Molecular and Cellular Cross Talk between Angiogenic, Immune and DNA Mismatch Repair Pathways

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1 SUMMARY

1.1 ENGLISH SUMMARY

VEGF is a main driver of tumor angiogenesis, playing an important role not only in the formation of new blood vessels, but also acts as a factor for cell migration, proliferation, survival and apoptosis. Angiogenesis is a universal function shared by most solid tumors and its inhibition was thought to have the potential to work across a broad patient population. Clinical evidence has shown that inhibiting pathological angiogenesis only works in a subset of patients and the identification of those patients is an important step towards personalized cancer care. The first approved antiangiogenic therapy was bevacizumab (Avastin®), a monoclonal antibody targeting VEGF in solid tumors including CRC, BC, NSCLC, RCC and others.

In addition to endothelial cells, VEGF receptors are present on a number of different cell types including tumor cells, monocytes and macrophages. The work presented in this thesis looked at the *in vitro* cellular changes in tumor cells and leukocytes in response to the inhibition of VEGF signaling with the use of bevacizumab. In the initial experiments, VEGF was induced by hypoxia in tumor cells to evaluate changes in survival, proliferation, migration and changes in gene or protein expression. There was a minimal direct response of VEGF inhibition in tumor cells that could be attributed to bevacizumab treatment, with minor variations in some of the cell lines screened but no uniform or specific response noted.

MMR deficiency often results in microsatellite instability (MSI) in tumors, as opposed to microsatellite stable (MSS) tumors, and accounts for up to 15% of colorectal carcinomas (CRCs). It has been suggested in clinical data that MMR deficient tumors responded better to bevacizumab regimens, therefore further research used isogenic paired CRC tumor cell lines (MMR deficient and proficient). Furthermore, a DNA damaging agent was added to the treatment regimen, the topoisomerase inhibitor SN-38 (the active metabolite of irinotecan). Inhibiting VEGF using bevacizumab significantly inhibited the ability of MMR deficient tumor cells to form anchor dependent colonies, however conversely, bevacizumab treatment before damaging cells with SN-38, showed a significant increase in colony numbers. Moreover, VEGF inhibition by bevacizumab pretreatment also significantly increased the mutation fraction in MMR deficient cells as measured by transiently transfecting a dinucleotide repeat construct, suggesting VEGF signaling may have an intrinsic role in MMR deficient cells. A number of pathways were analyzed in addition to changes in gene

expression profiles resulting in the identification of JNK as a possible VEGF targeted pathway. *JUN* expression was also reduced in these conditions reinforcing this hypothesis, however the intricate molecular mechanisms remain to be elucidated.

In order to remain focused on the clinical application of the findings, it was noted that some cytokines were differentially regulated by bevacizumab between MMR proficient and deficient cells. Treatment regimens employed *in vitro* attempted to mimic the clinical setting by inducing DNA damage, then allowing cells to recover with or without VEGF using bevacizumab treatment. Inflammatory cytokines, *CCL7* and *CCL8*, were found to have higher expression in the MMR deficient cell line with bevacizumab after DNA damage, therefore the cross talk via tumor derived factors to myeloid cells was analyzed. Gene expression changes in monocytes induced by tumor conditioned media showed *CCL18* to be a bevacizumab regulated gene by MMR deficient cells and less so in MMR proficient cells. CCL18 has been described as a prognostic marker in gastric, colorectal and ovarian cancers, however the significance is dependent on tumor type. CCL18 primarily exerts its function on the adaptive immune system to trigger a T_H2 response in T cells, but is also described to increase non-specific phagocytosis. The results of this study did show an increase in the phagocytic activity of macrophages in the presence of bevacizumab that was significantly more apparent in MMR deficient cells. Furthermore, after DNA damage MMR deficient cells treated with bevacizumab released a cytokine mix that induced monocyte migration in a bevacizumab dependent manner, showing a functional response with the combination of MMR deficiency and bevacizumab. In summary, the work in this thesis has shown evidence of immune cell modulation that is specific to MMR deficient tumor cells that may translate into a marker for the administration of bevacizumab in a clinical setting.

1.2 GERMAN SUMMARY

VEGF ist ein zentraler Regulator der Tumor-Angiogenese, und spielt eine wichtige Rolle nicht nur in der Bildung von neuen Blutgefäßen, sondern ist auch für die Migration, Proliferation, das Überleben und Apoptose von Tumorzellen essentiell. Angiogenese ist eine der universellen Funktionen, welche das Wachstum der meisten soliden Tumoren charakterisiert. Eine der klassischen therapeutischen Ideen wurde auf der Basis entwickelt, dass die spezifische Hemmung der Angiogenese das Potenzial hat in einer breiten Patientenpopulation einen klinischen Effekt zu zeigen. Die klinische Erfahrung und Anwendung hat jedoch gezeigt, dass die Hemmung der pathologischen Angiogenese nur in einem Teil der Patienten einen therapeutischen Nutzen aufweist. Somit stellt die Identifikation derjenigen Patienten, welche von der anti-angiogenen Therapie profitieren, einen wichtiger Schritt zur personalisierten Krebsbehandlung dar. Die erste zugelassene antiangiogene Therapie war Bevacizumab (Avastin®), ein monoklonaler Antikörper gegen VEGF, welcher unter anderem in soliden Tumoren wie CRC, BC, nicht-kleinzelligem Lungenkrebs (NSCLC) und dem Nierenzellkarzinom angewandt wird.

VEGF-Rezeptoren befinden sich nicht nur auf Endothelzellen, sondern sind auch auf einer Anzahl von verschiedenen Zelltypen, einschließlich Tumorzellen, Monozyten und Makrophagen nachweisbar. Die in dieser Arbeit vorgestellten Ergebnisse befassen sich mit den zellulären Veränderungen an Tumorzellen und Leukozyten als Reaktion auf die Hemmung der VEGF-Signalkaskade durch Bevacizumab *in-vitro*. In den Initialen Experimenten wurde VEGF durch Hypoxie in Tumorzellen induziert und Veränderungen der Überlebensrate, der Proliferation, Migration als auch in der Gen- oder Protein-Expression gemessen. Es konnte eine minimale direkte Reaktion der VEGF-Hemmung auf Tumorzellen beobachtet werden, welche auf die Bevacizumab Behandlung zurückgeführt werden könnte. Es zeigten sich aber auch geringfügige Abweichungen in einigen der verwendeten Zellinien, die keine einheitliche Interpretation erlauben oder auf eine uniformelle Reaktion hinweisen würden.

Das phänotypische Korrelat einer „Mismatch“ Reparatur (MMR)-Defizienz ist die Mikrosatelliteninstabilität im Gegensatz zu mikrosatellitenstabilen Tumoren und findet sich bei bis zu 15% der kolorektalen Karzinomen (CRC) wieder. Klinischen Daten deuten daraufhin, dass Bevacizumab besser in MMR-defizienten Tumoren wirkt. Daher wurden die weiteren Untersuchungen in gepaarten MMR stabilen und MMR instabilen CRC-Tumorzelllinien (MMR defizient und kompetent) durchgeführt. Weiterhin wurde ein DNA-schädigendes Agens, SN-38, ein Topoisomerase-Inhibitor (der aktive Metabolit von

Irinotecan) dem Behandlungsschema zugefügt. Es zeigte sich, dass die Hemmung von VEGF mittels Bevacizumab die Fähigkeit der MMR defizienten Tumorzellen Kolonien zu bilden signifikant inhibiert. Im Gegensatz dazu, hatte die Behandlung von Bevacizumab vor der Zugabe des DNA schädigenden Agens zu einer vermehrten Kolonienzahl geführt. Außerdem erhöhte die Vorbehandlung mit Bevacizumab deutlich die Mutationsrate in MMR-defizienten Zellen, was durch die transiente Transfektion eines Dinukleotid-Repeat-Konstrukts nachgewiesen werden konnte. Dies deutete darauf hin, dass VEGF eine intrinsische Rolle in der Signalkaskade des MMR-Systems haben könnte. Deshalb wurde eine Anzahl von Signalalkaskaden zusätzlich zu Veränderungen von Genexpressionsprofilen untersucht und JNK als mögliche Verbindungsstelle der beiden Signalkaskaden, VEGF und MMR, identifiziert. Diese Hypothese wurde zusätzlich unterstützt durch die Tatsache, dass die *JUN* Expression unter diesen experimentellen Bedingungen reduziert war. Die Aufklärung der komplexen molekularen Mechanismen der potentiellen Interaktion bleibt zukünftigen Untersuchungen vorbehalten.

In Hinblick auf die klinische Konsequenz der erhaltenen Ergebnisse war es auffällig, dass einige Zytokine durch Bevacizumab in den MMR defizienten Zellen im Gegensatz zu den MMR kompetenten Zellen unterschiedlich reguliert wurden. Die *in-vitro* verwendeten Behandlungsschemata waren den klinisch zur Anwendung kommenden Protokollen nachempfunden. Zuerst wurde ein DNA-Schaden gesetzt, und den Zellen ermöglicht, sich mit oder ohne Bevacizumab zu erholen. Es konnte gezeigt werden, dass die inflammatorischen Zytokine *CCL7* und *CCL8* eine höhere Expression in der MMR-defiziente Zelllinie in Kombination mit Bevacizumab aufweisen. Daher wurde ein möglicher Crosstalk zwischen von Tumorzellen sezernierten Faktoren und myeloischen Zellen weiter verfolgt. Veränderungen der Genexpression in Monozyten durch Tumorzell- konditionierte Medien zeigte *CCL18* als ein Bevacizumab reguliertes Gen in MMR-defizienten Zellen, aber nicht in MMR kompetenten Zellen. CCL18 übt seine Funktion primär im adaptiven Immunsystems aus um eine T_H2-Antwort in T-Zellen auszulösen Ausserdem wird eine Erhöhung der nicht-spezifische Phagozytose als weitere Funktion beschrieben. CCL18 wurde bereits als prognostischer Marker in Magen-, Dickdarm- und Eierstockkrebsarten beschrieben; die klinische Bedeutung ist jedoch abhängig von Tumortyp.

Die Ergebnisse dieser Arbeit zeigen, dass eine Erhöhung der phagozytischen Aktivität von Makrophagen in Gegenwart von Bevacizumab wesentlich deutlicher in MMR-defizienten Zellen ausgeprägt war. Weiterhin wurde gefunden, dass nach DNA-Schädigung in Bevacizumab behandelten MMR-defizienten Zellen Zytokine freigesetzt werden, welche eine Monozytenmigration in einer Bevacizumab-abhängigen Weise induzieren. Dies weist auf eine funktionelle Interaktion von MMR-Defizienz und Bevacizumab hin. Zusätzlich zeigen

die Ergebnisse dieser Arbeit eine Immunzellmodulation, die spezifisch für Mismatch-Reparatur defiziente Tumorzellen ist und in der klinischen Praxis als Marker für die Verabreichung von Bevacizumab verwendet werden könnte.

2 INTRODUCTION

2.1 CANCER

Cancer is a collective term for a heterogeneous population of diseases that are defined by uncontrolled growth with a disregard for regulatory queues from the surrounding environment. Malignancy, in the form of many epithelial derived tumors, arises from the transformation of cellular functions and an imbalance of controlling signals, resulting in abnormal cell growth and replication. Carcinogenesis is a multi-factorial process that results from a series of successive events of genetic modifications and natural selection of these cells. The initiating factor is commonly by gene mutation or epigenetic alterations in a tumor proto-oncogene or a suppressor gene, followed by sequential events promoting uncontrolled proliferation of an atypical cell.

The epithelial architecture forms protective layers of cells that create a barrier between organs and the basement membrane and subsequently the surrounding stroma, or in the case of skin, a barrier to the outside world. Some specialized epithelial cells secret factors into the surrounding tissue, ducts or channels that they border. These categories of epithelial cells, protective and secretory cells, generate different types of tumors, squamous cell carcinoma and adenocarcinoma respectively. Cancer develops predominantly in epithelial tissues, termed generally as carcinomas, presumably due to their exposure to extracellular insults, but cancer also manifests as sarcomas in connective tissue, leukemia and lymphomas in white blood cells and hematopoietic cells and tumors of the central nervous system.

In an effort to understand what drives cancer development an in depth analysis of cancer related genes was performed in 2004 by Futreal *et al.* with approximately 300 genes implicated in oncogenesis with functions relating to cell cycle progression, kinase activity or DNA repair amongst others (Futreal et al., 2004). Considerable progress was made to better understand genomic differences, as well as similarities, in cancer with the first publication from The Cancer Genome Atlas (TCGA) in 2008 (Cancer Genome Atlas Research, 2008). This approach continues to be further refined with the progress of genomic technologies that look more closely at tumor types and gene mutation combinations, which reveal certain cancer subtypes that relate specifically to clinical outcomes and risk factors (Budinska et al., 2013, Sadanandam et al., 2014, Celiku et al., 2014, Cancer Genome Atlas, 2012).

Two general classes of cancer genes have been identified, the first consisting of genes that control cell proliferation and cell growth, such as growth factors, signal transduction protein kinases and transcription factors. These genes are known as proto-oncogenes or gatekeeper genes, and when mutated or overexpressed, promote inappropriate proliferation of cells. The second class of genes control the stability of the genome and prevent the accumulation of mutations, termed tumor suppressor genes, antimutator or caretaker genes. Tumor suppressor genes include DNA repair genes, cell cycle checkpoint regulators and genes that maintain the fidelity of chromosome segregation. It is well established that particular genes within these classes are susceptible to mutations and promote tumorigenesis known as a mutator phenotype in more than one tumor type: for example suppressor genes *APC, PTEN, TP53,* oncogenes *BRAF, H-RAS, PI3KCA* or DNA repair genes *BRCA2* and *MSH2* (Baudot et al., 2010). For a tumor to evolve into metastatic disease, additional mutations accumulate within an aberrant cell through a succession of genetic and epigenetic changes. Supporting this theory of accruing mutations over time, the incidence of the majority of epithelial cancers increase with age (Rubin et al., 2010). A long-standing estimation in colorectal cancer is that 4-7 gene cellular modifications (genetic or epigenetic) are required to promote tumor development (Fearon and Vogelstein, 1990).

The vast majority of cancers are due to environmental factors or random replication errors, with approximately 5-10% as a result of inherited gene mutations. A number of inherited cancers are as a consequence of a cells inability to maintain the genetic exactitude necessary over time as a result of ineffective repair of DNA due to endogenous or exogenous insults (Table 1).

Table 1. Cancer predisposition dependent on non-functioning components of DNA repair pathways

DNA repair pathway	Implicated genes	Syndromes synonymous with mutations	Cancer predisposition
Mismatch Repair (MMR)	*MSH2, MLH1, MSH6, PMS1, PMS2, MLH3*	HNPCC	CRC
Nucleotide Excision Repair (NER)	*XPA, XPC, DDB1, ERCC4, ERCC5, POLH, ERCC2*	Xeroderma pigmentosum, Cockayne trichothiodystrophy, cerebro-oculo-facio-skeletal	Skin cancers, Squamous cell carcinoma, head and neck
Base Excision Repair (BER)	*POLB, FEN1*		Various
Homologous Recombination (HR)	*BRCA1, BRCA2, RAD54B, RAD51B, CtIP (RBBP8), WRN, BLM,RECQL4*	Bloom, Werner, Rothmund-Thomson	BC, OC, NHL, CRC, Lymphoma, uterine, leiomyoma, skin basal and squamous cell osteosarcoma
Non-homologous End Joining (NHEJ)	*MRE11, LIG4, Artemis (DCLRE1C)*	Ataxia telangiectasia-like disorder, LIG4, Omenn	CRC, leukemia, lymphoma
Interstrand Cross Link (ICL)	*FANCA, FANCC, FANCD2, FANCE, FANCG, FANCF, FANCL, FANCB, BRIP1, FANCM*	Fanconi anaemia	Various

2.1.1 Common Characteristics of Cancer (Hallmarks)

Regardless of tumor type, it has long been established that there are a number of common features shared by tumors, which was captured and summarized in the seminal paper by Hanahan and Weinberg in 2000 and later updated in 2011, describing the "Hallmarks of Cancer". Despite the enormous diversity in tumors, they succinctly highlighted uniform traits attributed to malignant disease (Figure 1): sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immorality, inducing angiogenesis, resisting cell death and more recent enabling hallmarks: genome instability and mutation and tumor-promoting inflammation, along with emerging hallmarks: reprogramming energy metabolism and evading immune destruction (Hanahan and Weinberg, 2011).

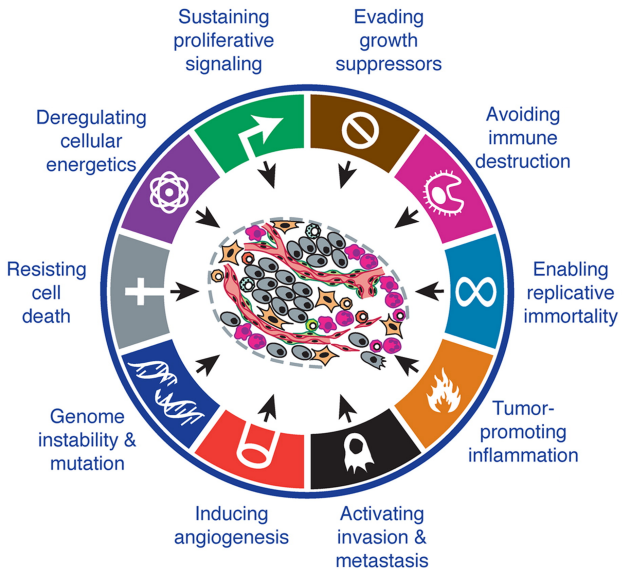


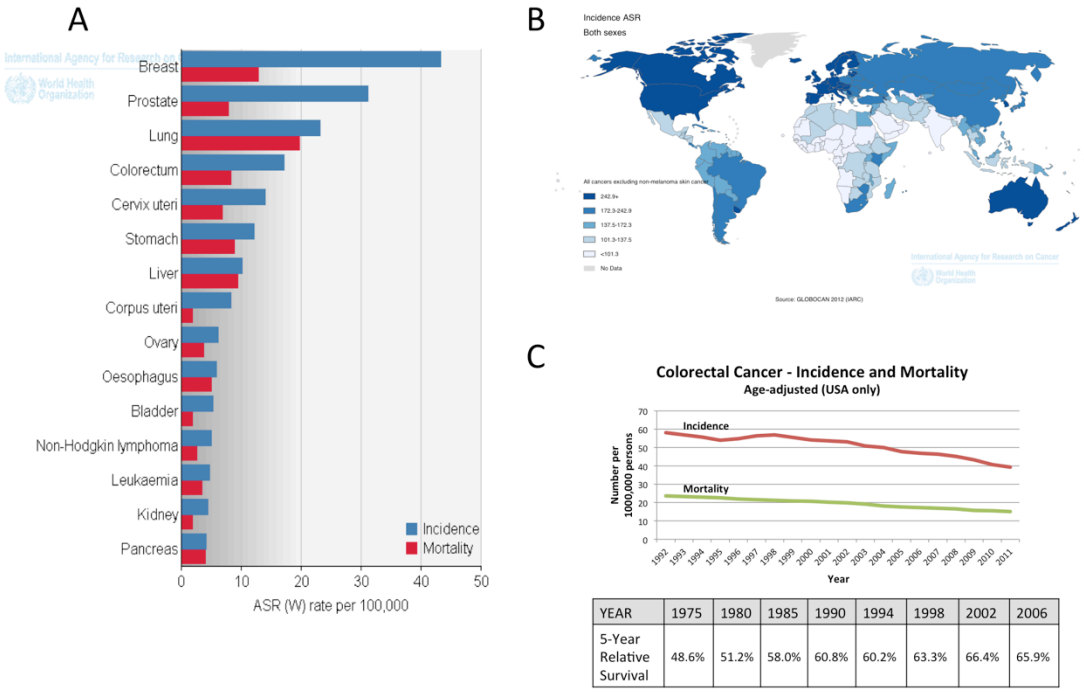
Figure 1. The next generation of cancer hallmarks. Used with permission and adapted from Hanahan and Weinberg 2011 (Hanahan and Weinberg, 2011).

Often viewed and studied as exclusive entities, the cell autonomous hallmarks of tumor cells have been widely detailed including initiating aberrant signaling driving uncontrolled proliferation or deregulation of apoptotic signaling. There has also been significant research at the boarders where these hallmarks intersect, where there is an interaction of tumor cells with for example endothelial cells in angiogenesis research and infiltrating immune cells in immune evasion and tumor promoting inflammation.

2.1.2 Cancer Incidence and Mortality

The burden of cancer is borne predominantly by industrialized nations in North America, Europe, Australia and New Zealand, with gender specific cancers having the highest incidence worldwide (Figure 2A-B). Lung cancer has the highest mortality with liver, stomach, colorectal and breast cancers being the major contributors to the over 8.2 million deaths from the disease in 2012 (Figure 2A WHO GLOBOCAN 2012). Prevention remains the most effective strategy in reducing the burden of cancer by avoiding known risk factors such as tobacco, alcohol, obesity and other known carcinogens. The World Health Organization (WHO) has reported that up to 30% of cancers could be avoided by modifying behaviours and avoiding mutagens. Early detection can also reduce mortality rates and screening for certain cancers has become routine in many countries. As the example for

colorectal cancer in Figure 2C shows, although incidence rates have decreased, mortality rates remain disproportionally high over the 20-year period measured. Despite the persistently high number of cancer related deaths, treatments have made some inroads to increase the 5-year survival by more than 17% in a 35-year time frame (NCI Cancer statistics 2011), indicating that treatment options extend life, but in general still fail to make a lasting impact in overall survival (OS).



YEAR	1975	1980	1985	1990	1994	1998	2002	2006
5-Year Relative Survival	48.6%	51.2%	58.0%	60.8%	60.2%	63.3%	66.4%	65.9%

Figure 2. Cancer Incidence and Mortality Rates. (A) Estimated age-standardized incidence and mortality rates of cancer worldwide in both genders, 2012. Source: WHO GLOBOCAN 2012 (IARC) Section of Cancer Surveillance. (B) Estimated cancer incidence worldwide, 2012. Source: WHO GLOBOCAN 2012 (IARC) Section of Cancer Surveillance. (C) USA rates of colorectal cancer over time. Source: National Cancer Institute (NCI) USA, Cancer Statistics 2011. ASR (W), Age-standardized rate (worldwide).

2.1.3 Colorectal Cancer

Colorectal cancer (CRC) is an insidious disease originating in the intestinal tract between the cecum to the rectum (Figure 3A), and is one of the most common and deadly cancers globally. Cancer of the colon or rectum is classified according to a number of systems including the Dukes' system, the modified Astler and Coller system and the TNM (Tumor/Node/Metastases) staging system. The Dukes' system was named after the British pathologist who developed the systematic grading of colorectal tumors and the modified

Astler and Coller system built upon the Dukes system by further dividing stages B and C. The current gold standard is the TNM staging system as it is considered the more precise and specific in the pathological description compared to the older classification systems. Not only is tumor classification descriptive (Figure 3B), but it is also indicative of prognosis with the later stages having a lower 5-year survival rate (Compton and Greene, 2004).

Key genetic malfunctions leading to the progression of colorectal tumors include the loss of genetic stability by either mutation or epigenetic silencing. There are three distinct pathways that have been recognized leading to genomic instability: 1) chromosomal instability (CIN), 2) microsatellite instability (MSI) and 3) CpG island methylator phenotype (CIMP) (Lengauer et al., 1998, Baylin and Ohm, 2006). The majority of CRC tumors are sporadic cancers displaying CIN, accounting for 75-85% of all CRC, with the remaining cases attributed to MSI, either from germline mutations or promoter methylation of genes charged with maintaining the integrity of the genome (Figure 3C) (Pancione et al., 2012, Poynter et al., 2008).

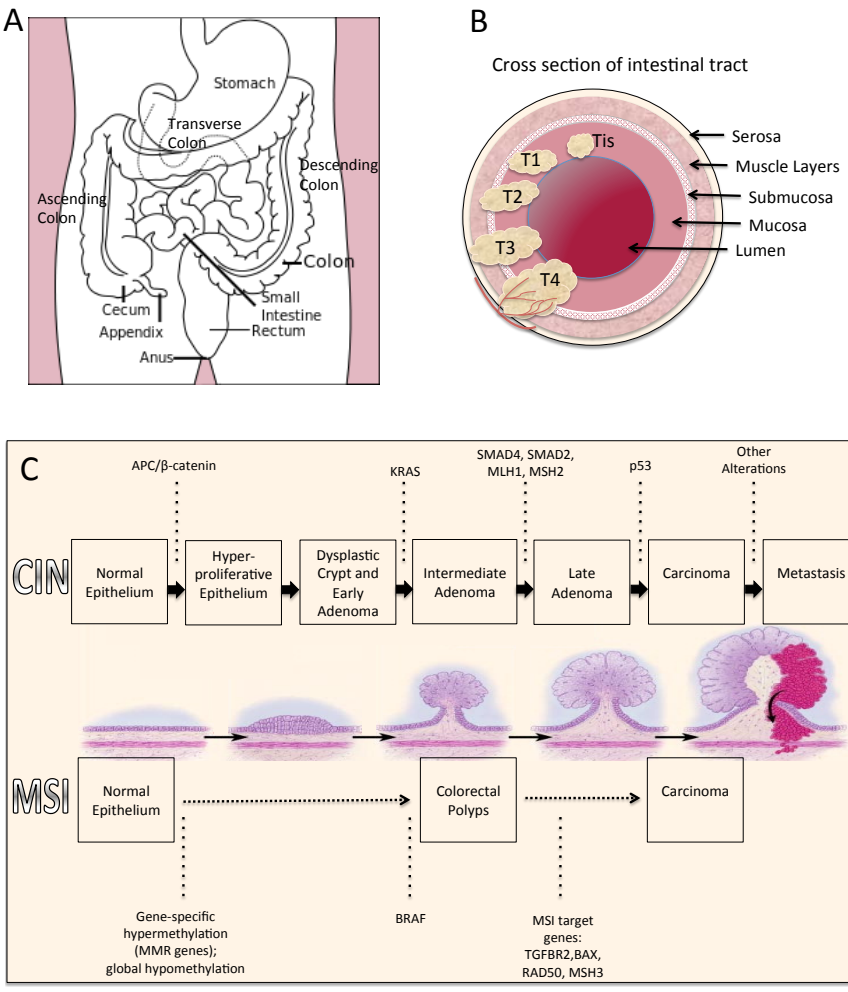


Figure 3. Colorectal Cancer Oncogenesis (A) Human gastrointestinal tract where CRC can develop. (B) Cross section of the intestines showing examples of tumor (T) staging. (C) Colorectal carcinoma progression via chromosomal instability pathway (CIN) and the microsatellite instability pathway (MSI). Used with permission and modified from (Janne and Mayer, 2000, Fearon and Vogelstein, 1990).

Tumors of the colon originate predominantly in the intestinal crypts and follow the loss of APC or β-catenin as well as deregulation of key pathways, such as the WNT, Hedgehog, Notch and bone morphogenetic proteins (BMP) signaling systems, which cooperate specifically to maintain intestinal stem cells (Figure 4) (Medema and Vermeulen, 2011). The highly proliferative nature of intestinal stem cells and the constant cellular turn over makes them particularly susceptible to malignant transformation allowing for sequential accumulation of genetic modifications. In addition, extracellular factors can also influence adenoma formation including consumed carcinogens. Furthermore there is evidence demonstrating that intestinal inflammation can induce mutations and the formation of numerous intestinal polyps via either the microsatellite instability (MSI) or chromosomal instability (CIN) pathways, increasing the predisposition to CRC (Tanaka et al., 2006, Colotta et al., 2009).

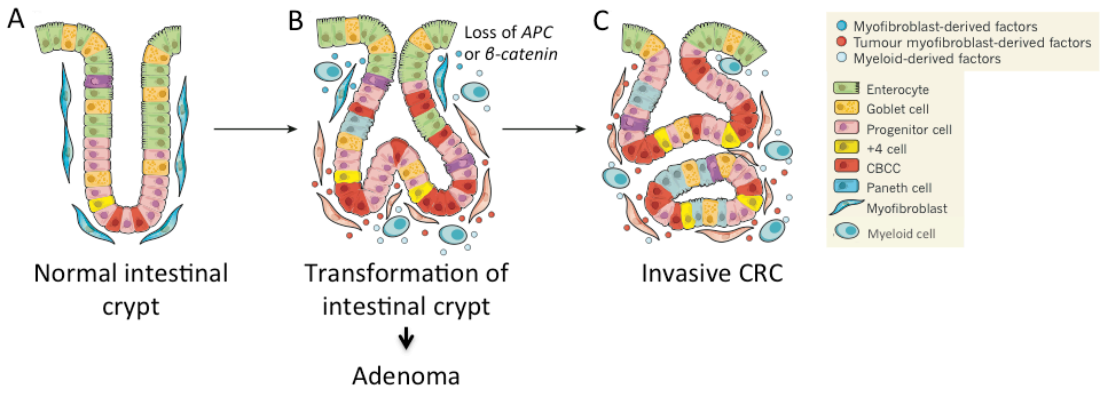


Figure 4. Stem cells in the progression of colorectal cancer in the crypts. (A) The cellular organization of a normal intestinal crypt. (B) Crypt changes due to loss of APC or β-catenin giving rise to cellular changes in appearance and behaviour. Cells take on a more immature phenotype, are located at irregular intervals and increase in proliferation as noted by more CBCCs and progenitor cells. Also myeloid cells start to accumulate around dysfunctional crypt. (C) Invasive CRC leading to tumor progression. Used with permission and modified from (Medema and Vermeulen, 2011). CBCC, Crypt base columnar cells; APC, Adenomatous Polyposis Coli.

2.2 DNA DAMAGE AND DNA REPAIR

In order to maintain the integrity of the genome, eukaryotic cells have evolved a number of pathways for ensuring genetic stability is preserved whether from the onslaught of exogenous insult, or from those driven by endogenous factors. Failure to repair DNA damage can have major consequences for cellular integrity and ultimately the organ system. In particular unrepaired damages can alter critical cellular functions, and in general have a detrimental effect on damaged cells. Figure 5 shows various types of DNA damage as well as the repair pathways that are primarily responsible for reducing the risk of certain mutations. Possible mutations that arise can be through base modifications, base mismatches, insertions or deletions, single or double stranded breaks (SSBs/DSBs) and DNA cross-linking either intra or inter strand (Lord and Ashworth, 2012, Hoeijmakers, 2001).

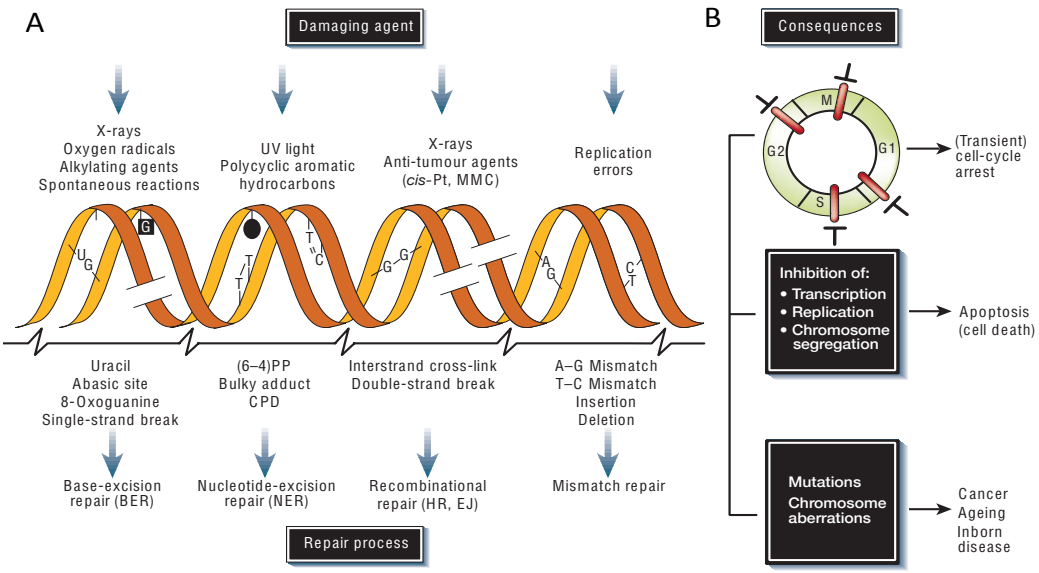


Figure 5. DNA Damage and DNA Repair Pathways (A) Types of DNA damage and responsible DNA repair pathways. (B) Cellular consequences of DNA damage. Used with permission from (Hoeijmakers, 2001). *cis*-Pt, cisplatin; MMC, mitomycin C; (6-4)PP, 6-4photoproduct; CPD, cyclobutane pyrimidine dimer; BER, base excision repair; NER, nucleotide excision repair; HR, homologous recombination; EJ, (non-homologous) end joining

2.2.1 DNA DAMAGE RESPONSE (DDR)

The DNA damage response (DDR) acts to impede tumorigenesis. The phenotypes of premalignant lesions generally share some common features that promote mutagenesis such as altered metabolism, potentially giving rise to reactive oxygen species (ROS); increased proliferation promoting replication stress; and genetic or epigenetic instabilities that alter gene structure or function (Bartkova et al., 2006, Halazonetis et al., 2008). In order to counter these factors, cells have developed mechanisms to trigger checkpoints along the cell cycle that prevent mutations from being passed on to the daughter cell (Figure 6). Depending on the degree of DNA damage, DNA repair can be initiated or alternatively when DNA damage is extensive, cell cycle arrest can become permanent as cells enter a senescent state or cell death can be induced.

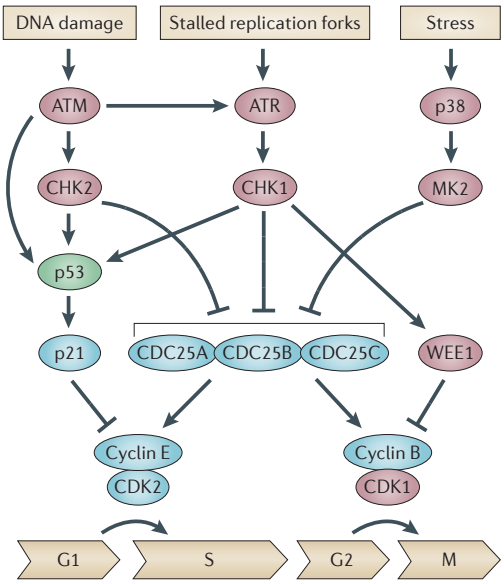


Figure 6. Cell cycle checkpoint activation induced by DNA damage and cell stress. Used with permission from (Bouwman and Jonkers, 2012).

2.2.2 DNA DAMAGE AND CELL STRESS SIGNAL TRANSDUCTION

Upon DNA damage, a myriad of signaling changes take place, with the type of DNA lesions or breakage of the phosphodiester backbone, dictating the engagement of alternate DDR pathways. In general there are two overlapping DDR signaling networks that are modulated by key sensor proteins from the phosphatidyl-inositol-3 kinase (PI3K) family, ataxia-

telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR) (Liu et al., 2006). These DNA damage sensors initiate a cascade of phosphorylation signaling events through mediator, transducer and effector proteins, which are triggered by single and double strand DNA breaks, base damage or mismatch, stalled replication forks and DNA strand cross-links. ATM is activated by double strand breaks (DSBs) through an interaction with a functional MRN complex to tether the strands (Uziel et al., 2003). ATM interacts directly with a subunit of the MRN complex, NBS1, and phosphorylates Ser-139 on the histone variant H2AX (Huang et al., 2004). The phosphorylation of H2AX activates proteins with a BRCA-1 c-terminus domain (BRCT) that appear to have a unifying function in the DNA damage response and checkpoint control, including adaptor proteins such as TOPBP1, PARP1, XRCC1 (Bork et al., 1997). The BRCT adaptor proteins recruit effector kinases and in the event of DSBs, this effector kinase is CHK2. CHK2 is involved in cell cycle arrest by subsequently activating the phosphatase CDC25A, which is degraded and therefore unable to dephosphorylate CDK2-Cyclin complex thereby arresting the cell cycle (Matsuoka et al., 1998). CHK2 also phosphorylates MDM2 and p53 that can then trigger an array of target gene transcription including DNA repair genes (e.g. *DDB2, BRCA-1*), cell cycle inhibitors (e.g. *p21*) or apoptotic initiator genes (e.g. *BAX*) (Adimoolam and Ford, 2003, Macleod et al., 1995, Haupt et al., 2003).

ATR is recruited to single strand breaks (SSBs) by RPA-coated ssDNA and forms a kinase complex together with ATRIP (ATR interacting protein) (Zou and Elledge, 2003). This complex is critical in the DDR for stalled replication forks, DNA strand cross-links as well as replication mediated DNA breaks. ATR is activated by an interaction with a number of factors including topoisomerase II binding protein (TOPBP1) and the 9-1-1 complex, which then mediates CHK1 activation. The CHK1 downstream substrates are similarly triggered as with CHK2, demonstrating partial redundancy between the two signaling networks, despite the two kinases being structurally unrelated (Bartek and Lukas, 2003). CHK1 is primarily active during S and G2 phases and is involved in other functions such as chromatin remodeling, DNA repair and replication regulation. CHK2 is stably expressed throughout the cell cycle, however is partially redundant in checkpoint regulation during replication and thought likely to be more important in the DNA damage induced apoptotic response to DSBs (Figure 7) (Lukas et al., 2001, Zhou and Bartek, 2004).

