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Expression and functional interactions of microRNAs with their target chemokine CCL20 in colorectal cancer

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Table of contents

Abbreviations

%	per cent
℃	degree Celsius
ABC	Avidin-Biotin-Complex
Adenoma	adenomatous polyp
AEC	3-Amino-9-Ethylcarbazole
AJCC	American Joint Committee on Cancer
APC	adenomatous polyposis coli
BCA	bicinchoninic acid protein assay
BCIP	5-bromo-4-chloro-3'-indolyphosphate
BSA	Bovine Serum Albumin
C. elegans	Caenorhapditis elegans
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CRC	colorectal carcinoma
CRLM	colorectal liver metastases
C_T	Cycle Threshold
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
dd H_2O	double distilled water
DGCR8	DiGeorge syndrome critical region gene 8
DMSO	Dimethylsulfoxid
DNA	desoxyribonucleic acid
dsRNA	double stranded RNA
E. coli	Escherichia coli
e.g.	for example
eIF	eucaryotic initiation factor
ELISA	Enzyme Linked Immunosorbent Assay
ELR	glutamic acid-leucine-arginine motif
ERK	extracellular signal-regulated kinase
Exon	expressed region
FFPE	formalin fixed paraffin embedded
g	gramm

G-protein	guanine nucleotide-binding protein
h	hour
HCC	hepatocellular carcinoma
HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
HRP	horseradish-peroxidase
ICD-O	international classification of diseases for oncology
IgG	Immunoglobulin G
IL	Interleukin
Intron	Intervening regions
JNK	Jun N-terminal kinase
kb	kilobases
kDa	kilo Dalton
LARC	liver and activation-regulated chemokine
LARII	Luciferase Assay Reagent II
LB	Luria-Bertani
min	minute
MIP-3α	macrophage inflammatory protein 3alpha
miRNA, miR	microRNA
ml	millilitre
mRNA	messenger RNA
NBT	nitro-blue tetrazolium
nm	nanometer
oncomiR	oncogenic miRNA
ORF	open reading frame
PACT	PKR activator
PAK	Primary Antibody
P-body	processing body
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pen	Penicillin
PI3K	phosphatidylinositol 3-kinase
PLB	passive lysis buffer
pre-miRNA	precursor-miRNA

pri-miRNA	primary-miRNA
qRT-PCR	Quantitative Realtime Polymerase Chain Reaction
RIPA	radio immunoprecipitation assay
RISC	RNA induced silencing complex
RNA	Ribonulceic Acid
RNase	Ribonuclease
RPM	revolutions per minute
RT	Room temperature
S	Svedberg
SAK	Secondary Antibody
SAPK	stress activated protein kinase
SEM	Standard error of the mean
siRNA	small interfering RNA
snoRNA	small nucleolar RNA
SSC	Standard saline-citrate
ß-ME	ß-Mercaptoethanol
Strep	Streptomycin
TBS	Tris Buffered Saline
TE	TRIS-EDTA
TNF	Tumour Necrosis Factor
TNM	tumour/node/metastases
TRBP	human immunodeficiency virus transactivating response RNA binding protein
U	(enzyme) unit
UTR	untranslated region
v/v	volume per volume
WHO	World Health Organization
x g	times gravity
yr	year

1. Summary

1.1 Summary

Colorectal cancer (CRC) is one of the most common cancer types worldwide. In recent years chemokines and miRNAs were found to be deregulated in CRC and some members were used as diagnostic- and prognostic markers. Chemokines are small secretory molecules involved in pro- and anti-tumour directed processes. MiRNAs are small non-coding RNA molecules which post-transcriptionally regulate gene expression thereby influencing oncogenic or tumour-suppressive effects dependent on the set of targeted genes.

Therefore, the present thesis aimed to analyze functional interactions between miRNAs and chemokine CCL20 and to characterize their interactions and expression profiles in CRC tissues. Target prediction tools were used to identify miRNAs, potentially regulating CCL20 expression. The predicted interactions were validated using luciferase assays and site directed mutagenesis. Functional interactions between specific miRNAs and the mRNA of their target gene CCL20 were tested in CRC cells by transfection of miRNA mimics and subsequent quantification of CCL20 expression on mRNA and protein level. CCL20 expression was also analyzed on mRNA and protein level in CRC tissue samples. In addition, miRNA expression was analyzed using qRT-PCR. Furthermore, localization of CCL20 and miRNA expression was investigated using immunohistochemistry and in situ hybridization, respectively. The results have shown that CCL20 gene expression is regulated by miR-21 in 3 of 4 CRC cell lines whereas miR-145 showed no interaction with CCL20 gene expression. Nevertheless, miR-21 expression and CCL20 mRNA and protein expression were demonstrated to be significantly up-regulated in CRC tissues in relation to corresponding normal colorectal tissues. However, CCL20 and miR-21 were observed to be predominantly expressed in mesenchymal elements in the tumour microenvironment, where miR-21 expression is restricted to tumour-associated fibroblasts while CCL20 is mainly expressed in infiltrating immune cells.

In summary, the results of this thesis demonstrated a functional interaction between miR-21 and CCL20 in CRC cells. However, investigating miR-21 and CCL20 expressions in CRC tissues revealed that the two molecules were predominantly

expressed in different cell types in the microenvironment of CRC tumours which indicates that miR-21 and CCL20 may not directly interact in CRC tissues. Therefore, a therapeutic application of miR-21 mediated CCL20 down-regulation would have to be applied in CCL20 expressing cells in the microenvironment of CRC tissues.

1.2 Zusammenfassung

Das kolorektale Karzinom (CRC) ist eine der häufigsten Krebsarten weltweit. In den letzten Jahren wurde erforscht, dass Chemokine und miRNAs im CRC dereguliert sind, wobei verschiedene Mitglieder dieser Molekülgruppen als diagnostische- und prognostische Marker untersucht wurden. Chemokine sind kleine, sekretorische Moleküle, die an pro- und anti-Tumor assoziierten Prozessen beteiligt sind. MiRNAs sind kleine nicht-kodierende RNA Moleküle, die die Genexpression post-transkriptionell regulieren und dabei in Abhängigkeit ihrer Zielgene eine onkogene oder Tumor supprimierende Wirkung entfalten.

Daher war es das Ziel dieser Arbeit, funktionelle Interaktionen zwischen spezifisch ausgewählten miRNAs und dem Chemokin CCL20 zu untersuchen und die Interaktionen und Expressionsprofile in CRC Gewebeproben zu charakterisieren. MiRNAs, die potentiell die Expression von CCL20 regulieren, wurden mit online verfügbaren Vorhersageprogrammen analysiert und die Interaktionen mittels Luciferase Assays und zielgerichteter Mutation validiert. Die funktionellen Interaktionen zwischen den so identifizierten miRNAs mit ihrem Zielgen CCL20, wurden in CRC Zellen durch Transfektion von miRNA-Mimics mit anschließender Expressionsanalyse von CCL20 auf mRNA und Proteinebene untersucht. Im nächsten Schritt wurde die Expression von CCL20 in CRC Gewebeproben auf mRNA und Proteinebene in Relation zu normalem, kolorekektalen Gewebe analysiert. Die Expression der miRNAs wurde ebenfalls mit qRT-PCR untersucht. Die Lokalisation der Expressionen von CCL20 und den entsprechenden miRNAs wurde durch Immunhistochemie und In-situ-Hybridisierung dargestellt. Die Ergebnisse zeigten, dass miR-21 die Expression des CCL20 Gens in 3 von 4 CRC Zelllinien reguliert während miR-145 keine regulatorische Funktion aufwies. Im CRC Gewebe wurde die Expression von miR-21 und CCL20 signifikant erhöht detektiert. Allerdings zeigte sich, dass CCL20 und miR-21 überwiegend in mesenchymalen Bereichen des

Tumor-Microenvironments exprimiert werden, wobei die miR-21 Expression vor allem in Tumor assoziierten Fibroblasten und die CCL20 Expression überwiegend in infiltrierenden Immunzellen stattfindet.

Zusammenfassend zeigten die Untersuchungen dieser Arbeit, dass miR-21 die CCL20 Expression in CRC Zellen reguliert. Expressionsuntersuchungen im Gewebe von CRC Patienten zeigten hierbei, dass miR-21 und CCL20 signifikant erhöht exprimiert werden. Allerdings findet ihre Expression vorwiegend im Tumor-Microenvironment statt und dort in jeweils unterschiedlichen Zelltypen. Dies zeigt, dass mutmaßlich keine direkte Interaktion zwischen miR-21 und CCL20 in CRC Gewebe stattfindet. Eine therapeutische Anwendung von miR-21 vermittelter CCL20 Regulation sollte daher in CCL20-exprimierenden Zellen im Tumor-Microenvironment von CRC Gewebe stattfinden.

2. Introduction

2.1 Cancer

2.1.1 Cancer – a general approach

In 2008, globally 12.7 million new cancer cases were diagnosed resulting in 7.6 million cancer deaths [Ferlay et al., 2008]. There are three distinct varieties of cancer which comprise leukaemia's and lymphomas, defined as cancers of the blood and blood-forming tissue, carcinomas, which are cancers of the epithelial cells and sarcomas, which are cancers of the mesodermal cells.

The most prevalent intersexual cancer types are solid tumours of the lung, colon/rectum and stomach. In females breast cancer is most prevalent and in males prostate cancer dominates the sex specific cancers [Jemal et al., 2011].

It is well documented that the incidence of cancer increases progressively with age in humans as well as in animals [Anisimov et al., 2009; Parkin et al., 2001; Balducci et al., 2005]. Thus, aging is associated with a number of events at the molecular, cellular and physiological level that influence carcinogenesis and cancer growth [Anisimov et al., 2009]. To explain the association of cancer and age, one simple explanation presumes that the high prevalence of cancer in older individuals may simply reflect a more prolonged exposure to carcinogens [Peto et al., 1985]. However, to list all recent arguments against this hypothesis would be beyond the scope of this thesis. To date, reputable scientific sources propose that age-related progressive changes in the internal milieu of the organism like proliferative senescence and many other changes may provide an increasingly favourable environment for the induction of new neoplasia as well as for the growth of latent malignant cells [Campisi, 2005; Anisimov, 2003]. Moreover, the combined effects of cumulative mutational load, increased epigenetic gene silencing, telomere dysfunction and altered stromal cell milieu may boost the development of age-related cancer [DePinho, 2000; Anisimov et al., 2009]. In addition to the major risk factor age, other risk factors for the development of cancer comprise heavy consumption of tobacco, alcohol, red meat and obesity. Thus, our diet can influence the risk of cancer. For example a diet which is high in fat like red meat has been linked to

increased risk of breast and colon cancer. Numerous environmental factors like industrial waste or cigarette smoke are also known to increase the risk of developing cancer. Thus, some chemicals such as asbestos may cause lung cancer and mesothelioma. Also several viruses can cause cancer, such as papillomavirus which causes cervical cancer in women or hepatitis B virus which can cause liver cancer. Moreover, inflammatory diseases like ulcerative colitis are known to cause colon cancer. Another risk factor for cancer is the exposure to radiation [Anand et al., 2008]. These risk factors can result in acquired genetic mutations and therefore contribute to the development of neoplasias which may be caused by the accumulation of several mutations [Mucci et al., 2001; Hahn et al., 2002]. The accumulation of mutations along with signals from the microenvironment may result in neoplastic changes which are characterized by cells that exhibit unregulated growth linked with the capability to invade the surrounding tissue and to spread to distant organs. To achieve such alterations, crucial cellular mechanisms need to be altered including the amplification of proliferative signalling, bypassing growth suppressors, avoiding cell death, gaining replicative immortality, increasing angiogenesis and achieving invasive properties [Hanahan et al., 2011]. The spread of tumour cells to distant organs leads to the formation of secondary tumours called metastases [Poste et al., 1980]. The alterations in genetics and behaviour of cancerous cells are also associated with a decrease in the differentiation state compared to neighbouring normal cells.

All these visible alterations in cancerous tissue and cancer cells as well as the incidence of secondary tumours are used by pathologists to determine the progression state of the cancer [American Joint Committee on Cancer, 2002; Wittekind et al., 2010]. Such categorization is generally used as a basis to apply suitable therapies and to predict the prognosis for the patients outcome.

2.1.2 Colorectal cancer (bowel cancer)

The development of colorectal cancer is viewed as a slow process which may progress over many years [Kozuka et al, 1975]. The most common preliminary stage of colorectal carcinomas (CRC) is the polyp. A special type of polyp designated adenomatous polyp (adenoma) is most susceptible to progress into a neoplastic

stage. However, only less than 10 % of adenomas become cancerous [Levine et al., 2006].

The progression of normal colorectal mucosa to colorectal cancer appears via the accumulation of genetic mutations in a multistep process (Figure 1) [Fearon et al., 1990]. The mutational landscape of CRC is complex and the alterations leading to CRC can occur in 20 different cancer-associated genes [Beerenwinkel et al., 2007]. Thus, one path compasses the inactivation of tumour-suppressor genes by mutation. For example, one pathway involved in CRC development which is often activated by mutations, is the Wnt-pathway [Morin et al., 1997]. The most common mutation leading to constitutive activation of this pathway is a mutation in the tumour-suppressor gene APC which regulates the oncoprotein β-catenin an activator of the Wnt-pathway [Korinek et al., 1997]. Another frequently affected pathway is the p53 pathway which is involved in cell cycle arrest, apoptosis and restriction of angiogenesis [Vazquez et al., 2008]. Inactivation of p53 is frequently found at the intersection from adenoma to carcinoma [Baker et al., 1990]. Finally, the TGF-β pathway is often deregulated by the mutation of tumour-suppressor genes. In a high number of CRCs the kinase domain of the TGFBR2 receptor is inactivated by mutations [Markowitz et al., 1995] but also mutations in downstream targets (e.g. SMAD4) are found [Takagi et al., 1996]. In addition to the inactivation of tumour-suppressor genes, the activation of oncogenic pathways is frequently involved in CRC development. Such activations often take place because of mutations in the RAS or BRAF genes which are activators of the MAPK pathway [Bos et al., 1987; Oliveira et al., 2007]. These two genes are also involved in DNA methylation processes [Nagasaka et al., 2008]. Another oncogenic pathway is the phosphatidylinositol 3-kinase (PI3K) pathway where the catalytic subunit PI3KCA frequently exhibits mutations as well as other regulators (like PTEN) in this cascade [Berg et al., 2010]. Therefore, some pathways and regulators are frequently affected in CRC and show effects associated with chromosomal instability (RAS and p53) or DNA methylation (RAS and BRAF). Such alterations can also have an impact on the expression of microRNAs (miRNA) which have an influence on cancer pathology including CRC [Lujambio et al., 2008].

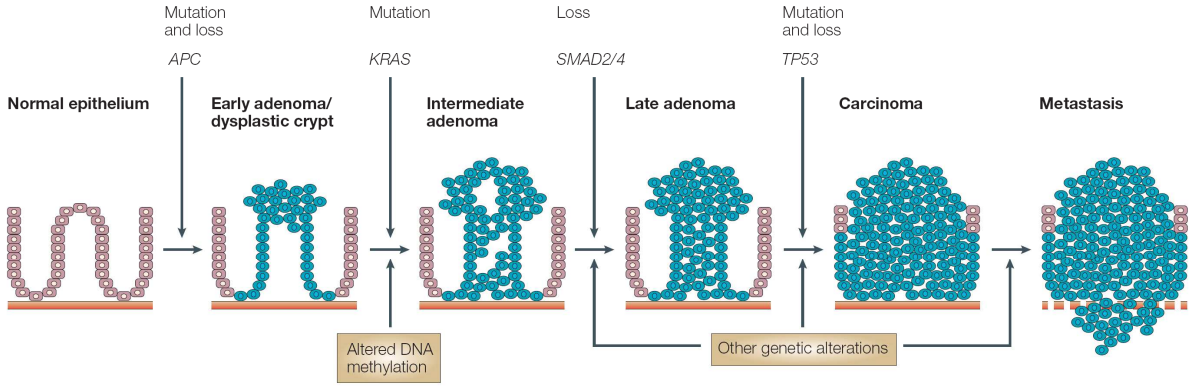

Figure 1 Development of CRC.
The progression from normal colorectal cells to cancerous and metastatic cells is linked to the accumulation of certain mutations. (Adapted from Davies et al., 2005)

MiRNAs are a relatively new group of molecules whose defective regulation has an impact on CRC development, progression and metastasis [Slaby et al., 2009; de Krijger et al., 2011].

Mutations in these pathways are associated with different hereditary diseases associated with an increased risk of developing CRC, e.g. familial adenomatous polyposis, Gardner's and Turcot syndromes and hereditary nonpolyposis colon cancer. However, only 5 % of CRCs are due to these syndromes [Ivanovich et al., 1999]. Beyond these hereditary risk factors also acquired factors have an impact on the risk for developing CRC. As described above for the general risk of developing neoplasias also acquired CRC risk factors comprise diets rich in fat or red meat, cigarette smoking, pelvic irradiation or inflammatory bowel disease [Lin, 2009].

According to histological tumour features distinct colorectal tumour types can be distinguished (Table 1). The most common (~96 %) colorectal tumour type is the adenocarcinoma which develops from the glandular in colonic tissue [Stewart et al., 2006].

Tumour Type	ICD-O Code
Adenocarcinoma	8140/3
Mucinous adenocarcinoma	8480/3
Signet-ring cell carcinoma	8490/3
Small cell carcinoma	8041/3
Squamous cell carcinoma	8070/3
Adenosquamous carcinoma	8560/3
Medullary carcinoma	8510/3
Undifferentiated carcinoma	8020/3

Table 1 Colorectal Tumour Types and ICD-O Codes (Adapted from Hamilton et al., 2000)

The progression state of colorectal cancer is primarily staged by two different systems. The older and simpler system is the Dukes Classification which classifies colorectal cancer into three classes (Table 2) [Dukes, 1932]. The second, newer and presently most common system for CRC staging is the TNM (tumour/node/metastases) system edited by the American Joint Committee on Cancer (AJCC) which uses different classes and their combinations (Table 2) [Wittekind et al., 2010].

Stage		**Dukes stage**
T stage: primary tumour		
TX	Primary tumour can not be assessed	
T0	No evidence of primary tumour	
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria	
T1	Tumour invades submucosa	A
T2	Tumour invades muscularis propria	A
T3	Tumour invades through muscularis propria into subserosa or into non-peritonealised pericolic or perirectal tissue	B
T4	Tumour directly invades other organs or structures and/or perforates visceral peritoneum	B
N stage: regional lymph nodes		
NX	Regional lymph nodes cannot be assessed	
N0	No regional lymph node metastasis	
N1	Metastasis in one to three regional lymph nodes	C (any T stage)
N2	Metastasis in four or more regional lymph nodes	C (any T stage)
M stage: distant metastasis		
MX	Distant metastasis cannot be assessed	
M0	No distant metastasis	
M1	Distant metastasis	
Stage grouping		
Stage 0	Tis N0 M0	
Stage I	T1 N0 M0 T2 N0 M0	
Stage II	T3 N0 M0 T4 N0 M0	
Stage III	Any T N1 M0 Any T N2 M0	
Stage IV	Any T Any N M1	

Table 2 Staging of colorectal cancer.
(Adapted from Treanor, 2007)

In addition, tumours can be graded according to the degree of differentiation based on a system from the WHO. Low grade carcinomas (G1 and G2) are well differentiated tumours whereas high grade carcinomas (G3 and G4) are low differentiated types.

2.2 Chemokines

2.2.1 Chemokines - a general approach

Chemokines are chemotactic cytokines which belong to a group of small secretory signal molecules. Commonly, chemokines have a length of 70-125 amino acid residues and a molecular weight of 8-14 kDa. According to their structural and functional characteristics they belong to a family of inflammatory and chemotactic active cytokines [Zlotnik et al., 2000]. Due to their secretion from the cell, chemokines control the migration of different cell types including lymphocytes, monocytes, neutrophils and fibroblasts and play a role in maturation and development of immune cells [Rossi et al., 2000]. Chemokines and their receptors are involved in a wide variety of biological processes such as angiogenesis, morphogenesis, autoimmunity and wound healing and also play a role in tumour cell growth and development of metastasis [Rossi et al., 2000]. Interestingly, the chemokine-chemokine receptor network was also shown to be involved in Human immunodeficiency virus (HIV)-infection and appear to take part in the process of HIV-resistance [Suresh et al., 2006].

Chemokines interact with specific chemokine receptors on the surface of their target cells. These receptors belong to a group of 7-transmembrane G-protein coupled receptors. The interaction of chemokines with their receptors activates an intracellular signal cascade which plays a role in the binding and extravasation of lymphocytes from the blood vessels into the corresponding tissues in physiologic and inflammatory processes [Locati et al., 2005]. Since many chemokines can interact with different chemokine receptors and most chemokine receptors bind different chemokines, the chemokine network is relatively complex (Figure 2). In addition, different cell types can produce and sustain different chemokine receptor sets and produce several chemokines [Mantovani, 1999]. According to the number and adjustment of the

characteristic cysteine residues at the amino-terminus, chemokines are subdivided into four subgroups (CC, CXC, CX3C and XC). The CC, CXC and CX3C group each contain four cysteine residues in the amino-terminal region whereas the XC group only contains two cysteines. In the CC-chemokine group the first two cysteines are directly neighboured whereas in the CXC-chemokine group the two cysteines are interspersed by any amino acid and in the CX3C-chemokine group these cysteines are interspersed by three arbitrary amino acids [Zlotnik et al., 2000]. According to a chemokine nomenclature reform the name of a chemokine consists of the name of the subgroup followed by L for ligand and consecutive numbers for the corresponding chemokine (e.g. CCL20) [International Union of Immunological Societies/World Health Organization Subcommittee on chemokine nomenclature, 2001]. Most of the human CXC-chemokine genes are coded on chromosome 4 whereas many CC-chemokine genes are predominantly found on chromosome 17 (Figure 2) [Zlotnik et al., 2000].

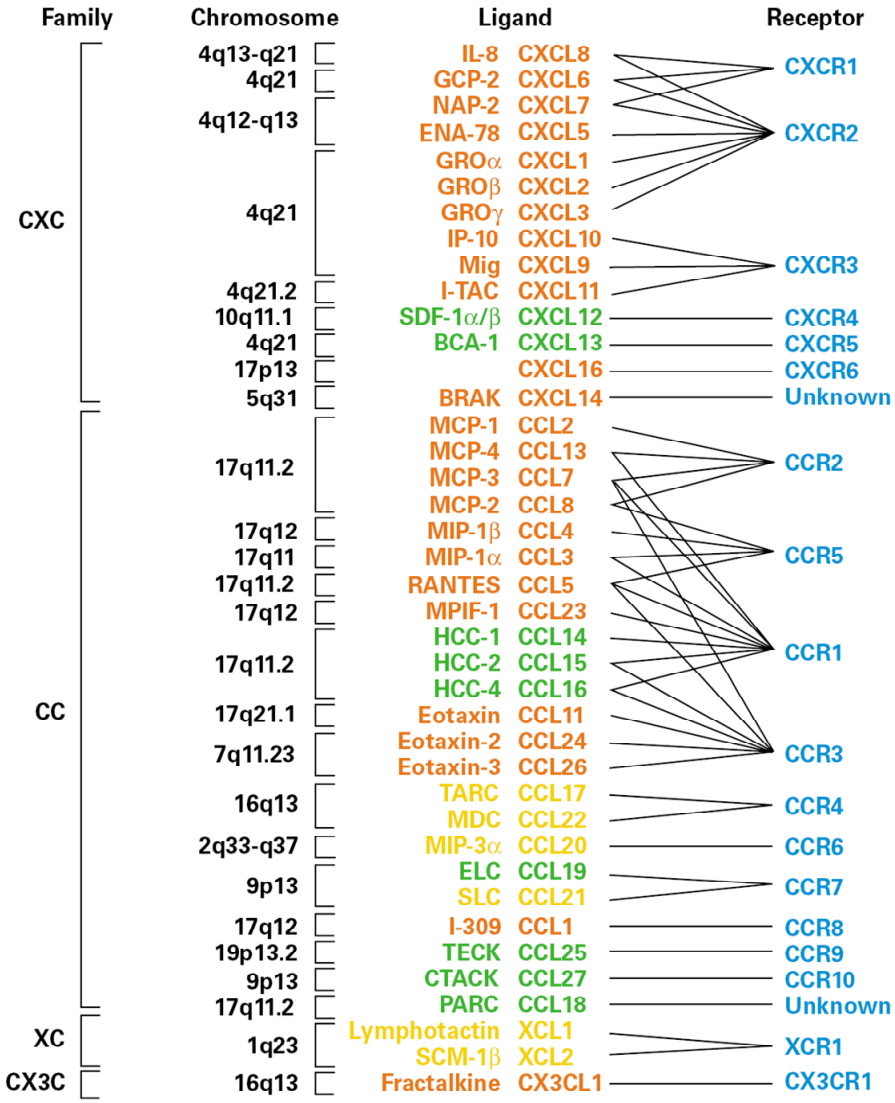

Figure 2 Human chemokines with their chromosomal location and their corresponding chemokine receptors.
Red labelled chemokines are inflammatory/inducible; green labelled chemokines are homeostatic; yellow labelled chemokines are both. (Adapted from Locati et al., 2005)

Furthermore, CXC-chemokines are classified according to the existence or absence of a characteristic motif of three amino acids (glutamic acid, leucin, arginin) referred to as the ELR-motif [Strieter et al., 1995]. CXC-chemokines which contain this ELR-motif are designated to induce angiogenesis whereas chemokines without this motif are angiostatic [Belperio et al., 2000]. In addition, CXC-chemokines containing the ELR-motif attract neutrophils by chemoattraction whereas CXC-chemokines without this motif attract lymphocytes [Luster, 1998]. Further subdivision of chemokines is achieved by their physiological characteristics into inflammatory and homeostatic chemokines. Homeostatic chemokines are constitutively expressed in certain tissues and are responsible for the infiltration of leukocytes into organs and thus being involved in activation, differentiation and maturation of leukocytes [Schaerli et al.,

2005]. Moreover, homeostatic chemokines play a role in angiogenesis and stem cell biology and have a direct proliferatory effect on cells. Contrarily, the main task of inflammatory chemokines is the recruitment of effector leukocytes to the site of inflammation [Moser et al., 2004]. Nevertheless, some chemokines appear to be involved in both inflammatory and homeostatic effects. Therefore, these chemokines cannot be categorized unambiguously as belonging to one of the two groups (Figure 2).

2.2.2 Chemokines and cancer

The potential to chemotactically attract immune- and tumour-cells made chemokines interesting targets for investigations on the development and progression of cancer. In recent years chemokines were therefore reported to play a role in different processes associated with cancer. CXCL1, CXCL2 and CXCL3 were shown to directly promote growth and tumourigenicity of melanoma cell lines [Balentien et al., 1991; Owen et al., 1997]. Furthermore, mutations in the corresponding receptor CXCR2 which renders this receptor constitutively active, are also capable of inducing oncogenic transformation [Burger et al., 1999]. In addition to these direct effects chemokines are also involved in angiogenesis of tumours. Thereby, CXCL8 enhances angiogenesis by interaction with CXCR2 [Gabellini et al., 1999] whereas CXCL4 [Yang et al., 2011b], CXCL9 [Addison et al., 2000] and CXCL10 [Sato et al., 2007] inhibit angiogenesis via CXCR3 signalling. Also in metastatic processes chemokines appear to play a role. CXCL8 is involved in increased matrix metalloproteinase expression in different cancers and thereby enhances invasiveness and metastatic potential [Watanabe et al., 2002; Inoue et al., 2000]. However, even in organ specificity of metastasis chemokines are involved. In this respect, the best investigated systems are the CXCL12-CXCR4 and CCL21/CCR7 pathways which guide CXCR4 and CCR7 positive cancer cells to organs expressing high amounts of CXCL12 and CCL21 [Müller et al., 2001]. As chemokines are key factors in leukocyte recruitment, these features also play a role in cancer. Thus, CCL2 was found to be the main attractor of macrophages in different cancers [Arenberg et al., 2000; Ueno et al., 2000]. In recent years, macrophage infiltration was shown to be associated with cancer progression, thereby affecting angiogenesis,

immunosuppression and metastasis [Sica, 2010]. The chemokines CCL3 [Maric et al., 1999], CCL5 [Lavergne et al., 2004] and XCL1 [Huang et al., 2004] were shown to attract effector T-cells as well as natural killer cells and thereby mediating anti-tumour effects. On the other hand, certain chemokines can attract regulatory T-cells which seem to be associated with decreased anti-tumour immunity [Maruyama et al., 2010; Shields et al., 2010]. Therefore, the subset of attracted T-lymphocytes is critical for pro- or anti-tumoural chemokine function. A third group of attracted lymphocytes are dendritic cells. Different chemokines were reported to mediate recruitment of immature dendritic cells to the tumour site. However, it is not clear if the accumulation of dendritic cells is pro- or contra-tumourigenic. Recent investigations propose dendritic cells to mediate tumour-induced immune tolerance [Janikashvili et al., 2011.].

As chemokines play such different roles in processes associated with cancer, chemokines are a potential starting point for cancer therapy. Some chemokine antagonists were already identified and were used in pre-clinical trials [Ishida et al., 2006].

2.2.3 CCL20 - a general approach

The chemokine CCL20 is also formerly known as liver and activation-regulated chemokine (LARC), macrophage inflammatory protein 3alpha (MIP-3α) or exodus-1 [Bacon et al., 2002]. CCL20 was first described in 1997 as a 96 amino acid residue long precursor and 70 amino acid residue long mature protein. It is a chemotactic cytokine of the group of CC-chemokines and is coded by a gene located on chromosome 2 locus q36.3 [Hieshima et al., 1997]. In contrast to most other chemokines consisting of three exons and two introns CCL20 contains four exons and three introns [Nelson et al., 2001]. Thus, CCL20 shows a relatively low sequence homology to other CC chemokines [Yoshie et al., 1997]. Expression of CCL20 was found in different mucosal-associated lymphoid tissues like liver, lung, peripheral lymph nodes, thymus, tonsils, colon and appendix [Schutyser et al., 2000]. Therefore, one important function of the CCL20 pathway seems to constitute an appropriate immunity in intestinal mucosal tissues [Kunkel et al., 2003]. In addition CCL20 is also expressed by immune cells like B-cells, dendritic cells, granulocytes, macrophages or

T-cells. The G-protein coupled CC-chemokine receptor CCR6 is the sole receptor for CCL20 and is not targeted by any other chemokine. Expression of CCR6 was mainly found in the appendix, fetal liver, lymph node and spleen tissue and in these tissues also in lymphocytes (T cells and B cells) and dendritic cells but not in monocytes [Baba et al., 1997].

CCL20 expression can be initiated by different factors or pathogens such as dsRNA, lipopolysaccharide or bacterial endotoxins [Schutyser et al., 2000]. In addition, CCL20 expression is also induced by pro-inflammatory cytokines including interleukin 1beta (IL-1β), tumour necrosis factor-alpha (TNF-α), or IL-17 [Kawashiri et al., 2009]. The regulatory role of CCL20 appears to be mediated in part through signalling via MAPK pathway(s) [Scapini et al., 2002] or the NF-κB pathway [Fujiie et al., 2001]. In contrast anti-inflammatory cytokines like IL-10 cause decreased expression of CCL20 [Rossi et al., 1997].

According to these activation pathways it was described that CCL20 can act as an inflammatory as well as a homeostatic chemokine due to the cellular conditions [Mantovani, 1999]. In immune responses CCL20 is proposed to be responsible for the attraction of cells specialized in antigen presentation and activation of the adaptive immune system. CCL20 is therefore involved in the attraction of CCR6 positive immature Langerhans cells, other immature dendritic cells and their precursors to locations of putative antigen invasion [Charbonnier et al., 1999; Iwasaki et al., 2000]. Upon maturation of these cells the expression of CCR6 and the chemotactic activity of CCL20 decrease [Charbonnier et al., 1999]. CCL20 seems to be involved in the enrichment of immature dendritic cells to the scene of infection [Dieu et al., 1998]. Additionally, memory B-lymphocytes in contrast to naive B-lymphocytes exhibit chemotactic movement towards CCL20 [Liao et al., 2002] and subsets of memory/effector T-lymphocytes are responsive to CCL20 rather by intracellular signalling than induction of chemotaxis [Liao et al., 1999]. Thereby the CCL20-CCR6 axis appears to be involved in the arrest of memory/effector T-cells on endothelial cells activated by TNF-α during the early phase of immune response [Fitzhugh et al., 2000]. In addition to these inflammatory associated functions of CCL20 the constitutive expression in mucosal tissue proposes a role for CCL20 in the general immune surveillance of the mucosa under normal conditions [Izadpanah et al., 2001]. This multifarious attraction of leukocytes enables the CCL20-CCR6 axis

to be involved in activation of the immune system as well as in immune tolerance [Comerford et al., 2010].
According to this dual function the expression and defective regulation of CCL20 has been reported to be involved in different human diseases like rheumatoid arthritis [Hirota et al., 2007] or inflammatory bowel disease [Kaser et al., 2004]. In rheumatoid arthritis CCL20 attracts memory T cells whereas in inflammatory bowel disease over-expressed CCL20 increases accumulation of both memory T cells and immature T cells [Kaser et al., 2004].

2.2.4 CCL20 and cancer

The expression of the pro-inflammatory chemokine CCL20 and its receptor CCR6 has been described in different types of cancer including pancreatic cancer [Rubie et al., 2010; Kleeff et al:, 1999], non-small-cell lung carcinoma [Kirshberg et al., 2011], breast [Bell et al., 1999], hepatocellular carcinoma (HCC) [Rubie et al., 2006a], CRC [McLean et al., 2011] and colorectal liver metastases (CRLM) [Rubie et al., 2006b; Rubie et al., 2006c].
Regulation of basal CCL20 in the CRC cell line Caco-2 is mediated by Sp1 whereas ESE-1 is required for basal and cytokine induced CCL20 expression in this cell line [Kwon et al., 2003]. Even in the colon tumour cell line CT26 the expression of CCL20 is regulated by the Toll-like receptor pathway and promotes tumour immune escape [Wang et al., 2008]. Another study that analyzed the role of the CCL20 receptor CCR6 in intestinal epithelial cells and CRC cells reported that CCR6 mediates ERK-1/2, SAPK/JNK, and Akt signaling resulting in proliferation and migration of CRC cells [Brand et al., 2006]. However, the role of CCL20 in tumour associated effects is discussed contradictorily. In experiments with the HCC cell line Huh7 stimulation with CCL20 resulted in increased growth via the p44/42 MAPK pathway [Fujii et al., 2004] and in a rodent tumour cell line CCL20 increased tumour growth and decreased anti-tumour immunity even though immature dendritic cells are attracted [Bonnotte et al., 2004]. It was shown that the attracted immature dendritic cells do not mature in the tumour which is a putative mechanism for tumour immune escape [Bell et al., 1999]. In addition, CCL20 increased adhesion to collagen and advanced growth in vitro and enhances invasion and tumour growth in vivo in a mouse xenograft model [Beider et

al., 2009]. In another mouse model of CRC the expression of CCL20 was found in tumour-associated macrophages and in turn promoted tumour growth by the recruitment of CCR6 positive regulatory T-cells [Liu et al., 2011a]. In human pancreatic cancer over-expression of CCL20 was present in pancreatic cancer cells as well as in tumour-infiltrating macrophages and promotes tumour growth and invasiveness in a autocrine or paracrine way [Kleeff et al., 1999]. Additionally, a high expression of CCL20 in HCC patients after resection was linked to poor prognosis [Ding et al., 2011]. Conversely in a mouse model decreased tumour growth was found upon intratumoural injection of CCL20 expressing adenovirus [Fushimi et al., 2000]. However CCL20 expression alone was not sufficient to induce anti-tumour reactions in B16 tumours [Crittenden et al., 2003; Furumoto et al., 2004].

