# CEA and its involvement in metastasis of HT29 human colon cancer cells

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# ZUSAMMENFASSUNG

Das Kolonkarzinom gehört zu den am häufigsten vorkommenden Krebsarten weltweit. Etwa 130 000 Menschen werden jährlich allein in den USA mit Dickdarmkrebs diagnostiziert. Statistisch gesehen werden 45 % von den diagnostizierten Menschen an den folgen des Dickdarmkrebs sterben. Die Chancen auf eine vollständigen Heilung hängt entscheidend von dem Stadium des Tumors ab zum Zeitpunkt der Diagnose.

CEA wurde bereits 1965 zum Ersten mal als oncofetales Antigen von Gold und Freedman beschrieben (GOLD, P. and FREEDMAN, S., 1965). CEA ist ein Glykoprotein, hat eine Grösse von ca. 180 kD und gehört zu der Familie Immunglobulingene. Es ist das Älteste bekannte tumorassoziierende Antigen. CEA ist in den vielen Karzinomen, wie zum Beispiel im Karzinomen des Dickdarms, der Brust, oder der Lunge, überexprimiert.

Klinische Studien zeigen, dass es eine Korrelation zwischen dem Tumorstadium und der allgemeinen Prognosis von Krebspatienten und der Konzentration von CEA im Serum gibt (GREM, J., 1997). Jedoch ist die Funktion von CEA und sein eventueller Einfluss an der Metastasierung von Kolonkarzinom Zellen noch unklar.

In der Literatur ist CEA bereits als ein Adhäsionsmolekül beschrieben worden (BENCHIMOL, S., et al. 1989, STANNERS, C.P., et al. 1998 und ZHOU, H., et al. 1993). Es wurde postuliert, dass Zellen, die leicht miteinander aggregieren, somit eine bessere Überlebenschance in der Blutzirkulation haben als Einzelzellen. Zum anderem wurde gezeigt, dass CEA an Kupffer Zellen der Leber bindet und die Freisetzung von Zytokinen stimuliert (GANGOPADHYAY, A., et al., 1998 und EDMINSTON, K., et al. 1997). Eine andere Forschungsatbeit postuliert eine mobilitätsfördernde/ metastasierende Funktion von CEA (VON KLEIST, S., et al. 1995).

Das Ziel dieser Arbeit war die Funktion von CEA und seine Bedeutung bei der Metastasierung zu klären. Um diese Fragestellung zu klären, haben wir die humane Dickdarmkrebszellinie HT29 mit Ribozymen (gerichtet gegen die mRNA von CEA) unter der Kontrolle eines Tet-off Promoter-Systems transfiziert.

Unter normalen Bedinnungen wird das Ribozym kontinuierlich synthetisiert, was zur einer Reduzierung der CEA Expression führte. Bei Hinzugabe von Tetrazyklin wird die Synthese des Ribozyms blockiert und eine normale (erhöhte) CEA Expression ist die Folge.
Diese Methode bringt uns drei wesentliche Vorteile gegenüber herkömmlichen Methoden: 1) die Benutzung von Ribozymen ermöglicht eine hohe spezifische Inhibition des Zielmoleküls. 2) Mit Hilfe des Tet-off Promoter-Systems ist es möglich, die Konzentration von CEA innerhalb eines Zellklones zu kontrollieren. 3) Es ermöglicht die Untersuchung von CEA innerhalb eines pathophysiologischen Kontextes.
Mit Hilfe des Tet-off Systems wurde die Expression von CEA um 50% auf der RNA sowie der Protein Ebene reguliert.

Zur Untersuchung des Einflusses von CEA auf die Aggregatenbildung von HT29 Zellen, führten wir einen Aggregationsassay durch. Wir konnten zeigen, dass bei Reduktion der CEA Expression die Aggregatenbildung von HT29 Zellen deutlich geringer war, als im Vergleich zu den HT29 Zellen mit normaler CEA Expression. Wir konnten somit die Funktion von CEA als ein homophile Adhäsionsmolekül (Tumor-Tumor Aggregatenbildung) zeigen.
Zur Identifikation CEA regulierter Gene, wurde ein cDNA Microarray durchgeführt. Der cDNA Microarray bestand aus 588 Gene (unterteilt in 13 Gruppen), die mit der Karzinogenese assoziiert sind. 134 von 588 Genen wurden durch CEA beeinflusst. Praktisch wurden Gene von allen 13 Gruppen beeinflusst - unter anderem Gene die zu den Apoptose regulierende Genen gehörten, wie auch Gene die zu den Zellzyklen regulierenden Genen gehörten.

Zur Überprüfung der Korrelation zwischen der Genexpression im cDNA Array und dem Phenotyp von HT29 Zellen *in vitro*, haben wir mit Hilfe von AnnexinV-Färbung die Apoptoserate von HT29 Zellen mit normaler und reduzierter CEA Expression verglichen.
Unter semikonfluenten Wachstumsbedinnungen war kein Unterschied in der Apoptoserate zwischen HT29 Zellen mit normaler CEA Expression und reduzierter CEA Expression beobachtet. Die Änderung der Wachstumsbedinnungen (z.B. Wachstum unter konfluenter Bedinnungen), führte zum Anstieg der Apoptoserate

in HT29 Zelllinie mit reduzierter CEA Expression signifikant, wobei die Apoptoserate der Zellen mit normaler CEA Expression nahezu gleich blieb. Dasselbe Ergebnis erhielten wir, durch Inkubation der HT29 Zellen entweder mit γ-Interferon , oder 5-FU. Zellen mit normaler CEA Expression hatten eine niedrigere Apoptoserate, als die Zellen mit reduzierter CEA Expression.
CEA hatte somit eine protektive Wirkung gegen Apoptose unter verschiedenen Stressbedinnungen.

Zur Untersuchung des Einflusses von CEA auf die Metastasierungsrate von HT29 Zellen *in vivo*, injizierten wir über die Schwanzvene von Nacktmäusen HT29 Zellen - einmal HT29 Zellen mit normaler CEA Expression (Tetrazyklin behandelt) und einmal HT29 Zellen mit reduzierter CEA Expression. 1 Stunde nach der Injektion von HT29 Zellen waren in der Lunge bei beiden Gruppen HT29 Zellen sichtbar. Quantitativ war kein Unterschied zwischen Zellen mit normaler CEA Expression und Zellen mit reduzierter CEA Expression zu sehen. Nach 24 Stunden waren optisch keine Tumorzellen in beiden Gruppen mehr sichtbar. Nach 6 Wochen jedoch waren in den Mäusen, die weiterhin mit Tetrazyklin behandelt wurden (normale CEA expression), Metastasen sichtbar, wärendessen in den Mäusen die nicht mit Tetrazyklin behandelt wurden (reduzierte CEA expression) eine wesentlich geringere Anzahl von Metastasen vorhanden waren. Das Ergebnis des Tierversuches deutet daraufhin, dass die Funktion von CEA als Aggregationsmolekül in den Prozess der Metastasierung nicht entscheidend ist, jedoch der Wachstumsvorteil der HT29 Zellen mit erhöhter CEA Expression (niedrigere Apoptoserate) gegenüber den Zellen mit reduzierter CEA Expression (erhöhte Apoptoserate) eine wesentliche Rolle spielt.

Zur Untersuchung, ob CEA fähig ist mit Proteinen zu interagieren, die eventuell Einfluss auf die Signaltransduktion von Dickdarmkrebszellen hat, haben wir eine Phage-Display-Analyse durchgeführt. Mit Hilfe einer cDNA Bibliothek –erstellt aus der Dickdarmkrebszelllinie LS174T – haben wir nach Kanditaten gesucht, die eventuell Einfluss auf die Signaltransduktion von Dickdarmkrebszellen hat. Insbesondere haben wir nach Kanditaten gesucht, die in Verbindung zur Apoptose stehen. CEA war fähig mit verschiedenen Proteinen zu interagieren und wir

konnten auch ein Protein identifizieren, dass in Verbindung zur Regulation der Apoptose steht.

Diese Arbeit zeigt, dass CEA ein multifunlktionelles Molekül ist. Erstens, wir konnten zeigen, dass CEA als ein homophiles Adhäsionsmolekül fungiert, welches die Aggregation von HT29 Zellen fördert. Des weiteren konnten wir zeigen, dass CEA ein protektives Molekül gegen Apoptose ist und dadurch die Metastasierung von HT29 Zellen in Nacktmäusen fördert. Zuletzt konnten wir mit Hilfe von Phage Display nachweisen, dass CEA fähig ist mit anderen Proteinen zu interagieren und somit auch Einfluss in die Signaltransduktion hat.

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# ABBREVIATIONS

5-FU = 5 fluoruracil

APC = Adenomatous polyposis coli

ATCC = American type culture collection

bp = base pair

BSA = Bovine serum albumin

cDNA = copy deoxyribonuclein acid

CEA = Carcinoembryonic antigen

CEACAM = carcinoembryonic antigen cell adhesion molecule

CMV = cytomegalovirus

DAB = 3,3'-Diamonobenzidine

DCC = deleted in colon cancer

dCTP = deoxycytosin triphosphate

$ddH_20$ = double distilled water

DEPC = Diethyl Pyrocarbonate

DNA = deoxyribonucelic acid

Dnase = Deoxyribonuclease

dNTP = deoxynucleoside triphosphate

E. coli = Escherichia coli

EDTA = Ethylenediaminetetraacetic acid

FACS = fluorescence activated cell sorting

FAP = Familial adenomatous polyposis

FBS = fetal bovine serum

GAPDH = Glyceraldehyde-3 phosphate dehydrogenase

GPI = Glycosyl phosphatidyl inositol

GTPase = Guanin triphosphatase

$H_2O_2$ = superperoxide

HNPCC = Hereditary nonpolyposis colorectal cancer

IFN-γ = Interferon gamma

Ig = Immunoglobulin

IGF = Insulin-like growth factor

IL = Interleukine

IMEM =

kDA = kilo Dalton

LB media = media for bacteria culture

MCC = mutated in colon cancer

MHC = Major histocompatibility complex

mM = milli Molar

mRNA = messenger ribonucleic acid

Mw = Molecular weight

NCA = Non-specific crossreacting antigen,

nt = nucleotide

PBS = phosphate buffered saline

PBST = Phosphate buffered saline with tween

PCR = Polymerase chain reaction

PI = probidium iodid

PS = Phosphatidylserine

PSG = Pregnancy specific glycoprotein

pTET = ribozyme expression plasmid

RNA = ribonucleic acid

Rz = ribozyme

SDS = Sodium dodecyl sulfate

SSC = Saline-sodium citrate

tet = Tetracycline

TNF = Tumor necrosis factor

tTA = tetracycline transactivating

UV = Ultra violet

VEGF = Vascular endothelial growth factor

# 1. INTRODUCTION

Peyton Rous opened his Nobel lecture of 1966 with the following passage: "Tumors destroy man in a unique and appalling way, as flesh of his own flesh which has somehow been rendered proliferative, rampant, predatory and ungovernable. [Tumors] are the most concrete and formidable of human maladies, yet despite more than 70 years of experimental study they remain the least understood... What can be the why for these happenings ?"

35 years after the speech of Rous a lot of progress and knowledge has been achieved in the field of cancer research. However, cancer is still a major problem, and one of the leading causes of death. It manifests itself as a population of cells that have lost their normal controls of growth and differentiation.

## 1.1. Colon Cancer

At least 50% of the western population develops a colorectal tumor by the age of 70 and in about 1 in 10 of these individuals, progression malignancy ensues. Colorectal cancer (CRC) accounts for approximately 1 of every 10 deaths from carcinoma and is as a result the second leading cause of cancer death (10 to 12%; HARMS, B. A. et al. 2001). Approximately 130.000 new cases will occur every year in the United States, alone, with many more cases worldwide (HARMS, B. A. et al. 2001), and 45% of these patient will die from their disease often despite the apparent complete surgical resection of their local disease (FEARON, E. R. 1995). 40 to 50% of colorectal cancer patients won't survive longer than 5 years. The main reason for mortality is metastatic disease which occurs most often in the liver, followed by the lung. The most important factor predicting recurrence is the stage of tumor at the time of resection. Patients whose tumors have only penetrated into the mucosa of the bowel but not further and have not spread to local lymph nodes (stage I or Dukes A) typically have a cure rate in of >90% (MINSKY et al. 1988). However, 20% of patients will suffer metastatic relapse when tumors have penetrated into, or through the muscularis propria of the bowl wall, but have not spread into the regional lymph nodes (tumors of stage II or Dukes B) (MINSKY et

al. 1988). If tumor spread is identified in the regional lymph nodes (stage III or Duke C) the odds for cure are reduced to 50% (MINSKY et al. 1988).

One of the most important developments in genetics over the past decade has been the proof that cancer is, in essence, a genetic disease (VOGELSTEIN, B. and KINZLER, K. W. 1996). First, cancer is, for the most part, caused by somatic mutations, whereas most of other genetic diseases of mammals are caused solely by germ-line mutations. Second, each individual cancer arises not from a single mutation, but from the accumulation of several mutations, even though there are differences in the amount of mutations needed. Statistical analyses of age-incidence curves have suggested three to four successive mutation-like changes for leukemia and six to seven for carcinomas (FARBER, E., & CAMERON, R. 1980 and KNUDSON, A. 1973). The only known exceptions are found in experimental viral oncology, where some of the retrovirus-transduced oncogenes can cause tumors in a single step (KLEIN, G., & KLEIN, E. 1985).

The most devastating aspect of neoplasia, however, is the spread from a primary site to distant organs (metastasis). Metastasis comes from Greek (from *methistanai,* to change), which literally means change of position, state, or form, as a transfer of a disease (-or disease-producing agency) from the site of disease to another part of the body. It was Virchow who, already in 1858 realized and coined the Latin aphorism by which the cell theory became known: omnis cellulae cellula – "all cells come from cells". Virchow pointed out that the cellular components of metastatic cancer generally resemble those of the primary tumor, as if they all might be relatives. The spread of tumors throughout the body is a pathophysiological process of profound clinical significance. The lethality of malignant neoplasms is attributable directly to their ability to develop secondary growths in organs at a distance from the primary tumor mass. The primary or localized tumor mass will usually pose little threat to the well being of the patient, unless it is located in a critical site within an organ such as the brain. In a case of a localized tumor, it is normally very probable that surgery will be curative at that stage.

Colorectal cancer develops through several histological well defined stages, reflecting the sequential acquisition of genetic alteration. Beginning with the

general mechanisms of oncogenesis of colorectal cancer, it is known that both inherited and environmental factors play an important role in carcinogenesis. Mutations in certain genes may be inherited, termed germ line mutations, or acquired, termed somatic mutations. Molecular experimental studies have demonstrated, that the progression of colorectal cancer from adenoma to carcinoma, results from the accumulation of molecular genetic alteration, involving mainly 3 factors: I) activation of oncogenes; II) inactivation of tumor suppressor genes; and III) abnormalities in genes involved in DNA mismatch repair (BRESALIER, R. S. et al. 1998).

Several frequently mutated genes have been identified which probably contribute to the development of both hereditary and somatic cancer (FEARON and VOGELSTEIN, 1990). Mutations have been able to observe in the earliest detectable stages of cancer development and it seems that specific genes tend to be mutated in a given order.

## Hereditary and non-hereditary colorectal cancer

It is believed, that approximately 15-20% of all colorectal carcinomas cases involve a hereditary component (KINZLER, K. W. & VOGELSTEIN, B. 1996). On the contrary, approximately 80% of colorectal cancer is considered 'sporadic'. Like all cancers, 'sporadic' colorectal cancer is the result of the accumulation of genetic mutations. Although any one gene mutation eventually can lead to cancer, research by Kinzler and Vogelstein has identified that there are at least seven genetic events that are required for colorectal cancer development and that these events occur most likely in a preferable order (KINZLER, K. W. & VOGELSTEIN, B. 1996).

In 1990, Fearon and Vogelstein proposed a model of successive genetic changes leading to colorectal cancer, in which a number of genes were involved, including the *APC* gene, *k-ras, DCC* gene and *p53* (FEARON and VOGELSTEIN, 1990).

Mutations in the *APC* gene are responsible for the FAP syndrome, which will be discussed in the hereditary colorectal cancer section below. In 'sporadic' colorectal cancer, such mutations or deletions also appear and are found to be essential in the beginning of the cascade in early adenoma formation.

The *k-ras* is one of the three mutant *ras* oncogenes that are found in human tumors. *K-ras* is found in approximately 40% of colorectal cancers. Mutation in the *k-ras*

oncogene normally appears at the border of small and large adenomas after *APC* loss, but before *DCC* and *p53* inactivation and leads to activation of a tyrosin kinase cascade, like for example *p21* activation (BOS, J. L., et al. 1987 and FORRESTER, K. et al 1987).

In 70 to 80% of colorectal cancer cases the long arm of chromosome 18 (containing the *DCC* gene) is deleted (VOGELSTEIN, B. et al. 1989). This deletion seems to appear a little later than *APC* and *k-ras*, but earlier than *p53* mutations. Also this is happening at the border of small and large adenomas. The *DCC* gene is a very large gene, and loss of function of this gene seems to impair normal cell adhesion. Some studies have shown that normal *DCC* is able to stop a cell in G2/M of the cell cycle and induce apoptosis (CHEN, Y. O. et al. 1999).

Regarding the *p53* gene, in most cases of colon cancer tissues (75 to 80%) at least one of the copies (which are located on chromosome 17q) of this region is deleted and the other allele found to be mutated (KINZLER, K. W. et al. 1991). It is hypothesized that a *p53* mutation appears in the later stages of tumor progression (late adenoma or early carcinoma) (DICATO, M. et al 2000).

In additions to the mutations in those genes mentioned above, there are other factors that are probably implicated in the cascade leading from normal colonic cells to invasive colon cancer. Some of them are known, such as *MCC* (mutated in colon cancer), but their exact role remains elusive. Some oncogenes like c-myc and *c-myb*, are amplified in some tumors and they are shown to induce transformation, however, their clinical relevance is not yet certain (D'EMILIA, J., et al 1989 and ERISMAN, M. D., et al. 1988).

Regarding hereditary colorectal cancer, two of the best defined familial forms of hereditary colorectal cancer are FAP (Familial Adenomatous Polyposis) and HNPCC (Hereditary Nonpolyposis Colorectal Cancer).

FAP is an autosomal, dominantly inherited disease that affects about 1 in 7000 individuals. Patients with FAP typically develop hundreds to thousands of, mainly benign, colorectal tumors (called adenomas or adenomatous polyps) during their second and third decades of life. Although these benign tumors are not individually life threatening, their large numbers virtually guarantee that some will progress to malignant lesions.

Adenomatous polyposis was first observed in the mid 18$^{th}$ century and its inherited nature was already recognized in1900 (KINZLER, K. W. and VOGELSTEIN, B.

1996). But it wasn't until the last decades that the cause of this disease was discovered. The first clue was a cytogenetically evident interstitial deletion of chromosome 5q in a patient with polyposis (HERRERA, L. et al. 1986). A Year later a tight linkage of the disease to markers on chromosome 5q21 was demonstrated by Bodmer et al and Leppert et al. (BODMER, W. et al. 1987 and LEPPERT, M. et al. 1987) and it became possible to identify the adenomatous polyposis coli (APC) gene, and to proof that this gene caused FAP (GRODEN, J. et al. 1991 and NISHISHO, I. et al. 1991).