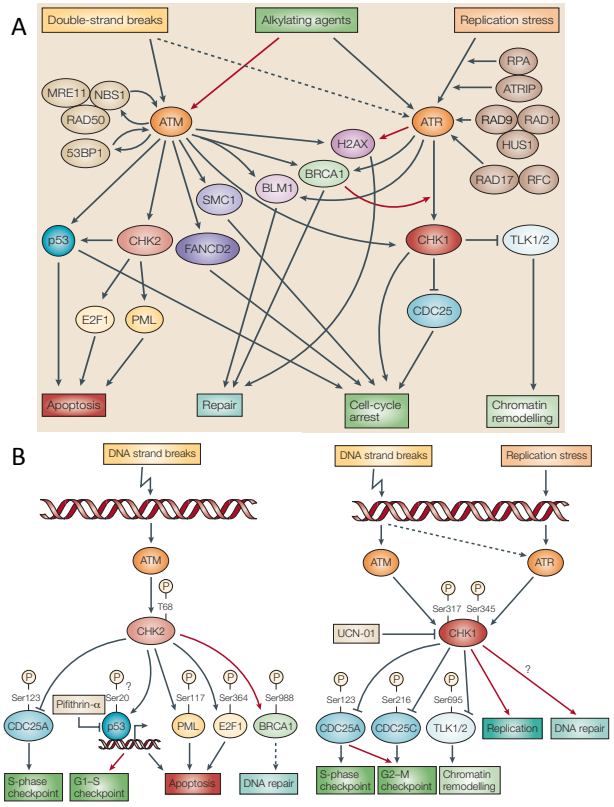


Figure 7. Signal transduction of the DNA damage response. (A) Activating sensor kinase pathways (B) Effector kinases and downstream cellular functions. Used with permission from (Zhou and Bartek, 2004).

The DDR isn't the only cell stress response, as cells can respond in a number of ways to resolve and recover from insult and stress. A cell's response is dependent on the type, amplitude and duration of attack, from cell death to activation of protective and survival responses. Environmental changes such as hypoxia and/or metabolic changes can trigger a number of stress responses including the UPR (unfolded protein response) that is mediated by the endoplasmic reticulum (ER), oxidative stress through generation of ROS and the heat shock response. Although distinct entities, these pathways converge in various ways by inducing overlapping gene transcription, activating chaperone proteins or altering metabolic function to coordinate cell fate (reviewed in (Fulda et al., 2010)).

Other key pathways of the cell stress response include NFκB and the mitogen-activated protein kinase (MAPK) including p42/p44 MAPK (aka ERK), p38 and JNK. These pathways convert extracellular stimuli such as cytokines, growth factors (e.g. EGF, VEGF), mitogens and cellular stressors into various cellular responses that modulate function and survival. MAPKs are a tiered phosphorylation network whereby each preceding kinase must be fully activated before the downstream cascade can continue. The target substrates orchestrate both a cytoplasmic and nuclear reaction to environmental cues that mediate or amplify the desired

response. Negative regulation of the MAPKs is through phosphatases, which return the pathway to the inactive state and ensure the signals remain in check (reviewed in (Johnson and Lapadat, 2002)).

In cancer, MAPKs can be constitutively activated, in particular p42/p44 MAPK, through deregulation of receptor tyrosine kinase by over expression (e.g. EGFR), activating mutations in *RAS* or *BRAF*, or increased ligand production to sustain autocrine or paracrine signaling (Dhillon et al., 2007). Should the DDR go awry by augmenting these signals, there is the potential for further malignancy as well as disrupting homeostasis and altering the surrounding tissues to support tumor progression.

ATM along with DNA repair proteins such as BRCA-1, BLM, MRE11, MLH1, MSH6, amongst others, have been described to form a super complex that acts as a DNA structure surveillance mechanism known as the BRCA-1 associated genome surveillance complex (BASC) (Wang et al., 2000). Over 40 proteins that form the super complex were identified in the study by Wang *et al.* and may indicate one possible mechanism how specific DNA repair pathways are uniquely set in motion.

2.2.3 DNA REPAIR PATHWAYS

Eliminating damaged DNA relies on accurate and appropriate activation of specific DNA repair pathways to counter different types to DNA damage (Figure 8). The role of base excision repair (BER) in the maintenance of genome stability is primarily to counter oxidative DNA damage, which generates 8-oxoguanine products (8-oxoG) but is also activated as a result of alkylation (e.g. methylation), deamination (e.g. cytosine to uracil mis-incorporation) or single strand breaks. Vital proteins involved in completing DNA repair by BER include XRCC1, PARP1, which have been implicated in both cancer susceptibility (e.g. XRCC1 SNPs) as well as targets for cancer treatment (e.g. PARP inhibitors). Deactivation of BER through bialleleic germline mutation in *MUTYH* has been identified in families with an increased incidence of colorectal cancers through a mutator phenotype inducing multiple polyposis (Wang et al., 2004b).

Nucleotide Excision Repair (NER) can repair a variety of bulky DNA damages including UV-light induced DNA photoproducts, crosslinking and alkylation adducts. NER consists of a multistep process in which the DNA lesions are recognized and demarcated by unwinding the DNA with over 30 proteins working sequentially from DNA damage recognition through to strand re-ligation. There are two distinct forms of NER: Global Genome NER (GG-NER)

and Transcription Coupled NER (TC-NER). The difference between the two sub-pathways is principally in their mechanisms of damage detection, as helical distortion and alteration of DNA chemistry appears to be the first structural elements that are recognized (van Hoffen et al., 2003). The nomenclature "XP" in many of the NER proteins, represent a condition known as xeroderma pigmentosum whose sufferers have hypersensitivity UV damage and a predisposition to skin cancer resulting from mutations in NER genes (Sugasawa, 2010, Marteijn et al., 2014).

Homologous Recombination (HR) is a critical process involved in chromosomal exchange during meiosis as well as in chromosome pairing in the repair of highly toxic or deleterious DNA damage by DSBs, single strand DNA gaps and interstrand crosslinks (Krejci et al., 2012). DNA repair by HR is timed during the cell cycle before mitosis, in S and G_2 phases, when DNA has been replicated and sister chromatids are present to provide an error free template of the damaged sequence. There are two main pathways whereby DNA can be repaired by HR, the double strand break repair model (DSBR) and the synthesis-dependent strand-annealing mode (SDSA). A number of crucial regulators of HR have been implicated in syndromes and diseases such as cancer, including positive regulators: BRCA2, RAD54 and negative regulators: BLM, FANCM. BRCA2 is central to the function of RAD51 and mutations in the *BRCA1* and *BRCA2* genes have been widely characterized for their role in breast cancer susceptibility. There is also an increased incidence of various cancers in those carrying mutations in MRE11, BLM, WRN and others, shown in Table 1.

Non-homologous end joining (NHEJ) is a critical DNA repair pathway with proposed tumor suppression functions in many tissues. NHEJ is most active during G_1 in the absence of a template chromatid for recombination where HR is otherwise preferentially activated during S and G_2 phases for repairing DSBs. As with other repair pathways, mutations in regulatory genes have the capacity to promote malignancies including CRC and cause sever combined immunodeficiencies and increase the susceptibility to lymphoma and leukemia. In addition, NHEJ defects promote translocations and other chromosomal abnormalities, similar to deficiencies in HR genes (Chappell et al., 2002).

Activation of interstrand crosslinks (ICL) repair works through the Fanconi anaemia (FA) pathway during S phase and is regulated by ubiquitination and a number of interacting proteins involved in the DNA damage response (Andreassen and Ren, 2009). HR, NER and translesion synthesis (TLS) resolves ICLs induced following exposure to environmental mutagens or ICL-based chemotherapy such as platinum based compounds. Mutations in FA genes cause hypersensitivity to platinum compounds and sufferers are at a high risk of developing hematological malignancies such as acute myeloid leukemia (AML), as well as

squamous cell carcinoma of the head and neck, and hepatocellular carcinoma (HCC) (D'Andrea, 2010).

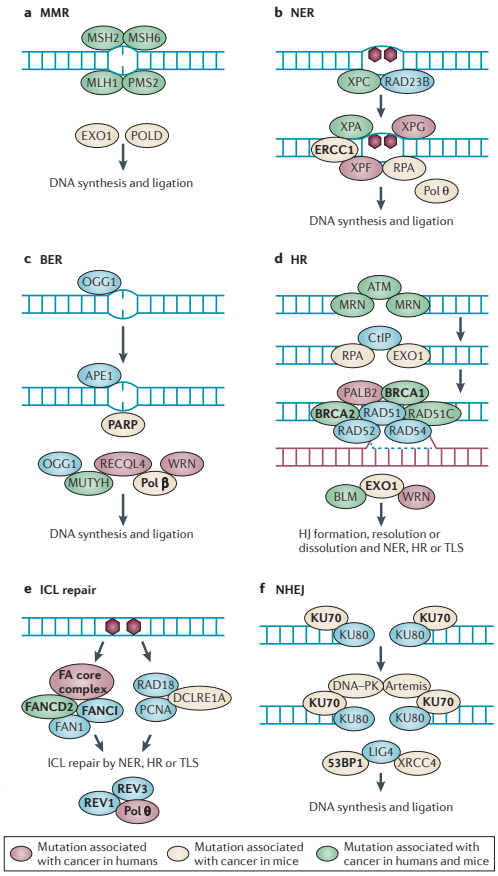


Figure 8. DNA Repair Pathways: Different DNA repair pathways and associated cancer susceptibilities from mutations in both human (red & green) and mice (yellow & green) genes. Used with permission from (Bouwman and Jonkers, 2012).

2.2.4 Mismatch Repair (MMR)

One of the most highly conserved DNA repair pathways from prokaryotes to eukaryotes is the mismatch repair (MMR) pathway. MMR is coupled to the replication process whereby it improves the fidelity of DNA replication by several orders of magnitude (Jiricny, 2006). It is initiated by detection of base-base mismatches and/or insertion/deletion loops (IDLs) by the binding of a MutS heterodimer and subsequent recruitment of MutL heterodimers (Figure 9). MutS exists in two forms MutSα, composed of MSH2 and MSH6 and MutSβ, comprising of MSH2 and MSH3, which have specific roles for repairing 1-2 bps IDLs or larger IDLs,

respectively. There is partial redundancy between MutSα and MutSβ as demonstrated by the various mutator phenotypes in *Msh2*-, *Msh3*- and *Msh6*- null mice (see Table 2).

Table 2. Mutator phenotype of MMR deficiency. Adapted from (Hewish et al., 2010, Jiricny, 2006)

MMR genes	Contribution to hereditary CRC (%)	Tumor phenotype in null mice
MutS		
MSH2	39	Severe
MSH6	14	Moderate
MSH3	-	Moderate (but amplifies phenotype in double knockouts)
MutL		
MLH1	32	Severe
PMS2	15	Moderate (no GI tumors)

MutL also exists in various forms: the primary complex MutLα consisting of MLH1 and PMS2, supports repair initiated by both MutSα and MutSβ; MutLβ is a heterodimer of MLH1 and PMS1, however its function is not well understood; MutLγ formed by MLH1 and MLH3 contributes mainly to meiotic recombination, however it has also been implicated in repair of a subset of IDLs, further supported by knockout studies that have shown a weak instability in mononucleotide microsatellites (Flores-Rozas and Kolodner, 1998).

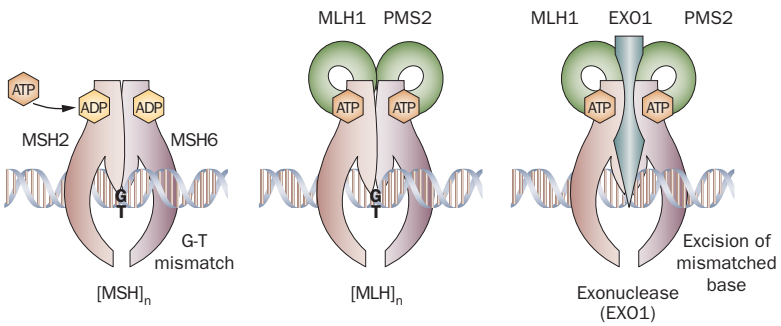


Figure 9. Mechanisms of mismatch repair. Used with permission from (Vilar and Gruber, 2010).

In addition to post-replicative repair, MMR proteins have also been described in the repair of oxidative damage outside of S phase as well as contributing to class switch recombination (CSR). CSR is a process that switches immunoglobulins from one class to another to enable interactions with alternative effector molecules. Along with other repair factors mostly involved in non-homologous end joining, it has been shown that Msh2-deficient mice had a two to 10-fold reduction in isotype switching (Ehrenstein and Neuberger, 1999) suggesting proteins of the MMR pathway have evolved a range of functions.

A deficiency in the MMR pathway is a high-risk indicator in the development of a number of cancers including colorectal, endometrial, gastric and sebaceous carcinoma as well as glioblastomas and lymphomas (Vilar and Gruber, 2010). Deficient MMR tumors display a mutator phenotype in particular at sites with simple sequence repeats (SSRs). The instability in these sequences, in the genome also known as microsatellites, is termed microsatellite instability (MSI) and is characteristic of MMR deficient tumors as discussed in more detail in section 2.3.2 below.

2.3 GENOMIC INSTABILITY

It has long been established that genomic instability is a characteristic of almost all human cancers dating back to the early 1900's and summarized as one of the enabling hallmarks of cancer by Hanahan & Weinberg (Hanahan and Weinberg, 2011, Boveri, 1914, Boveri, 2008). Advances in the detection of gene mutations have resulted in large data sets of mutation profiles in numerous cancer types by point mutations, insertions or deletions or translocations (Sadanandam et al., 2014, Sjoblom et al., 2006, Wood et al., 2007, Jones et al., 2008, Parsons et al., 2008). These data have highlighted the mutator phenotype in sporadic cancers and interestingly, that only a few genes are mutated with a high frequency across tumor types including genes such as *ATM, TP53, PTEN, CDKN2A, EGFR* and *RAS* amongst others (Negrini et al., 2010). Although single genes do not appear to be preferentially targeted, the pathways in which they are involved do, suggesting disabling of the cellular function is the primary objective to drive tumor development.

Genomic instability through mutation of tumor suppressor genes or oncogenes is proposed via oncogene-induced DNA replication stress (Halazonetis et al., 2008), in particular at common fragile sites that are prone to DNA breakage during replication (Durkin and Glover, 2007). DNA replication is a tightly controlled process with a myriad of checkpoints and regulatory mechanisms in place to ensure meticulous reproduction of DNA in daughter cells. Activated

oncogenes, such as activated *RAS* signaling by BCR-ABL translocations or overexpression of EGF receptors, can induce DNA damage through replicative stress and trigger a DNA damage response (DDR). Senescence or apoptosis are commonly resulting from the DDR as a protective mechanism against tumorigenesis, however circumventing these pathways can allow for selective pressure in acquiring mutations in tumor suppressor genes or oncogenes that promote further expansion. Subsequently, genomic instability and malignant transformation can ensue (Hills and Diffley, 2014, Negrini et al., 2010). Different forms of genomic instability have been described in colorectal cancer including chromosomal instability (CIN) (Lengauer et al., 1998), microsatellite instability (MSI) (Ionov et al., 1993, Thibodeau et al., 1993) and CpG island methylator phenotype (CIMP) (Weisenberger et al., 2006).

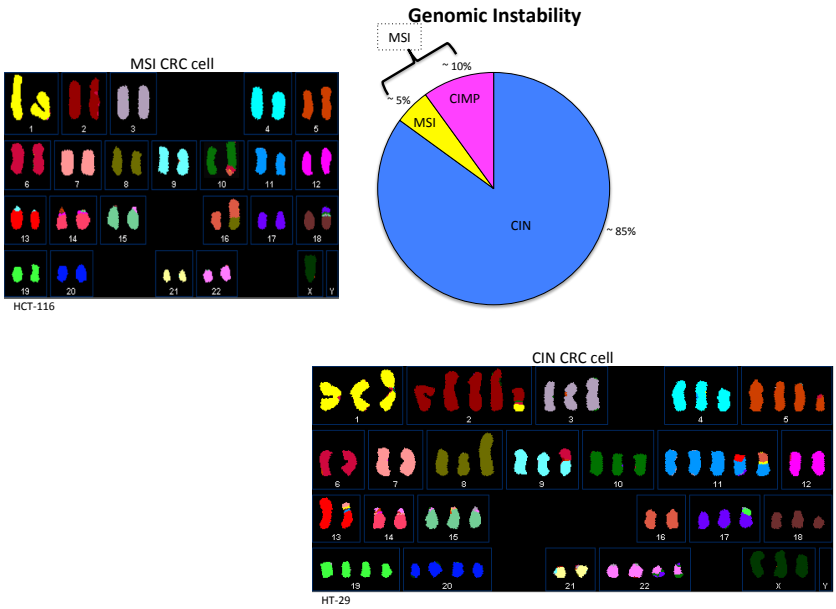


Figure 10. Frequency of genomic instability and karyotype examples of MSI (diploid) and CIN (polyploidy). Example karyotypes detected in tumor cell lines HCT-116 (MSI) and HT-29 (CIN) (Abdel-Rahman et al., 2001). MSI shows a normal diploid karyotype with some chromosomal rearrangements and CIN have an abnormal number of chromosomes also with translocations. Images are publically available for use from www.path.cam.ac.uk. MSI, microsatellite instability; CIMP, CpG island mutator phenotype; CIN, chromosomal instability; CRC, colorectal cancer.

2.3.1 CHROMOSOMAL INSTABILITY

The majority of tumors have chromosomal instability (CIN), defined as anomalies in abundance or altered structural features of chromosomes in a cell (Lengauer et al., 1998). Alterations in number of chromosomes in cancer cells presents a long-standing question of

cause or consequence with some suggesting it as a primary driver of cancer (Duesberg and Li, 2003), whilst others consider aneuploidy as a result of oncogenic transformation (Zimonjic et al., 2001). Although CIN and aneuploidy are not always synonymous, as Down syndrome demonstrates cells can have abnormal karyotypes but remain genetically stable, however for the discussion of aneuploidy in cancer, it commonly refers to evidence of genomic instability as a rate of karyotypic change: CIN leads to aneuploidy, but not all aneuploidy cells exhibit CIN (Beroukhim et al., 2010, Gordon et al., 2012).

Whole chromosomal instability (W-CIN) is defined by genetic alterations in genes such as spindle-assembly checkpoint components (Dikovskaya et al., 2007) that promote defects in mitosis and apoptosis and continuously induce abnormal karyotypes. This is opposed to a model of random errors in mitosis that might drive aneuploidy in individual cells. Another mechanism by which tumor cells acquire instability is through structural changes in chromosomes (S-CIN) such as translocations as in cases of chronic myeloid leukemia (CML) with the BCR-ABL gene fusion (Hehlmann et al., 2007) that drive oncogenic transformation as described previously.

Hereditary cancers can contribute to chromosomal instability through, for example, germline mutations in breast cancer susceptibility gene 1 (*BRCA1*) and other genes linked to repair of DNA (Ripperger et al., 2009). Several syndromes have been described that show an increased level of spontaneous chromosomal abnormalities, such as Fanconi anaemia (FA), Bloom syndrome (BS) Nijmegen breakage syndrome (NBS), ataxia telangiectasia (A-T) and ataxia telangiectasia-like disorder (ATLD). A common feature of these syndromes is that they are predominantly as a result of mutations in DNA repair genes, specifically those involved in double stranded breaks (DSBs) repair genes and have an increased susceptibility to various forms of cancer (Table 1) (Taylor, 2001).

2.3.2 MICROSATELLITE INSTABILITY

Microsatellites are simple sequence repeats (SSR) (also known as short tandem repeats) that occur ubiquitously throughout the genome, particularly in euchromatin, and to a lesser extent in heterochromatin (Schlotterer, 2000). The number of repeat nucleotides can be from 1 to 6 bps and the nomenclature is dependent on the length of the repeating sequence (Table 3).

Table 3. Microsatellite (SSR) examples and terminology.

Sequence repeats		
Mononucleotide:	$(A)_n$	AAAAAAAAAAA…
Dinucleotide:	$(AT)_n$	ATATATATATATATAT…
Trinucleotide:	$(GAT)_n$	GATGATGATGATGATGAT…
Tetranculeotide:	$(TCAG)_n$	TCAGTCAGTCAGTCAGTCAG…
Pentanucleotide:	$(GATTC)_n$	GATTCGATTCGATTCGATTC…
Hexanucleotide:	$(CCATGG)_n$	CCATGGCCATGGCCATGG…
Imperfect microsatellite:		GTGTGTGTGTGT**A**GTGTGTGT…
Interrupted microsatellite:		GTGTGTG**AAA**GTGTGTG…
Compound microsatellite:		ATATATATAT**CTCTCTCTC**…

SSRs in DNA are inherited markers and as such appear in unique patterns that are specific to an individual. For this reason SSRs are used to determine familial relationships and in comparative analysis. The repeat sequences can also impact the secondary structure of DNA, for examples trinucleotide repeats are more prone to form hairpins (e.g. $(CCG)_n$ in fragile X), mismatched duplexes and single stranded loops (Li et al., 2004). Furthermore, it has been proposed that proteins involved in recombination, such as RAD51, bind preferentially to dinucleotide repeats, due in part to induced Z-DNA formation or other secondary DNA structures (Biet et al., 1999).

Paradoxically, many DNA mismatch repair (MMR) genes that are responsible for maintaining microsatellite stability during replication, contain mononucleotide repeats $(A)_n$ in the coding region of their genes (Chang et al., 2001). These genes are subsequently vulnerable to spontaneous insertion or deletion mutations, inducing frameshift loss-of-function mutations at higher than normal frequencies (Moxon et al., 1994). Evidence suggests that the distribution of microsatellites throughout the genome is not random and are more commonly found in non-coding DNA, resulting only in approximately 14% of proteins containing repeat sequences (Marcotte et al., 1999) (examples of genes shown in Table 4). These regions of the genome are susceptible to errors during replications as DNA polymerases have difficulty binding to these motifs and escape their intrinsic proofreading activity. DNA MMR is responsible for detecting mismatched bases during replication, however if this mechanism fails or is not present, then the errors will persist and can give rise to mutations. If an SSR is not faithfully replicated, the consequence of mutations in these regions is termed microsatellite instability (MSI).

Table 4. Microsatellite sequences within human genes, detailing function and phenotypic effect attributed to microsatellite instability. Adapted from (Li et al., 2004, Marcotte et al., 1999)

Repeat sequence	Gene	Function and Phenotype	Reference
$(A)_n$	***MMR genes:*** *MSH2, MSH6, PMS1, PMS2, MBD4/MED1*	Inactivation increases prevalence of various types of cancer, incl. CRC.	(Duval and Hamelin, 2002, Vassileva et al., 2002, Yamada et al., 2002, Chang et al., 2001)
$(A)_n$	*TGFRII, IGFIIR, ACTRII, WISP, GRB-14, AXIN-2*	Signal transduction genes with tumor suppressor functions	(Markowitz et al., 1995, Souza et al., 1996)
$(A)_n$	*BAX, CASP5, APAF-1, BCL-10, FAS*	Apoptosis related genes with tumor suppressor functions	(Rampino et al., 1997, Schwartz et al., 1999)
$(A)_n$	*TCF-4, CDX2*	Transcriptional regulation genes with tumor suppressor functions	(Duval et al., 1999)
$(A)_n$	*B2M*	Immune surveillance gene with tumor suppressor function	(Bicknell et al., 1996)
$(A)_n$	*PTEN, RIZ, Hg4-1*	Cell cycle genes with tumor suppressor function	(Guanti et al., 2000, Zhou et al., 2002, Duval and Hamelin, 2002)
$(A)_n$	*BLM, CHK1, RAD50*	DNA damage response genes with tumor suppressor function	(Duval and Hamelin, 2002)
$(CAG)_n$	*HD*	Huntington's disease (HD)	(Zoghbi and Orr, 2000)
$(CAG)_n$	*DRPLA*	Dentatorubro-pallidoluysian atrophy (DRPLA)	(Nakamura et al., 2001)
$(CAG)_n$	*KR*	Spinobulbar muscular atrophy (SBMA)	(Mao et al., 2002)
$(CAG)_n$	*SCA*	Spinocerebellar ataxias (SCA types 1-7)	(Mao et al., 2002)
$(CAG)_n$	*Androgen receptor (AR)*	Hepatitis B virus (HBV)-related hepatocellular carcinoma Prostate cancer AR activity SBMA with partial androgen insensitivity	(Buchanan et al., 2001, Coetzee and Irvine, 2002, Dejager et al., 2002)
$(CGC)_n$	*PABP2*	Poly(A)-binding protein related to oculopharyngeal muscular dystrophy	(Brais et al., 1998)

MSI is commonly detected using the Bethesda reference panel that assesses five microsatellite loci, comparing nucleotide repeats in tumor cells with adjacent normal tissue. In order to improve sensitivity and stratify high MSI (MSI-H) with low MSI (MSI-L) and microsatellite stable (MSS) tumors, the panel has undergone modifications and expanded in recent years, however the established criteria remains unchanged, defined as: MSI-H with >30% loci (or two or more) unstable, MSI-L with 10-30% of loci (or one loci) and MSS <10% loci (or no loci) (Boland et al., 1998, Umar et al., 2004).

MSI can trigger a mutator phenotype whereby oncogenes and tumor suppressor genes with sequence repeats can be targeted, in particular *BRAF, RAD50, TGFBRII, IGFRII, BAX, HDAC2, PTEN* and other MMR genes (Table 4) (Duval and Hamelin, 2002). The genes targeted have diverse biological functions from growth factors, pro-apoptotic factors to DNA repair genes, however mutations in these genes are not exclusive to MSI tumors. MSI tumors have 100-1,000-fold higher mutation rates compared to tumors with functioning MMR (Bhattacharyya et al., 1994, Parsons et al., 1993), and the secondary mutations after MMR loss of function, are what increase the susceptibility to oncogenic transformation in various cancer types including CRC and endometrial cancer amongst others.

MSI through loss of MMR function in CRC, is predominantly through epigenetic modifications with ~10-13% of all CRC attributed to CpG island methylator phenotype (CIMP) and ~2-5% associated with germline mutations of MMR genes, as seen in hereditary non-polyposis colon cancer (HNPCC). MMR can also be deactivated by somatic mutations (Thibodeau et al., 1993, Ionov et al., 1993). Hypermethylation of CpG islands in DNA, commonly results in the loss of MLH1 expression (Ogino and Goel, 2008) and is strongly associated with oncogenic BRAF ($BRAF^{V600E}$) signaling (Rajagopalan et al., 2002). In addition, *KRAS* mutations, which are detected more frequently but not exclusively in CIN tumors and considered to be a key transforming event in sporadic CRC, are mutually exclusive with *BRAF* mutations (Rajagopalan et al., 2002, Fearon and Vogelstein, 1990). Not only are there molecular differences in tumorigenesis between MSI and MSS tumors, but they also present differently clinically. MSI tumors are characterized by location, being predominantly in the proximal (right) colon; are poorly differentiated and have a low pathological stage; present with excess of mucinous, signet cell and medullary subtypes; are lymph node negative; and have a high proportion of infiltrating lymphocytes (Vilar and Gruber, 2010, Hewish et al., 2010). Patients with MSI tumors generally have a better prognosis and a reduced likelihood of metastasis (Vilar and Gruber, 2010).

There have also been recent reports of an additional subset of tumors that are CIMP positive, but remain MSS. This subset is proposed to consist of serrated tumors that have a stem-like phenotype, an EMT gene signature and high Wnt signaling with an overall poor prognosis (De Sousa et al., 2013, Sadanandam et al., 2014). There have been a number of studies attempting to delineate other subtypes of CRC by gene expression profiling in an attempt to identify prognostic or predictive gene signatures and correlating subtype to therapy response (Sadanandam et al., 2014, Perez-Villamil et al., 2012, Cancer Genome Atlas, 2012, De Sousa et al., 2013). The gene signatures have reiterated the genetic heterogeneity of colorectal cancer that may give rise to better understanding and targeting tumors more efficiently.

2.4 ANGIOGENESIS

The formation of new vessels from pre-existing ones – angiogenesis - is a vital process during development, in addition to performing a necessary function in pregnancy, wound healing and the restoration of blood supply to damaged tissue. Angiogenesis is maintained by pro- and anti-angiogenic factors that when disrupted can lead to a myriad of diseases, with excessive angiogenesis promoting cancer, rheumatoid arthritis as well as diabetic eye disease. Conversely, poor circulation from insufficient angiogenesis contributes to coronary heart disease, stroke and delayed wound recovery (Carmeliet, 2005).

Angiogenesis is a well-established factor in cancer development, so much so that it lends its characteristic pattern to cancer terminology derived in some of the earliest recorded history of tumors in the 7th century AD. Carcinoma (karkinos: crab in ancient Greek), is derived from the crab-like features of "veins stretched on all sides [of a tumor] as the animal the crab has its feet" (Moss, 2004). In order for tumors to grow beyond 1-2 mm and metastasize, tumors require nutrients via a blood supply (Gimbrone et al., 1972). New vessels can be produced through different mechanisms, by vessel elongation, intussusception, incorporation of circulating endothelial progenitor cells (EPCs) and the classical method of vessel sprouting. Furthermore, tumor cells can also induce a phenotype that mimics endothelial cells and form part of the endothelium, suggesting a novel mechanism in tumor vasculature (Risau, 1997, Rafii et al., 2002) (Figure 11).

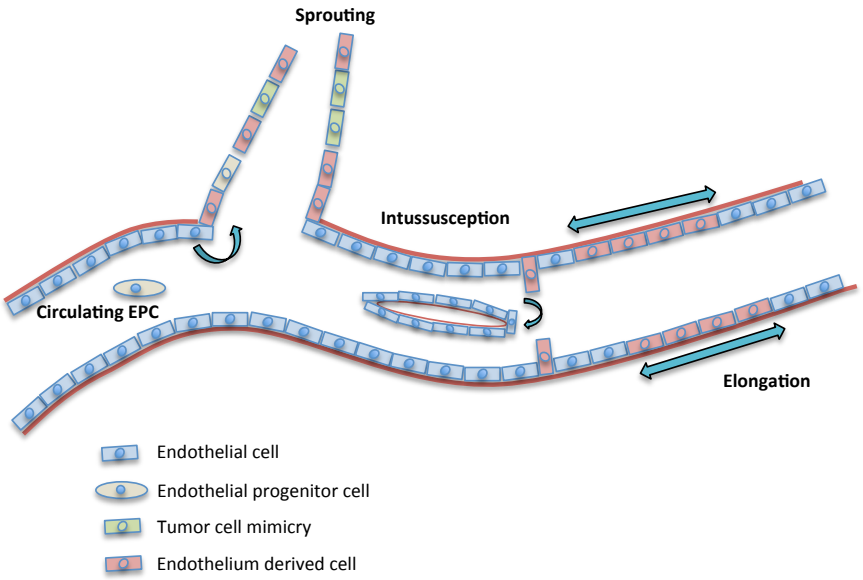


Figure 11. Primary mechanisms of angiogenesis. Adapted and modified from (Gargett and Rogers, 2001)

Tumor associated vessel remodeling has been traditionally defined as the sprouting of endothelial cells from existing vasculature, in response to a gradient of pro-angiogenic factors (Folkman, 2002). Tumor associated vasculature has morphological abnormalities in terms of structural irregularities, incorporation of bone marrow-derived endothelial progenitor cells and lack of pericyte or smooth muscle cell (SMC) coverage, leading to leakiness and impaired blood flow (McDonald and Baluk, 2002, Santarelli et al., 2006). The tumor microenvironment largely governs a tumor cell's ability to trigger angiogenesis and can be triggered by factors that can either be tumor-derived or originate from surrounding cell types such as inflammatory cells (e.g. macrophages) (Grivennikov et al., 2010). Cytokines and growth factors form the milieu in which the balance of pro- versus anti-angiogenic factors can instigate the "angiogenic switch" and a cascade of events that give rise to new vessels. A hypoxic environment is a major cause of prolific angiogenesis in tumor biology.

2.4.1 HYPOXIA

Hypoxia is a key modulator in tumor angiogenesis, which develops as malignant cells outgrow the limitation of simple perfusion of oxygen and other nutrients, and begin the process of securing their own blood supply. Hypoxia induces metabolic changes in cells from oxidative to glycolytic metabolism through the increased expression and stabilization of hypoxia-inducible factor (HIF-1) subunit HIF-1α, with HIF-1β. HIF-1 is critical transcription factor that controls the adaptive changes required for the cells to survive in an environment of lower oxygen availability, by up-regulating gene expression of target genes with a HIF-1 recognition sequence in their promoter. HIF-1 belongs to the basic helix-loop-helix (bHLH) family of transcription factors and is regulated via proteasomal degradation. Oxygen sensors such as PHDs (prolyl hydroxylase domain) prevent accumulation of HIF-1 by utilizing the oxygen as a substrate to hydroxylate prolyl residues in HIF-1α (Mazzone et al., 2009). In the absence of oxygen, degradation does not take place and HIF-1 is stabilized to allow for transcription of target genes, including vascular endothelial growth factor (VEGF) and glycolysis modulating genes such as glucose transporter 1 (GLUT1) (Lu et al., 2008, Sowter et al., 2001).

There are a number of other adaptive changes induced by hypoxia to promote tumor cell survival including enhancing receptor tyrosine kinase signaling by increased endocytosis (Wang and Ohh, 2010); genomic selection of hypoxia-tolerant clones (e.g. TP53 mutations) (Graeber et al., 1996); induction of epithelial-to-mesenchymal (EMT) transition promoting invasiveness and metastasis (Chang et al., 2011, Hill et al., 2009, Pennacchietti et al., 2003).

Furthermore, genetic stability can be corrupted by the increase in reactive oxygen species (ROS) production and down regulation of repair pathways (Bristow and Hill, 2008, Guzy et al., 2005).

2.4.2 VEGF AND VEGF RECEPTORS

Vascular endothelial growth factors (VEGFs) are some of the best characterized modulators of angiogenesis triggered by hypoxia and exist as five related homologous glycoproteins referred to as: VEGFA (also known as vascular permeability factor (VPF) and commonly referred to as VEGF), VEGFB, VEGFC, VEGFD and PlGF. Further proteolytic processing of the mature isoforms results in other, smaller isoforms that alter affinities for receptor binding, for example VEGFC must first be proteolytically cleaved to bind to VEGFR2 (Joukov et al., 1997). VEGFA, herein referred to as VEGF, has been identified as a critical factor in angiogenesis and is expressed in alternatively spliced isoforms indicative of protein amino acids, as VEGF-121, -145, -165, -189 and -206 (Figure 12A-B). All isoforms except $VEGF_{121}$, bind to heparin or heparan sulfate (HS) at exon 6 or 7 (Figure 12C), which enables vascular patterning through controlled location and release of ligands. This binding can also modify signal intensity in terms of amplitude and intensity, when activation of VEGF is for example presented *in trans* by heparan sulfate proteoglycans (HSPGs) to an adjacent cell expressing VEGFR2 (Jakobsson et al., 2006) thereby preventing receptor endocytosis.

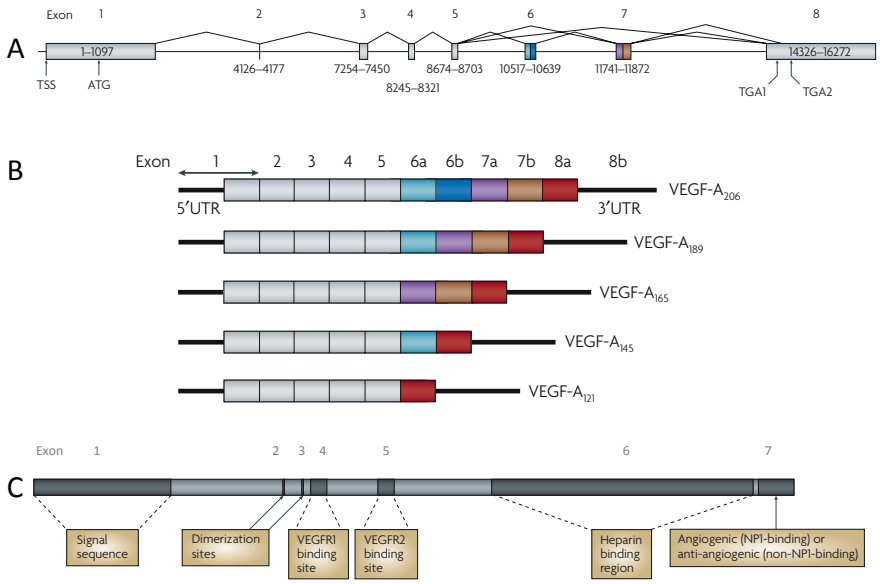


Figure 12. VEGF alternative splicing. Adapted from (Harper and Bates, 2008) with permission.