2.3 MicroRNAs (miRNA)

2.3.1 MicroRNAs - general approach

The miRNA family comprises a class of small, non-coding RNAs in a range of 21-25 nucleotides which play an important role in post-transcriptional gene regulation [Bartel, 2004]. The negative regulation of post-transcriptional expression conducted by miRNAs is mediated either by inhibition of translation or degradation of the target mRNA [Kim, 2005]. The fact that miRNAs are endogenously expressed as imperfect stem loop structures distinguishes miRNAs from siRNAs which are generated from long, double stranded RNA (dsRNA) molecules [Zeng et al., 2003]. The first miRNA discovered, lin-4, is complementary to parts of the 3'UTR of lin-14 mRNA and thereby regulating lin-14 expression in Caenorhapditis elegans (C. elegans) which is involved in timing of embryonic development [Lee et al., 1993]. Since their discovery miRNAs were described as an abundant class of gene regulatory elements in many different organisms including worms [Ambros, 2003], flies [Ambros, 2003], plants [Reinhart et al., 2002] and mammals [Lagos-Quintana et al., 2002]. In this respect, miRNAs play a role in the regulation of a wide variety of cellular processes involved in development [Agrawal et al., 2009], differentiation [Tarantino et al., 2010], proliferation [Pogue et al., 2010] and apoptosis [Ge et al., 2011]. In order to accomplish these multifaceted functions, miRNAs are expressed in distinct patterns in different tissue types. If these

patterns become defective, the aberrant expression of miRNAs can cause or contribute to diseases [Geekiyanage et al., 2011] as well as to cancer development and progression [Chang et al., 2011b]. Efforts to predict the number of miRNA genes in humans estimated that there are >1000 different miRNAs [Berezikov, 2011]. Therefore miRNAs are the most prevalent group of regulatory molecules. As every miRNA can regulate the expression of multiple target genes and miRNAs can cooperatively regulate one gene [Krek et al., 2005] it was assumed that miRNAs take part in the post-transcriptional regulation of approximately 30 % of human genes [Lewis et al., 2005].

2.3.2 MicroRNA biogenesis

MiRNA genes are spread in clusters over the genome but most frequently found in introns of protein coding genes or intergenic regions [Kim et al., 2007; Borchert et al., 2006]. Therefore miRNAs which reside in introns are expressed with the according gene whereas miRNAs in intergenic regions have their own promoters.

The miRNA genes are transcribed either by polymerase II or polymerase III according to the location of the gene [Borchert et al., 2006; Lee et al., 2004]. MiRNAs transcribed by polymerase II result in several kilobases (kb) long primary-miRNAs (pri-miRNAs) which are capped and poly-adenylated and contain a stem loop (Figure 3) [Lee et al., 2004]. Such pri-miRNAs can be mono- or poly-cistronic and are processed by the microprocessor complex consisting of the RNAse III type protein Drosha and the protein DiGeorge syndrome critical region gene 8 (DGCR8) to smaller ~65-75 nucleotide long hairpin-structured molecules which are designated precursor-miRNAs (pre-miRNAs) [Han et al., 2004]. In addition to this common pathway there is a less common one in which pre-miRNAs are created by direct splicing from intronic structures called mirtrons. These mirtrons are independent from the Drosha/DGCR8 microprocessor complex but in turn have special splicing sites and are therefore dependent on the functional splicing machinery and debranching enzymes [Okamura et al., 2007]. However, not all miRNAs coded in introns undergo this processing pathway and it is not terminally clarified whether pre-miRNAs created from mirtrons enter the normal downstream miRNA processing machinery or if they are processed in a separate pathway [Sibley et al., 2012]. Following the nuclear

processing steps the pre-miRNAs are exported to the cytoplasm in an energy consuming Ran-GTP dependent step mediated by Exportin 5 which is part of the nuclear transport receptor family (karyopherins) [Katahira et al., 2011]. In the cytoplasm the pre-miRNAs are cleaved ~22 nucleotides distant from the 5' end of the pre-miRNA by the Dicer complex consisting of the cytoplasmic RNAse III type protein Dicer, human immunodeficiency virus transactivating response RNA binding protein (TRBP) and PKR activator (PACT) [Kok et al., 2007] in order to create miRNA:miRNA* duplexes [Park et al., 2011]. After cleavage by Dicer the miRNA:miRNA* duplexes are loaded to Ago proteins. To date, four Ago proteins (Ago 1-4) are known in humans, but the sets of miRNAs that bind to each of them obviously overlap among all Ago proteins [Su et al., 2009]. The Ago proteins are a central part of the RNA induced silencing complex (RISC) [Yoda et al., 2010]. After incorporation to the RISC complex the dsRNA is unwound and only one strand (designated as the guide strand) remains in the complex whereas the other strand is degraded. Thus, the thermodynamic features of the 5' end of both strands are responsible for strand selection [Khvorova et al., 2003]. However, the mechanism of strand loading and selection is not completely understood as in some rare cases both strands produce functional miRNAs [Yang et al., 2011a]. Once loading and assembly of the RISC complex is completed, the regulatory events are mediated.

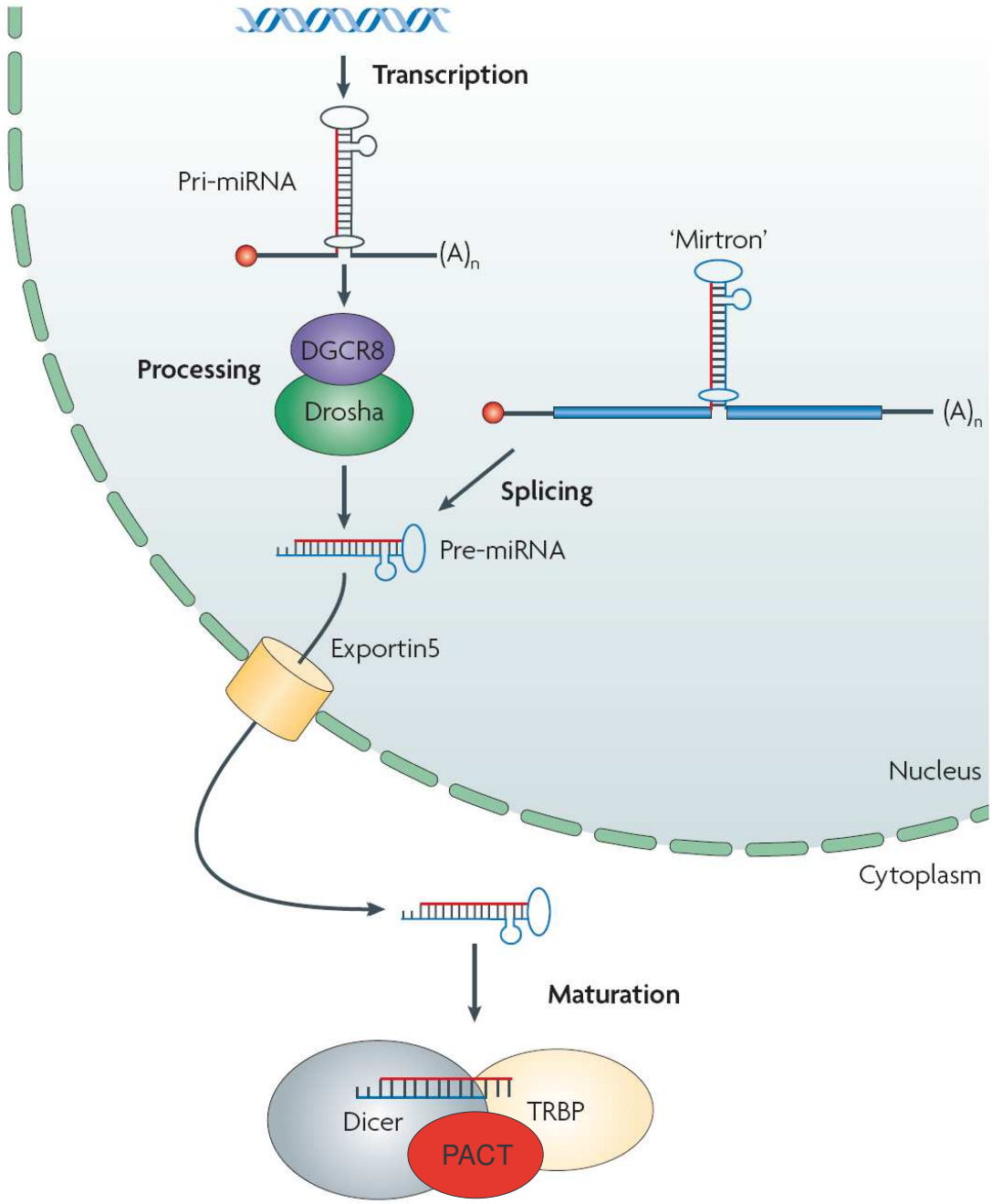

Figure 3 Overview of the biogenesis of miRNAs.
MiRNAs are primarily transcribed as pri-miRNAs in the nucleus and further processed by Drosha/DGCR8 to pre-miRNAs. Besides this pathway pre-miRNAs can also be generated by splicing from mirtrons. The pre-miRNAs are exported to the cytoplasm by Exportin 5. In the cytoplasm the pre-miRNAs are processed by the Dicer-complex which results in miRNA-duplexes and the strand with the weaker 5' base pairing is incorporated into the RISC complex. (Adapted from Filipowicz et al., 2008)

2.3.3 MicroRNA regulatory interactions

The guide strand incorporated into the RISC complex is the key element for the recognition of the target mRNAs [Parker et al., 2006]. Recognition is mediated by

complementarity between the miRNA and parts of the 3'UTR of the target mRNA. Some rare cases also reported that miRNA target sites are present in the open reading frame (ORF) or 5'UTR of target mRNAs [Moretti et al., 2010]. The degree of complementarity between the miRNAs and their target sequences is considered as a predictor for the respective regulatory mechanism. Complete or nearly complete complementarity labels the target mRNA for proteolytic degradation whereas a lower degree of complementarity labels the mRNA for processes resulting in decreased translation or mRNA destabilization [Bartel, 2009]. Nevertheless, there are some exceptions in which low complementarity also leads to degradation of the target mRNA [Bagga et al., 2005]. In both cases target recognition is mediated by perfect base pairing between the "seed" sequence, present at position 2-8 at the 5' end of the miRNA and the target mRNA. Another interaction which possibly takes part in target recognition is 3' end pairing of the miRNA which is primarily important for miRNAs with weaker "seed" interaction. This effect is referred to as "compensatory 3' end binding" [Brennecke et al., 2005]. In addition to "seed" complementarity as the most common mechanism there are some functional miRNAs reported which do not have such a "seed" interaction.

In most cases miRNAs are only partially complementary to their target sequences. To date, various mechanisms are discussed by which miRNAs possibly or actually mediate the repression of target mRNA translation (Figure 4) [Fabian et al., 2010; Filipowicz et al., 2008]. There are different steps in which the translation can be blocked by miRNAs. The first step is translation initiation. Competition of AGO 2, which contains certain homology to the binding site of the initiation factor eIF4E, with eIF4E for cap binding [Kiriakidou et al., 2007] or interruption of the association of active 80S ribosomes by the RISC-complex [Chendrimada et al., 2007] can lead to blockage of initiation. Also mechanisms at post-initiation steps like poly(A) tail shortening and decapping of the target mRNA which leads to mRNA degradation [Behm-Ansmant et al., 2006], retarded translation speed and subsequent ribosome drop-off [Petersen et al., 2006] or degradation of synthesized protein during translation [Nottrott et al., 2006] can contribute to translational repression.

It was shown that miRNA-mediated translationally repressed mRNAs are directed to P-bodies by the RISC-complex where they are either stored or degraded [Liu et al., 2005].

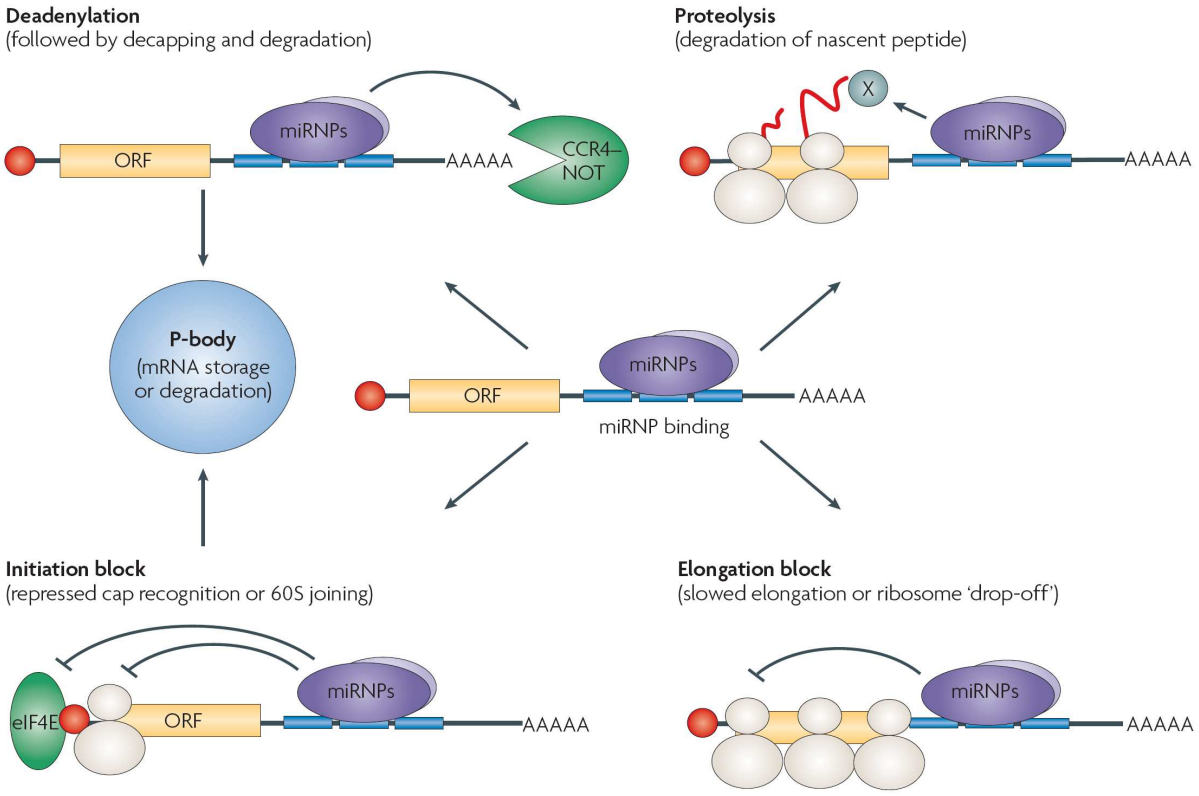

Figure 4 Possible mechanisms for miRNA mediated post-transcriptional target gene repression.
The translation of mRNAs can be blocked at different stages. The initiation can be blocked by inhibition of 5' cap recognition or ribosome assembly. Post-initiation the translation can be blocked by deadenylation, decapping or retardation of translation speed which results in ribosome drop-off. At last it was proposed that the RISC complex is directly involved in degradation of the nascent protein. (Adapted from Filipowicz et al., 2008)

2.3.4 MicroRNAs in cancer

The process of carcinogenesis is characterized by altered regulation of gene expression. Thus, special interest is focused on altered expression of tumour-suppressor genes and oncogenes which normally are involved in many cellular processes such as development, proliferation, differentiation, metabolism or apoptosis. As miRNAs are involved in post-transcriptional regulation of gene expression, they were proposed to have an impact on tumour-associated gene expression.

In recent years, a wide variety of interactions between miRNAs and cancer associated genes were found. The first interaction of miRNAs with cancer was shown in B-cell chronic lymphocytic leukemia where the loss of the 13q14 locus is responsible for the decreased expression of miR-15a and miR-16-1 [Calin et al., 2002]. Hence miRNAs can also act as tumour-suppressor genes (tumour-suppressor

miRs) or oncogenes (oncomiRs) [Cowland et al., 2007] by interacting with protein coding oncogenes or tumour-suppressor genes. This is in line with the investigations that miRNA signatures can also be used as markers of cancer [Fabbri, 2010] and the expression patterns of miRNAs can actually be applied for the determination of different cancer types and tissue origin of tumours [Su et al., 2001].

Altered regulation of miRNAs and reduced global miRNA expression evolved as a characteristic of cancer [Davalos et al., 2010]. Along the global down-regulation of miRNAs, also up-regulated expression of distinct miRNAs is found to be associated with tumour development [Li et al., 2012b]. As all tissue types have their specific miRNA expression patterns [Liang et al., 2007a] it is not surprising that miRNAs are misregulated in cancer. When normal cells are transformed into malignant cells they experience changes with respect to their phenotype and their state of differentiation which distinguishes them from normal cells and endows them with characteristic capabilities [Hanahan et al., 2011.]. The dysregulation of miRNA expression patterns can therefore be causative or a consequence of these processes.

Reasons for the altered expression of miRNAs in different kinds of cancer can be diverse. A wide variety of miRNA genes are coded in fragile genomic regions and/or in regions associated with distinct kinds of cancer [Calin et al., 2004]. Therefore, genomic instability can be one cause of increased or decreased miRNA expression associated with cancer [Braude et al., 2006]. However, alterations in miRNA expression can also occur when parts of the miRNA processing machinery or its regulators are deficient [Kumar et al., 2007]. Thus, a defect of the proteins Drosha, DICER1 or Exportin 5 was shown to be associated with global down-regulation of miRNAs in cancer [Thomson et al., 2006; Kumar et al., 2009; Melo et al., 2010]. Apart from mutational/genomic changes also epigenetic changes like histone modifications or DNA methylation patterns have an impact on the expression levels of miRNAs in diverse cancer types [Sampath et al., 2012; Saito et al., 2006]. In selected cancer types it was shown that several miRNA transcription units are located in CpG islands which are major targets of DNA methylation processes [Suzuki et al., 2011]. In addition to the mechanisms described above, polymorphisms in the sequence of mature miRNAs or pre-miRNAs can also have an impact on miRNA expression [Jazdzewski et al., 2008]. However, this is rather rare due to the shortness of miRNA sequences.

Apart from an altered miRNA gene expression profile, failure in miRNA function can also lead to altered gene expression. Thus, polymorphisms in miRNA binding sites of target genes can have an impact on miRNA mediated regulation in cancer [Schetter et al., 2011].

In recent years miRNAs aberrantly expressed in different cancer types were related to certain classical oncogenic and tumour-suppressor pathways. A miRNA known to act as an oncomiR is miR-155 which is over-expressed in different cancer types and regulates among others the tumour suppressor gene SOCS1 [Volinia et al., 2006; Jiang et al., 2010]. Also the miR-17 cluster (containing miR-17-5p, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92) is up-regulated in several cancer types, [Volinia et al., 2006] where it appears to be activated by Myc, targeting the expression of PTEN [Olive et al., 2009] and E2F1 [O'Donnell et al., 2005] and also decreasing TGF-β signalling [Dews et al., 2010]. Similarly, miR-21 plays a role as an oncomiR and is up-regulated in a wide variety of cancer types including stomach, prostate, pancreas, lung, colon and breast [Vicinus et al., 2012; Volinia et al., 2006]. Thereby miR-21 is activated by RAS through AP-1 and represses the expression of PDCD4 [Talotta et al., 2009.].

In contrast, miRNAs can also act as tumour suppressor miRNAs. Known tumour suppressor miRNAs are miR-15a and miR-16-1 whose down-regulation is coupled to loss of 13q14 in different cancers [Calin et al., 2002; Dong et al., 2001]. These miRNAs down-regulate BCL-2, CCND1 and WNT3A which are involved in tumourigenic processes [Bonci et al., 2008]. Another miRNA with tumour suppressor activity is let-7 which regulates the oncogene RAS, E2F2 and CCND2 [Johnson et al., 2005; Dong et al., 2010]. At last, miR-143 and miR-145 are predominantly down-regulated in cancer of the colon and breast [Slaby et al., 2007] where their down-regulation is involved in altered expression of the KRAS and EGFR receptors [Chen et al., 2009; Cho et al., 2011].

Thus, the small selection of miRNAs incorporated upstream or downstream of oncogenic and tumour-suppressor pathways show their potential role in these tumourigenic pathways. However, as miRNAs can regulate a wide variety of target genes and as to date not all targets of a distinct miRNA are identified, it cannot reliably be predicted if a miRNA acts pro- or contra-tumourigenic.

2.3.5 MicroRNAs in colorectal cancer

The first study related to altered regulation of miRNAs in CRC was published in 2003 [Michael et al., 2003]. Subsequently, several high throughput profiling studies on miRNAs expression patterns in CRC identified more than 100 miRNAs with altered gene expression. The miRNAs miR-20, miR-21, miR-31 and miR-99b were consistently up-regulated whereas expressions of miR-143, miR-145 and miR-192 were found to be decreased [Bandrés et al., 2006; Volinia et al., 2006; Slaby et al., 2007; Schetter et al., 2008; Chen et al., 2009]. Thereby, miR-31 showed increased expression in higher stages and miR-21 was correlated to metastasis and tumour stage [Slaby et al., 2007]. Down-regulation of miRNAs in CRC can be caused by epigenetic silencing as 47 miRNAs of 37 primary transcription units were potential targets of these mechanisms [Suzuki et al., 2011]. Another mechanism contributing to altered miRNA expression in CRC is the p53 pathway which is frequently mutated in CRC [Scott et al., 1991]. MiRNA profiling in CRC cell lines identified p53 putative binding sites present in ~45 % of miRNA promoter regions [Xi et al., 2006b]. Direct targets activated by p53 in CRC are the miRNAs miR-34a [Chang et al., 2007], miR-192 and miR-215 [Georges et al., 2008] which play a role in cell cycle arrest and other cancer relevant processes.

Certain miRNAs deregulated in CRC were shown to target pathways often altered in CRC (Figure 5). MiRNA-143, which is frequently down-regulated in CRC was shown to regulate KRAS and therefore decreased miR-143 expression was shown to promote growth in CRC cells [Chen et al., 2009]. The regularly up-regulated miRNA-135 targets APC, a member of the Wnt/β-catenin pathway, whose expression is often altered in initial steps of CRC [Nagel et al., 2008]. In the PI3K pathway miR-126 decreases p85-beta whereas the up-regulated miR-21 regulates PDCD4, an inhibitor of the PI3K pathway [Guo et al., 2008; Asangani et al., 2008].

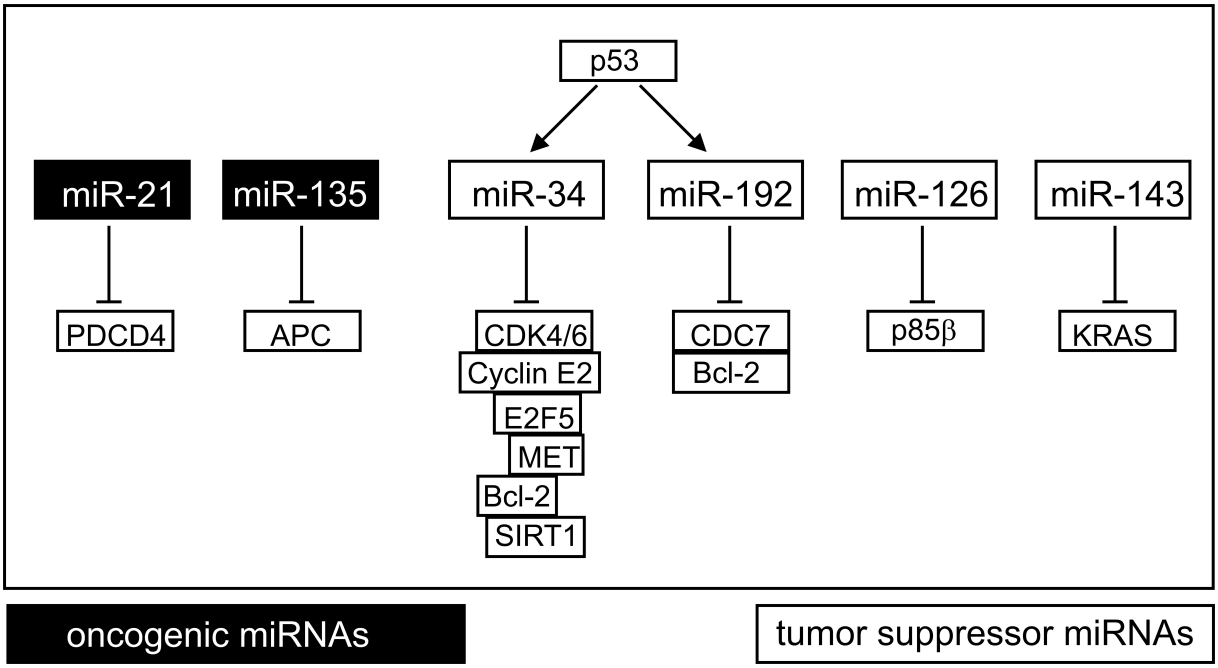

Figure 5 Overview of miRNAs involved in central pathways in CRC.
The presented miRNAs influence key pathways of CRC carcinogenesis like Wnt/β- catenin, PI3K, p53 or KRAS signalling. (Adapted and modified from Faber et al., 2009)

It is known that the dysregulation pattern of miRNAs is characteristic for different cancer types and the origin of various tumours. Therefore, distinct investigations on miRNA dysregulation patterns are presently conducted for the diagnosis of CRCs. Thus, the expression of certain miRNAs was found to be altered in plasma (miR-17-3p, miR-92, miR-95, miR-135b and miR-222) or stool samples (miR-21 and miR-106a) of CRC patients compared to normal controls [Ng et al., 2009; Link et al., 2010]. Therefore, miRNAs can possibly serve as a non-invasive diagnostic tool of CRC. The expression levels of miR-21 [Schetter et al., 2008], miR-200c [Xi et al., 2006a], miR-106a [Díaz et al., 2008] and miR-215 [Karaayvaz et al., 2011] were correlated with prognosis of survival. To date, preliminary studies were conducted with respect to the application of miRNAs in therapeutics. Thereby, local or systemic administration of miR-145 led to reduced proliferation in a mouse xenograft model of CRC [Ibrahim et al., 2011].

2.4 Aims of this study

In various cancer entities chemokines were reported to be deregulated and reported to be associated with tumour development and progression [Wang et al., 2009b].

Thereby CCL20 was identified as a chemokine which may play a role in cancer associated processes. Similarly, various miRNAs were reported to be deregulated in colorectal carcinoma [Dong et al., 2011]. As recently both groups of molecules gained importance with respect to cancer development and progression, the aim of the present thesis was to identify and characterize a functional link between certain miRNAs and the chemokine CCL20 with a special interest in CRC.

As no information on miRNAs interacting with CCL20 was known at the starting point of this thesis, computational prediction of interactions together with experimental validation of functionality were applied. Therefore, it was aimed to investigate the regulatory correlation between the identified miRNAs and CCL20 by transfecting CRC cells with the specific miRNAs. In addition, it was scheduled to analyze the expression profiles of CCL20 and the respective miRNAs in tissue samples from patients with CRC in order to detect expression alterations compared to normal non-affected tissues and to identify potential correlations between miRNA expression and CCL20 mRNA and/or protein expression. To further characterize the role of the identified miRNAs and CCL20 with respect to CRC pathology and progression it was intended to localize the expression patterns of these molecules by immunhistochemistry (CCL20) and in situ hybridization (miRNAs).

The results obtained from the present study aimed to identify miRNAs which may have the potential to regulate the expression of CCL20, thereby shedding light on the role of miRNA-chemokine interactions and impact on CRC pathogenesis.

3. Material and Methods

3.1 Material

3.1.1 Instrumentation

Instrumentation	**Manufacturer**
Analysis scale	Sartorius
Autoclave, VARIOKLAV®	Thermo Fisher Scientific
Centrifuge, Avanti™ J-20 XP Centrifuge	Beckman Coulter™
Centrifuge, Biofuge primo	Hereaus Holding GmbH
Centrifuge, Centrifuge 5417R	Eppendorf
Centrifuge, Centrifuge 5810R	Eppendorf
Exthaust hood	Köttermann GmbH
Homogenizer Ultra-Turrax ®T8	IKA® -Werke GmbH Co. KG
Incubator (37 °C; 5,0 % CO_2) Nunc™	RS Biotech
Incubator shaker, INNOVA® 44	New Brunswick Scientific
Laminar flow, s@feflow 1.2	Nunc™
Luminometer, Lumat LB9501	Berthold Technologies GmbH & Co.KG
Multipipette® Plus	Eppendorf
PCR-Cycler, Gene Amp® PCR System2700	Applied Biosystems
pH-meter	WTW GmbH
Photometer, Biophotometer	Eppendorf
Photometer, Microplate Absorptions-Reader ELx800	BioTek Instruments
Pipettes (10/20/200/1000µl)	Eppendorf
Pipetting aid, Accu-Jet® Pro	VWR International GmbH
Sequence Detection System, 7900HT ABI PRISM	Applied Biosystems
Shaker, Unimax 2010	Heidolph Instruments GmbH & Co.KG
Thermomixer compact	Eppendorf
Ultrasonic bath, RK100	Bandelin-Sonorex
Vortex $2x^3$	UniEquip Laborgerätebau und Vertriebspartner GmbH
Water bath, WB22	Labortechnik Medingen
Tweezers	Carl Roth GmbH + Co KG
Humidity chamber	Thermo Fisher Scientific
Millipore water dispenser Arium® 611	Sartorius

Instrumentation	Manufacturer
Neubauer counting chamber	Bender & Hobein
Microscope	Olympus
Microtome	Leica Microsystems
Waterbath	Kunz Instruments
Heating cabinet HIR12 (for hybridization)	Grant Boekel

3.1.2 Consumables

Product	Manufacturer
Cell culture multiwell plates, CELLSTAR® (6 und 24 well)	Greiner Bio-One GmbH
Centrifuge tubes (50 ml)	Greiner Bio-One GmbH
Centrifuge tubes, SuperClear™ (14 ml)	VWR International GmbH
Culture flask, CELLSTAR® (25 cm^2)	Greiner Bio-One GmbH
Cuvette, UVette®	Eppendorf
Disponsable cell scraper	Greiner Bio-One GmbH
Disponsable Weighing pan, Rotilabo®	Carl Roth GmbH + Co. KG
ELISA-plates, Immuno 96 Microwell™ Solid Plates	Nunc™
MicroAmp® 96-Well Optical Adhesive Film	Applied Biosystems
MicroAmp® Optical 96-Well Reaction Plate	Applied Biosystems
Microtest-plates, Rotilabo®	Carl Roth GmbH + Co. KG
Optical adhesive film for ELISA-plates	Nunc™
Parafilm M, Laboratory Film	American National Can
PCR-tubes, 0.2 ml	VWR International GmbH
Petri dish, 8.5 cm	Greiner Bio-One GmbH
Pipette tips (0.1-100/200/1000 µl)	Greiner Bio-One GmbH
Pipettes, Serologic Rotilabo® Pipettes (1/2/5/10/25 ml)	Carl Roth GmbH + Co. KG
Polypropylen tubes, round bottom (12 ml)	Greiner Bio-One GmbH
Polystyrol tubes, round bottom	Greiner Bio-One GmbH
Reaction tubes (1.5/2 ml)	Eppendorf
Ritips® professional, non-sterile (500 µl/2.5 ml /5 ml)	Ritter GmbH
Weighing paper	Carl Roth GmbH + Co. KG

Product	Manufacturer
Coblin jar	Carl Roth GmbH + Co. KG
Staining insert	Carl Roth GmbH + Co. KG
Microscopic slides Superfrost® / Plus	Carl Roth GmbH + Co. KG
Coverglasses	Carl Roth GmbH + Co. KG
Fixogum	Marabu

3.1.3 Chemicals and Biochemicals

Product	Manufacturer
Absolute ethanol	VWR
Albumin Fraktion V, Protease-frei	Carl Roth GmbH + Co. KG
Complete, Proteaseinhibitor cocktail tablets	Roche
DharmaFECT®1, Transfection Reagent	Thermo Scientific
Dimethylsulfoxid (DMSO)	Carl Roth GmbH + Co. KG
Dinatriumhydrogenphosphat (Na_2HPO_4)	Carl Roth GmbH + Co. KG
DPBS	GIBCO® (Invitrogen™)
EDTA (Dinatriumsalz Dihydrat)	Carl Roth GmbH + Co. KG
Ethanol 99 % denatured	Zentrales Chemikalienlager Saarbrücken
Foetal Bovine Serum Gold	PAA Laboratories GmbH
HPLC-H_2O ROTISOLV® HPLC Gradient Grade	Carl Roth GmbH + Co. KG
Kanamycin	Sigma-Aldrich®
Lipofectamine™ 2000 Reagent	Invitrogen™
N, N-Dimethylformamide (DMF)	Sigma-Aldrich®
NP-40 Alternative 100 %	Calbiochem®
Pen/Strep, Penicillin Streptomycin	GIBCO® (Invitrogen™)
PMSF	AppliChem GmbH
Sodium chloride (NaCl)	Merck
Sodium deoxycholate (DOCA)	Carl Roth GmbH + Co. KG
Sodium dihydrogen phosphate (NaH_2PO_4)	Carl Roth GmbH + Co. KG
Sodium fluoride (NaF)	Carl Roth GmbH + Co. KG
Sodium hydroxide (NaOH)	Sigma-Aldrich®
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich®
ß-Mercaptoethanol	Merck
Sulphuric acid H_2SO_4, reinst 98 %	Zentrales Chemikalienlager Saarbrücken
TaqMan® Universal Master Mix II	Applied Biosystems
Tris Ultra Qualität (Tris–(hydroxylmethyl-) aminomethan)	Carl Roth GmbH + Co. KG

Product	Manufacturer
TrypsinEDTA (10x)	Invitrogen™
Tween®-20	Carl Roth GmbH + Co. KG
Potassium chloride (KCl)	Carl Roth GmbH + Co. KG
Hydrochloric acid	Riedel-de Haën®
Xylene	Carl Roth GmbH + Co. KG
SSC solution 20 x	Ambion®
NBT/BCIP Ready-to-Use Tablets	Roche Applied Science
Sheep serum	Santa Cruz Biotechnology Inc.
Nuclear Fast Red	Sigma-Aldrich®
RNaseZap®	Ambion®
Levamisole hydrochloride	Sigma-Aldrich®
Eukitt® quick-hardening mounting medium	Sigma-Aldrich®
HiPerFect® Transfection reagent	Qiagen
DEPC	AppliChem GmbH
Aquatex	Merck KGaA
Mayers Hämalaun	AppliChem GmbH
Hydrogen peroxide (H_2O_2)	VWR
Antibody diluent	DAKO
Target Retrieval Solution	DAKO

3.1.4 Applied Kits

Product	Manufacturer
BCA™ Protein Assay Kit	Thermo Fisher Scientific
Dual-Luciferase® Reporter Assay System	Promega Corporation
DuoSet® ELISA, human MIP-3α/CCL20	R&D Systems®
GenElute™ HP Plasmid Maxiprep Kit	Sigma-Aldrich®
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems
mirVana™ miRNA Isolation Kit	Ambion®
RNeasy® Mini Kit	QIAGEN
Substrate Reagent Pack	R&D Systems®
TaqMan® MicroRNA Reverse Transcription Kit	Applied Biosystems
VECTASTAIN® Elite ABC Kit (Goat IgG)	Vector Laboratories
DIG Wash and Block Buffer Set	Roche Applied Science
miRCURY LNA™ microRNA ISH Optimization Kit (FFPE)	Exiqon
QuikChange® II XL Site-Directed Mutagenesis Kit	Agilent Technologies

3.1.5 Enzymes

Product	Manufacturer
MultiScribe™ Reverse Transkriptase, 50 U/µl (part of High Capacity cDNA Reverse Transcription Kit)	Applied Biosystems
RNAse–Inhibitor (Part of TaqMan® MicroRNA Reverse Transcription Kit)	Applied Biosystems
Proteinase K buffer and reagent (part of the miRCURY LNA™ microRNA ISH Optimization Kit (FFPE)	Exiqon

3.1.6 Probes and nucleic acids

All TaqMan® Gene Expression Assays, TaqMan® MicroRNA Assays and Pre-miR™ miRNA Precursor were purchased from Ambion® Life Technologies (Darmstadt, Germany).