The normal function of the *APC* gene is believed to be tumor suppression. The most common mutation results in a 'stop codon' that leads to a truncated protein product (POWELL, S. M. et al. 1993). More than 300 different germline *APC* mutations have been described in FAP patients and there are evidence for correlation between the exact location of the mutation on the gene and the resulting phenotype expressed (OLSEN, S. J. et al. 2000).

Hereditary nonpolyposis colorectal cancer is the most common hereditary colon cancer syndrome. It is dominantly expressed and accounts for 5-10% of all colorectal cancer cases (BURKE, W. et al. 1997). Despite its name, it also is characterized by the presence of colon polyps. The number of polyps present in hereditary nonpolyposis colorectal cancer is much less than those seen in FAP, that is because, in contrast to FAP, patients with HNPCC do not have abnormal *APC* alleles and therefore do not develop extensive polyps (HARMS, B. A. et al. 2001).

However, although their lesions are more commonly right sided, clinically they represent identically to patients with sporadic colon carcinoma. The exact definition of HNPCC is problematic owing to various clinical phenotypes and the multiplicity of associated genes.

## Treatment of colorectal cancer

The main form of treatment for colorectal cancer is surgical resection (ROUGIER, P. and MITRY, E. 2000). Nevertheless, as many as 30% of patients who undergo "successful" surgery for colon carcinoma will develop recurrent disease, either locally or at distant sites, within 5 years of surgery (HARMS, B. A. et al. 2001). Additional systemic treatment, known as "adjuvant" therapy is nowadays widely used. However, there are variabilities in the use of adjuvant therapy after curative

surgery. In the United States it is used very selectively for patients with stage B2 colorectal carcinoma, and is generally recommended for patients with lymph node involvement (Dukes C) without other significant comorbidities, while in Japan for example, adjuvant therapy has been used for all patients except those with stage A tumors (TOMINAGA, T. et al. 1996).

5-Fluorouracil (5-FU), a thymidylate inhibitor and analog of uracil, is the main chemotherapeutical agent in the treatment of colorectal cancer (HARMS, B. A. et al. 2001). It is an analog of uracil, and it has been used now already for more than 40 years. However, used alone it has limited activity. Nevertheless, improvements have been made in the last 15 years that justify its role as the main agent.

In the United States the most widely used adjuvant treatment for metastatic colorectal cancer is the combination of 5-FU plus leucoverin (MOERTEL, C. G. 1994). Other combination therapies used are 5-FU in combination with Oxalplatin, or 5-FU in combination with Irinotecan, a topoisomerase 1 inhibitor (ROUGIER, P. and MITRY, E. 2000).

## 1.2. Carcinoembryonic Antigen (CEA)

Little more than 35 years ago two groups, Gold and Freedman in Canada, and von Kleist and Burtin in France, described independently the presence of a tumor associated antigen in colonic carcinomas and their metastases (GOLD, P. and FREEDMAN, S. O. 1965, and VON KLEIST, S. BURTIN, P. 1966). This tumor associated antigen was called carcinoembryonic antigen (CEA) because it was also shown to be present in the homologous embryonic tissues. The name is *strictu senso* incorrect, because CEA is produced not only during the first trimester of gestation, but throughout pre-natal life and even after birth.
CEA is physiologically found to be expressed apical-wise of glandular epithelial tissue and it is also strongly expressed by the intestinal mucosa (ÖBRINK, B. 1997). CEA has been shown to bind to certain strains of Escherichia coli (THOMPSON, J. A. et al. 1991). According to Thompson et al. this binding may facilitate bacterial colonization of the intestine (THOMPSON, J. A. et al. 1991). Hammerstrom, on the other hand suggested that CEA may play a role in protecting the colon from microbial infection, possibly by binding and trapping infectious microorganisms (HAMMERSTROM, S. 1999).

Analysis of the amino acid sequence of CEA (and other members of the CEA family) revealed, that CEA belongs to the immunoglobulingene-superfamily (Figure1).

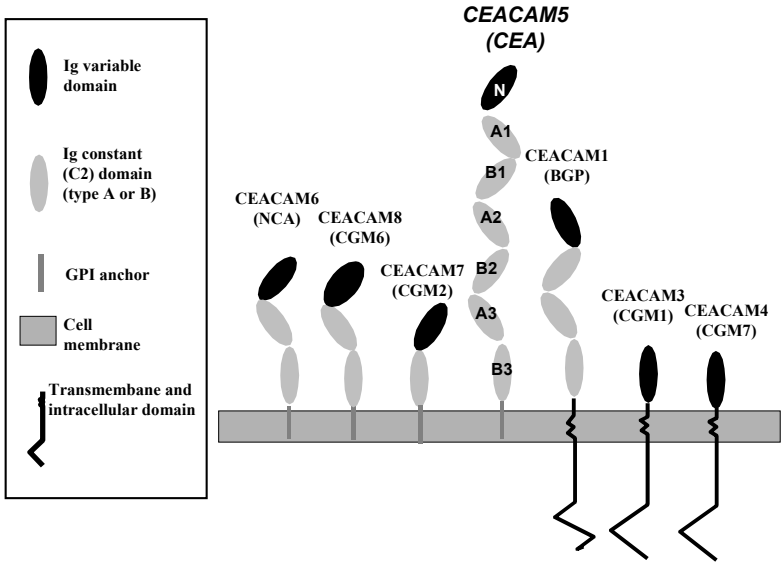



**Figure 1:** The CEA family. CEA itself is anchored with a GPI anchor to the cell membrane (compared to BGP for example CEA does not have a transmembrane and a intercellular domain).

29 different genes and pseudogenes have been identified which are related to CEA. All of these genes are clustered to the chromosome 19q13.2 within a region of 1.8 Mb. The CEA family can be divided into three subgroups. First, the *CEA subgroup*, containing 12 members, where 7 genes are expressed and 5 are pseudogenes. Second, the *pregnancy specific glycoprotein* (*PSG*) *group* consisting of 11 expressed genes. And third, a group of *six pseudogenes* (Hammerstrom, S. 1999).

All members of the *CEA subgroup*, CEACAM1 (biliary glycoprotein, BGP), CEACAM3 (CGM1) and CEACAM4 (CGM7), have a transmembrane domain followed by a cytoplasmic domain, while CEACAM5 (carcinoembryonic antigen, CEA), CEACAM6 (non-specific crossreacting antigen, NCA), CEACAM7 (CGM2) and CEACAM8 are anchored in the cell membrane via a glycosyl phosphatidyl inositol (GPI) moiety (Hammerstrom, S. 1999).

CEA is a glycoprotein which contains approximately 50% carbohydrate and has a molecular weight of approximately 180 kDa. It consists of a N-terminal domain of 108 amino acids homologous to the Ig variable domain (IgV-like) and six domains homologous to the Ig constant domain of the C2 set (IgC2-like). The six constant

domains are divided in 3 type A IgC2 domains containing each 93 amino acids and 3 type B IgC2 domains which contain 85 amino acids. A signal peptide of 34 amino acids precedes the N-domain but is cleaved off from the mature protein following transport to the cell membrane.

CEA is taken rapidly up from the circulation by the liver and catabolized in the lysosomes. Peter Thomas group studied the clearance of CEA in rat liver. They found out, that about 70% of 125-labeled intact CEA was cleared by the rat liver already within 1 hour and that CEA is probably initially taken up by Kupffer cells, where it is modified for removal and transferred afterwards to the hepatocytes. 10% of CEA added to an isolated perfused liver appeared in bile (THOMAS, P. et al. 1983).

CEA is the oldest known and most frequently found tumor associated antigen in cancer such as colorectal-, gastric-, pancreatic, lung-, and breast cancer. While CEA is nowadays the most commonly used tumor marker for malignancies of the gastrointestinal tract, it has never proved useful as a screening test for early cancer (HARMS, B. A. et al. 2001). Primary colorectal cancers are commonly CEA positive. In fact 90% of these tumors can be shown to produce CEA (GOLD, P. et al. 1978 and CUTAIT, R. et al. 1991). While CEA is found on the apical side of normal epithelial cells it has lost its polarized expression in malignant tumors. Furthermore it is secreted in the extracellular space. Thereby, it has access to blood capillaries, and hence, can be found in the serum. CEA can be used as a tumor marker, by measuring CEA levels in the serum. However, low levels of CEA can also be found in blood of healthy people and elevated levels can occur under various non-malignant disorders such as chronic inflammatory diseases. CEA can be modestly elevated in about 19% of smokers who do not have cancer and in 3% of normal population. Certain benign liver diseases impair liver function, and thus, the clearance of CEA. These false elevation, however, are almost always less than 10ng/ml and remain stable during serial testing, in contrast to CEA produced by recurrent tumor. Therefore, in patients with appropriate symptoms and a highly increased concentration (e.g. >5 times the upper limit of normal) should be considered suggestive for the presence of cancer in that particular patient.

Multiple studies have shown that patients with high preoperative concentrations of CEA have a worse outcome than those with low concentrations of the marker (GREM, J. 1997).

When CEA levels are elevated in patients, preoperatively for the primary colorectal cancer, they predict a worse prognosis. The sensitivity of CEA as a monitoring test varies from 43 to 89%, with a specificity of 70 to 90%. Elevation of CEA in primary tumors correlates with Dukes' stage, 45% of Dukes C tumors and 25% of Dukes B tumors (HARMS, B. A. et al. 2001).

Persistent elevation of CEA 1-month postoperatively suggests the presence of occult metastatic disease and predicts for early development of recurrence. For those patients with a normal preoperative CEA and patients whose CEA returns to normal within 4 to 6 weeks of the resection of their primary tumor, a CEA increase is most often the first warning of metastatic disease. A significant and progressively increasing CEA is associated with 75% of metastatic colorectal cancer (MAYER, R. J., et al. 1978).

**Biological function of CEA**

Even though it has been shown that CEA correlates with stage of tumor, the role of CEA in malignant conditions is barely understood. Ever since it was discovered that the CEA gene family is a member of the Ig-supergene family, to which belongs a whole array of prominent molecules involved in cell-adhesion or cell/cell-recognition (e.g. ICAM-1, or ICAM-2), studies have been done to determine the role of CEA as an adhesion molecule. Benchimol et al. suggested CEA to function as an intercellular (homophilic) adhesion molecule. They found that aggregate formation of single cells correlates with the level of CEA. Adding of an anti-CEA antibody inhibited aggregate formation. However, they could not exclude that adhesion might also rely on interactions with other CEA family members such as CEACAM6 or CEACAM1. To exclude the interaction of CEA with other CEA family members, they transfected chinese hamster ovary cells, which do not express CEA-related molecules, with a CEA expression vector. In doing so, they could prove an adhesive function of CEA (BENCHIMOL, S. et al. 1991). Because alterations in cell adhesion are causally involved in cancer invasion and metastasis, it was suggested that CEA may play a role in these processes. However, Landuzzi

et al. did similar studies using a rhabdomyosarcoma cell line and demonstrated results which were the opposite of those demonstrated by Benchimol et al. (LANDUZZI, L., et al. 1996). CEA overexpressing cells showed reduced adhesion and a reduction of the metastatic rate, suggesting that CEA molecules could interfere with adhesion maintained by other adhesion molecules, such as E-cadherin, MUC18 and N-CAM (LANDUZZI, L., et al. 1996).

Beside homophilic cell adhesion function, CEA has also been implicated as a heterophilic intercellular binding molecule which mediates the colonization of liver and lung by colon cancer cells. Recently, Hostetter et al. showed that after transplantation of colorectal tumors into nude mice the number of liver metastases increased from 2% to 48% following injection of mice with CEA (HOSTETTER, R.B. et al 1990). There is, however no direct evidence that CEA causally was involved in the process of metastasis. Thomas et al. overexpressed MIP-101, a poorly differentiated human colon cancer cell line, with CEA. They found an increased metastatic rate from high CEA expressing clones and described strong CEA staining of Kupffer cells adjacent to tumor cells in the liver (THOMAS, P. et al. 1995). The potential interaction of Kupffer cells and CEA expressing tumors was also described by Bayon et al. They injected a syngeneic colon cancer cell line into a mesenteric vein of rats. They suggested that Kupffer cells play a relevant role in arresting circulating tumor cells at the liver sinusoid, maybe through binding of CEA to Kupffer cells (BAYON, L. G., et al. 1996).

Further studies suggest that CEA might increase the metastatic capability of cancer cells by immunemodulation. It was found that CEA could induce the production of cytokine, by binding of CEA to Kupffer cells. Binding of CEA to HnRNP M4, a recently described receptor of CEA on Kupffer cells (BAJENOVA, O. V. et al. 2001), was shown to induce the release of IL-6, TNF-α, IL-1α and IL-1β. This might downregulate the immune response against tumor cells and thereby enhance the metastatic rate *in vivo* (EDMINSTON, K. et al. 1997). The release of cytokines from Kupffer cells (caused by binding of CEA to Kupffer cells) might also modiy the adhesion molecule pattern of endothelial cells in a way that colon cancer cells can better bind and, thereby, enhance their metastatic potential (GANGOPADHYAY, A. et al. 1998).

Some groups have shown that CEA modulates the susceptibility of human colorectal carcinoma cells to cytotoxic lymphocytes (RIVOLTINI et al. 1992;

KAMMERER and VON KLEIST, 1994). Kammerer and von Kleist further demonstrated, that the density of the CEA molecules on the tumor cell surface has a determining influence on its protective function (for example protection from LAK-cell lysis), whereas CEA released by tumor cells had no protective effect for CEA negative cells (KAMMERER and VON KLEIST, 1996).
Finally, Ordonez et al. reported that overexpression of CEA can protect tumor cells from undergoing anoikis, i.e. apoptosis induced by loss of cell contact with the extracellular matrix (ORDONEZ, C. et al. 2000).

Even though a lot of functions have been implicated for CEA, the role of CEA in normal and pathological human physiology remains unknown. The purpose of this study was to determine the role of CEA for growth and metastatic behavior of colon cancer cells. If CEA is involved in the pathway of metastasis the next question would be how.
We assume that CEA's function is tissue type dependent and depends on the interaction with other molecules, either with cancer cells, or with its microenvironment. In order to define the specific function of CEA within its pathophysiological context we choose CEA-targeting ribozymes under control of a tet-off promoter system.

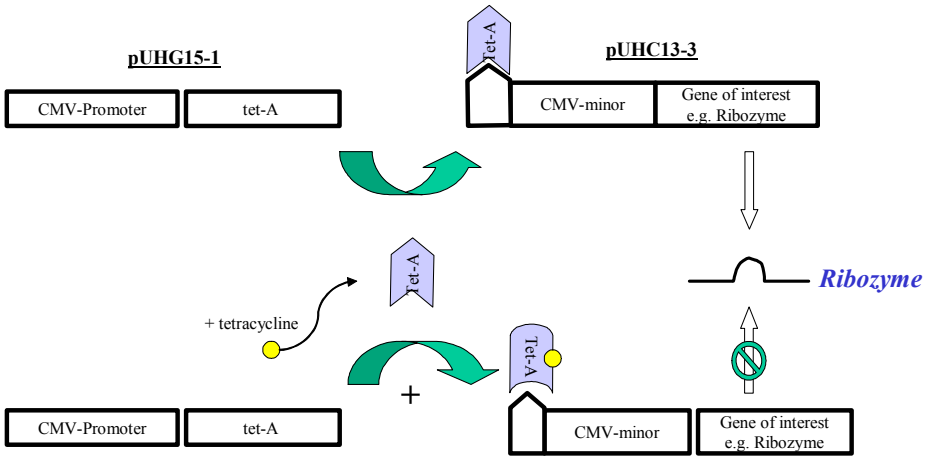



**Figure 2:** The tet-off system. Tetracycline binds to the Tet-A protein causing its change of confirmation. The Tet-A protein is no longer able to bind to the CMV-minor promoter region resulting in blocking of ribozyme synthesis.

This model has three major advantages compared to other methods used before. First, because of the high specificity of ribozyme molecules (UHLENBECK, O. et al 1995) it is able to target exclusively the gene of interest (CEA) and inhibit the translation of the mRNA to its actual protein, resulting in a specific inhibition of endogenous CEA expression. Secondly, by using the tet-off promoter system the CEA levels within identical cancer cell clones can be regulated. And third, by using the tet-off system it enables a comprehensive analysis of CEA-mediated effects within an intact pathophysiological cellular context.

In this study we investigated the impact of CEA on tumor cell aggregate formation and the impact of CEA-dependent apoptosis on tumor growth *in vitro* and *in vivo*.