In addition to HS, vascular endothelial growth factors bind to three VEGF receptor with varying ligand specificities: VEGFR1 (also known as FLT1), VEGFR2 (also known as KDR) and VEGFR3 (also known as FLT4) (Figure 13). The VEGF receptors are receptor tyrosine kinases (RTKs) and are structurally similar, consisting of an extracellular immunoglobulin-like (Ig-like) domain, transmembrane domain (TMD), juxtamembrane domain (JMD), tyrosine kinase domain (TKD) and a C-terminal tail. VEGFC and VEGFD bind to VEGFR3 homo- and heterodimers and after processing, also to VEGFR2. PlGF and VEGFB bind only to VEGFR1 homo- and herterodimers in both the soluble and membrane bound forms. VEGF is the most promiscuous ligand binding to all VEGF receptors with the greatest affinity to VEGFR1, however with the most obvious activity regulated via VEGFR2. VEGF-VEGFR2 mediated signaling controls endothelial cell differentiation, proliferation, migration and structural formation of vascular tubes (Gille et al., 2001).

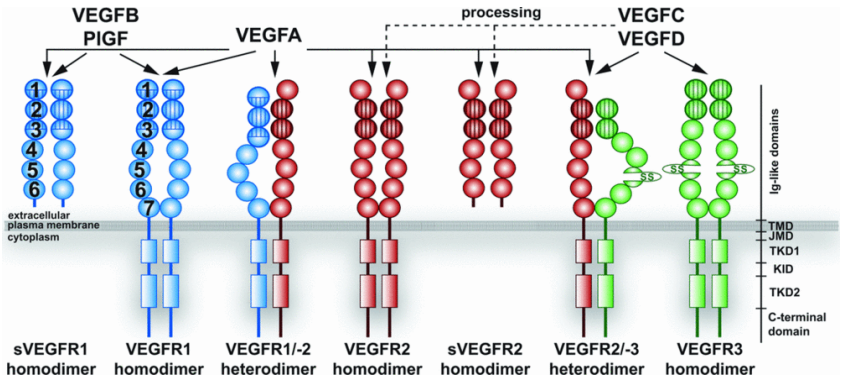


Figure 13. VEGF family members and respective binding partners. From (Koch et al., 2011). Soluble VEGFRs (sVEGFR1 and sVEGFR2) lack the seventh Ig-like domain. JMD, juxtamembrane domain; KID, kinase insert domain; TMD, transmembrane domain; TKD1/2, Tyrosine kinase domain.

VEGF signal transduction has been elucidated in endothelial cells with a focus mainly on VEGFR2 as it displayed the most obvious phenotype in response to ligand binding (Figure 14). A number of pathways are activated by VEGF including PI3K, Akt and p42/p44 MAPK that regulate endothelial cell survival and proliferation, Src and FAK regulating endothelial cell migration and PKC and p38 for vascular permeability (reviewed in (Olsson et al., 2006)).

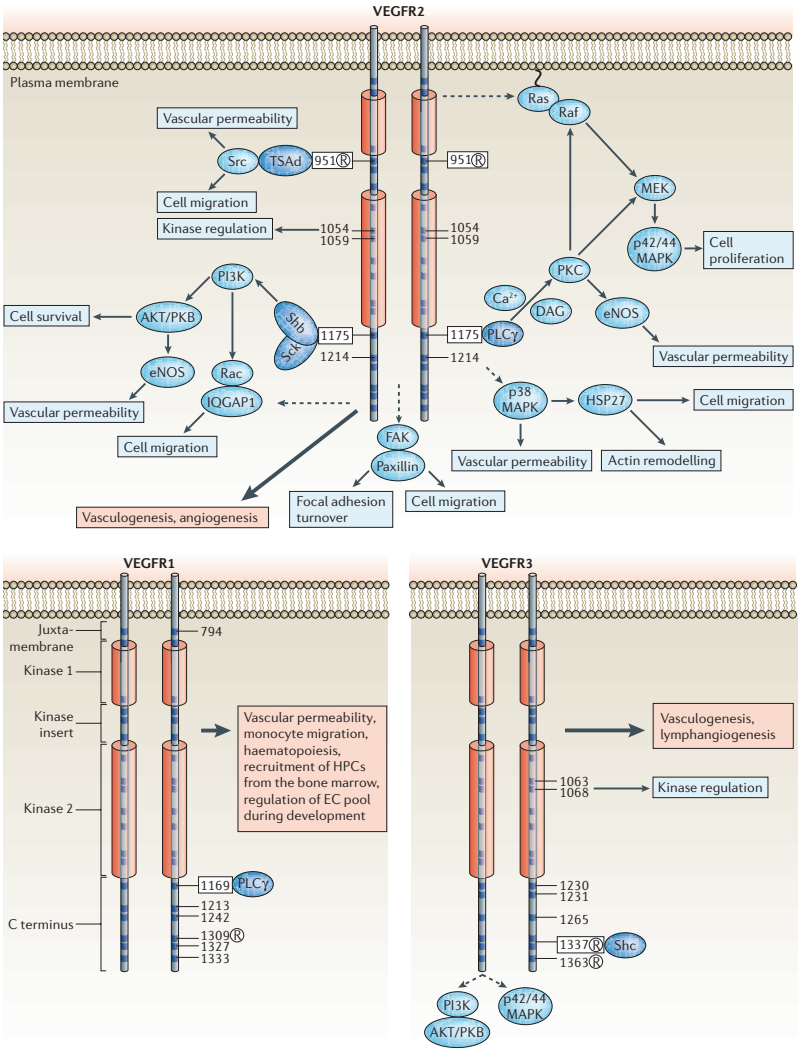


Figure 14. VEGF intracellular domains and signal transduction in endothelial cells. Tyrosine phosphorylation sites are indicated in the kinase domain of the VEGF receptors and functional regulation is described for each of the receptors. VEGFR2 controls many of the functions in endothelial cells involved in angiogenesis including survival, proliferation and migration, whereas VEGFR1 is known to regulate hematopoietic cells and VEGR3 controls lymphangiogenesis. Used with permission from (Olsson et al., 2006).

The necessity for VEGF family members, receptors and the various isoforms in development is apparent by knockout phenotypes seen in mice. Vegfa$^{-/-}$, Vegfa$^{+/-}$, Vegfc$^{-/-}$, Vegfr1$^{-/-}$, Vegfr2$^{-/-}$, Vegfr3$^{-/-}$ and Nrp1$^{-/-}$ are all embryonic lethal where as Vegfa$^{164/164}$, Vegfd$^{-/-}$ and Plgf$^{-/-}$ knockouts develop normally. Interestingly, $VEGF_{164/5}$ is not essential in development, however considered as the primary isoform necessary in pathological angiogenesis, as it is strongly induced by solid tumors (Ferrara et al., 1992). Recognition of $VEGF_{164/5}$ signal transduction pathways elucidated functions including vessel permeability and endothelial cell modulation by hypoxic tumors. These discoveries led to the hypothesis that tumors could be controlled by limiting the availability of growth factors and ability for metastatic spread

through VEGF signal blockade. With this, targeted therapies were developed against VEGF and VEGF signaling, the first of which was the neutralizing antibody bevacizumab (Avastin ©) (Ferrara, 2005, Hurwitz et al., 2004) see section 2.6.2.

2.4.3 *Angiogenesis-Independent Activity of VEGF*

With the multitude of research into VEGF biology in recent years, it has been discovered that VEGF can mediate functions in cell types other that just endothelial cells. With regards to cancer research, it has been shown that tumor cells not only up-regulate VEGF, but also express VEGF receptors (Hein and Graver, 2013, Amaya et al., 1997, Duff et al., 2006, Kajita et al., 2001, Donnem et al., 2007, Ryden et al., 2003, Ghosh et al., 2008, Wulfing et al., 2005, Decaussin et al., 1999, Fan et al., 2005, Wang et al., 2004a) suggesting that an autocrine or paracrine mechanism may contribute to tumor cell survival, although direct evidence supporting VEGF as a critical factor in tumor cells *in vivo* is not conclusive. It has for example been shown *in vitro* that blocking VEGFR1 inhibited tumor cell migration, invasion and colony formation (Fan et al., 2005) as well as inhibiting the transition of mesenchymal from epithelial phenotype (EMT) (Yang et al., 2006). Furthermore, breast cancer xenographs relied onVEGFR1 intracellular signaling for growth and survival (Wu et al., 2006) and CRC tumor cells have demonstrated VEGF as a differential survival factor in response to hypoxia and chemo resistance (Samuel et al., 2011, Calvani et al., 2008).

2.5 Immunology and Tumor Immunogenicity

The concept that inflammation is linked to cancer has for many years been an accepted paradigm of some cancer types dating back to Virchow's observation in the 19th century (Table 5). Epidemiological studies suggest that depending on geographical location, cancer can be attributable to infections or chronic inflammation, from 3% in more developed regions (e.g. North America) to 33% in less developed regions (e.g. Sub-Saharan Africa) suggesting environmental factors as well as bacterial or viral infections influence cancer development (de Martel et al., 2012). Further to the predisposition of cancers by the inflammatory response, it also later forms the environment of most neoplastic tissues regardless of causality. Tumor related inflammation is one of the recent emerging and enabling hallmarks of cancer and there has been a resurgence of interest in how tumors can influence the immune system and re-educate immune cells for malignant purposes.

Table 5. Association between inflammation and cancer risks (Balkwill and Mantovani, 2001, de Martel et al., 2012)

Malignancy	Inflammatory stimulus condition
Colorectal	Inflammatory bowel disease (IBD)
Cervical	Papillomavirus
Ovarian	Pelvic inflammatory disease/tissue remodeling
Bladder	*Schistosoma haematobium*
Gastric	*H pylori* induced gastritis
Stomach	*H pylori*
Oesophageal	Barrett's metaplasia
Hepatocellular	Hepatitis virus (B and C)
Bronchial	Silica, asbestos, cigarette smoke
Mesothelioma	Asbestos
Hodgkin's lymphoma	Epstein-Barr virus (EBV)
Kaposi's sarcoma	Human herpes virus type 8

Immune cells that feature prominently in cancer related inflammation include cells from the myeloid lineage such as macrophage (MΦ), neutrophils, dendritic cells (DCs), natural killer (NK) cells, myeloid-derived suppressor cells (MDSCs) and cells from lymphoid progenitors such as B-cells and T cells. Cells of the immune system are highly plastic and have the ability to facilitating tumor growth by suppressing the immune response, or control malignant disease by promoting the activation of the immune response.

The immune response to tumors varies considerably, however it is characteristic of most solid tumors to have some degree of infiltration of different immune cells types. Macrophages, mast cells, granulocytes and myeloid derived suppressor cells (MDSCs) are found infiltrating tumors as well as at the invasive margins, whereas lymphocytes appear more specific. B cells and T cells, in particular CD8+ cytotoxic T cells, are mostly located at the invasive front, however can also be in the tumor core (Fridman et al., 2012). In colorectal cancer, the T stage (from TNM staging) is inversely proportional to the density of infiltrating CD4+ memory T cells and CD8+ cytotoxic T cells, that is the higher the T stage the lower the density of T cells (Halama et al., 2011). The fact that immune cell composition in and around tumors differ from tumor type to tumor type as well as from patient to patient, it prompts the question if the immune contexture of tumors could also impact the clinical outcome to different treatments.

2.5.1 Cell-Mediated Immune Response – Macrophages and T Cells

Macrophages, along with DCs and neutrophils, are phagocytes and are critical elements in the bridge between the innate and adaptive immune systems. Macrophage precursors go from the bone marrow into the blood stream as monocytes and migrate into tissues such as the lung (alveolar macrophages) or intestines, to perform important homeostatic functions. Alternatively monocytes respond to specific chemokines, infiltrating damaged or infected tissue and differentiate according to environmental cues. Monocytes can be activated, or polarized, either classically into what is known as the M1 phenotype typically induced by pro-inflammatory effectors such as GM-CSF and IFN-γ; or alternatively into the M2 phenotype induced by factors such as IL4 or IL10 and have anti-inflammatory properties (Figure 15) (Leidi et al., 2009). M1 macrophages are considered pro-inflammatory and target direct pathogen clearance in addition to indirect recognition via Fc receptors. M2 macrophages are further subdivided into M2a, M2b and M2c and are generally considered immune-regulating and commonly thought to have anti-inflammatory properties (Hao et al., 2012). Tumor-associated macrophages (TAMs) are usually classified as M2 macrophages as they express the CD163 marker. However due to the unique microenvironment of tumors they do not form a single uniform population and have been shown to display overlapping features of the macrophage subsets. In general, TAMs are considered as a poor prognostic marker as they have been shown to promote tumor progression and metastasis (Mantovani, 2002), however this can be tumor type dependent as TAMs in CRC can be a good prognostic marker (Dumont et al., 2008, Nardin and Abastado, 2008, Ong et al., 2012). In contrast to the accepted theory of M2 being immunosuppressive, it has also been shown that under certain conditions they have an increased ability for non-specific phagocytosis (Leidi et al., 2009) suggesting an overlap of some functional abilities.

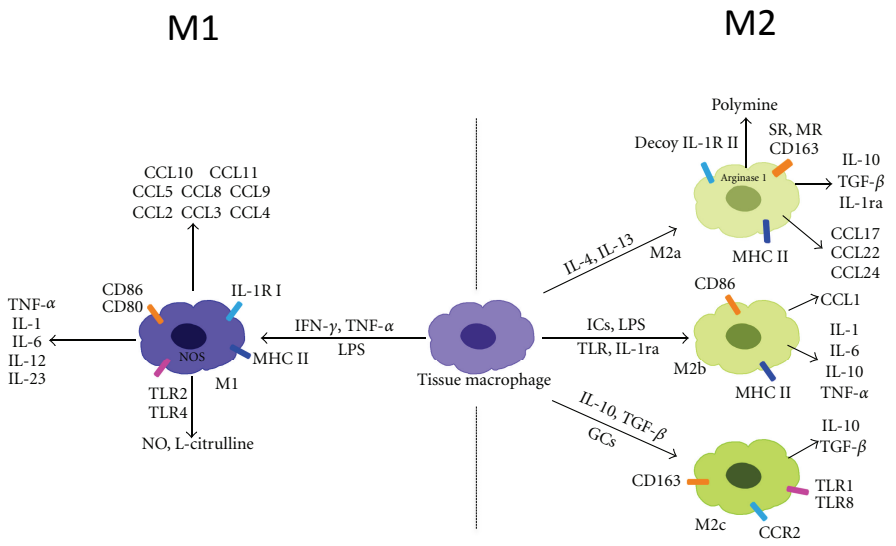


Figure 15. Classically activated (M1) macrophages and alternatively activated (M2) macrophages. Each sub-set of macrophages are polarized by different cytokines and express different combinations of cytokines. M2 macrophages can be further divided into M2a, M2b and M2c macrophages depending on both inducing stimuli as well as expressing cytokines. Adapted from (Hao et al., 2012).

Once phagocytes have engulfed their target they present antigens using (major histocompatibility complex) MHC class II molecules to naïve helper T cells (T_H) in the branch of the adaptive immune response. T cells recognize the antigen via the T cell receptor (TCR), thereby activating the T cell and inducing proliferation through autocrine IL2 secretion becoming T_H0 cells. T_H0 cells then differentiate into either T_H1 or T_H2 cells depending on the cytokine microenvironment, and are involved in the cell-mediated immune response with CD8+ cytotoxic T cells (Figure 16). There are also other minor T cell subtypes with distinct effector and regulatory functions including T_{regs}, T_H17, $T_H\gamma\delta$ and T_H9 (Wilson et al., 2009). In tumor biology, T_H1 is considered anti-tumor and T_H2 and T_{regs} are thought of as immunosuppressive and are activated by M1 or M2 macrophages respectively. This interaction between macrophages and T cells operates bi-directionally, activating a type of feedback that educates the microenvironment to support or destroy tumor development. However these cell types are not terminally differentiated and can exist in an intermediate state, being skewed towards one phenotype but not with all characteristic features of the phenotypic extremes (Biswas and Mantovani, 2010).

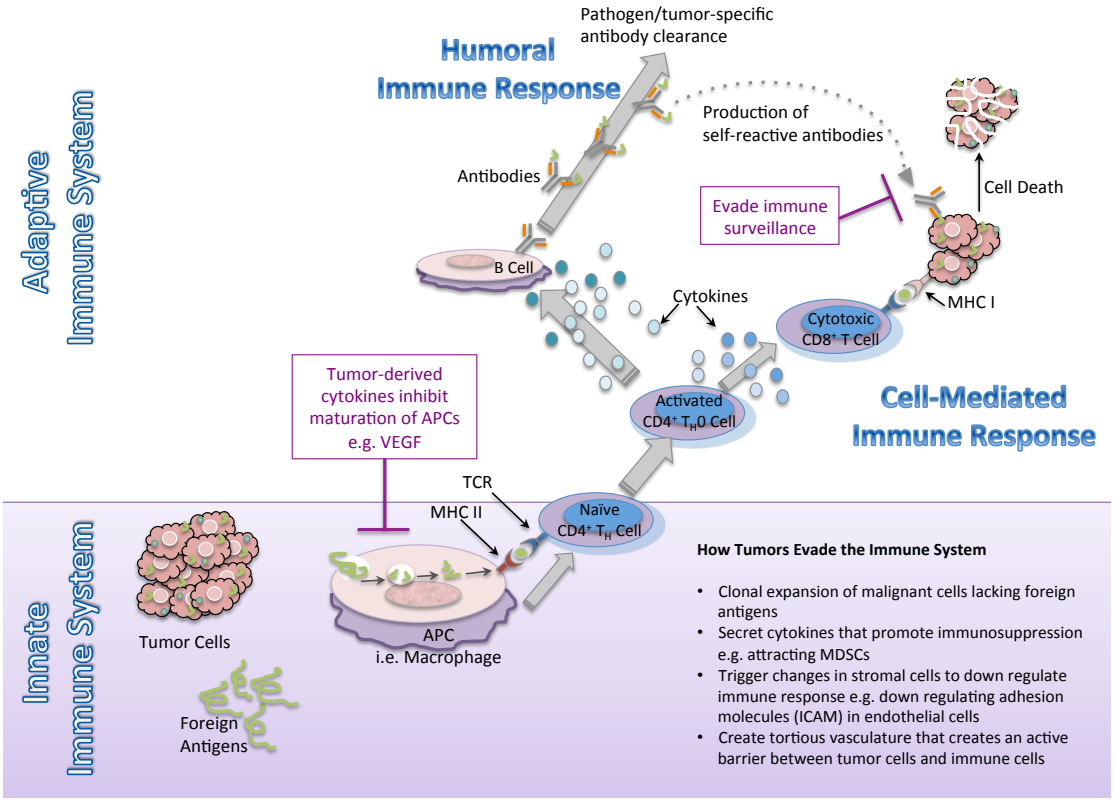


Figure 16. Activation of the immune system and how tumors evade destruction. MHC I/II, Major Histocompatibility Complex I/II; APCs, Antigen Presenting Cells; TCR, T Cell Receptor

2.5.2 *VEGF and the Immune System*

There is accumulating evidence that VEGF can have a significant effect on the immune system. Immune cells have a high degree of plasticity and are broadly categorized as either proinflammatory or immunosuppressive. VEGF can modulate the immune system within both of these realms, which Broxmeyer and colleagues showed in *in vitro* studies by adding VEGF to bone marrow derived myeloid progenitor cells. VEGF could enhance the colony growth of mature granulocytes and macrophage populations however, suppressed the expansion of more immature progenitor cells (Broxmeyer et al., 1995). In corollary studies, VEGF is described as negatively affecting the maturation potential of dendritic cells (Gabrilovich et al., 1996) and these immature dendritic cells subsequently failed to stimulate a T cell response (Almand et al., 2001). VEGF also acts as a mitogen for monocytes, hematopoietic progenitor cells and transendothelial polymorphonuclear neutrophils (Barleon et al., 1996, Bautz et al., 2000, Lee et al., 2002), suggesting a function in the potential activation of the immune system.

VEGF is a crucial regulator of the tumor microenvironment, necessary for the function of various cell types in addition to endothelial cells, that express VEGF receptors such as

dendritic cells (DC) (Gabrilovich et al., 1998), T cells (Ohm et al., 2003, Terme et al., 2013b, Terme et al., 2013a) monocytes and macrophages (MΦ) (Linde et al., 2012). These cell types not only respond to VEGF, but in some cases also regulate its expression in either an autocrine or paracrine manner.

VEGF is a critical factor in hematopoiesis and hematopoietic stem cell survival. Proliferation and differentiation of hematopoietic progenitor cells has been associated with increasing levels of VEGF expression that induced reciprocal GM-CSF expression in bone marrow derived endothelial cells, likely resulting in a paracrine loop supporting the survival and function of both cell types (Bautz et al., 2000). Furthermore, adding to the complex biology in pathological conditions, it has also been established that tumor-derived VEGF can modulate extramedullary hematopoiesis in the liver and spleen (Xue et al., 2009) and promote stemness of cancer stem cells (CSCs) in perivascular niches (Beck et al., 2011).

2.6 Treatment of Colorectal Cancer

Standard of care for colorectal cancer depends largely on the stage of the tumor, from surgical resection with a curative intent in early stages to palliative radiotherapy and/or chemotherapy to prolong survival in more advanced stages. Chemotherapy forms the backbone of systemic treatment, in addition to targeted agents such as antibodies against growth factors (Van Cutsem et al., 2014). The treatment combination prescribed is dependent on many factors including the treating physician, stage of tumor, previous chemotherapy, adverse events or tumor specific mutations, for example *KRAS* mutations are contraindicated for EGFR inhibitors (Lievre et al., 2006). Together with endogenous assaults that continually damage DNA, many anti-cancer drugs are genotoxic or target the cell cycle and induce DNA lesions in hyperproliferative cells, therefore preferentially targeting tumor cells. In the treatment of cancer, these agents attempt to inhibit further replication and transcription of malignant cells and activate cell death in an endeavor to control the disease.

There are a multitude of drugs used to treat cancer that are DNA damaging agents, engaging tumor cell's DNA repair pathways: MMR, NER, BER, HR, NHEJ, and FA. Importantly if tumor cells are deficient in any of these repair pathways, this can have an impact on the sensitivity to certain types of treatments, both positively and negatively (Figure 17). Promising target candidates in development have been those directed towards inhibiting DNA repair (e.g. PARP inhibitors) that show improved outcomes in subsets of patients. For example, hereditary *BRCA1* and *BRCA2* mutations are important predictors of therapy with

platinum compounds and PARP inhibitors through synthetically lethal mechanisms (Edwards et al., 2008, Sakai et al., 2008).

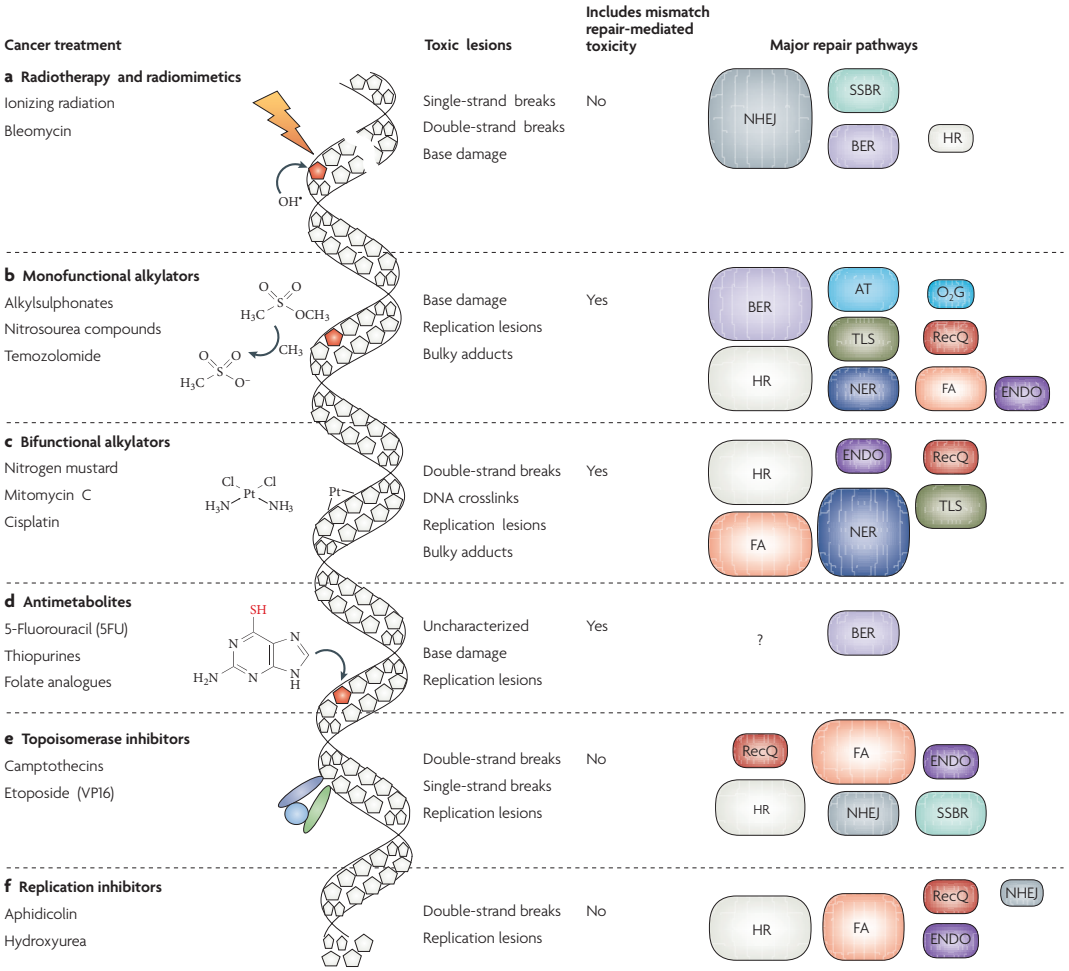


Figure 17. Common cancer treatments and DNA repair pathways responsible for repairing specific toxic lesions. Used with permission from (Helleday et al., 2008)

A number of inhibitors targeting DDR pathways have been or are in development, working by sensitizing tumor cells to cytotoxic agents to promote apoptosis through bypassing cell cycle effector kinases such as ATM or CHK1. However, likely due to the broad activity of these key pathways, lack of specificity of the compounds and the unfavourable cytotoxicity profile, this has not translated beyond preclinical studies or early clinical trials (Sausville et al., 2014, Matthews et al., 2007).

As part of the multi-modality treatment of cancer, drugs targeting survival pathways have been developed such as growth factor inhibitors, including EGFR and VEGF antibodies. By

using a combination of drug targets, the aim is to synergistically enhance the mode of action compared to each individual drug, to more selectively target cancer cells. Two of the most common combinations of chemotherapy for CRC are FOLFOX (5-Fluorouracil (5-FU), Leucovorin (LV) and Oxaliplatin) and FOLFIRI (5-FU, LV and irinotecan) with or without bevacizumab (Avastin).

Colorectal cancer, as well as other cancer types, is no longer considered a single disease entity and steps have been taken to further refine the definition of CRC subtypes and subsequently, fine-tune treatment options for patients. MSI CRC has been a recognized as a distinct molecular subtype since the early 1990's (Aaltonen et al., 1993, Ionov et al., 1993, Thibodeau et al., 1993) and a number of studies have evaluated if MSI tumors respond differently to certain treatments compared to MSS tumors (Hemminki et al., 2000, Elsaleh et al., 2000, Ribic et al., 2003, Jover et al., 2006, Lamberti et al., 2007, Des Guetz et al., 2009). There is evidence that MSI tumors have a predominantly inflammatory phenotype although as Sadanandam et al. elegantly described, it is not excusive to this subtype (Figure 18B).

Different subtypes of CRC not only have variable disease free survival (Figure 18A), but also respond differently to certain chemotherapy regimens. Figure 18C shows that the stem-like and inflammatory subtypes have higher response rates with FOLFIRI chemotherapy compared to other subtypes and other studies have also described tumors displaying MSI respond better to irinotecan containing therapy (Kim et al., 2011, Vilar et al., 2008, Jacob et al., 2001, Magrini et al., 2002, Sadanandam et al., 2013). The improved response is thought to be due to inactivation of DSB repair genes through MSI in genes such as *MRE11A* and *RAD50* (Vilar et al., 2008).

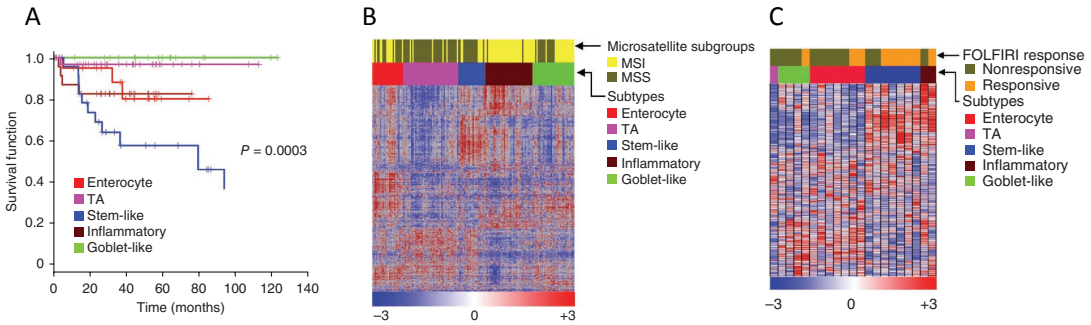


Figure 18. Molecular subtypes of CRC. Enterocyte: high expression of enterocyte specific genes. Transit amplifying (TA): heterogeneous subtype with variable expression of stem cell and Wnt target genes. Stem-like: found predominantly in crypt base with high expression of Wnt signaling targets, stem cell, myoepithelial and mesenchymal genes and low expression of differentiation markers. Inflammatory: comparatively high expression of chemokines and interferon related genes. Goblet-like: high MUC2 and TFF3 expression. (A) Disease free survival (DFS) Kaplan-Meier survival curves of CRC subtypes of untreated patients. (B) Microsatellite status relative to CRC subtype. (C) Response to FOLFIRI chemotherapy associated with CRC subtypes. TA, Transit amplifying. Modified with permission from (Sadanandam et al., 2013).

Certain challenges remain in classifying subtypes, in particular defining and reaching a consensus of candidate biomarkers to accurately and consistently identify different tumor classifications and therefore direct clinical treatment.

2.6.1 IRINOTECAN

Irinotecan (aka CPT-11) forms part of the FOLFIRI chemotherapy regimen and is a derivative of the natural alkaloid camptothecin that inhibits the activity of topoisomerase I. Irinotecan is a pro-drug that is converted into an activated form, SN-38, by plasma carboxypeptidase (Creemers et al., 1994, Rothenberg et al., 1993). Topoisomerase I relaxes supercoiled DNA during replication and transcription and it has been shown that levels of topoisomerase I are significantly higher in colon cancers compared to normal mucosa (Giovanella et al., 1989). Inhibition of topoisomerase I by irinotecan induces replication fork collapse eventually leading to DSBs, which preferentially triggers DSB repair by NHEJ or HR repair pathways (Helleday et al., 2008).

2.6.2 BEVACIZUMAB

In pathological angiogenesis, it as been postulated that restricting the ability of tumors to attract blood vessels subsequently reduces the availability of nutrients as well as any vehicle for metastases. The angiogenic pathway, primarily focusing on VEGF and it's receptors, has been the focus of much research to date, resulting in the approval of bevacizumab (Avastin ®) for the treatment of metastatic colorectal cancer (mCRC), metastatic renal cell carcinoma (mRCC), glioblastoma multiforme (GBM), cervical carcinoma, metastatic non-small cell lung cancer (mNSCLC) and metastatic breast cancer (mBC) (Hurwitz et al., 2004, Miller et al., 2007, Sandler et al., 2006, Tewari et al., 2014, Friedman et al., 2009, Escudier et al., 2007). Bevacizumab is a monoclonal antibody with a circulating half-life of ~20 days and sequesters the VEGF ligand from the VEGF receptors and various co-receptors. It has shown limited benefit as a monotherapy and as such, is used in combination with chemotherapeutics, or interferon in mRCC (Ferrara et al., 2005, Hurwitz et al., 2004). Additional compounds were later developed including small molecule RTKIs (receptor tyrosine kinase inhibitors) such as

Sorafenib and Sunitinib that are selective for VEGFRs, as well as VEGFR traps and neutralizing aptamers (Sennino and McDonald, 2012, Olsson et al., 2006).

In clinical trials, bevacizumab has shown the greatest efficacy in mCRC with an increase in PFS of over 4 months and just under 4 months in cervical cancer, compared to other tumor types such as NSCLC and BC that have more modest improvements in response (Ellis and Hicklin, 2008, Tewari et al., 2014). A number of studies have attempted to find biomarkers to predict the response to anti-angiogenic therapies by looking at both tumor as well as host factors. Markers investigated include circulating endothelial or tumor cells, circulating proteins such as VEGF, in particular small isoforms of VEGF, ANG2 and sVEGFR1, and SNP (single nucleotide polymorphism) analysis of genes within the VEGF signaling pathway such as *VEGFR1*. Tumor specific markers have also been evaluated such as abundance of $CD31^+$ vasculature within tumors along with receptor and adhesion molecule expression (e.g. NRP1 & ICAM1) (Lambrechts et al., 2013, Sennino and McDonald, 2012). Despite some prognostic relevance of some markers (e.g. SNPs), a predictive marker has not yet been validated in correlation with bevacizumab or other anti-angiogenic therapy.

Often vasculature differs between different cancers and also within the same cancers from adjuvant to the metastatic setting. This is perhaps one reason why trials in adjuvant CRC have generally failed to meet their primary end point of improved OS, although secondary endpoints of increased PFS/DFS were met (reviewed in (Van Cutsem et al., 2011)). Recently, a study in adjuvant CRC (NSABP C-08) that failed to meet its primary endpoint of improved OS, reported a post hoc Cox regression analysis that found a molecularly defined subset of patients did significantly benefit from the addition of bevacizumab to the therapy regimen. The study analyzed MMR status by IHC of MLH1 and MSH2 as well as $BRAF^{V600E}$ mutation status, and determined that MMR deficient tumors derived a significant benefit with bevacizumab with a hazard ratio of 0.52 (95% CI = 0.29 to 0.94; p = 0.03). Moreover, they reported the combined markers of deficient MMR and $BRAF^{V600E}$ mutation derived the most benefit (HR = 0.27; 95% CI = 0.08 to 0.94; p = 0.03), however this was a small subset of patients (n = 51) and there is a known association between MMR status and $BRAF^{V600E}$ so the analysis was purely exploratory (Pogue-Geile et al., 2013).

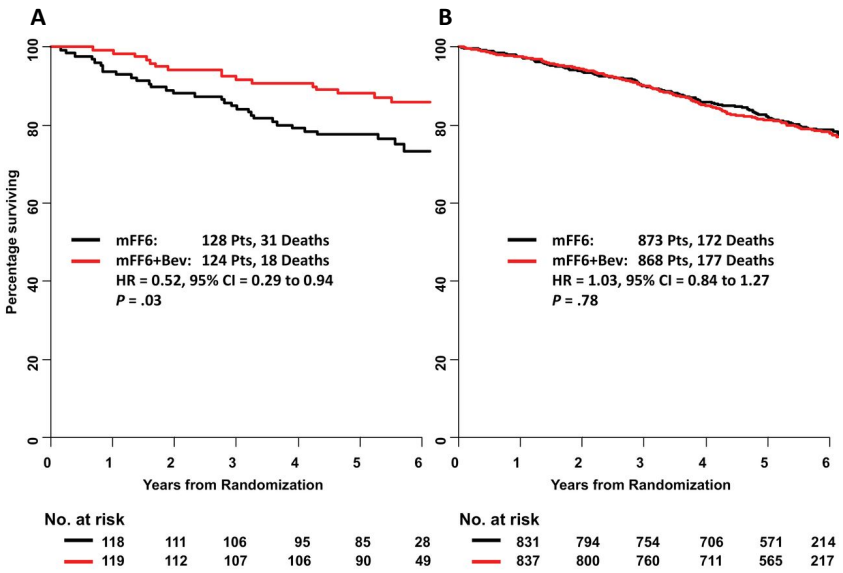


Figure 19. Overall survival in patients treated with bevacizumab in combination with chemotherapy dependent on MMR status: (A) MMR deficient and (B) MMR proficient. mFF6, modified FOLFOX6; Bev, bevacizumab; HR, hazard ratio; CI, confidence interval. Used with permission (Pogue-Geile et al., 2013).

The analysis of Pogue-Geile et al. and others highlights the necessity of better understanding CRC subtypes and the potential in improving treatment options that are selective for intrinsic resistance (Lievre et al., 2006) or a predictive beneficial response (Vilar et al., 2008, Pogue-Geile et al., 2013) based on an accepted classification system. The data also suggest that there is an underlying mechanism between MMR status and VEGF signaling, which warrants further exploration.