TaqMan® Gene expressions assays:

B2m – Gene Expression Assay

CCL20 – Gene Expression Assay

TWF1 – Gene Expression Assay

TaqMan® microRNA assays:

hsa-miR-21 MicroRNA Assay

hsa-miR-145 MicroRNA ssay

RNU48 MicroRNA Assay

Pre-miR™ miRNA Precursors:

hsa-miR-21 Pre-miR™ miRNA Precursor

hsa-miR-145 Pre-miR™ miRNA Precursor

hsa-miR-1 Pre-miR™ miRNA Precursor (Positive control)

Negative Control # 1 Pre-miR™ miRNA Precursor (Negative control)

All miRCURY LNA™ microRNA Detection Probes for in situ hybridization (ISH) were purchased from Exiqon (Vedbaek, Denmark).

hsa-miR-21	miRCURY LNA™ Detection probe, 5`-DIG and 3`-DIG labeled
hsa-miR-145	miRCURY LNA™ Detection probe, 5`-DIG and 3`-DIG labeled
U6 hsa/mmu/rno	Control, miRCURY LNA™ detection probe, 5`-DIG and 3`DIG labeled
scramble-miR	Control, miRCURY LNA™ detection probe, 5`-DIG and 3`DIG labeled

For the site-directed mutagenesis of the miR-21 target site the following primer were applied and purchased from Thermo Scientific (Waltham, MA, USA):

Forward Primer:

```
5'-CTGTGTTTAGCTATTTAATACTAATTTTCCGTGAGCTATTTTGGTTTAGTGCAAAGTATAAAA-3' Primer sequence
   |||||||||||||||||||||||||||||| | ||||||||||||||||||||||||||||||
3'-GACACAAATCGATAAATTATGATTAAAAGGTATTCGATAAAACCAAATCACGTTTCATATTTT-5' CCL20 3'UTR
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Reverse Primer:

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5'-TTTTATACTTTGCACTAAACCAAAATAGCTCACGGAAAATTAGTATTAAATAGCTAAACACAG-3' Primer sequence
   |||||||||||||||||||||||||||||| | ||||||||||||||||||||||||||||||
3'-AAAATATGAAACGTGATTTGGTTTTATCGAATACCTTTTAATCATAATTTATCGATTTGTGTC-5' CCL20 3'UTR
```

3.1.7 Culture media, Buffers and Solutions

3.1.7.1 Culture media for bacteria- und human cell lines

DMEM + GlutaMAX™	GIBCO® (Invitrogen™)
MEM + GlutaMAX™	GIBCO® (Invitrogen™)
McCoy' s 5A + GlutaMAX™	GIBCO® (Invitrogen™)
Leibovitz's L-15, GlutaMAX™	GIBCO® (Invitrogen™)
OPTI- MEM® I + GlutaMAX™	GIBCO® (Invitrogen™)
LB Broth	Sigma-Aldrich®
LB Agar	Sigma-Aldrich®

3.1.7.2 Buffers and Solutions

(1) Saturation buffer (ELISA):

Tris-(hydroxymethyl)-aminomethan	12.1 g
Adjust pH with HCl to a value of 7.5	
BSA	5 g
Fill up with dd H_2O to a final volume of 500 ml	

(2) Stock Complete™

25 x Complete™ Protease Inhibitor Cocktail	1 tablet
Dissolve in 2 ml of dd H_2O	

(3) Sodium fluoride (200 mM):

NaF	840 mg
Dissolve in 100 ml dd H_2O and autoclave the solution	

(4) PMSF (100 mM):

PMSF	87.1 g
Isopropanol	5 ml

(5) RIPA Lysis Buffer:

0.5 M Tris-HCl pH 7.4	10 ml
100 % NP-40 Alternative	1 ml
2.5 % DOCA	10 ml
1.5 M NaCl	10 ml
100 mM EDTA pH 7.4	1 ml
dd H_2O	58 ml
On the day of the experiment add:	
200 mM NaF	5 µl
200 mM Na_3VO_4	5 µl
25 x Stock Complete™	80 µl
Directly before use add 10 µl PMSF (100 mM)	

(6) TE Buffer:

0.121 g Tris-HCl (10 mM)
0.029 g EDTA (1 mM)
Dissolve in 100 ml dd H_2O and adjust pH to 8.0

(7) 0.5 M Tris-HCl:

12.11 g Tris-HCl
Dissolve in 100 ml H_2O dest.
Adjust pH to 7.4
Fill up with dd H_2O to a final volume of 200 ml

(8) Washing buffer for ELISA 0.01 M PBS + 0.1 % Tween:

0.01 M Na_2HPO_4 x $2H_2O$	3.5598 g
0.01 M NaH_2PO_4 x $2H_2O$	3.1202 g
0.15 M NaCl	17.529 g

Adjust pH to 7.4 with NaOH
Add 1 ml of Tween20 and fill up with dd H_2O to a final volume of 2000 ml

(9) 10x PBS for ISH:

10.9 g Na_2HPO_4
3.2 g NaH_2PO_4
90 g NaCl
Dissolve in 1000 ml H_2O and autoclave

(10) PBS-T for ISH:

1000 ml 1x PBS
1 ml Tween®-20
autoclave

(11) KTBT buffer for ISH:

7.9 g Tris-HCl
8.7 g NaCl
0.75 g KCl
Dissolve in 1000 ml DEPC H_2O

(12) 1 M Tris-HCl pH 7.4:

121.1 g Tris-HCl
Dissolve in DEPC H_2O, adjust pH to 7.4 with HCl and fill to final volume of 1000 ml

(13) 10 mM Tris-HCl pH 7.5:

0.121 g Tris-HCl
Dissolve in DEPC H_2O, adjust pH to 7.5 with HCl and fill to final volume of 100 ml

(14) 0.5 M EDTA:

186.1 g EDTA
Dissolve in DEPC H_2O, adjust pH to 8.0 with NaOH and fill to a final volume of 1000 ml

(15) 5 M NaCl:

146.1 g NaCl
Dissolve in 500 ml DEPC H_2O

(16) Proteinase K buffer:

5 ml of 1 M Tris-HCl (pH 7.4)
2 ml of 0.5 M EDTA
0.2 ml of 5 M NaCl
Adjust with DEPC H_2O to a final volume of 1000 ml and autoclave

(17) DEPC H_2O:

1000 ml dd H_2O

1 ml DEPC

Mix for 6 h on a magnetic stirrer and then autoclave

3.1.8 Antibodies

Product	Manufacturer
Capture Antibody, mouse anti-human MIP-3α/CCL20 (part of DuoSet® ELISA)	R&D Systems®
Detection Antibody, biotinylated goat anti-human MIP-3α/CCL20 (part of DuoSet® ELISA)	R&D Systems®
Standard, recombinant human MIP-3α/CCL20 (part of DuoSet® ELISA)	R&D Systems®
IHC/ICC antibody: goat anti-human CCL20/MIP-3α (AF360)	R&D Systems®
biotinylated anti-goat secondary antibody (part of VECTASTAIN® Elite ABC Kit (Goat IgG)	Vector Laboratories
Anti-Digoxigenin-AP, Fab fragments from sheep	Roche Applied Science

3.1.9 Cell lines

The cell line Caco-2 (ATCC number: HTB-37):
The cell line Caco-2 is a human CRC cell line which was isolated and characterized from a primary colon adenocarcinoma by J. Fogh in the year 1974 [Fogh et al., 1975]. Caco-2 cells are immortally and adherent growing cells. Under normal growth conditions these cells become differentiated and polarized which results in a morphological, physiological and functional similarity to enterocytes of the human small intestine [Pinto M et al., 1983].

The cell line HT29 (ATCC number HTB-38):
The cell line HT29 was isolated from a primary tumour of a colorectal adenocarcinoma by J. Fogh in the year 1964 [Fogh et al., 1975]. These cells are

immortally and adherent growing cells which attain morphological characteristics of absorptive epithelial cells growing in a polarized monolayer under specific growth conditions [Le Bivic et al., 1988].

The cell line SW480 (ATCC number: CCL-228):
The cell line SW480 was isolated from a primary adenocarcinoma and characterized by A. Leibovitz [Leibovitz et al., 1976]. SW480 cells are hyperdiploid and grow as small clusters of epithelial cells and bipolar cells.

The cell line SW620 (ATCC number: CCL-227):
The cell line SW620 was isolated from lymph nodes of recurring colorectal adenocarcinoma and characterized by A. Leibovitz [Leibovitz et al., 1976]. SW 620 cells are hyperdiploid, highly dedifferentiated and grow as a mixture of spherical cells and bipolar cells.

The cell line 293T (ATCC number: CRL-11268):
The cell line 293T, a kidney epithelial cell line, is a variant of the cell line HEK-293 (Human Embryonic Kidney) which was primarily transformed with fractions of Adenovirus 5 DNA [Graham et al., 1977]. 293T cells in addition express the SV40 large T-antigen which allows these cells to replicate episomal plasmids containing the SV40 origin of replication. The 293T cell line is widely used for reporter expression assays [DuBridge et al., 1987].

3.1.10 Patient material

All tissue samples applied in the present thesis were surgically resected and stored at the Department of General, Visceral, Vascular and Paediatric Surgery of the University of the Saarland. The study was permitted by the local ethics committee of the Ärztekammer des Saarlandes and informed written agreement for tissue procurement was obtained from all patients. Fourty-six patients with colorectal adenocarcinoma were included in the present work. For all patients corresponding non-affected normal colorectal tissue was available. The clinical data and patient

characteristics were obtained from a prospective database and were summarized in Table 3.

Characteristic	CRC (n=46)
Localization of primary tumour	
Colon	23
Rectum	23
Gender	
Male	28
Female	18
Age at surgery (yr)	
Median	68.3
Range	44-88
Tumour (T)-stage of primary tumour	
T1	8
T2	12
T3	16
T4	10
Lymph node status	
Positive	26
Negative	20
Clinical stage of primary tumour	
I	16
II	7
III	17
IV	6
Grade	
G1	4
G2	19
G3	21
G4	2

Table 3 Clinical features of CRC patients

3.1.11 Vectors

3.1.11.1 Reporter construct containing the 3'UTR of CCL20

The miTarget™ microRNA 3'UTR target clones in mammalian expression vector pEZX-MT01 (GeneCopoeia™; Rockville, MD, USA) containing two luciferase reporter genes allows the identification of miRNA-targets and the functional validation of predicted targets by detection of regulatory interactions. In the present thesis a

construct from GeneCopoeia™ was chosen which contains the 3'UTR of CCL20. In the reporter vector the 3'UTR of CCL20 is arranged directly downstream of the Firefly–luciferase gene (hLuc) (Figure 6). Therefore a hybrid-mRNA of the coding region of the firefly-luciferase and the 3'UTR of CCL20 is transcribed and the interaction of miRNAs with the 3'UTR of CCL20 can be measured by firefly-luciferase activity. The reporter vector contains another luciferase gene coding for Renilla-luciferase (hRLuc) whose expression is independent from the investigated target sequence and served for the normalization for general expression and transfection differences. In addition the vector contains a Kanamycin/Neomycin resistance gene required for cloning of the vector.

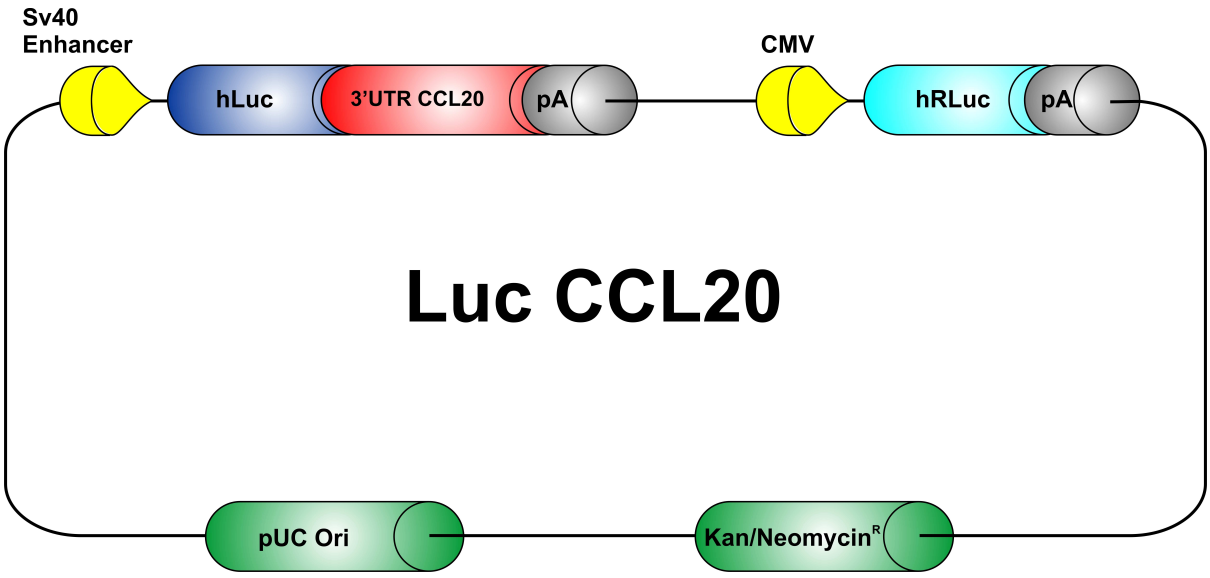

Figure 6 Luciferase Reporter Vector
The reporter vector contains two luciferase genes. The firefly luciferase gene is fused to the 3'UTR of CCL20. Furthermore, the vector contains a pUC origin and a Kanamycin/Neomycin resistance gene for amplification. hLuc = Firefly luciferase gene; hRLuc = Renilla luciferase gene, pA = poly Adenin signal; Sv40 and CMV = promoter sequences; pUC Ori = origin of replication; Kan/Neomycin = Resistence genes; 3'UTR CCL20 = untranslated region of CCL20 gene

3.1.11.2 Reporter construct containing a mutated 3'UTR of CCL20

In order to demonstrate that the interaction of miRNAs with the 3'UTR of CCL20 is specific the 3'UTR of CCL20 in the expression vector was mutated at the binding site of the according miRNA (Figure 7).

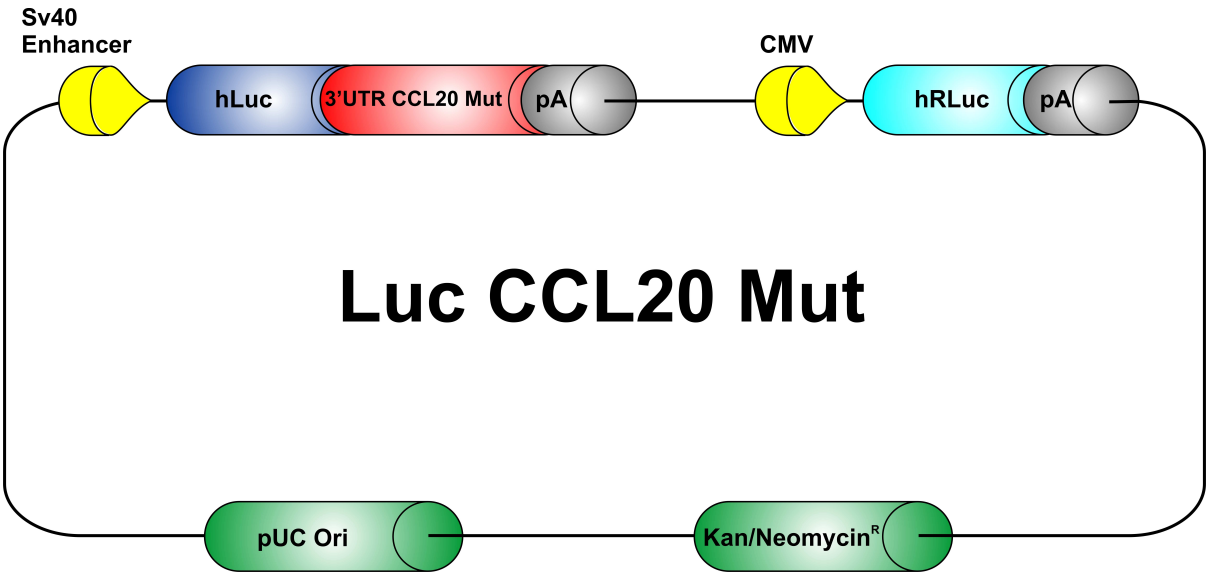

Figure 7 Luciferase Reporter Vector with mutated 3'UTR of CCL20
Two nucleotides in the predicted target site of miR-21 were mutated in the 3'UTR of CCL20. hLuc = Firefly luciferase gene; hRLuc = Renilla luciferase gene, pA = poly Adenin signal; Sv40 and CMV = promoter sequences; pUC Ori = origin of replication; Kan/Neomycin = Resistence genes; 3'UTR CCL20 Mut = mutated untranslated region of CCL20 gene

3.1.11.3 Control vector

As a negative control vector the pEZX-MT01 vector form GeneCopoeia^{TM} was used which contain no 3'UTR dowstream of the Firefly-luciferase gene (Figure 8). Also in this vector a second firefly gene for the Renilla–firefly was available for expression and transfection normalization. A kanamycin/neomycin resistance gene served for vector cloning.

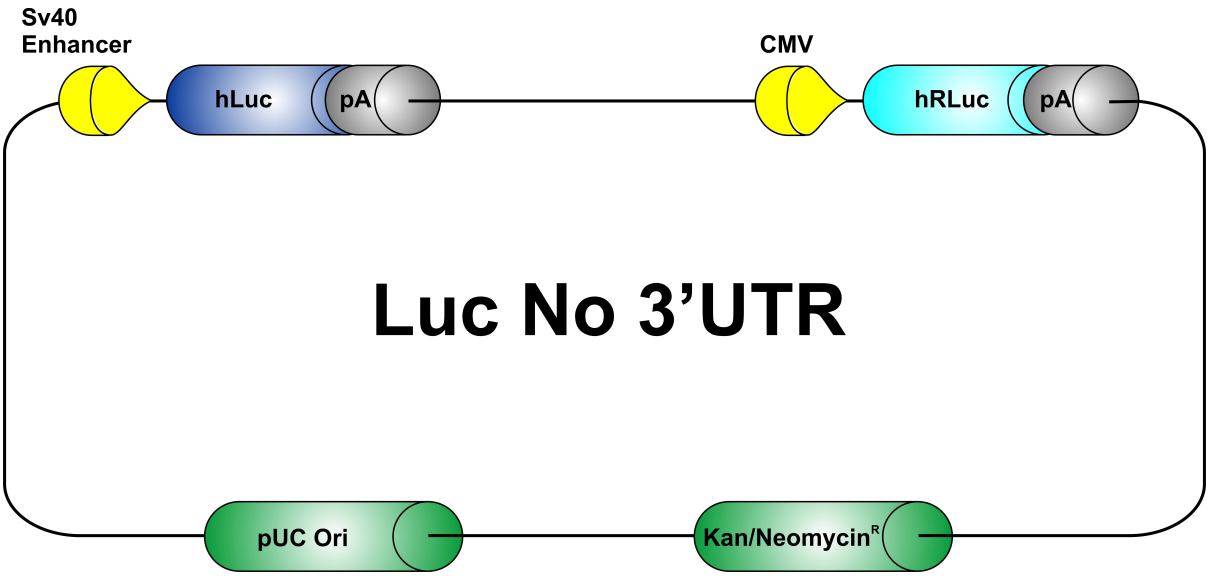

Figure 8 Luciferase Reporter Vector Control (without 3'UTR)
hLuc = Firefly luciferase gene; hRLuc = Renilla luciferase gene, pA = poly Adenin signal; Sv40 and CMV = promoter sequences; pUC Ori = origin of replication; Kan/Neomycin = Resistence genes; 3'UTR CCL20 Mut = mutated untranslated region of CCL20 gene

3.1.12 Bacteria

For amplification of vector DNA the competent Escherichia coli (E. coli) bacteria GC10 from Sigma-Aldrich® were used.
During the mutagenesis procedure the ultracompetent bacteria XL10-Gold (contained in the QuikChange® II XL Site-Directed Mutagenesis Kit) were applied.

3.2 Methods

3.2.1 miRNA Target prediction programmes

In order to identify miRNAs potentially interacting with the 3'UTR of CCL20 and therefore regulate its expression different online available target prediction programmes were applied using diverse input data (Table 4).

Prediction Tool	Input data
TARGETSCANHUMAN 4.2 (http://www.targetscan.org/vert_42)	TargetGene Name
MICRORNA.ORG (http://www.mircoRNA.org)	TargetGene Name
PICTAR (www./pictar.mdc-berlin.de/cgi-bin/PicTar_vertebrate.cgi)	TargetGene Name
EBI MICROCOSM TARGETS (http://www.ebi.ac.uk/enrightsrv/microcosm/htdocs/targets/v5)	TargetGene Name
MIRDB (http://mirdb.org/miRDB/)	TargetGene Name
PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html)	TargetGene AND *microRNA* Name
RNA22 (http://cbcsrv.watson.ibm.com/rna22.html)	Target 3'UTR sequence AND *microRNA* sequence
TAREF (http://scbb.ihbt.res.in/TAREF/index2.html)	Target 3'UTR sequence
MICROINSPECTOR (http://bioinfo.uni-plovdiv.bg/microinspector/)	Target 3'UTR sequence AND *microRNA* sequence
STARMIR (http://sfold.wadsworth.org/cgi-bin/starmir.pl)	Target 3'UTR sequence AND *microRNA* sequence
ElMMO (http://www.mirz.unibas.ch/ElMMo2/)	TargetGene sequence AND *microRNA* Name
TARGETRANK (http://genes.mit.edu/targetrank/)	*microRNA* Name

Table 4 Overview of the applied Target Prediction Programmes

Input Data:

Target Gene Name: CCL20

miRNA Name: hsa-miR-21 or hsa-miR-145

miRNA sequence: 5'_uagcuuaucagacugauguuga_3' (hsa-miR-21)

5'_guccaguuuucccaggaaucccu_3' (hsa-miR-145)

Target 3'UTR sequence: CCL20:

5'_AAACTGTGGCTTTTCTGGAATGGAATTGGACATAGCCCAAGAACAGAAAGAACCTTGCTG
GGGTTGGAGGTTTCACTTGCACATCATGGAGGGTTTAGTGCTTATCTAATTTGTGCCTCACT
GGACTTGTCCAATTAATGAAGTTGATTCATATTGCATCATAGTTTGCTTTGTTTAAGCATCA
CATTAAAGTTAAACTGTATTTTATGTTATTTATAGCTGTAGGTTTTCTGTGTTTAGCTATTT
AATACTAATTTTCCATAAGCTATTTTGGTTTAGTGCAAAGTATAAAATTATATTTGGGGGGG
AATAAGATTATATGGACTTTCTTGCAAGCAACAAGCTATTTTTTAAAAAAACTATTTAACAT
TCTTTTGTTTATATTGTTTTGTCTCCTAAATTGTTGTAATTGCATTATAAAATAAGAAAAAC
ATTAATAAGACAAATAT_3'

TargetGene sequence: NCBI Reference sequence NM_004591

3.2.2 Quality of target prediction programmes

The quality of the different target prediction programmes was assessed by comparing the number of correct predicted miRNA target gene interactions with the

combinations of features applied by the diverse programmes.

3.2.3 Bacterial-Techniques

3.2.3.1 Transformation and storage of bacteria

For the amplification of plasmids competent E. coli GC10 bacteria were transformed with the according plasmid and cultivated. The applied vector plasmids were produced by GeneCopoeia™ and provided dried on a “filter plate”. Dried vector DNA was resolved in 50 µl of TE-buffer pH 8.0 for 1 h. Afterwards 50 µl of GC10 competent bacteria were thawn in 15 ml tubes on ice, 5 µl of vector DNA solution were added and incubated for 30 min on ice. Then competent bacteria were heat shocked for 45 sec at 42 °C in a water bath. Directly after the heat shock 450 µl of SOC-medium were added and the bacteria were aerobically incubated for 1 h at 37 °C on an incubator shaker at 250 RPM. Accordingly the transformed bacteria were spread on two LB-Agarplates containing 50 µg/ml Kanamycin and incubated at 37°C over night. Transformation success was checked by optical assessment of the agarplates for colonies. For short-term storage of bacteria the agar plates were sealed with Parafilm® and retained at 4 °C in a refrigerator.

3.2.3.2 Small-scale isolation of plasmid-DNA

In order to analyze the DNA sequence of transformed plasmid-DNA small-scale isolation was performed using the QIAGEN Plasmid Mini Kit. The base for this kind of plasmid purification is the alkaline lysis followed by plasmid-DNA binding to anion-exchange resin columns which bind plasmid-DNA under appropriate low-salt and pH conditions and thereby allows the detachment of proteins and other low molecular contaminants by different washing buffers (medium salt). At the end the plasmid-DNA is eluted with an elution buffer (high salt).

For plasmid isolation 7 ml LB medium were inoculated with the desired bacterial clone and incubated at 37 °C on a shaker incubator over night. The next day 3 ml of the culture were transferred to two tubes and centrifuged at 6000 x g for 15 min. The

supernatants were discarded and the pellets were resuspended in 300 µl resuspension buffer P1 which contained RNAse A. The two resuspended pellets were mixed and 300 µl of lysis buffer P2 were added, mixed by inversion of the tube and then incubated for 5 min. Subsequently, 300 µl of precipitation buffer P3 were added and mixed thoroughly in order to precipitate cell debris, proteins and genomic DNA. The lysat was incubated for 5 min on ice and then centrifuged at 14000 RPM and 4 °C for 10 min. During this step the QIAGEN-columns were equilibrated with 1 ml QBT buffer. After centrifugation the supernatant was loaded to the equilibrated column and due to the low salt concentration and pH-conditions the plasmid-DNA bound to the matrix. Following two wash steps with 800 µl of QC-buffer the plasmid-DNA was eluted with 800 µl of QF-buffer. Subsequently 560 µl of Isopropanol were added to precipitate the DNA and after mixing the sample was centrifuged at 14000 RPM at 4°C for 30 min. Afterward the supernatant was discarded and the plasmid-DNA was washed with 1 ml of 70 % ethanol. The sample was centrifuged at 14000 RPM at RT for 10 min and the supernatant was discarded. After 5 to 10 min of drying the DNA pellet was resuspended in 20 µl of HPLC-H_2O and the concentration was photometrically determined. Until further processing the plasmid-DNA was stored at -20 °C.

3.2.3.3 Large-scale isolation of plasmid-DNA

In order to obtain a large amount of plasmid-DNA of high quality the GenElute™ HP Plasmid Maxiprep Kit from Sigma-Aldrich® (Taufkirchen, Germany) was used. The base of this method is a lysis of the grown bacteria with a SDS containing alkaline buffer to set the plasmid-DNA free which is subsequently purified via a silica-membrane. For bacterial culture 500 ml of LB media containing 100 µg/ml Kanamycin were inoculated with 1 ml of miniprep bacteria and incubated over night at 37 °C in an incubator shaker. The bacterial culture was centrifuged at 5000 x g for 10 min and the supernatant was discarded. The bacterial pellet was resuspended by pipetting up and down in 12 ml of resuspension buffer which contained RNAse A and transferred to a 50 ml tube. After addition of 12 ml lysis buffer the mixture was agitated by inverting the tube several times and then incubated for 5 min at RT. The SDS in the buffer destroyed the bacterial cell membrane and thereby lysed the

bacterial cells and released cellular components including the vector DNA. Accordingly, 12 ml of neutralization buffer were added and carefully mixed by inverting the tube several times in order to precipitate proteins, lipids, chromosomal DNA and cell debries. After addition of 9 ml binding buffer the mixture was transferred into a filter syringe and incubated for 5 min. During this incubation step 12 ml of column preparation solution were pipetted to the GenElute HP column and subsequently centrifuged for 2 min at 3000 x g. The flow through was discarded and one half of the bacterial lysate was added to the column through the filter syringe. After centrifugation for 2 min at 3000 x g the rest of the bacterial lysate was added and centrifuged in the same way. Due to the specific salt- and pH-conditions in the binding buffer the plasmid-DNA was bound to the matrix of the column whereas all other bacterial components passed the column. After two wash steps with wash buffer the plasmid-DNA was eluted with 3 ml of elution buffer pH 8.5 and centrifugation at 3000 x g for 5 min. The concentration of the isolated plasmid-DNA was determined photometrically at 260 nm.

3.2.4 Cell culture techniques

3.2.4.1 Cultivation of permanent adherent human cell lines

Permanent human cell line cells were cultured in cell culture flasks (25 cm^2) as monolayer. The morphology and viability of the cells was checked every day. The cultivation was performed under standard conditions in a humidified incubator at 37 °C with 5 % CO_2 or without CO_2 supplementation dependent on used cell line. All cultivation steps were carried out under the laminar flow with sterile reagents.
In the present thesis the permanent human cell lines HT29, Caco-2, SW480, SW620 and 293T were used.

3.2.4.1.1 HT29 cell line

The CRC cell line HT29 was maintained in McCoy's + GlutaMAX™ medium supplemented with 10 % (v/v) Fetal Bovine Serum and 1 % (v/v) Penicillin/Streptomycin. Cells were incubated at 37 °C and 5 % CO_2 in a humidified

incubator. HT29 cells were splitted 1:6 two times a week.

3.2.4.1.2 Caco-2 cell line

The CRC cell line Caco-2 was cultivated in MEM + GlutaMAX™ medium comprising 10 % (v/v) Fetal Bovine Serum and 1 % (v/v) Penicillin/Streptomycin and incubated at 37 °C and 5 % CO_2. Caco-2 cells were splitted 1:4 two times a week.

3.2.4.1.3 SW480 and SW620 cell lines

The CRC cell lines SW480 and SW620 were grown in Leibovitz's L-15, GlutaMAX™ medium comprising 10 % (v/v) Fetal Bovine Serum and 1 % (v/v) Penicillin/Streptomycin. Cell lines were incubated in a humdified incubator at 37 °C without CO_2 supplementation. SW480 and SW620 were splitted 1:6 two times a week.

3.2.4.1.4 293T cell line

The human kidney epithelial cell line 293T was cultivated in DMEM + GlutaMAX™ medium comprising 10 % (v/v) Fetal Bovine Serum and 1 % (v/v) Penicillin/Streptomycin. Cells were incubated at 37 °C and 5 % CO_2. 293T cells were splitted 1:10 two times a week.

3.2.4.2 Thawing of adherent cell lines

Stocks of the different cell lines were stored in a cryocontainer at -170 °C in liquid nitrogene. For thawing of cells 9 ml of prewarmed medium were pipetted into a round bottom tube and cells were taken from the liquid nitrogene container. Subsequently the cells were thawed in a waterbath transferred to the prewarmed medium and centrifuged for 5 min at 1000 RPM to get rid of the DMSO. After centrifugation the

supernatant was discarded and cells were resuspended in 1 ml medium. Cell suspension was transferred to a cell culture flask comprising 5 ml of medium. The medium was changed after 24 h of incubation.

3.2.4.3 Splitting of adherent cells

The splitting of the different cell lines was performed according to the proliferative and metabolistic condition of the cells. According to the confluency of the cells either the medium was renewed or the cells were splitted. Normally splitting was conducted when the confluence of the monolayer was around 80-90 %. Therefore the medium was detached and the cell monolayer was rinsed with 5 ml of PBS to remove remaining medium. Afterwards 1 ml Trypsin/EDTA was added to the cell layer and incubated for 5 min at 37 °C in order to detach the cells from the flask. Trypsin was inactivated by the addition of serum-containing medium and the cell suspension was transferred to a round bottom tube. After the cell suspension was centrifuged for 5 min at 1000 RPM the supernatant was removed and the cell pellet was resuspended in 1 ml of medium. Subsequently, the cells were splitted in the according relation (HT29 1:6; Caco-2 1:4; SW480 1:6; SW620 1:6; 293T 1:10), transferred into a cell culture flask containing fresh medium and incubated at the according conditions.

3.2.4.4 Counting of cells

In order to determine the number of cells approximately 20 µl of cell suspension were applicted to a Neubauer counting chamber. Subsequently, the cells were counted under the microscope and the cell number was calculated.

3.2.4.5 Transfection of eukaryotic cells

The process of transfection is described as the insertion of exogeneous nucleic acids into eucaryotic cells analog to transformation in bacterial cells. There are different techiques for transfection but in the present thesis the transfection was performed by

cationic lipids. For the lipofection synthetic cationic lipids were used that form unilamellar vesicles. These liposomes can aggregate spontaneously with negative charged phosphate groups in nucleic acids by ionic interactions with the positively charged parts of the cationic lipids. These aggregates have the potential to fuse with the cellular membrane of eucaryotic cells and thereby insert the bound nucleic acid into the cytoplasm of the cell [Felgner et al., 1987]. For optimization of liposome formation and membrane fusion the cationic lipids can be used either alone or with uncharged lipids [Chesnoy et al., 2000].
For the transfection of the cell lines applied in the present thesis the transfection reagents Lipofectamine™ (293T), DharmaFECT® (HT29 and Caco-2) and HiPerFect® (SW480 and SW620) were used.