# 2. MATERIALS AND METHODS

## 2.1. Materials

### 2.1.1. Biological materials

**Animals**

| | |
|---|---|
| • Ner nude mice | Harlan Sprague-Dawley, Indianapolis, USA |

**Bacterials**

| | |
|---|---|
| • BLT 5615 (*E.coli*) | Novagen, Madison, USA |
| • DH5α-competent cells (*E. coli*) | Invitrogen, Rockville, USA |

**Cell lines**

| | |
|---|---|
| • human colon cancer cell line HT29 | ATCC, Manassas, USA |
| • murine colon cancer cell line MC38 | kindly provided by Dr. J. Shively |
| • C15A3 (MC38 cell line transfected to express CEA) | kindly provided by Dr. J. Shively |
| • HUVEC (human umbilical vein endothelial cells) | ATCC, Manassas, USA |

**Enzymes**

| | |
|---|---|
| • Restriction enzymes (NotI, PSTI, Apa I) | Roche, Indianapolis, USA |

| | |
|---|---|
| • Superscript™ II-Reverse Transcriptase | Invitrogen, Rockville, USA |
| • Taq-DNA-Polymerase [5 U/µl] | Invitrogen, Rockville, USA |

**Nucleic acids**

| | |
|---|---|
| • [α-$^{32}$P*]-dCTP-Nucleotide | Amersham, Piscataway, USA |
| • 100 bp-DNA-ladder | Invitrogen, Rochville, USA |
| • dNTP-Nucleotid-Mix, 10 mM | Invitrogen, Rockville, USA |
| • pBluescript II KS vector | Invitrogen, Rockville, USA |
| • pUHG15-1 and pUHC13-3 expression vector | Dr. Bujard, Heidelberg, Germany |
| • Random Hexamers | Invitrogen, Rockville, USA |
| • RNA-ladder | Invitrogen, Rockville, USA |
| • Salmon sperm | Invitrogen, Rockville, USA |

**Phage**

| | |
|---|---|
| • T7 Select10-3 (bacteriophage) | Novagen, Madison, USA |
| • cDNA library of LS174T | kindly provided by Prof. Dr. Wellstein |

**Antibodies**

primary antibodies

- Anti-human CD66e (CBL54), mouse monoclonal antibody — Cymbus, Biotechnology, Hampshire, UK
- Anti-human Cytokeratin (Kl-1) mouse monoclonal antibody — Cymbus Biotechnology, Hampshire, UK

secondary antibodies

- Fluorescein (DTAF)-conjugated AffiniPureGoat Anti-Mouse IgG +IgM — Dianova,Hamburg, Germany
- Peroxidase-conjugated AffiniPure Rabbit Anti-Mouse IgG + IgM — Dianova, Hamburg, Germany

**Other**

- Doxycycline enriched food (220mg/kg) — Bioserv, Frenchtown, USA

## 2.1.2. Chemicals, reagents and medium

- 1 x Trypsin — Invitrogen, Rockville, USA
- Agarose — Invitrogen, Rockville, USA
- Ampicillin — Invitrogen, Rockville, USA
- Bacto™Agar — Becton&Dickinson, Franklin Lakes, USA

| | |
|---|---|
| • Chloroform | Mallingkrodt Chemicals |
| • Cytoseal Mounting medium | Stephens scientific, Kalamazoo, USA |
| • DEPC (Diethylpyrocarbonate) | Sigma, St. Louise, USA |
| • Diethanolamin | Sigma, St. Louise, USA |
| • Dimethylsulfoxide (DMSO) | Invitrogen, Rockville, USA |
| • ECL™ Westerb blotting detection reagents | Amersham, Piscataway, USA |
| • EDTA (Ethylene diaminetetraacetic acid) | Biofluids, Rockville, USA |
| • Ethidiumbromide | Sigma, St. Louise, USA |
| • Fetal bovine serum | Invitrogen, Rockville, USA |
| • Formaldehyde (37 %) | Mallingkrodt Chemicals, |
| • Formamide | Mallingkrodt Chemicals, |
| • Geneticin® (G418-Sulfat) | Invitrogen, Rockville, USA |
| • Glutamine (200mM) | Invitrogen, Rockville, USA |
| • Harris Hematoxylin solution | Sigma, Rockville, USA |
| • IMEM (cell culture medium) | Invitrigen, Rockville, USA |
| • LB broth base | Invitrogen, Rockville, USA |
| • LipofectAMINE™ | Invitrogen, Rockville, USA |
| • Magnesium Chloride (50mM) | Invitrogen, Rockville, USA |
| • Metofane (Methoxyfluorane) | Schering Plough, New York, USA |
| • Mineral oil | Sigma, St. Louise, USA |

| | |
|---|---|
| • PCR 10 x buffer | Invitrogen, Rockville, USA |
| • Tris-(hydroxymethyl-)Aminomethan (Tris) | Roche, Madison, USA |
| • Trypanblue, (0,4 %) | Invitrogen, Rockville, USA |
| • Tween20 | Bio-Rad Laboratories, Hercules, USA |
| • WST-1 | Roche, Madison, USA |
| • Zeocin™ | Invitrogen, Rockville, USA |

### 2.1.3. Buffers and Solutions

| | |
|---|---|
| • 1 x PBS (phosphate buffered saline) | Invitrogen, Rockville, USA |
| • 10 % SDS (Sodium dodecyl sulfate) | Biofluids, Rockville, USA |
| • 20 x SSC | Biofluids, Rockville, USA |
| • Tris-HCl 1M (pH 7.5) | Biofluids, Rockville, USA |

DAB solution

180 ml 1 x PBS

20 ml Tris-HCl (pH 7.5)

250 mg diaminobenzidine in 50 ml of $ddH_20$

25 µl $H_2O_2$

Agarose for Soft agar plates

100 ml IMEM

50 ml 2 x IMEM

20 ml FBS

50 ml Agarose (1.2g agarose in 50 ml $dH_2O \rightarrow$ autoclave)

1 % Agarose Gel

1g Agarose

99g $dH_2O$

1µl Ethidium bromide

PCR Reaction Mix (for 11 reactions)

| | |
|---|---|
| Water | 368.5µl |
| PCR 10 x Buffer | 55µl |
| $MgCl_2$ (50mM) | 33µl |
| dNTP (10mM) | 11µl |
| Primer up | 11µl |
| Primer down | 11µl |
| Taq polymerase | 5.5µl |
| | **45µl** $\rightarrow$ into a PCR tube + 5µl of DNA template |

## 2.1.4. Kits

| | |
|---|---|
| • Apoptag® | Invitrogen, Rockville, USA |
| • Atlas™ cDNA expression array | Clontech, Palo Alto, USA |
| • Qiaquick gel extraction kit | Qiagen, Valencia, USA |
| • *redi*prime™II (random prime labelling system) | Amersham, Piscataway, USA |
| • RNASTAT-60 | Tel.Test-Inc., Friendswood, USA |
| • TACS™ Annexin V-FITC kit | Trevigen, Gaithersburg, USA |
| • PKH26 Red Fluorescent Cell Linker Kit | Sigma, St. Louise, USA |
| • PKH2 Green Fluorescent Cell Linker Kit | Sigma, St. Louise, USA |

## 2.1.5. Equipment and other materials

**Equipment**

| | |
|---|---|
| • 0.5-10, 10-100 and 100-1000µl pipettes | Eppendorf, Westbury, USA |
| • Dry bath incubator | Fisher scientific, Needham-Heights, USA |
| • DU 640® Spectrophotometer | Bekton Coulter, Sommerset, USA |
| • EasyCast™ horizontal electrophoresis system | Owl seperation systems, Portsmouth, USA |
| • FACStar flow cytometer | Becton & Dickinson, Franklin Lakes, USA |

| | |
|---|---|
| • Microcentrifuge 5417 C/R | Eppendorf, Westbury, USA |
| • Mini Trans-Blot® Electrophoretic transfer cell | BioRad, Hercules, USA |
| • Mini-Protean® II electrophoresis cell | BioRad, Hercules, USA |
| • Power Pac 300 (power supply) | BioRad, Hercules, USA |
| • RoboCycler® Gradient 40 temperature cycler | Stratagene, La Jolla, USA |
| • Rocker II | Boekel, Feasterville, USA |
| • Rotina 46 R (cell culture centrifuge) | Hettich, Tuttlingen, Germany |

**Other materials**

| | |
|---|---|
| • Falcon 100 x 15mm cell culture dishes | Becton & Dickinson, Franklin Lakes, USA |
| • Falcon 15ml tubes | Becton & Dickinson, Franklin Lakes, USA |
| • Falcon 50ml tubes | Becton & Dickinson, Franklin Lakes, USA |
| • Falcon 6-well-cell culture pates | Becton & Dickinson, Franklin Lakes, USA |
| • Falcon 96-well-cell culture plates | Becton & Dickinson, Franklin Lakes, USA |
| • ImmobilonP™ transfer membrane | Millipore, |
| • Nylon membranes (positively charged) | Roche, Madison, USA |
| • Falcon T25 and T75 cell culture flasks | Becton & Dickinson, Franklin Lakes, USA |
| • Falcon Ten-twenty-nine™ petri dishes | Becton & Dickinson, Franklin Lakes, USA |
| • Tris-HCl 4-15 % pre-cast gels | BioRad, Hercules, USA |
| • Falcon 0.5ml microcentrifuge tubes | Becton & Dickinson, Franklin Lakes, USA |

- Falcon 1.5ml microcentrifuge tubes — Becton & Dickinson, Franklin Lakes, USA
- Falcon 2,5,10 and 25ml aspiration pipettes — Becton & Dickinson, Franklin Lakes, USA

## 2.2. Methods

### 2.2.1. Generation of Constructs

The ultimate goal was to generate a clone were we are able to regulate the levels of CEA by treating the cells with or without tetracycline.

Plasmids expressing the tetracycline transactivating (tTA)/VP16 fusion protein (pUHG15-1) and the tTA/heptameric operator binding site (tet-O; pUHC13-3) were obtained from Dr. Bujard (Heidelberg, Germany). The ribozyme expression plasmide (pTET) was derived from pUHC13-3 were the CEA mRNA-targeted ribozyme Rz2113 was expressed under the control of the tTA/heptameric operator binding site and a cytomegalosvirus (CMV) minimal promoter. (Gossen M. and Bujard H. 1992).

The major portion of the luciferase gene and the simian virus 40 polyadenylation site in the pUHC13-3 plasmid were deleted by *Hind*III/*Hpa*I digestion and replaced with the Rz2113/bovine growth hormone polyadenylation *HindIII-Pvu*II fragment from the pRz2113 expression vector (Schulte A et al 1996). The remaining luciferase start codon was replaced by *Sal*I/*Cla*I/*Hind*III cassette to yield the construct pTET/Rz2113.

The following ribozyme coding sense and antisense oligonucleotides were annealed and ligated into the HindIII- and NotI-restriction site of pTET:

5’agctt**TGCTCTT***CTGATGAGTTCCGTTAGGACGAA***ACTATGGA**gggcc-3’ (sense) and

5’-c**TCCATAGT***TTCGTCCTAACGGACTCATCAG***AAGAGCA**a-3’ (antisense)

Lower case letters indicates HindIII-/*Not*I restriction site overhangs, bold capital letters shows CEA specific antisense regions and italic capital letters indicates the hammerhead ribozyme core sequence.

The resulting ribozyme expression plasmid pTET/Rz2113 contains CEA specific antisense flanking regions of 7 nt on 5' and 8 nt on 3' ends of the 22 nt catalytic hammerhead ribozyme core sequence, that target it to the B3 domain of CEA.
Additionally, the ribozyme DNA was ligated into the pRc/CMV vector which allows performance of an *in vitro* cleavage assay.

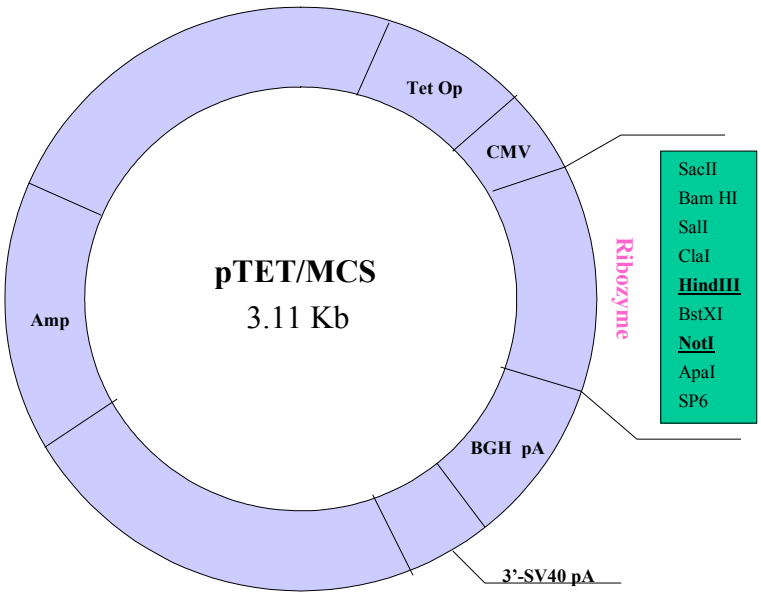



**Figure 3:** Ribozyme expression plasmid pTET/Rz2113.

## 2.2.2. *In Vitro* Cleavage Assay

The cleavage assay was done in order to demonstrate that our ribozyme is actually cleaving our gene of interest (the mRNA of CEA). In order to do that a smaller *in vitro* transcripts of the full length CEA sequence was generated and incubated it for different amount of time (from 0 up to 12 hours). Aliquots were taken out of the reaction and visualized by silver staining.
To generated smaller *in vitro* transcripts, the full length CEA sequence which was ligated into the pBluescript II KS (+/-) vector was cut with *Not*I and *PST*I

restriction enzymes. This yielded a 765 bp CEA fragment containing the recognition sequence for Rz2113 which was religated in a pBluescript SK (+/-) vector and linearized by *Not*I. *Apa*I was used to linearize the pRc/CMVRz2113 vector. The enzymes were heat inactivated and the transcripts were refined using a Chroma SPIN-30+DEPC-$H_2O$ column. A run-off transcription reaction for the ribozyme and target RNA was carried out with T7 RNA polymerase using a MAXIscript Transcription Kit. After DNA digestion (DnaseI treatment) transcripts were refined with Chroma columns. The purified RNA products were combined (100 fold molar excess of ribozyme transcripts) and resuspended in a 50µl reaction volume containing 50mM Tris-HCl (pH 7.5) and 1mM EDTA and heated 3 minutes at 95°C and quick-cooled on ice. The reaction was started by addition of $Mg_2Cl_2$ (0-20mM) following incubation at 37°C (Palfner et al). As a negative control the same amount of CEA RNA was incubated under the same conditions without the ribozyme.

Aliquots of 10µl were removed after 0.5, 1, 2, 4, and 12 hours and the reaction was stopped by the addition of Ambion Loading buffer II including 40mM EDTA and stored at -80°C. Samples were visualized by silver staining according the manufacturers protocol with the exception that 2mg/l $Na_2S_2O_3$ was added to the developing reaction to reduce background staining.

## 2.2.3. Cell Lines and Transfection

Human HT29 colon cancer cells were obtained from the American Type Culture Collection (ATCC) and were maintained in continuous culture at 37°C/5% CO2 using IMEM supplemented with glutamine 1% and 10% heat-inactivated fetal bovine serum (FBS). Murine MC38 colon cancer cells and human CEA expressing MC38 cells were kindly provided by Dr. J. Shively. MC38 cells were stable transfected by electroporation using a eukaryotic expression vector (neomycin resistance gene) which contained the full length cDNA of human CEA. CEA expressing clones were obtained after G418 selection. CEA expression levels exceeded the CEA expression of HT29 cells by a factor of 2 determined by FACS analysis.

HT29 cells were transfected in a 96-well-plate using LipofectAmine. Cells at 50-70% confluency were incubated for 5 hours with plasmid DNA mixed with LipofectAmine in a ratio of 1:10 (9µl LipofectAmine/1µg plasmid DNA) in serum-free Opti-MEM medium at 37°C in 5% $CO_2$. The transfection medium was the replaced with normal growth medium and 36 hours later supplemented with the respective drugs for selection of stable integrants. HT29 stable expressing tetracycline regulated CEA targeted ribozymes were generated in a two-step transfection protocol. In a first step, HT29 cells were transfected with 9µg of pUHG15-1 plasmid DNA and 1µg of pRc/CMV plasmid DNA to provide Geneticin (G418) resistance. After selection for stable integrants in the presence of G418 at 0.7mg/ml individual tTA expressing clones were isolated. To test the clones for tTA expression and tetracycline regulation, a luciferase assay was performed.
Clone HT29/tTA-5 demonstrating the best tetracycline regulation of luciferase activity was used for further transfections with the ribozyme expression plasmids. HT29/tTA-5 cells were then transfected with 9µg of pTET/Rz2113 mixed with 1µg of pZeo to provide Zeocin resistance. Clones, obtained after selection with 0.4mg/ml Zeocin and 1µg/ml tetracycline, were screened for tetracycline regulated CEA expression using FACS analysis. Each clone was screened three times in order to get reliable results. Two clones, showing best tet-regulation of CEA (HT29/Rz4 and HT29/Rz4-2), and were used for further experiments.

## 2.2.4. Luciferase Assay

Luciferase refers to a family of enzymes that catalyse the oxidation of various substrates (e.g. luciferin), resulting in light emission. Luciferases are used in expression assays, where the luciferase acts as a "reporter" for the activity of any regulatory elements that controls its expression.
To test the clones for tTA expression and tetracycline regulation, the cells were transiently transfected with pUHC13-3 plasmid DNA that contains a luciferase cDNA under the control of the tet-O binding site and cultured in the absence and presence of 1µg/ml tetracycline, respectively. For this cells were plated overnight at 60-70% confluency in six-well plates and then transfected in Optimem with 1µg of

DNA per well using 7µl of LipofectAmine. After 5 hours transfection medium was replaced by fresh culture medium and the cells were incubated for another 36 hours. After incubation cells were harvested, washed, lysed in 0.25 M Tris·HCl (pH 7.8) and freeze-thawed three times. 5-20µl of the lysate was mixed thereafter with 350µl of 0.1 M potassium phosphate/15 mM $MgCl_2$/5 mM ATP at pH 7.8 and assayed for luciferase activity using 1mM D-luciferin as a substrate. A cytomegalovirus-driven vector was used to control for transfection efficacy (Schulte A et al).

## 2.2.5. Northern Analysis

Total cellular RNA was isolated by using the commercially available RNA STAT-60 method and protocol. 30µg of total RNA were mixed in RNA loading mix, 17.5 % formaldehyde, 50 % formamide, 10 % RNA-formaldehyde loading buffer heated to 65°C for 10 minutes and separated by electrophoresis in 1.6 % formaldehyde-agarose gel and then blotted onto nylon membranes. The blots were prehybridized in 6 x SSC, 0.5 % (w/v) SDS, 5 x Denhardt's solution for 4 hours at 68°C and hybridized overnight at 68°C in hybridization solution (same composition as the prehybridization solution with addition of 0.01 M EDTA (pH 8.0)) containing a [α-32P]dCTP-labeled CEA cDNA probe (541 nt PstI fragment) This probe was prepared by using a commercially available random primed DNA labeling kit.
After hybridization, blots were washed once with 1 x SSC and 0.1 % SDS for 20 minutes, once with 0.2 x SSC and 0.1 % SDS for 20 minutes at room temperature, and once for 20 minutes at 68°C (Fang WJ et al). Blots were then autoradiographed using intensifying screens at -70°C for 16 hours. To correct for variability in loading 18s RNA bands were used or blots were stripped and re-probed with a Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) cDNA probe. Relative band intensities were measured by densitometry.

**RNA isolation**

RNA was isolated from cell lines using RNA-STAT60, which is based on the single step method of RNA isolation developed by Chomczynsky and Sacchi (CHOMCZYNSKY, P. and SACCHI, N., 1987).
Cells were grown in 15 cm tissue culture dishes to approximately 80 % confluence. Medium was aspirated and 1ml of RNA-STAT60 was added onto the cells. Cells were scraped off the plate, transferred to a microcentrifuge tube and allowed to incubate at room temperature for 5 minutes. 0.2ml of chloroform was added to each sample and vortexed for 10 seconds and incubated at room temperature for 3 minutes. Next, a 15 minute centrifugation at 15.000g was performed at 4°C. After centrifugation the RNA-containing aqueous phase was transferred to a fresh microcentrifuge tube. To precipitate the RNA, 0.5 ml of isopropyl alcohol was added, vortexed shortly, incubated at room temperature for 5 minutes and centrifuged at 15.000g for 10 minutes. The visible pellet (RNA) was washed with 80 % ethanol and centrifuged for 5 minutes at 7.500g. Supernatant was discarded and dry pellet was dissolved in 20-50µl of DEPC-treated water and stored at -80°C. RNA concentration was determined via UV spectrophotometry.

**Preparation of radioactive probes**

CEA probe

The CEA probe was prepared from a pBluescript II KS vector, which contained full length cDNA of CEA. The plasmid was kindly provided by M. Neumaier University of Hamburg. A fragment of 541 nt was isolated by restriction enzyme digestion with PSTI followed by separation on a 1 % agarose gel. The DNA fragment was then cut out of the gel and extracted from the gel with a commercially available kit (QiaQUICK gel extraction kit).

Agarose gel extraction

Extraction of DNA fragments from an agarose gel was performed using the QiaQUICK gel extraction kit. DNA was sliced out from the gel and weighed. 3 volumes of a provided solubilizing buffer were added per 1 volume of gel. Next, a 10 minutes incubation at 50°C was carried out. Afterwards samples were centrifuged and the supernatant was discarded. The pellets were washed once with a high salt buffer to remove residual agarose and twice with ethanol containing buffer to remove salt contaminants. Residual ethanol was removed by air drying. DNA was eluted by resuspending the pellet in water. The concentration of the purified DNA was measured by UV spectrophotometry.