3 OBJECTIVES

The response to anti-angiogenic therapy in cancer, has failed to show an extensive or lasting response in unselected patient populations. Initial endeavours to identify those patients who have a beneficial response, have focused on the obvious targets of the angiogenic pathways as well as changes in tumor microvessel density. Tumor cells express the VEGF receptors and the VEGF ligand, suggesting that the tumor cells could also be instructive or directly affected by the inhibition of angiogenic pathways. Thus, the primary objective of the work presented in this thesis was to determine if patients who respond to anti-angiogenic treatments could be identified based on tumor specific molecular markers. The aim was to elucidate if the *in vitro* inhibition of VEGF by bevacizumab had a cell autonomous effect on tumor cells in an autocrine or paracrine manner and identify the molecules involved in such interactions. Furthermore, a potential synergistic effect with a DNA damaging agent was explored in terms of tumor cell survival, apoptosis, DNA repair and signaling changes to evaluate the contribution of VEGF signaling in these mechanisms. Factors that are responsible for these effects and interactions are expected to serve as candidates for novel and improved biomarkers

4 MATERIAL & METHODS

4.1 REAGENTS:

10 x PBS: 1.4 M NaCl, 27 mM KCl, 80 mM Na_2HPO_4, 18 mM KH_2PO_4

Potassium phosphate buffer: Prepare two solutions: (1) 100 mM K_2HPO_4 (MW = 174.17 g/mol) and (2) 100 mM KH_2PO_4 (MW = 136.09 g/mol). Add solution (2) to solution (1) until it reaches a pH of 7.8, resulting in a 100 mM Kphosphate buffer.

Luciferase Assay Buffer: 20 mM Tricine (MW = 179.2 g/mol), 1.07 mM $(MgCO_3)_4Mg(HO)_2.5H_2O$ (MW = 485.7 g/mol), 2.67 mM $MgSO_4$ (MW = 246 g/mol), 0.1 mM EDTA, 33.3 mM DTT in dH_2O.

Luciferase Assay Reagent: 500 µM CoA (MW = 821.3 g/mol), 500 µM Luciferin (MW = 280.3 g/mol), 1 mM ATP (MW = 507.2 g/mol) in luciferase assay buffer. Stored at -80 ° C .

CoA 100x stock (50 mM): 100 mg in 4.88 ml H_2O

ATP 50x stock (50 mM): 100 mg in 3.94 ml H_2O

4.2 CELL CULTURE AND REAGENTS:

Cell lines were obtained from ATCC (American Type Culture Collection) and cultured routinely in complete RPMI (RPMI-1640 media (Gibco, UK) supplemented with 10 % FBS, 1 % Penicillin/Streptomycin & 1 % Glutamax). Cells were routinely monitored for mycoplasma detection by PCR. Once the cells reached ~80 % confluency, they were washed with PBS and de-attached with Trypsin/EDTA. Cells were cultured either under normoxic conditions (5 % CO_2) or hypoxic conditions (BioSpherix, C-Chamber and adapter plate) (1 % O_2) at 37°C.

CRC	BC	RCC	NSCLC
COLO-205	BT-549	786-O	A549
HCC-2998	HS-578T	A498	EKVX
HCT-15	MCF7	ACHN	HOP-62
HCT-116	MDA-MB-231	CAKI-1	HOP-92
HT-29	MDA-MB-468	RXF-393	LXFL529
KM12	T-447D	RXF-631	NCI-H23
KM20L2		SN12C	NCI-H226
SW-620		SN12K1	NCI-H460
		TK-10	NCI-H522
		UO-31	

As part of the research pertained to DNA repair, HCT-116 was used that is MMR deficient due to a hemizygous mutation in MLH1 resulting in a truncated, non-functioning form of the MLH1 protein. For comparison, the isogenic MMR proficient sub-line HCT-116+ch3 was used, complemented with chromosome 3 containing the wild type MLH1. The presence of MLH1 was verified by western blot analysis. HCT-116+ch3 cell line was kindly provided by Prof. Dr. Winfried Edelmann at the Einstein College of Medicine NY, USA.

4.3 Generation of Stable Tumor Cell Lines

Stable tumor cell lines expressing a luciferase construct driven by a CMV promoter, were generated by transfecting the tumor cells with a commercial luciferase lentivirus plasmid (EX-hLUC-Lv114, GeneCopoeia, USA). At approximately 70% confluency in a 6 well plate, 500 µl of lentivirus was added to 1 ml of complete RPMI (minus antibiotics) and 1.5 µl polybrene for 12 hours, media was then changed to complete RPMI (minus antibiotics) for a further 3 days in controlled conditions.

4.4 Proliferation Assay

Cells were seeded from 2,000-10,000 cells/well in 96 well plates and allowed to adhere for 24 hrs. Media was changed at 0hrs to relevant conditions. Each time point was processed by

incubation for 2 hrs with 5mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) (Sigma, Germany). The media was removed and the resulting formazan crystals were dissolved with 10 % (4 mM) HCl, 0.1 % NP40 in isopropanol or DMSO for 1-2 hrs. The plates were read in ELISA reader at wavelength of 570 nm with a background filter of 650 nm.

4.5 mRNA Extraction

Cells were homogenized using peqGOLD TriFast (PeqLab, Germany) and the separation of phases was performed with chloroform. The solution was centrifuged for 5 minutes at 12,000 x g at RT. The upper phase was transferred to a new eppendorf tube and 500 µl of isopropanol was added and incubated for 5-15 minutes on ice to precipitate RNA. The solution was centrifuged for 10 minutes at 4 ° C at 12,000 g. The pellet was washed twice with 1 ml 75% ethanol. After centrifugation, the pellet was resuspended in DEPC-H_2O and incubated for 10 minutes at 50 ° C before RNA concentration was calculated using NanoDrop 1000 (Thermo Scientific).

For macrophages, RNA extraction was performed using RNeasy mini kit (Qiagen #74106) as per manufacturers instructions. 400 ng of RNA was reverse transcribed into cDNA using Superscript III First Strand Kit (Invitrogen #4309155) prior to use for qPCR.

4.6 cDNA Transcription

Total RNA (1-2 µg) was reverse transcribed to cDNA for real time polymerase chain reaction using RevertAid^{TM} First strand cDNA Synthesis Kit (Thermo Scientific, Germany) according to manufacturers instructions. Briefly, 1-2 µg/ml of total RNA was made up to 11 µl with 1 µl of oligo (dT)18 primer added and incubated at 65 ° C for 5 minutes. 4 µl of 5x Reaction Buffer, 1 µl RiboLock RNase Inhibitor (20 U/µl), 2 µl 10 mM dNTP Mix and 1 µl of RevertAid M-MuLV Reverse Transcriptase (200 U/µl) was added to each sample and incubated at 42 ° C for 60 minutes. The reaction was terminated by heating to 70 ° C for 5 minutes.

4.7 RT-qPCR

Each well contained 25 mN dNTPs, 15 U/µl Taq-Polymerase, 10 pmol/µl of both reverse and forward primers, SYBR green (1:2000, Ambrex Bioscience, Germany) Repro Fast buffer (10x: 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl (pH 8.8) 100 mM KCl, 20 mM $MgSO_4$, 1 % Tritonx100, 1 % BSA) and cDNA. The cDNA was amplified by qRT-PCR using a 40-cycle two-step program on an Eppendorf Masercycler *ep realplex* (95°C for 15 s denaturing, annealing and extension at 60° for 30 s terminated with a melting curve 50-95°C for 5 min).

4.8 qPCR

qPCR amplification in macrophages was performed using TaqMan Fast Universal PCR Master Mix (Life Technologies #4352042) with primer mix purchased from Integrated DNA Technologies. Beta-Actin was used as a housekeeping gene to compare relative expression. Per reaction 5 µl of Taqman fast universal PCR master mix, 0.5 µl primer mix, 2.5 µl RNase free H2O and 2 µl of cDNA per well in duplicate were performed. The PCR cycler was set with 95 °C 20 sec, then 40 cycles of 95 °C 3 sec and 60 °C for 30 sec.

4.9 Primers

Primers were designed using the NCBI reference sequence (ncbi.nlm.nih.gov) of the gene of interest ensuring product spanned an exon-exon junction (if applicable). Sequence specificity was assessed using the Basic Local Alignment Search Tool (BLAST) and ordered from Sigma-Aldrich (Germany).

Table 6: Primers for qRT-PCR

Gene	Sequence
HPRT	F:AAGATGGTCAAGGTCGCAAG R:GTCAAGGGCATATCCTACAACAA
VEGF	F:TACCTCCACCATGCCAAGTG R:GCTGCGCTGATAGACATCCA
$VEGF_{189}$	F:TATAAGTCCTGGAGCGTTCCC R:CTCGGCTTGTCACATCTGC
$VEGF_{165}$	F:AGATAGAGCAAGACAAGAAAATCCC R:CTCGGCTTGTCACATCTGC
$VEGF_{121}$	F:GTGTGTGCCCACTGAGGAG R:GCCTCGGCTTGTCACATTT
VEGFR1	F:CTTCACCTGGACTGACAGCA R:ACAGCTGGAATGGCAGAAAC
VEGFR2	F:ACAACCAGACGGACAGTGGT R:AGTCAGGCTGGAGAATCTGG
NRP1	F:CAAAACCAGCAGACCTGGAT R:CATTATGCCAACAGGCACAG
GLUT1	F:GCTTTGTGGCCTTCTTTGAA R:CAGAACCAGGAGCACAGTGA
CXCR4	F:GGATATAATGAAGTCACTATGGGAAAA R:GGGCACAAGAGAATTAATGTAGAAT
TLR3	F:GCCGTCTATTTGCCACACAC R:CATGATTCTGTTGGATGACTGCT
CCL2	F:TCTGTGCCTGCTGCTCATAG R:GGGCATTGATTGCATCTGGC
CCL7	F:ATGAAAGCCTCTGCAGCACT R:TAATCCCAACTGGCTGAGCA
CCL8	F:GCTCAGCCAGATTCAGTTTCCA R:TCTTGTGTAGCTCTCCAGCC
CXCL12	F:TGCCAGAGCCAACGTCAAG R:CAGCCGGGCTACAATCTGAA
ICAM1	F:CGGCATTACTGCACACGTCAGCCG R:GTTCCCTGGACGGGCTGTTC
IL8	F:AGGGTTGCCAGATGCAATAC R:GCAAACCCATTCAATTCCTG
JUN	F:GGACAGGCTTGTTAGCTTCG R:ATTCCCCAGCCTGCTTATTT

Primers used to measure expression of genes in macrophages, were commercially obtained from Integrated DNA Technologies (IDT – Leuven, Belgium):

Table 7: PCR Primers for gene expression in macrophages

Gene	IDT Probe Number
ACTB	45430259 94763534
CXCL9	81505346
NOS2	81505362
CCL18	81505342
CXCR4	81505350
CXCL10	81505370
MRC1	81505378

4.10 Protein Extraction of Subcellular Fractions

Cells grown in a monolayer were gently scraped using PBS and spun down for 5 min at 1000 rpm. Cells were resuspended in 400 µl of hypotonic lysis buffer (10 mM Hepes (pH 7.9), 10 mM KCl, 100 µM EDTA, 100 µM EGTA in H_2O). After resuspension, NP-40 was added to a final concentration of 0.6 % and centrifuged at 10,000 rpm for 30 s at 4°C. Supernatant (cytoplasmic fraction) was removed and nuclei were resuspended in buffer (20 mM Hepes (pH 7.9), 400 mM NaCl, 25 % glycerol, 100 µM EDTA, 100 µM EGTA in H_2O). Solution was placed on a shaker for 15 min at 4°C. Nuclear fraction was recovered after centrifugation at 14,000 rpm for 5 min at 4°C. Proteins were used immediately or stored at -80°C.

4.10.1 Protein Concentration Determination (BCA Method)

BCA protein assay kit from Thermo Scientific Peirce (USA) was used as per manufactures instructions. Briefly, a standard curve was determined from a 2 mg/ml albumin standard in PBS (starting concentration was 800 µg/ml) and samples were diluted in PBS up two 100 µl, then incubated with BCA reagent for 30 mins. The 96 well plate was read on a

spectrophotometer/plate reader at 550 nm and protein concentration for each sample was determined against the standard curve.

4.11 Western Blot

For full cell lysates, cell pellets were lysed in 20 nM HEPES (pH 7.8), 500 mM NaCl, 5 mM $MgCl_2$, 5 mM KCL, 10 µg/ml Leupeptin, 10 µg/ml Aprotinin, 1 mM PMSF, 200 µM Na_3VO_4 and 0.1 M NaF for up to 4 hrs on ice. Protein concentration was measured using the Bradford solution (Thermo Scientific) based on a standard curve of known protein concentrations. Proteins were denatured by heating to 95 ° C for 5 minutes, before equal amounts were added to each well of a 8-14 % SDS-PAGE gel (dependent on the size of the protein of interest) with loading buffer (0.25 % Bromophenol blue, 10 mM EDTA, 15 % Ficoll). The samples were separated by electrophoresis and run towards the positive electrode in running buffer (50 mM Tris, 38.5 mM Glycine and 0.1 % SDS) to separate proteins by size. Proteins were transferred from the gel onto a nitrocellulose membrane by electroblotting in blotting buffer (25 mM Tris, 192 mM Glysine and 20 % methanol). To reduce the background as well as unspecific binding, membranes were blocked for 1 hr at room temperature in blocking buffer (10 mM Tris (pH 7.9), 150 mM NaCl, 0.1 % Tween (TBS-T) and 5 % BSA). Primary antibodies were diluted at given concentrations in blocking buffer and incubated overnight at 4 ° C. After rinsing membranes in TBS-T, a species specific secondary POD coupled antibody was incubated for 1 hr at room temperature before visualization by enhanced chemiluminescence (ECL) on the Kodak Image Station 4000MM. Where indicated band intensity was quantified using Carestream Molecular Imaging software (v 5.4.2) against the internal standard.

4.11.1 Antibodies

The following antibodies were used and purchased from:

Cell Signaling Technology (Germany): phospho-Akt (Ser473), phospho-H2AX (Ser139), phospho-p53 (Ser15), phospho-PKC (pan), phospho-Chk1 (Ser345), DAPK1, phospho-p73 (Tyr99), phospho-JNK (Thr183/Tyr185), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), VEGFR2 (55B11)

Santa Cruz (USA): Neuropilin (H-286), VEGFR1 (Flt1) (C17), β-Actin (C4)

Sigma Aldridge (Germany): α-Tubulin (T6074), Vinculin (V9131)

BD Bioscience (Germany): Cleaved PARP (Asp214) (552596)

Chemicon (Germany): anti-mouse IgG (AP124P), anti-Rabbit IgG H+L

4.12 Flow Cytometry (Florescence-activated cell sorting – FACS):

4.12.1 Propidium Iodide

Cells were treated as indicated and trypsinized prior to being fixed in 80 % ice-cold ethanol. Samples were stored at -20 ° C for up to 48 hrs. Samples were resuspended and permeabilized in 38 mM sodium citrate, 24 µg/ml RNaseA and incubated for 30 minutes at 37 ° C then kept on ice. Just prior to detection, samples were labeled with 54 µM of the DNA-binding fluorescent molecule propidium iodide (Sigma-Aldrich, Germany) for cell cycle analysis, on the premise that equal amounts of dye bind proportionally to the amount of DNA in a cell. Cells were detected on a Beckman Coulter FC500, using CXP Cytometer software.

4.12.2 Cell Surface Protein Detection

Tumor cells were harvested using Accutase™ solution (Sigma-Aldrich, Germany) to maintain a single cell suspension before being stained with antibodies for Neuropilin-1 and VEGFR2 (CD304 (BDCA-4/Neuopilin-1)-APC and CD309 (VEGFR2/KDR)-APC) (Miltenyi Biotec, Germany) in cold buffer (PBS (pH 7.2), 0.5 % BSA, 2 mM EDTA). Measurements were performed using BD FACS Canto II flow cytometer and FlowJo software (v 8.8.6).

To determine T cell sub populations after indicated treatments, cells were harvested with ice cold PBS and centrifuged at 1500 rpm for 5 min at 4 ° C. Cells were resuspended in 100 µl PBS (2% FBS) and non-specific antibody binding was blocked by 2 µl of FCγII/IIIR (CD16/32 2.4G2 clone, BD Bioscience, US) per $1x10^5$ cells for 15 min on ice. 1 µl of each antibody was added to cells and incubated up to 30 min in the dark, (eBioscience: CD4 – FITC (0CT4 clone), CD25 – APC (BC96 clone), from BioLegend CXCR3 (CD183) – Pacific Blue (clone TG1) and CCR4 – PE from R&D Systems). Cells were rinsed with 4 ml of PBS (2%FBS) and centrifuged at 1500 rpm 5 min 4 ° C. Cells were resuspended in 200 µl PBS (2% FBS) and analyzed within 1 hour on a BD FACSAria II (BD Bioscience).

4.13 Soft Agar Growth Assay

Equal volumes of 1.0 % agar dissolved in H_2O, and 20% FCS RPMI media was mixed to form a 0.5% base layer of agar in a 6-well plate. Upon polymerization, a top layer of agar was added with an equal amount of 0.7% agar and 20% RPMI containing $2.5x10^4$ of treated cells per well. The polymerized soft agar was supplied with 100 µl of RPMI media with and without 0.25 mg/ml of bevacizumab every 2 days. After 14 or 28 days of growth under standard culture conditions (37°C, 5 % CO_2), then stained with 500 µl of 0.005 % crystal violet. The numbers of cell colonies were counted using a dissecting microscope at 20-fold magnification. Images were converted to binary 8-bit data files and colonies were counted using ImageJ (v1.48s).

4.14 MicroArray

Equal amounts of extracted RNA were used for microarray analysis using GeneChip Human Gene 2.0 ST Array from Affymetrix (CA, USA). RNA quality and purity was determined by electrophoretic Bioanalyzer with the RNA Integrity Number (RIN) in all samples above 9.30 (on a scale of 1(lowest) – 10 (highest)) as an indication of RNA integrity, with an average of 9.83 across all samples. The arrays were scanned using a GeneChip Scanner 3000 from Affymetrix and results were analyzed and assessed for quality using different R packages from the Bioconductor project (www.bioconductor.org).

4.15 Senescence

Cellular senescence was determined by the β-galactosidase assay, whereby treated cells were rinsed with PBS then fixed in freshly prepared 3.7 % formaldehyde (in PBS) at room temperature for 5 mins. After washing twice with PBS, X-gal substrate staining solution (1 mg/ml X-gal in dimethylformamide (DMF), 40 nM citric acid ($C_6H_8O_7$), 5 mM potassium ferrocyanide ($K_4Fe(CN)_6$), 5 mM potassium ferricyanide ($K_3Fe(CN)_6$), 150 mM NaCl and 2 mM $MgCl_2$) was added and incubated at 37 ° C (5% CO_2) overnight. Cells were viewed in PBS on a bight field microscope.

4.16 Mutation Assay

The pCAR-OF and pCAR-IF reporter vectors were a gift from the lab of Bert Vogelstein (Addgene plasmid #16627 & 16628) (Nicolaides et al., 1998). Cells were transfected with the reporter vectors as well as a firefly luciferase construct as a transfection control. Transfection was performed using X-tremeGENE (Roche, Germany) according to manufactures instructions 48 hrs prior to treatment. pCAR-OF contains a dinucleotide (CA) repeat that when transcribed without error, will not result in β-galactosidase expression i.e. β-galactosidase remains out of frame (OF). β-galactosidase expression is restored in pCAR-OF transfected cells from a frame-restoring mutation, such as an insertion or deletion. pCAR-IF is used as a control, which has β-galactosidase in frame (IF) and results in constitutive expression (Figure 20). Both plasmids are under the control of a CMV promoter and β-galactosidase expression was measured using the Dual-Light® System chemiluminescent reporter gene assay system for the combined detection of Firefly Luciferase and β-Galactosidase from Applied Biosystems (MA, USA). Mutation fraction was calculated as a ratio of β-galactosidase activity of pCAR-OF transfected cells to pCAR-IF activity.

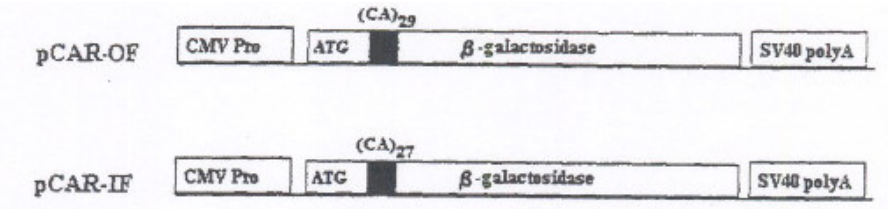


Figure 20. Reporter vector constructs of pCAR-OF and pCAR-IF

4.17 Macrophage isolation

4.17.1 Murine peritoneal macrophage collection

Macrophages were isolated from the peritoneal cavity of C57BL/6 wt mice. The animals were anesthetized with 50 µl of intramuscular injection of Nembutal before the addition of 5 ml of complete RPMI using a 5 ml syringe with a 26 G needle. The macrophages were collected after approximately 5 minutes of gentle friction from the peritoneum. The animals were

sacrificed by cervical dislocation. The Institutional Animal Care and Research Advisory Committee of the K.U. Leuven approved all mouse procedures.

The macrophages were collected in media were spun down for 5 minutes at 1100 rpm at 4 °C before being pooled and counted. At this stage, any samples with an indication of contamination (i.e. from blood) were excluded. 800,000 macrophages were seeded per well in a 24 well plate and allowed to adhere for 4 hours. 30 mins before conditioned media was added, 1 µl of Fcγ Block (CD 16/CD 32) was added per well to avoid unspecific binding activity. After 16 hrs of incubation, 350 µl of RTL lysis buffer (Qiagen, Germany) was added to the macrophages and frozen at -80 ° C.

4.17.2 Human monocytes isolation from whole blood (PBMC – peripheral blood mononuclear cells) – Healthy Volunteers

4.17.2.1 Quick protocol

Monocytes were isolated by positive selection. CD 14 Dynabeads (Invitrogen, Norway) were washed in isolation buffer (Ca/Mg free PBS, 0.1% BSA, 2 mM EDTA, pH 7.4) in a 15 ml falcon tube. Tube was placed in DynaMag (Invitrogen) magnet and supernatant was aspirated. CD 14 beads were resuspended in original amount of buffer before use. Peripheral blood was extracted from healthy volunteers and resuspended in 62 µl of dynabeads per 10 ml of blood and incubated at 4 ° C for 20 minutes on a rotating carousel. Tubes were then place in the DynaMag for 2 minutes and the supernatant was aspirated. The beads/monocytes were resuspended in buffer and transferred to a new tube. The wash step was repeated 2 further times before counting and seeding for use or differentiation on sterilin plates. Approximately 1 x 10^6 monocytes were extracted per 10 ml of whole blood.

4.17.2.2 Long protocol

Whole blood was diluted 1:2 with PBS/EDTA (DPBS Ca^{2+}/Mg^{2+} free, 1 mM EDTA) then 20 ml was added extremely slowly to 10 ml of Lymphoprep™ (Axis Shield, Norway), which allows cell separation by isoosmotic density centrifugation. Erythrocytes and granulocytes sediment through the Lymphoprep ™ medium and the mononuclear monocytes and lymphocytes remain at the medium/sample interface after 20 mins RT centrifugation at 1200

g (acceleration 6, deceleration 1). The peripheral blood mononuclear cells (PBMCs) at the interphase were collected and washed with PBS/EDTA then centrifuged 250 g for 12 minutes at 4 ° C (with deceleration set at 3). The cells were washed twice more with cold Buffer M (DPBS (Ca^{2+}/Mg^{2+} free), 2 mM EDTA and 0.5 % BSA) and centrifuged at 300 g for 10 minutes at 4 ° C before being pooled (to the equivalent of 20 ml of starting whole blood into one 15 ml falcon tube – approximately 3-5x10^6 monocytes/ml). After final wash, cells were resuspended in 100 µl of cold Buffer M and 25 µl of CD14 Microbeads (MACS Miltenyi Biotech, Germany) were mixed by pipetting and incubated for 15 minutes at 4 ° C. Cells were made up to a total volume of 2 ml then centrifuged at 300 g for 10 minutes at 4 ° C. The pellet was resuspended in 500 µl of Buffer M. LS columns (MACS Miltenyi Biotech, Germany) were placed in the MACS Multistand magnet and prepared by rinsing with 3 ml of cold Buffer M before the cells were added. Negative cells were washed through the column with 4 x 3 ml of Buffer M (flow-through of unlabeled/CD14 negative cells kept for further isolation). Column was removed from the magnetic stand and 5 ml of buffer was added and immediately flushed through the column with the plunger to isolate CD 14^+ cells. Monocytes were counted and either seeded for immediate use or allowed to differentiate into macrophages for 48-72 hrs in IMDM (10% FCS, Glutamate, 1% penicillin/streptomycin) (Gibco, Germany). Average yield per 10 ml of whole blood was 2.5 - 5 x 10^6 monocytes. Purity was determined by running a full blood count on an aliquot of isolated cells and noted to be routinely > 90% monocytes

4.18 $CD4^+$ T Cell Isolation

T-cells were isolated by negative selection using the EasySep® Human $CD4^+$ T Cell Enrichment kit. Whole blood was processed as above (long protocol) and $CD14^+$ cells (as monocytes are also weakly positive for CD4) were removed from PBMCs. The CD14 negative cells collected were centrifuged at 300 g for 10 minutes at 4 ° C and pooled (equivalent to 40ml of whole blood) to a total volume of 1 ml of Buffer M in a 5 ml round bottom falcon tube. 50 µl/ml of EasySep® Human $CD4^+$ T Cell Enrichment Cocktail was added to the cells and incubated at room temperature for 10 minutes. EasySep® D Magnetic Particles were vortexed before adding 100 µl/ml to the cells and incubated for a further 5 minutes at room temperature. Cell suspension was made up to a total volume of 2.5 ml with Buffer M and gently mixed. Tube was placed in the Purple EasySep® Magnet for 5 minutes then in one continuous motion, the magnet and tube were inverted pouring the desired negative cells into a fresh tube for further use. Approximate yield from 10 ml of whole blood

was 5 x 10^6 T cells. Purity was determined by running a full blood count on an aliquot of isolated cells and noted to be > 95% lymphocytes.

4.19 $CD8^+$ T Cell Isolation

CD8 positive cells were purified using the human $CD8^+$ T Cell Isolation Kit (MACS Miltenyi Biotec, Germany) as per manufacturers protocol. PBMC were isolated by lymphoprep gradient centrifugation and washed in PBS (1 mM EDTA). After centrifugation at 300 x g for 10 min at 4 ° C, cells were resuspended in 40 µl of Buffer M and 10 µl of $CD8^+$ T Cell Biotin-Antibody Cocktail was added per $1x10^7$ total cells. After cells were incubated for 5 min at 4 ° C, a further 30 µl of Buffer M was added with 20 µl of $CD8^+$ T Cell MicroBead Cocktail and incubated for 10 min at 4 ° C. Volume was adjusted to 500 µl before proceeding to magnetic separation with MACS LS columns. The flow-through of the cell suspension was collected after one 3 ml wash with Buffer M, representing the enriched CD8+ T cells.

4.20 Phagocytosis Assay/Luciferase Assay

To determine treatment-dependent phagocytic activity and macrophage-dependent cytotoxicity, treated tumor cells (5 x 10^3 cells/well), which were stably transfected with luciferase construct (EX-hLUC-Lv114, GeneCopoeia, USA), were incubated overnight (18 hrs) in a 96 well plate with and without differentiated macrophages (5 x 10^4 cells/well). Measure was luciferase luminescence of tumor cells. After incubation, supernatant was discarded and wells were rinsed with PBS before adding 35 µl of luciferase lysis buffer (100 mM potassium phosphate buffer pH 7.8, 0.2% Triton X-100 and 0.5 mM DTT added fresh before use) and frozen at -80 ° C. Upon thawing on ice, a 1:3 ratio of sample to substrate ratio was used to determine cell number using an automated injector after a 5 sec delay. To normalize each well, cell viability was determined with WST-1 (Roche, Germany).

As a control for phagocytosis, the same cells were seeded without macrophages to get the phagocytic index (= (luciferase luminescence + MΦ)/(luciferase luminescence – MΦ)) to give rise to remaining viable tumor cells.

4.21 T Cell Suppression Assay

Freshly isolated CD4+ T Cells were added to increasing concentrations of differentiated macrophages (+/- tumor conditioned media). After 24 hrs, cells were pulsed with ^{3}H-thymidine (PerkinElmer) and incubated for another 18 hrs before incorporated radioactivity was measured.

4.22 Monocyte Migration Assay/Transwell Migration Assay

Migration was assessed applying a modified Boyden chamber assay consisting of cell culture inserts and a polycarbonate filter (polyethylene Terephthalate (PET), pore size 8 μM; Cell Biolabs, Heidelberg, Germany). One hundred microliters of freshly isolated monocytes suspension (2.5×10^5 cells) was added to the upper wells in 1% RPMI. Chambers were incubated for 12 h at 37°C in a 5% CO_2 atmosphere with tumor conditioned media in the lower chamber. As monocytes do not adhere to the membrane, membranes were scraped and cells in lower chamber were counted for quantification.

4.23 Transient Transfection of siRNA

Cells were seeded in a 6 well plate to approximately 60-80% confluency at time of transfection. The first solution of 50 μl lipofectamine (Invitrogen, USA) and 250 μl Opti-mem (Gibco, Germany) was mixed with 200 nM of siRNA (prevalidated siRNA: siRelA #s72859, or scrambled siRNA (#46-2002) Ambion - Invitrogen) in 250 μl Opti-mem and incubated for 15 mins at RT. The mix was added drop wise to the cells in 2 ml of complete RPMI (minus antibiotics) for 12 hours before media change to complete RPMI. Cells were used 24-48 hrs after transient transfection.

4.24 TransAM® NFκB Family Transcription Factor Assay Kit

Activation of NFκB subunits was assayed using an ELISA-based kit according to manufacturer's instructions (Active Motif, Belgium). Briefly, the kit contains an NFκB consensus binding site immobilized on a 96-well plate. Nuclear extracts with activated NFκB

hetero- or homodimers bind specifically to the oligonucleotide and are detected by antibodies targeted directed against the NFκB subunits (p65, p50, p52, c-Rel or RelB). The addition of a secondary antibody conjugated to horseradish peroxidase (HRP) provided a colorimetric readout detected by spectrophotometry at 450 nm with a reference wavelength of 655 nm.

5 RESULTS

5.1 THE EFFECTS OF BEVACIZUMAB MONOTHERAPY ON TUMOR CELL LINES

Bevacizumab was developed to target tumor vasculature as a universal mechanism to halt tumor progression, by way of inhibiting VEGF activity in endothelial cells. Various studies have also established VEGF receptor expression on tumor cells in addition to hypoxic-induction of VEGF, leading to the hypothesis that VEGF may act as in a paracrine or autocrine manner on tumor cells. As a first step to determine if bevacizumab alone had any effect on tumor cells, a series of experiments were employed to measure known VEGF-induced functions derived from the large body of literature in endothelial cells. Tumor cell lines were selected derived from various tumor types based on preliminary experiments as well as publically available microarray data of high relative gene expression within the angiogenic pathway, of both receptors and ligands (Shankavaram et al., 2007). In light of these data, the following cell lines were selected for further analysis: NSCLC: H522 & HOP62, CRC: HCT-116, HT-29 & KM-12, RCC: A498 and BC: HS-578T & MDA-MB-231. The results pertaining to data published in (Hein and Graver, 2013) have been included here in full, however experiments using CRC and NSCLC cell lines were performed by Melanie Hein and included in this thesis with permission in the interest of providing a complete data set.

5.1.1 VEGF INDUCTION UNDER HYPOXIC CONDITIONS

In an effort to promote a VEGF-dependent response, the cells were treated in hypoxia to induce maximal VEGF expression in order to stimulate any potential autocrine or paracrine mechanisms. All experiments were performed in media with reduced additive nutrients to stimulate autocrine or paracrine mechanisms. In order to determine if the cells responded to hypoxia, the induction of known HIF1 targets, *VEGF* and *GLUT1*, were investigated. It was established that the majority of cell lines responded to hypoxia with the up-regulation of *VEGF* and/or *GLUT1* (Figure 21A-B). It was found that two cell lines MDA-MB-231 and A498, did not show regulation of either gene, indicating a lack of sensitivity to the hypoxic environment. In the set of selected cell lines, cells were identified that were sensitive to hypoxia by up-regulating *GLUT1* and *VEGF*, those who responded to hypoxia with increased *GLUT1* expression but not *VEGF*, and those that did not modify the expression of either gene.

With these results we investigated how the comparable expression of VEGF and subsequent inhibition with bevacizumab could affect cellular functions.

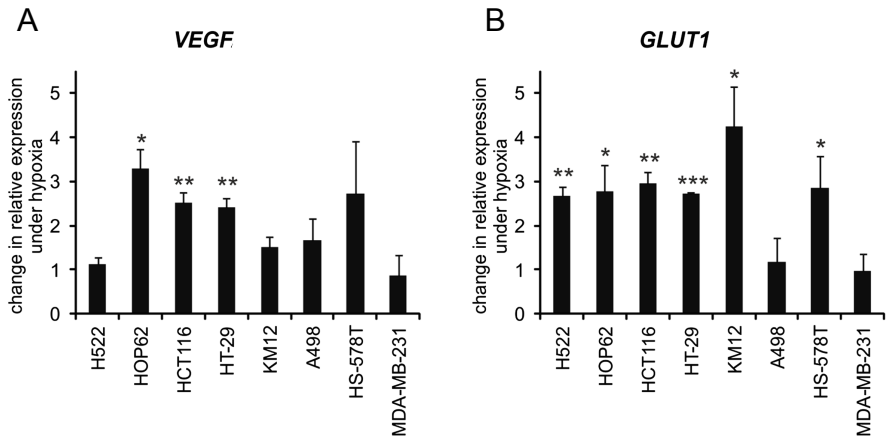


Figure 21. Induction of VEGFA (A) and GLUT1 (B) expression under hypoxic conditions. The expression is a measure relative to the normoxic control. A similar figure was published in (Hein and Graver, 2013). Results of NSCLC and CRC cell lines were kindly provided by Melanie Hein and for the sake of completeness, additionally presented in this thesis.

5.1.2 VEGF Receptor Expression on Tumor Cells

For the ability of the induced VEGF to promote an autocrine or paracrine effect, VEGF receptor expression was evaluated. Clear evidence of receptor expression was demonstrated of at least one known VEGF receptor or co-receptor (VEGFR1, VEGFR2 and Neuropilin 1 (NRP1)) on each of the cell lines. We showed protein expression from full cell lysates cultured in normoxia, hypoxia and hypoxia with bevacizumab treatment. It has been described that the majority of VEGF activity is triggered by VEGFR2 activation and four of our cell lines showed VEGFR2 protein expression, H522, HOP62, HCT-116 and MDA-MB-231 (Figure 22 (A)). It was difficult to deduce changes in VEGFR2 by western blot so potential changes in cell surface protein expression by flow cytometry were evaluated (Figure 22 (B&C)). The results did not detect any regulation of VEGFR2 in terms of localization or abundance by either hypoxia or hypoxia with bevacizumab treatment.

Most of the cell lines had VEGFR1 expression, except H522, and all tumor cell lines showed NRP1 protein expression to varying degrees detected by western blot analysis. NRP1 expression did not significantly change by either hypoxia or hypoxia with bevacizumab in any of the cell lines in terms of cell surface expression or overall quantity and was in general more limited in expression on the cell surface compared to VEGFR2 (Figure 22 A-D).

Hypoxia up-regulated VEGFR1 expression in A498 by 1.8-fold however this was not attenuated by inhibition of VEGF by bevacizumab treatment. Concurring with the lack of effect seen with bevacizumab treatment, A498 did not show transcriptional regulation of either GLUT1 or VEGF, inferring VEGF does not regulate VEGFR1 under these conditions.