3.2.4.5.1 Co-transfection with Lipofectamine™

The co-transfection with Lipofectamine™ was performed in 24 well cell culture multiwell plates. One day before transfection cells were seeded (5 x 10^4 for 293T and 8 x 10^4 for HT29) in 800 µl of medium per well and grown over night. For transfection 50 pmoles (293T) or 75 pmoles (HT29) of Pre-miR™ miRNA Precursor (Ambion® Life Technologies) or Pre-miR™ Negative Control #1 (Ambion® Life Technologies) respectively and 50 ng of the luciferase reporter vector DNA were diluted in OPTI-MEM® I Medium to a final volume of 50 µl. In another tube 1 µl (293T) or 2 µl (HT29) Lipofectamine™ were diluted in OPTI-MEM® I Medium to a final volume of 50 µl. After incubation for 5 min the contents of the two tubes were mixed and incubated for further 20 min at RT. The medium on the cells was changed to 800 µl of medium without serum and Pen/strep and subsequently 100 µl of transfection mixture per well were added dropwise to the cells. The cells were cultivated at 37 °C and 5 % CO_2 for 48 h and then collected for Luciferase-reporter assay.

3.2.4.5.2 Transfection with DharmaFECT®

For transfection of the adherent growing cell lines HT29 and Caco-2 one day before transfection the cells were seeded. Per well of a 6 well plate 5 x 10^5 cells were adopted in 2.5 ml medium to reach a confluency of around 60 % at the day of

transfection. In the first tube 200 pmoles of Pre-miR™ miRNA Precursor were diluted in OPTI-MEM® I medium to a final volume of 200 µl. In another tube 6 µl of DharmaFECT® were also diluted in OPTI-MEM® I medium to a final volume of 200 µl. The two tubes were incubated apart for 5 min and then mixed with each other by gentle vortexing. Afterwards the mixture was incubated for 20 min at RT and the medium on the cells was changed to 1.6 ml of medium without serum and Pen/Strep. Past incubation, per well 400 µl of the transfection mixture were dropwise added to the cells. The cells were incubated for 48 h at 37 °C and 5 % CO_2 in an incubator and then prepared either for RNA isolation or protein isolation.

3.2.4.5.3 Transfection with HiPerFect

One day before transfection 5 x 10^5 cells per well of the adherent cell lines SW480 and SW620 were seeded in a 6 well plate in order to reach a confluency of around 60 % at the day of transfection. For transfection 100 pmoles of Pre-miR™ miRNA Precursor were mixed with OPTI-MEM® I medium before 12 µl of HiPerFect were added to a final volume of 400 µl. Subsequently, the samples were incubated for 10 min to allow formation of transfection complexes. The growth medium on the cells was changed to 1.6 ml of medium without serum and Pen/Strep and accordingly 400 µl transfection mixture per well were added dropwise to the cells. The cells were incubated at 37 °C and 5 % CO_2 for further 48 h.

3.2.4.6 Cryoconservation of cell lines

Cells were cryoconserved according to a standard protocol. Thereby the cells were detached as done for splitting procedure and counted subsequently. After a centrifugation step the cells were resuspended in DMSO and transferred to cryotubes. The cells were kept at -80 °C over night and then transferred to -170 °C in liquid nitrogen for longterm storage.

3.2.5 Luciferase-Assay

The luciferase-assay is a kind of a reportergene-assay which enables investigations on the expression of a specific target gene as well as cellulare processes. Key feature of this assay is the capacity of the Firefly- and Renilla-luciferases to transubstantiate luciferin in the presence of oxygen, ATP and Mg^{2+} or Coelenterazine, respectively, to light. A quantitative evaluation of the luciferase reportergene-assay is possible because the emitted light is proportional to the amount of luciferase expressed. For investigations on miRNA-target gene interactions the 3'UTR of the target gene is cloned downstream of the Firefly-luciferase gene so that the interaction of the according miRNAs with the 3'UTR influences Firefly-luciferase expression.

The applied Dual-Luciferase® Reporter Assay from Promega allows the detection of Firefly-luciferase and Renilla-luciferase activity in one single assay. In the first step the activity of the Firefly-luciferase is detected using a substrate specific for this kind of luciferase: Luciferase Assay Reagent II (LARII). After measurement of the emitted light the activity of Firefly-luciferase is blocked and the activity of Renilla-luciferase is started by addition of the Stop & Glo® substrate.

In the present thesis a reporter construct from GeneCopoeia™ was used which contains the 3'UTR of CCL20. A reporter construct, which contains no 3'UTR (GeneCopoeia™), served as control. In the first step 5 x 10^4 293T cells per well or 8 x 10^4 HT29 cells per well were seeded in 24 well plates. The following day the cells were co-transfected with the according reporter constructs and Pre-miR™ miRNA Precursor (as described 3.2.4.5.1). After 48 h the cells were lysed in 200 µl (293T) or 100 µl (HT29) of 1 x passive lysis buffer (PLB) for 25 min on a shaker. For measurement of the 293T lysate the lysates were further pre-diluted 1:50 in 1 x PLB whereas the HT29 lysates kept undiluted. After 50 µl of LARII are dispensed in luminometer tubes 10 µl of cell lysate was added and the light emission was measured for 10 sec. Subsequently, 50 µl of Stop & Glo® substrate were added and the light emission was again detected for 10 sec. For evaluation, the activity of the Firefly-luciferase was normalized to the Renilla-luciferase activity of the same sample and the samples to be compared were related to each other.

3.2.6 Site-directed Mutagenesis

For functional validation of the 3'UTR of CCL20 as a target of the investigated miRNA the predicted target sequence was mutated with the help of the QuikChange® II XL Site-Directed Mutagenesis Kit. This kit was optimized for the site-directed mutagenesis of sequences in big vector constructs (8-14 kb). The according mutation was introduced using specifically designed primers (as described 3.1.6). As central step the mutagenic replication of complementary plasmid-DNA strands was carried out with highest accuracy using the *PfuUltra* HF DNA polymerase. After replication of mutated plasmid-DNA the parental DNA was digested by *Dpn1* (1 µl) for 1 h at 37 °C. The endonuclease *Dpn1* is an enzyme which specifically digests methylated DNA molecules. Therefore the methylated parental plasmid-DNA becomes digested and the new amplificated, non-methylated mutated DNA-strands remain intact. The reaction mixture for the PCR is depicted in Table 5 and the according PCR programme is described in Table 6.

Component	**Amount / volume**
10 x reaction buffer	5 µl
dsDNA template	10 ng
forward primer	125 ng
reverse primer	125 ng
dNTP mix	1 µl
QuickSolution	3 µl
ddH_2O	Adjust to final volume of 50 µl
PfuUltra HF DNA Polymerase	1 µl

Table 5 Pipetting scheme for Site-directed mutagenesis

Temperatur	Time / Duration	
95 °C	1 min	
95 °C	50 sek	18 x
60 °C	50 sek	
68 °C	8.5 min (1 min / kb)	
68 °C	7 min	
4 °C	∞	

Table 6 Cycler programme for Site-directed mutagenesis

After digestion of the PCR products with *Dpn1* ultracompetent XL-Gold® cells were transformed with 2 µl of the DNA. Therefore the ultracompetent cells were thawed on ice and 45 µl of bacteria were dispensed in pre-chilled tubes. Afterwards 2 µl of ß-Mercaptoethanol (ß-ME) were added to the cells and incubated on ice for 10 min. Subsequently, 2µl of the DNA were pipetted to the cells and the mixture was incubated on ice for 30 min. Then the cells were heat shocked in a waterbath at 42 °C for 30 sec and incubated on ice for further 2 min. After addition of 500 µl preheated SOC medium (SOC Outgrowth Medium) cells were incubated at 37 °C on a shaker for 1 h. Then the cells were spread on LB-agar plates containing kanamycin and incubated in an incubator at 37 °C over night. A suspension culture was inoculated in 7 ml LB-medium containing kanamycin.
As mutation control the QuikChange® II XL Site-Directed Mutagenesis Kit provided the control plasmid (pWhitescript plasmid with according Primers) and as transformation control the control plasmid (pUC18) was used. The mutation was validated by DNA sequencing by the company GATC (Konstanz, Germany). The interaction of the according miRNA with the mutated construct was investigated with the luciferase assay.

3.2.7 RNA Technologies

3.2.7.1 Isolation of total RNA from tissue samples

For isolation of total RNA comprising miRNAs from human tissue samples of patients with CRC the mirVana™ miRNA Isolations Kit from Ambion® Life Technologies was

applied. The method is based on the organic extraction and immobilisation of RNA to glass-fiber filters in order to purify total RNA. In the first step 700 µl Lysis/Binding buffer is pre-dispensed in a round bottom tube. Afterwards 60 mg of tissue were weighed without thawing, added to the lysis buffer and homogenized using the Ultra-Turrax ® T8. Subsequently, the homogenate was transferred to a 2 ml tube, 70 µl of Homogenate Additive were added and incubated on ice for 10 min. Adjacently, 700 µl of Acid-Phenol:Chloroform were pipetted to the homogenate and vortexed for 60 sec. For phase separation of the organic and aqueous phase the mixture was centrifuged for 20 min at 10000 x g at RT. The aqueous solution which contained the RNA was taken off, 1.25 volumes of 100 % Ethanol were added and mixed. Subsequently, 700 µl of the mixture were applied to a filter column and centrifuged for 20 sec at 10000 x g. The flow-through was discarded and the last step was repeated until the whole lysate got loaded on the column. After the complete lysate was loaded three wash steps were done. Therefore 700 µl of Wash Solution 1 were added to the column, centrifuged for 20 sec at 10000 x g at RT and the flow-through was discarded. For the second and third wash step 500 µl of Wash Solution 2/3 were used followed by centrifugation. Another centrifugation step for 1 min at 10000 x g was done to get rid of remaining wash buffer. For the elution of the RNA from the filter the column was placed into a new 1.5 ml tube, 50 µl of 95 °C warm nuclease-free water were added and centrifuged for 30 sec at maximal speed. After elution the column was discarded and the concentration and purity of RNA was determined by photometric measurement. The isolated RNA was stored at -80 °C until further processing.

3.2.7.2 Isolation of RNA from cultivated cells

Isolation of RNA from cultivated cell lines was performed using the RNeasy® Mini Kit from Qiagen. The method is based on two technologies: the specific binding of RNA to a silica-membrane under specific buffer conditions and the microspin technology. In the first step 5 x 10^5 cells per well were seeded in 6 well plates. The following day cells were transfected with Pre-miR^{TM} miRNA Precursor (described in 3.2.4.5.2 or 3.2.4.5.3). After 48 h the cells were harvested and the RNA was isolated. Therefore the growth medium was removed and the cells were washed once with PBS. Subsequently, the cells were lysed with RLT buffer (part of the kit) which was

replenished with β-ME. β-ME irreversibly denaturates RNAses which are released during the lysis of cells. In combination with the Guanidiniumisothiocyanat contained in the RLT buffer all RNAses were completely inactivated. Past an incubation step of 2 min, the lysate was pipetted to a 2 ml tube and 600 µl of 70 % ethanol were added and mixed. The addition of ethanol optimizes the binding condition for the RNA to the silica-membrane. Adjacently, the ethanol-lysate mixture was added to the filter-column and centrifuged for 20 sec at 10000 RPM. The flow-through was discarded and in the following four wash steps proteins, salts and cell elements were removed. In the first wash step 700 µl of RW1-buffer were applied and centrifuged for 20 sec at 10000 RPM. The next three wash steps were performed each with 500 µl of RPE-buffer which was complemented with ethanol. After each wash step the column was centrifuged for 20 sec at 10000 RPM and the flow-through was discarded. An additional centrifugation step of 1 min at 14000 RPM was done after the last wash step to get rid of remaining wash buffer. Subsequently, the column was transferred to a new 1.5 ml tube and the RNA was eluted from the silica-membrane using 35 µl of RNAse-free HPLC-H_2O. After incubation for 1 min the column was centrifuged for 1 min at 10000 RPM to drain the column. The isolated RNA now resided in the tube. Quality and amount of RNA was analyzed using a photometer. Until further application the RNA was stored at -80 °C.

3.2.8 Synthesis of cDNA

In order to quantify the gene expression in a quantitative real-time PCR (qRT-PCR) the miRNA and RNA respectively have to be reverse transcribed to cDNA. The cDNA was either directly applied for further applications or stored at -20 °C.

3.2.8.1 Synthesis of cDNA of specific miRNAs

The reverse transcription of miRNAs was performed using the TaqMan® MicroRNA Reverse Transcription Kit and the 5 x Reverse Transcription-Primer of the according MicroRNA Assay (Applied Biosystems). For cDNA synthesis 10 ng of total RNA was applied. Therefore the concentration of RNA was measured after thawing, a dilution

of 1:100 was done and the volume containing 10 ng RNA was calculated. Subsequently, the Mastermix was prepared (Table 7) and 7 µl per reaction were pre-dispensed in each PCR-tube. The amount of RNA which contains 10 ng was added and filled up with HPLC-H_2O to a final volume of 12 µl. Last 3 µl of 5 x Reverse Transcription-Primer were pipetted to each tube and the reverse transcription was carried out in a thermocycler with the following programme (Table 8).

Component	**Amount / volume**
dNTPs	0.15 µl
Reverse Transkription buffer	1.50 µl
Reverse Transkriptase	1.00 µl
RNAse-Inhibitor	0.19 µl
Nuklease free H_2O	4.16 µl

Table 7 Pipetting scheme for cDNA-synthesis of miRNAs

Temperature	**Time / Duration**
16 °C	30 min
45 °C	30 min
85 °C	5 min
4 °C	∞

Table 8 Temperature scheme for cDNA-synthesis of miRNAs

3.2.8.2 Synthesis of cDNA of mRNAs

The synthesis of cDNA from mRNAs was performed using the High Capacity cDNA Reverse Transcription Kit. Thereby all mRNAs present in the RNA sample were reverse transcribed to cDNAs by the use of random primers. At first the concentration of the RNA was measured after thawing and the volume containing 1 µg of RNA was calculated. The calculated volume was pipetted into a PCR-tube and HPLC-H_2O was added to a final volume of 17.5 µl. The Mastermix was combined according to Table 9. In each tube 7.5 µl of Mastermix was added. In addition a negative control was performed containing no RNA in order to detect possible contaminations. The tubes were processed in a thermocycler according to the following programme (Table 10).

Component	Amount / volume
Reverse Transcription Buffer	2.5 µl
dNTPs	1 µl
Random Primer	2.5 µl
Reverse Transkrictase	1.5 µl

Table 9 Pipetting scheme for cDNA-synthesis of mRNAs

Temperature	Time / Duration
25 °C	10 min
37 °C	120 min
85 °C	5 sek
4 °C	∞

Table 10 Temperature scheme for cDNA-synthesis of mRNAs

After the PCR each sample contained a total volume of 25 µl with a concentration of 40 ng/µl. For further application the samples were diluted 1:1 with HPLC-H_2O to obtain a concentration of 20 ng/µl.

3.2.9 Quantitative real-time-PCR

The qRT-PCR is a technique to detect the expression levels of miRNAs and mRNAs, respectively. This method is based on a conventional PCR but in addition enables the quantification of the amplified cDNA in real time. After every PCR-cycle the fluorescence signal is measured which is equivalent to the cDNA quantity. In the present thesis TaqMan^{TM}-probes complementary to the sequence of the investigated cDNA were applied. These probes consist of oligonucleotides with a reporter-fluorescence dye at one end and a fluorescence-quencher at the other end. In this state the probe does not emit fluorescence because of the adjacency of fluorescence dye and quencher. Thereby the fluorescence energy of the dye is transfered to the quencher which in turn emits light not measured (Figure 9 A). During the PCR cycles the probe hybridizes with the complementary DNA-strand. As the applied polymerase also have a 5'-3' exonuclease activity the probe becomes abolished when the polymerase reaches the point where the probe has bound (Figure 9 A). Subsequently, the probe is degraded and the fluorescence dye becomes locally

separated from the quencher and measurable fluorescence is emitted (Figure 9 B and C). The fluorescence intensity increases proportional with the amount of amplificated PCR-product. This allows a precise quantification of gene expression [Bustin, 2000].

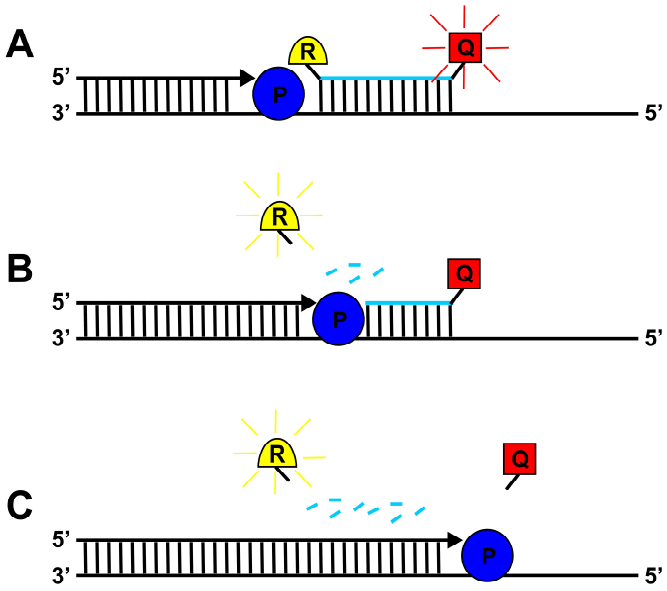

Figure 9 Theory of Taqman Assay.
P = polmerase; R = Reporter dye; Q = quencher

The quantified target genes were normalized to endogenous housekeeping genes during the gene expression analysis. These housekeeping genes are characterized by a relatively constant expression level in all tissue types and under all conditions investigated. Therefore in theory no significant change in expression should be visible between normal unaffected tissue and pathologically modified tissue. Nevertheless, in practice it was shown that certain expression variability is present. Rubie et al. indicated in a comparative study of different commonly used house keeping genes that the consistency of the expression of such house keeping genes could not be adopted in general among different tissue types or pathological modifications [Rubie et al., 2005]. According to the results of this study the housekeeping genes were chosen.

The analysis of the qRT-PCR was done using either the absolute standard curve method or the $\Delta\Delta C_T$ (Cycle Threshold)-method for the quantification of differences in expression levels between the samples.

For the standard curve method the expression of the target gene was determined with a standard curve applied on the same plate. Therefore known concentrations of

the target gene in different concentrations were amplified on the same plate. The known concentrations of the standard points were plotted against the resulting C_T -values and in turn the concentrations of the investigated samples can be read from the slope of the standard curve [Holzapfel et al., 2007].

The $\Delta\Delta C_T$ -method is used for relative quantification and gives no assertion on the copy number of the investigated cDNA. Such an assertion is sufficient when comparison of two groups (normal and cancerous) is performed. For this method the C_T -values of the target gene and the constantly expressed housekeeping gene are substracted to get ΔC_T -values and then the ΔC_T -values of the two groups are substracted to get the $\Delta\Delta C_T$ -value. In order to receive the expression disparity between the two groups the $\Delta\Delta C_T$ -value is adopted to the formula $2^{-\Delta\Delta C_T}$.

3.2.9.1 qRT-PCR for miRNA expression analysis

In order to investigate the expression profiles of different miRNAs in samples of CRC patients in addition to the target-miRNAs the endogeneous control gene RNU48 was applied. RNU48 belong to the so called small nucleolar RNA (snoRNA) family and has a function in the 2' O-Ribose methylation of its target RNAs [Galardi et al., 2002]. In a study performed by Applied Biosystems and other studies it was demonstrated that snoRNAs suits as endogeneous control gene for studies on the expression of miRNAs [Wong et al., 2007; Chang et al. 2010].

In the first step of qRT-PCR a Mastermix was prepared containing the miRNA specific TaqMan® Gene Expression Assay, TaqMan® Universal Master Mix II and HPLC-H_2O (Table 11). Subsequently, 2.79 µl of the according miRNA-cDNA was pre-dispensed in a tube and filled up with 39.21 µl of Mastermix to a final volume of 42 µl. The samples were vortexed, centrifuged and pipetted to MicroAmp® Optical 96-Well reaction plates in duplicates. Afterwards, the plate was sealed with MicroAmp® 96-Well Optical Adhesive Film and run in a Sequence Detection System, 7900HT ABI PRISM with the cycling programme in Table 12.

Component	**Amount / Volume (per duplicate sample)**
TaqMan® Gene Expression Assay	2.1 µl
TaqMan® Universal Master Mix II	21 µl
HPLC-H_2O	16.11 µl

Table 11 Pipetting scheme of qRT-PCR Mastermix

	Enzym activation	PCR	
		Cycles (40 cycles)	
Step	HOLD	Denature	Annealing / Elongation
Temperature	95 °C	95 °C	60 °C
Time / Duration	10 min	15 sec	60 sec

Table 12 Temperature cycles of qRT-PCR

3.2.9.2 qRT-PCR for CCL20 mRNA expression analysis

For the investigations on expression profiles of the chemokine CCL20 in samples from CRC patients the housekeeping gene *b2m* was used as reference gene. For the CCL20 gene expression assay performed in tissue samples a standard curve was used. The Mastermix for the standard curve was pipetted as indicated (Table 13).

Component	**Amount / volume (per duplicate sample)**
TaqMan® Gene Expression Assay	2.1 µl
TaqMan® Universal Master Mix II	21 µl

Table 13 Pipetting scheme for the standard curve Mastermix of qRT-PCR

Subsequently, the appropriate amount of cDNA was pre-dispensed into a tube and HPLC-H_2O was added to a final volume of 18.9 µl (Table 14). Then 23.1 µl of standard curve Mastermix was added.

	50 ng	20 ng	10 ng	1 ng	0.1 ng
cDNA	5.25 µl	2.1 µl	1.05 µl	1.05 µl	1.05 µl
Predilution	X	X	X	1:10	1:100
HPLC-H_2O	13.65 µl	16.80 µl	17.85 µl	17.85 µl	17.85 µl

Table 14 Pipetting scheme for cDNA dilutions of the standard curve

In the next step the Mastermix for the investigated samples was prepared as follows (Table 15). For reaction preparation 2.1 µl of according cDNA was pre-dispensed in a tube und subsequently 39.9 µl of Mastermix were added to each tube to a final volume of 42 µl. The samples for the qRT-PCR were vortexed gently, centrifuged to bring down and then pipetted in duplicates to a MicroAmp® Optical 96-Well reaction plate. The plates were sealed with MicroAmp® 96-Well Optical Adhesive Film and the samples were processed in a 7900HT ABI PRISM with the same cycling programme used for miRNA expression analysis (Table 12).

Component	**Amount / volume (per duplicate sample)**
TaqMan® Gene Expression Assay	2.1 µl
TaqMan® Universal Master Mix II	21 µl
HPLC-H_2O	16.8 µl

Table 15 Pipetting scheme for the qRT – PCR Mastermix of investigated samples

3.2.10 Protein technologies

3.2.10.1 Isolation of protein

Protein lysates were isolated from colorectal tissue samples and cultivated cell lines using RIPA-buffer. RIPA-buffer is a highly denaturating lysis buffer due to its ingredients Sodium deoxycholate and Sodium dodecyl sulfate. This buffer therefore allows the extraction of cytoplasmic, nuclear and membrane proteins.

3.2.10.1.1 Isolation of proteins from tissue samples

For isolation of proteins from tissue samples 60 mg of tissue were transferred to a round bottom tube containing 1 ml of RIPA-buffer (as described in 3.1.7.2). Subsequently, the tissue was homogenized with the help of the Ultra-Turrax ® T8. For the control of total homogeneity and further break up of cell structures the round bottom tube was taken to an ultrasonic bath for 30 sec. After total homogeneity was reached the samples were transferred to a 1.5 ml tube and centrifuged for 10 min at 13000 RPM and 4 °C. After the centrifugation step the supernatant containing the protein was aliquoted in two new 1.5 ml tubes and the pellet containing cell debris was discarded. Until further processing the protein lysates were stored at -20 °C:

3.2.10.1.2 Isolation of proteins from cultivated cell lines

Also for isolation of proteins from cultivated cell lines the RIPA-buffer was applied. At first 5 x 10^5 cells per well were seeded in a 6 well plate. The following day the cells were transfected with Pre-miR^{TM} miRNA Precursor (as described in 3.2.4.5.2 or 3.2.4.5.3). After incubation for further 48 h the growth medium was removed and the adherent cells were washed once with PBS. Afterwards 1 ml of RIPA-buffer were added to the cells for 5 min. The lysed cells were pipetted to a 1.5 ml tube and centrifuged for 10 min at 13000 RPM and 4 °C. Then protein containing supernatant was aliquoted in two new 1.5 ml tubes and stored at -20 °C until further processing.

3.2.10.2 Determination of protein concentration using the BCA-assay

In order to determine the protein concentration of the protein extracts the bicinchoninic acid protein assay (BCA)-method was applied. This method is based on the reduction of Cu^{2+}-ions to Cu^{+}-ions through proteins in an alkaline environment. Thereby the amount of produced Cu^{+}-ions is proportional to the amount of protein in the solution. These monovalent copper ions form a purple coloured complex together with bicinchoninic acid. This colour complex can be detected photometrically at a wavelength of 562 nm. The absorption is proportional to the protein concentration in the according sample [Smith et al., 1985]. The absolute protein concentration can be

determined using standard-dilution series with known concentrations of Bovine Serum Albumin (BSA) in the range of 0 to 2 mg/ml.

In the first step protein lysates were diluted with dd H_2O. The lysates from tissue samples were used in a dilution of 1:5 and 1:10 whereas the cell lysates were used undiluted and in a dilution of 1:2. Then the dilutions for the standard series were prepared with BSA (Table 16). Subsequently, 10 µl per well of the standard dilutions or the samples were pipetted to a 96 well plate. In order to start the quantifiable reaction, proportional to protein concentration, a reagent containing 50 parts BCA and 1 part 4 % Copper (II) sulfate was prepared and 200 µl of this solution were added to each well. The 96 well plate was shaken for 30 sec and afterwards incubated in an incubator at 37 °C for 30 min. Thereafter, the absorption at a wavelength of 562 nm was measured. The absolute protein concentration was calculated according to the standard curve.

Concentration of BSA-solution	**Contents**
2 mg/ml	undiluted
1.5 mg/ml	25 µl Aqua dest. + 75 µl BSA (2 mg/ml)
1 mg/ml	50 µl Aqua dest. + 50 µl BSA (2 mg/ml)
0.75 mg/ml	50 µl Aqua dest. + 50 µl BSA (1.5 mg/ml)
0.5 mg/ml	50 µl Aqua dest. + 50 µl BSA (1 mg/ml)
0.25 mg/ml	50 µl Aqua dest. + 50 µl BSA (0.5 mg/ml)
0.125 mg/ml	50 µl Aqua dest. + 50 µl BSA (0.25 mg/ml)
0.025 mg/ml	90 µl Aqua dest. + 10 µl BSA (0.25 mg/ml)

Table 16 Pipetting scheme of standard-dilution series of BCA assay

3.2.10.3 Enzyme-linked Immunosorbent Assay (ELISA)

The ELISA is a method to quantitatively detect specific proteins in protein lysates. In the present thesis the principal of Sandwich-type ELISA was applied. Thereby two target protein specific antibodies are required: (I) a so called “Capture antibody” and (II) a different “Detection antibody”. These two antibodies have to bind to different epitops on the antigen to avoid reciprocal inhibition. In the first step of the assay a 96

well ELISA-plate is coated with the “Capture antibody” which means that it is bound to the bottom of the plate. After an adequate incubation time a saturation buffer is applied which saturates all non-specific binding sites and avoids undesirable binding to uncoated areas. Subsequently, the samples are applied to the plate and the according antigen is bound to the “Capture antibody” at the recognized epitope. In order to remove unbound antigens a washing step is applied. In the next step a biotinylated “Detection antibody” is added which binds to a different epitope of the antigen and thereby accumulates to an antibody-antigen-antibody-complex which is the reason for the designation “Sandwich-type” ELISA. With the help of a further wash step excessive “Detection antibody” is removed. Subsequently, streptavidin which is conjugated to the enzyme horseradish-peroxidase (HRP) is added. Streptavidin shows an affinity binding to biotin and increases thereby the sensitivity for small protein amounts. The antigen can be quantified through the transformation of a chromogenic substrate (Tetramethylbenzidin) specific for the according enzyme. Thereby a colorimetric reaction occurs which is stopped by addition of 2 M sulphuric acid (H_2SO_4). The quantification is performed by measuring the absorption at a wavelength of 450 nm in a photometer. As reference the wavelength is measured at 562 nm. With the help of a standard curve consisting of known protein concentrations the content of the specific protein in each sample can be calculated.

For the detection of CCL20 in human colorectal tissue samples and protein lysates from cultivated cell lines a specific ELISA DuoSet® from R&D Systems® was used. In the first step the 96 well ELISA plates were coated with mouse anti-human MIP-3α/CCL20 “Capture antibody”. Therefore the antibody was diluted with PBS to a final concentration of 1 µg/ml. In each well 100 µl of the antibody solution were pipetted. The plate was sealed with adhesive film and incubated at 4 °C over night. The following day the plate was washed three times with washing buffer and then 200 µl of saturation buffer were added per well. The saturated plates can be used after incubation at 37°C for 2 h or can be stored for up to one week at 4 °C. Prior to application of the samples under investigation every sample was diluted with PBS/0.1 % BSA (50 ml PBS + 0.05 g BSA). Normally, tissue samples were diluted 1:5 and 1:10 whereas cell lysates were diluted 1:2 and kept undiluted. For the standard curve the following dilution series was made with recombinant human CCL20 protein: 1000 pg/ml, 500 pg/ml, 20 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.25 pg/ml, 15.625 pg/ml and 0 pg/ml (Blank). After sample preparation the ELISA-plate was washed three times with

washing buffer and 100 µl of sample or standard, respectively, were loaded in duplicates. Subsequently, the plate was sealed and incubated for 2 h at RT (or at 4 °C over night). After incubation the plate was washed three times and then 100 µl of biotinylated goat anti-human MIP-3α/CCL20 “Detection antibody” (50 ng/ml) was added and incubated for 2 h at RT (or at 4 °C over night). Following incubation the plate was washed three times and then Streptavidin-HRP conjugate (1:200 in PBS/0.1 % BSA) was added. After incubating the plate for 30 min in the dark the plate was washed three times and accordingly 100 µl of substrate solution (1:1 solution A : solution B) were added per well. Past a development time of 20 min the colorimetric reaction was stopped by the addition of 50 µl stopping solution (H_2SO_4). With the help of a plate reader the absorptions at the wavelengths 450 nm and 562 nm were detected.

3.2.11 Imaging technologies

3.2.11.1 Immunohistochemistry (IHC)

IHC methods are used for the detection of antigens (e.g. proteins) in tissue sections on cellular or even subcellular resolution. Thereby this method is widely used in diagnosis as well as basic research investigating localization of biomarkers. The IHC employs primary antibodies which specifically bind to the investigated antigen. As a detection system secondary antibodies are used which are conjugated to fluorescent dyes or enzymes (e.g. alkaline phosphatase) that have the potential to catalyze a colour generating reaction. These secondary antibodies were raised against the species the primary antibody was originated from and therefore specifically bind to the primary antibody. Therefore the fluorescent dyes or conjugated enzymes are located to the place where the investigated antigen is found. This in turn means that the fluorescence signal or coloured precipitate which appears after addition of an enzyme specific substrate indicates the cellular or subcellular location where the investigated antigen resides. Besides the system applying two antibodies there are also systems where the detection system is conjugated directly to the primary antibody but the system with two antibodies increases sensitivity because it allows an amplification of the signal as more than one secondary antibody can bind to the

primary antibody. The tissue sections stained with IHC subsequently can be analyzed using either a fluorescence microscope or a conventional light microcope.

For investigation of tissue samples from colorectal cancer patients formalin-fixed, paraffinem-bedded (FFPE) tissue was used. Therefore, sections of 4 µm were cut using a microtome. Subsequently, the sections were incubated in a 37 °C water bath and then mounted on microscope slides. After drying the sections were stored at 37 °C until further processing.

For the staining procedure the sections had to be deparaffinized and rehydrated. The sections were deparaffinized and rehydrated by passaging them from xylene via different ethanol steps to TBS (Table 17).

Step	**Solution**	**Time / duration**
1	Xylene	10 min
2	Xylene	10 min
3	Xylene	10 min
4	99.9 % ethanol	immerse 10 x
5	99.9 % ethanol	immerse 10 x
6	99.9 % ethanol	10 min
7	96 % ethanol	immerse 10 x
8	96 % ethanol	10 min
9	70 % ethanol	immerse 10 x
10	70 % ethanol	10 min
11	Aqua dest.	5 min
12	TBS	5 min

Table 17 Deparaffinization and rehydration of tissue sections

The rehydated tissue sections were transferred to a staining cuvette containing 1 x Target Retrieval buffer in order to unmask the antigens. The Target Retrieval Buffer containing the tissue sections was heated in a microwave to approximately 96 °C for 20 min. Afterwards the solution was allowed to cool down at RT for 20 min. Then the sections were transferred to a new staining cuvette and washed two times with dd H_2O for 3 min. Subsequently the endogenous peroxidases were blocked by incubating the sections in 3 % H_2O_2 for 10 min. After two washing steps with TBS for 5 min unspecific binding sites were blocked by addition of rabbit serum diluted in PBS

(1 drop of serum + 3.33 ml PBS) for 20 min. Next, the serum was removed and the endogeneous biotin was blocked by addition of Avidin D-solution for 15 min followed by washing for 5 min in dd H_2O and further incubation in Biotin-solution for 15 min. Afterward, the slides were washed 5 min in TBS and then the CCL20 specific primary antibody (PAK; goat anti human CCL20/MIP3α) diluted in antibody diluent (15 µg/ml) was applied to the sections and incubated in a humidity chamber at 4 °C over night. The next day the PAK was removed and the sections were washed two times with TBS for 5 min. Subsequently, the biotinylated secondary antibody (SAK; rabbit-anti goat) solution (3 drops serum + 10 ml PBS + 1 drop SAK) was added and incubated in a humidity chamber for 30 min at RT. At the beginning of the SAK incubation step the ABC-reagent was prepared (1 drop reagent A + 2.5 ml PBS + 1 drop reagent B) and incubated for 30 min. After SAK incubation the slides were washed two times for 5 min in TBS and then the ABC-reagent was applied for 25 min. Subsequent two further 5 min wash steps with TBS the AEC-solution (5 ml Aqua dest. + 1 drop acetate buffer + 2 drops AEC concentrate + 2 drops 3 % H_2O_2) was added and left for 7 min. The colour reaction was stopped by rinsing the slides in dd H_2O. In the next step the sections were counterstained by incubating them for 7 min in Mayers Hämatoxylin-solution. Subsequently, the slides were washed two times in dd H_2O and then rinsed for 7 min with tap water. Accordingly, the slides were rinsed in dd H_2O and finally aqueous embedded with two drops of Aquatex.