GAPDH probe

GAPDH cDNA probes were used to standardize for RNA loading in Northern analyses. A 1.1 kb fragment for GAPDH cDNA was purchased from Clonetech.

CEA and GAPDH probes were generated by random prime labeling.

**Random prime labeling of cDNA**

The commercially available labeling system was used to generate radioactive labeled probes from cDNA. 50ng of cDNA was brought to a final volume of 45µl and boiled for 5 minutes (denaturation of cDNA) and then put on ice for 2 minutes. Next, the cDNA was added to a reaction mix containing dNTP, random primers and Klenow enzyme. 5µl of α-32P-dCTP was added to the reaction, which was then incubated at 37°C for 30 minutes. The unincorporated nucleotides were removed by Sephacryl HR resin columns. Activity was measured in a scintillation counter.

## 2.2.6. Fluorescence Activated Cell Sorting (FACS)

FACS analysis is the measurement of cellular properties as they are passing through a laser. The emitted light from the excitated fluorochrome (target) is registered and quantified on a graphic.

For the FACS analysis cells were detached using 0.02 % EDTA in PBS, washed with ice cold PBS containing 7.5mM sodium azide and 5 x $10^5$ cells were incubated with 2µg of anti-CEA antibody for one hour at 4°C. The cells were then washed three times with ice cold PBS and then incubated for an additional 30 minutes with 1:100 diluted DTAF-conjugated goat anti-mouse IgG + IgM antibody in the dark and at 4°C. After two final washings, cells were resuspended in 300 µl of PBS and fixed by the addition of 100µl of 4 % paraformaldehyde. The mean values of the fluorescence intensity were determined by FACS analysis. Unlabeled cells and cells labeled only with the secondary antibody served as a negative control.

## 2.2.7. Western Blot Analysis

The western blot is a method to detect and quantify proteins from cells or tissues. Proteins are blotted onto a membrane and visualized by antibodies which bind to their target.

Cells were grown to approximately 80 % confluency and harvested by scraping them in ice cold PBS on ice. They were pelleted by centrifuging at 100g for 2 minutes and lysed in lysis buffer. Cells were incubated on ice for 10 minutes, vortexed briefly and spun down at 15000 g and 4 °C for 10 minutes. The supernatant, containing the protein, was transferred to a clean 1.5ml Eppendorf tube and protein levels were measured by Bradford assay.

Equal amount of total protein (40µg), mixed in a 1:3 ratio in 3 x loading buffer, were loaded into pre-cast 4-20 % gradient Tris-glycine polyacrylamide gels and gels were run at 130 V in Running buffer.

Gels were transferred onto Immobilon-P nylon membranes for 3 hours at 150 mA per gel, the membranes were dried overnight, rehydrated, and blocked for 1 hour in PBST and 5 % nonfat dry milk.

Membranes were probed with a 1:500 diluted monoclonal mouse antibody to CEA for two hours at room temperature. Membranes were washed 4 times in PBST 5 min. each, followed by incubation with 1:5000 diluted rabbit anti-mouse IgG antibody conjugated to horseradish peroxidase. Immunoreactive bands were visualized by enhanced chemiluminescence

## 2.2.8. Aggregation Assay

To determine aggregate formation a modified soft agar assay described below (**2.2.14**) was performed. Tumor cells were incubated under continuous agitation in complete medium and fixed after the incubation time in a layer of soft agar.
Therefore, single cell suspensions of HT29 cells were prepared by drawing cells through a 30G x “needle. Cells were kept in suspension for 20 minutes in a rotating advice at 37°C to allow aggregation. After incubation period cells were seeded into liquid agar at 42°C. The soft agar solidified an image analyzer was used to determine the number of aggregates larger than 60 μm in diameter which corresponds to a cluster of at least 10 cells.

## 2.2.9. Tumor/Endothelial Cell Adhesion Assay

This assay was performed to determine the effect of endogenous CEA on the binding of HT29 cells to endothelial cells (HUVEC).
HT29/Rz4 cells and HUVEC cells were labeled with different commercially available fluorescent dyes (red and green) and recultivated over night to recover the cells from the labeling procedure. The following day single cell suspension of cells were prepared and kept in suspension for 0, 20 minutes, 1 and 2 hours in an rotating advice at 37°C to allow adhesion. Adhesion of HT29 cells to HUVEC cells were determined by running cell suspension after different time points through a flow cytometry and counting red and green fluorescent cell clumps.

## 2.2.10. cDNA Microarray

The principle of a microarray is that mRNA from a given cell line or tissue is used to generate a labeled sample, which is hybridized in parallel to a large number of DNA sequences immobilized on a solid surface.

For cDNA microarray analysis, the "Atlas™ Human Cancer cDNA Expression Array" was used which covers 588 cancer related genes spotted on positively charged nylon membranes and arranged in 13 functional groups (cell cycle/growth regulators, intermediate filament markers, apoptosis, oncogenes/tumor suppressors, DNA damage response/repair and recombination, cell fate and development, receptors, cell adhesion and motility, angiogenesis, invasion regulators, cell-cell interactions, Rho family and small GTPases, growth factors and cytokines). Plasmid and bacteriophage DNAs are included as negative controls to confirm specificity, along with several housekeeping cDNAs as positive controls. The microarray analysis was performed according to the manufactures guidelines. HT29/Rz4 cells were cultivated in culture medium. 12, 24, and 48 hours before harvesting equivalent amounts of cells were distributed in 6 culture flask and 1 μg/ml tetracycline was added in 3 flask to block ribozyme expression. Cells were detached by 0.02 % EDTA/PBS and cells with and without tetracycline treatment were pooled. The cells in all flasks were at comparable levels of confluence at the time of harvesting. HT29/tTA5 cells were treated in the same way and served as a control.

RNA was extracted according to the manufacturers protocol, which is a phenol:chloroform based method, followed by a 30 minutes incubation at 37 °C with DNAse I. A Northern Blot for CEA was performed to confirm intact RNA and regulation of CEA levels before experiment were continued.

For cDNA synthesis 50μg total RNA was used which was converted to $^{32}$P-labeled first strand cDNA by means of SuperScriptII reverse transcriptase. Unincorporated nucleotides were removed by CHROMA SPIN-200 column chromatography. The first two fractions with highest activity were pooled. Equivalent amounts of cpm were used to minimize loading differences. Pre-hybridization of the membrane was done for 30 minutes with continuous agitation at 68°C in ExpressHyb supplemented with 200μg/ml salmon sperm DNA. After pre-hybridization heat

denaturated $^{32}$P-labeled probe was added. Hybridization was performed over night at 68 °C with continuous agitation. The following day the membrane was washed four times with 200 ml or pre-warmed Wash Solution 1 for 30 minutes at 68°C with continuous agitation. After washing the membrane four times with Wash Solution 1, membrane was washed one time with Wash Solution 2 for 30 minutes at 68°C again with continuous agitation. A final wash of 5 minutes with 2 x SSC was done at room temperature.
The hybridization was analyzed by autoradiography followed by phosphoimager analysis for quantification.

## 2.2.11. Cell Cycle Analysis

Cell cycle analysis was performed by the Vindelov staining method (Vindelov et al 1983), which is a method based on detergent lysis of cell membranes and production of stained nuclei in monodisperse suspension in a single step.
Tetracycline treated and untreated cells were harvested, 2 x $10^6$ cells were resuspended in 100µl of 40mM citrate/DMSO buffer. Solution A (1800µl) was added to 200µl of the cell suspension in citrate buffer and the tube was inverted to mix the contents gently. After 10 minutes at room temperature, during which the tube was inverted five to six times, 1500µl solution B was added. The solutions were again mixed by inversion of the tube and after additional 10 minutes at room temperature 1500µl ice-cold solution C was added. The solutions were mixed and the samples were filtered through a 30µm nylon mesh into tubes wrapped in aluminum foil for light protection of the PI. Cell cycle analysis was performed by flow cytometry.

## 2.2.12. Proliferation Assay

Cell numbers were determined by a colorimetric assay, based on the cleavage of the tetrazolium salt WST-1 in viable cells, according to the protocol of the

manufacturer. Tetrazolium salts are metabolically reduced by cellular enzymes to highly colored end products called formazans.

5 x $10^3$ cells were cultured in microtiter plates (96 well plates) in a final volume of 100µl/well culture medium and standard culture conditions. Cells were divided into two groups. One group being treated with 1µg/ml tetracycline per well and the other group without tetracycline treatment. After incubation period of 3 days 10µl of WST-1 reagent was added into each well and incubated for 30 minutes in a humidified atmosphere at 37°C. The absorbances of the samples were determined at 450nm using ELISA microreader. A well with 100µl culture medium and 10µl WST-1, but without cells worked as a background control.

A time kinetic over 3 days was also performed in the same way as described above.

## 2.2.13. Determination of Apoptosis

Apoptosis is known to be an important factor in tumor growth. Determination of apoptosis was performed by using a commercially available AnnexinV kit. AnnexinV is a calcium-dependent phospholipid binding protein, with a high affinity for phosphatidylserine (PS). During apoptosis, an early and ubiquitous event is the exposure of phosphatidylserine at the cell surface. Since necrotic cells are leaky enough to give access to inner membrane PS, apoptotic cells were differentiated from necrotic cells by simultaneous staining with Annexin V and the DNA stain propidium iodid.

HT29/Rz4 cells (1 x $10^6$), treated with and without tetracycline, were harvested, washed twice with 500µl cold PBS and resuspended in 100µl of propidium iodide-AnnexinV-FITC dual staining solution as described in the manufacturers manual and incubated in the dark for 15 minutes at room temperature. 400µl 1 x binding buffer was added to cell suspension and cells were analyzed by flow cytometry.

## 2.2.14. Colony Formation Assay

To determine colony formation a modified soft agar assay described previously (JUHL et al 1997) was performed.

24 hours before the actual assay, cells were divided into two groups, one group receiving 1 μg/ml tetracycline. 5 x $10^3$ single cells in 0.35 % agar were layered on top of 1ml of solidified 0.6 % agar a layer in a 35mm dish. Growth media (IMEM) with 10 % FBS were included in both layers. In addition 1μg/ml tetracycline was added into the agar of tetracycline receiving group. Colonies more than 80μm in diameter were counted after 0, 1, and 2 weeks of incubation time using an image analyzer.

## 2.2.15. Animal Experiments

4-6 weeks old Ner nude mice were obtained from the NCI (Frederick, MD). Mice were housed at standard temperature (22°C), humidity (50-60%) and light (light-dark cycle 12:12 hours) environment with free access to food and water. Animals were kept at least three days according to the standards of good animal practice before use in experiments. All experiments were approved by the Ethics Committee for Animal Experiments, Georgetown University.

HT29/Rz 4 cells, which were treated 24 hours before the assay with or without tetracycline to modulate CEA levels, were resuspended into 200μl complete medium and injected into the tail vein of mice. To block ribozyme expression one group of mice obtained doxycycline enriched food (a tetracycline analogue which is absorbed by the intestine). The second group obtained regular non-doxycycline enriched food. After 30minutes, 24 hour and 6 weeks mice were sacrificed, the lung were removed and embedded in paraffin.

The numbers of metastatic lesions were visualized and quantified by cytokeratin (Kl-1) staining using light microscopy (10 x magnification) on all 5 different lung section per mice.

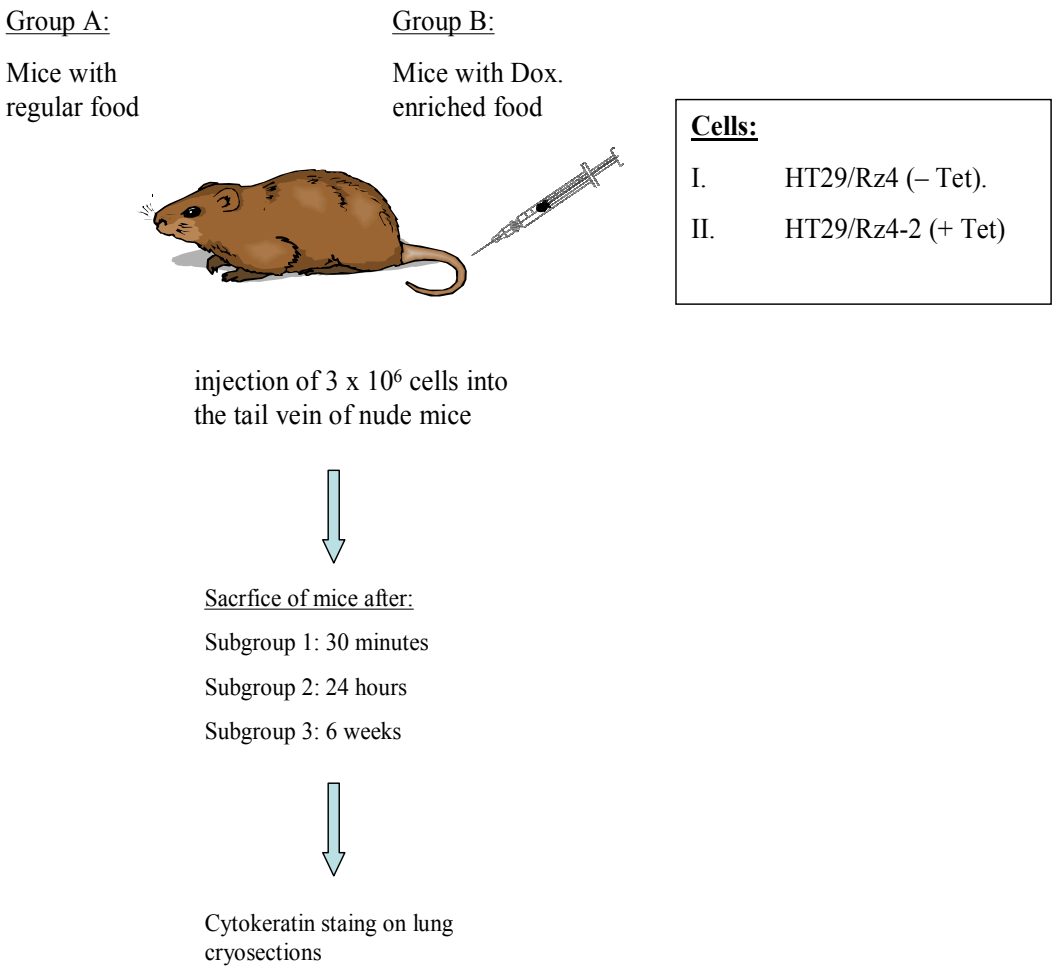



**Figure 4:** Schematic of animal experiment. Group B was divided into two subgroups. The first subgroup was injected with non-tetracycline pre-treated HT29/Rz4 cells, and the second subgroup was injected with HT29/Rz4 cells pre-treated with tetracycline.

## 2.2.16. Immunohistochemistry

Tissue slides were obtained from the Histopathology and Shared Tissue Resource of Georgetown University Medical Center as formalin-fixed, paraffin-embedded sections.

Paraffin sections (5µm) were performed at 5 different regions of the lung. Immunostaining was used to identify metastatic lesions. Kl-1 anti-human

cytokeratin antibody which does not cross react with murine cells was applied to identify human epithelial cells.
Slides were baked at 56°C over night, microwaved for 3 minutes and deparaffinized by xylene and stepwise passages through decreasing concentration of ethanol.

- 2 x 5 minutes in xylene
- 2 x 5 minutes in 100 % ethanol
- 1 x 10 minutes in 100 % ethanol
- 1 x 1 minute in 90 % ethanol
- 1 x 1 minute in 80 % ethanol
- 1 x 1 minute in 70 % ethanol
- 1 x 1 minute in dd$H_2O$

Then slides were placed into a 0.3 % $H_2O_2$ solution for 20 minutes at 4°C to quence endogenous peroxidase, followed by a washing step of 3 times in PBS for 5 minutes. Slides were then blocked in 1 % BSA/PBS for 15 minutes at room temperature and incubated with the primary antibody (Kl-1) diluted 1:250 in 1 % BSA/PBS for 2 hours at room temperature. After washing the slides 3 times with PBS for 5 minutes slides were incubated with the secondary antibody (peroxidase conjugated rabbit anti-mouse antibody) diluted 1:1000 in 1 % BSA/PBS for 1 hour at room temperature. Next, slides were washed 3 times with PBS for 5 minutes and incubated for 5 minutes in a diaminobenzidine (DAB) solution. Slides were counterstained with hematoxylin, washed in dd$H_2O$, dehydrated and mounted in mounting medium.

## 2.2.17. Phage Display

The mean of a phage display is to analyze the interaction of the target protein with other, already known or unknown proteins. A peptide or protein is displayed on the surface of a phage as a fusion protein that is normally found in the phage particle. Phage display has been used to identify peptides that bind to receptors, substrates or inhibitors of enzymes, epitopes, improved antibodies, altered enzymes and cDNA

clones (O'NEIL, K. T. wt al, 1995). The cDNA of a human colon cancer cell line (LS174 T) was screened for interaction of CEA with other proteins. The cDNA library was prepared and kindly provided by A. Wellstein.

**Preparation of bacteria culture**

BLT 5615 (*E.coli)* were grown in 15 % LB media. For each experiment fresh bacteria culture was prepared. LB media was prepared, autoclaved and stored at 4°C. Before use of the bacteria a little amount of bacteria was put into 6 ml of LB media in a 15 ml tube and incubated for 3 hour at 37 °C under continuous strong agitation.

**Phage display**

500ng of purified CEA was immobilized on a 6-well plate containing 2 ml of 1 x PBS over night at 4°C. The following day wells were washed three times with 2 ml of 1 x PBS. After washing, wells were blocked for 4 hours with LB media. Wells were washed five times with 1 x PBS after blocking with LB media. $10^{12}$ phages were put into each well and incubated for 20 minutes at room temperature. After 20 minutes 2 ml of freshly prepared bacteria culture was added into each well and incubated for additional 10 minutes. An aliquot of10µl was taken from each well and used for the titration series. The rest of the phage/bacteria culture was taken out from each well and amplified separately over night in freshly prepared LB media at 37°C.

This procedure was repeated five times (round 1-5). After the last round phages were amplified over night and lysed for PCR.

**Amplification of Phages**

For amplification, phages were incubated over night in a 15 ml tube in 6 ml freshly prepared bacteria culture. Incubation was performed at 37°C under continuous strong agitation. After the incubation period bacterial culture with the phages were

centrifuged at 10.000g for 3 minutes to separate the bacteria lysate from the phage. The supernatant was aliquoted into fresh 2ml tubes and stored at -80°C.

**PCR with Phages as DNA template**

15µl of amplified phages were mixed with 35µl of EDTA resulting in a final concentration of EDTA of 10mM (pH 8). Mixture was heat inactivated for 10 minutes at 65°C. 5µl of the mixture was put into the PCR reaction mix (see material part) and run for 38 cycles. 1 Cycle:

1. 95°C for 60 sec. (denaturation)
2. 37 °C for 60 sec.(annealing)
3. 72°C for 60 sec. (extension)

The final extension was applied for 5 min. and stopped by cooling down to 4°C.

# 3. RESULTS

## 3.1. Cleavage of CEA mRNA

To determine the capability of the ribozyme to cleave the mRNA of CEA the ribozyme was incubated in a 100-fold molar excess with the CEA mRNA for various time points (0.5, 1, 2, 4 and 12 hours). Already after 0.5 hour it was possible to detect the cleaving products of the 814 nt CEA mRNA transcript. The CEA mRNA transcript was completely cleaved by Rz4 in a 412 nt and a 393 nt long fragment after 12 hours (Figure 4).