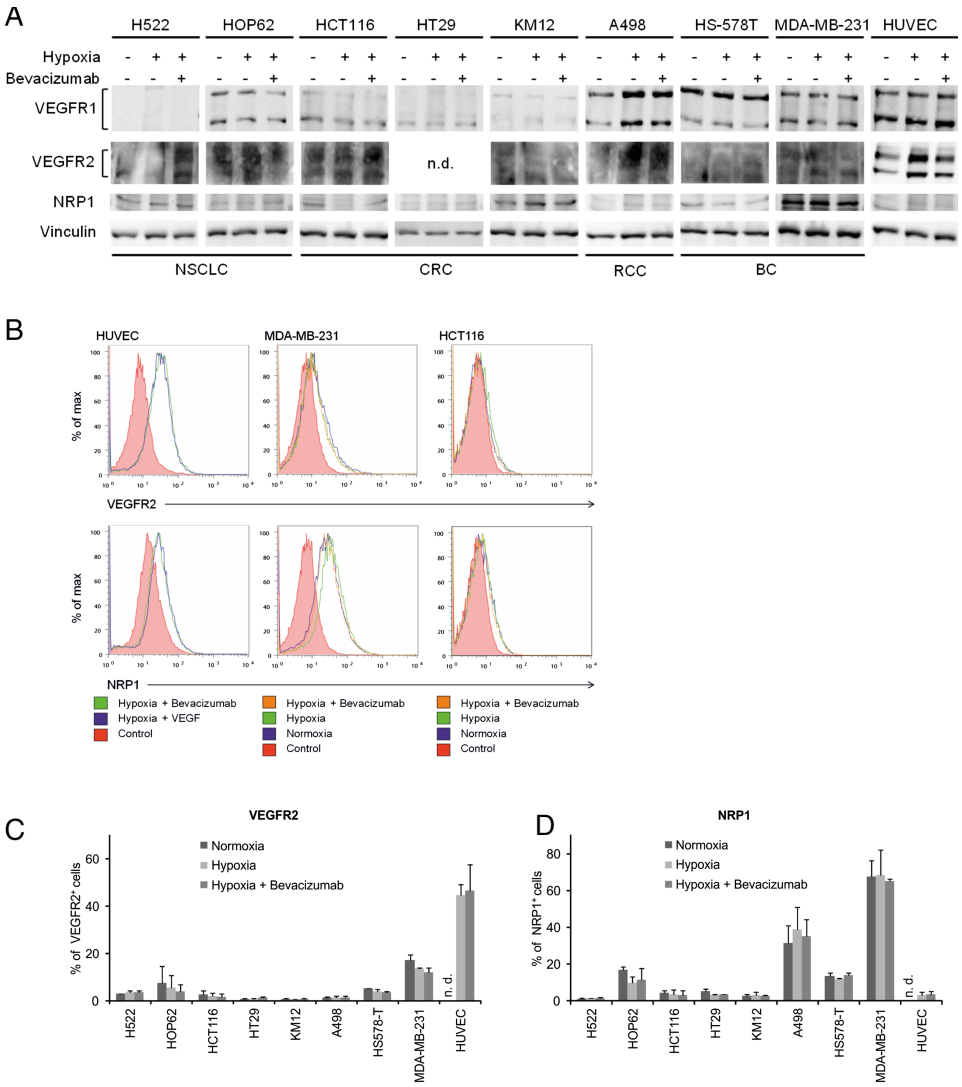


Figure 22 Expression of VEGF receptors and hypoxic VEGF induction in tumor cells. (A) Protein expression of VEGFR1, VEGFR2 and NRP1 was determined in tumor cells under normoxia and after 24 hours of hypoxia with or without bevacizumab. Vinculin was used as a loading control. (B) Cell surface expression of VEGFR2 and NRP1 as analyzed by flow cytometry. Quantification of VEGFR2+ (C) and NRP1+ (D) cell surface expression. A similar figure was published in (Hein and Graver, 2013). Results of NSCLC and CRC cell lines were kindly provided by Melanie Hein and for the sake of completeness, additionally presented in this thesis.

5.1.3 *Gene Expression Changes with Bevacizumab Treatment*

Failing to see significant effects of receptor proteins both in terms of overall abundance as well as localization, regulation of mRNA expression was evaluated for receptors and VEGF isoforms. The aim was to determine if regulation was induced by VEGF, by incubating cells in hypoxia to promote VEGF expression, then treating with bevacizumab to inhibit any potential response. Consistent with the protein analysis, there was also no marked change in mRNA expression in the genes evaluated including by those cell lines that significantly up-regulated *VEGF* under hypoxia. The VEGF isoforms were not regulated with the inhibition of VEGF by bevacizumab in any cell line, nor in HUVECs (Figure 23A). There was one exception of HS-578T that did significantly down regulate *NRP1* expression with bevacizumab in hypoxia 3-fold (Figure 23C). Other cell lines showed comparable down regulation, however did not meet the threshold of significance, including MDA-MD-231 (1.7-fold), HOP62 (1.9-fold) and HCT-116 (1.6-fold). This regulation did not correlate with any pattern of receptor expression or gene induction and no other discernible pattern was evident.

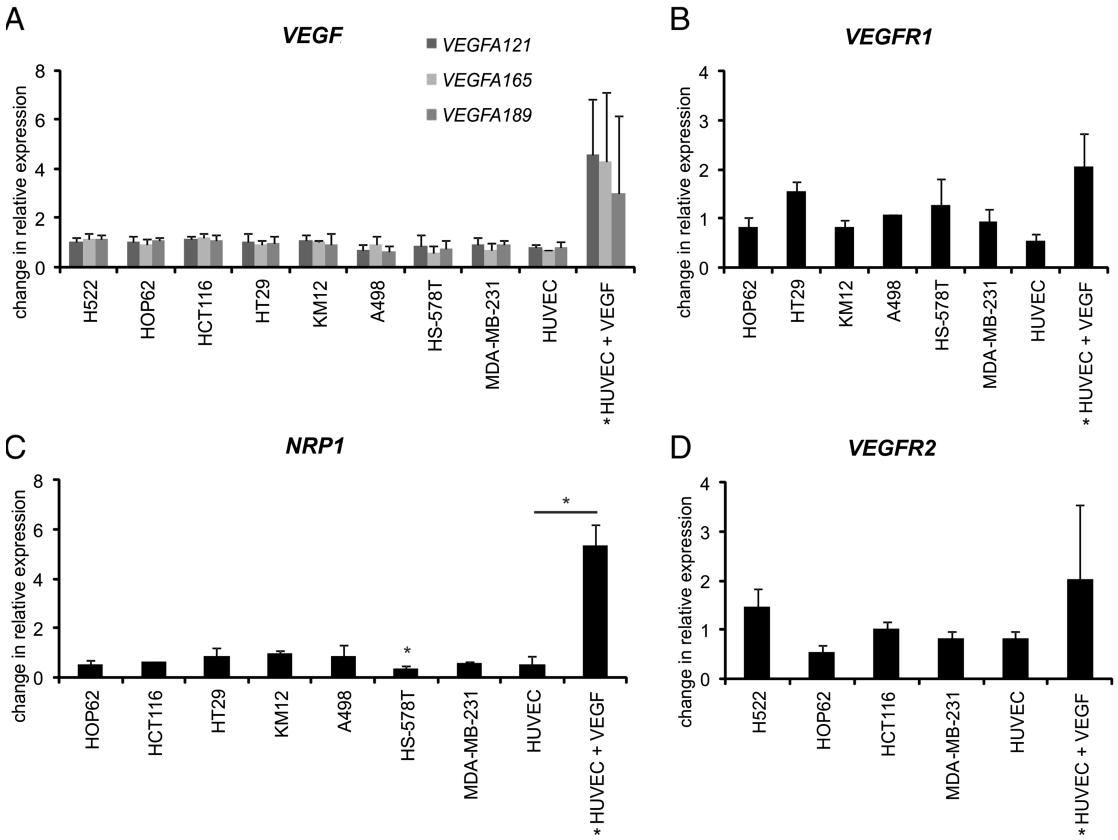


Figure 23. Gene expression analysis in bevacizumab treated tumor cells. Change in relative expression of (A) VEGF isoforms, (B) VEGFR1, (C) NRP1 and (D) VEGFR2 in bevacizumab treated cells after 24 hours of hypoxia versus untreated hypoxic cells. Only cell lines with detectable expression are included. * indicates

HUVECs were in addition stimulated with recombinant human VEGF (rhVEGF) in the absence of bevacizumab and normalized against untreated controls. A similar figure was published in (Hein and Graver, 2013). Results of NSCLC and CRC cell lines were kindly provided by Melanie Hein and for the sake of completeness, additionally presented in this thesis.

5.1.4 Tumor Cell Survival with Bevacizumab Treatment

As families of growth factors generally have a function in maintaining cell viability, the impact of VEGF as a survival factor in tumor cells was assessed and VEGF inhibition with bevacizumab. Both cleaved PARP (Poly-[ADT-Ribose]-Polymerase) and sub G1 fraction by FACS analysis of PI conjugated DNA were evaluated (Figure 24 A-C) as indicators of cell death. We showed that the apoptotic rate of the tumor cells was not significantly changed by bevacizumab, however the NSCLC cell lines H522 and HOP62, did interestingly appear to have decreased cleaved PARP and a lower percentage of cell in the sub G1 fraction with bevacizumab indicating that the inhibition of VEGF improved survival in hypoxic conditions to a minor degree. These results once again did not reach the level of significance and any conclusion was difficult to make beyond observation. With these data, it was analyzed if these moderate differences in survival as well as some expression changes could functionally alter cellular behaviour.

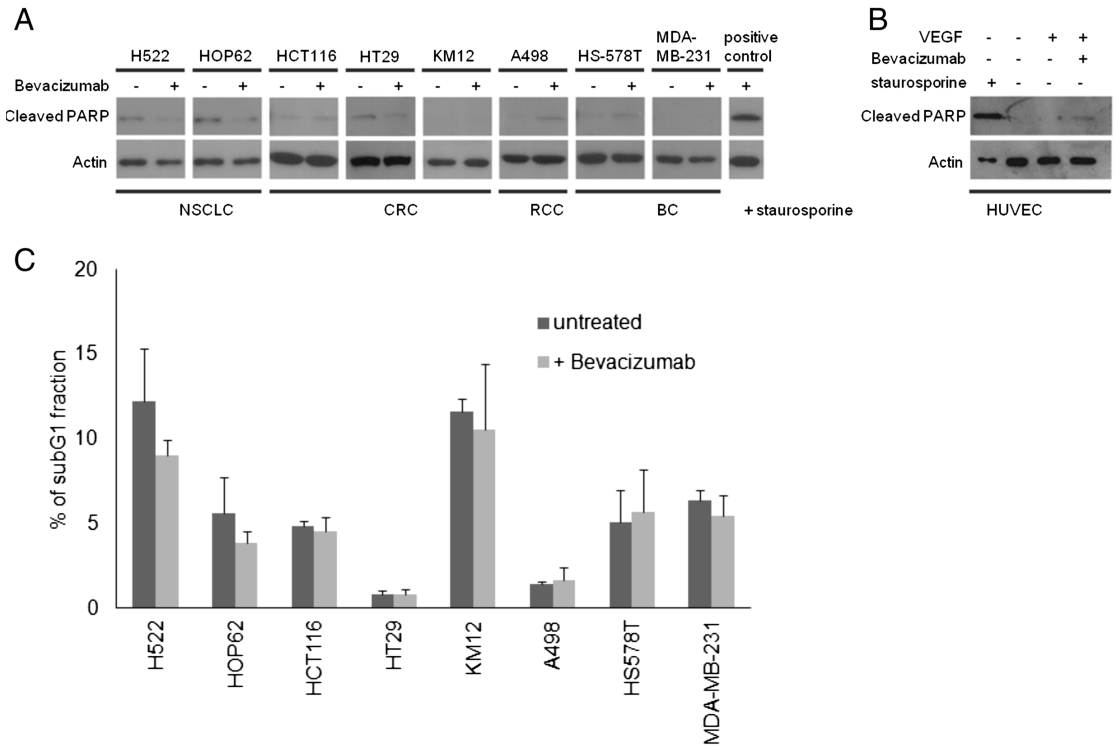


Figure 24. Tumor cell survival after bevacizumab treatment. Levels of apoptosis were determined in bevacizumab treated cells after 48 hours by (A) western blot analysis using an antibody against cleaved PARP. β-actin served as a loading control. As a positive control, cells were treated with staurosporine (0.15 µM) for 24 hours to induce apoptosis, which is shown for the tumor cell line KM12 and (B) HUVECs as an example. (C) Quantification of cellular sub G1 fraction after 48 hours of bevacizumab treatment. Cells were conjugated with propidium iodide and analyzed by flow cytometry. A similar figure was published in (Hein and Graver, 2013). Results of NSCLC and CRC cell lines were kindly provided by Melanie Hein and for the sake of completeness, additionally presented in this thesis.

5.1.5 Proliferation Changes with Bevacizumab Treatment

The activity of VEGF induced under hypoxia on the proliferation of tumor cells through VEGF receptors was assessed. The analysis was expanded beyond the initial cell lines and a panel of tumor cell lines was tested from the NCI-60 panel of well-characterized tumor cell lines. The proliferation rate of these cells was evaluated in hypoxia after 72h of continuous bevacizumab treatment. Of the 30 cell lines (Figure 25), only one cell line, HT-29 (CRC), showed reduced proliferation compared to the untreated control. Once again, there were some moderate differences that did not meet significance indicating that effects of VEGF inhibition by bevacizumab in tumor cells may be a gradual or time dependent effect.

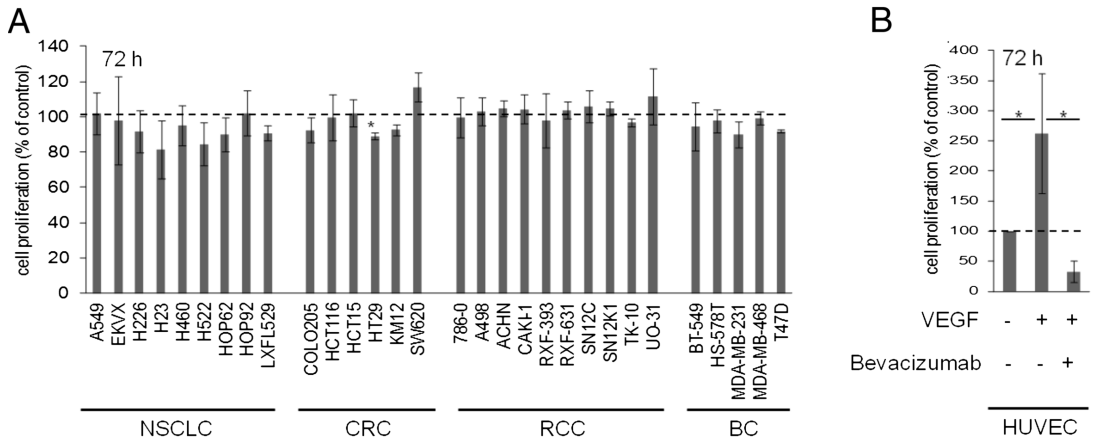


Figure 25. Tumor cell proliferation after continuous bevacizumab treatment for 72 hrs. (A) Tumor cell proliferation as a percentage of control under hypoxic conditions. Cells were cultured under hypoxia and serum starved for 72 hours. For comparison, (B) HUVECs were stimulated with rhVEGF and treated with bevacizumab as a control. A similar figure was published in (Hein and Graver, 2013). Results of NSCLC and CRC cell lines were kindly provided by Melanie Hein and for the sake of completeness, additionally presented in this thesis.

5.1.6 VEGF Dependent Cell Motility

VEGF has an established role in endothelial cell migration and this function was tested in the selected NCI-60 panel of cell lines in hypoxic conditions (Figure 26). Invasion of tumor cells *in vivo* relies on cellular motility, resultant and dependent on signaling from cytokines within the extracellular environment and has been linked to hypoxic conditions (Annabi et al., 2003, Fujiwara et al., 2007). It was established that by sequestering VEGF with bevacizumab, tumor cell migration did not diverge from the controls in the tumor cell line panel after either 6 hours (for highly motile cells), or 24 hours (for less motile cells).

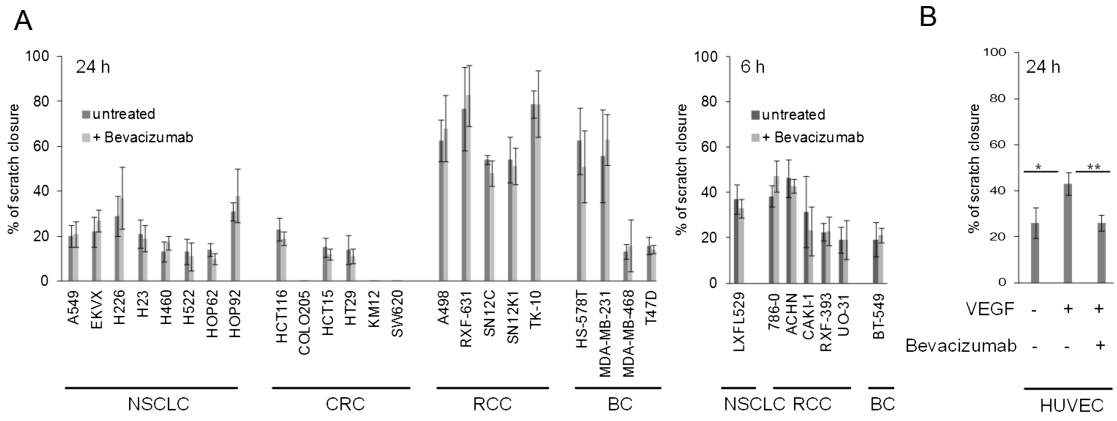


Figure 26. Tumor cell migration. (A) Migration of bevacizumab treated versus untreated cells after 24 or 6 hours of hypoxia. (B) HUVECs were treated with rhVEGF as an experimental control. A similar figure was published in (Hein and Graver, 2013). Results of NSCLC and CRC cell lines were kindly provided by Melanie Hein and for the sake of completeness, additionally presented in this thesis.

In the series of experiments, transcriptional changes, protein expression or localization changes or functional effects of inhibiting VEGF with bevacizumab in hypoxic conditions could not be established and subsequently the initial hypothesis was revisited.

5.2 The Effects of Combined Therapy on Tumor Cells – SN-38 and Bevacizumab

The conclusions of the tumor cell panel screen showed that there was not a universal function that could go in some way to explain the varied response seen to bevacizumab in a clinical

setting. To further advance the understanding of any potential cell autonomous effect of bevacizumab on tumor cells, the approach was reassessed to include a chemotherapeutic compound (SN-38 – a camptothecin derivative and the active metabolite of irinotecan) in the regimen. The rationale for which was derived from clinical practice whereby bevacizumab is administered in combination with various chemotherapies. It was decided to focus on CRC and in addition, MSI and MSS cell lines were selected, as there was a suggestion from unpublished data (at the time through personal communication with Prof. Paik) that mismatch repair status could influence the bevacizumab response seen in patients. In light of this new premise, an experimental plan was developed using and MSI cell line (HCT-116) and MSS cell lines (HCT-116+ch3 & HT-29).

The experiments first set out to investigate if administration sequence of VEGF inhibition by bevacizumab could sensitize tumor cells to the DNA damaging agent SN-38. A number of sequence options were evaluated, including pre-treatment of tumor cells with bevacizumab before DNA damage with SN-38, to determine if there was any sensitization of the cells to SN-38 by inhibiting VEGF. Furthermore, as MSI was a consideration, which is commonly as a consequence of non-functioning MMR, it was attempted to establish if VEGF inhibition by bevacizumab interfered with tumor cell stress responses or DNA repair, by treating cells during recovery from DNA damage. The treatment schedules employed are detailed in figure 27.

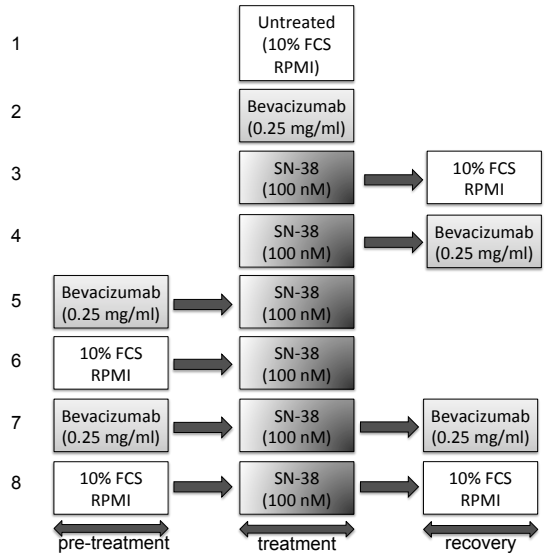


Figure 27. Tumor Cell Treatment Schedules. Pre-treatment of cells aimed to determine if VEGF inhibition sensitized tumor cells to SN-38 and treatment during recovery looked at VEGF activity in DNA repair.

5.2.1 *Tumor Cell Dose Response to SN-38*

Initial experiments with SN-38 focused on determining the appropriate dose *in vitro*. A dose response curve was established for the 3 selected colorectal carcinoma cell lines HCT-116 (MSI), mismatch repair proficient isogenic matched cell line derived from HCT-116, HCT-116+ch3 (MSS) and HT-29 (MSS) (Figure 28 A-C). Signaling pathways activated were evaluated in a dose dependent manner including phosphorylation of H2AX to detect DNA strand breaks, cleaved PARP1, which is indicative of apoptotic signaling, and phosphorylated p53 which acts as an axis in signal transduction in response to DNA damage and/or cell stress (Figure 28D). Cleaved PARP1, γH2AX and phospho-p53 all showed a dose dependent response to SN-38, however as the interest was in sensitization of tumor cells or DNA repair during recovery, for further experiments the dose 100 nM close to the IC_{50} of all cell lines was selected. This dose allowed the evaluation of any augmentation or attenuation of the apoptotic response with the inclusion of bevacizumab to the treatment schedule, without immediately triggering apoptosis.

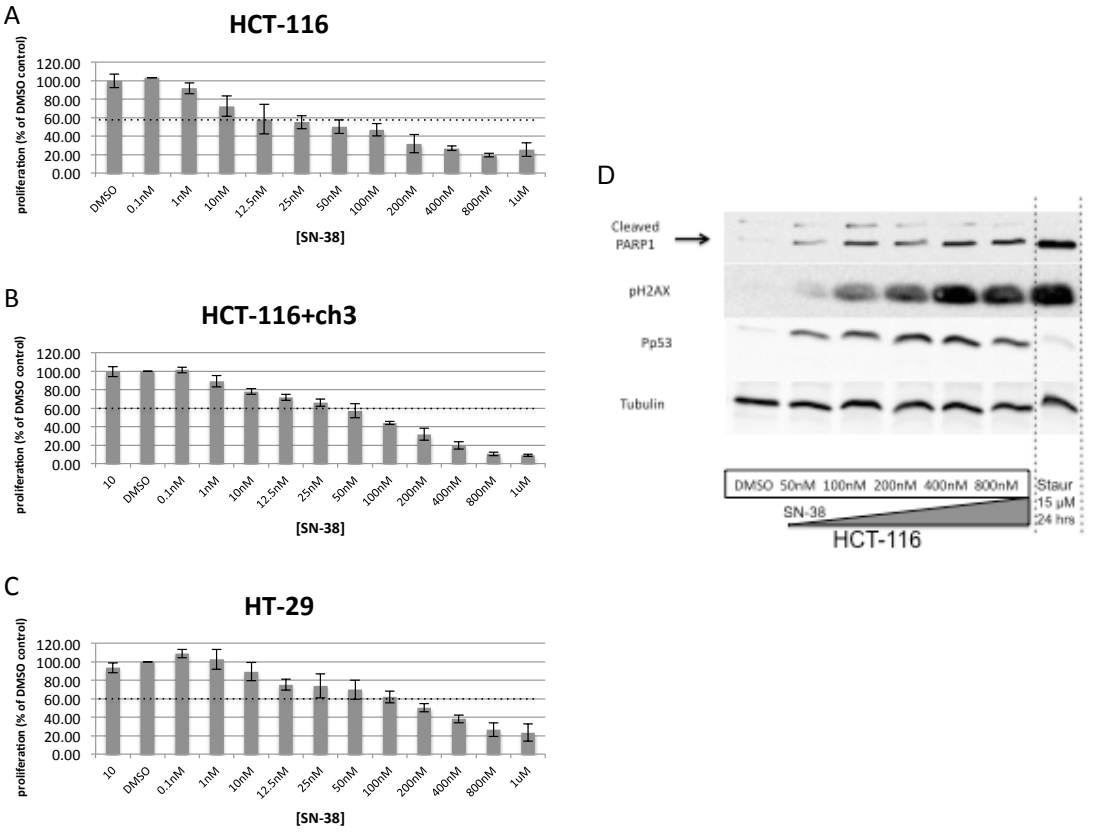


Figure 28. Dose response curve to SN-38 after 48 hours of continuous treatment of (A) HCT-116 (MSI), (B) HCT-116+ch3 (MSS) and (C) HT-29. (D) Phosphorylation of key cellular stress and DNA damage signaling proteins as a response to a dose escalation of SN-38 in HCT-116 (MSI). Staurosporine 15 µM for 24 hours as a control for the induction of apoptosis. Staur, staurosporine;

5.2.2 *Bevacizumab Before and After DNA Damage with SN-38*

In order to determine if there was an effect of bevacizumab in either priming tumor cells, or influencing the recovery of tumor cells different types of DNA damage were induced by SN-38, H_2O_2, TNFα treatment or UV exposure. SN-38 and UV activate the DNA damage response and DNA repair pathways, whereas H_2O_2 induces ROS dependent cell stress and TNFα activates the MAPK pathways, in particular p38, JNK as well as NFκB. To this end, tumor cells were either pre-treated for 24 hrs with bevacizumab, or DNA damage was induced with SN-38, oxidative stress with H_2O_2 and DNA breaks with UV, then allowed the tumor cells to recover with or without bevacizumab. The concentrations of each agent were a sub-lethal concentration selected to allow analysis of viable cells. Figure 29 shows the results of sequential experiments of all the treatment options showing no significant change in proliferation, either by priming the cells or through interference of the process of DNA repair by bevacizumab.

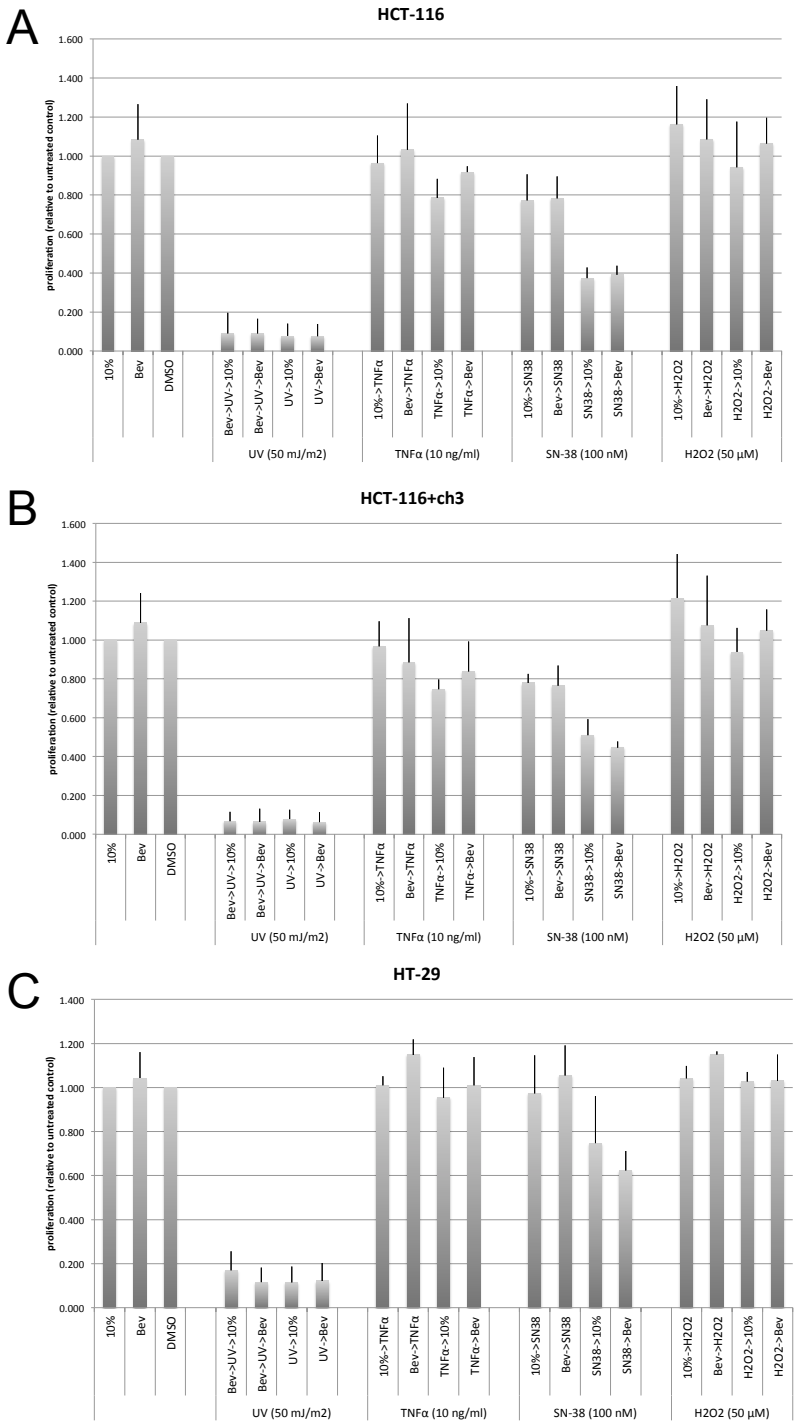


Figure 29. Cell proliferation in response to various types of stimuli measured by the MTT assay, by priming the cells as a pre-treatment (24 hrs) or altering recovery as a post-treatment (24 hrs) with bevacizumab. Tumor cell lines (A) HCT-116, (B) HCT-116+ch3 and (C) HT-29 were treated with UV-irradiation, TNFα, SN-38 and H_2O_2. None of the cell lines showed that bevacizumab could significantly alter proliferation as pre- or post-treatment.

It was attempted to establish if there was an induction of apoptosis in sub-confluent cells, under the same conditions either by sensitization through pre-treatment or in hindering DNA

repair as a post-treatment with bevacizumab. Fluorescence-activated cell sorting (FACS) analysis was performed on synchronized tumor cells under the various combinations of treatment with bevacizumab and SN-38. Overall, the analysis did not display any significant variation in cell cycle phases or rate of apoptosis compared to cells without pretreatment (not shown). When cells were subjected to short term SN-38 treatment (2 hours) then allowed to recover in normal media or media containing bevacizumab (Figure 30 A-B), a difference was noted in the HCT-116 cell line. Cellular recovery with bevacizumab appeared to reduce cells in G_1 and increase the number of Sub G_1 cells, indicative of fragmented DNA from apoptotic cells. However, after up to 5 independent experiments, this failed to reach significance. Furthermore, this effect was only seen after 72 hours and at earlier time points this trend was not noted. The other cell line HT-29 did not show the same trend.

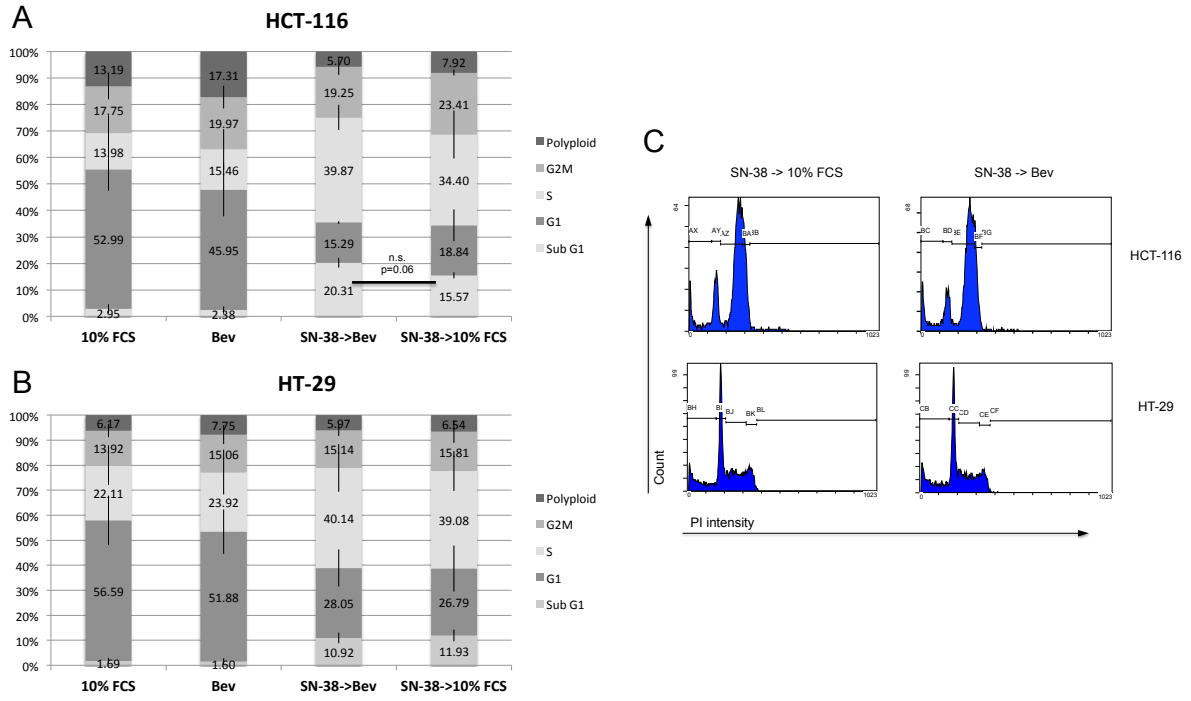
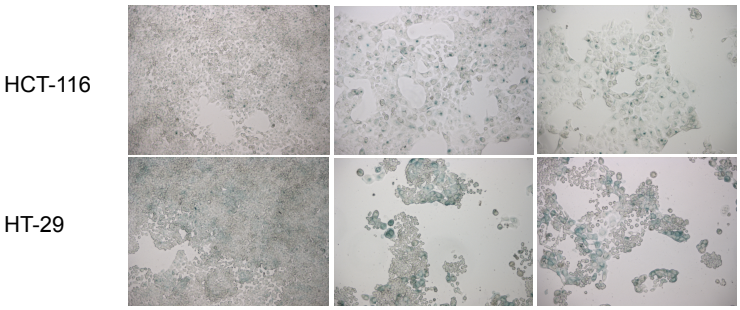


Figure 30. FACS analysis of synchronized cells treated with SN-38 (100 nM) to induce DNA damage. Cells were harvested and PI-conjugated 6, 24, 48 and 72 hours post DNA damage with recovery +/- bevacizumab (0.25 mg/ml). (A) HCT-116 and (B) HT-29 show percentage of cells in each phase of the cell cycle as a measure of PI intensity 72 hours post DNA damage. (C) Representative FACS profiles of cellular recovery at 72hrs. Bev, bevacizumab.

5.2.3 *Cellular Senescence after DNA damage*

It was clear from the FACS analysis that the cells arrested in G_2M in HCT-116 and G_1 in HT-29. Therefore it was questioned if the induction of senescence was in anyway modulated by bevacizumab treatment. β-galactosidase was analyzed staining to determine if bevacizumab could prematurely induced senescence in tumor cells or if it could be abrogated. Representative results for HCT-116 (MSI) and HT-29 (MSS) are shown (Figure 31). Whilst there were differences between cell lines consistent with the FACS analysis of cell cycle arrest, it did not demonstrate a change in β-galactosidase expression between equivalent treatments with or without bevacizumab during recovery.



	Recovery			
SN-38 (100 nM) +	10% FCS		X	
	Bevacizumab (250 µg/ml)			X
DMSO		X		

Figure 31. Representative β-galactosidase staining for HCT-116 (MSI) and HT-29 (MSS) +/- bevacizumab after recovery from DNA damage by 2 hours of 100 nM SN-38 treatment.

5.2.4 *Colony Formation of Tumor Cells after DNA Damage*

As many of the results indicated potential effects of bevacizumab over longer treatment periods, experiments were designed to expose tumor cells with and without DNA damage to extended bevacizumab therapy. The soft agar assay, which supports 3-dimensional colony formation of tumor cells, enabled the ability to show that bevacizumab pre-treatment in HCT-116 (MSI) significantly promoted an increase in colony numbers over a period of 28 days after the induction of DNA damage (100 nM SN-38 treatment) (Figure 32). Interestingly, continued treatment with bevacizumab decreased the number of colonies formed, however was still significantly higher than colonies formed after DNA damage in the control, with no

exposure to bevacizumab. Furthermore, without DNA damage, HCT-116 (MSI) tumor cells treated for 14 days with bevacizumab, displayed significantly lower numbers of colonies compared to the untreated control. There were no significant differences in HCT-116+ch3 (MSS) colony numbers between treatments with or without bevacizumab, however there was a trend of decreasing colony formation. HT-29, having shown a decrease in proliferation after 72 hours of bevacizumab, did not translate to a change in colonies numbers with bevacizumab alone, however, bevacizumab after DNA damage did attenuate colony numbers. This decrease was abrogated with bevacizumab pre-treatment, whereby colony numbers subsequently increased to that of the control. These results indicated that potential cell signaling changes may have evidently taken place with bevacizumab either as a pre-treatment before DNA damage, or during cell recovery that affect the ability of cells to form colonies.

