well as the more differentiated progeny. Unravelling why CSCs are resistant to therapy, can assist in finding new therapies that target these CSCs.

Consequently, the aim of this thesis was to study inter- and intra- tumor heterogeneity in CRC and describe the findings related to potentially new combination therapies that can improve chemotherapy efficacy.

In **chapter 1** and **2** we reviewed the current knowledge in the field of CRC development, progression, and therapy. In addition, in **chapter 2**, an overview is given on the role of CSCs in the resistance to tumor therapy and the current efforts to target these therapy resistant cells.

In **chapter 3** we set out to understand why some stage II CRC patients have a poorer prognosis compared to other CRC patients and thus a higher propensity to show tumor regrowth after successful surgery. We performed gene expression profiling of CRC stage II patients with the concept that gene expression profiles related to CSCs would potentially predict the likelihood of recurrence. The generation of a CSC gene signature, which was derived from *in vitro* cultured CRC stem cell cultures, revealed a strong dependence on WNT signalling activity in the CSCs and thus a relation between WNT target gene expression and stemness. However, comparison with the patient gene-expression profiles revealed an unexpected finding that suggested that patients that display an association with this CSC gene signature, i.e. expression of WNT targets, had a good prognosis. Previous data point to a crucial role for WNT signalling pathway in (cancer) stem cells, and has shown it to be the driving force in CRC. Our unexpected correlation therefore suggested that tumors that do not show expression of WNT target genes rather have a poor outcome. Further insight into this contradiction came from the observation that in this subgroup of stage II CRC patients expression of WNT target genes was repressed by CpG island methylation. Methylation is a process by which a methyl group (CH3) is attached to the DNA and this is an effective means to silence gene expression. In agreement, our further work shows that the methylation status of some WNT target genes (*LGR5, DKK1, APCDD1, AXIN2*, and *ASCL2*) predicts prognosis in stage II CRC. Moreover, treatment of cells *in vitro* or mice subcutaneously injected with colon cancer cells with demethylation agents leads to re-expression of these WNT target genes and tumor growth inhibition *in vitro* and *in vivo*. Thus, the data presented in **chapter 3** reveal that promoter methylation of a set of key WNT target genes is a strong predictor for recurrence of CRC.

In cancer, the balance between proliferation and cell death is disturbed. Most cancerous cells resist apoptosis and this appears even more prominent in CSCs. The aim of
Chapter 4 was to study if targeting the apoptotic machinery and thereby upsetting the balance is sufficient to kill colon-CSCs. Cell death-inducing signals, like chemotherapy, increase pro-apoptotic or decrease anti-apoptotic BCL2 signals resulting subsequently in mitochondrial outer membrane permeabilization. This is followed by activation of caspase 9 and other downstream caspases that will ultimately result in cell death. To directly study whether CSCs can be targeted by caspase activation we made use of an exogenously activatable system that allows us to induce caspase 9 activity in colon-CSCs by the simple addition of a small molecule. In vitro and in vivo experiments showed that activation of caspase 9 induces apoptosis very effectively and results in tumor regression in xenografts indicating that apoptotic signalling downstream of mitochondria is functional in colon-CSCs. Chapter 4 therefore shows that targeting the apoptotic machinery in the intrinsically resistant colon-CSCs can be an interesting approach to kill these cells.

The study into the mechanism by which this resistance occur is described in Chapter 5. At the time that we initiated these studies tools to measure apoptosis specifically in colon-CSCs were not available. To satisfy this unmet need, we first developed FACS-based assays that allowed us to measure cell death in colon-CSCs and differentiated tumor cells simultaneously. These assays showed that under the same conditions colon-CSCs are more resistant to various chemotherapies compared to their differentiated progeny. The underlying reason for this resistance appeared to be a result of lowered apoptotic priming, which is determined by pro- and anti-apoptotic signals within a cell, and thus pointed to an elevated apoptotic threshold in colon CSCs. In agreement, treatment of colon-CSCs with various inhibitors of anti-apoptotic BCL2 molecules, revealed that colon-CSCs are sensitive to a BCLXL specific inhibitor. Moreover, BCLXL inhibition was sufficient to sensitize colon-CSCs towards chemotherapy. Surprisingly, BCL2 inhibition had no effect on these cells. This suggests that colon-CSCs utilize the anti-apoptotic molecule BCLXL, but not BCL2, for their selective chemotherapy resistance and treatment with compounds targeting BCLXL can improve chemotherapy response.

In Chapter 6 we aimed to extend this insight on the elevated apoptotic threshold of CSCs and queried the role of the microenvironment in this resistance. In CRC patients, tumor cells are surrounded by stromal myofibroblasts. In this so called microenvironment, stromal cells can communicate with tumor cells by direct cell-cell contact as well as by secretion of different factors that can either repress or promote tumor growth. Myofibroblasts are abundantly present in the CRC microenvironment. In Chapter 6 we confirm our previous findings that these myofibroblasts secrete factors that induce stemness in differentiated tumor cells. More importantly, de-differentiation renders
these cells chemotherapy resistant. Similar to our findings in CSCs, we observe that resistance is dependent on an elevated apoptotic threshold and can be perturbed by using BCLXL inhibitors. This indicates that BCLXL is also determining chemotherapy-resistance in fibroblast-induced de-differentiated tumor cells.

In chapter 7 we set out to circumvent apoptotic resistance of colon-CSCs and screened for compounds that could sensitize these cells towards chemotherapy. Out of all compounds tested treatment with histone deacetylase (HDAC) inhibitors gave the most pronounced sensitization towards chemotherapy. HDAC inhibitors are best known for their effect on histones acetylation, which has a role in DNA compacting. This posttranslational modification of the histone tail reduces the affinity for DNA and increase transcription factor access to DNA. Therefore, acetylated histones are associated with transcriptionally active chromatin. So HDAC inhibition leads to increased acetylation and potentially increased transcription. However, histones are not the only targets of HDACs and it is important to mention that also other proteins, such as transcription factors can be acetylated, which can have a plethora of effects on these proteins. Our ongoing experiments to unravel the mechanism by which HDAC inhibitors sensitize colon-CSCs revealed that these compounds differentiate colon-CSCs. In line with this, HDAC inhibition sensitizes cells towards BCLXL targeting compounds, while ectopic overexpression of BCLXL rescues HDAC inhibitor-induced sensitization. Hence HDAC inhibitor induces differentiation of colon-CSCs and thereby it lowers the apoptotic threshold and sensitizes resistant colon-CSCs towards chemotherapy.

Finally, in chapter 8 we employed a method to force colon-CSCs to differentiate that was previously reported to drive differentiation of normal intestinal stem cells. This method depends on the activation of the unfolded protein response (UPR) pathway, which appears incompatible with stemness. This pathway gets activated when, for example, misfolded proteins accumulate in the endoplasmic reticulum (ER) due to increased protein synthesis or DNA mutations that lead to ER-stress. To induce ER-stress in colon-CSCs, we made use of two known ER stress-inducing compounds, bacterial toxin SubAb and Salubrinal. Using this approach we observed that ER-stress induction strongly induces colon-CSCs to differentiate. More importantly, this differentiation rendered the cells sensitive to chemotherapy both in vitro and in vivo.

In conclusion, this thesis provides novel insights into the role of inter- and intra-tumor heterogeneity in CRC and shows various means to target chemotherapy resistant colon-CSCs. Modulation of apoptotic signalling by using BCL2 family member inhibitors or by forcing colon-CSCs to differentiate are promising approaches for targeting colon-CSCs and improving therapy response in CRC patients.