As a negative control, the same amount of CEA mRNA was incubated under the same condition without the ribozyme. The mRNA was stable and did not degrade during the 12 hours of incubation time. Of note, studies have been done to document the specificity of ribozymes. Werner, M. et al studied the effect of base mismatches in the substrate recognition helices of hammerhead ribozymes on binding and catalysis. They demonstrated that ribozymes lack already cleavage activity if there is a mismatch of two or more nucleotides (WERNER, M. et al 1995).

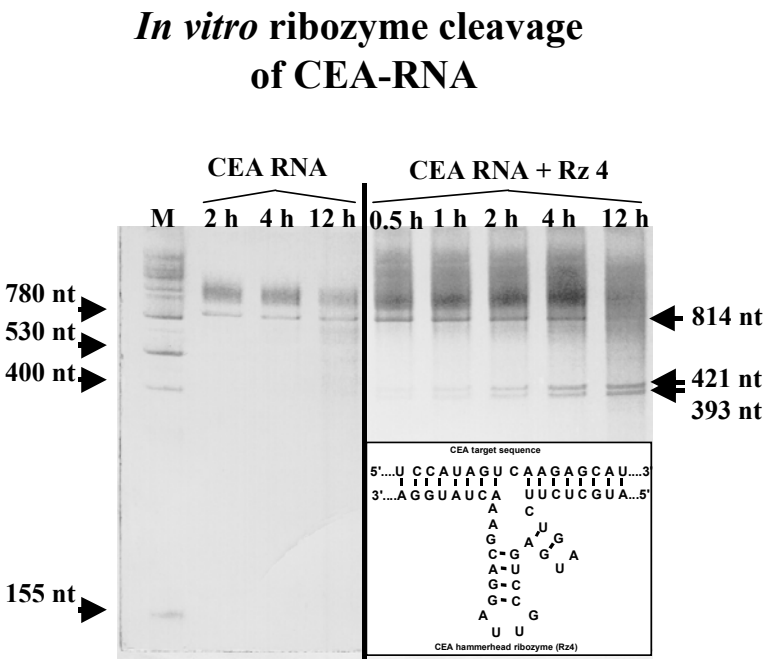



**Figure 5:** *In vitro* cleavage assay to determine the activity of CEA-targeted ribozyme Rz4 (sequence shown in the insert). A CEA transcript of 814 nt was coincubated with 100-fold excess of Rz4 for various time intervals. The expected cleavage products of 421 nt and 393 nt lenght became visible after 1 hour. As a control served CEA RNA without addition of Rz (M= molecular weight marker).

## 3.2. Tetracycline Dependent Regulation of the tTA Protein

A luciferase assay was done in order to determine tetracycline dependent regulation of gene expression in stably transfected HT29 colon cancer cell clones.

After transient transfection of the luciferase gene inserted in the tTA binding vector (pUHC13-3 plasmid) we demonstrated very efficient tetracycline dependent regulation. Luciferase activity was measured 36 hours after transient transfection in the absence and presence of tetracycline.

Figure 5 shows a luciferase assay of HT29 colon cancer cells which stable express the tTA expression vector and enable tetracycline regulated expression of the luciferase reporter gene. From various HT29/tTA clones, which demonstrated tetracycline dependent regulation, clone HT29/tTA-5 showed most efficient tetracycline dependent regulation (by a factor of 100) and was used for further transfection with the ribozyme plasmid.

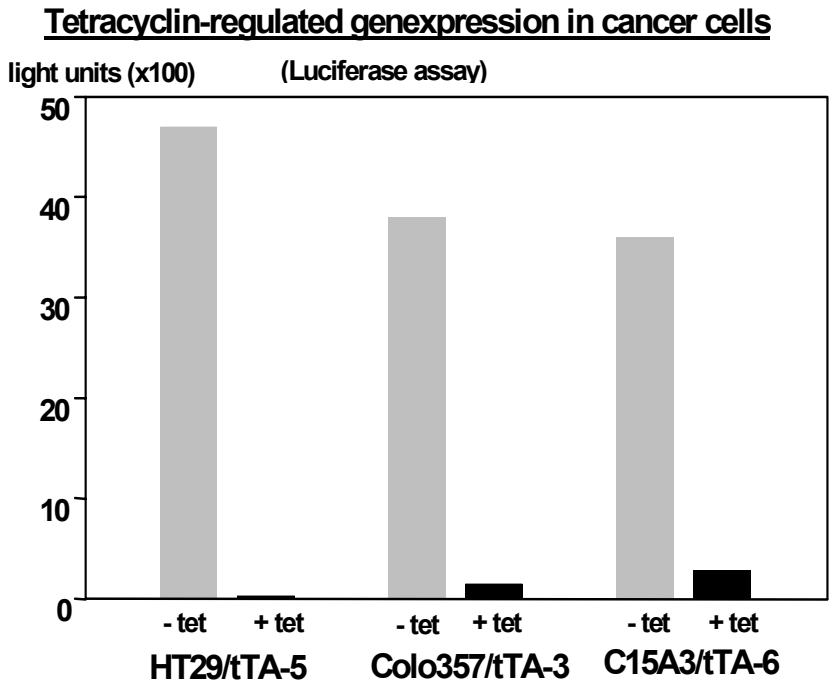



**Figure 6**: Luciferase assay. HT29 cells expressing the tetracycline Transactivator (tTA) protein were transiently transfected with pUHC13-3 plasmid DNA that codes for luciferase under the control of the tet-O binding site. Luciferase activity was measured 36 hours after transfection in the absence and presence of tetracycline (1μg/ml).

## 3.3. Reduction of CEA on the Protein Level

After transfection of HT29/tTA-5 clone with the ribozyme cells were screened for tetracycline dependent CEA regulation by FACS analysis. Each clone was screened three times for reliable results. From two separate transfection experiments 9 out of a total of 70 clones, demonstrated tetracycline dependent CEA down-regulation ranging from 20-50 % by FACS analysis. Two out of those 9 clones showed CEA regulation in a tetracycline dependent manner of ~50 % on the protein level and were therefore chosen for further experiments. Clones were named HT29/Rz4 and HT29/Rz4-2 and both of them were transfected with ribozyme 4.

The tetracycline regulated reduction of CEA expression in HT29/Rz4 and HT29/Rz4-2 cells was confirmed on the protein level by FACS and Western blot analysis in several independent experiments.

Figure 6 shows the results from a FACS analysis. Overall, both clones showed a ribozyme dependent decrease of CEA expression by approximately 50 %. Western blot analysis showed even a reduction of CEA by 70 % (figure 7).

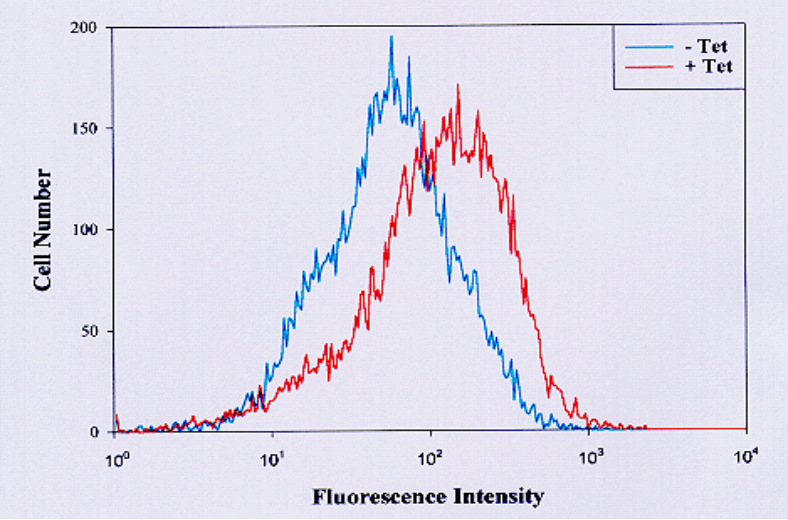



**Figure 7**: FACS-analysis of HT29/Rz4 cells.

To determine the time kinetics of ribozyme activity with CEA translation (protein expression), a Western blot was performed measuring the CEA levels in HT29/Rz4 clone at various time intervals after addition of tetracycline (figure 7).

The Western blot showed antibody staining of exclusively one specific CEA band of Mw 180 kDa. Half of the CEA expression was reversed after 9-12 hours of tetracycline treatment and maximal CEA levels were seen after 24 hours. As a control, CEA levels were measured in HT29/Rz4 cells continuously cultured without tetracycline. It is shown, that these cells had consistently low levels of CEA.

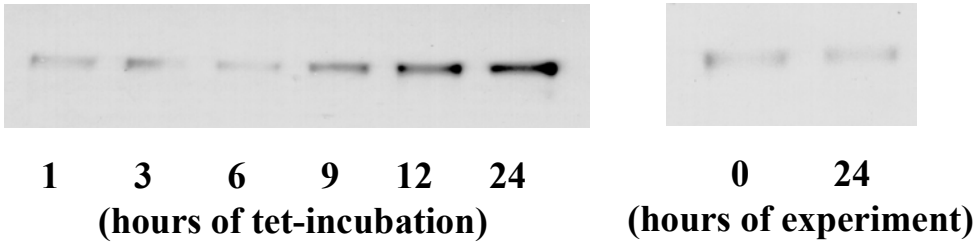



**Figure 8**: Western blot analysis to demonstrate regulation of CEA level in HT29/Rz4 cells. Ribozyme expression reduced CEA levels by approximately 70% (difference between the first and the last time point). Addition of tetracycline inhibited ribozyme expression, and subsequently, restored CEA protein levels. As a control, HT29/Rz4 cells were kept in culture in the absence of tetracycline.

## 3.4. Reduction of CEA on the RNA Level

In accordance with the results from the FACS analysis and Western blot assay we performed a Northern blot to see a tetracycline dependent regulation of CEA on the RNA level. Figure 8 shows a Northern blot analysis with a 50 % reduction of the CEA RNA. As a negative control CEA-non-expressing MC38 murine colon cancer cells were used. MC38 cells stably transfected with a CEA expression vector were used as a positive control. One can see on the picture that the molecular weight of CEA transcript in murine cells were slightly higher than CEA mRNA in human HT29 cells. This is possible because of a longer poly(A)-tail in the murine cell CEA mRNA.

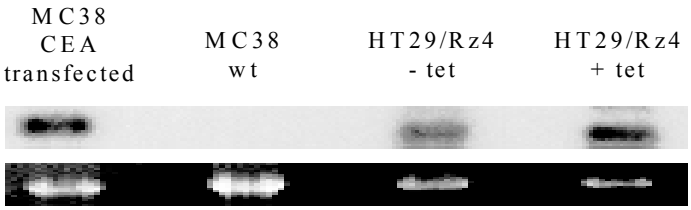



**Figure 9:** Northern Blot analysis. 1 = MC38 murine colon cancer cell line overexpressed with human CEA cDNA, 2 = MC38 cells (CEA negative), 3 = ribozyme expressing HT29/Rz4 (-tet.) and 4 = ribozyme inhibited (+tet.) HT29/Rz4 cell line. Ribozyme expression reduced CEA levels by approximately 50%.

## 3.5. CEA's Role as an Adhesion Molecule

### 3.5.1. Involvement of CEA in Tumor-Tumor Cell Adhesion

Figure 9 demonstrates that CEA contributes to aggregate formation of HT29 colon cancer cells. For determination of aggregate formation a modified soft agar assay was performed. Aggregates >40μm (cluster of at least 4 cells) and aggregates >60μm (clusters of at least 10 cells) were determined. A dramatic reduction of aggregate formation (up to approximately 70 %) of CEA reduced HT29 cells was observed. Tetracycline itself did not affect aggregation formation in HT29/tTA5 wild type cells.

**A.**

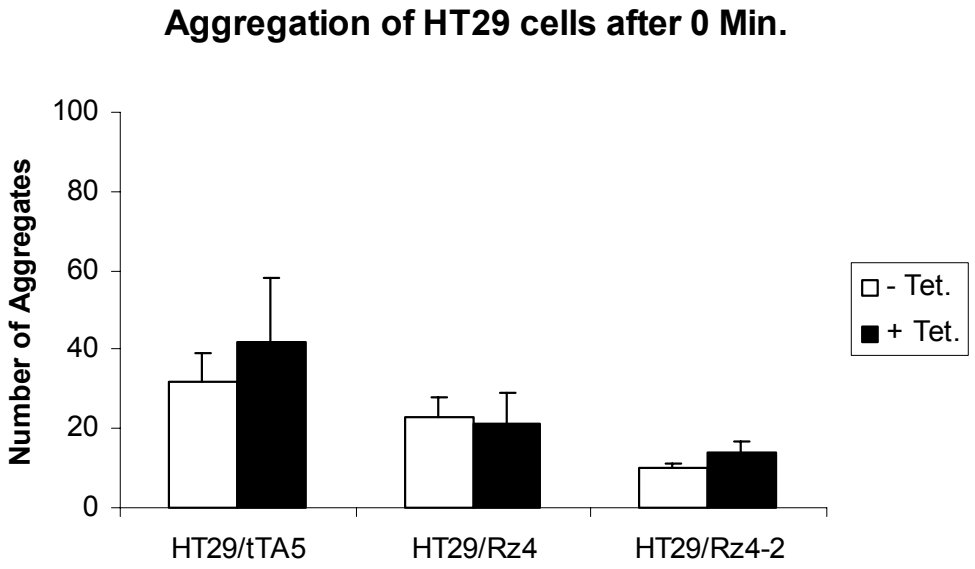



**Figure 10 A:** In vitro aggregation assay to determine CEA dependent aggregate formation of HT29 colon cancer cells. HT29/Rz4 and HT29/Rz4-2 cells were treated with or without tetracycline to modify CEA levels. Cells were incubated for 0 h at 37°C under continuous agitation and seeded in soft agar. The number and size of aggregates were determined. Cell clusters of 60 >μm diameters were determined.

**B.**

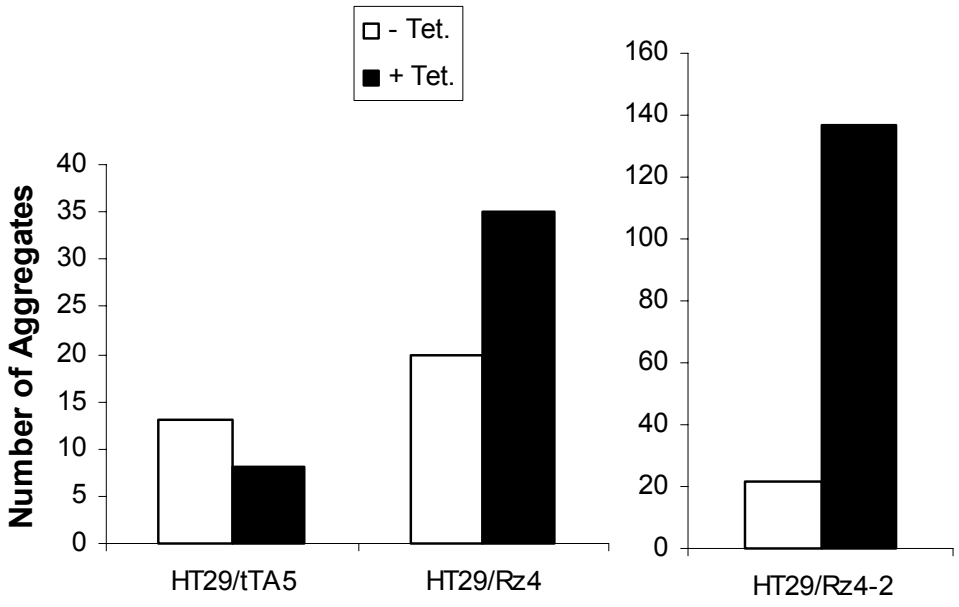



**Figure 10 B:** In vitro aggregation assay to determine CEA dependent aggregate formation of HT29 colon cancer cells. HT29/Rz4 and HT29/Rz4-2 cells were treated with or without tetracycline to modify CEA levels. Cells were incubated for 2 h at 37°C under continuous agitation and seeded in soft agar. The number and size of aggregates were determined. Cell clusters of 60 >μm diameters were determined.

## 3.5.2. Involvement of CEA in Tumor/Endothelial Cell Adhesion

To determine the ability of HT29 cells to adhere to endothelial cells (HUVEC cells) we established a method to determine the adhesive function of CEA to HUVEC cells. We labeled each cell line with a different commercially available fluorescent marker. HT29 cells were labeled with a red fluorescent marker and the endothelial cells with a green fluorescent marker and run through an FACS analyzer to determine the number of red/green labeled aggregates. Regardless of CEA levels tumor cells increasingly bound to endothelial cells.

However, when endothelial cells were treated with IFN-γ for 24 hours before the actual adhesion assay there was a decrease of adhesion over time in those cells which had normal CEA levels (cells treated wit tetracycline). CEA HT29 reduced cells showed increased endothelial cell binding (figure 11). In the control group tetracycline itself did not have any effect on adhesion to endothelial cells.