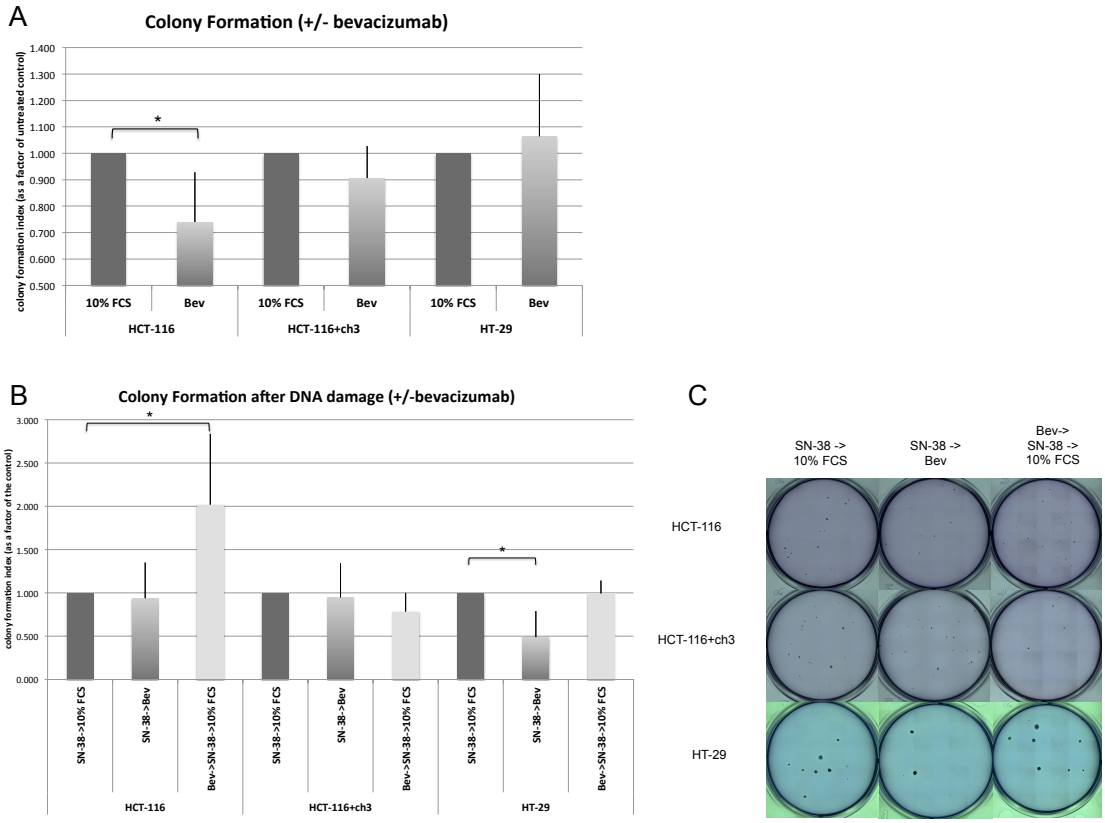


Figure 32. Colony formation potential measured by the soft agar assay over a period of (A) 14 days with Bevacizumab alone and (B) 28 days during recovery from DNA damage. (C) Representative examples of changes in colony numbers after bevacizumab pretreatment. Cells were treated with SN-38 (100 nM) for 24 hrs prior to seeding in soft agar +/- bevacizumab (0.25 mg/ml). * = $p < 0.05$

5.2.5 VEGF IN DNA REPAIR

The role of VEGF in the ability of cells to repair the DNA damage induced by SN-38 was evaluated by transient transfection with a reporter construct containing an out of frame $(CA)_{29}$ repeat sequence (pCAR-OF). When there was a frame shift or an insertion or deletion, it reinstated β-galactosidase in frame and was subsequently expressed. A control vector was transfected in parallel experiments, which contained in frame $(CA)_{27}$ repeats (pCAR-IF). The mutation fraction was determined and illustrated in Figure 33. H_2O_2 was used as a positive control, as it had previously been shown to induce frameshifts in similar experiments (Gasche et al., 2001) and the results shown in Figure 33 are comparable in percentage (for the same cell line).

In line with the colony formation assay, pretreatment of HCT-116 (MSI) cells with bevacizumab appeared to reduce the ability of the tumor cells to repair, thereby increasing the rate of mutation represented here as an increase in the β-galactosidase activity. In addition, bevacizumab during cellular recovery from DNA damage (100 nM of SN-38) also significantly increased the rate of mutation compared to the untreated control, but not when compared to the cells with DNA damage minus bevacizumab. In contrast, HCT-116+ch3 (MSS) showed, as expected, a general reduction in the propensity to induce mutations and did not show a decrease in the repair capacity when bevacizumab was included in the treatment schedule in any form.

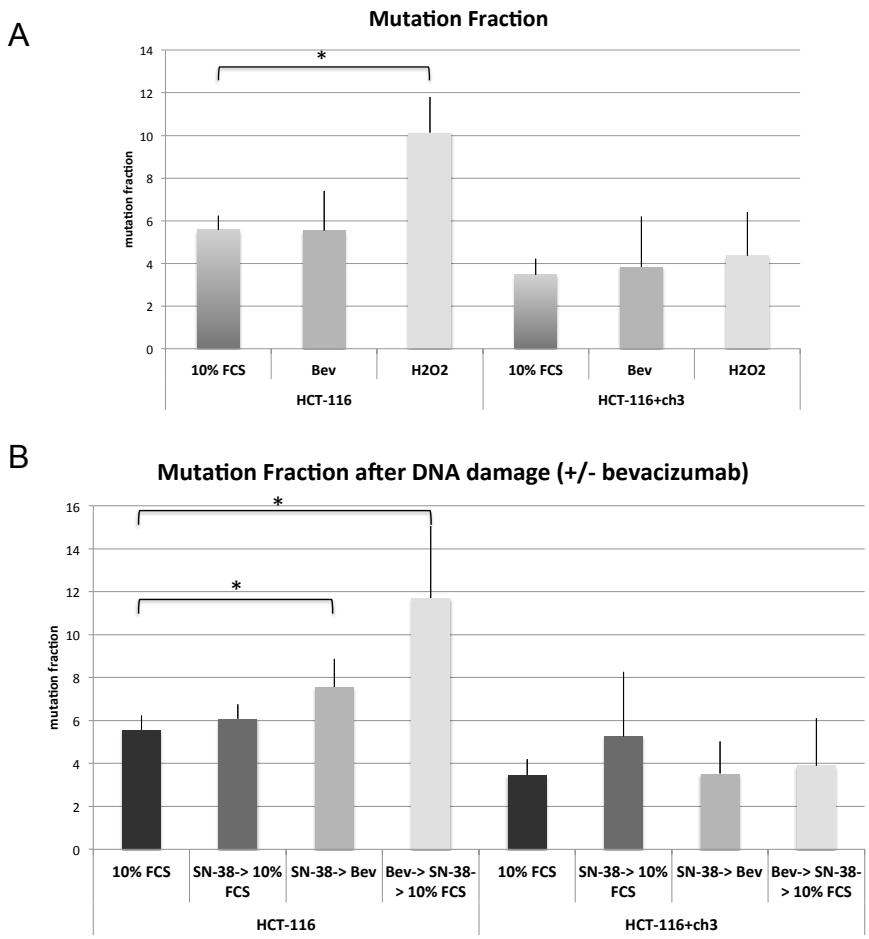


Figure 33. Induction of mutagenesis by DNA damage +/- bevacizumab. Mutation fraction for (A) controls and (B) after DNA damage. Cell lines were transfected separately with pCAR-OF or pCAR-IF, as well as a luciferase construct to act as a transfection control. β-galactosidase and luciferase expression was detected by ELISA and normalized to the untreated control. Mutation fraction was calculated using pCAR-OF luciferase as a factor of pCAR-IF for each condition.

5.2.6 Signaling Changes in Response to Bevacizumab Treatment in Tumor Cells

VEGF-induced signaling is known to activate the Akt pathway in endothelial cells, therefore phospho-Akt (Ser 473) was evaluated in response to bevacizumab in tumor cells. Figure 34A shows HCT-116 (MSI) and HCT-116+ch3 (MSS) both decreased phosphorylation of Akt that was partially in a dose-dependent manner. This decrease wasn't evident in HT-29 (MSS) possibly be due to the low cell surface expression of VEGF receptors. Analysis of downstream targets of the Akt pathway did not indicate obvious pathway regulation in any cell line (Figure 34 (B)). Furthermore, phosphorylation sites on Serine 129 and Threonine 308

of Akt were not evaluated, whereby it is possible that under certain circumstances phosphorylation of these sites may be further inhibited by bevacizumab.

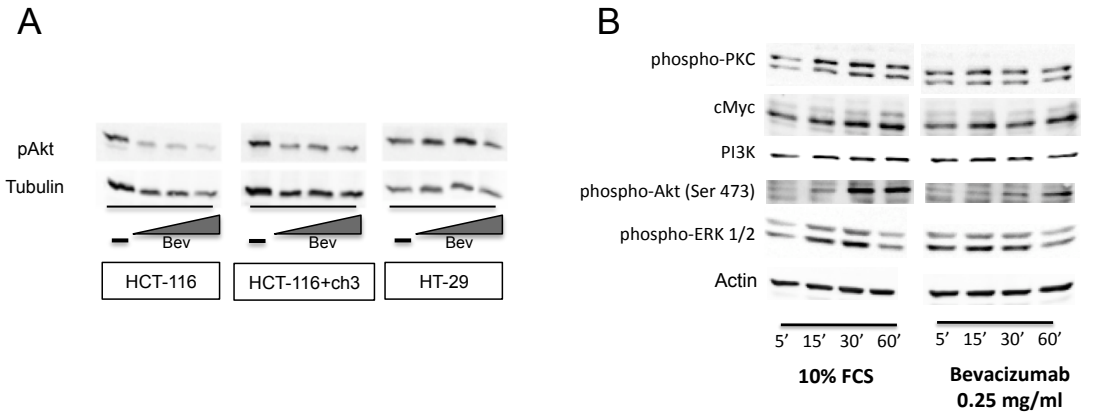


Figure 34. Akt Signaling. (A) Inhibition of phospho-Akt (Ser 473) after 1 hour of bevacizumab treatment in HCT-116, HCT-116+ch3 and HT-29 cell lines. (B) Delayed and attenuated activation, however not complete inhibition of Akt (Ser 473) by bevacizumab in HCT-116 (MSI) cells. PI3K, PKC and ERK activation was not affected by bevacizumab. Other cell lines showed no effect of bevacizumab.

Signaling changes through the inhibition of VEGF with bevacizumab was assessed and DNA damage and cell stress signaling pathways were investigated such as γH2AX, p53, p73, Chk1, Chk2, DAPK1 (Figure 35A). There were no apparent changes in phosphorylation status of key pathway effectors in response to bevacizumab and DNA damage. It was found however that pretreatment, and not combined treatment, of bevacizumab with SN-38 abrogated the phosphorylation of JNK (cJun N-terminal kinase) a key stress response kinase (Figure 35B). Functional studies previously demonstrated by our lab, showed that key mismatch repair genes such as MSH2, contains an active c-Jun binding site after DNA damage (Scherer et al., 2000). Activation of c-Jun in response to DNA damage in mismatch repair deficient cells has also been associated with resistance to apoptosis by cisplatin (Nehme et al., 1997). Our results however, did not show increased sensitivity to DNA damage by abating JNK phosphorylation through bevacizumab pretreatment.

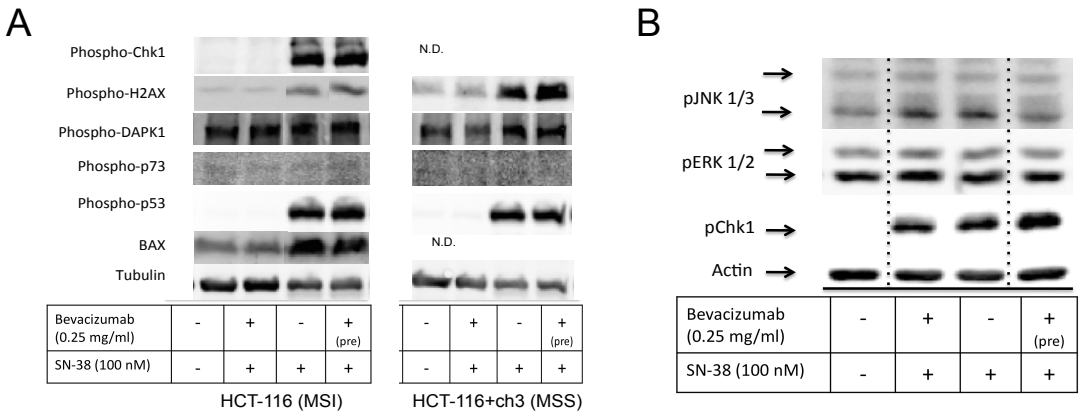


Figure 35. DNA damage response and cell stress response pathways in (A) HCT-116 (MSI) and HCT-116+ch3 (MSS) with bevacizumab 24 hours prior to DNA damage by SN-38. N.D. = not done. (B) HCT-116 (MSI) cells pre-treated with bevacizumab before DNA damage with SN-38.

5.2.7 Gene Expression Changes with Bevacizumab Treatment in Tumor Cells

As a strategy to further evaluate the changes that took place inducing cellular changes with the inclusion of bevacizumab, a microarray was performed on MSI (HCT-116) and MSS (HCT-116+ch3) cell lines. The treatment regimens included bevacizumab alone and bevacizumab both before and after DNA damage with SN-38 (100 nM). Through bioinformatics, stress response pathways were assessed including DNA repair pathways and genes responsible for inducing inflammation.

As a decrease in the phosphorylation of JNK was detected, AP1 target genes were assessed (Figure 36). JNK phosphorylates c-Jun, which forms hetero- and homodimers that make up the AP1 transcription factor and it was found that *JUN* was differentially regulated in both of the cell lines with bevacizumab alone and as a pretreatment before DNA damage. However in the analysis of all genes, no enrichment was found for the AP1 transcription factor at the transcriptional level. Further experiments would be necessary to evaluate the role of VEGF and cJun in any potential role relating to repair mechanisms, in particular in MMR.

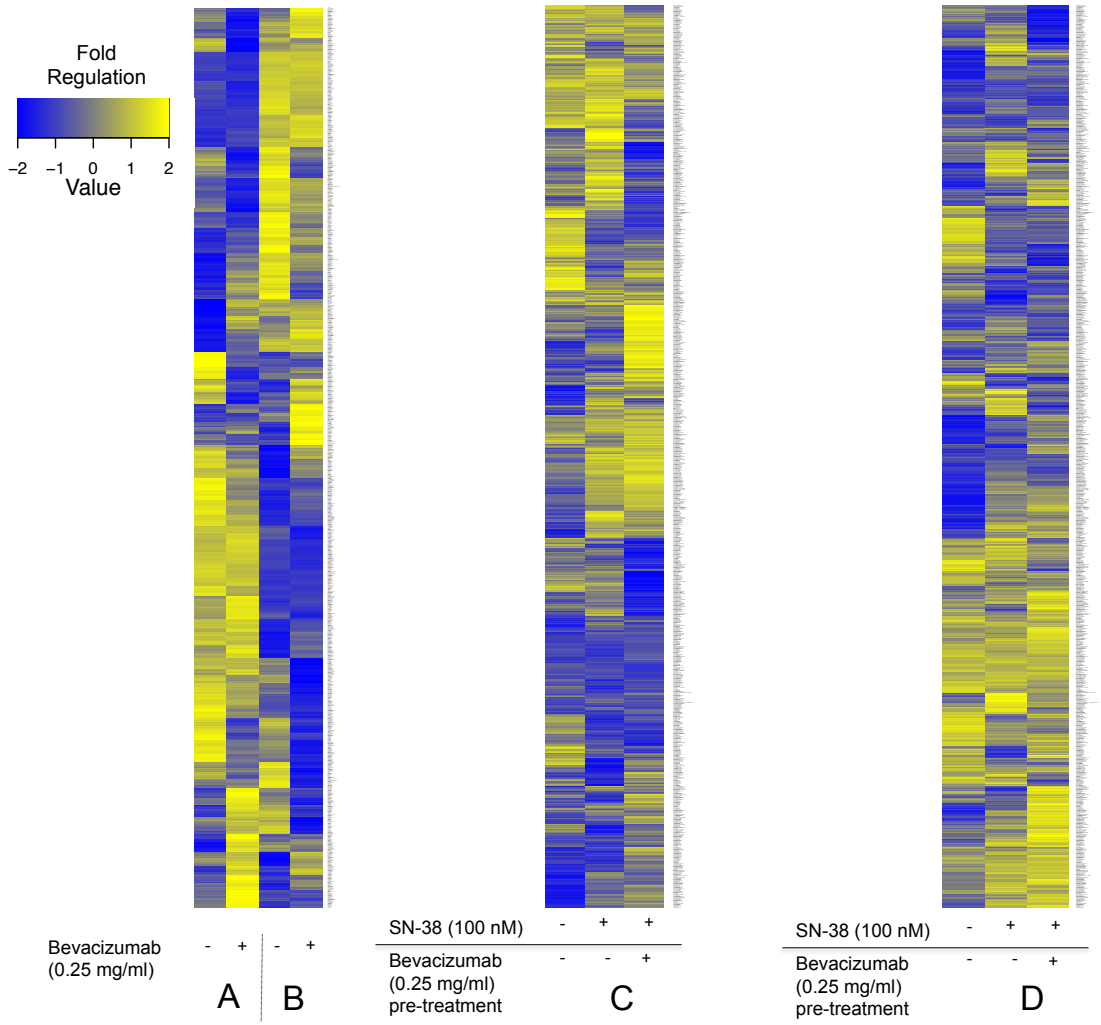


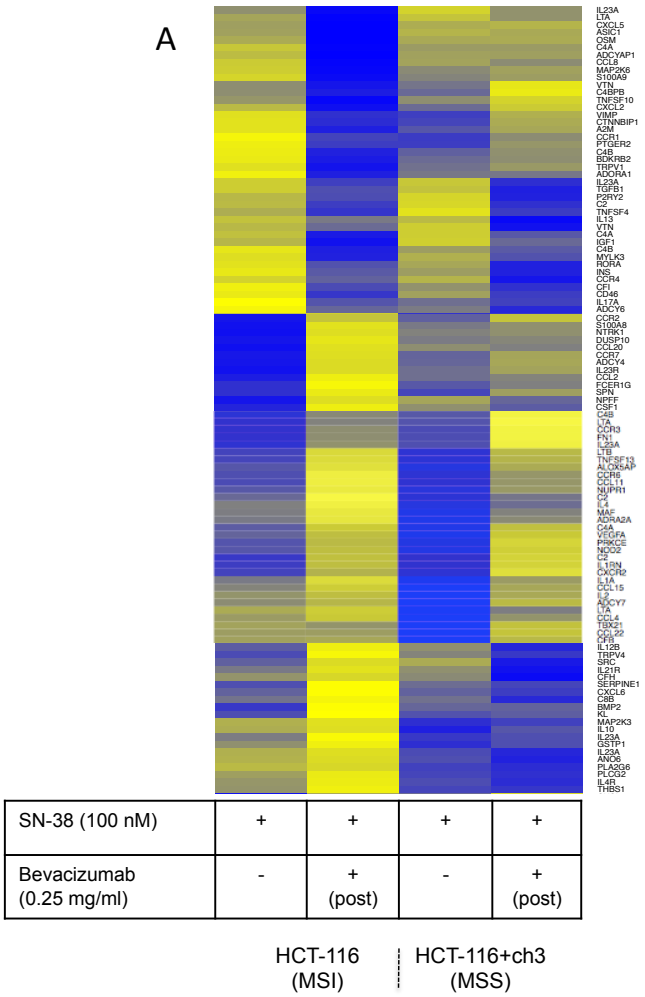
Figure 36. AP1 transcription factor enrichment analysis of microarray gene expression data. (A) AP1 target genes with bevacizumab treatment in HCT-116 (MSI). (B) AP1 target genes with bevacizumab treatment in HCT-116+ch3 (MSS) (C) Bevacizumab pretreatment before DNA damage in HCT-116 (MSI) and (D) Bevacizumab pretreatment before DNA damage in HCT-116+ch3 (MSS)

In the evaluation of the microarray data, there were a number of histones that were regulated by bevacizumab pretreatment before DNA damage (Table 8) predominantly in HCT-116 (MSI), however also in HCT-116+ch3 (MSS).

Table 8. Regulation of histones and histone modifiers by bevacizumab pretreatment in HCT-116. (*) Indicates genes also regulated by bevacizumab in HCT-116+ch3 (MSS).

Up regulated	Log fold change	Down regulated	Log fold change
1H2AA	0.66	2H3D	-1.62
		1H1T (*)	-0.91
		1H4D	-0.79
		3H2A (*)	-0.91
		HDAC4	-0.63
		BORC	-0.76

CXCR4 appeared to be regulated in both cell lines after DNA damage that was augmented by bevacizumab. Furthermore, a number of inflammatory chemokines that were differentially regulated by bevacizumab treatment *after* DNA damage (Figure 37 A-B).



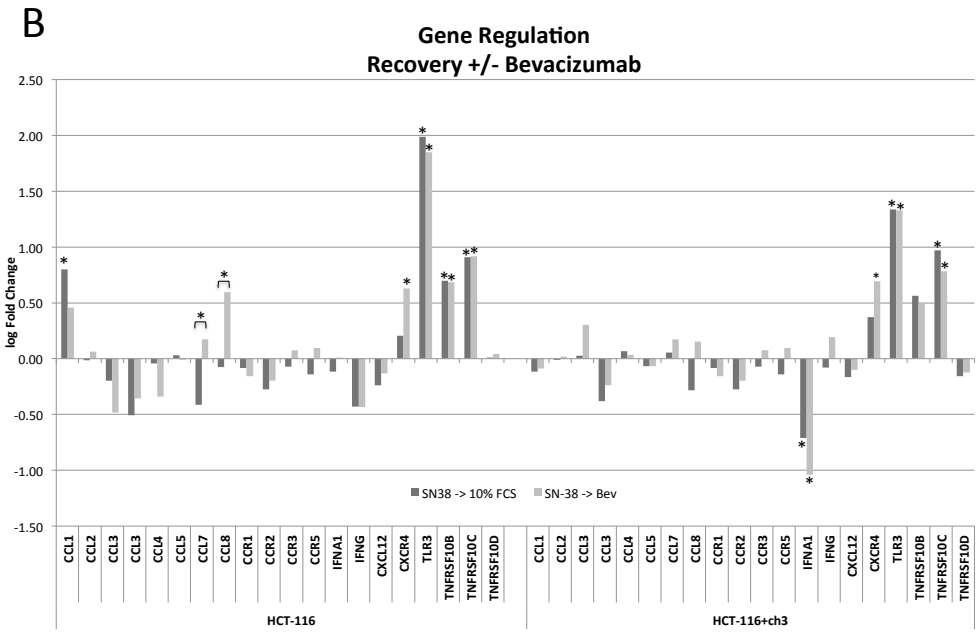


Figure 37. Gene regulation in tumor cells of inflammatory genes after DNA damage. (A) Inflammatory gene regulation of both HCT-116 (MSI) and HCT-116+che (MSS). (B) Cytokine gene regulation

It was postulated that a clinical response could be as a result of modulation of immune cells by bevacizumab, in particular tumor associated macrophages (TAMs).

5.3 THE EFFECT OF BEVACIZUMAB IN MODULATING IMMUNE CELLS

Some differentially regulated genes after DNA damage in HCT-116 (MSI) tumor cells treated with bevacizumab included CCL7 and CCL8, also known as monocyte-specific chemokine 3 (MCP3) and monocyte-specific chemokine 2 (MCP2) respectively. The concept that bevacizumab could perhaps influence a clinical response by signaling through tumor cells to accomplice cell types beyond endothelial cells, to cells of the immune system was further investigated. As CCL7 and CCL8 are monocyte-specific chemokines, a direct response of bevacizumab was investigated on monocytes and/or macrophages, as well as indirectly through conditioned media from tumor cells treated with bevacizumab +/- DNA damage with SN-38.

5.3.1 ACTIVATION OF NFκB

NFκB is a primary regulator of the cellular immune and inflammatory response therefore it was decided to assess the activation of NFκB by bevacizumab treatment is a series of assays. In addition to other chemokines, there was some enrichment, although not significant, of NFκB target genes including *GADD45B*, *IFNA13* and *SMAD4* in the microarray. The phosphorylation of NFκB was inconclusive when investigated by western blot, so a more sensitive assay was performed. An ELISA of the p65/RelA and p50 subunits of NFκB analyzed changes in homo- or heterodimer formation. Using TNFα as a positive experimental control, there was an increase in binding of both p65/RelA and p50 in both cell lines (Figure 38 A-B). However, there was no significant change in binding of either subunit in the tumor cell nuclear extracts under any of the treatment conditions. In concurrent experiments, p65/RelA was knocked down in both cell lines (Figure 38 E-F) and determined if this altered tumor cell survival or ability to proliferate with bevacizumab and/or DNA damage. As seen in Figure 38 C-D, there was no significant difference in the proliferation of p65/RelA knockdown treated cells compared to the control (scrambled) siRNA. It was concluded that NFκB activation did not contribute to potential change in immune cytokine expression or sensitization of tumor cells to bevacizumab.

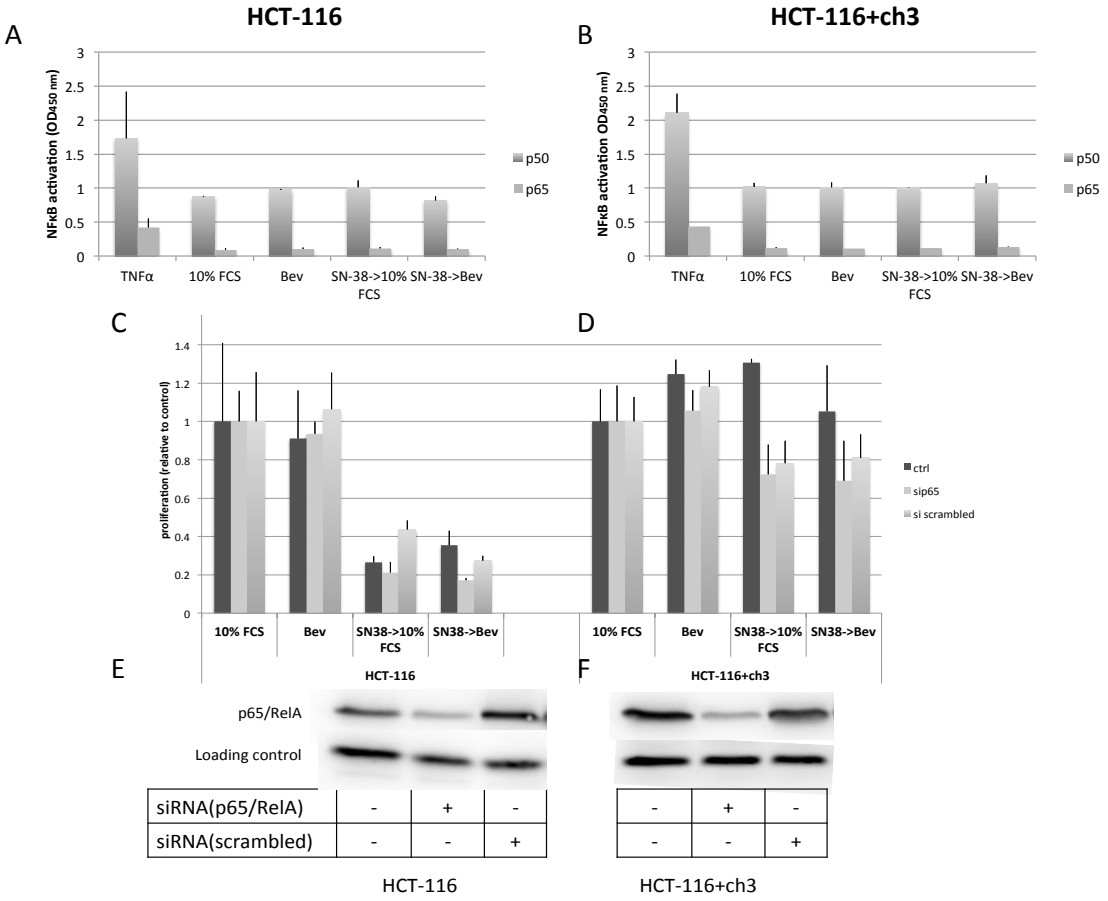


Figure 38. Activation of NFκB by bevacizumab. Activation of NFκB in nuclear extracts of tumor cells of the p65/RelA and p50 subunits measured by ELISA in (A) HCT-116 and (B) HCT-116+ch3. Proliferation of tumor cells after knockdown of p65/RelA in (C) HCT-116 and (D) HCT-116+ch3. Western blot of p65/RelA knockdown in (E) HCT-116 and (F) HCT-116+ch3.

5.3.2 Induction of VEGF after DNA damage

VEGF has been shown to induce changes in monocytes/macrophages *in vivo* such as monocyte migration, where localization is dependent on VEGF expression and vascularization (Giraudo et al., 2004, Lewis et al., 2000, Yan et al., 2011). It was first investigated if there was a difference in VEGF secretion between cell lines that could impact the monocytes/macrophages, in addition to changes due to DNA damage. The results demonstrated equal amounts of VEGF detected by the ELISA assay in both HCT-116 (MSI) and HCT-116+ch3 (MSS) in the absence of DNA damage. There was a significant reduction of VEGF secreted by the cells after DNA damage in both cell lines, but more so in HCT-116 (MSI) (Figure 39).

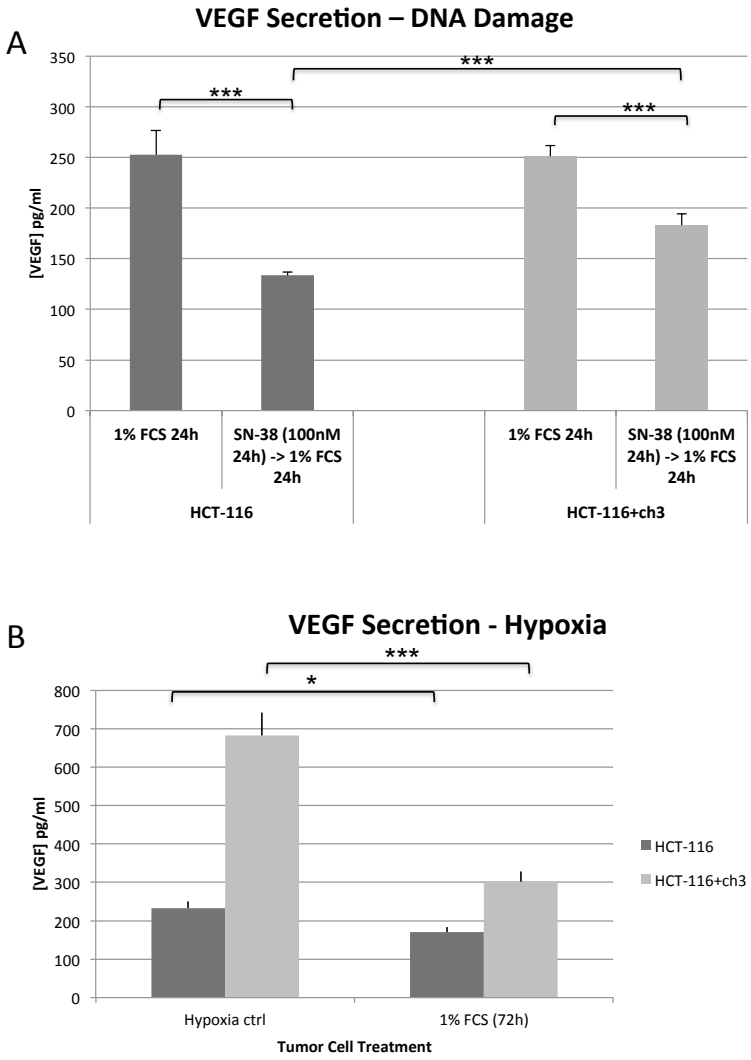


Figure 39. Detection of VEGF secreted by tumor cells and measured by ELISA. (A) Secretion of VEGF from tumor cells after 24 hours. Cells were treated in 1% FCS to detect secretion of VEGF at basal levels as well as changes in VEGF secretion after DNA damage with SN-38. Values were normalized to cell viability, measured by the cleavage of tetrazolium salts by cellular enzymes of metabolically active cells (B) Hypoxic induction of VEGF secreted into media after 72 hrs.

5.3.3 Macrophage/Monocyte Gene Expression Induced by Tumor Conditioned Media

Tumor educated macrophages form a critical component of the tumor microenvironment and are suggested to play an essential role in tumor suppression as well as promoting tumor progression through induction of specific gene expression towards an M1 or M2 phenotype. In light of *CCL7* and *CCL8* regulation selectively by bevacizumab after DNA damage in HCT-116 (MSI) tumor cells, cellular cross talk from tumor cells to macrophages was evaluated in terms of induction of gene expression. In the initial experiments, the standard *in*

vivo model of C57BL/6 wt mice was used. Peritoneal macrophages were extracted and stimulated with tumor conditioned media. The well characterized murine genes selected for analysis were *Nos2* & *Cxcl9*, which indicated macrophages polarized towards the M1 phenotypes and *Agr1* & *Mrc1* as an indication of M2 activation. There appeared to be gene regulation by bevacizumab, particularly of M1 markers *Nos2* and *Cxcl9* (Figure 40). In the analysis of the results and in evaluating the contextual importance of the selected genes, it was considered that there can be a number of discrepancies in translating findings from murine models to human diseases, particularly relating to the immune system (Mestas and Hughes, 2004). It was therefore decided to determine if similar regulation could be demonstrated in human derived myeloid cells.

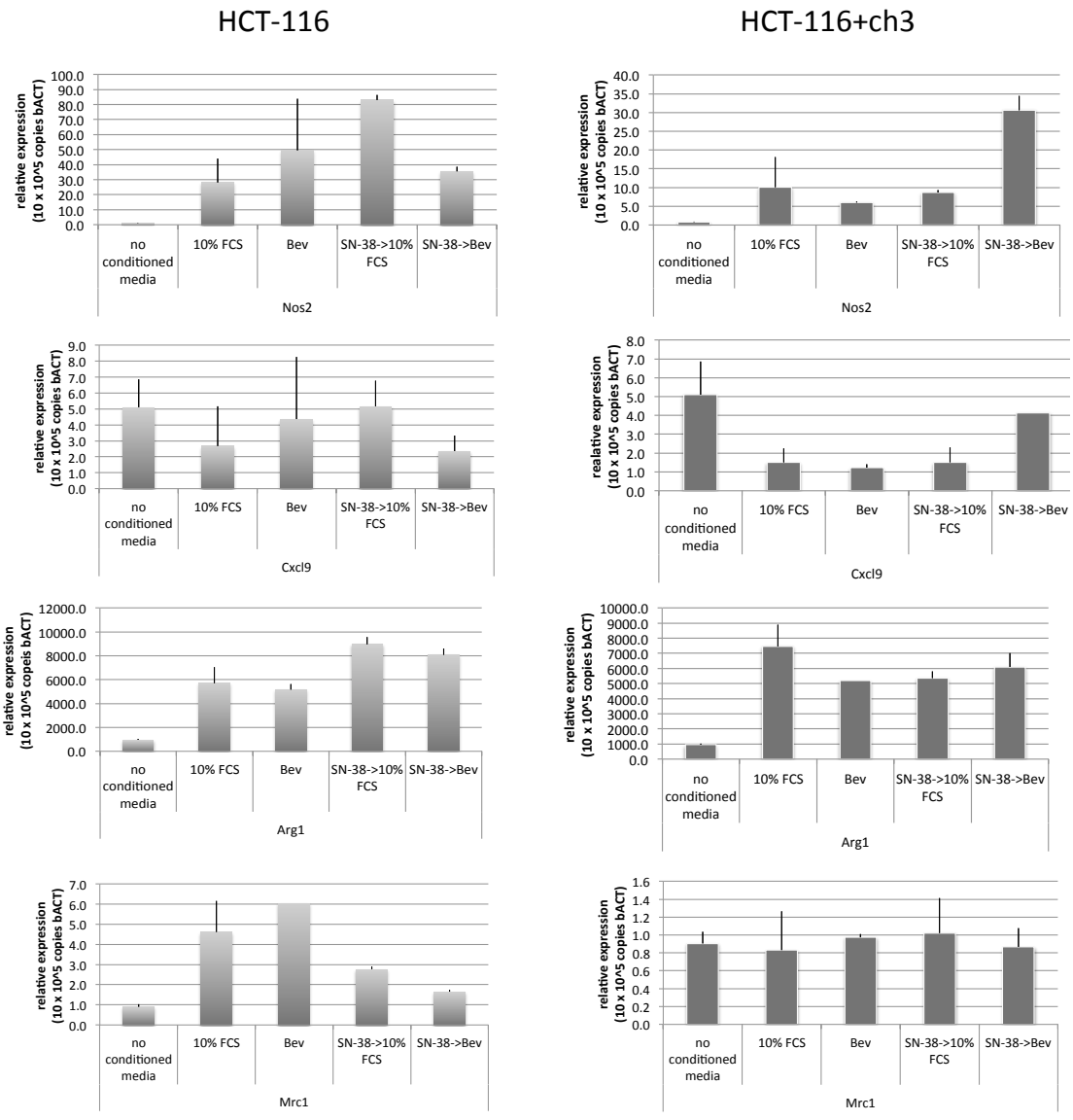


Figure 40. Gene expression in murine macrophages in response to tumor conditioned media. Nos2 & Cxcl9 are indicators of M1 polarization whereas Arg1 and Mrc1 are M2 markers. Macrophages were treated for 12 hrs in tumor conditioned media before extraction of RNA. The experiment was performed in duplicate therefore there were insufficient data points to calculate significance accurately.