3.2.11.2 In Situ hybridization (ISH)

ISH is a method which allows an assignment on localization and distribution of nucleic acids in a portion or section of tissue (in situ). Thereby, the resolution of localization is relatively high and can distinguish between tissues, cells, cell compartments or even chromosomes. Since the ISH was first described in 1969 [John et al., 1969; Gall et al., 1969] it became a routine method in diagnostics and is also applied for embryologic, cell biological, genetic or pathologic issues.

The method of ISH applies RNA or DNA probes which are complementary to the nucleic acid sequence under investigation. This marks a major difference with respect to the method of IHC which detects proteins. The nucleic acid probes can be marked with radio-, fluorescent- or antigen-labelling. To date the probes for ISH with

emphasis on the detection of miRNAs are modified with LNA™ (Locked Nucleic Acid) a nucleic acid analogue which increases affinity, specificity and biostability of the probes. For the detection of mRNAs or miRNAs RNAse-free thin tissue sections are required. Thereby paraffin sections or frozen sections may be used. In the case of paraffin sections the first step in ISH is deparaffinization and rehydration followed by cell opening with proteinase K. Then the specific probe is applied under the according temperature pH- and salt-conditions [Jin et al., 1997]. With various wash steps at probe specific hybridization temperature non-specific interactions of the probe are removed. If radio- or fluorescent-labelling of the probe is used, the samples can be directly analyzed by autoradiography or fluorescence microscopy. When the probes were antigen labelled an immunohistochemical detection has to be performed before microscopic analysis of the sample.

For the detection of miR-21 and miR-145 in tissue samples from CRC patients 6 µm thick sections were cut under RNAse-free conditions one day before ISH procedure. After stretching of the sections in a 37 °C DEPC water bath the sections were dried for 1 h at RT, heated to 60 °C for 40 min and then stored at 4 °C until use. The first step of the ISH protocol constitutes deparaffinization and rehydration of the sections. These steps were performed in the same way as described for IHC (Table 17) except that PBS was used instead of TBS. Following the PBS step the slides were placed in a humidified chamber and 300 µl of proteinase K (15 µg/ml) in proteinase K buffer were applied to the sections and incubated for 24 min at 37 °C. Afterwards, the slides were washed two times with PBS and then dehydrated applying increasing ethanol concentrations (Table 18).

Solvent	**Time / Duration**
70 % Ethanol	Immerse 10 times
70 % Ethanol	1 min
96 % Ethanol	Immerse 10 times
96 % Ethanol	1 min
99.9 % Ethanol	Immerse 10 times
99.9 % Ethanol	1min

Table 18 Dehydration procedure of In situ-hybridization

After dehydration the slides were dried for 10 min at RT on a clean paper towel. During the drying phase the hybridization mixtures were prepared: For U6 snRNA 4

µl of probe stock (0.5 µM), for miR-21 and miR-145 12 µl of probe stock (25 µM), respectively, were transferred to different 2 ml tubes and heated at 90℃ for 4 min. Hence, 2 ml of 1 x microRNA ISH buffer were added to each tube containing different probes. After the drying phase the slides were placed on a flat surface and 30 µl of the according hybridization mixture were pipetted on different sections. In the following step a sterile, RNAse-free cover glass was applied on each section to avoid air bubbles and the edges were sealed with Fixogum (rubber cement). The sealed slides were incubated for 1 h at the hybridization temperature of the respective probe (Table 19).

Probe	**Hybridization temperature**
U6 snRNA	57 ℃ or 59℃
miR-21	57 ℃
miR-145	59 ℃

Table 19 Hybridization temperature of applied probes

After hybridization the Fixogum was removed using tweezers and the cover glasses were carefully detached to avoid damaging the tissue. Subsequently, the slides were incubated in a Coplin jar containing 5 x SSC at room temperature followed by several wash steps in different concentrations of SSC buffer (Table 20).

Step	**Buffer**	**Time / duration**	**Temperature**
1	5 x SSC	5 min	Hybr. Temp.
2	1 x SSC	5 min	Hybr. Temp.
3	1 x SSC	5 min	Hybr. Temp.
4	0.2 x SSC	5 min	Hybr. Temp.
5	0.2 x SSC	5 min	Hybr. Temp.
6	0.2 x SSC	5 min	RT

Table 20 Stringency wash steps with different concentrations of SSC buffer

After the washing procedure the slides were transferred to a Coplin jar with PBS and then placed in a humidifying chamber. The slides were dried with a clean towel around the section to create a hydrophobic barrier. Hence, 150 µl of blocking solution (10 x blocking solution diluted with 1 x Maleic acid to 1 x concentration) were added to each section and incubated for 15 min at RT. The blocking solution was removed and 50 µl of anti-DIG reagent (1 x blocking solution + 2 % sheep serum + 1:800 anti-

DIG antibody) were applied to the tissue sections for 60 min at RT. After washing three times for 3 min in PBS-T the AP substrate was prepared. A NBT-BCIP tablet was solved in 10 ml dd H_2O and levamisole was added to a final concentration of 0.2 mM. Slides were taken to a humidifying chamber and dried around the sections prior to addition of 100 µl AP substrate. After incubating the AP substrate for 1 h at 30 °C the slides were transferred to a Coplin jar with KTBT-buffer and incubated two times for 5 min to stop the reaction. Subsequently, the slides were washed two times for 1 min in dd H_2O and then 200 µl of Nuclear Fast Red solution were added for 1 min to the sections. Accordingly, the slides were transferred to a Coplin jar with tap water and rinsed for 10 min with tap water. Afterwards the sections were dehydrated with different concentrations of Ethanol (Table 18) and then placed in Xylene. Finally, the slides were transferred to a clean paper towel and 1-2 drops of Eukitt mounting medium were added per section and a cover glass was applied. After over night settlement of the precipitate the sections were microscopically analysed.

4. Results

4.1 Target prediction of miRNAs

To predict miRNAs which potentially interact with the target gene CCL20 five online available target prediction programmes were applied (3.2.1). For continuative investigations only miRNAs were used which were predicted by two or more of the programmes (Table 21). Hence, four of the programmes predicted miR-21 to potentially interact with the 3'UTR of CCL20 whereas only two programmes predicted miR-145 to potentially be involved in CCL20 post-transcriptional regulation. For notice, target prediction programme *PICTAR* did not contain the sequence data of CCL20 and therefore a prediction was not possible.

Prediction Tool	hsa-miR-21	hsa-miR-145
TARGETSCANHUMAN 4.2	**X**	
MICRORNA.ORG	**X**	**X**
PICTAR		
EBI MICROCOSM TARGETS	**X**	**X**
MIRDB	**X**	

Table 21 Overview of miRNA target prediction based on CCL20 sequence
Predicted interaction of the according miRNA with CCL20 is marked by X.

In order to further investigate the feasibility for an interaction of the chosen miRNAs with the target gene CCL20 seven other target prediction programmes were applied which depend on sequence information of both the target gene and the respective miRNA (3.2.1 and Table 22). Thereby, four of these programmes confirmed an interaction of miR-21 with CCL20 and three of the programmes confirmed an interaction of miR-145 with CCL20.

Prediction Tool	hsa-miR-21	hsa-miR-145
PITA	**X**	**X**
RNA22	**X**	**X**
TAREF		
microinspector		
STARMIR	**X**	
EIMMO	**X**	**X**
TARGETRANK		

Table 22 Overview of miRNA target prediction based on CCL20 and miRNA sequence
Predicted interaction of the according miRNA with CCL20 is marked by X.

According to most of the available target prediction programmes miR-21 has one binding site in the 3'UTR of CCL20 with a seed sequence located at position 261-268 (Figure 10). In addition, two programmes predicted other binding sites: *StarMir* (position 172-179) and *RNA22* (position 189-195). In case of miR-145 four of five programmes predicted position 24-31 as the seed binding site whereas only *PITA* predicted an interaction at position 195-202. For further investigations only target sites predicted by the majority of the programmes were applied.

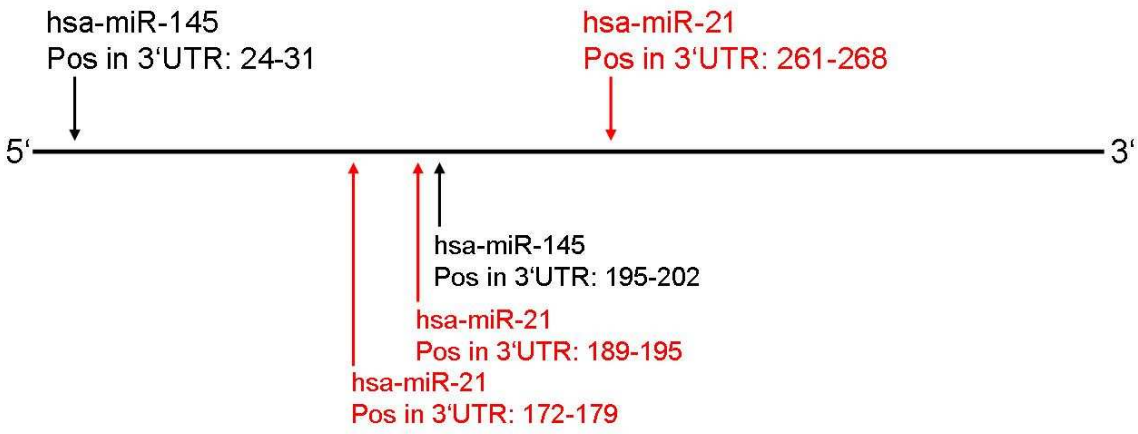

Figure 10 Predicted miRNA binding sites in the 3'UTR of CCL20 (390 bp)
Target sites indicated above the line were predicted by more than one programme. Target sites beneath the line were predicted by only one programme.

The sequence alignments of the miRNAs with the 3'UTR of CCL20 are depicted for the binding site predicted by the majority of the respective target prediction programmes presented in Figure 11.

Binding site alignment of miR-21 at position 261-268

```
3′  AGUUGUAGUCAGA--CUAUUCGAU  5′  hsa-miR-21
    |:|||:| :| |:|   ||||||||
5′ …TTAATACTAATTTTCCATAAGCTA… 3′  CCL20 3'UTR
```

Binding site alignment of miR-145 at position 24-31

```
3′  CCCUAAGGACCCU--UUUGACCUG  5′  hsa-miR-145
    ||  ||:|||| |  :||:|||||
5′ …GGCTTTTCTGGAATGGAATTGGAC… 3′  CCL20 3'UTR
```

Figure 11 Target site alignment
Shown are the target positions predicted by most target prediction programmes under investigation. The alignment is adapted from the programme *MICROCOSM TARGETS*. Lines indicate base pairing between miRNA and target sequence, dots indicate wobble pairing and spaces indicate no pairing.

4.2 Luciferase assay

In order to validate the predicted interactions of the two miRNAs with the target gene CCL20 a luciferase assay was applied. In this assay, two luciferase reporter constructs manufactured by Gene Copoeia™ were applied. The first construct contained the 3'UTR of the target gene CCL20 directly downstream of the firefly luciferase gene (Luc CCL20). The second construct served as a control vector and contained no 3'UTR downstream of the firefly luciferase gene (Luc No CCL20). The vector maps of the two constructs are depicted in Figure 6 and Figure 8. For normalization of transfection efficiency the constructs contain another luciferase gene (Renilla luciferase) under the control of a constitutive promoter. The luciferase assay was performed with the Dual-Luciferase® Reporter Assay from Promega (3.2.5). In case of a functional interaction between the 3'UTR of CCL20 and one of the investigated miRNAs a decreased expression of the firefly luciferase is expected and detectable by luminometric measurement.

4.2.1 Impact of miR-21 on CCL20-3'UTR dependent luciferase activity in 293T cells

Initially, luciferase assays were performed in cell line 293T which is commonly used for luciferase reporter assays. In this assay cells were co-transfected with the reporter vectors and pre-miR precursors of miR-21 or a negative control (NK) pre-miR precursor, respectively, as described in chapter 3.2.4.5.1. In Figure 12 the mean values of luciferase activity after miR-21 pre-miR precursor co-transfection relative to NK pre-miR precursor transfected cells is shown. Data were obtained from five independent experiments with co-transfected 293T cells and presented with the according standard error of mean (SEM).

Co-transfection of 293T cells with the reporter construct Luc CCL20 and the miR-21 precursor resulted in a significant ($P < 0.05$) reduction of luciferase activity in relation to 293T cells which were co-transfected with Luc CCL20 and the NK pre-miR precursor. Co-transfection of 293T cells with Luc No CCL20 and miR-21 precursor resulted in no significant alteration of luciferase activity related to cells co-transfected with NK pre-miR precursors. Furthermore, a significant difference was observed ($P < 0.05$) between 293T cells co-transfected with Luc CCL20 and miR-21 precursor and cells co-transfected with the Luc No CCL20 construct and the miR-21 precursor. Thus, our experiments demonstrate that miR-21 precursors regulate the expression of luciferase activity in a vector containing the 3'UTR of CCL20. Therefore, our results allow to draw the conclusion that miR-21 regulates the expression of the target gene CCL20 through a regulatory element present in the 3'UTR of CCL20 mRNA.

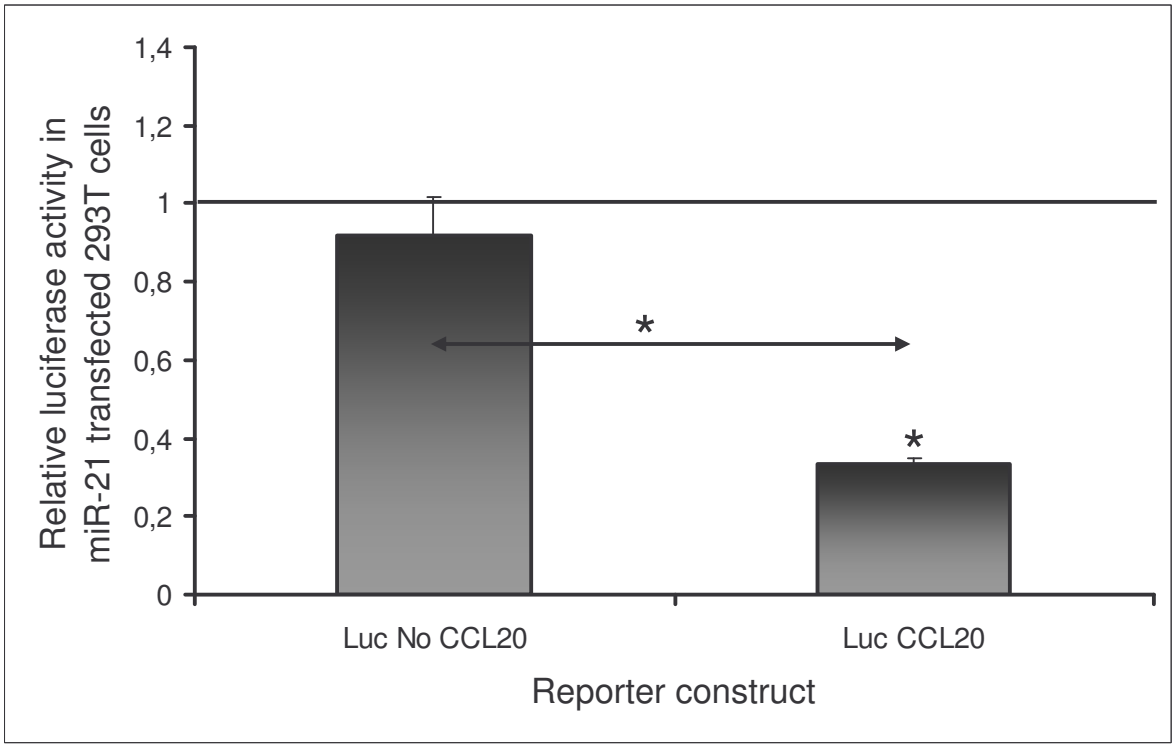

Figure 12 Relative luciferase activity in miR-21 transfected 293T cells
Luciferase activity in 293T cells co-transfected with Luc No CCL20 vector and Luc CCL20 vector, respectively, and miR-21 pre-miRNA precursor relative to cells co-transfected with negative control (NK) pre-miRNA precursor. Data are presented as mean +/- SEM (n=5). Only co-transfection of miR-21 pre-miRNA precursor with Luc CCL20 vector resulted in a significantly decreased luciferase activity *($P < 0.05$). Fold decrease below 1 indicates down-regulation of luciferase activity in miR-21 co-transfected cells related to NK miR precursor transfected cells.

4.2.2 Impact of miR-145 on CCL20-3'UTR dependent luciferase activity in 293T cells

In order to analyze the predicted interaction of miR-145 with the 3'UTR of CCL20 also 293T cells were co-transfected with the according constructs and pre-miRNA precursors. Co-transfection of miR-145 precursor with the Luc CCL20 reporter construct did not result in decreased luciferase expression in relation to 293T cells co-transfected with NK miRNA precursors (Figure 13). Likewise, no difference in luciferase expression was observed with respect to the co-transfection of Luc No CCL20 and miR-145 precursors in relation to 293T cells co-transfected with NK miRNA precursors. Therefore, it may be concluded that there is no direct regulatory interaction between miR-145 and the 3'UTR of CCL20.

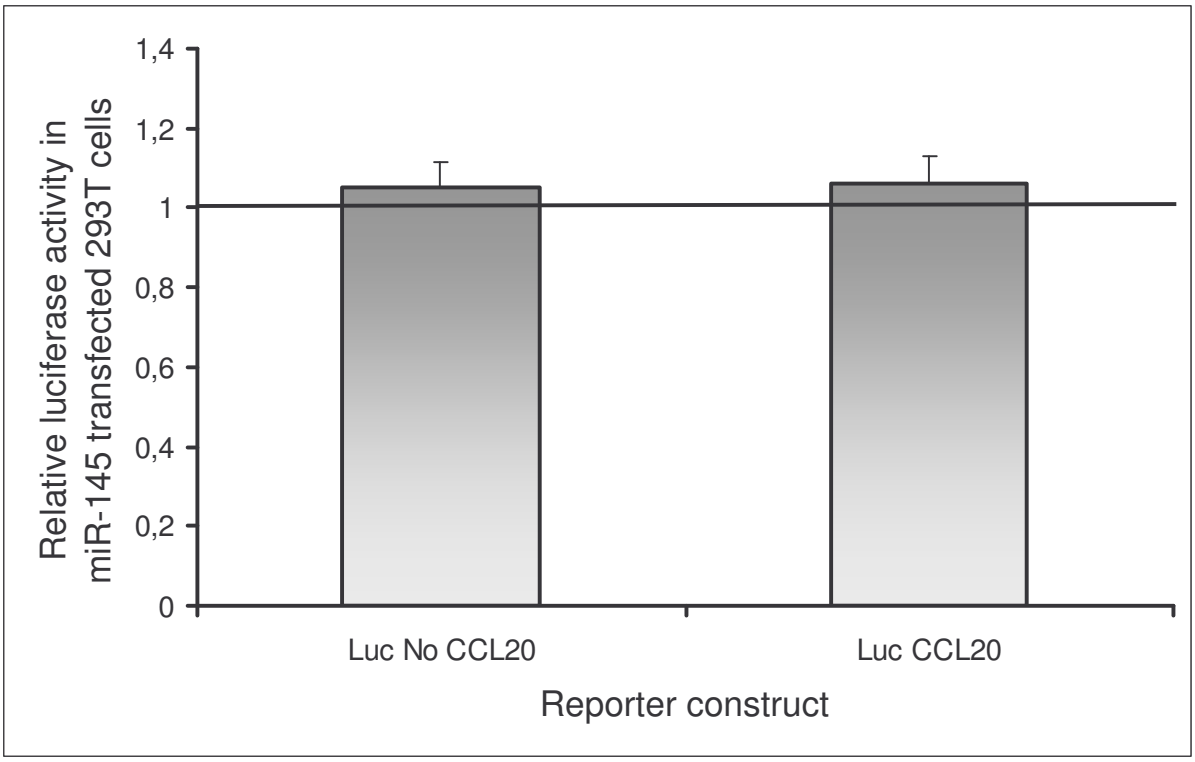

Figure 13 Relative luciferase activity in miR-145 transfected 293T cells
Luciferase activity in 293T cells co-transfected with Luc No CCL20 vector and Luc CCL20 vector, respectively, and miR-145 pre-miRNA precursor relative to cells co-transfected with negative control (NK) pre-miRNA precursor. Data are presented as mean +/- SEM (n=5). Neither co-transfection of miR-145 precursor with Luc CCL20 nor with Luc No CCL20 resulted in altered luciferase expression in relation to co-transfection with NK miRNA.

4.2.3 Impact of miR-21 on CCL20-3'UTR dependent luciferase activity in HT29 cells

As cell lines are immortalized cells some cell lines exhibit alterations in their genome which can interfere with cellular regulatory mechanisms. To avoid a bias introduced by the choice of the applied cell line, luciferase assays were performed in an additional cell line. For this purpose HT29 was chosen as this cell line constitutes a CRC cell line leading to results which may be comparable to results obtained in cancerous tissue.

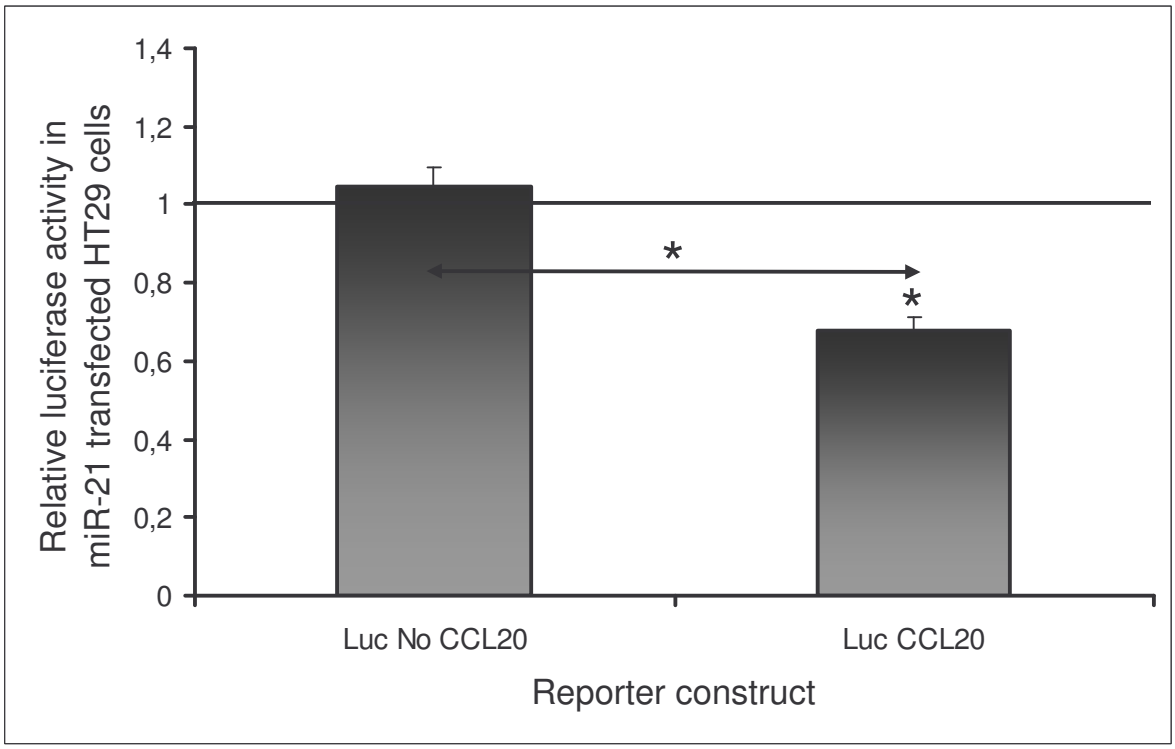

Figure 14 Relative luciferase activity in miR-21 transfected HT29 cells
Luciferase activity in HT29 cells co-transfected with Luc No CCL20 vector and Luc CCL20 vector, respectively, and miR-21 pre-miRNA precursor relative to cells co-transfected with negative control (NK) pre-miRNA precursor. Data are presented as mean +/- SEM (n=5). Only co-transfection of miR-21 pre-miRNA precursor together with the Luc CCL20 vector resulted in a significantly decreased luciferase activity *($P < 0.05$). Fold decrease below 1 indicates down-regulation of luciferase activity in miR-21 co-transfected cells related to NK miR precursor transfected cells.

The results obtained in cell line HT29 were similar to the results received in cell line 293T. Co-transfection of Luc CCL20 with miR-21 pre-miRNA precursor resulted in significantly decreased luciferase activity related to cells co-transfected with the same reporter construct together with the NK pre-miRNA precursor ($P < 0.05$) (Figure 14). Co-transfection of Luc No CCL20 with miR-21 pre-miRNA precursor had no effect on luciferase activity compared to cells co-transfected with NK pre-miRNA precursor molecules. Likewise, a significant difference in relative luciferase activity was observed between HT29 cells co-transfected with miR-21 pre-miRNA precursors and Luc CCL20 and Luc No CCL20, respectively. These results support the conclusion that miR-21 regulates CCL20 gene expression by a miR-21 specific target site in the 3'UTR.

4.2.4 Impact of miR-145 on CCL20-3'UTR dependent luciferase activity in HT29 cells

The interaction of miR-145 with the 3'UTR of CCL20 was also tested in a luciferase assay performed in cell line HT29. Likewise, the results obtained in cell line HT29 were very similar to the results received in cell line 293T. Neither co-transfection of miR-145 with either Luc No CCL20 nor the vector containing the 3'UTR of CCL20 showed an alteration in luciferase activity related to cells co-transfected with the NK pre-miRNA precursor (Figure 15). Therefore, it may be concluded that miR-145 plays no role in direct regulation of CCL20 gene expression by direct interaction with the 3'UTR of CCL20.

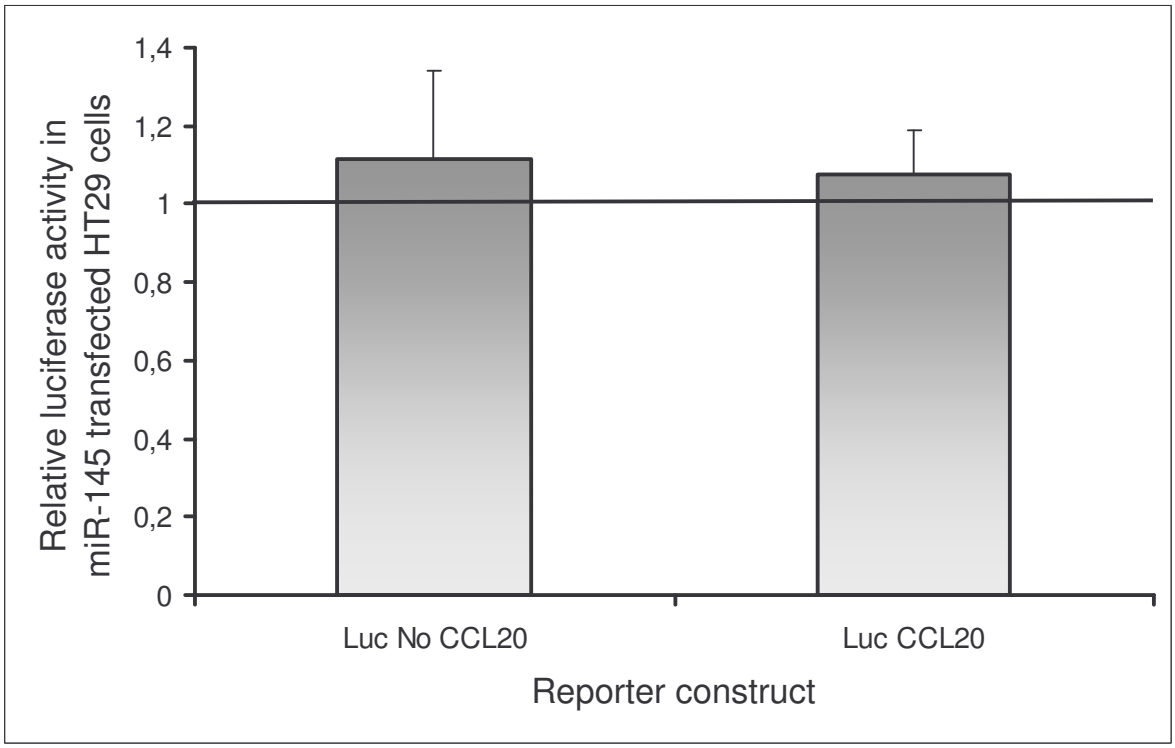

Figure 15 Relative luciferase activity in miR-145 transfected HT29 cells
Luciferase activity in HT29 cells co-transfected with Luc No CCL20 vector and Luc CCL20 vector, respectively, and miR-145 pre-miRNA precursor relative to cells co-transfected with negative control (NK) pre-miRNA precursor. Data are presented as mean +/- SEM (n=5). Neither co-transfection of miR-145 precursor with Luc CCL20 nor with Luc No CCL20 resulted in altered luciferase expression in relation to co-transfection with NK miRNA.

4.3 Site directed mutagenesis of miR-21 binding site

Applying the luciferase reporter gene assay it was demonstrated that miR-21 may reduce luciferase activity by binding to the 3'UTR of CCL20. To further investigate this interaction a site directed mutagenesis of the miR-21 binding site was performed. Target of the site directed mutagenesis approach was the region of the 3'UTR which was complementary to the seed sequence of the miR-21 target site predicted by most programmes. As the sequence complementary to the seed region of the according miRNA is the most important part for miRNA binding, two nucleotides in the sequence complementary to the seed sequence of the CCL20 3'UTR were mutated. The 3'UTR sequence ATAAGCTA was changed to **G**T**G**AGCTA by the use of primers containing mismatches at the according positions. The outcome of the mutations in the new construct (Luc CCL20 Mut) was confirmed by DNA sequencing of the mutated region performed by GATC (Figure 16).

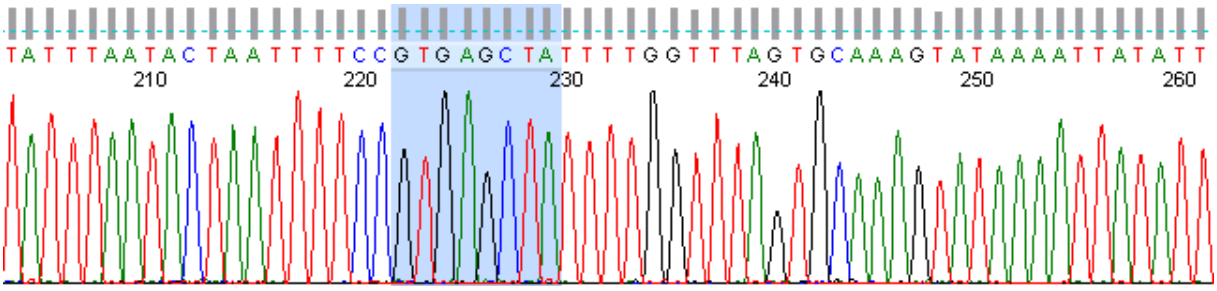

Figure 16 Mutated luciferase reporter construct
Luciferase reporter construct containing two point mutations in the site complementary to the seed region of miR-21.

4.4 Luciferase assay with mutated target site

As miR-21 may reduce luciferase activity by binding to the 3'UTR of CCL20, a reporter construct with the mutated seed sequence of the CCL20 3'UTR was applied to investigate whether the observed regulatory effect is due to a direct interaction of miR-21 with the specific site in the 3'UTR of CCL20. Therefore, luciferase assays were repeated applying three constructs, Luc CCL20, Luc No CCL20 and the construct with the mutated, predicted target site of miR-21. Thus, it was aimed to

monitor if the introduced mutations may reduce or avert the miR-21 mediated 3'UTR dependent down-regulation of luciferase activity.

4.4.1 Luciferase assay with mutated target site in 293T cells

The mutated Luc CCL20 Mut construct was applied in a luciferase assay along with miR-21 pre-miR precursors in 293T cells. In samples co-transfected with the vector containing the mutated 3'UTR of CCL20 (Luc CCL20 Mut) in combination with miR-21 pre-miR precursors a significantly decreased luciferase expression was observed with respect to samples co-transfected with NK pre-miR precursors (Figure 17). Also with respect to samples co-transfected with Luc No CCL20 and miR-21 pre-miR precursors a significantly decreased luciferase expression was observed in the Luc CCL20 Mut samples as presented in Figure 17. However, in comparison to Luc CCL20 samples co-transfected with miR-21 pre-miR precursors a significantly increased luciferase expression was observed in the Luc CCL20 Mut samples (Figure 17). Therefore, data resulting from luciferase assays with the mutated seed side of miR-21 indicate that mutating the predicted target site represses the inhibitory effect of miR-21 on CCL20 3'UTR dependent luciferase expression but they also show that this repression is not complete.

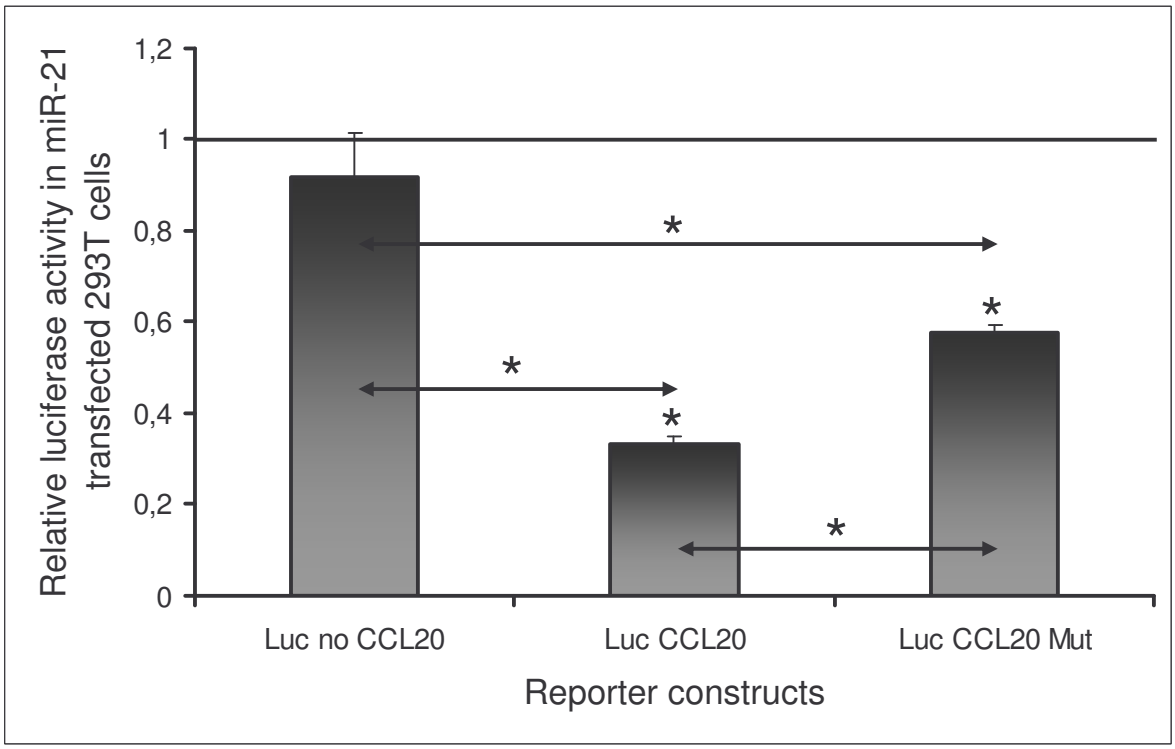

Figure 17 Luciferase assay with mutated target site in 293T cells
Luciferase activity in 293T cells co-transfected with Luc No CCL20 vector, Luc CCL20 and Luc CCL20 Mut vector, respectively, and miR-21 pre-miRNA precursors relative to cells co-transfected with negative control (NK) pre-miRNA precursors. Data are presented as mean +/- SEM (n=5). Co-transfection of miR-21 pre-miRNA precursor / Luc CCL20 or miR-21 pre-miRNA precursor / Luc CCL20 Mut resulted in significantly decreased luciferase activities *($P < 0.05$) related to co-transfection of NK pre-miR precursors, respectively. Significant decreased luciferase activities were also found in miR-21 pre-miRNA precursor / Luc CCL20 and pre-miRNA precursor / Luc CCL20 Mut co-transfected cells compared to miR-21 pre-miRNA precursor / Luc no CCL20 cotransfected cells. Thereby, significant increased luciferase activities were observed in Luc CCL20 Mut / miR-21 pre-miRNA precursor co-transfected cells in comparison to Luc CCL20 / miR-21 pre-miRNA precursor co-transfected cells, respectively *($P < 0.05$).