**A.**

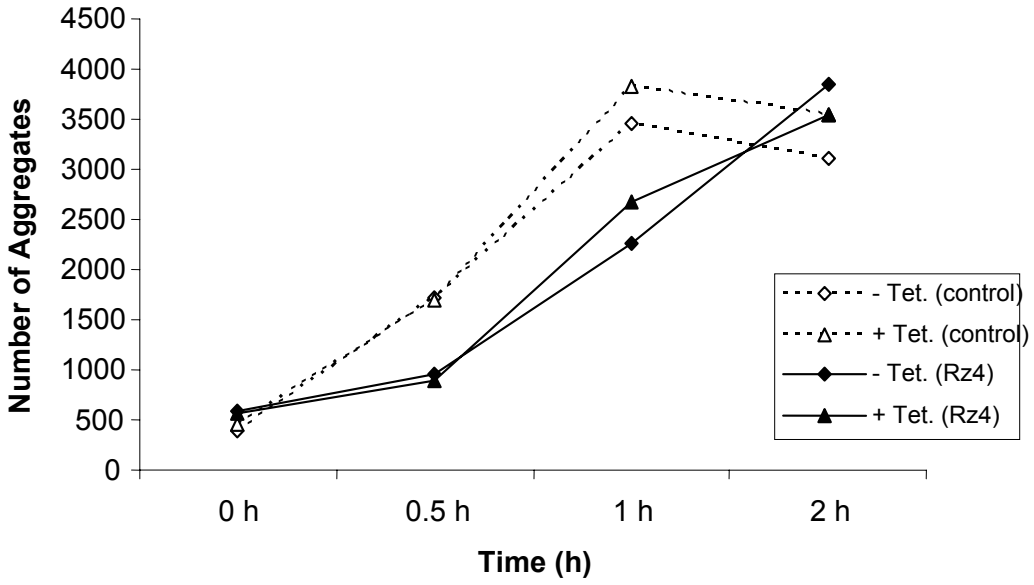
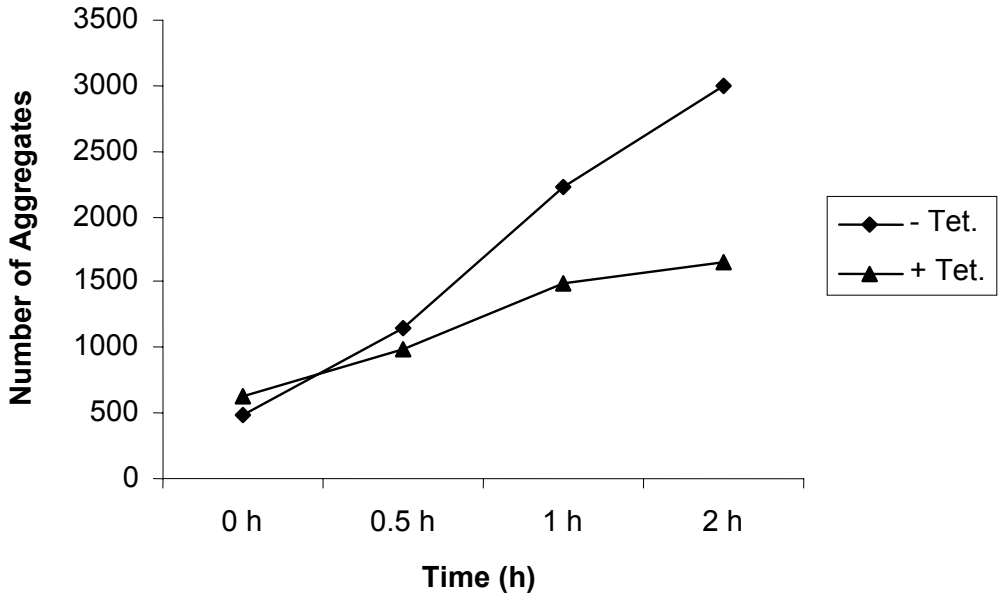



**Figure 11 A:** Adhesion of HT29/Rz4 cells to HUVEC cells treated with 1000 U of Interefron-γ. Each cell line was labeled with a fluorescent marker (red or green). HT29/Rz4 cells were treated with or without tetracycline and incubated for 0 to 2 hours with HUVEC cells at 37°C under continuous agitation. Cell clusters (red and green) were determined with a FACS analyzer. **A)** HT29/Rz4 with HUVEC cells, without Interefron-γ treatment

**B.**




**C.**

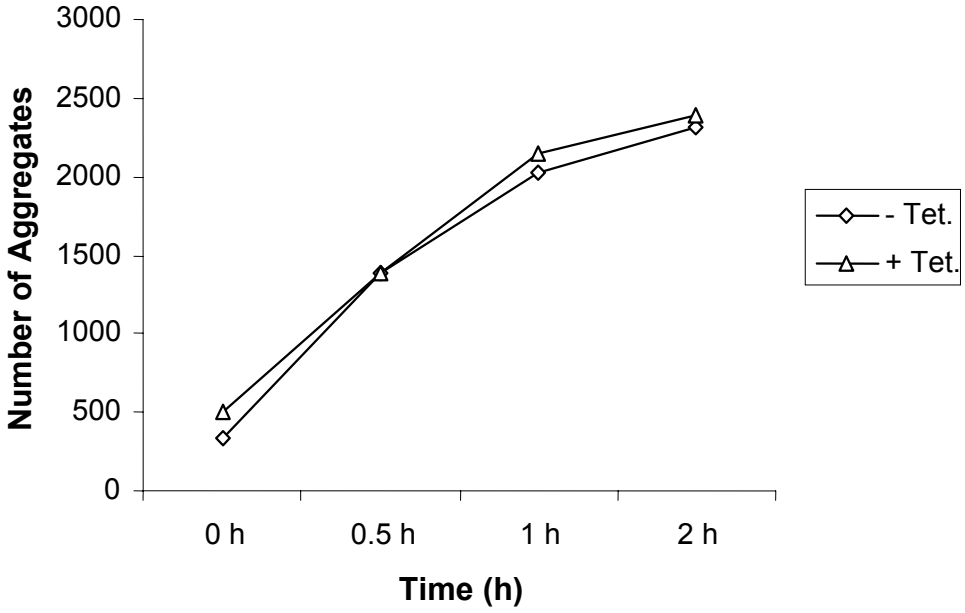



**Figure 11 B + C:** Adhesion of HT29/Rz4 cells to HUVEC cells treated with 1000 U of Interefron-γ. Each cell line was labeled with a fluorescent marker (red or green). HT29/Rz4 cells were treated with or without tetracycline and incubated for 0 to 2 hours with HUVEC cells at 37°C under continuous agitation. Cell clusters (red and green) were determined with a FACS analyzer. **B)** HT29/Rz4 with HUVEC cells treated with Interefron-γ. **C)** Adhesion of HT29/tTA-5 cells (control cells without ribozyme) to HUVEC cells treated with 1000 U of Interefron-γ.

## 3.6. CEA Dependent Gene Expression

The gene expression of HT29 cells were studied using the cDNA gene expression array. The mRNA levels of HT29/Rz4 cells were analyzed comparing ribozyme expressing cells (cells without tetracycline treatment) and ribozyme depressed cells (cells treated with tetracycline) (figure12).

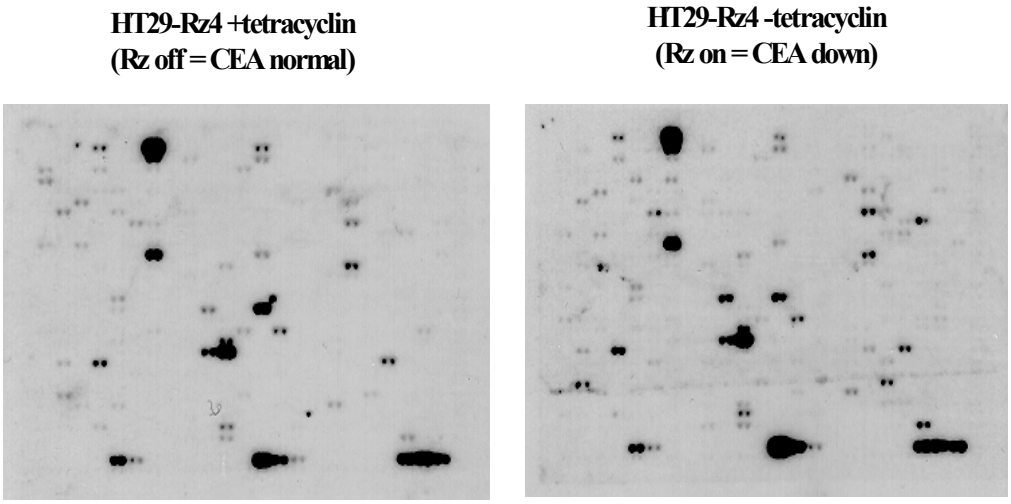



**Figure 12:** Microarray with HT29/Rz4 cells. Cells were incubated 24 hours before RNA extraction with or without tetracycline. Two seperated microarrays were performed (one with cells treated with tetracycline and one array with cells without tetracycline treatment).

To exclude potential influence of tetracycline on gene expression, we analyzed the wild type cells treated with and without tetracycline in parallel. Signals with an intensity >1000 were regarded as reliable signals.

Reliable signals were available for 273 out of 588 genes. The signal intensity on each membrane was adjusted to houskeeping genes. A shift of gene expression by a factor of 1.5 was regarded as significant. In that case 134 genes were affected by CEA. The change of CEA level resulted in a different gene expression in virtually all group of genes, including: cell cycle, cell fate development regulators, growth factors/cytokines, Rho family, invasion regulators, angiogenesis regulators, cell

adhesion/motility and invasion, receptors, DNA damage response, apoptosis and the intermediate filament markers. Table 1 gives the detailed data for genes which we which showed a >2-fold CEA dependent difference of expression. These effects were not seen in the wild type cells when tetracycline alone was added.

**Table 1:** Microarray. List of genes and gene groups which were affected by CEA.

| | **- Tet.** | **+ Tet.** | **- Tet./+Tet.** |
|---|---|---|---|
| **Cell cycle/ proliferation** | | | |
| Cell divisio protein kinase 4 | 3420 | 13572 | 0.25 |
| Cell division protein kinase 5 | 2524 | 9372 | 0.27 |
| E2F-1 pRBbp | 1000 | 2923 | 0.34 |
| Cyclin A | 23107 | 12858 | 1.80 |
| C-myc bp | 2591 | 1000 | 2.59 |
| ERK6 | 15588 | 5962 | 2.61 |
| PIN1 | 3315 | 1000 | 3.32 |
| s/t-PK PCTAIRE-3 | 3426 | 1000 | 3.42 |
| Cyclin D3 | 5210 | 1000 | 5.21 |
| s/t-PK PCTAIRE-1 | | | |
| **Intermediate filaments** | | | |
| Cytokeratin 2E | 17292 | 2426 | 7.13 |
| **Apoptosis** | | | |
| TRAF6 | 3163 | 1513 | 2.09 |
| CRAF1 | 3273 | 1154 | 2.84 |
| casp9 prec | 2970 | 1000 | 2.97 |
| PDCD2 | 6206 | 1000 | 6.21 |
| **Oncogenes/tumorsuppresors** | | | |
| MDMX | 2841 | 1000 | 2.84 |
| STAT5B | 2840 | 1000 | 2.84 |
| **DNA damage and repair** | | | |
| DNA repair protein XRCC1 | 6110 | 1463 | 4.18 |
| **Cell fate** | | | |
| smoothened | 2821 | 1000 | 2.82 |
| dishevelled homo. | 10418 | 3179 | 3.28 |
| dishevelled (DVL)+DVL3 | 5322 | 1296 | 4.11 |
| Notch4 | 4432 | 1000 | 4.43 |
| WNT-8B | 4635 | 1000 | 4.64 |

| | | | |
|---|---|---|---|
| Jagged2 | 6633 | 1000 | 6.63 |
| **Receptors** | | | |
| RARalpha 1 + PML-RAR protein | 1495 | 3865 | 0.39 |
| RARgamma | 6414 | 3264 | 1.97 |
| HER3 | 2876 | 1000 | 2.88 |
| FAU | 46754 | 8609 | 5.43 |
| IGFBP6 | 9451 | 1000 | 9.45 |
| **Invasion regulators** | | | |
| MMP-11; stromelysin-3 | 2881 | 1339 | 2.15 |
| metastasis-associated MTA1 | 2969 | 1136 | 2.61 |
| platelet basic protein precursor PBP | 2620 | 1000 | 2.62 |
| TIMP-2 | 5377 | 1144 | 4.70 |
| MMP-17 | 6443 | 1000 | 6.44 |
| Emmprin | 32669 | 1000 | 32.67 |
| **Cell adhesion** | | | |
| laminin 3kDA receptor | 29457 | 13110 | 2.25 |
| netrin-2 | 3853 | 1000 | 3.85 |
| LAR | 4400 | 1000 | 4.40 |
| integrin a3 chain | 5039 | 1000 | 5.04 |
| **Angiogenesis** | | | |
| STK-1 | 3778 | 962 | 3.93 |
| tie-1 precursor | 4959 | 797 | 6.22 |
| Cystein-rich FGFR | 2552 | 1219 | 2.09 |
| **Rho family/small GTPases** | | | |
| Rho GDP-dissociating inhibitor 1 | 70418 | 210956 | 0.33 |
| rhoB | 10829 | 3355 | 3.23 |
| **Growth factors/cytokines** | | | |
| IFN gamma inducible chem IP-10 | 1000 | 5076 | 0.17 |
| PDGF A-chain | 1371 | 4488 | 0.31 |
| IL-10 | 1175 | 2665 | 0.44 |
| IL-7 | 2518 | 1093 | 2.30 |

| | | | |
|---|---|---|---|
| MIF | 48530 | 20330 | 2.39 |
| Macrophage stimulating protein MST-1 | 2786 | 1000 | 2.79 |
| VEGF | 3202 | 1000 | 3.20 |
| pre IL-1 | 3283 | 1000 | 3.28 |
| IL-12 (NKSF p40) | 3348 | 1000 | 3.35 |
| Metallothionein-III | 7392 | 2127 | 3.48 |
| hepatoma der.GF | 4116 | 1000 | 4.12 |
| KROX24 | 37616 | 6800 | 5.53 |
| Kidney EGF precursor | 7507 | 1000 | 7.51 |
| NT-4 (NT-5) + NT-6 | 4683 | 616 | 7.60 |
| Endothelin ET2 | 10886 | 1000 | 10.89 |

Out of all gene groups we focused on two, the cell cycle/proliferation and the apoptosis gene group, because an imbalance in those two pathways is known to affect tumor growth. When HT29/Rz4 cells were treated with tetracycline (ribozyme inhibition), CEA seemed to affect the cell cycle gene expression in an bi-directional and balanced fashion, whereas the apoptotic genes were shifted in one direction (figure 13). 9 out of 10 apoptotic genes were down-regulated. Tetracycline did not affect the expression of these genes, as determined in parallel experiments.

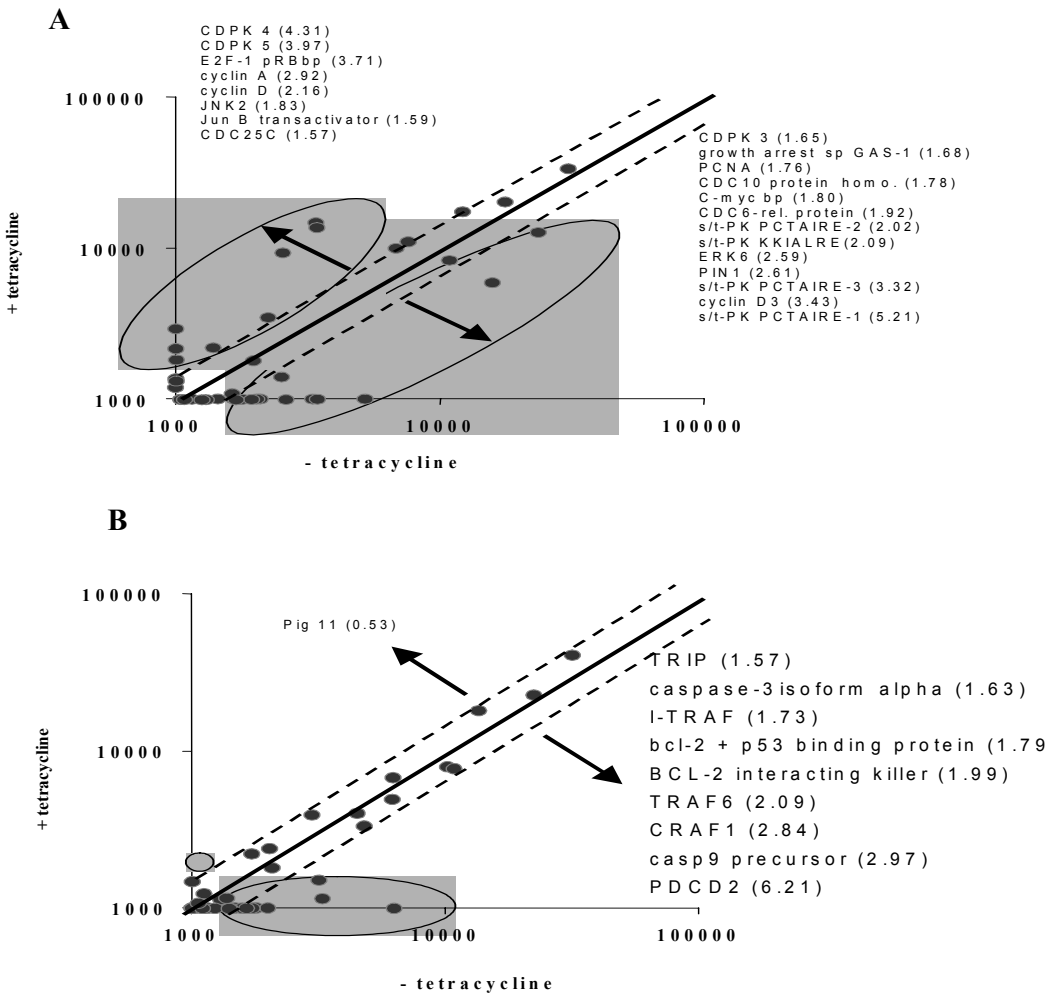


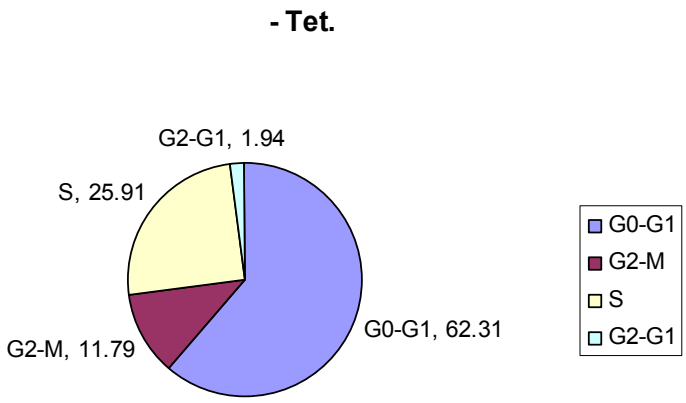

**Figure 13:** Correlation of the gene expression of HT29/Rz4 cells in relation to CEA expression. **A)** genes belonging to the cell cycle/proliferation group and **B)** genes belonging to the apoptotic genes group. Indicated are genes which showed a 1.5-fold CEA dependent difference of expression.

## 3.7. Analysis of the Cell Cycle

We saw a bi-directional and balanced shift of the genes on the cDNA array, belonging to the 'cell cycle and proliferation gene group'. To see if these changes on the microarray had an effect *in vitro*, we analyzed the influence of CEA on the cell cycle using the Vindelov method. Both clones, HT29/Rz4 and HT29/Rz4-2 were used to determine the influence of CEA on the cell cycle.

Differences in CEA levels did not affect the cell cycle in HT29 colon cancer cells. Both clones did not demonstrate differences in the cell cycle (Figure 14).

**A.**




**B.**

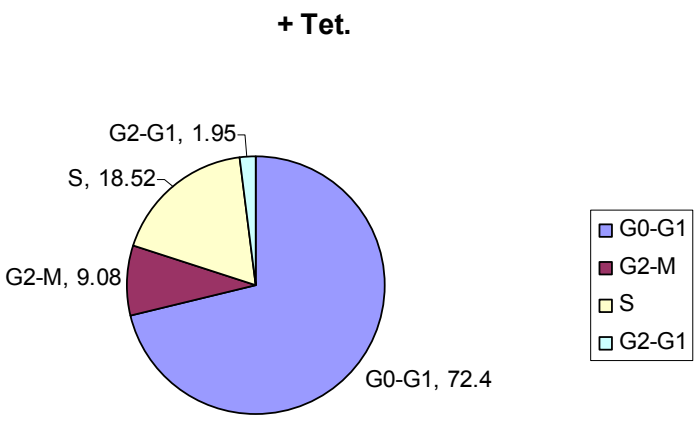



**Figure 14 A + B:** Cell cycle assay with HT29/Rz4 cells measured after **A)** 0h and **B)** 12 h. There was no significant effect of CEA on cell cycle of HT29/Rz4 cells.

## 3.8. Influence of CEA on Proliferation of HT29 Cells

Cell numbers were determined at different time points by a colorimetric assay, based on the cleavage of the tetrazolium salt WST-1 in viable cells. CEA did not affect the proliferation rate of HT29 cells in both clonal cell lines. There was no difference in proliferation at any time point (figure 15).