Monocytes were extracted from the whole blood of healthy volunteers and, similar to treatment of the murine macrophages, monocytes were treated with tumor conditioned media for 12 hrs. In addition to the tumor conditioned media, monocytes were also treated with bevacizumab as a single agent in standard media to evaluate bevacizumab specific induction of gene expression compared to the effects of bevacizumab on tumor cells. Three genes were selected to denote M1 polarization, *NOS2*, *CXCL9* and *CXCL10*. The gene expression analysis did not provide conclusive data of the M1 markers, in that expression levels were very low and as such, the standard deviation high.

The M2 markers however, did show not only bevacizumab specific regulation but also significant differences between the different tumor cell conditioned media. *CCL18*, *CXCR4* and *MRC1* genes are indicators of M2 activated macrophages. *CXCR4* and *MRC1* were not regulated by bevacizumab alone but *CCL18* did show significant regulation (Figure 41 A-C). Tumor conditioned media significantly induced expression of *CCL18* and *MRC1* in monocytes by media from both cell lines compared to the controls without conditioned media (as noted by the relative expression levels). There was a strong inhibition of *CXCR4* by conditioned media from HCT-116 (MSI) compared to the control of monocytes without conditioned media. Expression levels of *CXCR4* in monocytes remained high in monocytes from HCT-116+ch3 (MSS) tumor conditioned media. Single agent Bevacizumab treatment of tumor cells from either cell line did not in general further enhance or inhibit gene expression in monocytes by use of the conditioned media.

After DNA damage, *CCL18* was induced in a bevacizumab specific manner in the conditioned media of HCT-116 (MSI) (Figure 41D). Conversely, in the absence of bevacizumab after DNA damage *CCL18* was down regulated by the tumor conditioned media of both cell lines (Figure 41 D&G). Although the tumor conditioned media from HCT-116 (MSI) decreased the expression levels of *CXCR4* and increased *MRC1* compared to monocytes treated with no conditioned media, the treatment options failed to induce further changes in the monocyte gene expression (Figure 41 E-F). The soluble factors released by HCT-116+ch3 (MSS) DNA damaged treated tumor cells did translate into changes in gene expression, however there was no universal response of the selected M2 markers. In response to DNA damage in HCT-116+ch3 (MSS) tumor cells, *CCL18* and *CXCR4* were both down regulated in monocytes, which bevacizumab abrogated, however only significantly in *CXCR4*

(Figure 41 G-H). *MRC1* on the other hand was significantly upregulated by conditioned media from DNA damaged HCT-116+ch3 (MSS) tumor cells and once again bevacizumab countered the regulation.

The gene expression analysis indicated that bevacizumab could influence the cellular cross talk in a tumor cell specific manner that could also therefore potentially translate to changes in monocyte/macrophage function.

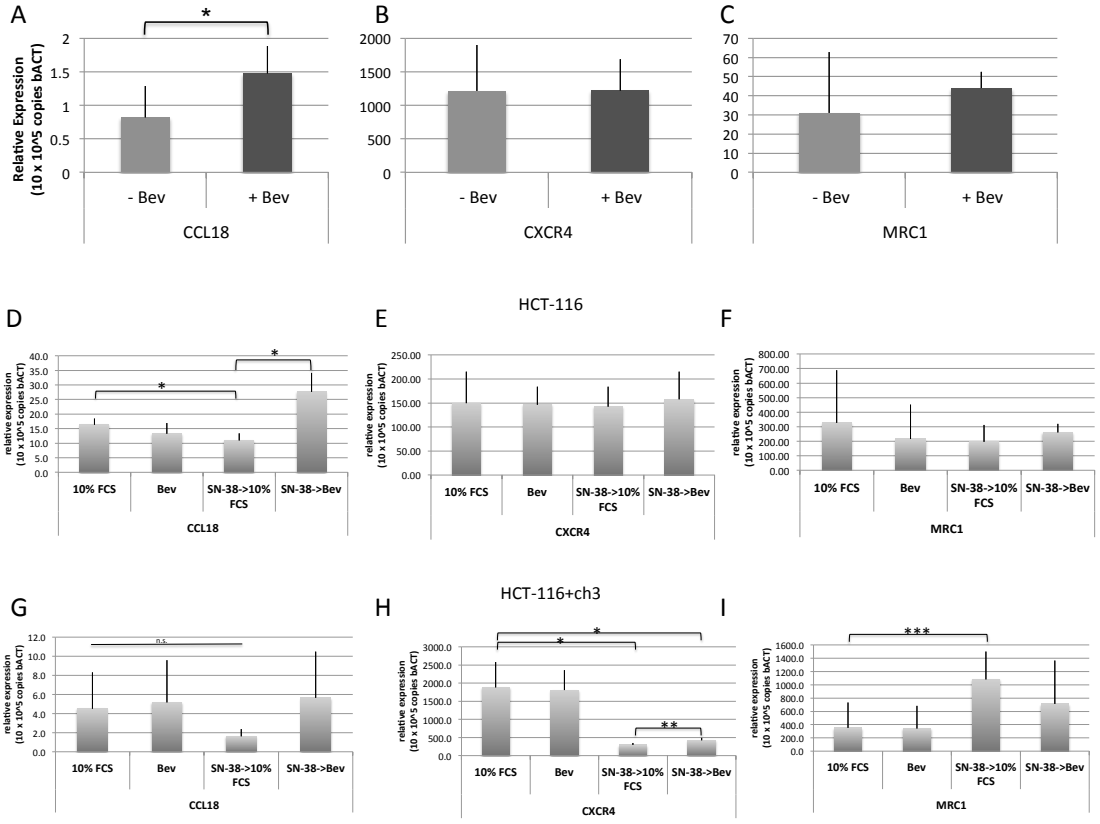


Figure 41. Monocyte gene expression induced by tumor conditioned media. (A-C) Controls show bevacizumab specific induction of gene expression by treating monocytes in standard media +/- bevacizumab. Gene expression induced by tumor conditioned media from (D-F) HCT-116 (MSI) and (G-I) HCT-116+ch3 treated tumor cells.

5.3.4 Monocyte Migration by VEGF and Tumor Conditioned Medium

The first functional experiment performed to evaluate both tumor cell and bevacizumab dependent effects on monocytes, was to investigate the induction of migration. The migration of monocytes was induced by rhVEGF (Figure 42A) and bevacizumab was able to significantly reverse this effect. When the monocytes were exposed to tumor conditioned

media, there were opposing effects between the 2 different cell lines. Monocytes significantly increased migration towards HCT-116 (MSI) conditioned media after DNA damage, which is expected as monocytes/macrophages can be recruited to clear damaged cells (Savill et al., 2003). However, with the addition of bevacizumab after DNA damage, the migration of monocytes decreased and did not reach significance compared to the control (Figure 42B). HCT-116+ch3 (MSS) in contrast, did not show an effect on monocyte migration after tumor cells sustained DNA damage with SN-38. HCT-116+ch3 (MSS) conditioned media did however show a trend of a slight increase in migration with the inclusion of bevacizumab (Figure 42C) with and without DNA damage, however this did not reach significance after repetition.

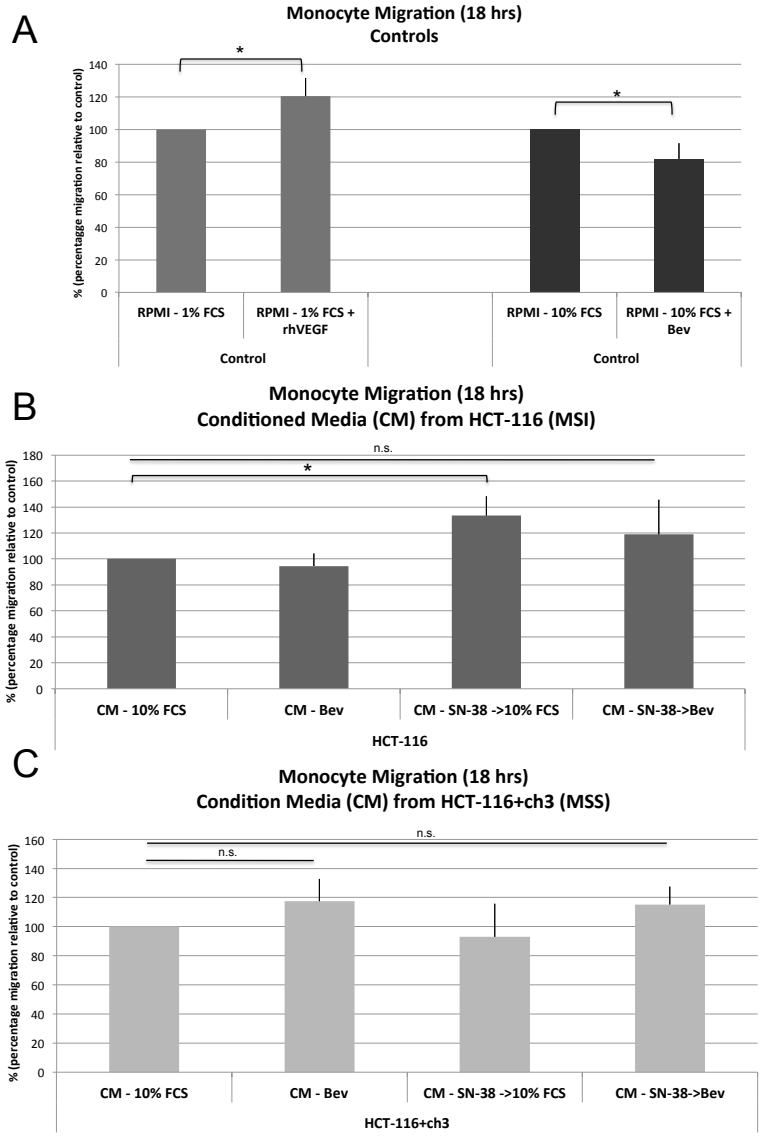


Figure 42. Transwell migration assay measuring monocyte migration towards conditioned media of (A) controls (non-conditioned media), (B) HCT-116 (MSI) and (C) HCT-116+ch3 (MSS). Monocytes were added to inserts in 1% FCS media and counted after 18 hours incubation. CM, conditioned media, n.s., not significant

5.3.5 PHAGOCYTIC ACTIVITY OF MACROPHAGES

In an attempt to determine further functional effects in the stimulated macrophages, the tumor cells were co-cultured with human macrophages to evaluate any cytotoxic or phagocytic activity. Tumor cells were stably transfected with a constitutively active luciferase-containing lenti-virus and measured to be indicative of viable tumor cell numbers (Figure 43A). As a control for phagocytosis, the same cells were seeded without macrophages to get the phagocytic index to give rise to remaining viable tumor cells; therefore the lower the phagocytic index (i.e. luciferase luminescence), the lower the number of viable cells.

In the initial experiment, the cells were treated for 2 hrs with SN-38 prior to seeding for co-culture +/- bevacizumab. It very clearly showed no significant phagocytic activity after 12 hrs incubation (not shown). It was determined that the extent of DNA damage was not effective enough to induce a response from the macrophages, therefore for the follow up experiment, cells were treated for 24 - 48 hrs prior to co-culture (24 hrs DNA damage + 24 hrs recovery). There were two facets of the experiment that could be assessed; firstly the differences in phagocytosis between different treatments of tumor cells, and secondly, the impact of bevacizumab on the mature macrophages with the addition at the time of co-culture.

The results showed in general that HCT-116 (MSI) tumor cells were more susceptible to phagocytosis (Figure 43B), in particular after DNA damage. Interestingly in this cell line, phagocytosis was enhanced regardless of the treatment of the tumor cells by the addition of bevacizumab at the time of co-culture. The co-culture of macrophages with HCT-116+ch3 (MSS) (Figure 43C) cells was less responsive and showed limited significance overall, however mirrored the same trend as HCT-116 (MSI).

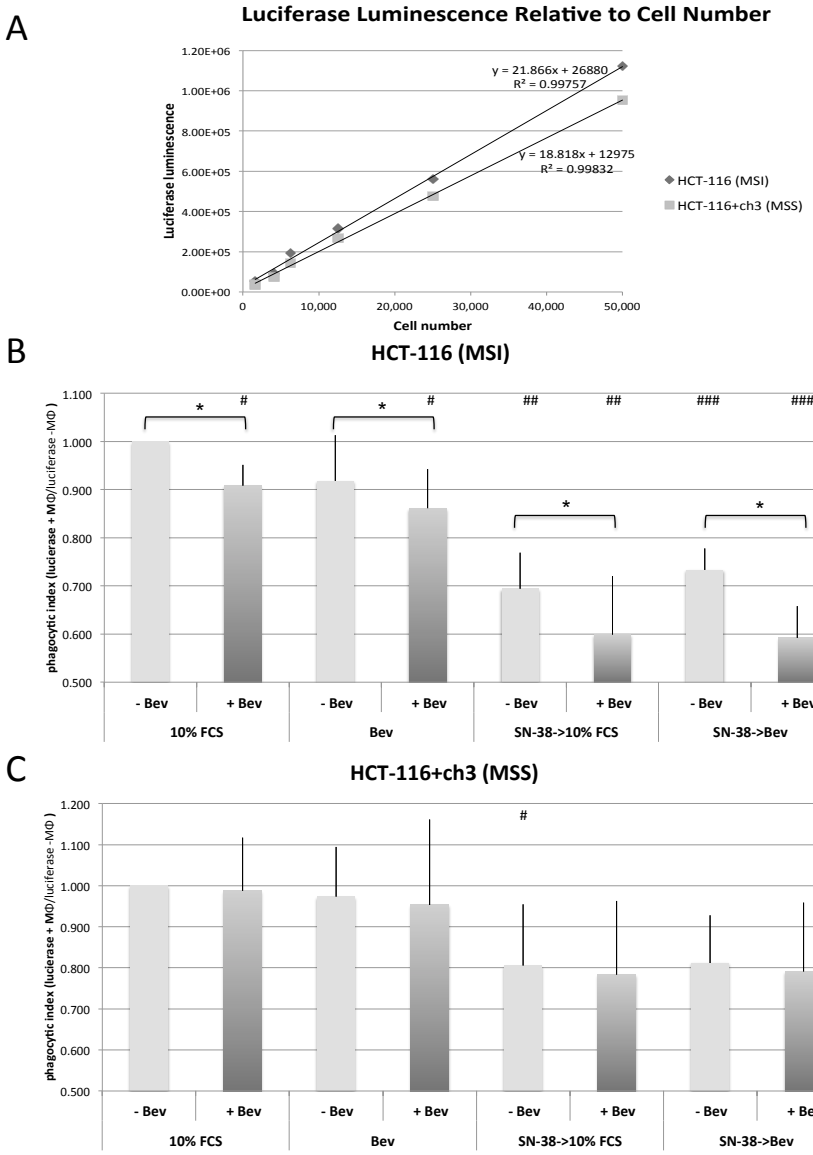


Figure 43. Macrophage cytotoxic/phagocytic activity on tumor cells (A) Luciferase expression is shown to be relative to number of tumor cells. Phagocytic activity in (B) HCT-116 and (C) HCT-116+ch3. Values were calculated as a measure of tumor cell expression of luciferase in the presence of MΦ / tumor cell luciferase expression without MΦ. Each experiment was normalized to the luciferase expression to the control (phagocytic activity on tumor cells without treatment 10% FCS – Bev) to accommodate inter-experimental variation of luciferase expression. Macrophages were differentiated for 72 hrs in standard media. Tumor cells were treated as described on the x-axis: untreated, bevacizumab (0.25 mg/ml) for 24 hrs or with SN-38 (100 nM) for 24 hrs, then recovery for 24 hrs +/- bevacizumab. Each condition was plated twice with (dark grey bars) or without (light grey bars) bevacizumab and at the time of co-culture with the differentiated macrophages. * = p value < 0.05; # = p value <0.05 compared to the control (10% FCS – bev); ## = p value <0.01 compared to the control (10% FCS – Bev); ### = p value <0.001 compared to the control (10% FCS – bev), Bev, bevacizumab; MSI, microsatellite instability; MSS, microsatellite stable, MΦ, macrophages.

5.3.6 *Tumor Conditioned Macrophages and T Cell Suppression*

Following the sequence of the immune response, and having seen an effect of bevacizumab on macrophages/monocytes, changes in antigen presenting cells (APC) was investigated and how it could follow through to the suppressive activity on CD4+ T cells. The mixed lymphocyte reaction (MLR) was assayed step-wise (Figure 44), where monocytes were differentiated in tumor conditioned media, then macrophages were co-cultured with CD4+ T cells for 24 hours. 0.33 µCi of [^{3}H]thymidine was added for a further 18 hours before reading radioactivity (CPM). CD4+ T cell suppression was determined by a decrease in the incorporation of [^{3}H]thymidine.

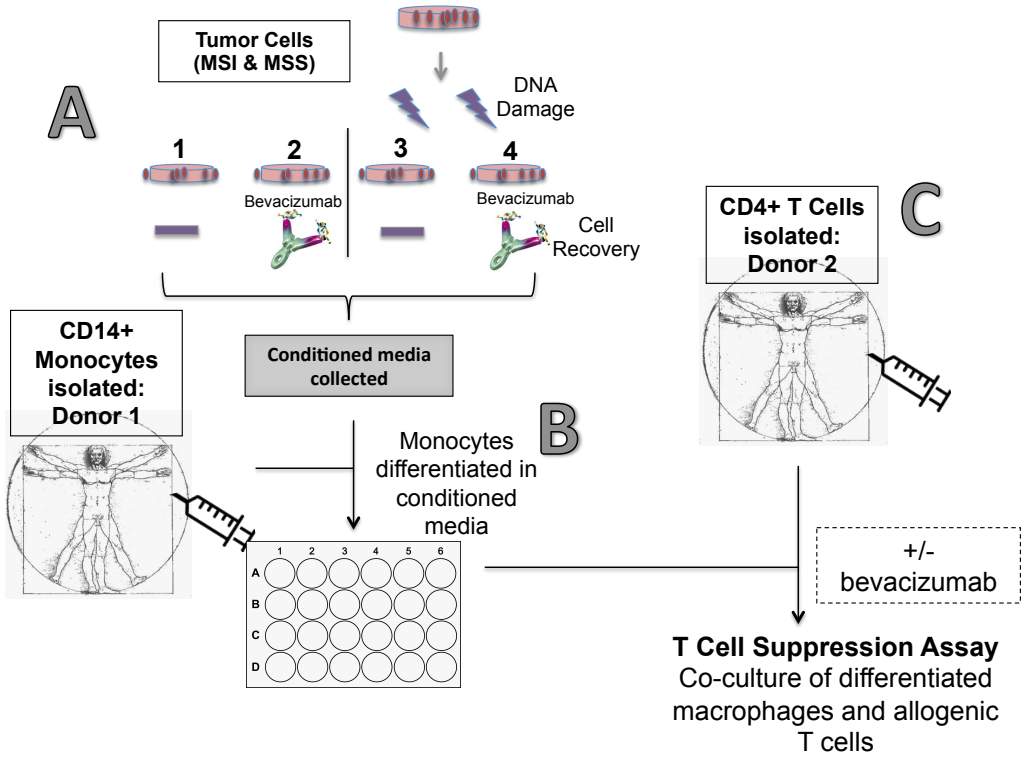


Figure 44. 2-step experimental set-up for T cell suppression assay: (A): tumor cells were treated as outlined (B): freshly isolated CD14+ monocytes were added to conditioned media from tumor cells (MSI & MSS). After 72 hours differentiation in conditioned media, (C): macrophages were co-cultured with freshly isolated T cells from a second donor that have been treated +/- bevacizumab. Different donors were used to stimulate T cell expansion without the need for CD3 or CD28 stimulation. In the absence of tumor conditioned media, controls included bevacizumab treatment during monocyte differentiation in addition to at the time of co-culture of macrophages and T cells to determine an effect on either mature macrophages or T cells directly.

In order to capture the effects on macrophages of the tumor derived factors, the monocytes were activated in conditioned media to macrophages before the MLR. This primed the APCs depending on cytokine and chemokine release from the treated tumor cells, which in turn

provokes an outcome in CD4+ T cells. Figure 45 A and B demonstrate the effects on the APCs and subsequently CD4+ T cells, by the conditioned media from HCT-116 (MSI) and HCT-116+ch3 (MSS) tumor cells respectively. Although the results of a series of experiments did not establish significance according to the student's t-test with conditioned media, there was a definite trend of CD4+ T cell suppression in combination with HCT-116 (MSI) treated macrophages and bevacizumab in the MLR. The HCT-116+ch3 (MSS) treated macrophages conversely, displayed the opposing effect. After co-culture of T cells with macrophages that have been polarized in the presence of bevacizumab (not conditioned media), there was a strong suppression of CD4+ T cells (Figure 45C). This suppression was further enhanced by continued bevacizumab treatment at the time of the MLR. These data suggest that both polarization of APCs by specific tumor cell derived cytokines, in addition to bevacizumab is required for CD4+ T cell suppression

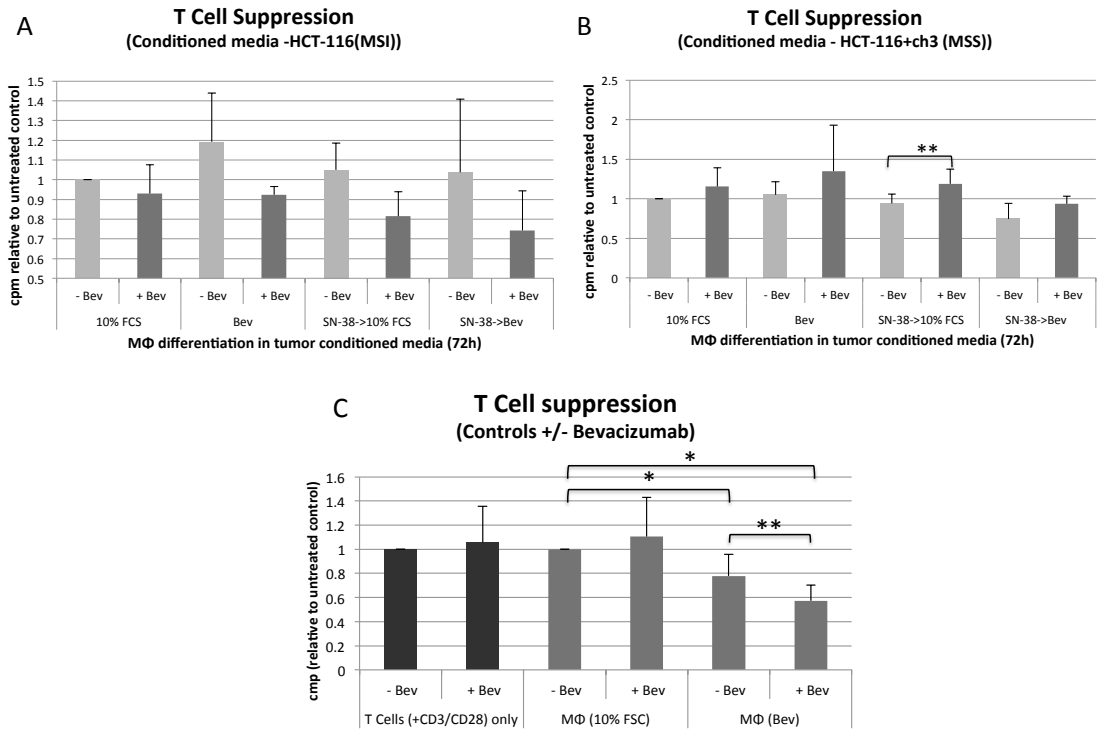


Figure 45. CD4+ T cell suppression in a mixed lymphocyte reaction with macrophages polarized by (A) HCT-116 (MSI) conditioned media and (B) macrophages polarized by HCT-116+ch3 (MSS) conditioned media, (C) macrophages polarized by RPMI 10% FCS +/- bevacizumab

5.3.7 T CELL ACTIVATION WITH BEVACIZUMAB

As with macrophages, T cells can exist in a variety of states that are termed either pro-inflammatory or anti-inflammatory, T_H1 and T_H2 respectively. In addition, Tregs commonly form a significant portion of a tumor microenvironment and CD8+ T cells are considered positive prognostic indictors when surrounding tumor cells. In order to determine the effect of the conditioned macrophages on T cell activation/suppression, macrophages were cultured from monocytes over 72 hrs with or without bevacizumab and cell surface expression of T cell markers were evaluated. No conditioned media from tumor cells was used due to the high number of samples in each experiment, so it was only possible to evaluate bevacizumab effects on the cell mediated immune response. Markers used to identify T cell sub types were: CD25 represented Tregs, CXCR4 indicated T_H2 T cells, CXCR3 on the surface of T cells was a T_H1 marker and CD8 was a marker for CD8+ cytotoxic T cells.

The two left bars in each graph show the effect of bevacizumab on expression of markers on T cells, without macrophages (Figure 46). In each instance, there was no significant change in expression of any marker, suggesting bevacizumab doesn't have any intrinsic effect on T cells. The co-culture of macrophages with T cells did demonstrate a slight induction of CXCR4 overall, although this did not prove to be significant. Further more CXCR3 and CD8 appeared to be down regulated on the cell surface compared to the untreated control. There was a significant effect of bevacizumab on both the monocytes and mature macrophages, translating to a reduction of T cell CXCR3 expression on the adaptive arm of the immune response. A reduced T_H1 response correlates with the T cell suppression assay with macrophages differentiated in the presence of bevacizumab.

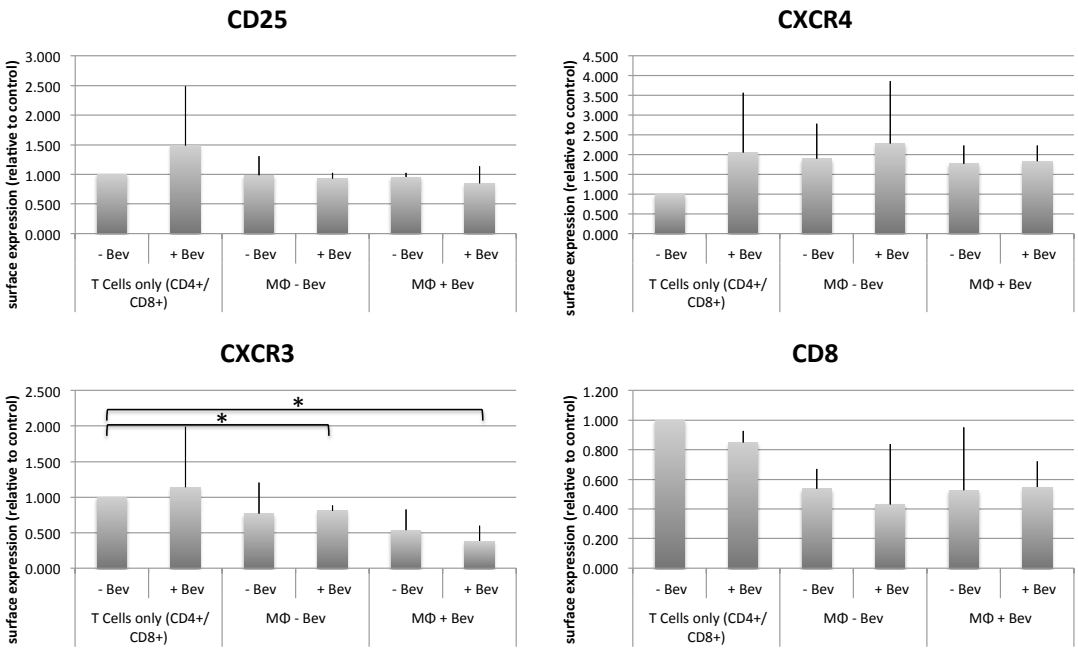


Figure 46. Cell surface expression of T cell markers measured by FACS analysis. CD25: Tregs, CXCR4: T_H2, CXCR3: T_H1, CD8 cytotoxic T cells.

6 DISCUSSION

6.1 EFFECTS OF BEVACIZUMAB ON TUMOR CELLS

Growth factor signaling activates effector kinases to translate extracellular messages within cells and trigger numerous associated functions, predominantly relating to cellular growth, proliferation and survival. Vascular endothelial growth factor (VEGF) falls into this category, stimulating survival associated functions in, as the name suggests, vascular endothelial cells. Various cells secrete VEGF including tumor cells and myeloid cells. In tumor biology, this elevated VEGF in the microenvironment induces angiogenesis to avascular and hypoxic regions of a solid tumor, in addition to hypervascularizing regions in and around a tumor. To counter this, anti-angiogenic compounds have been developed on the premise that universal inhibition of VEGF could stop angiogenesis and subsequently starve tumor cells from oxygen as well as other nutrients to halt cancer progression and potentially trigger tumor regression. Bevacizumab (Avastin ®) is a monoclonal antibody directed against VEGF, which has had moderate success clinically in that progression free survival (PFS) improved, but meaningful benefits in overall survival (OS) remain elusive.

Independent of angiogenesis and vascular permeability functions, a number of studies have also shown VEGF to be a survival factor in some tumor cells, including a number of tumor cell lines used in this thesis (Lee et al., 2007, Samuel et al., 2011). Based on this evidence, along with clinical observation of a varied response to anti-VEGF containing therapy, the work set out to establish if there was a characteristic biomarker in tumor cells that made them more susceptible to inhibition of VEGF signaling. Using selected cell lines representing tumor entities in which anti-VEGF therapy has shown clinical relevance from the well established NCI-60 panel of tumor cell lines, it was established that factors (e.g. VEGF receptors) relating to an auto- or paracrine mechanism were expressed. In order to promote a VEGF-dependent response in the tumor cells and to mimic neo-angiogenesis in hypoxic areas, tumor cells were initially incubated in a hypoxic environment to induce VEGF expression. It was found that not all tumor cells responded uniformly to hypoxia, with some cell lines up-regulating both HIF1 target genes analyzed, *VEGF* and *GLUT1*, others only selectively up-regulating *GLUT1* and not *VEGF*, and 2 cell lines (MDA-MB-231 & A498) that were not sensitive to the hypoxic environment by this measure. These results concur with previous studies showing the contrasting response of tumor cells to hypoxia, with adaptive changes in terms of gene regulation, survival as well as resistance to DNA damage (Yao et al., 2005, Vaupel and Harrison, 2004, Kunz and Ibrahim, 2003, Selvakumaran et al., 2013). In

the tumor cells with different genetic backgrounds, HT-29 and HCT-116, Selvakumaran *et al.* demonstrated an increase in resistance to a DNA damaging agent under hypoxia, whereas KM12 did not show this change in sensitivity. The results in our study highlight the differences in theses cell lines in response to hypoxia, with HT-29 and HCT-116 both up-regulating HIF1 target genes and KM12 selectively up-regulated *GLUT1* but not *VEGF*.

It was further established that the selected cell lines expressed at least one VEGF receptor (VEGFR1 and/or VEGFR2) or the co-receptor NRP1 that would be able to mediate any VEGF activity. The expression of VEGFRs on tumor cells, whilst initially contentious, has become a generally accepted phenomenon and the results in this study concur with the growing body of evidence for VEGF receptors on tumor cells both *in vitro* and *in vivo* (Bates et al., 2003, de Jong et al., 1998, Fan et al., 2005, Badalian et al., 2007, Ghosh et al., 2008, Giatromanolaki et al., 2007, Goel et al., 2012, Jubb et al., 2012, Parikh et al., 2004). Protein analysis by immunoblot identified one cell line displayed a hypoxia-dependent induction of VEGFR1 expression (A498), which was not reversed by bevacizumab treatment, inferring this was not VEGF-dependent. This concurs with the lack of induction of *VEGF* under hypoxic conditions in this cell line, however *GLUT1* was up-regulated illustrating cellular changes were evident by incubation in limited oxygen supply.

There is some evidence that hypoxia regulates receptor expression for example of NRP1 in embryonic stem (ES) cells in a HIF1-depedent manner (Brusselmans et al., 2005), VEGFR1 in endothelial cells (Ulyatt et al., 2011) and soluble VEGFR2 in HEK293 (human embryonic kidney cells) and endothelial cells (Collet et al., 2014). HUVECs did show regulation of VEGFR2 by hypoxia, however receptor mapping of cell surface analysis was inconclusive in determining if an abundance of receptor protein would be available for binding of extracellular VEGF. It was however noted that bevacizumab treatment did not significantly attenuate VEGFR2 either in total levels or localization, indicating this is not likely to be VEGF regulated. Protein analysis by immunoblot did not reveal clear regulation in other cell lines of NRP1 or VEGFRs by either hypoxia or bevacizumab treatment. Furthermore, flow cytometry of cell surface expression demonstrates that although some cell lines expressed receptors, it did not necessarily translate to cell surface expression, thereby limiting a potential VEGF-dependent auto- or paracrine effect. Localization of receptors did not appear to be affected by hypoxia or bevacizumab and therefore not likely to be a VEGF regulated event. It appears that although VEGF regulates activation of VEGFRs and NRP1 upon binding (Ferrara et al., 1992), there is no feedback loop regulating expression in the cell lines in this study.

It has been reported that VEGF can selectively up-regulate NRP1 in a VEGFR2 dependent manner (Oh et al., 2002), however in the cell lines used in this study with both VEGFR2 and NRP1 (H522, HOP62, HCT-116 & MDA-MB-231), did not reveal an obvious induction at a protein level. In fact HCT-116 showed some down regulation of NRP1 by hypoxia although *VEGF* was significantly induced in these conditions and MDA-MB-231 was less sensitive to hypoxia by measure of *VEGF* and *GLUT1* induction. Although not all cell lines were investigated regarding VEGF secretion, we did show after 72 hrs incubation that the induction of *VEGF* gene expression in HCT-116 did translate to a significant increase of VEGF collected from the culture supernatant and measured by ELISA. In the results by Oh *et al.* it was noted that NRP1 was induced in BRECs (bovine retinal endothelial cells) maximally after incubation with 25 ng/ml of VEGF. Two possible reasons for the discrepancy between the results of Oh *et al.* and this study could be firstly, that the maximum VEGF secretion that was measured in this study was significantly lower (i.e. pg/ml as opposed to ng/ml) and secondly, the VEGF biology between endothelial cells and tumor cells are not expected to be always comparable.

To further define the effect of bevacizumab on tumor cells, activation of receptors by phosphorylation specific immunoblotting of VEGFR1 and VEGFR2 could go some way in determining if signaling changes take place. Although NRP1 lacks a kinase domain, looking at macromolecular complexes including NRP by co-immunoprecipitation could shed some light on compensatory pathways that may be activated, as NRPs act as a co-receptor to a number of other RTKs such as MET receptors (Matsushita et al., 2007), EGFR (Rizzolio et al., 2012) and TGFβ receptors (Glinka et al., 2011).

Having ascertained the required cast of signaling players were present to varying degrees in all of the selected cell lines, the effect of limiting hypoxic induction of VEGF signaling through the use of bevacizumab was evaluated in a series of functional assays as well as gene regulation by qRT-PCR. As the experimental control, HUVECs showed a clear trend of up-regulation of the VEGF family of genes following the addition of recombinant VEGF (50 ng/ml), however only reaching significance in *NRP1* expression in agreement with published data (Oh et al., 2002, Brusselmans et al., 2005). A dose dependent response to VEGF was not evaluated in HUVECs to establish if increasing concentrations of VEGF could augment other genes, however this could potentially show more significant regulation of the selected genes.

Analysis of gene expression changes in tumor cells by blocking VEGF signaling with bevacizumab in hypoxia identified only one cell line (HS-578T) having a significant down regulation of *NRP1*. Many other cell lines (HOP62, HCT-116 & MDA-MB-231) showed a trend in the down regulation of *NRP1*, however all other genes were unchanged. The cell

lines with decreased *NRP1* expression did not share any patterns of receptor expression abundance or localization, nor induction of HIF1 regulated genes. However, down-regulation after bevacizumab treatment suggests this could be partially a VEGF driven mechanism. It is possible that the levels of VEGF induction were insufficient in fully driving NRP1 regulation and analysis with the addition of recombinant VEGF could further elucidate this principle.

It has been described that VEGF acts as a survival factor in MDA-MB-231 in a NRP1-dependent autocrine loop (Bachelder et al., 2001) as well as through VEGFR1 (Lee et al., 2007) and in HCT-116 (Samuel et al., 2011) via VEGFR2 (Calvani et al., 2008). As NRP1 not only showed expression in all cell lines but also some regulation with bevacizumab treatment, we evaluated tumor cell survival under the same conditions. None of the cell lines, including MDA-MB-231 and HCT-116, showed sensitivity to apoptosis by inhibiting VEGF with bevacizumab. We evaluated apoptotic signaling triggering PARP cleavage in addition to sub G1 fractions by flow cytometry, to also potentially detect cells that may have undergone necrosis. Our results emphasize the difference in VEGF regulation by blocking extracellular signaling with bevacizumab, and VEGF inhibition by siRNA or genetic knockdown, where intracellular signaling could be a factor. This also pertains to the tumor cell proliferation results, whereby the studies mentioned previously, found a decrease in proliferation that was VEGF dependent. Our results with bevacizumab in a larger screen of 30 cell lines did not establish any evidence of a dependence on extracellular VEGF, with the exception of one cell line (HT-29). HT-29 consistently showed approximately a 10% decrease in proliferation, however this could not be attributed to changes in VEGFRs or NRP1 expression nor to an increase apoptosis; this cell line remained an outlier. This could possibly be due to an induction of senescence as bevacizumab has been described to influence permanent cell cycle arrest both *in vitro* (although not specifically in this cell line) and *in vivo* (Hasan et al., 2011). Depletion of VEGF over a longer period of time may allow further studies relating to this mechanism.