4.4.2 Luciferase assay with mutated target site in HT29 cells

Luciferase assays with the construct containing the mutated seed site of CCL20 were also perfomed in CRC cell line HT29. Also in this cell line co-transfection of miR-21 pre-miR precursors with Luc CCL20 Mut resulted in a significant reduction of luciferase activity relative to the luciferase activity in cells co-transfected with NK pre-miR precursors and Luc CCL20 Mut ($P < 0.05$) (Figure 18). Likewise, the reduction of luciferase activity in cells co-transfected with miR-21 pre-miR precursors and Luc CCL20 was significantly higher compared to cells co-transfected with miR-21 pre-miR precursors and Luc CCL20 Mut ($P < 0.05$). Relative luciferase activities of both Luc CCL20 and Luc CCL20 Mut were significantly lower compared to cells co-transfected

with the control vector Luc No CCL20 and miR-21 pre-miR precursors (Figure 18). As co-transfection of miR-21 pre-miR precursors with Luc No CCL20 showed no significant change in luciferase activity with respect to co-transfection of NK pre-miR precursors/Luc No CCL20, it was concluded that miR-21 does not interfere with luciferase activity if the 3'UTR of CCL20 is absent. Therefore, also results obtained in HT29 cells indicate that there is a 3'UTR dependent regulatory interaction between HT29 and luciferase or CCL20 expression, respectively.

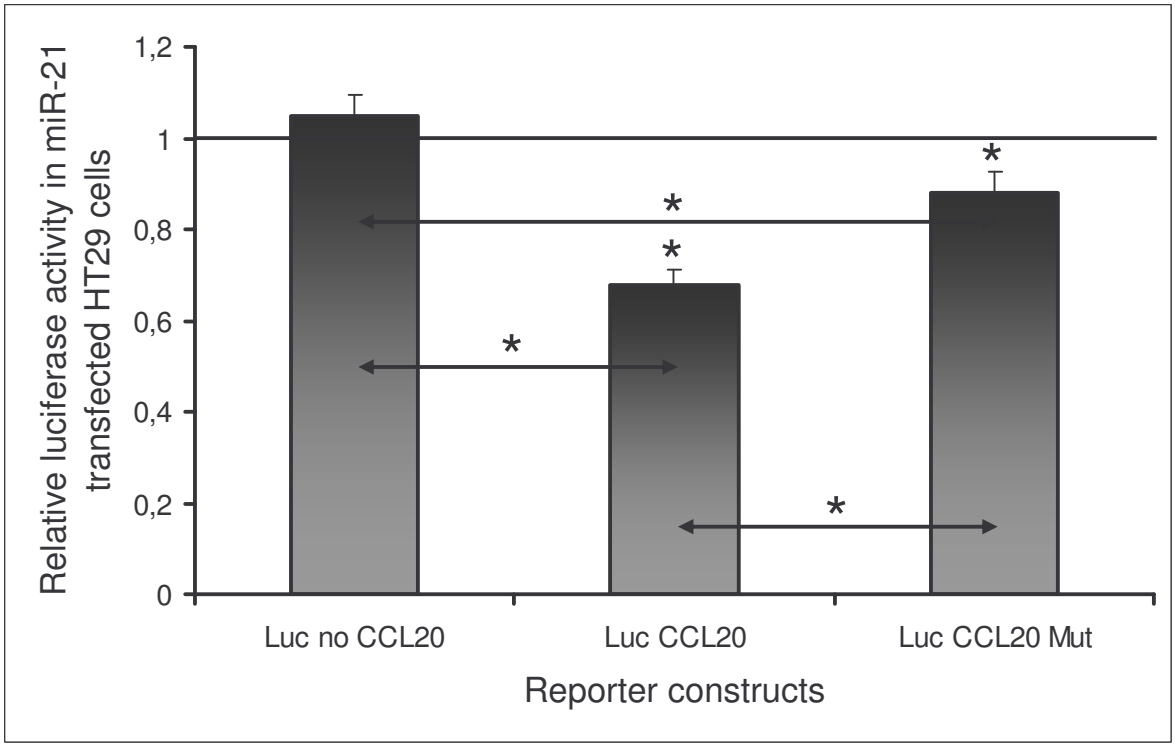

Figure 18 Luciferase assay with mutated target site in HT29 cells
Luciferase activities in HT29 cells co-transfected with Luc no CCL20, Luc CCL20 and Luc CCL20 Mut vector, respectively, and miR-21 pre-miR precursors relative to cells co-transfected with negative control (NK) pre-miRNA precursors. The data are presented as mean +/- SEM (n=5). Co-transfection of miR-21 pre-miRNA precursor / Luc CCL20 or miR-21 pre-miRNA precursor / Luc CCL20 Mut resulted in significantly decreased luciferase activities *($P < 0.05$) related to co-transfection of NK pre-miR precursors, respectively. Significant decreased luciferase activities were also found in miR-21 pre-miRNA precursor / Luc CCL20 and pre-miRNA precursor / Luc CCL20 Mut co-transfected cells compared to miR-21 pre-miRNA precursor / Luc no CCL20 cotransfected cells. Thereby, significant increased luciferase activities were observed in Luc CCL20 Mut / miR-21 pre-miRNA precursor co-transfected cells in comparison to Luc CCL20 / miR-21 pre-miRNA precursor co-transfected cells, respectively *($P < 0.05$).

4.5 Quality of target prediction according to luciferase assay results

With respect to data obtained from luciferase assays only one of the predicted miRNAs, namely miR-21, was successfully shown to regulate CCL20 gene expression by a miR-21 specific target site in the 3'UTR. Therefore, the quality of prediction was assessed. The target prediction tools *TargetScanHuman 4.2*, *microRNA.org*, *MiRDB*, *PITA* and *StarMir* predicted a positive interaction for both miRNAs, miR-21 and miR-145 (Figure 19). The tools *TAREF*, *TargetRank*, *EBI MicroCosmTargets*, *EIMMo* and *RNA22* predicted correctly one of the miRNA target gene interactions whereas *microinspector* correctly predicted no interaction between the miRNAs and the respective target gene. *PicTar* did not predict any interaction due to the absence of CCL20 in its database. Most of the target prediction tools applied "Seed complementarity" in combination with other features (Figure 19). The only exception constituted *StarMir* which predicted miRNA target gene interaction according to thermodynamic features. The usage of further features for predicting miRNA target gene interactions seemed to be more efficient in most cases according to the results of the present thesis.

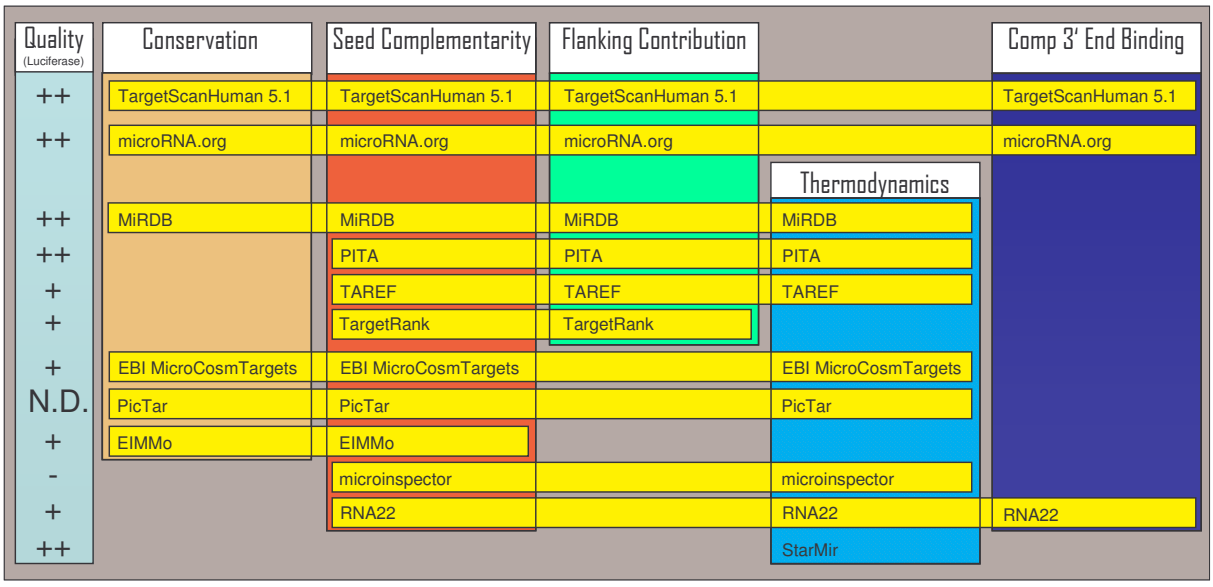

Figure 19 Target Prediction Tools Features and Quality.
Number of correct predictions in accordance with the results of the luciferase assay (luciferase) ++ = correct predictions for both *miRNA*s, miR-21 and miR-145, + = correct prediction for one *miRNA*, - = no correct prediction, N.D. = no prediction was possible.

4.6 Transfection of eukaryotic cells with pre-miRNA mimics

After demonstrating miR-21 interaction with the 3'UTR of CCL20 in luciferase assays, it was investigated if artificial over-expression of miRNAs in different CRC cell lines and the kidney cell line 293T may influence the expression of CCL20 on the mRNA and/or protein level. Therefore, four CRC cell lines, Caco-2, HT29, SW480 and SW620 as well as 293T were transfected with pre-miR precursor molecules of miR-21, miR-145 and NK pre-miR precursor, respectively. After 48 h the expression of CCL20 was measured on the mRNA level by qRT-PCR and on the protein level using ELISA.

4.6.1 Pre-miR-21 mimics

In the previous sections a regulatory interaction of miR-21 with the 3'UTR of CCL20 was shown and validated by the use of a luciferase assay. Thus, CCL20 expression was expected to be down-regulated after transfection of miR-21 pre-miR precursors into the CRC cell lines or the kidney cell line 293T. However, not all cell lines under investigation fulfilled this expectation.

In cell line Caco-2 transfection with miR-21 pre-miR precursors showed no effect on CCL20 mRNA expression whereas on the protein level a significant 40 % down-regulation of CCL20 protein expression was observed ($P < 0.05$). In contrast, artificial over-expression of miR-21 by transfecting cell line SW480 caused a significant down-regulation of CCL20 expression both on the mRNA and the protein level ($P < 0.05$). As shown in Figure 20 A and B CCL20 mRNA expression was reduced to approximately 60 % and CCL20 protein expression was decreased to approximately 40 % with respect to NK pre-miR precursor transfected cells. Likewise, CCL20 mRNA and protein expression were significantly reduced after transfection of cell line SW620 with miR-21 pre-miR precursor molecules. Here, CCL20 mRNA expression was significantly decreased to approximately 45 % and protein expression was significantly reduced to approximately 60 % related to cells transfected with NK pre-miR precursors ($P < 0.05$) as shown in Figure 20 A and B. In contrast to the first three cell lines under investigation CRC cell line HT29 showed no significant alteration in CCL20 expression upon transfection with miR-21 pre-miR precursors

neither on the mRNA nor on the protein level (Figure 20 A and B). However, in kidney cell line 293T CCL20 mRNA expression was also significantly down-regulated after artificial over-expression of miR-21 whereas CCL20 protein expression was not detectable at all, neither in NK transfected cells nor in miR-21 transfected cells.

In summary, CCL20 mRNA expression was significantly down-regulated in two out of four CRC cell lines under investigation whereas CCL20 protein expression was significantly decreased in three CRC cell lines. Moreover, in kidney cell line 293T CCL20 mRNA expression was significantly down-regulated and CCL20 protein expression was not measurable.

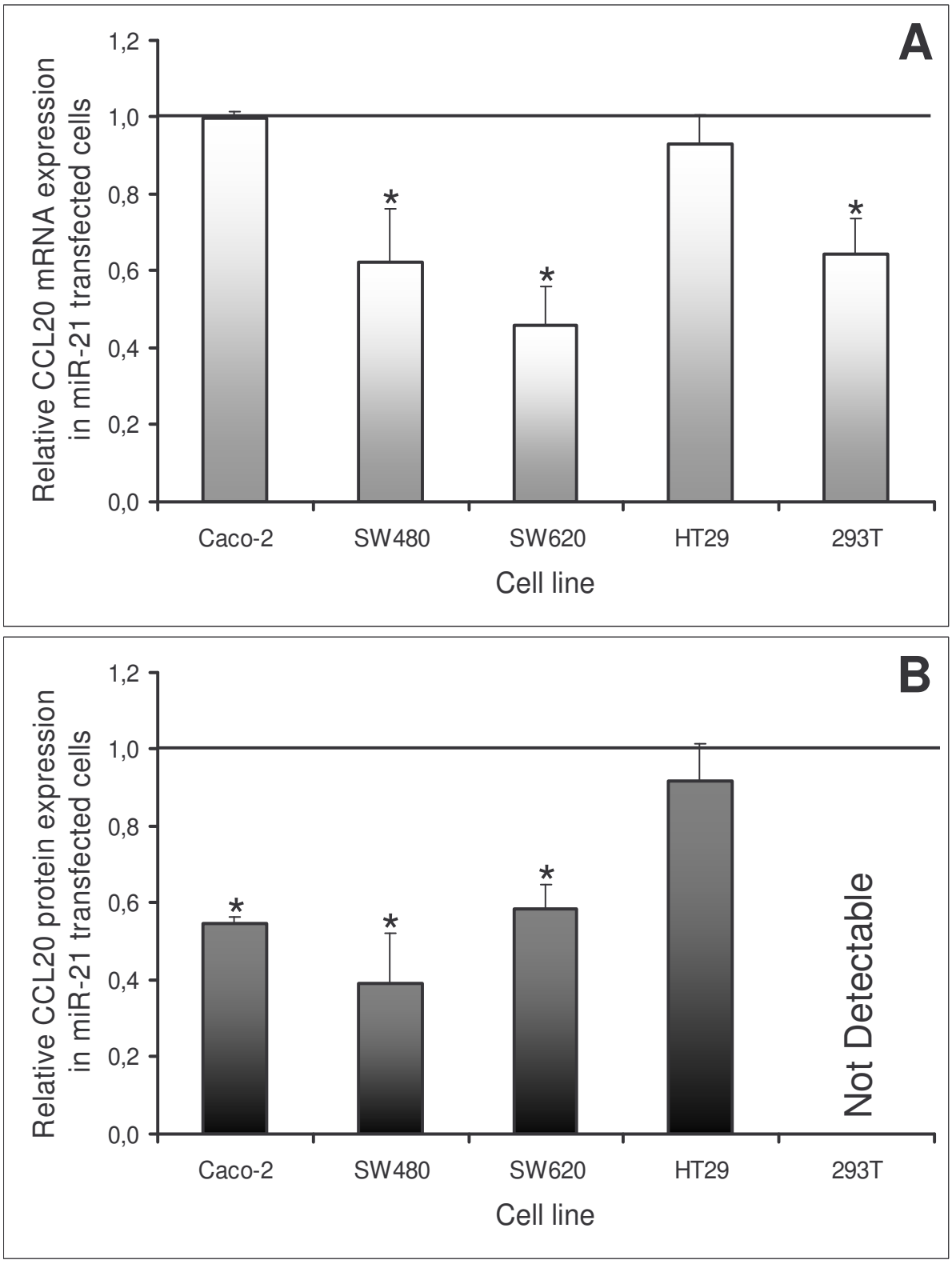

Figure 20 CCL20 expression analysis in cell lines transfected with miR-21 pre-miR presursor
(A) Expression of CCL20 mRNA in different cell lines after transfection with miR-21 pre-miR precursors or NK pre-miR precursors. Data are presented as mean +/-SEM (n=6). Fold decrease below 1 indicates down-regulation of CCL20 mRNA in the respective cell lines relative to NK transfected cells *($P < 0.05$). (B) Expression of CCL20 protein in different cell lines after transfection with miR-21 pre-miR precursors or NK pre-miR precursors. Protein data are presented as mean +/-SEM (n=6). Fold decrease below 1 indicates down-regulation of CCL20 protein expression related to NK pre-miR precursor transfected cells *($P < 0.05$).

4.6.2 Pre-miR-145 mimics

Based on the luciferase assay no direct interaction between miR-145 and the 3'UTR of CCL20 has been indicated. However, in analogy to miR-21 transfection studies experiments with pre-miR precursor molecules of miR-145 were performed on CRC and 293T cells to test for an indirect regulatory effect of miR-145 on CCL20 mRNA and protein expression. As expected, no significant alteration in CCL20 mRNA or protein expression was observed in either cell line with respect to NK pre-miR precursor transfected cells (Figure 21).

In summary, pre miR-145 transfection experiments have demonstrated that artificial increase of miR-145 has no effect on CCL20 expression, neither on the mRNA nor on the protein level. Therefore, miR-145 expression is not expected to influence CCL20 expression in a direct or indirect manner.

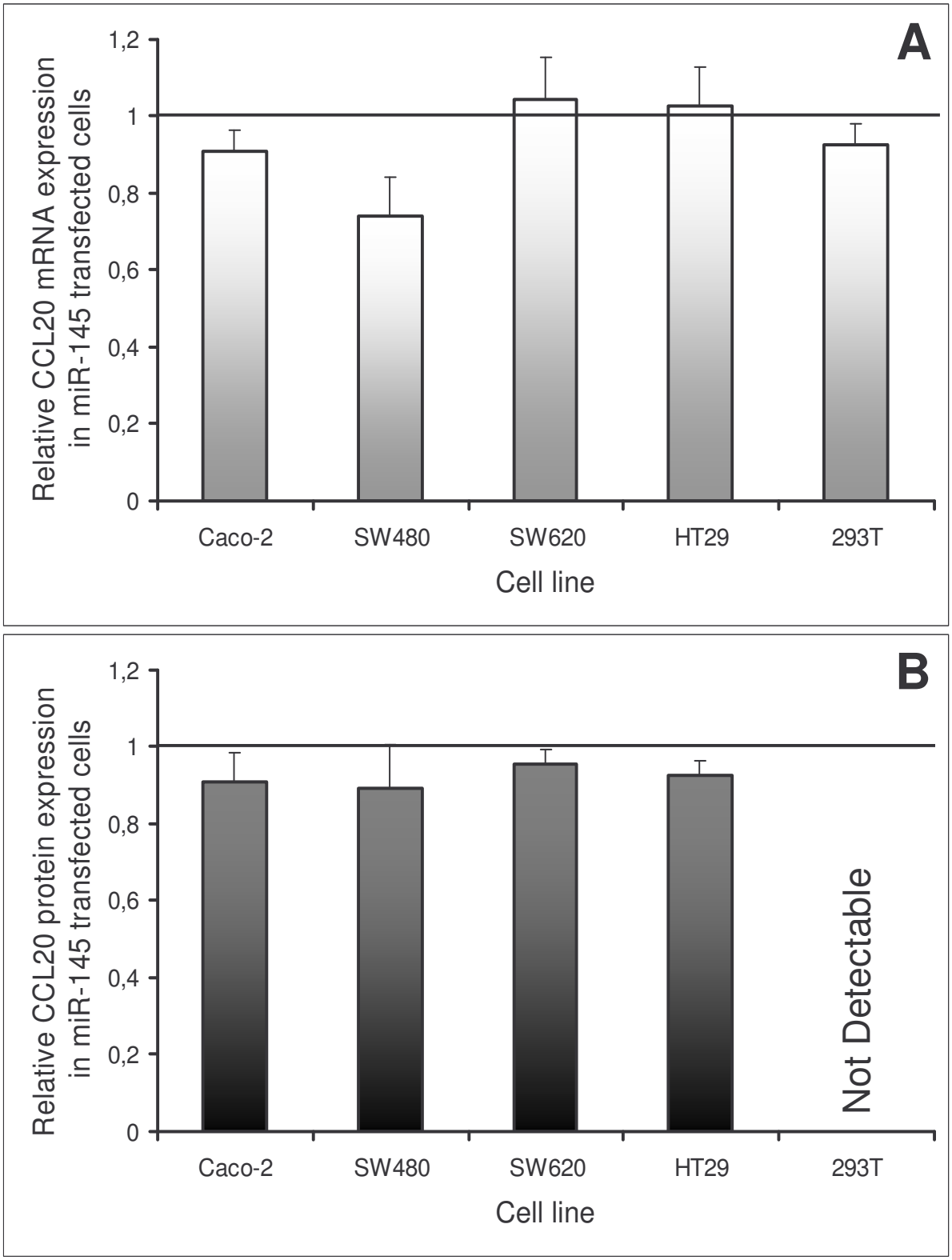

Figure 21 CCL20 expression analysis in cells transfected with miR-145 pre-miR precursors
(A) Expression of CCL20 mRNA in different cell lines after transfection with miR-145 pre-miR precursors or NK pre-miR precursors. Data are presented as mean +/-SEM (n=6). Fold decrease below 1 indicates down-regulation of CCL20 mRNA expression in the respective cell lines relative to NK transfected cells. (B) Expression of CCL20 protein in different cell lines after transfection with miR-145 pre-miR precursors or NK pre-miR precursors. Protein data are presented as mean +/- SEM (n=6). Fold decrease below 1 indicates down-regulation of CCL20 protein expression related to NK pre-miR precursor transfected cells.

4.7 Expression of the chemokine CCL20 in CRC tissue samples

After demonstrating that artificial over-expression of miR-21 down-regulates CCL20 expression in various CRC cell lines, the next set of experiments aimed to investigate CCL20 gene expression in CRC tissues. To study CCL20 gene expression on mRNA and protein level, tissue samples from patients with different clinicopathological features of CRC were analyzed using qRT-PCR and ELISA.

4.7.1 CCL20 mRNA expression analyzed by qRT-PCR

Investigating CCL20 mRNA expression in CRC tissues comprised samples from 46 CRC patients. Quantification of mRNA levels was performed using qRT-PCR. Data were normalized to the housekeeping gene *b2m*. In Figure 22 the expression of CCL20 mRNA is depicted as n-fold expression relative to the corresponding normal colorectal tissue. The mean of all patients was calculated and the standard error of mean was indicated (Figure 22 A). In addition, the patients were grouped according to the characteristics of their tumour (Figure 22 B-C). With respect to corresponding normal tissue in tumourous tissue a significantly 14-fold increased expression of CCL20 mRNA was observed ($P < 0.05$) (Figure 22 A). This increase in expression was also observed when patients were grouped according to the T-stage of the tumour ($P < 0.05$) (Figure 22 B). Dividing the patients into subgroups according to clinical Staging indicated a nearly equal significant increase in CCL20 mRNA expression in both subgroups: Stage I+II and Stage III+IV ($P < 0.05$) (Figure 22 C). Also in patients of G1/G2 and G3/G4 a significantly increased CCL20 mRNA expression was observed related to the corresponding normal tissue ($P < 0.05$). (Figure 22 D).

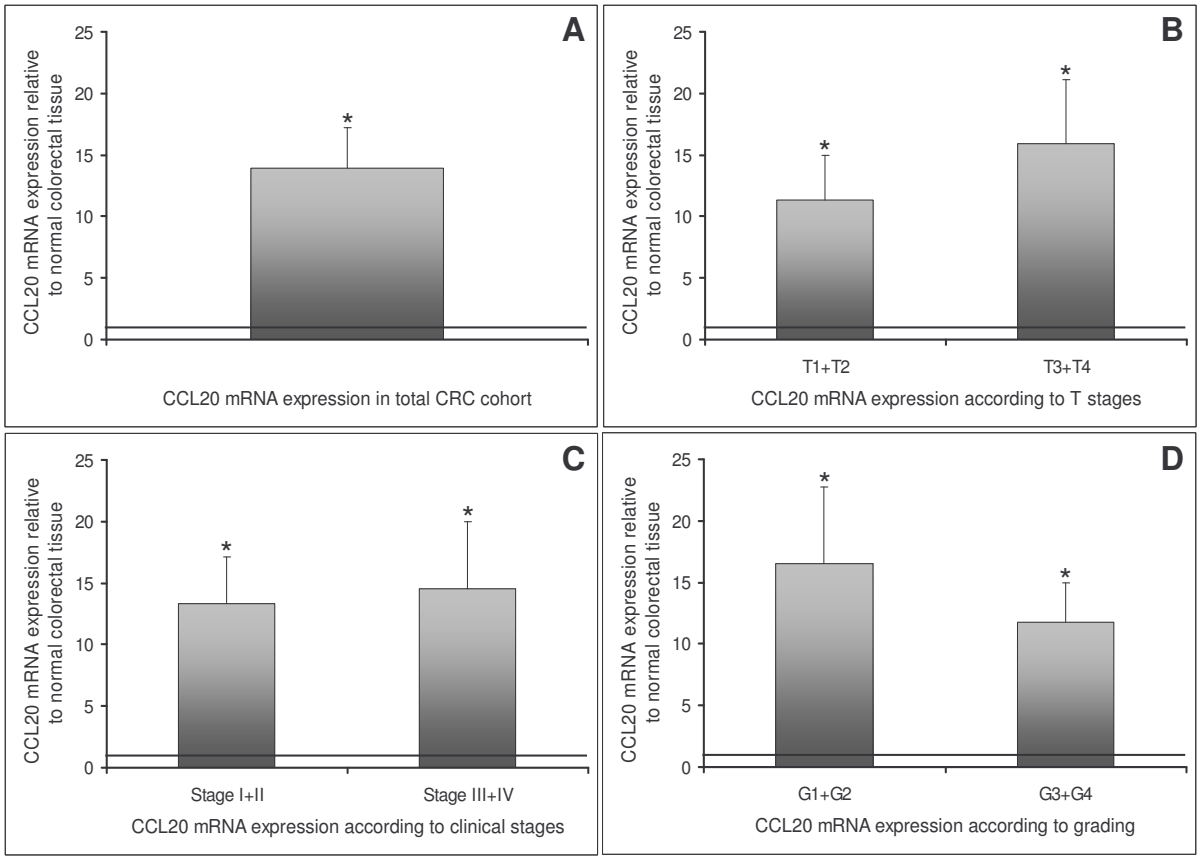

Figure 22 CCL20 mRNA expression in CRC tissue
CCL20 mRNA expression levels were analyzed by qRT-PCR and depicted as relative expression with respect to normal colorectal tissue. Data are presented as mean +/- SEM *($P < 0.05$). (A) Mean data of all investigated patients. (B) Scoring of patient data according to T-stages. (C) Scoring of patient data according to clinical staging. (D) Scoring of patients according to G-stages.

4.7.2 CCL20 protein expression analyzed by ELISA

Investigations on CCL20 protein expression were carried out on tissue samples obtained from 36 patients with CRC. Quantification of protein levels was performed applying the ELISA technique. Data were normalized to the absolute protein content analyzed by a BCA-assay. In the total CRC cohort a significant increase in CCL20 protein expression of CRC tissues was found compared to CCL20 protein expression in corresponding normal colorectal tissues ($P < 0.05$) (Figure 23 A). Likewise, significant CCL20 protein up-regulation was detected independently of T- and G–stages or clinical staging ($P < 0.05$) as presented in Figure 23 B-D

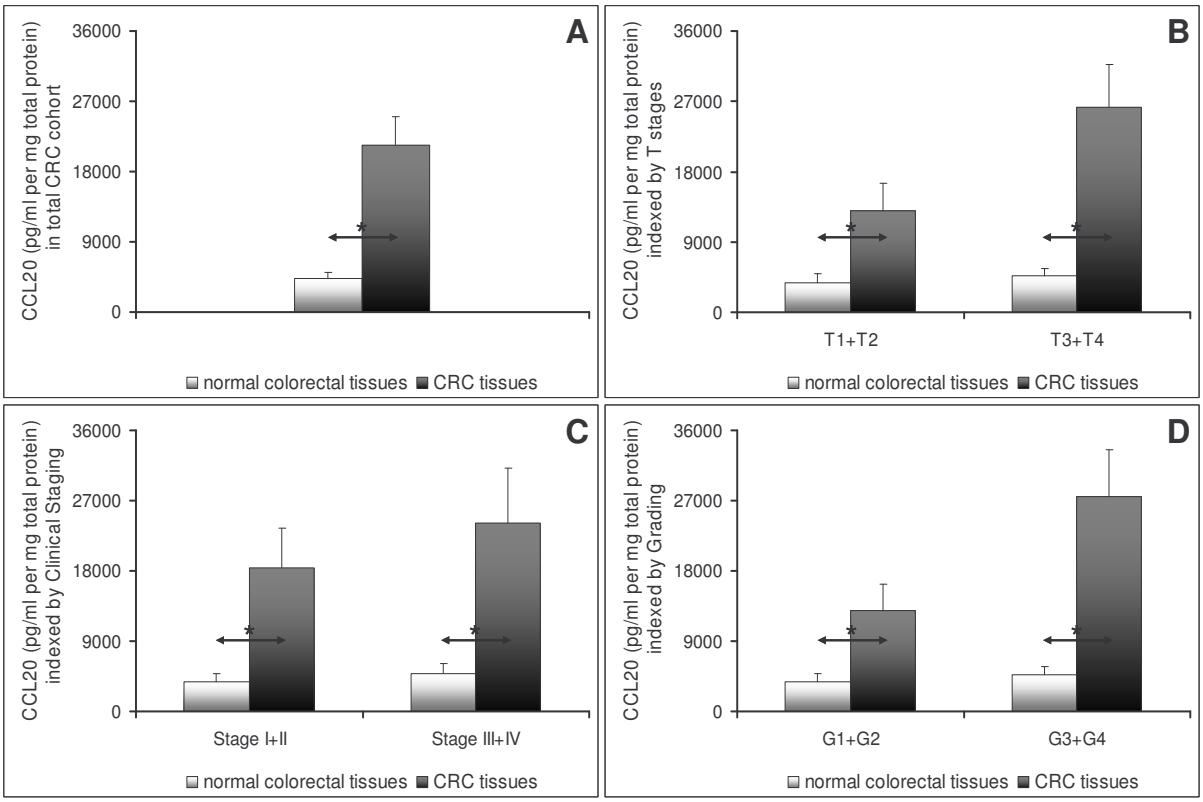

Figure 23 CCL20 protein expression in CRC tissue
CCL20 protein expression levels were analyzed by ELISA analysis and depicted as absolute CCL20 expression normalized to total protein expression. Data are presented as mean +/- SEM *($P < 0.05$). (A) Absolute CCL20 expression in CRC tissues and corresponding normal colorectal tissues of all investigated patients. (B) Scoring of patient data according to T-stages. (C) Scoring of patient data according to clinical staging. (D) Scoring of patients according to G-stages.

4.8 Expression of miRNAs in CRC tissue samples

As miRNAs regulate the expression of their target genes by direct interaction with the 3'UTR of these target genes, it may be concluded that miRNAs regulating CCL20 gene expression are down-regulated in CRC samples. Therefore the expression levels of miR-21 and miR-145 were analysed in CRC tissue samples from 46 patients using qRT-PCR with special miRNA detection assays. The expression data obtained were normalized to the expression of RNU48, which is a housekeeping gene used for miRNA analysis, and relative miRNA expression in tumour samples was depicted as n-fold expression relative to corresponding normal colorectal tissue.

4.8.1 Analysis of miR-21 in CRC using qRT-PCR

Mean miR-21 expression was significantly ($P < 0.05$) increased in the total cohort relative to corresponding non affected tissues (Figure 24 A). With respect to clinical features like T-stages, clinical staging or G-stages only late stages showed significantly up-regulated miR-21 expression ($P < 0.05$) (Figure 24 B-D).