**A.**

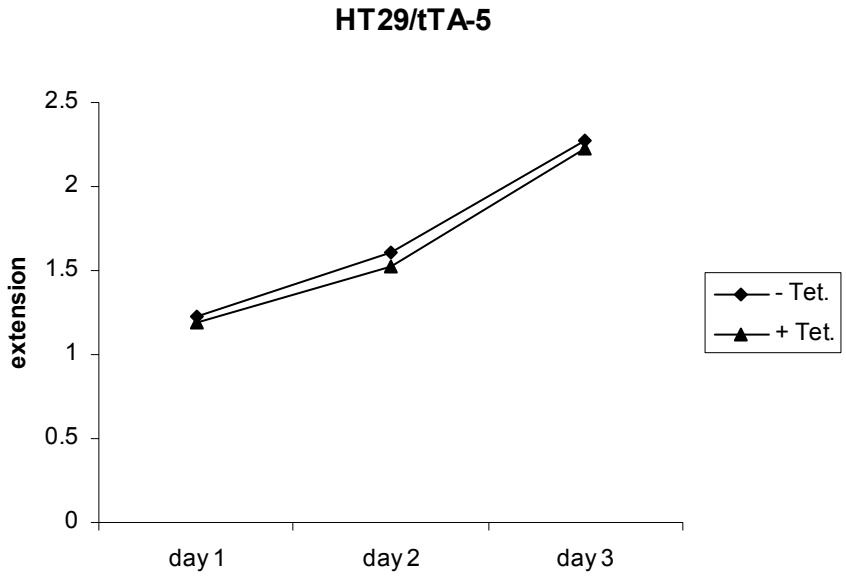
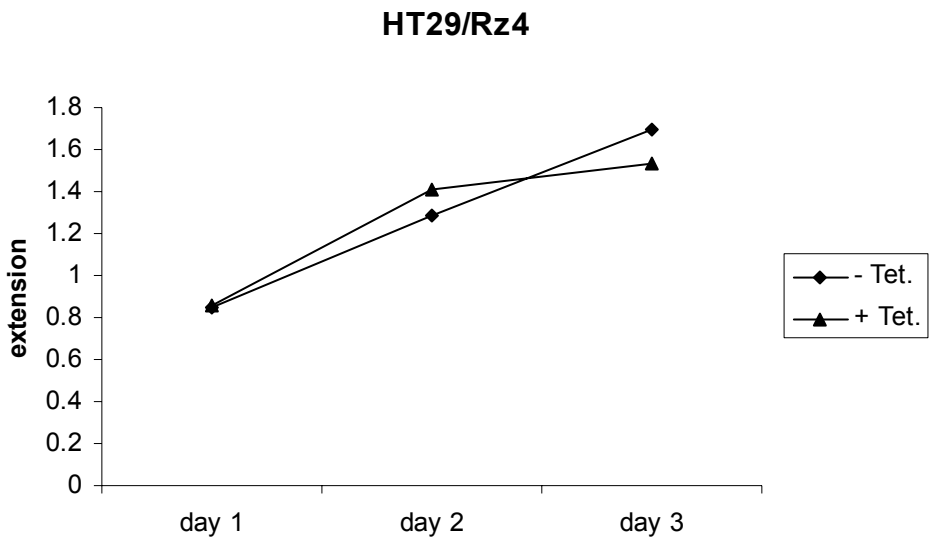



**Figure 15 A:** Proliferation assay with control cells HT29/tTA-5 (cells without ribozyme).

**B.**




**C.**

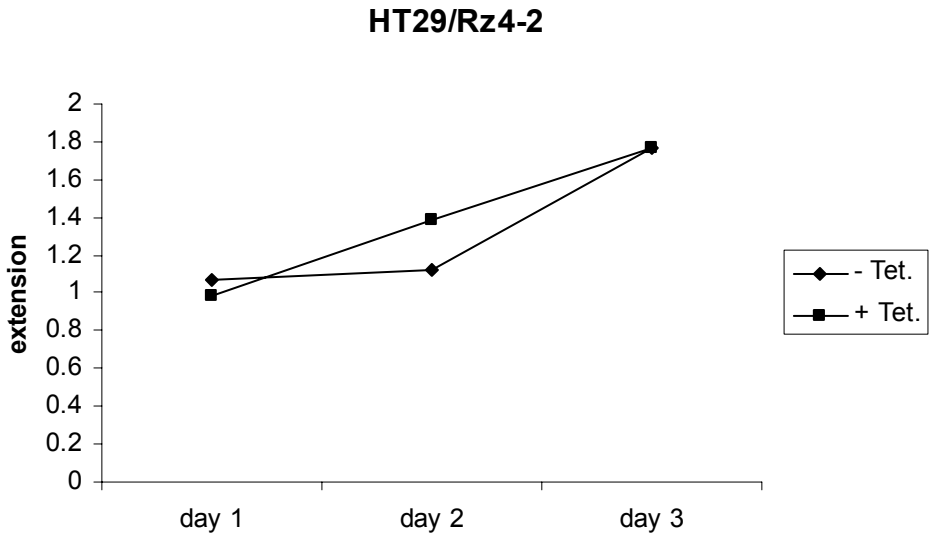


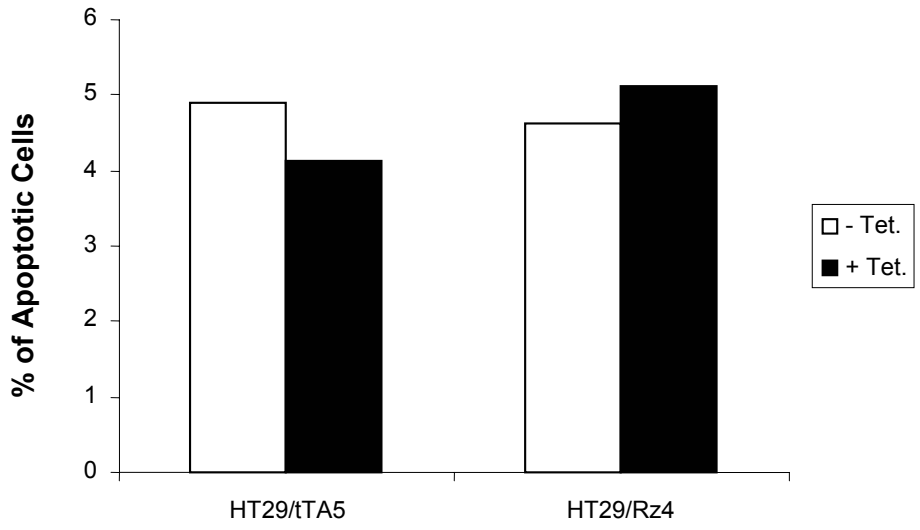

**Figure 15 B+C:** Proliferation assay with HT29/Rz4 and HT29/Rz4-2 cells. CEA did not affect the proliferation rate of HT29 cells. **B)** HT29/Rz4 **C)** HT29/Rz4-2

## 3.9. Protection of CEA Against Apoptosis

Because of the fact, that the cDNA array demonstrated a shift in the apoptotic genes, the apoptotic rate of cultured HT29/Rz4 cells with low and normal CEA levels were determined by using the AnnexinV/propidium iodid staining method. Initially, the apoptotic rates of HT29/Rz4 cells 24 hours after tetracycline treatment in semiconfluent growing cells were analyzed. When HT29/Rz4 cells were grown to a semiconfluent stage, cells with normal CEA levels (tetracycline treated cells) had a slightly higher apoptotic rate than the cells with reduced CEA levels (cells without tetracycline treatment) (Figure 16 A). With clone HT29/Rz4-2 there was no difference between cells with normal CEA levels, and cells with reduced CEA levels.

However, under confluent conditions the apoptotic rate in CEA reduced cells increased, whereas tetracycline treated cells (normal CEA levels) were not affected (figure 16 B). We were able to confirm these results also in HT29 IV 2 cells, with virtually the same results (figure 16 B).

**A.**



**B.**

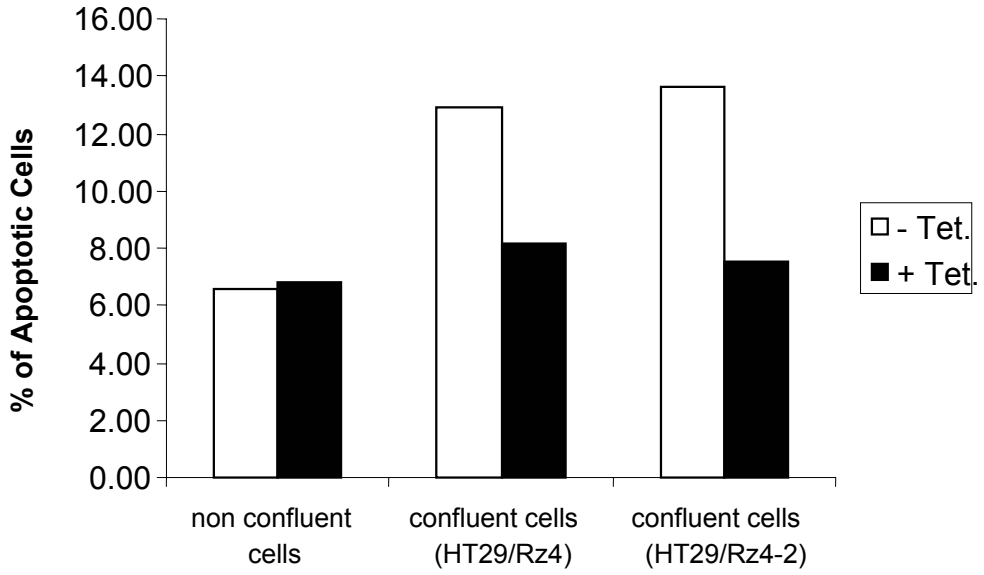



**Figure 16:** Analysis of the apoptotic rate of HT29/Rz4 and HT29/Rz4-2 cells under **A)** semiconfluent and **B)** confluent growing conditions. Cells, which were stained positively for both, Annexin V and propidium iodid, were measured

To see if CEA is able to protect the cells also under different conditions, we studied the protective effect of CEA against different apoptotic stimuli including UV light, IFN-γ and 5-FU. Regarding to HT29/Rz4 cells exposed to 200 J UV light, CEA seemed to protect cells from undergoing apoptosis. Cells with decreased CEA levels (cells not treated with tetracycline) had a significant elevated apoptotic rate compared to the cells with normal CEA levels (cells treated with tetracycline) ($p<0.05$) (figure17).

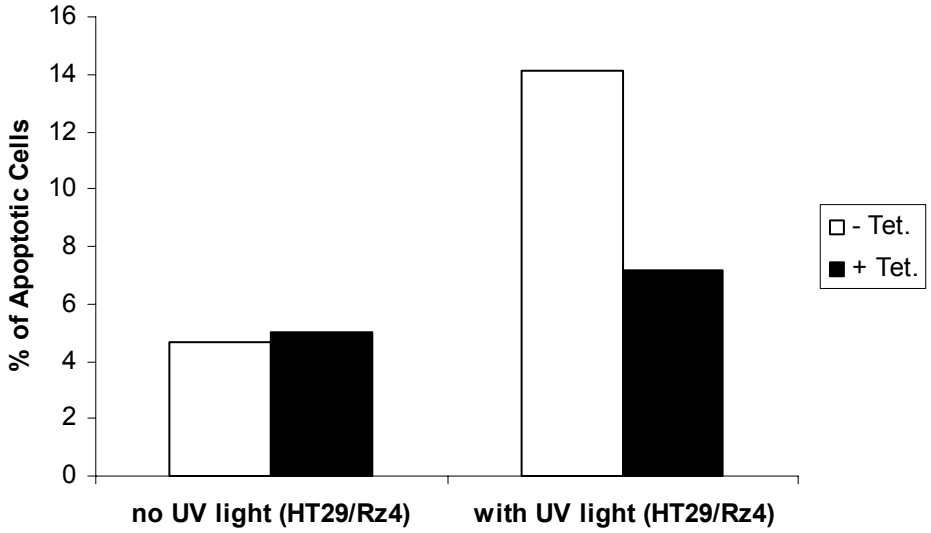
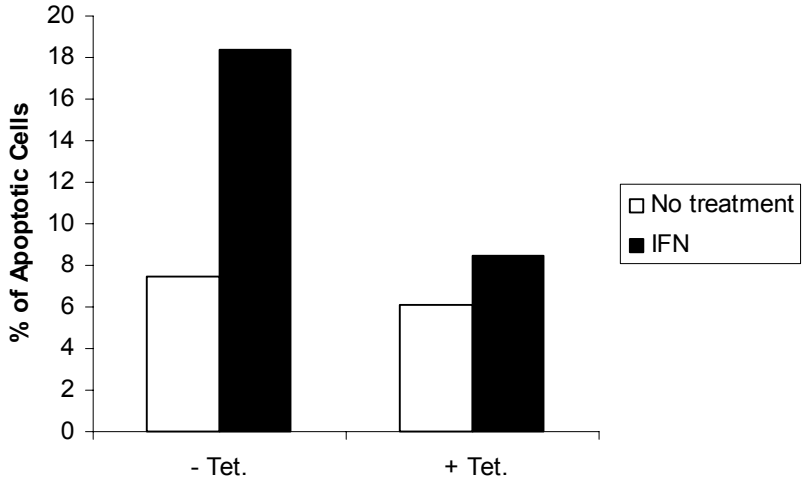



**Figure 17**: Analysis of the apoptotic rate of HT29/Rz4 cells treated with 200 J of UV-light. Apoptosis was measured 24 hours after UV-light treatment. Cells, which were stained positively for both, Annexin V and propidium iodid, were measured. As a control we used HT29/tTA5 cells (cells without ribozyme).

Also, when HT29/Rz4 cells were treated with 25 units/ml of IFN-γ cells increased their apoptotic rate 2.5-fold in CEA down-regulated cells but had no significant impact on the apoptotic rate of cells with normal CEA levels (Figure 18 A).

Finally, when HT29/Rz4 cells were treated 24 hours before the assay with 50 μM 5-FU cells with normal CEA levels were protected from undergoing apoptosis, whereas the apoptotic rate increased 2.8-fold in the CEA down-regulated cells (Figure 18 B).

**A.**




**B.**

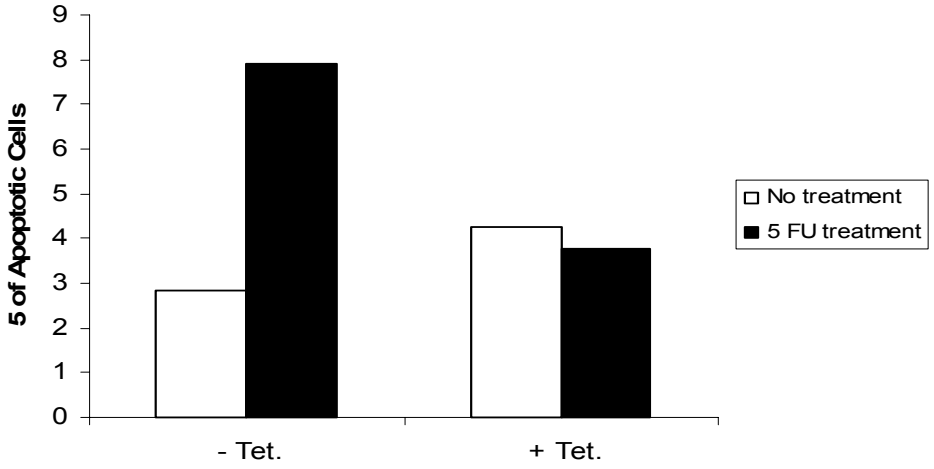



**Figure 18:** Annexin V-PI staining with HT29/Rz4 cells. **A)** HT29/Rz4 cells that were treated 48 hours with 25 U/ml IFN-γ. **B)** HT29/Rz4 cells that were treated 48 hours with 50μM/ml 5-FU.

Tetracycline alone did not affect the apoptotic rate in the wild type cells and had no influence on the apoptotic rate when cells were treated with UV light, IFN-γ, or 5-FU.

## 3.10. Colony Formation of HT29 Cells

Because we did not see a difference in the proliferation rate between the tetracycline treated and non-tetracycline treated cells within 72 hours we wanted to determine whether CEA's antiapoptotic function has a long term effect on tumor cell growth. For that purpose we used a soft agar colony formation assay to determine the growth of HT29/Rz4 and HT29/Rz4-2 cells in relation to CEA over a period of 2 weeks. We found that, in both clones, cells with normal CEA levels (cells treated with tetracycline) developed 30 to 50 % higher colony numbers compared to cells with reduced CEA levels (cells not treated with tetracycline)(figure 19).

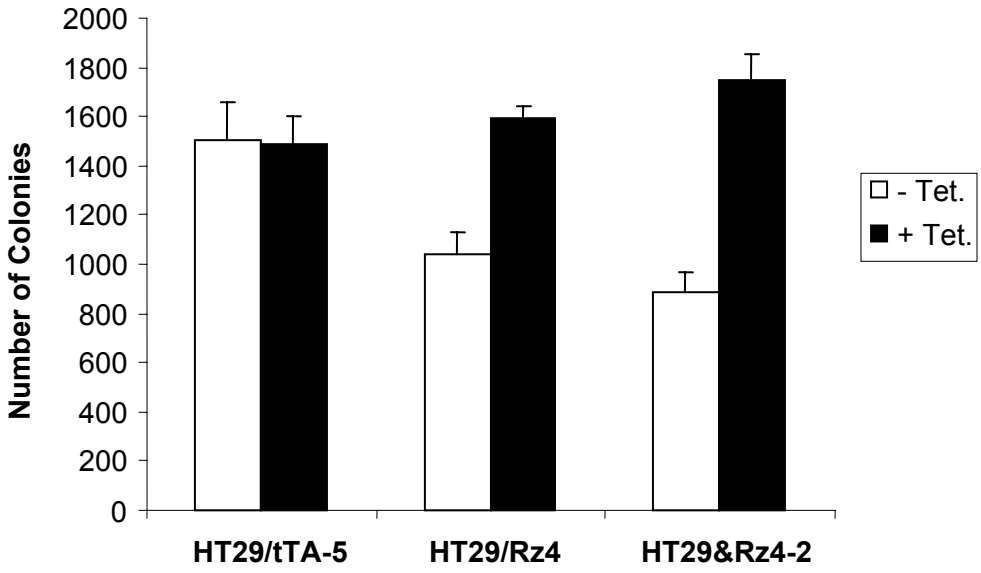



**Figure 19:** Colony formation assay. HT29 cells were seeded as single cells into soft agar. Number of colonies (>80μm in diamter) were counted after two weeks. As a control we used HT29/tTA 5 cells (cells without ribozyme).

## 3.11. CEA Dependent Tumor Cell Seeding and Growth *in Vivo*

We demonstrated that CEA is not only involved in aggregate formation, but also protects against apoptosis and thus, promotes colony formation in HT29 cells in vitro. To see if those effects in vitro has any influence on the tumor growth, in vivo studies were performed by tail vein injection of HT29/Rz4 cells, pretreated and untreated with tetracycline, in nude mice.