Keeping with the expanded analysis in 30 cell lines, the final functional assay was to determine if blocking hypoxia induced VEGF using bevacizumab could alter tumor cell migration. Epithelial-mesenchymal transition (EMT) requires cells to acquire both morphologic and phenotypic changes, including an increase in motility that contributes to a more metastatic phenotype. Both hypoxia and VEGF have been identified as regulators of tumor cell migration prompting EMT (Indelicato et al., 2010, Soker et al., 2001, Fujiwara et al., 2007). Conversely, genetic deletion of tumor cell specific *Vegf* in RIP1-Tag2 pancreatic neuroendocrine tumor bearing mice have shown a more invasive phenotype, but also with increased tumor hypoxia, HIF1α stabilization and potentially compensatory c-Met activation (Paez-Ribes et al., 2009, Sennino et al., 2012). Furthermore, it has also been shown that short-

term treatment with anti-angiogenic therapy with VEGF receptor TKI's elicits accelerated metastases *in vivo*. Some of these studies attributed tumor progression to rapid regrowth of tumor vasculature (Mancuso et al., 2006) and other associate decreased survival to increased metastatic tumor burden (Ebos et al., 2009).This work set out to determine if bevacizumab treatment could induce or inhibit migration of tumor cells, perhaps hinting if hypoxic induced VEGF might contribute to a more aggressive phenotype. Although the cell lines displayed a high variation in migration ability, we were unable to establish that bevacizumab was able to promote or inhibit migration. The difference with hypoxic treated cells with those incubated in normoxia was not assessed, therefore we cannot conclude if hypoxia modulated migration in any of the tumor cell lines analyzed. In other studies, alternative pathway activation has been shown to be essential to invasion and metastasis, which was reduced by concurrent inhibition of both VEGF and c-Met, the tyrosine kinase receptor for hepatocyte growth factor (HGF) (Sennino et al., 2012). These findings support a role for VEGF signaling in invasion and metastasis. However, the *in vitro* 2D scratch assay, whilst simple and inexpensive, may lack some sensitivity and is unable to assess invasion through any matrix, which the Boyden chamber maybe able to determine and which may be important in VEGF regulation of migration.

Interestingly, a study by Fan *et al.* described CRC cell lines that were chronically exposed for 3 months to the same dose of bevacizumab as our treatments, had enhanced expression of VEGF family members, namely VEGF, PlGF and VEGFC in addition to the VEGF receptors VEGFR1 and NRP1. Furthermore, they demonstrated that the tumor cells (including HCT-116) chronically exposed to bevacizumab were more invasive and migratory, likely dependent on VEGFR1 signaling (Fan et al., 2011). Collectively, the results imply that prolonged exposure conditions cells inducing increased metastatic potential and raises interesting questions on what regulatory mechanisms control these features and how this could translate into the clinical setting.

The hypoxic-induction of VEGF expression to varying degrees in tumor cells showed through a series of functional assays that there was a limited effect of blocking para- and or autocrine VEGF by bevacizumab over a short period of exposure (Hein and Graver, 2013). In an effort to use treatments that could be more relevant to the clinical setting, later experiments subsequently focused on the treatment of tumor cells (in normoxia and with complete FCS) with both bevacizumab as well as a DNA damaging agents, as clinically bevacizumab is commonly given together with chemotherapy. Furthermore, we focused on CRC cell lines and based the premise on data suggesting that DNA repair deficient tumor types, in particular mismatch repair deficient, showed a beneficial response to bevacizumab clinically (Pogue-Geile et al., 2013). Isogeneic cell lines were therefore used, one mismatch repair deficient

(MLH1$^{-/-}$) HCT-116 and the other mismatch repair proficient HCT-116+ch3 (MLH1^{wt}) that carries the stably transferred chromosome 3, in addition to HT-29. It has recently been reported that sequential administration of an EGFR inhibitor (erlotinib) and a DNA damaging agent (doxorubicin) could enhance apoptosis in a subset of breast cancer cell lines (Lee et al., 2012). The experimental set up aimed to address if sequential administration of bevacizumab had an impact in sensitizing the tumor cells to DNA damage, or inhibiting DNA repair after DNA damage.

As discussed above, VEGF is shown to be critically involved in not only endothelial cell biology, but also in the survival of colon cancer cells, in particular in the sensitization to DNA damaging agents (Calvani et al., 2008, Samuel et al., 2011). This has been demonstrated by abrogating HIF-1α as well as up-regulation of apoptotic mediators including Bax, PARP and caspase-3. Different types of DNA damage induce alternative repair pathways and it was investigated if a response was dependent on any synthetic lethality with a deficient MMR cell line compared to the proficient cell lines. The cells were pretreated with bevacizumab, to determine if there is any sensitization, as well as post treated after DNA damage to investigate if repair specific modifications could be altered. DNA damaging agents used were: 1) SN-38 (active metabolite of irinotecan) which activates repair via HR and FA through the generation of both SSBs and DSBs (Voigt et al., 1998); 2) UV irradiation that produces bulky DNA adducts that are preferentially repaired by NER or BER (Sinha and Hader, 2002); 3) H_2O_2, a form of ROS, triggers base damage that is repaired by BER that is associated to Ca^{2+} fluxes or MMR, particularly Adenine or Thymine oxidative products (Korzets et al., 1999, Bridge et al., 2014); and 4) TNFα that activates MAPK pathways (p38 (Li et al., 2005) and JNK (Wicovsky et al., 2007)) as well as regulating expression of NRP and VEGFRs (Yang et al., 2004).

The treatment of bevacizumab alone in the tumor cells was consistent with the previous findings and published data of a limited effect in terms on proliferation (Shi and Siemann, 2004, Ortholan et al., 2010, Hein and Graver, 2013). Interestingly however, HT-29 that showed a bevacizumab-dependent reduction in proliferation in hypoxia and serum reduced conditions, was not evident with complete serum in normoxia. It is possible that other growth factors in the complete serum compensate for the loss of VEGF signaling with bevacizumab treatment such as increasing binding of alternative binding partners of VEGFRs such as PlGF (Loges et al., 2009, Carmeliet et al., 2001) or activation of alternative pathways such as FGF/FGFR (Crawford and Ferrara, 2009).

UV induced damage from 50 J/m^2 was selected based on the activation of signaling pathways such as p53 and JNK at a sub-lethal dose. Although the metabolic activity, which was

measured by the proliferation reagent (MTT), was significantly decreased, there were sufficient cells present for analysis. JNK is a key signaling pathway that can both regulate VEGF via the AP1 binding sites on the *VEGF* promoter (Pages and Pouyssegur, 2005), in addition to VEGF induced activation of cytokine release in a JNK-dependent manner in lung carcinoma cells (Lo et al., 2013). Furthermore, excision repair induced by UV damage, investigated a potential mechanism whereby repair could be assessed. Analysis showed that bevacizumab did not provide a protective or synergistic effect with UV damage and there was no evidence that activation of UV-induced pathways synergized with depleted VEGF.

The generation of ROS by H_2O_2 is a form of endogenous insult on DNA that can oxidize DNA bases in both nuclear DNA as well as mitochondrial DNA. Exogenous H_2O_2 can partially mimic a cellular response to changes in ROS levels by other stress factors (Dickinson and Chang, 2011). Moreover, VEGF via VEGFR2 signaling can induce ROS from NAD(P)H oxidases and has a critical role in vascular pathophysiology as well as hematological malignancies (Maraldi et al., 2010, Ushio-Fukai, 2007). Once again, with the reliance on both MMR and BER to repair oxidative damage, the role of VEGF in this context was assessed. It was noted that bevacizumab treatment after H_2O_2 might have a protective effect, however this did not prove to be significant and pre-treatment did not sensitize tumor cells to ROS mediated damage.

TNFα has a primary function in an inflammatory response, activating pathways such as NFκB and the MAPK pathways, in particular JNK. However TNFα can also activate death pathways under certain conditions via the TNFR-TRADD-FADD pathway (Gaur and Aggarwal, 2003). VEGF is positively regulated by activated NFκB in endothelial cells (Kim et al., 2001) and macrophages (Kiriakidis et al., 2003). Our results did not show an enhanced dependence on VEGF relating to proliferation by sensitization or post-treatment. As with the treatment with H_2O_2, there did appear to be some protective effect of inhibiting VEGF signaling through the treatment with bevacizumab, however not to a statistically significant degree.

Chemosensitization to DNA damaging agents has been described with the use of TKIs targeting EGFR (Lee et al., 2012) and synergistic apoptotic effects with Trk family receptor inhibitors (Strock et al., 2006) and a VEGFR selective TKI together with SN-38 treatment in a pancreatic cancer cell lines (Canu et al., 2011). The mechanisms by which synergies occur are varied from gene regulation of VEGFR2 to preventing efflux of the DNA damaging agent. Although a VEGFR selective TKI could synergize with SN-38, bevacizumab failed to mimic these results and did not show a change in the apoptotic rate. Differences in the mechanism of action between a monoclonal antibody that is specific for VEGF, compared to

a RTK inhibitor that is selective for VEGFRs but also partially inhibit other pathways including for example PDGFR signaling, could be a reason why the results are not equivalent.

Bevacizumab is generally given throughout chemotherapy clinically and is usually continued beyond the completion of cytotoxic treatment. Because of this, apoptotic cells were analyzed in addition to apoptotic signaling pathways but the results were unable to show an enhancement of chemosensitivity to SN-38 by sequestering VEGF with bevacizumab even after longer time points. According to a report suggesting cellular senescence could be induced by bevacizumab in a p16 dependent fashion (Hasan et al., 2011), it was evaluated if after DNA damage, senescence was differentially induced dependent on mismatch repair status. All treatment schedules analyzed did not reveal a significant impact on growth arrest. The results for cell line HCT-116 that was used in both sets of experiments was consistent in that bevacizumab did not induce senescence. Furthermore, the additional cell lines we selected were different to those of Hasan and colleagues and finally the discrepancy between results may be due to the fact that DNA damage was induced, which in itself can be a strong inducer of cellular senescence and bevacizumab was unable to show an additive effect.

Published data suggested that the consequences of events such as growth factor knock out or inhibition was triggered after expansion of several generations in MMR deficient cells (de las Alas et al., 1997). In order to investigate long-term effects of bevacizumab in relation to DNA damage or repair, the mutation rate of the tumor cells was evaluated as well as the ability of the tumor cells to form colonies after DNA damage after up to 28 days. It could be shown that pre-treatment with bevacizumab enhanced tumor cell's abilities to form colonies as well as to increase the rate of mutation. It was found that this pretreatment decreased the phosphorylation of JNK, a key mediator of the stress response transcription faction AP1. Although there was no enrichment for the AP1 target genes detected in the microarray, it does not exclude that persistent small changes in gene expression have an effect over a longer period of time, which was not discovered molecularly in the scope of these experiments. This is particularly common with epigenetic changes, as opposed to genomic changes whereby epigenetic modifications may require daughter cells to effect significant changes within the total population of cells. With regards to epigenetic changes, the microarray data showed a number of histone genes were down regulated by bevacizumab pretreatment along with the histone modifier HDAC4. HDAC4 knock down has recently been shown to inhibit deacetylation of target proteins (Isaacs et al., 2013) that control cell cycle as well as angiogenesis genes (Kehat et al., 2011). Continued activation of genes related to the cell cycle, along with DNA damage could explain why the mutation rate increases over time. These data may also concur with publications describing accelerated progression of pre-clinical tumors after short-term tumor angiogenesis inhibition (Ebos and Kerbel, 2011, Paez-

Ribes et al., 2009). The clinical relevance relating to changes in the disease course was analyzed in patients discontinuing bevacizumab (or placebo) and the meta analysis from 4,205 patients with BC, CRC, RCC and pancreatic cancer did not support increased mortality or an altered disease progression pattern between bevacizumab and placebo treated patients (Miles et al., 2011). Therefore, the preclinical results of short-term treatment may not translate to the clinical setting as pooled analysis describes, and these findings require further investigation and additional studies to establish a conclusive role for VEGF.

It was concluded that a direct effect of bevacizumab on tumor cells is possible after prolonged exposure and likely to involve JNK and/or cJUN. Furthermore, the intracellular activity of VEGF is potentially more significant than extracellular signaling through the VEGFRs in tumor cells that may produce a more obvious phenotype that could be studied with a greater degree of significance. A number of additional experiments are required to fully elucidate this potential mechanism such as AP1 reporter assays, inhibiting the activation of JNK and VEGF, NRP1 or JUN knock down.

6.2 Tumor-Derived Cytokines and Immune Cell Cross-Talk with Bevacizumab Treatment

As a significant but subtle effect of bevacizumab on tumor cells was detected, in order to be able to contribute to the translational aspects of antiangiogenic treatments in a clinical setting, we narrowed in on some of the data found in the microarray that showed gene expression changes in cytokines related to an inflammatory response. High-grade MSI tumors have been reported to have an increased lymphocytic infiltration (Kumar et al., 2009) that related to an improved overall survival compared to MSS tumors. In particular, activated CD8+ tumor infiltrating lymphocytes were more abundant in MSI tumors (Houston et al., 2008), suggesting MSI tumors are more immunogenic than MSS tumors, perhaps in part due to the production of truncated proteins and subsequent HLA antigen presentation. In addition, MSI tumors have a greater propensity for the molecular classification of an inflammatory subtype, which is marked by an increase in chemokine and interferon-related gene expression (Sadanandam et al., 2013, Kumar et al., 2009). Combining such data with the knowledge that many immune cell type express VEGF receptors, it was contemplated that the cytokine release of MSI tumors in addition to the inhibition of VEGF signaling through the treatment of bevacizumab, may differentially activate an immune response that is anti-tumorigenic.

Cancer-related inflammation is a potential mediator between malignancy and metastases and commonly occurs simultaneously with angiogenesis in response to various stimuli. The role of VEGF in the inflammatory process continues to unravel with current knowledge suggesting it is responsible for leukocyte adhesion on the endothelium via ICAM-1 and/or -2 (Tromp et al., 1998); preventing dendritic and other myeloid cell maturation (Gabrilovich et al., 1998, Dikov et al., 2005, Osada et al., 2008); accumulation of regulatory T cells (Terme et al., 2013a, Terme et al., 2013b); and induces recruitment of monocytes/macrophages to tumors (Shurin et al., 2006, Linde et al., 2012).

As bevacizumab is generally given clinically at the same time, as well as after chemotherapy, the further investigations focused on the question if bevacizumab altered immune mediated functions in combination with tumor-derived factors after DNA damage. In the gene regulation analysis of tumor samples during cellular recovery from the DNA damaging agent, regulation by bevacizumab of key macrophage chemokines, *CCL7* and *CCL8* were detected in HCT-116 (MSI) cells but not HCT-116+ch3 (MSS) cells.

Tumor associated macrophages (TAMs) are a key cellular subset in amplifying the inflammatory response in the tumor microenvironment (Colotta et al., 2009) in addition to contributing to angiogenesis. Macrophages have been shown to express VEGF receptors and co-receptors that influence monocyte/tumor associated macrophages migration, infiltration and tumor metastases via VEGFR1(Barleon et al., 1996), VEGFR2 (Dineen et al., 2008), VEGFR3 (Espagnolle et al., 2014) and Nrp1 (Casazza et al., 2013). We assessed bevacizumab not only directly on monocytes/macrophages, but also in conjunction with tumor derived factors by the use of tumor conditioned media. We wanted to determine if bevacizumab was able to modify the behavior of monocytes/macrophages in the context of tumor-secreted factors.

Macrophage polarization was assessed using known markers for M1- or M2-type macrophages, considered anti-tumor (pro-inflammatory) and pro-tumor (immune suppressive) respectively. Under the experimental conditions M1 markers were not detected, however M2 markers did appear to be influenced by bevacizumab. *CXCR4* (Chemokine (C-X-C motif) receptor 4) was influenced by bevacizumab, however only in the MSS cell line (HCT-116+ch3), which had a much higher expression level, suggesting a potential negative regulation. CXCR4 signaling enhances monocyte-driven angiogenesis via chemoattraction as well as differentiation (Seta et al 2013). In addition, we found that *CCL18* (Chemokine (C-C motif) ligand 18) could be induced in macrophages by bevacizumab alone and also by tumor derived factors after DNA damage with bevacizumab. This was consistent in both cell lines, however the MSI cell line (HCT-116) had approximately 2.5 fold higher expression levels.

CCL18, also known as pulmonary and activation-regulated chemokine (PARC), dendritic cell (DC)-chemokine 1 (DC-CK1), alternative macrophage activation-associated CC chemokine-1 (AMAC-1) and macrophage inflammatory protein-4 (MIP-4), is secreted by APCs but primarily exerts its effector functions on the adaptive immune system that preferentially trigger a T_H2 response in T cells via the receptor CCR8 (Schraufstatter et al., 2012, Islam et al., 2013). CCL18 is implicated in chronic inflammatory conditions such as systemic sclerosis, rheumatoid arthritis and idiopathic pulmonary fibrosis, and is a prognostic marker in some cancers (Chen et al., 2011, Prasse et al., 2007, Schutyser et al., 2005, Yuan et al., 2013, Leung et al., 2004).

To further assess functional adaptations of monocytes in response to tumor derived factors with and without bevacizumab inhibiting VEGF signaling, monocyte migration, macrophage phagocytic activity and T cell suppression were investigated. It was found that rhVEGF did induce migration of monocytes as per previous reports (Barleon et al., 1996, Linde et al., 2012, Roland et al., 2009), which bevacizumab was able to significantly inhibit. By including tumor-derived factors in addition to sequestering VEGF by bevacizumab, migration was not consistently influenced compared to results using recombinant VEGF. MSI tumor conditioned media after DNA damage did show a significant increase in monocyte migration after DNA damage from HCT-116 (MSI) conditioned media, which was partially attenuated by bevacizumab. Concurring with these results, the DNA damage response has been associated with inflammatory cytokine release (Rodier et al., 2009), and as a decrease in VEGF secretion after DNA damage was detected, the other cytokines secreted by the tumor cells are likely to be more critical in monocyte migration. Furthermore, Tripathi et al. demonstrated hypoxic tumor cells more strongly induced monocyte migration in conditioned media, compared to the normoxic control (Tripathi et al., 2014), which would perhaps suggest that only after a strong induction, is VEGF able to affect the induction of monocyte migration. As an increase in *CCL7* and *CCL8* with bevacizumab post DNA damage in HCT-116 (MSI) was detected, it was expected that monocyte migration would increase compared to DNA damaged conditioned media without bevacizumab. One explanation for this not being the case could be related to the expression levels of *CCL7* and *CCL8* detected, as they were quite low in terms of relative expression. In order to establish if CCL7 or CCL8 were key inducible proteins in response to bevacizumab treatment after DNA damage, a selective ELISA assay to measure the secretion of both chemokines would be necessary to elucidate the significance in tumor cell-immune cell cross talk. MSS tumor conditioned media had no significant effect on monocyte migration, however there was a trend that migration increased with bevacizumab, independent of DNA damage ($p=0.07$). It was concluded that *in vitro* analysis of monocyte migration could not directly correspond to clinical benefits and it is

therefore likely, that other cytokines or combinations of cytokines are more instructive factors regarding monocyte migration. These data suggest that monocyte migration towards tumor conditioned media is not significantly impacted by bevacizumab and by definition, VEGF, indicating other factors are likely to be instrumental in attracting monocytes. Most of the *in vitro* data showing VEGF to be a crucial factor for monocyte migration used recombinant VEGF (Linde et al., 2012, Roland et al., 2009), excluding other tumor-derived factors, which in this set up appear to be important.

The ability of macrophages to induce phagocytosis that was dependent on the treatment of tumor cells was evaluated. We found that when DNA damage was induced, the rate of phagocytosis increased in both cell lines but was significant only in MSI tumor cells (HCT-116). This follows the well-known pathway of clearing apoptotic or damaged cells by professional scavenger cells, including macrophages. The treatment of the tumor cells with bevacizumab prior to the co-culture with the macrophages did not greatly enhance the phagocytic activity, either with or without DNA damage. An interesting finding however was that macrophage co-culture with MSI tumor cells (HCT-116) showed a significant increase in the phagocytic rate when bevacizumab was added at the time of the co-culture. This would suggest that the activity was distinctly targeted at the macrophages. However, as this was not the case in the MSS (HCT-116+ch3) co-culture and it is conceivable that other tumor derived factors specific to MSI tumor cells (HCT-116) also contributed to the response. It is possible that CCL18, which was identified as a bevacizumab regulated gene, could be significant in this context, as it has previously been shown that CCL18-stimulated macrophages, at least 48 hours after culture of monocytes (and not freshly isolated monocytes), had increased non-specific phagocytosis (Schraufstatter et al., 2012).

Boussiotis *et al.* have shown that alternatively activated macrophages that expressed high levels of CCL18, were able to critically effect T cell functions including viability, cytokine production, clonal expansion and effector function via the B7/CD28 pathway (Boussiotis et al., 1996). In light of these results, a 2-step experimental set up was designed, whereby monocytes were first differentiated in tumor conditioned media, then bevacizumab was added to co-cultures with differentiated macrophages and T cells. In the controls (i.e. without conditioned media) monocytes differentiated with bevacizumab clearly show significant T cell suppression in a bevacizumab dependent manner, that was further enhanced by adding bevacizumab at the time of co-culture with T cells. This indicated clonal expansion of T cells is impeded by a direct effect of bevacizumab on monocytes/macrophages, potentially resulting in more naive T cells. There was no significant effect on T cell suppression dependent on the tumor-conditioned media in which the monocytes were differentiated. However, when bevacizumab was added at the time of co-culture (i.e. to mature

macrophages), there was a clear trend in T cell suppression that was only evident in macrophages from MSI tumor conditioned media. This suggests there are important tumor derived factors in addition to bevacizumab-induced activity in mature macrophages when compared to the controls. Macrophages from MSS tumor conditioned media showed the opposite effect, however experimental conditioned need to be further optimized to determine if these effects are more significant. Stimulating T cell clonal expansion with CD3/CD28 before incubation with differentiated macrophages may elucidate clearer mechanisms that could be further studied.

Interestingly CCL18 is reported to significantly attract freshly isolated T cells *in vitro* and has also been described to not only to promote the accumulation of CD4+, but also CD8+ T cells *in vivo* (Schutyser et al., 2002, Guan et al., 1999, Schutyser et al., 2005). Further experiments looking at the clonal expansion of CD8+ T cells warrants further investigation, particularly in the context of tumor conditioned media to determine if CD8+ T cell activity is influenced by either MSI tumors and/or VEGF. Our preliminary experiments looking at the detection surface markers of different subgroups of T cells using macrophages differentiated with or without bevacizumab did not alter CD8+, CD25 (Tregs) or CXCR4 (T_H2) populations. The results did however show a reduction of the T_H1 marker CXCR3 that appears to be reduced in a bevacizumab-dependent manner, giving an overall net effect of an increase the immunosuppressive arm of the adaptive immune response.

CCL18 may have a significant prognostic role in oncology that is dependent on tumor type. CCL18 recovered from ascites of ovarian cancer patients and has been described as an immune suppressor and correlated to poor overall survival (Duluc et al 2009). However, CCL18 is likely to be relative to conditions and/or cancer type, as gastric cancer (Leung et al 2004) and colorectal cancer (Yuan et al 2013) studies have shown it to be a favorable prognostic marker.

VEGF has a role in cell differentiation; inducing functional endothelium from embryonic stem cells (Nourse et al., 2010), inhibiting dendritic cell maturation (Gabrilovich et al., 1998) and tumor cell dedifferentiation through NRP1 and downstream effector Ras (Cao et al., 2012). The inhibition of VEGF in a pathological disease such as cancer, can subsequently affects numerous cell types beyond the obvious target of endothelial cells. In these experiments using tumor-derived factors and inhibiting VEGF with bevacizumab, we have shown evidence of immune cell modulation that is dependent on a specific subtype of CRC, MSI, and as well as an effect of bevacizumab directly on tumor cells. These findings may contribute to a better understanding of the molecular mode of action that could illuminate the key between DNA repair, anti-angiogenesis treatment and immune cell modulation.

7 CONCLUSIONS

Angiogenesis in cancer has been considered a universal target in patients with solid tumors, however the clinical use of anti-angiogenic treatments has not shown a universal response to compounds targeting angiogenic pathways such as VEGF. Amongst others, tumor specific factors have been considered to contribute to the response to anti-angiogenic compounds such as bevacizumab. VEGF is proving to be a functionally promiscuous protein, inducing not only endothelial cell proliferation and migration to form new vessels, but also affecting myeloid cell differentiation and tumor cell survival.

In this thesis it was found that inhibition of VEGF signaling using bevacizumab in tumor cells, had only a limited cell autonomous effect as a single agent, failing to significantly impact tumor cell survival, apoptosis, migration or expression of VEGF family members. A potential feed back loop of VEGF was evident in the regulation of NRP1, however the degree to which this was measurable in tumor cells was variable. There appears to be some effect of VEGF inhibition before DNA damage in reducing the activation of the JNK pathway, however clear mechanisms are yet to be fully elucidated.

Tumor derived factors from MSI tumors in addition to the inhibition of VEGF by bevacizumab, were shown to elicit significant changes in immune cell modulation potentially creating an anti-tumor microenvironment. *CCL18* was found to be significantly induced by bevacizumab, to a greater degree in MSI tumor cells than in to MSS tumor cells. High levels of CCL18 have been shown to induce functional changes in immune cells that was mirrored by the use of bevacizumab in conjunction with tumor derived factors from MSI tumors. These data propose that CCL18 has the potential to act as a marker for bevacizumab therapy and link a subtype of CRC to the benefit from a targeted anti-VEGF therapy.

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9 APPENDICES

9.1 ABBREVIATIONS

(6-4)PP	6-4photoproduct
5-FU	5-Fluorouracil
8-oxoGua	8-oxo-7,8-dihydroguanine
A-T	Ataxia telangiectasia
ACTB	Beta actin
ACTRII	Actin-related protein 2
AML	Acute myeloid leukemia
ANG2	Angiopoietin 2
APAF-1	Apoptotic peptidase activating factor 1
APC	Adenomatous polyposis coli
APE1 (APEX1)	AP endonuclease 1
AR	Androgen receptor
ARG1	Arginase 1
ASR (W)	Age-standardized rate (worldwide)
ATLD	Ataxia telangiectasia-like disorder
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
B2M	Beta-2-microglobulin
BASC	BRCA1-associated genome surveillance complex
BAX	BCL2-associated X protein
BC	Breast cancer
BCA	Bicinchoninic acid assay
BCL-10	B-cell lymphoma/leukemia 10
BCR-ABL	Breakpoint cluster region - Abelson murine leukemia oncogene
BER	Base excision repair
Bev	Bevacizumab
BIR	Break-induced replication
BLM	Bloom syndrome RecQ helicase-like
BMP	Bone morphogenetic protein
BNIP3	BCL2 adenovirus E1B 19-kDa interacting protein 3
bps	base pairs
BRAF	B-raf proto-oncogene serine/threonine kinase
BRCA1/2	Breast cancer susceptibility gene 1/2
BRIP1	BRCA1 interacting protein C-terminal helicase 1
BS	Bloom syndrome
CASP3/5	Caspase 3/5
CBCC	Crypt base columnar cells
CCL18	Chemokine (C-C motif) ligand 18
CCL7/MCP3	Chemokine (C-C motif) ligand 7
CCL8/MCP2	Chemokine (C-C motif) ligand 8
CDC25A/B/C	Cell division cycle 25A/B/C

CDK1/2	Cyclin-dependent kinase 1/2
CDKN2A	Cyclin-dependent kinase inhibitor 2A
cDNA	Complementary DNA
CDX2	Caudal type homeobox 2
CEN2	Centrin-2
CHK1/2	Checkpoint kinase 1/2
CI	Confidence interval
CIMP	CpG island methylator phenotype
CIN	Chromosome instability
cis-Pt	Cisplatin
CML	Chronic myleoid leukemia
CPD	Cyclobutane pyrimidine dimer
CRC	Colorectal cancer
CSA/B	Cockayne syndrome A/B
CSC	Cancer stem cells
CSR	Class switch recombination
CtIP (RBBP8)	Retinoblastoma blinding protein 8
CXCL10	Chemokine (C-X-C motif) ligand 10
CXCL9	Chemokine (C-X-C motif) ligand 9
CXCR3	Chemokine (C-X-C motif) receptor 3
CXCR4	Chemokine (C-X-C motif) receptor 4
DC	Dendritic cells
DCLRE1A	DNA cross-link repair gene 1A
DCLRE1C/Artemis	DNA cross-link repair gene 1C
DDB1/2	Damage-specific DNA binding protein 1/2
DDR	DNA damage response
DFS	Disease free survival
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DRPLA	Dentatorubro-pallidoluysian atrophy
DSB	Double strand break
DSBR	Double strand break repair
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
EMT	Epithelial to mesenchymal transition
EPC	endothelial progenitor cells
ER	Endoplasmic reticulum
ERCC1	Excision repair cross-complementation group 1
ERCC2	Excision repair cross-complementation group 2
ERCC4/XPF	Excision repair cross-complementation group 4
ERCC5/XPG	Excision repair cross-complementation group 5
ERK (p42/p44 MAPK)	Mitogen-activated protein kinase 1
EXO1	Exonuclease 1
FA	Fanconi anaemia

FACS	Florescence-activated cell sorting
FAK	Focal adhesion kinase
FAN1	FANCD2/FANCI-associated nuclease 1
FANC (A-G/L/M)	Fanconi anaemia (A-G/L/M)
FAS ligand/FASLG	TNF ligand superfamily 6
FAS receptor/TNFRSF6	TNF receptor superfamily 6
FEN1	Flap structure-specific endonuclease
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FOLFIRI	5-FU, LV, Irinotecan (Chemotherapy)
FOLFOX	5-FU, LV, Oxaliplatin (Chemotherapy)
GADD45	Growth arrest and DNA damage inducible protein 45
GBM	Glioblastoma multiforme
GG-NER	Global genome NER
GLUT1	Glucose transporter 1
GM-CSF	Granulocyte macrophage colony-stimulating factor
H-RAS	GTPase HRas
H2AX	H2 histone family X
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HD	Huntington's disease
HDAC2/4	Histone deacetylase
HIF	Hypoxia-inducible factor
HNPCC	Hereditary non-polyposis colon cancer
HR	Homologous recombination
HR (stat)	Hazard ratio
HR23B	Rad23 homolog B
HSPG	Heparan sulfate proteoglycans
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM	Intercellular adhesion molecule 1
ICL	Interstrand crosslink
IF	In Frame
IFN	Interferon
IGF2R	Insulin-like growth factor 2 receptor
IHC	Immunohistochemistry
IL8	Interleukin 8
JMD	Juxtamembrane domain
JNK	cJUN N-terminal kinase
JUN	jun proto-oncogene
KRAS	GTPase Kras
KU70/XRCC6	X-ray repair complementing defective repair in Chinese hamster cells 6
KU80/XRCC5	X-ray repair complementing defective repair in Chinese hamster cells 5
LDHA	Lactate dehydrogenase A
LIG4	Ligase IV

LV	Leucovorin
MAPK	Mitogen-activated protein kinase
MBD4/MED1	Methyl-CpG binding domain protein 4
MDM2	Mouse double minute 2 homolog/E3 ubiquitin-protein ligase
MDSC	Myeloid-derived suppressor cells
MET	MET proto-oncogene receptor tyrosine kinase
mFF6	Modified FOLFOX6
MHC	Major histocompatibility complex
MK2	Mitogen-activated protein kinase-activated protein kinase 2
MLH1	MutL homolog 1
MLH3	MutL homolog 3 (S. Cerevisiae)
MLR	Mixed leukocyte reaction
MMC	Mitomycin C
MMR	Mismatch repair
MRC1	Mannose receptor C type 1
MRE11	Meiotic recombination 11 homolog A
MRN	Mre11-Rad50-Nbs1
mRNA	Messenger RNA
MSH2	MutS homolog 2
MSH3	MutS homolog 3
MSH6	MutS homolog 6
MSI	Microsatellite instability
MSI-H	MSI-High
MSI-L	MSI-Low
MSS	Microsatellite stable
MYH (MUTYH)	MutY homolog
MΦ	Macrophages
NBS	Nijmegen breakage syndrome
NCI	National Cancer Institute (USA)
NEIL1-3	nei endonuclease VIII-like 1-3
NER	Nucleotide excision repair
NFκB	Nuclear factor kappa-light-chain enhancer of activated B cells
NHEJ	Non-homologous end joining
NHL	Non-Hodgkin lymphoma
NIH	National Institute of Health
NK	Natural killer cells
NOS2	Nitric oxide synthase 2, inducible
NRP1/2	Neuropilin-1/2
NSCLC	Non-small cell lung cancer
OC	Ovarian cancer
OF	Out of Frame
OGG1	8-oxoguanine DNA glycosylase
OS	Overall survival
p65/RelA	NFκB subunit
PABP2	Poly(A)-binding protein 2
PALB2	Partner and localizer of BRCA2

PARP	Poly ADP ribose polymerase
PBMC	Peripheral blood mononuclear cells
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDK1	Pyruvate dehydrogenase (PDH) kinase isozyme 1
PFS	Progression free survival
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PI3KCA	PI3K catalytic alpha polypeptide
PKC	Protein kinase C
PlGF	Placental growth factor
PMS1/2	Post meiotic segregation increased 1/2 (*S. Cerevisiae*)
PNKP	Polynucleotide kinase-phosphatase
POLB	Polymerase beta
POLD	Polymerase (DNA directed) delta 1
POLH	Polymerase eta
POLQ	Polymerase theta
PTEN	Phosphatase and tensin homolog
qPCR	quantitative polymerase chain reaction
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RAD18	RAD18 E3 ubiquitin protein ligase
RAD51/B/C	RAD51 recombinase/paralog B/C
RAD52	RAD52 homolog (*S. Cerevisiae*)
RAD54	Homologous recombination protein family
RAD54B	RAD54 paralog B
RAS	Protein member of the RAS family
RECQL4	RecQ protein-like 4
REV1	REV1 polymerase (DNA directed)
REV3	REV3-like polymerase zeta catalytic subunit
RIZ	PR domain containing 2 with ZFN domain
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPA	Replication protein A
RTK	Receptor tyrosine kinase
RTKI	RTK inhibitor
S-CIN	Structural CIN
SBMA	Spinobulbar muscular atrophy
SCA	Spinocerebellar ataxias
SDSA	Synthesis-dependent strand-annealing
siRNA	Small interfering RNA
SMAD4	Mothers against decapentaplegic homolog 4
SMC	Smooth muscle cells
SNP	Single-nucleotide polymorphism
SSB	Single strand break
SSBR	SSB repair
SSR/STR	Simple sequence repeats/short tandem repeats

TA	Transit amplifying
TAM	Tumor associated macrophages
TC-NER	Transcription coupled NER
TCF-4	Transcription factor 4
TCGA	The Cancer Genome Atlas
TCR	T cell receptor
TGF	Transforming growth factor
TGFRII	TGF beta receptor 2
TKD	Tyrosine kinase domain
TLR3	Toll-like receptor 3
TLS	Translesion synthesis
TMD	Transmembrane domain
TNFα	Tumor necrosis factor alpha
TNM	Tumor/Node/Metastasis
TOP1	Topoisomerase 1
TOPBP1	Topoisomerase II binding protein
TP53	Tumor protein p53
TP53BP1	TP53 binding protein 1
UNG	Uracil-DNA glycosylase
UPR	Unfolded protein response
UV	Ultraviolet
UVSSA	UV-stimulated scaffold protein A
VEGF/VEGFA	Vascular endothelial growth factor A
VEGFB	Vascular endothelial growth factor B
VEGFC	Vascular endothelial growth factor C
VEGFD	Vascular endothelial growth factor D
VEGFR1	Vascular endothelial growth factor receptor 1
VEGFR2	Vascular endothelial growth factor receptor 2
VEGFR3	Vascular endothelial growth factor receptor 3
W-CIN	Whole CIN
WEE1	WEE1 G2 checkpoint kinase
WHO	World Health Organization
WISP	Sorting nexin 9
WNT	Secreted protein of the WNT signaling pathway
WRN	Werner syndrome RecQ helicase-like
XP	Xeroderma pigmentosum
XPA	XP complementation group A
XPC	XP complementation group C
XRCC1	X-ray repair complementing defective repair in Chinese hamster cells 1
XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4

9.2 COPYRIGHT AUTHORIZATION

9.3 PUBLICATIONS

Hein M, **Graver S**, (2013) Tumor cell response to bevacizumab single agent therapy *in vitro*. *Cancer Cell International* 2013 13:94

Graver S, Mazzone M, Schartl M, (*manuscript in preparation*) Modulation by bevacizumab of an anti-tumor immune response in combination with MSI tumor-secreted factors in CRC

9.4 CURRICULUM VITAE

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9.6 AFFIDAVIT

Affidavit

I hereby confirm that my thesis entitled **The Molecular and Cellular Cross Talk between Angiogenic, Immune and DNA Mismatch Repair Pathways** is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore I confirm that the thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

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