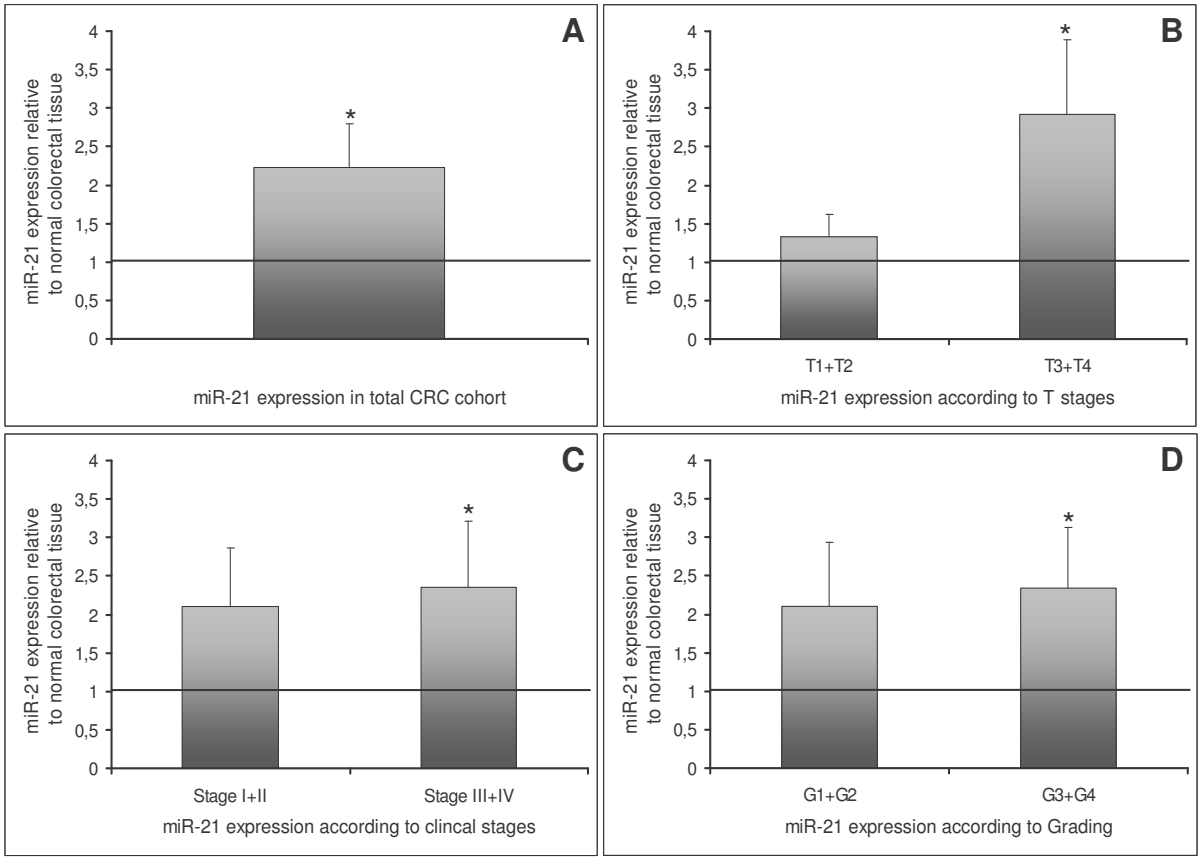

Figure 24 Expression of miR-21 in CRC tissue samples
The expression levels of miR-21 in samples from CRC patients were analyzed using qRT-PCR. miR-21 expression was normalized to RNU48 and depicted as relative expression with respect to normal colorectal tissue Expression levels are presented as mean +/- SEM *($P < 0.05$). (A) Expression of miR-21 in all investigated patient samples. (B) miR-21 expression according to T-stage of tumour. (C) Expression according to clinical staging. (D) Expression of miR-21 according to G-stage of tumour.

4.8.2 Analysis of miR-145 in CRC using qRT-PCR

Also gene expression of miR-145 was analyzed in CRC tissue samples by qRT-PCR resulting in significant down-regulation in the total CRC panel related to corresponding normal tissue ($P < 0.05$) (Figure 25 A). Subdivision of tumour samples

according to T-stages, clinical staging and grading resulted in significantly decreased miR-145 expression in all subgroups ($P < 0.05$) (Figure 25 B-D).

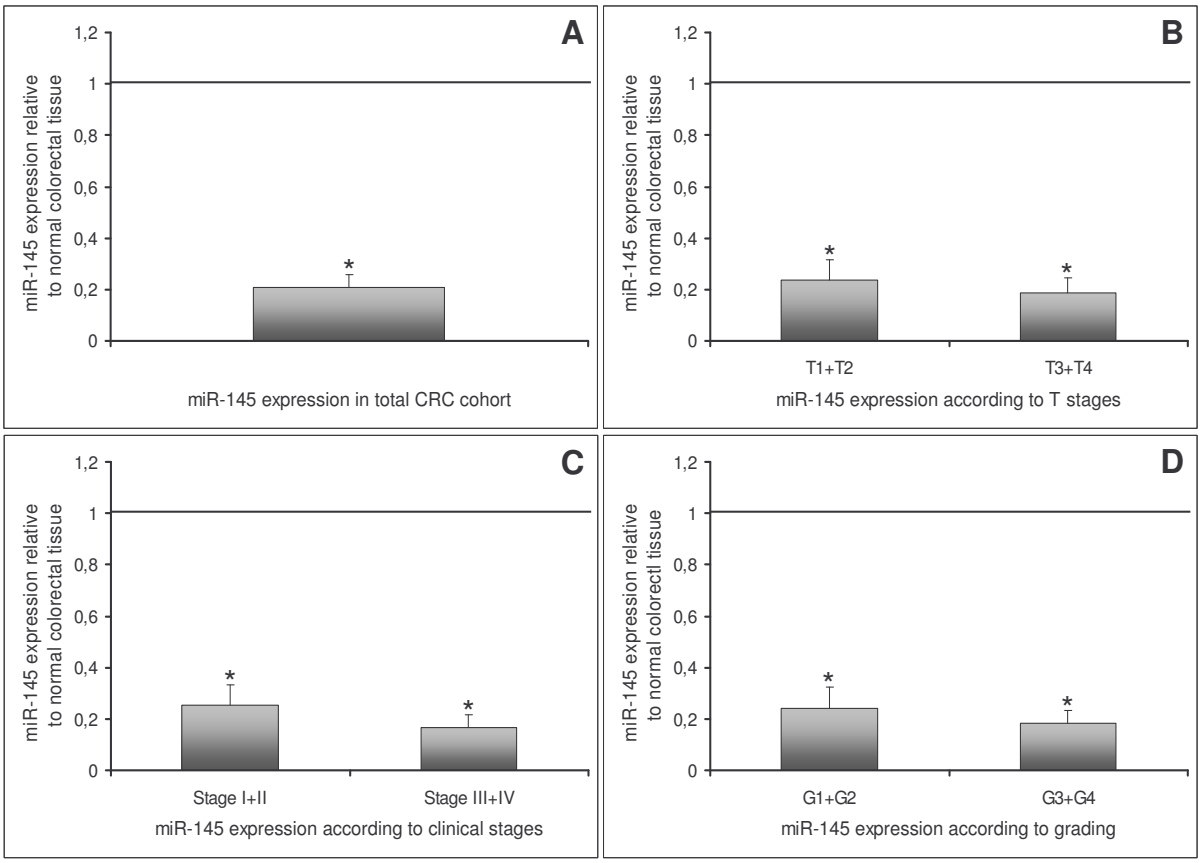

Figure 25 Expression of miR-145 in CRC tissue samples
The expression levels of miR-145 in CRC tissue samples were analyzed using qRT-PCR. The expression of miR-145 was normalized to the endogenous control RNU48 and is presented relative to the expression in normal colorectal tissue. Data are presented as mean +/- SEM *($P < 0.05$). (A) Relative expression of all analyzed patients. (B) Expression according to T-stage of tumour. (C) Expression according to Staging of tumour. (D) Expression for groups with different G-stage.

4.9 Tissue localization of CCL20, miR-21 and miR-145 expression

If a functional interaction of a miRNA with a target gene takes place, an inverse expression correlation of the respective miRNA and target gene would be expected. With respect to previous results concerning functional miR-21/CCL20 interactions an antidromic expression pattern in CRC tissues was expected. However, gene expression analysis revealed that the expressions of miR-21 and the target gene CCL20 were both significantly up-regulated in CRC tissue samples. In contrast,

expression of miR-145 was shown to be significantly down-regulated. Thus, the next step aimed to investigate if the expressions of miR-21 and miR-145 as well as the target gene CCL20 were localized to the same cell type or even localized to the same cells within the CRC tissues.

4.9.1 Localization of CCL20 expression using IHC

Immunohistochemical presentation of normal colonic tissue samples of CRC patients (n=8) illustrated that CCL20 is primarily expressed in perinuclear regions of mucosal epithelial cells as shown in Figure 26 A. In addition, positive CCL20 signals were found in mesenchymal cells (Figure 26 A). In 7 out of 8 samples under investigation the tumour was negative for CCL20 reactivity. In these cases predominantly infiltrating immune cells like macrophages or lymphocytes in the microenvironment of the tumour showed CCL20 associated signalling (Figure 26 B). However, in one case a positive reactivity against CCL20 was also observed in adenocarcinoma cells of a CRC tumour sample (Figure 26 C). Also this specimen exhibited CCL20 associated signalling in infiltrating immune cells (Figure 26 C). Therefore, it may be speculated that in the majority of patients under investigation the significantly increased CCL20 expression was due to an increase in mesenchymal cells associated with infiltration of immune cells like macrophages or lymphocytes to the site of the tumour.

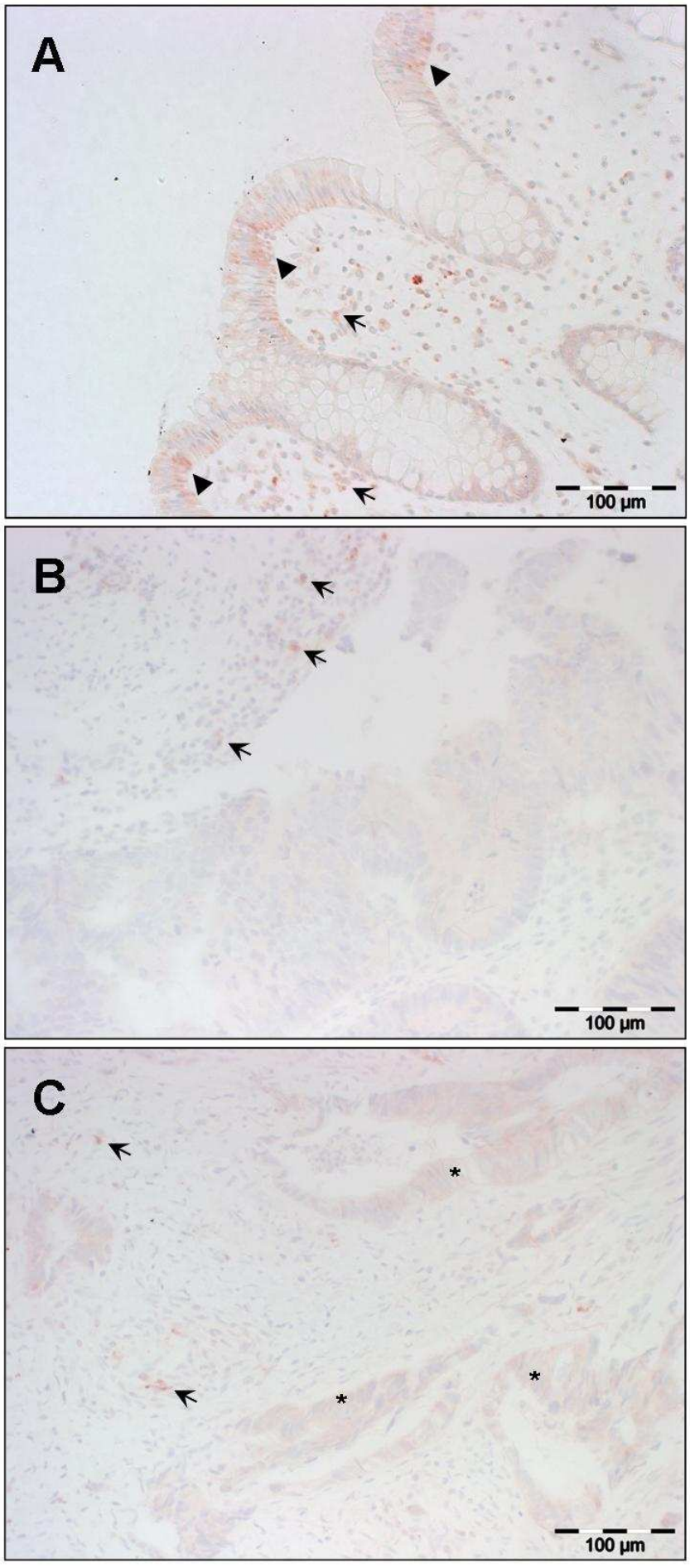

Figure 26 Localization of CCL20 protein expression in CRC
Representative specimens (thickness: ~4µm) of the regular mucosa as well as of adenocarcinomas of the colon and rectum, were probed with antibodies against CCL20 (15 µg/ml; ABC method). [A] Perinuclear regions of mucosal epithelial cells (arrowheads) and some mesenchymal cells (arrows) revealed immunoreactivity for CCL20. [B] Most CRC specimens displayed immunoreactive signals for CCL20 only in mesenchymal parts (arrows) while no anti-CCL20 signals were observed in adenocarcinoma cells while one specimen [C] showed immunoreactivity for CCL20 in both adenocarcinoma cells (black asterisks) and mesenchymal elements (black arrows).

4.9.2 Localization of miRNA expression using In Situ hybridization

For localizing miRNAs in CRC tissues ISH was employed on FFPE biopsies of approximately 1 mm diameter. As larger embedded tissue specimens (up to 1.5 cm) have shown positive in situ reactivity only in the outer layers of the sections and not in the center of the sections, only biopsies were applied for ISH experiments. Since this method has not been applied in our laboratory, ISH concerning miR-21 and miR-145 has been established in the frame of this thesis. In the course of such establishing experiments subsequent optimization steps relating to changes in hybridization temperature and probe concentration were employed until an optimized hybridization result was achieved.

A probe complementary to U6snRNA was used as a positive control and a scrambled probe not known as being complementary to any endogeneous miRNA was applied as a negative control. U6snRNA is a small nucleic acid present in abundancy in all cells and therefore easy to detect. As the scrambled probe is not complementary to any known nucleic acid in the cells this probe is not expected to produce any signal. Accordingly, no signal was detected in the negative controls (Figure 27 A), while ISH with the probe for U6snRNA resulted in a positive reactivity in nearly all nuclei without substantial background reactivity (Figure 27 B).

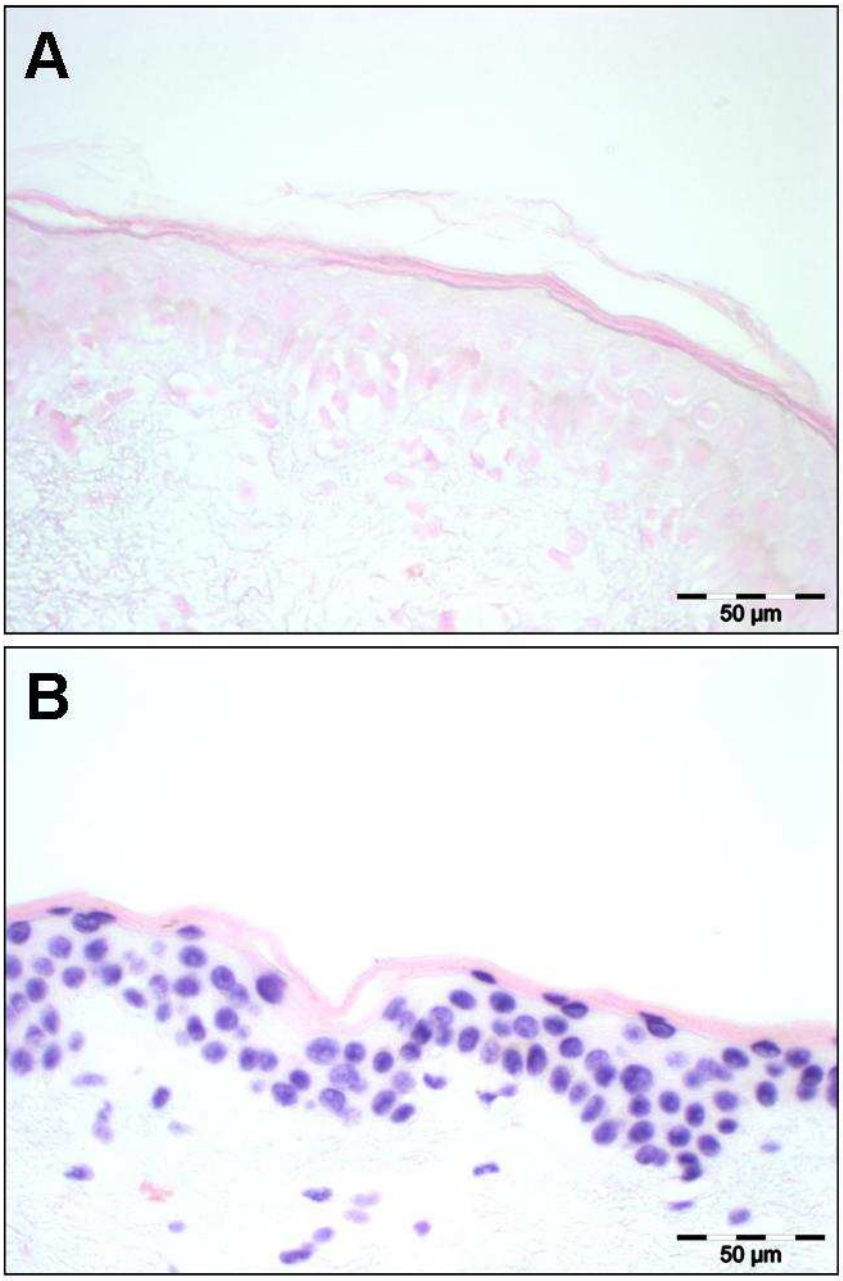

Figure 27 In situ hybridisation controls
Sections of formalin fixed paraffin embedded colorectal tissue were stained with [A] a scrambled probe (25 nM) as negative control and [B] a probe complementary to the U6snRNA (1 nanoMolar) present in the nucleus of all cells. Hybridization temperature was 57°C in both cases. Both probes were double DIG labeled and colour reaction was performed by alkaline phosphatase. Tissue was counterstained with nuclear fast red.

4.9.2.1 Localization of miR-21 expression in CRC samples

As preliminary tests concerning thickness of sections revealed inferior results for sections with 4 µm, FFPE biopsy samples with a thickness of 6 µm were applied for all further experiments. Optimal performance of miR-21 probes was achieved at a hybridization temperature of 57°C and a probe concentration of 150 nM where positive colour reaction without substantial background reactivity was detected.

Positive reactivity with miR-21 probes was restricted to mesenchymal areas in the microenvironment of CRC tissues (Figure 28 A). Therefore, predominantly fibroblasts exhibit a considerable amount of staining in their cytoplasmic compartments whereas lymphocytes and macrophages exhibit miR-21 staining to a minor extent (Figure 28 B). However, tumour cells of CRC patients displayed no miR-21 reactivity indicating that miR-21 expression in tumour cells may be under the detection limit of the ISH experiments (Figure 28 A and B). Accordingly, miR-21 expression may be restricted to mesenchymal cells like tumour associated fibroblasts as well as macrophages or lymphocytes which are recruited to the site of tumour.

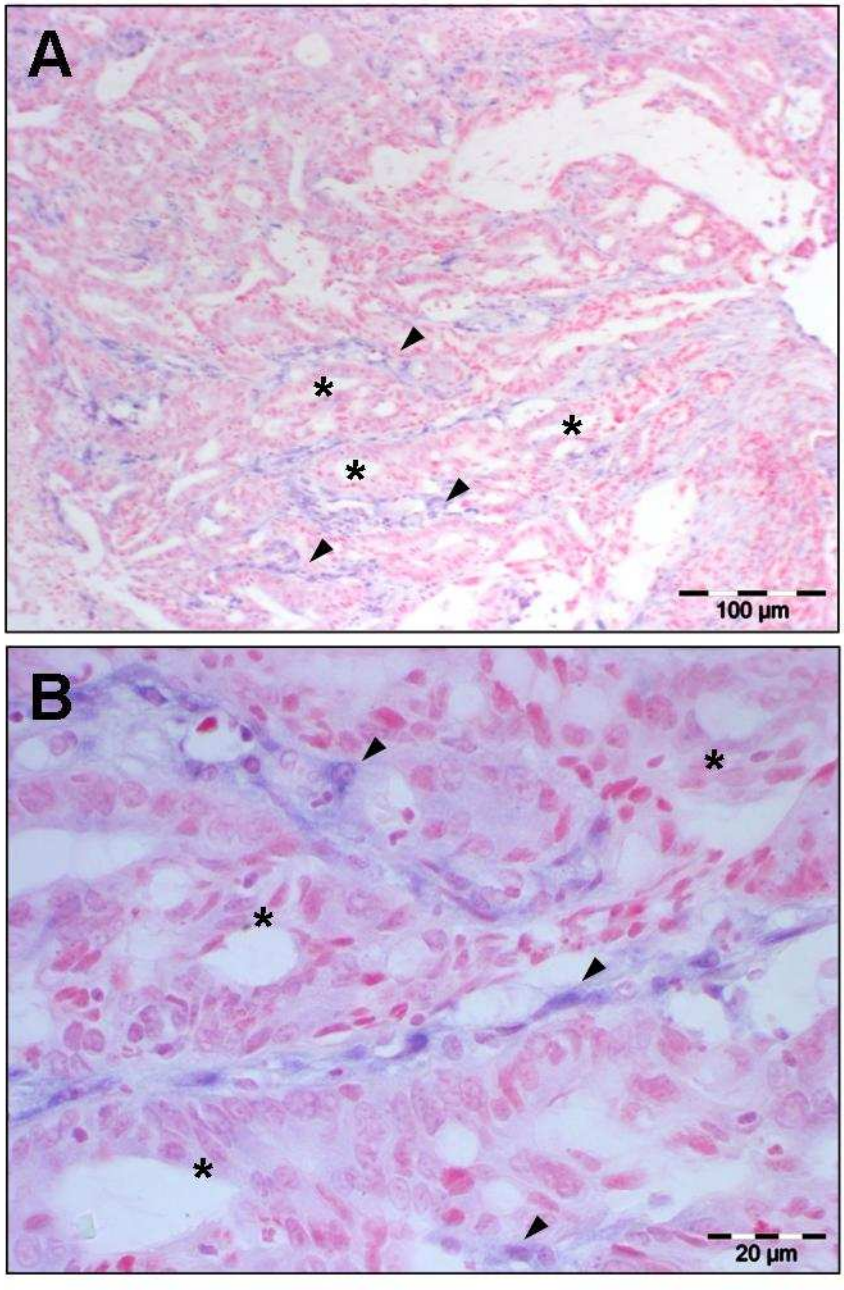

Figure 28 Localisation of miR-21 expression in CRC biopsies
In situ hybridization with a double DIG labelled probe to miR-21 (150 nM) at a hybridization temperature of 57°C. Colour reaction was performed using alkaline phosphatase. Tissue was counterstained with nuclear fast red. Arrowheads indicate mesenchymal areas with positive miR-21 associated signalling in the cytoplasm of stromal cells (fibroblasts, macrophages). Asterisks indicate tumour areas which are negative for miR-21 staining. [A] low magnification [B] detail.

4.9.2.2 Localization of miR-145 expression in CRC samples

Also for detecting miR-145 in CRC tissues, biopsies of 1mm diameter and 6 µm thickness were applied. ISH experiments were performed at an optimized temperature of 59°C and a probe concentration of 150 nM. ISH for miR-145 was performed at a higher temperature because lower hybridization temperatures resulted in increased background activity. Using the optimized hybridization conditions resulted in colour reactions with strong specific reactivity for miR-145 and low background. Positive reactivity for miR-145 probes was observed in mesenchymal areas in the microenvironment of CRC tumours including fibroblasts, macrophages or lymphocytes (Figure 29 A and B). In addition, smooth muscle fibres or myofibroblasts showed positive reactivity for miR-145 probes (Figure 29 A and B). In contrast, tumour cells were negative for miR-145 reactivity (Figure 29 A and B) implying that miR-145 signals detected by molecular biological methods were due to miR-145 expression in mesenchymal cells associated to the microenvironment of the tumour. It may be concluded that tumour cells do not contribute to miR-145 expression in CRC tissues of the patients under investigation.

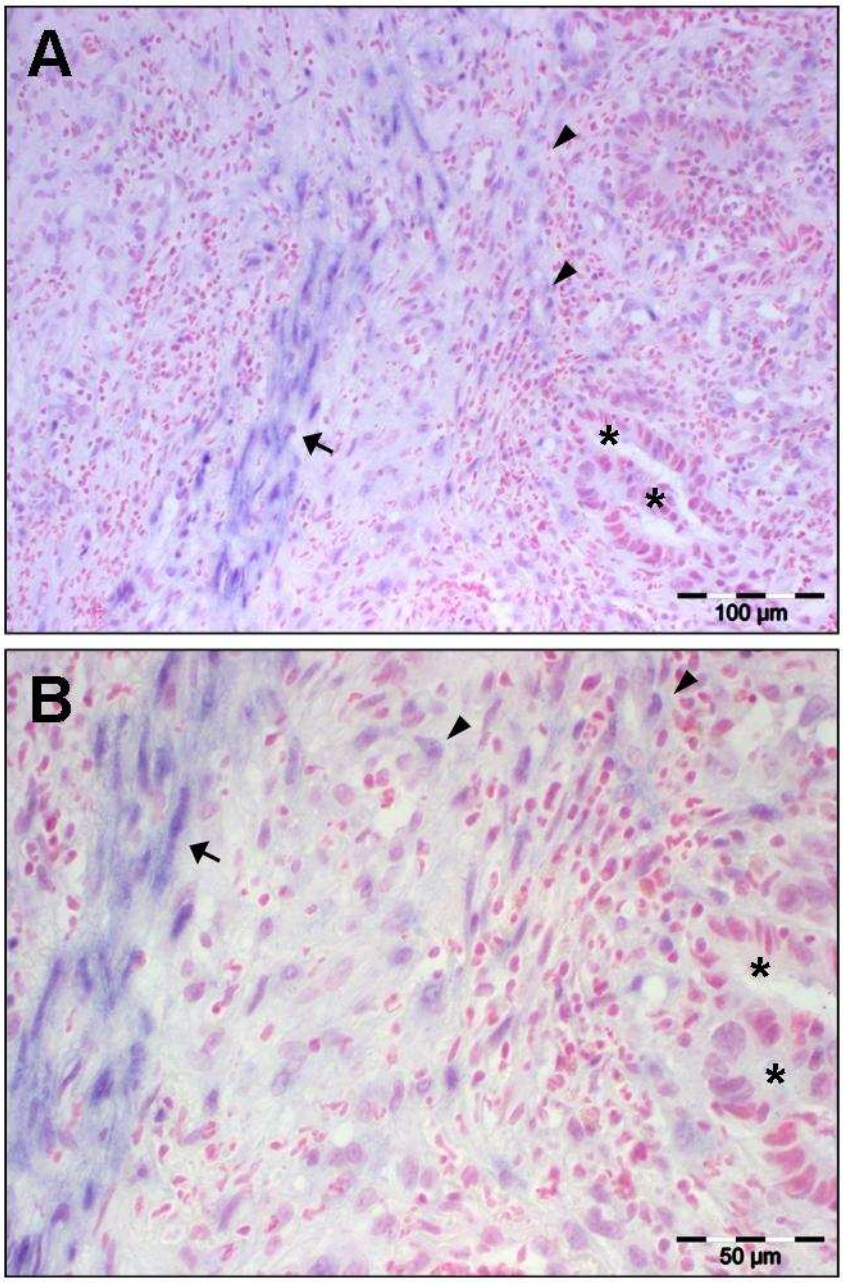

Figure 29 Localization of miR-145 expression in CRC biopsis
In situ hybridization with a double DIG labelled probe to miR-145 (150 nM) at a hybridization temperature of 59°C. Colour reaction was performed using alkaline phosphatase. Tissue was counterstained with nuclear fast red. Arrowhaeds indicate mesenchymal areas with positive miR-145 associated signalling in the cytoplasm and nucleus of stromal cells (fibroblasts, macrophages). In addition, smooth muscle fibres showed positive miR-145 staining (arrows). Asterisks indicate tumour areas which were negative for miR-145 staining. [A] low magnification [B] detail

5. Discussion

In the present thesis the functional interaction of miRNAs with chemokine CCL20 gene expression was investigated. Chemokines gained in importance in investigations on proliferation and development of cancer. Also studies conducting anti-tumoural effects of malignant tumours due to their ability to attract tumour and immune cells contributed to the importance of chemokines in recent years. Chemokine CCL20 was formerly shown to be misregulated in different cancer entities like endometrial cancer or pancreatic cancer [Wallace et al., 2011; Rubie et al., 2010]. Thereby, the role of CCL20 in cancer development is discussed controversially as there have been studies conducted indicating a proliferative role of CCL20 as well as anti-tumour effects [Wallace et al., 2011; Choi et al., 2010]. At the same time, designated miRNAs have gained in importance concerning the development and progression of cancer due to their altered expression in a wide variety of cancer types including pancreatic cancer [Bloomston et al., 2007], gastric cancer [Guo et al., 2009] as well as CRC [Arndt et al., 2009]. Accordingly, it was the first goal of this thesis to predict miRNAs that show an interaction with the expression of CCL20. After direct interactions have been investigated applying luciferase assays, precursors of the identified miRNAs were transfected in CRC cell lines in order to account for a functional effect on CCL20 gene expression in CRC cells. Furthermore, the expression of the identified miRNAs and CCL20 was analyzed in tissue samples from patients with CRC and the cellular localization of miRNAs and CCL20 expression was investigated by IHC and in ISH.

5.1 Prediction of miRNAs potentially interacting with CCL20

To date, no easy and clear convention exists for procedures applied for the experimental identification of miRNAs targeting a specific gene of interest. Most commonly a combination of computational prediction followed by experimental validation is applied [Kuhn et al., 2008]. Thereby, computer assisted analysis estimated that miRNAs control approximately more than 60 % of human coding genes on the post-transcriptional level [Friedman et al., 2009]. Nevertheless,

presently only a small part of these regulatory interactions have been approved and therefore the need for tools able to predict and validate these interactions is very important for the understanding of miRNA biology.

To date, a wide variety of strategies have been developed and used, both in *in vivo* and *in vitro* experiments, in order to identify functional targets of miRNAs for which no targets have been identified yet. Since bioinformatics provides the instruments to accumulate and handle large amounts of data and to merge diverse data sources, computer-assisted miRNA target gene interaction analysis is often applied. The theory behind those algorithms is dependent on the relative feasibility of *in vitro* experimentation as well as on the availability of statistical analysis. According to these basic principles diverse target prediction algorithms and data bases have been developed e.g. TargetScan [Grimson et al., 2007], PicTar [Krek et al., 2005], miRanda [Enright et al., 2003; Enright et al., 2005], PITA [Kertesz et al., 2007], RNA22 [Miranda et al., 2006] or DIANA-microT [Maragkakis et al., 2009]. These algorithms can be grouped into diverse categories according to the applied method: weighted dynamic programming algorithms, (e.g. miRanda) [Cormen et al., 1990], the usage of thermodynamic modelling, (*e.g.* TargetScan) [Zuker et al., 1981], Bayesian probabilistic algorithms, (*e.g.* PicTar) [Cooper et al., 1992], support vector machine based algorithms, (*e.g.* TAREF) [Shawe-Taylor et al., 2000], algorithms dealing with pattern recognition or string matching, (*e.g.* MICROINSPECTOR) [Rusinov et al., 2005] or systems that use the combination of diverse methods adapted from a retrieval engine and the use of a specific database structure, (*e.g.* MAGIA) [Raghavan, 1997]. All these target prediction tools have in common that they use various parameters, derived from the sequences of validated miRNA:target mRNA interactions or expression experiments as well as thermodynamic features, for the prediction of miRNA target sites in the 3'UTR of the mRNA under investigation. Thus, most target prediction tools used for predicting CCL20-miRNA interactions in this work, apply the following factors in different combinations and varying rating: conservation of target site, complementarity in the seed region of the miRNA, flanking sequence of the predicted target site, thermodynamic stability of the predicted interaction and the presence of compensatory 3' end binding of the miRNA (Figure 19). In addition to these features also various other features are commonly used in order to refine prediction results [Sturm et al., 2010]. However, factors independent of binding sites may influence miRNA:target mRNA interactions and thereby influence the precise

prediction of these interactions negatively. Hence, prediction can be complicated by the impact of endogenously expressed miRNAs as well as by the mode of interplay between the miRNA and target mRNA with regard to target repression by mRNA degradation or inhibition of translation [Ruike et al., 2008]. Another factor that impedes prediction of regulatory miRNA interactions constitutes cooperative binding of different miRNAs to one mRNA which has an impact on inhibition of expression [Doench et al., 2004]. Such refinement of miRNA:target gene interactions is of great importance as all target prediction programmes relate to many false positive interactions and also miss true miRNA:target mRNA interactions [Mazière et al., 2007].

To use the full spectrum of available methods and algorithms and to achieve the highest quality of prediction, in the present thesis 12 target prediction tools were applied. However, the results obtained were not consistent. Thus, eight of the applied target prediction tools identified miR-21 and five tools identified miR-145 as a potential regulator of CCL20 gene expression. Using combinations of features for their prediction of targets, may explain why not all of these programmes predict the same miRNAs. The features building the base of the prediction programmes include seed complementarity, compensatory 3' end binding, site conservation, thermodynamic features and flanking sequences of the target site. The different programmes employ these features in different combinations and weighting which may explain the prediction of varying miRNA interactions predicted by the different programmes. Notably, target prediction programmes using no seed complementarity or not using seed complementarity as the primary feature for their predictions predicted different target sites with respect to programmes which apply seed complementarity as a primary feature. Therefore, application of programmes using seed complementarity not as the first step in prediction may advance the search for miRNA target gene interactions by the detection of additional miRNA target sites.

5.2 Validation of direct miRNA:target gene interactions

Due to the fact that the miRNA target prediction programmes do not work with high accuracy, predicted interactions have to be validated experimentally. To date, different experimental methods for the validation of regulatory interactions between

miRNAs and a potential target mRNA are available. These experimental methods comprise gene silencing [Meng et al., 2007], co-immunoprecipitation assays [Boll et al., 2012] and reporter gene assays, e.g. luciferase assays [Zhu et al., 2007]. The latter two assays are used to validate direct interactions between miRNAs and a potential target mRNA whereas gene silencing experiments also identify indirect interactions. As the luciferase assay is one of the most common methods for validating direct miRNA:target mRNA interactions, in the present thesis this assay was applied to identify direct interactions of the predicted miRNAs, miR-21 and miR-145, respectively, with target gene CCL20. To identify a precise binding site for miR-21 in the 3'UTR of CCL20, the luciferase assay was applied in combination with site directed mutagenesis of the predicted target site [Brennecke et al., 2005; Yang et al., 2008].

As demonstrated in this thesis miR-21 regulates luciferase expression in a reporter construct containing the 3'UTR of CCL20 mRNA directly downstream of the luciferase gene. In contrast, miR-145 showed no effect on luciferase activity in this assay. These results were obtained in two different cell lines, 293T and HT29, respectively. However, down-regulation of luciferase upon miR-21 addition was more prominent in 293T cells compared to HT29 cells. This may be explained by the fact that the two cell lines express different sets of miRNAs. Thereby, the expression levels of miRNAs binding cooperatively with miR-21 to the 3'UTR of CCL20 may impact the luciferase expression level [Doench et al., 2004]. Therefore, the results indicate that miR-21 regulates CCL20 gene expression by an interaction with a regulatory binding site in the 3'UTR of CCL20. Validation of the precise binding site was achieved by mutation of the target site predicted by most of the prediction programmes. However, this mutation could not totally inhibit the regulatory effect of miR-21 on luciferase expression/activity as shown by luciferase assays performed with the mutated construct. These findings may be explained by additional binding sites predicted by individual target prediction programmes (*StarMir* {172-179} and *RNA22* {189-195}) or further miR-21 target sites not identified by any of the prediction programmes. Some studies report that mutating one of several binding sites does not result in a total reversion of miRNA mediated regulation of gene expression [Otsubo et al., 2011; Szczyrba et al., 2010]. Nevertheless, it is not clear if the target sites predicted by *StarMir* and *RNA22* are functional ones. Therefore, the mutation of all additional functional target sites of miR-21 in the 3'UTR of CCL20 mRNA would be

likely to completely inhibit miR-21 associated expression inhibition. Another explanation for the incomplete reversion of miR-21 down-regulation of CCL20 expression suggests that the mutation of two nucleotides in the seed region in the 3'UTR of CCL20 mRNA may be insufficient to totally circumvent the regulatory interaction of miR-21 with the 3'UTR. Nevertheless, some studies report that mutating 3-5 nucleotides in the seed region is sufficient to circumvent regulatory interactions of miRNA with target mRNAs [Yi et al., 2010]. Therefore, the mutation of more nucleotides in the binding region of miR-21 or total deletion of the binding region may result in complete inhibition of miRNA:target mRNA interactions and thereby impede miRNA mediated expression regulation. Such an effect was characterized for the interaction of miR-27b with the mRNA of PPARγ [Jennewein et al., 2010]. The aim of the present study was defined to identify functional, regulatory interactions between miRNAs and CCL20 mRNA and not to characterize miRNA binding sites. Thus, lack of complete reversion of repression by mutation is not an essential feature for the conclusion that only miR-21 may interact with CCL20. If miR-21 interactions with CCL20 are aimed to be applied for cancer therapy, further studies will be needed for more accurate characterization of the respective interaction and binding sites.

5.3 Quality of target prediction related to validated interactions

The luciferase assay constitutes a common method for the validation of miRNA targets [Nicolas, 2011; Zhu et al., 2007]. Thus, the luciferase assay results presented in this thesis have shown that the features "flanking contribution" and "complementary 3' end binding" performed best in target prediction. Nevertheless, this finding seemed to be due to the fact that these features were used more seldom than others and only in combination with other features. Therefore "flanking contribution" and "compensatory 3' end binding" rather played a role in refinement of target prediction. In addition target prediction tools which applied the combination of more different features had a better performance than tools which applied less features. An exception of this observation is *StarMir* which used only a single feature (thermodynamics) and resulted in correct prediction of both investigated

miRNAs in the present thesis. Besides the applied features also the difference in the prediction mechanism/algorithms used by the different tools could be a reason for prediction performance.

Therefore, online available target prediction tools are to date only helpful to decrease the number of potential miRNAs which interact with CCL20 gene expression. However, these programmes also indicate false-positive as well as false-negative miRNA target gene interactions. Due to these limitations in computer miRNA target gene prediction, it is still essentially required to experimentally validate the predicted interactions.

5.4 Transfection of eukaryotic cells with pre-miRNA precursors miR-21 or miR-145

In CRC cell lines SW480, SW620 and kidney cell line 293T artificial over-expression of miR-21 by transfection of miR-21 precursor molecules resulted in significantly decreased expression of CCL20 on the mRNA level. In the other two CRC cell lines Caco-2 and HT29 no decrease in CCL20 expression was found on mRNA level. These results suggest that CCL20 expression is down-regulated by miR-21 due to degradation of the respective mRNA in two CRC cell lines and one kidney cell line.

Degradation of mRNA is one common mechanism used by miRNAs to decrease target gene repression as reported by different studies [Kazenwadel et al., 2010; Jennewein et al., 2010]. However, another common regulatory mechanism applied by miRNAs is the inhibition of translation. Protein expression of the target gene constitutes a more suitable indication for functional interaction of miRNAs with the target mRNA [Borchert et al., 2011]. Upon transfection with miR-21 CCL20 expression was significantly down-regulated in three of five cell lines, namely Caco-2, SW480 and SW620, on the protein level. Contrarily, in cell line HT29 CCL20 expression was not altered on the protein level and in 293T cells the expression of CCL20 protein was not high enough to be measured in untransfected cells as well as in miRNA transfected cells. These findings may indicate that the repression mechanism which is responsible for down-regulation of CCL20 in the non-metastatic CRC cell line Caco-2 is different from the regulation mechanism in the metastatic

CRC cell lines SW480 and SW620. As miRNAs have the capability to inhibit the expression of their target genes either by mRNA degradation or translational repression, both mechanisms may contribute to decreased target protein expression. Thus, deciding which mechanism to apply depends on the degree of complementarity between the miRNA sequence and the target sequence in the 3'UTR of the target mRNA [Huntzinger et al., 2011]. A high degree of complementarity between the sequences results in degradation of the target mRNA [Hutvágner et al., 2002] whereas a low degree of complementarity leads to inhibition of target mRNA translation [Sun et al., 2010]. According to this correlation it may be assumed that a mutation in the target site for miR-21 in the 3'UTR of CCL20 mRNA has the potential to change the regulatory mechanism from mRNA degradation to repression of translation or totally avert a regulatory interaction. As cancer is characterized by genomic instability the feasibility for mutations is increased in cancer cell lines [Hoeijmakers, 2001] and mutations in the target sites of miRNAs were already shown to be associated with diseases [Hughes et al., 2011]. Therefore, such mutations may constitute a potential explanation for the fact that in Caco-2 cells CCL20 expression is down-regulated only on the protein level and not on the mRNA level in contrast to SW480 and SW620 cells and that no regulatory effect was visible in cell line HT29. To analyze possible causes for such differential regulatory interactions, the 3'UTR of CCL20 may be sequenced in different CRC cell lines so that putative mutations in the binding site of miR-21 may be detected.

As expected, transfection of the various cell lines with precursors of miR-145 resulted in no alterations in CCL20 expression neither on mRNA level nor on protein level.

Therefore, the transfection experiments confirmed the results obtained by the luciferase assays. However, functional interaction between miR-21 and CCL20 expression was observed in the CRC cell lines and it may be speculated that the mode of interaction varies between certain cell lines

5.5 Expression of CCL20 in CRC

CCL20 expression is primarily found in mucosal-associated lymphoid tissues including liver, lung, peripheral lymph nodes, thymus, tonsils, colon and appendix. Also cells of the immune system like B-cells, dendritic cells, granulocytes,

macrophages or T-cells express and secrete CCL20 [Schutyser et al., 2000]. Chemokines were shown to be involved in different pro-tumour associated processes including tumour growth [Bandapalli et al., 2012; Owen et al., 1997], oncogenic transformation [Burger et al., 1999], angiogenesis [Gabellini et al., 1999] or metastasis [Müller et al., 2001] but also in contra-tumour associated processes like attraction of activated immune cells [Lavergne et al., 2004; Huang et al., 2004]. Therefore, the expression of chemokines in tumours and the microenvironment of tumours is important for the progression of cancerous lesions. Thus, CCL20 expression was investigated in CRC by analyzing CCL20 expression patterns in tumour-free and tumour-infiltrated colorectal tissue samples on mRNA and protein level. The results of the expression analysis of the present thesis have shown that CCL20 gene expression is significantly increased in tumour-infiltrated colorectal tissues related to corresponding normal tumour-free tissue samples. Also a study by McLean et al. demonstrated CCL20 up-regulation in adenocarcinoma of the colonic mucosa compared to normal unaffected colonic mucosa. This up-regulation was also found in adenoma and proposed to be due to the inflammatory microenvironment of the tumour [McLean et al., 2011]. In contrast, Brand et al. demonstrated ambiguously expressed CCL20 expression in CRC based on the observation that CCL20 expression was significantly down-regulated or lost in ~60 % of CRC samples indicated by semiquantitative IHC [Brand et al., 2006]. Nevertheless, the methods used in the present study are more sensitive for detecting differences in gene expression on the mRNA and protein level in contrast to the methods applied by Brand et al. Several other studies also reported significant up-regulation of CCL20 expression in inflammatory diseases of the colon and rectum [Kwon et al., 2002] which also constitute risk factors for CRC. Therefore, it may be speculated if CCL20 expression could be associated with the induction of CRC and correlated with early stages of CRC. In order to investigate such a correlation, patient samples were investigated according to T-stage and clinical staging. While no significant differences in CCL20 expression were found between tumours of different clinicopathological features, Brand et al. made the observation that tumours of higher TNM stage exhibit significantly lower CCL20 expression as measured by semiquantitative immunohistochemical analysis [Brand et al., 2006]. In addition sorting samples according to tumour grading showed no significant differences of CCL20 protein expression between tumours of low grade (G1+G2) and tumours of high grade

(G3+G4). This in turn indicates, that the differentiation state of the tumour does not influence the expression of CCL20 neither on the mRNA nor on the protein level.

Since CCL20 was shown to be significantly over-expressed in CRC tissues compared to corresponding normal tissues, it would be a scientifically interesting question if this over-expression promotes or inhibits tumour growth and metastasis. To date, only few studies are available which investigated CCL20 expression in CRC and their results are contradictory. Thus, it was shown that CCL20 has the potential to induce ERK1/2 and SAPK/JNK kinases and the Akt pathway by signalling via CCR6 thereby stimulating proliferation and migration of CRC cells (SW480) [Brand et al., 2006]. Both mechanisms are also characteristic for tumour progression and metastasis. In addition, increased CCL20 levels in the microenvironment of CRC tumours were shown to be associated with recruitment of regulatory T-cells which results in an increase in tumour growth and a reduction of anti-tumour immunity [Liu et al., 2011a; Clarke et al., 2006]. In contrast, in a mouse model it was found that increased expression of CCL20 mediated by the therapeutic rofecoxib may increase immune response towards the tumour by attraction of dendritic cells, a mechanism known to be involved in the reduction of tumour immune escape [Walmesley et al., 2007]. Besides these mechanisms which directly influence the primary tumour it was also reported that the CCL20/CCR6 axis is involved in organ specific (liver) metastasis of CRC [Rubie et al., 2006c; Ghadjar et al., 2006].

Significantly altered CCL20 gene expression was not only observed in CRC but also in various other cancer types. In HCC over-expression of CCL20 is associated with increased infiltration of regulatory T-cells which in turn are linked to worse prognosis [Chen et al., 2011]. Additionally, CCL20 induced growth of hepatocellular cell line Huh7 by signalling via the ERK1/2 pathway [Fujii et al., 2004]. Previous works of our group have demonstrated significant up-regulation of CCL20 in HCC tissues which correlate with tumour grade suggesting that the CCL20/CCR6 system may be involved in hepatocarcinogenesis [Rubie et al., 2006a]. Moreover, high level expression of CCL20 and CCR6 were also observed in CRLM with marked up-regulation of CCL20 expression in CRLM in relation to HCC tissues [Rubie et al., 2006b]. These results may indicate involvement of the CCL20/CCR6 axis in carcinogenesis and progression of hepatic malignancies. In addition, our group identified significant over-expression of CCL20 in pancreatic cancer [Rubie et al., 2010]. Recently, endometrial cancers were shown to over-express CCL20 and CCR6

compared to unaffected endometrial tissues and CCL20 was demonstrated to induce proliferation in endometrial cancer cell lines [Wallace et al., 2011]. While CCL20 expression was shown to be related to proliferation, some studies also associated CCL20 expression with invasion and migration in nasopharyngeal and pancreatic cancer [Chang et al., 2008; Kimsey et al., 2004]. In contrast, in renal cell carcinoma CCL20 was shown to be involved in recruitment of immature dendritic cells which can be activated by CCL19 in the tumour margin thereby mediating anti-tumour immunity [Middel et al., 2010].

Therefore, CCL20 over-expression observed in these experiments in CRC tissues was also detected in other studies as well as in investigations on tumours of different entities indicating that increased CCL20 expression is a common event in neoplasia. Nevertheless, functional investigations on CCL20 expression did not identify a clear pro- or anti-tumour activity of CCL20 as this molecule influences tumour cells as well as immune cells infiltrating the tumour thereby modulating the immune response to the tumour.

5.6 Expression of miRNAs in CRC

In recent years investigations on miRNA expression patterns were intensified and this in turn led to the identification of certain miRNA expression patterns associated with different kinds of diseases including cancer [Thai et al., 2010]. The first association between altered miRNA expression and cancer was shown by Calin et al. in 2002 [Calin et al., 2002]. Since then several studies identified characteristic miRNA expression patterns associated with various kinds of cancer entities and different states of disease progression [Cohn et al., 2010; Vaksman et al., 2011]. In this respect, down-regulation as well as up-regulation of certain miRNAs was found to be involved in cancer associated processes and therefore miRNAs can act either as tumour suppressor miRNAs or as oncomiRs according to the set of targeted genes [Volinia et al., 2006]. Also in CRC some miRNAs were found to be up-regulated while others were shown to be down-regulated and correlated to tumour development, progression and metastasis [Schetter et al., 2008]. Based on these observations the expression profiles of miR-21 and miR-145 predicted to regulate the expression of

CCL20 were analyzed in tissue samples of patients with CRC.

5.6.1 Expression of miR-21 in CRC

First, miR-21 expression was analyzed in CRC using qRT-PCR specific for this miRNA. Expression patterns among the CRC patients under investigation were divergent. Most patients showed significant over-expression of miR-21 in tumour tissues but there were also a considerable number of patients showing no altered expression or even lower expression of miR-21. These results may indicate that miR-21 over-expression is not essential for the development of CRC but may constitute a risk factor and could therefore play a role in the progression of CRC. Kulda et al. also observed increased miR-21 expression in CRC tissues compared to normal adjacent colon tissue. MiR-21 over-expression was also observed in CRLM samples, associated with shorter disease free survival and suspected to have oncogenic potential [Kulda et al., 2010]. Also another study observed miR-21 over-expression in CRC tissues compared to polyps and normal tissue. These authors also demonstrated down-regulation of the putative miR-21 target gene PDCD4 on mRNA level [Chang et al., 2011a]. In order to analyze, if miR-21 over-expression is dependent on the degree of local invasion of the investigated tumours, the samples were sorted into two groups according to the T-stage of the resected tumours. Analysis of the two groups resulted in a significant increase only in tumours of higher T-category. This may in turn indicate, that miR-21 expression is associated with increased local invasion of CRC. This is in contrast to a study by Slaby et al. which demonstrates significantly over-expressed miR-21 expression in all T-categories [Slaby et al., 2007]. Sorting patient samples by clinical stage indicated a significantly increased miR-21 expression compared to normal adjacent tissue only in the subgroup of tumours with higher clinical stages (III+IV). This is also in contrast to the study by Slaby et al. where significant over-expression of miR-21 was found in all clinical stages although tumours of higher clinical stages present highest miR-21 expression levels compared to lower clinical stages [Slaby et al., 2007]. However, the number of investigated patients per clinical stage (3, 11, 6 and 9 respectively) was relatively low in the study by Slaby et al. The findings are also in line with a study by Vickers et al., which described that miR-21 expression was associated with clinical

progression of CRC [Vickers et al., 2012]. Nevertheless, the study by Vickers et al. predominantly compared samples of patients with metastasis with samples of patients without metastasis. As samples from patients with metastasis constitute stage IV, the results of this study are not directly comparable with stage III+IV tumours analyzed in the present thesis. Also Liu and colleagues found a significant over-expression of miR-21 in CRC tumour samples compared to normal adjacent tissue with elevated miR-21 expression in stage III compared to samples of stage II [Liu et al., 2011b]. In contrast to the present study Liu et al. only included tumours of stage II or III whereas the present study compares groups comprising stage I+II or stage III+IV. Moreover, the investigated tumours were divided by their differentiation state (G stage) to investigate their impact on miR-21 expression. Accordingly, a significantly increased expression was observed only in tumours with lower differentiation stages (G3+G4) indicating that increased miR-21 expression is potentially causative or a consequence of de-differentiation of CRC tumour cells. This result is well in line with other studies of Liu et al. investigating a difference in miR-21 expression between well and moderately differentiated tumours and poorly differentiated tumours [Liu et al., 2011b]. Likewise, other studies observed an increase in miR-21 expression concurrently with decreased differentiation of the tumour [Slaby et al., 2007].

However, miR-21 up-regulation was not only reported in tissue samples of CRC patients but also in stool samples of CRC patients. As recent studies reported also significantly increased miR-21 levels in patients with adenoma but not in patients with polyps [Wu et al., 2012; Link et al., 2010], it was suggested that expression analysis of miR-21 may be used as a non-invasive screening tool for CRC. However, miR-21 expression analysis in stool samples of CRC patients may not always lead to significant differences between CRC patients and healthy persons as Li et al. reported [Li et al., 2012a].

Corresponding to findings described in literature also the results obtained in the present thesis indicate miR-21 over-expression in CRC with higher expression levels in more progressive stages. Nevertheless, further studies are required to apply miR-21 expression for a prognosis of CRC disease outcome.

MiR-21 up-regulation was not only reported in CRC patients but also in tumours of other entities. Thus, in non-small cell lung cancer expression of miR-21 was shown to be up-regulated and high miR-21 expression was also correlated with various

clinicopathological features including higher tumour stages, metastatic status, lymph node positivity and poor survival [Zhang et al., 2010; Gao et al., 2010]. Similarly, miR-21 was reported to be up-regulated in breast cancer and correlated with advanced proliferation, higher clinical stage and lymph node positivity [Huang et al., 2009; Yan et al., 2008]. Likewise, in gastric cancer miR-21 over-expression was reported to be associated with the differentiation state and metastasis [Zhang et al., 2012].

As miR-21 is over-expressed in a wide variety of tumours it may be speculated that altered miR-21 expression is a common process in the malignant transformation of cells of different tumour entities.

5.6.2 Expression of miR-145 in CRC

Also the expression of miR-145 was analyzed in tumour tissues in relation to normal adjacent tissues using quantitative real-time PCR. As the results of this thesis have shown miR-145 expression is significantly down-regulated in tumour tissues compared to corresponding normal tissues. In contrast to miR-21 expression, significantly decreased expression of miR-145 was found in most patients under investigation. This may indicate that down-regulation of miR-145 is an early event in the development of CRC. In addition, decreased expression of miR-145 in CRC was found in two other studies which reported miR-145 down-regulation in CRC cell lines [Bandrés et al., 2006; Arndt et al., 2009]. Also in a microarray study on miRNA expression in CRC it was found that miR-145 is down-regulated in relation to normal tissue [Motoyama et al., 2009]. Sorting patient samples by T-category, clinical stage or grading into two groups, respectively, indicated that down-regulation of miR-145 is not changing during progression or dedifferentiation of CRC. Similar results were obtained by Wang et al. where miR-145 expression did not correlate with any of the investigated clinicopathological features [Wang et al., 2009a]. Therefore, it may be speculated that events leading to decreased miR-145 expression take place in early stages of CRC development. This theory is fortified by another study which demonstrated that miR-145 expression is also decreased in ulcerative colitis which in turn increases expression of K-RAS, API5 and MEK-2, molecules involved in cell regulatory processes as well as in apoptosis [Pekow et al., 2012]. Apart from a

decreased miR-145 expression in tumour tissues, reduced miR-145 expression was also detected in stool samples of CRC patients where it may be applied as a non-invasive marker for colorectal neoplasia [Li et al., 2012a].

MiR-145 dysregulation is not only known to play a role in CRC pathogenesis, but also reported as a common mechanism that is involved in malignant neoplasia of different organs. Thus, in gastric cancer decreased miR-145 expression was observed by Takagi et al. [Takagi et al., 2009]. This study also reported that artificial increase of miR-145 in gastric cancer cells inhibits their proliferation. Also in prostate cancer down-regulated miR-145 expression was detected together with increased miR-145 promotor methylation in prostate cancer cells which indicated epigenetic silencing of miR-145 [Zaman et al., 2010; Suh et al., 2011]. In addition other cancer entities like esophageal cell carcinoma [Kano et al., 2010], lung cancer [Yanaihara et al., 2006] and breast cancer [Iorio et al., 2005] were reported to exhibit down-regulation of miR-145. In breast cancer cells it was also found that activation of miR-145 expression is dependent on p53 [Spizzo et al., 2010]. As p53 is a molecule regularly mutated and inactivated early in CRC development further studies will show if this pathway is responsible for miR-145 down-regulation.

Recently it was reported that miR-145 is down-regulated in CRC as well as in most other solid cancers. In some cancer types down-regulation is associated with methylation of the promoter region of miR-145 or aberrant expression of p53. Hereby, it will be important to identify the specific causes for miR-145 down-regulation in CRC development. Investigating the causes of decreased miR-145 expression in cancer is of particular interest as miR-145 is known to act as a tumour suppressor and targets of miR-145 are established oncogenes. Among these the best established target is FSCN1 which is over-expressed e.g. in bladder cancer [Chiyomaru et al., 2010]. Restoration of miR-145 expression in bladder or prostate cancer cells was reported to result in higher FSCN1 expression and decreased cell viability, proliferation, migration and invasion [Chiyomaru et al., 2010; Fuse et al., 2011]. Another known oncogenic factor down-regulated by miR-145 is FLI1 and artificial expression restoration of this factor results in decreased CRC cell proliferation [Zhang et al., 2011]. Further oncogenic molecules targeted by miR-145 in different cancer cell lines were SWAP70, p70S6K1, RTKN, JAM-A and fascin thus influencing oncogenic processes like tumour growth, invasiveness or apoptosis [Chiyomaru et al., 2011; Xu et al., 2012; Wang et al., 2009c; Götte et al., 2010].

5.7 Expression of CCL20 in tumour microenvironment detected by IHC

As tumours are heterogeneously composed, not only consisting of tumour cells but also of stromal and infiltrating immune cells, it is of great interest to detect the specific compartment where CCL20 is expressed. Hence, an antibody specific for CCL20 was used to detect CCL20 expression by IHC in CRC tissue samples as well as in unaffected colonic mucosa. In the present study it was found that CCL20 expression in CRC specimens is heterogeneously expressed with respect to tumour and microenvironment. In most colorectal tumours CCL20 expression was found in (mesenchymal) elements of the microenvironment comprising macrophages and lymphocytes. This is of particular interest as it was shown in a mouse model that over-expression of CCL20 by macrophages is involved in recruitment of CCR6 positive regulatory T-cells and tumour development [Liu et al., 2011a]. In the present thesis it was also found that CCL20 is expressed in epithelial cells of normal colorectal mucosa whereas most colorectal tumour cells do not express CCL20. This is well in line with another study that investigated CCL20 and CCR6 expression in CRC demonstrating differential expression intensities of CCL20 with respect to colorectal tumours from different patients [Brand et al., 2006]. Thus, over-expression of CCL20 in colorectal tumour cells may only be characteristic for a specific subset of colorectal tumours. Nevertheless, no clinicopathological features were reported to correlate with the expression of CCL20 in CRC. Similarly, in various cancer entities like pancreatic cancer CCL20 expression was detected in cancer cells and also in tumour associated stromal cells [Rubie et al., 2010; Kimsey et al., 2004]. Anyway most pancreatic cancer specimens exhibit CCL20 expression in malignant cells [Campbell et al., 2005]. In addition, CCL20/CCR6 expression was also described in tumour cells of hepatocellular- and prostate cancer specimens [Ghadjar et al., 2008; Rubie et al., 2006a]. As in the present study it was shown that CCL20 is preferentially expressed in mesenchymal elements in the tumour microenvironment and CCR6 was formerly shown to be expressed in CRC cells it may be presumed that a paracrine pathway may account for CCL20/CCR6 mediated regulation in CRC. These considerations are well in line with studies reporting that exogeneous administration of CCL20 enforce colorectal tumour cell growth and migration [Brand et al., 2006].

Therefore, expression of CCL20 in mesenchymal elements (e.g. macrophages) may impact CRC by two different mechanisms. The first one is recruitment of regulatory T-cells which could mediate tumour immune escape and the second is direct promotion of cancer cell proliferation and migration.

5.8 Expression of miRNAs in tumour microenvironment detected by ISH

Detection of miRNAs using ISH brought up certain problems. Using slices from FFPE tissue samples resulted in positive staining only at the outer margin of the slices. The centres of the regions showed no staining. This may partly be due to fixing and embedding procedures of the tissue samples. Thus, the interfusion time of formalin during fixation is subject to the sample size. In case of larger samples the time till the fixation dye reaches the centre is relatively long and therefore RNAses could possibly degrade mRNA and miRNA in this area which may explain the loss of miRNA signal in the centre of the tissue samples. This theory is supported by the fact that using FFPE biopsies which are far smaller in diameter showed a continuous and consistent staining of the complete tissue slice. In addition to problems with fixation interfusion in a study based on mouse tissue it was shown that fixation of samples with formaldehyde leaded to loss of miRNAs in tissue sections [Pena et al., 2009]. Also another study reported negatively on formalin fixation for ISH experiments [Yan et al., 2010]. Nevertheless, some studies based on FFPE tissue slices showed a uniformly and consistent staining [Hansen et al., 2012; Jørgensen et al., 2010]. Hence, appropriate fixation and embedding procedures are critical for the preservation of miRNAs in FFPE tissue samples.

5.8.1 Expression of miR-21 in tumour microenvironment

As tumours are heterogeneously composed and contain not only malignant but also stromal and other cells like infiltrating immune cells, in situ detection of miR-21 was

performed in order to examine the distribution of miR-21 expressing cells in CRC tissue sections. As reported in the present thesis miR-21 expression is primarily found in tumour associated mesenchymal/stromal compartments (e.g. fibroblast like cells) but not in tumour cells. This result is well in line with a recent study by Nielsen et al. which indicated that miR-21 expression is predominantly found in fibroblast-like cells in the stroma. In contrast to the present study only tumours of stage II were included in this study [Nielsen et al., 2011]. Nevertheless, it was also reported that miR-21 expression in CRC is found not only in the tumour associated fibroblasts in the stroma but also directly in tumour cells [Yamamichi et al., 2009]. However, recently miR-21 expression was reported to be exclusively expressed in colonic epithelial cells within the tumour of CRC patients [Schetter et al., 2008]. While various studies investigating the localization of miR-21 expression in CRC reported rather heterogeneous results, the findings of this thesis support miR-21 expression exclusively in the tumour microenvironment. Also studies in different tumour entities reported heterogeneous miR-21 localization. Whereas tumours within colon and breast exhibit miR-21 expression primarily in tumour-associated fibroblasts, tumours of pancreas, prostate and lung were shown to express miR-21 directly in tumour cells [Sempere et al., 2010]. Thus, pancreatic cancer cells, [Dillhoff et al., 2008; du Rieu et al., 2010] prostate cancer cells [Li et al., 2012c] as well as fibroblast expression in breast cancer [Rask et al., 2011] show a more homogeneous expression pattern of miR-21.

5.8.2 Expression of miR-145 in tumour microenvironment

Investigating the localization of miR-145 expression this study revealed primarily miR-145 expression in mesenchymal/stromal compartments of the tumour and smooth muscle cells whereas the tumour cells showed no detectable miR-145 reactivity. In the literature miR-145 expression is described in normal colonic tissue primarily in colonocytes of the crypts. In contrast, CRC tissues are reported to express reduced miR-145 levels [Schepeler et al., 2008]. Recently another study demonstrated that miR-145 expression is also down-regulated in colonocytes of the crypts in ulcerative colitis [Pekow et al., 2012]. Thus, altered miR-145 expression might play a role in inflammation induced CRC development. As shown in the present

study, miR-145 is present in smooth muscle cells, whereas miR-145 expression analysis in lung cancer revealed significantly reduced miR-145 expression in smooth muscle cells, the main miR-145 expressing cells in normal lung tissue [Liu et al., 2009]. In addition, other studies showed that in prostate and bladder cancer miR-145 is down-regulated in tumours and microenvironment including epithelial cells, connective tissue and lymphocytes [Wach et al., 2012; Dyrskjøt et al., 2009].

Although probes against miR-145 only detect mature miRNAs which are found in the cytoplasm, the findings of this thesis report miR-145 expression not only in the cytoplasm of stromal cells but also perinuclearly and to a minor extent nuclearly. Nevertheless, these findings were also present in other studies which applied the same probe against miR-145 [Wach et al., 2012; Jørgensen et al., 2010]. It was proposed that this effect is due to the fact that this probe does not discriminate between the mature form and precursor forms of miR-145. Consequently, miR-145 staining may be considered as a sum signal of mature and immature forms of miR-145.

5.9 Regulatory interactions of miRNAs with CCL20 in CRC

In the present thesis it was demonstrated that both miR-21 and its functional target chemokine CCL20 exhibit significantly up-regulated expression in CRC tissue samples compared to normal non affected colorectal tissue. Initially, this result may be conflicting with the presumption that miRNAs and their target genes are expressed in an inverse manner, as reported in different studies [Xiao et al., 2012]. This conflict was elucidated by the finding that miR-21 as well as CCL20 is rarely expressed in CRC cells but predominantly in stromal compartments of colorectal tumours. In the stromal microenvironment miR-21 was predominantly found in fibroblast-like cells whereas CCL20 expression was primarily detected in infiltrating immune cells, like macrophages and lymphocytes. Hence, the main part of expression of miR-21 and CCL20 detected by molecular biological methods potentially does not exist in the same cells/cell types and therefore miR-21 and CCL20 in CRC do not interfere with each other. To further analyse this assumption, it would be recommendable to perform double staining experiments with miR-21 and

CCL20 on the same section. However, in this thesis separate expression analyses were performed for miR-21 and CCL20. Thus, the observations presented in this thesis indicate that over-expression of a miRNA and its functional target gene in cancerous tissues is possibly due to expression of both molecules in different types of cells. However, tumour tissues consist not only of tumour cells but also of tumour stroma constituted of different cell types which may influence the outcome of molecular biological experiments.

As it was demonstrated in the present thesis that miR-145 does not regulate the expression of CCL20 we expected that the expression patterns of miR-145 and CCL20 in CRC tissues are not associated with each other. Expression of miR-145 was found predominantly in smooth muscle cells or myofibroblasts along with other cell types in the tumour microenvironment. It was previously shown that miR-145 is highly expressed in smooth muscle cells [Cheng et al., 2009]. Thus, miR-145 and CCL20 are likely to be expressed by different cell types.

5.10 Conclusion and perspective

The present thesis describes for the first time that the chemokine CCL20 is a target gene of miR-21 and artificial over-expression of miR-21 has the potential to suppress the expression of CCL20 in CRC cell lines [Vicinus et al., 2012]. Nevertheless this observation was not true for all investigated CRC cell lines which implicates the assumption that this interaction is fragile in CRC cell lines. Furthermore it was demonstrated that both CCL20 and miR-21 exhibit significant over-expression in CRC tissues whereas miR-145 exhibits significantly decreased expression in colorectal tumours compared to normal colorectal tissue. This in turn indicates that these molecules may be potentially involved in CRC associated processes.

Thus, CCL20 and miR-145 showed significantly altered expression profiles in early as well as in late stages of CRC in comparison to corresponding normal tissues implicating a role in tumour development. The miRNA miR-21 was found to be primarily over-expressed in tumours of higher T-stages (T3+T4), higher clinical stages (III+IV) and lower differentiation stages (G3+G4) which implies that altered miR-21 expression may be causative for or a result of CRC progression.

Furthermore, it was shown for the first time that CCL20 is predominantly expressed in the tumour microenvironment but not in tumour cells. Over-expression of CCL20 as indicated by molecular biological experiments therefore may be due to CCL20 expression in lymphocytes and macrophages infiltrating the tumour. Also for miR-21 exclusive expression was found in the tumour microenvironment predominantly in tumour associated fibroblast-like cells. It may be speculated that miR-21 expression is induced in these cells by CRC cells or other cell types in the microenvironment. Expression of miR-145 was primarily found in smooth muscle cells in mesenchymal compartments of the tumour but not in tumour cells.

Over-expression and involvement of CCL20 was shown in CRC as well as in different other cancer entities [Rubie et al., 2006b; Wallace et al., 2011; Chang et al., 2008; Kimsey et al., 2004]. Recently, it was demonstrated that down-regulation of other chemokines like CCL2 in prostate cancer cells reduced tumour progression and metastasis [Shi et al., 2011]. Furthermore down-regulation of the CXCL12/CXCR4 pathway with an artificial miRNA resulted in lower invasion potential and metastasis of breast cancer cells [Liang et al., 2007b]. Hence, miR-21 mediated down-regulation of CCL20 in tumour tissues may be viewed as a potential future therapy option for CRC as well as for other CCL20 expressing cancer types. However, down-regulation of CCL20 directly in tumour cells only makes sense in tumours that express CCL20 in tumour cells. As the present thesis demonstrated that CCL20 is predominantly expressed in the microenvironment in CRC, down-regulation may be expected to take place in cells in the microenvironment. Nevertheless, miRNAs target a wide variety of genes and as long as not all regulated genes are identified, therapies which restore or block specific miRNAs are risky. Thus, mechanisms which identify miRNA-target gene interactions in a fast and reliable way are required to use the therapeutic potential of miRNAs.

6. Literature

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7. Appendix

7.1 Publications

Parts of the present thesis and results of other projects I participated in were published in the following scientific articles:

Vicinus B, Rubie C, Stegmann N, Kölsch K, Kauffels A, Ghadjar P, Wagner M, Glanemann M. (2012) miR-21 and its target gene CCL20 are both highly over-expressed in the microenvironment of CRC tumors: consequences for their regulation. *submitted*

Vicinus B, Rubie C, Faust SK, Frick VO, Ghadjar P, Wagner M, Graeber S, Schilling MK. (2012) miR-21 functionally interacts with the 3'UTR of chemokine CCL20 and down-regulates CCL20 expression in miR-21 transfected colorectal cancer cells. *Cancer Lett 316:105-112*

Rubie C, Frick VO, Ghadjar P, Wagner M, Justinger C, Faust SK, **Vicinus B**, Gräber S, Kollmar O, Schilling MK. (2011) CXC receptor-4 mRNA silencing abrogates CXCL12-induced migration of colorectal cancer cells. *J Transl Med 9:22*

Rubie C, Frick VO, Ghadjar P, Wagner M, Grimm H, **Vicinus B**, Justinger C, Graeber S, Schilling MK. (2010) CCL20/CCR6 expression profile in pancreatic cancer. *J Transl Med 8:45*

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8. Curriculum vitae

Persönliche Angaben

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Ausbildung

06/1990 - 06/1999	Wilhelm-von-Humboldt-Gymnasium Degree: Abitur
09/1999 - 09/2000	Civilian Service at the Katholische Gesamtkirchengemeinde Ludwigshafen
10/2000 - 08/2007	Academic studies in Biologie with focus on Cellulare Biology at the Ruprecht-Karls-Universität Heidelberg Degree: Diplom Major Subject: Cellulare Biology Minor Subjects: Molecular Biology, Biochemistry Subject of Diploma Thesis: „Interfering with nuclear ERK1/2- signaling in cultured hippocampal rat neurons“
09/2007 - 03/2008	Graduate assistant with degree at the Ruprecht-Karls-Universität Heidelberg
11/2008 - 08/2009	Administrator telemarketing at Alpha Informationsgesellschaft mbH in Lampertheim
Seit 09/2009	Resaech associate and graduation at the laboratory of the Department of General, Visceral, Vascular and Paediatric Surgery, University of the Saarland

Hiermit versichere ich an Eides statt, dass ich die Arbeit selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Heßheim, August 2012

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Benjamin Vicinus