To detect and quantify the tumor cells or eventually metastatic lesions in the lung we performed an anti-human cytokeratin staining. One hour after cell injection we sacrificed the first group of mice. This was done in order to see if CEA is involved in the attachment of HT29 cells to the endothelium of the capillary system. After 24 hours the second groups of mice were sacrificed. This was done in order to see how many tumor cells actually survived and attached to the organ, because it is known that over 99 % of cells which are injected into the bloodstream are removed within hours. After six weeks the last groups of mice were sacrificed to determine how many metastases developed.

One hour after tumor cell injection 230 ± 20 single cells and small cell clusters per slide were detected in mice receiving tetracycline in contrast to 235 ± 29 cells/cell clusters per slide in the untreated mice. After 24 hours only 3 ± 2 tumor cells per slide were found in the tetracycline treated group, but non in the untreated group. However, after 6 weeks all five mice of the tetracycline treated group showed metastatic lesions (14.5 ± 4.6 lesions per mouse) whereas in the untreated group only one of five mice had one metastatic lesion (0.2 ± 0.2 lesions per mouse) (figure 20).

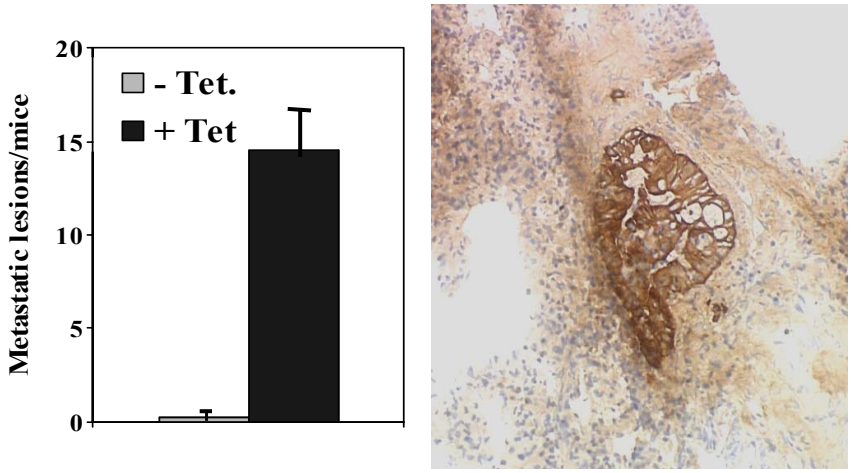



**Figure 20:** *In vivo* experiment with HT29/Rz4 cells. Cells were injected into the tailvein of nude mice. After six weeks mice were sacrificed and metastatic lesions were detected immunohistochemically. Mice which received tetracycline enriched food had more metastatic lesions than mice without tetracycline treatment.

As a control we injected wild type cells into the mice. Irrespective of tetracycline treatment there was no difference between the tetracycline untreated and tetracycline treated group.

## 3.12. Interaction of CEA With Other Proteins

To discover interaction between CEA and other proteins, especially those which are involved in the signal transduction cascade (e.g. proteins belonging to the apoptosis regulating cascade), we performed a phage display study by using the cDNA library of a human colon cancer cell line (LS174T). CEA was immobilized on a 6-well plate. Phages which bound to CEA were amplified and used for the next round. Figure 21 shows the binding of the phages to CEA after each round.

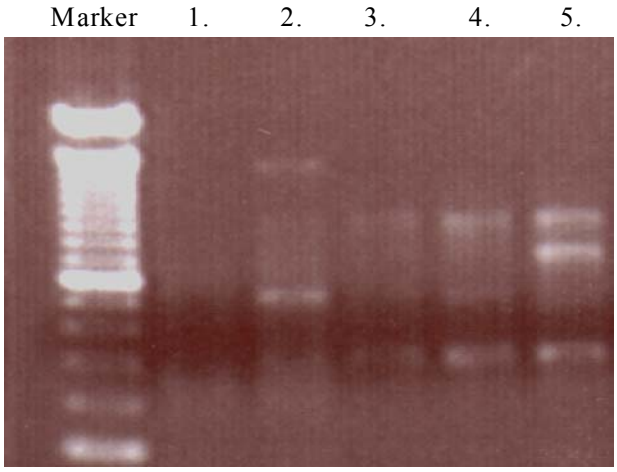



**Figure 21:** Round 1 – 5 of a Phage display. CEA was immobilized using 6-Well plates. The cDNA library of a human colon cancer cell line (LST174) was screened.

The meaning of each round was to get rid of unspecific binding. After the fifth round 40 clones were selected and amplified. Clones were purified and a PCR was run for each clone in order to identify if the clones had an insert or not. The PCR product was run on an agarose gel. Out of 40 clones we selected 12 clones, which contained a cDNA fragment and gave a positive PCR signal (figure 22).

**Figure 22:** PCR of selected clones from the phage display.

Some of these 12 clones coded for the same protein, resulting in 8 clones coding for different proteins.

For each clone a binding assay was performed in parallel to a control protein (as a negative control we used LB) to see if binding of phages to CEA is specific or unspecific. For each clone the binding assay was repeated three times. Figure 23 shows an example of a binding assay.

**control** **CEA**

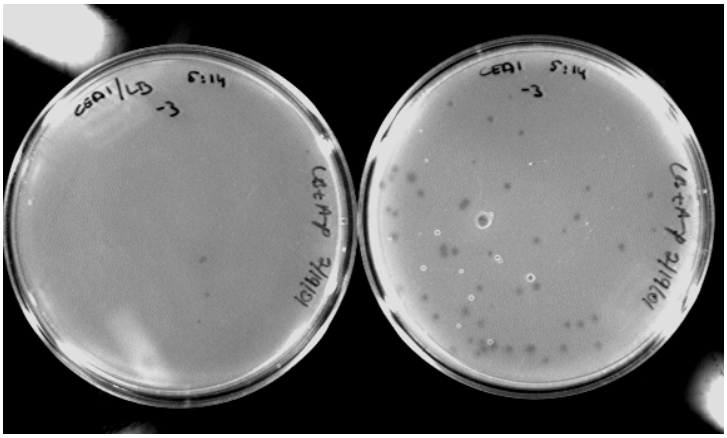


**Figure 23:** Titration series with clone 5:14. As a control we used LB. Clone 5:14 shows specific binding to CEA, demonstrated by the number of plaques shown on the agar plates. No binding was seen with the control plate.

In addition, in order to identify these proteins, a PCR was performed from these clones and run on a gel. The insert (or product from the PCR) was cut out of the gel and purified for sequencing. Table 2 gives a list of proteins we could identify after sequencing.

**Table 2:** Selected clones from phage display.

| Clone # | Corresponding protein |
|---|---|
| 5:5 | Homo sapiens, CGI-120 protein |
| 5:6 | Homo sapiens, similar to ribosomal protein L5 |
| 5:8 | Homo sapiens histone deacetylase 2 (HDAC 2) |
| 5:11 | Homo sapiens, clone IMAGE: 3611719 |
| 5:14 | Homo sapiens, ribosomal protein L24 |
| 5:19 | Homo sapiens, nuclear phosphoprotein p130 |
| 5:28 | Homo sapiens clone RP11-12N15 |
| 5:38 | Homo sapiens chromosome 10 clone RP11-302K17 |

# 4. DISCUSSION

The aim of this study was to elucidate the pathophysiological function of CEA in the pathway of metastasis. Because of the fact that CEA overexpression is one of the most common events in the development of adenocarcinoma, it is interesting to know, if CEA is also involved somehow in the pathway of metastasis.

To analyze the function of CEA, specific CEA-targeted hammerhead ribozymes were designed and expressed under the control of the tet-off system. Compared to other approaches this has three major advantages: First, because of the high specificity of ribozyme molecules (UHLENBECK, O. et al 1995) it is able to target exclusively the gene of interest (CEA) and inhibit the translation of the mRNA to its actual protein, resulting in a specific inhibition of endogenous CEA expression. Secondly, by using the tet-off promoter system the CEA levels within identical cancer cell clones can be regulated. And third, by using the tet-off system it enables a comprehensive analysis of CEA-mediated effects within an intact pathophysiological cellular context.

CEA was first described in 1965 (FREEDMAN, S. O. et al, 1965) and is the oldest known tumor-associated antigen. Numerous clinical studies demonstrate that CEA level correlates with the clinical outcome of colorectal (BRÜNNER, N. et al 1998), gastric (HIOKI, K. et al 1994), lung (ESTAPE, J. et al 1998) and breast cancer (SEIDEL, D. et al 1999).

Since CEA has been known already from the mid sixties a lot of different hypothesis about the function of CEA has been proposed. C.P. Stanner's lab suggested CEA functioning as an intercellular adhesion molecule (BENCHIMOL et al, 1989). In their study they demonstrated that CEA mediated Ca2+-independent, homotypic aggregation of cultured human colon adenomacarcinoma cells (LS-180). This could result in a greater surviving chance to form metastasis, since cell aggregates better survive in the blood stream than single cells. GRIFFIN, T. W. et al. studied the adherence of cell lines to microtiter wells coated with CEA and well described adhesive molecules (like laminin, collagen and fibronectin). He showed that the CEA positive cell line LoVo and the CEA devoid cell line H-Meso-1 did not differ in adherence to the extracellular matrix proteins laminin, collagen and fibronectin, whereas LoVo cells adhered to CEA in a specific manner, while H-

Meso-1 showed no adhesion to CEA (GRIFFIN, T. W. et al 1991). However, P.-L. Lollini performed also some adhesion studies on CEA and found the opposite result using a human rhabdomyosarcoma cell line (P.-L. LOLLINI et al 1996). They demonstrated that at least for cells not metastatic to the liver CEA could interfere with the intercellular adhesion and therefore acts as an anti-metastatic molecule. They concluded that from the fact that the impairment of metastatic potential correlated with a reduction in homotypic adhesion properties of these cells. These conflicting data suggest that the function of CEA depends in part on its interaction with other membrane molecules and might be also tissue type dependent. However, so far studies about the adhesive function of CEA have only been done in cells which express CEA at high density or with cells which have been transfected with a CEA expression vector. The adhesive function has never been demonstrated in human colon cancer cells which show moderate or low CEA levels such as HT29 cells. In addition, so far no one was able to give proof that the adhesive function of CEA plays any role under physiological *in vivo* conditions at all.

By using the tet-off system we were able to analyze the impact of physiological CEA levels on tumor cell aggregation. We were able to regulate the CEA levels within HT29/Rz4 and HT29/Rz4-2 cell clones by treating them with or without tetracycline. Ribozyme mediated reduction of CEA levels by 50 % resulted in a 70 % decrease in cellular aggregate formation of HT29 cells. By treating the cells with tetracycline we could completely reverse the ribozyme effect. Tetracycline itself did not affect aggregate formation in HT29 cells.

We could demonstrate that the aggregate formation is dependent on the density of CEA molecules. However, in addition to that it is possible that the adhesive function of CEA is also tissue type dependent and that the intercellular binding depends not only on the CEA density, but would also include the interaction between CEA and other molecules. Renkonen and his group demonstrated that CEA is also expressed on human umbilical vein endothelial cells (HUVEC) by using immunohistochemistry (RENKONEN, R. et al 1994). They also demonstrated that CEA expression on cultured endothelial cells could be enhanced by TNF-$\alpha$ or IFN-$\gamma$. We hypothesized, that if CEA functions as a homophilic adhesion molecule, meaning that CEA binds to CEA, we could probably expect binding of CEA expressing cells to HUVEC cells. We labeled HT29/Rz4 cells with a red and HUVEC cells with a green fluorescent marker and performed an adhesion

assay. The results showed, that overall there was an increase of binding of HT29/Rz4 cell to HUVEC cells over time, but surprisingly binding was not dependent on CEA levels in HT29 cells. There was no difference between CEA-depleted cells and cells with normal CEA levels. Maybe the density of CEA on HUVEC cells is too low, or the difference between normal CEA and low CEA levels in the HT29 cells were not enough to cause a difference. The resulting step was to treat the HUVEC cells with IFN-γ to enhance CEA expression. After treating the HUVEC cells for 24 hours with IFN-γ we repeated the adhesion assay. The resulting data were surprising. HT29/Rz4 cells with low CEA levels adhered more to HUVEC cells than the HT29/Rz4 cells with normal CEA levels. This experiment was repeated several times with virtually the same results, whereas in the wild type showed no difference between tetracycline treated and untreated cells. These results could confirm the hypothesis, that binding of HT29 cells is not only dependent on the density of CEA on the cell surface (like that would be the case in homophilic binding CEA to CEA binding), but is also very much dependent on the interaction of CEA to other molecules. However, these data are not fully conclusive. We don't know the possible effects IFN-γ has on HUVEC cells, in addition to enhancement of CEA expression. It is very likely, that IFN-γ affects HUVEC cells in many different ways. In addition to the enhancement of CEA expression, there are most likely also many other cell surface molecules affected by IFN-γ. These cell surface molecules might be up-, or down-regulated as a result to IFN-γ treatment. In any case, these changes affect their properties to interact with CEA.

CEA has also been implicated as a heterophilic intercellular binding molecule that mediates the colonization of liver and lung by colon cancer cells. Jessup, J.M. and his group transfected CEA into a poorly differentiated human colorectal cancer cell line MIP-101 and found out that the clones which expressed high levels of CEA were tumorigenic when injected subcutaneously into nude mice compared to the parent cell. They also had an increased incidence of hepatic tumors when injected intrasplenic into nude mice. In addition, they observed strong CEA staining in Kupffer cells in the normal liver adjacent to the CEA producing tumors. (JESSUP, J.M., et al 1995).

Further studies suggest that CEA might increase the metastatic capability of cancer cells by inducing paracrine effects on normal cells. It has been found that binding of CEA to Kupffer cells induces the release of various cytokines including Il-6, TNF-α, Il-1α and Il-1β (EDMINSTON, K. H., 1997). These cytokines are known to affect the microenvironment by enhancement of the expression of different molecules (for example different adhesion molecules) and making the microenvironment therefore more suitable for tumor cells. This interaction of CEA to Kupffer cells is believed to happen through binding to hnRNP M4, a recently described receptor of CEA (BAJENOVA, O. V et al, 2001).

To identify genes that are potentially affected by CEA, we screened for CEA-mediated molecular effects by performing a cDNA microarray, which covers 588 cancer related genes arranged in 13 functional groups.

So far microarrays have been used to investigate problems in cell biology in various ways. At one end, single changes in gene expression, that might be the key to a given phenotype or its alteration, can be discovered. At the other end, an overall pattern of gene expression can be studied with cDNA microarrays.

Our intention was not to look for single genes, which might affect tumorigenecity of HT29 cells, but to get an overall idea if changes in gene expression are related to CEA.

Our finding was surprising. 273 genes out of 588 generated reliable signal and were evaluated for the effect of CEA on their expression level. A sensitive parameter for evaluating differences in gene expression is the “fold-change sensitivity”. The “fold-change sensitivity” is the ability of hybridization arrays to reliably determine a certain magnitude difference in gene expression. The claimed fold-change sensitivity for radioactivity based methods varies from 1.5- to 0.66 fold (FREEMAN, W. M, et al, 2000).

Using a 1.5-fold change in gene expression as a cutoff approximately half of the 273 genes, which gave reliable signals, changed their expression level when levels of CEA were modified. When using a 2-fold change in gene expression as a cutoff, differences in CEA dependent gene expression was found in 46 genes. Even when using a 2-fold change in gene expression as a cutoff the CEA dependent gene expression was affecting virtually all gene groups. As examples for genes affected by CEA: CDPK4 and 5, belonging to the cell cycle group, Jagged2, belonging to

the fate development regulators, Interleukin12 and IFN-inducible chemokine IP-10, belonging to the growth factors/cytokine, rhoB, belonging to the Rho family, MMp-17 and Emmprin, belonging to the invasion regulators, VEGFbeta, belonging to the angiogenesis regulator, laminin-3kDA receptor, belonging to the cell adhesion/motility and invasion group, IGFBP6, belonging to the receptor group, PDCD2, belonging to the apoptosis group and cytokeratin 2E, belonging to the intermediate filament markers.

The problem with microarray experiments is that they are highly capable of generating long lists of genes with altered expression, but they provide little information as to which of these changes are important or results in a given phenotype. It is fair to say, that it is hard to predict the change of a given phenotype, when we see a shift of a single gene. Nevertheless, a dysregulation of genes within a functional group implies phenotypic changes, in particular when a significant shift occurs in an unidirectional manner.

In this study we focused especially onto two gene groups, the one of the cell cycle/proliferation and apoptosis, because the balance of both functionally groups were significantly affected by ribozyme mediated modification of CEA levels. In addition, we focused our study on those two gene groups, because the balance of these two pathway is known to significantly affect tumor growth.

However, the change of gene expression in the cell cycle/proliferation group and in the apoptosis group differed from each other. While in the cell cycle /proliferation group genes shifted in a bidirectionally manner, apoptotic genes were shifted in a unidirectional manner in the CEA down-regulated cells. None of the observed changes were seen in the tetracycline-treated control cell line (HT29/tTA-5).

Because we saw a CEA dependent shift in gene expression in the cell cycle/proliferation group and in the apoptosis gene group the next step, we were pursuing, was to determine if that would result in a change in the phenotype of HT29 cells in vitro. Interestingly, the vast majority of apoptosis regulators (down-regulated in the CEA expressing cells) are proapoptotic genes. However, at this point it is difficult to draw conclusions regarding particular genes affected by CEA and how CEA may interact within the apoptotic cascade.

Studies regarding the apoptosis regulating function of CEA has been done already previously by Ordonez, C. et al. They described for the first time, that CEA might play a potential role of CEA in regulation of anoikis, a special type of apoptosis,

which is induced by loss of cell contact with the extracellular matrix. They demonstrated that forced overexpression of CEA and CEACAM6 on the surface of different cell lines (L6 rat myoblasts, MDCK epithelial cells, and human SW1222 and Caco-2 colorectal cancer cells) inhibited their anoikis *in vitro*. They hypothesized, that the antiapoptotic effect could be a result of intercellular interactions mediated by binding of CEA to CEACAM6 on the cell surface (ORDONEZ, C. et al, 2000). However, the influence of CEA alone as an antiapoptotic molecule was not studied.

In this study we demonstrated that CEA alone may have an important regulatory role in apoptosis. We are able to demonstrate a significant interaction between CEA and apoptosis–related gene expression and that endogenous CEA protects human HT29 cells from a variety of apoptotic stimuli. We suggest that CEA has a direct role in the regulation of apoptosis, but is also very much dependent on external factors including cell proximity to other CEA-expressing cells. For example, dense cell growth resulted in a significantly (2.5-fold) higher apoptotic rate in CEA-depleted cells, whereas semiconfluent conditions did not significantly affect the apoptotic rate. Interestingly, when semiconfluent growing cells were treated with various inducers of apoptosis, including UV-light, IFN-γ and 5-FU (which is the main cancer drug for treatment of colorectal cancer), we could demonstrate a protective role of CEA. Basically, the protective and stabilizing effect of CEA could only be shown under condition of external stress. Otherwise, under normal condition there was no significant affect of CEA to the apoptotic rate of HT29 cells. Additional studies are needed to define the exact mechanism by which CEA protects against apoptosis. Because of the fact, that the expression of CEA on the cell surface of cancer cell is without an intracellular signaling moiety, we hypothesize that CEA may interact with other membrane molecules. For example, cell signaling (phosphorylation) could happen by binding of CEA to another protein ( for example a receptor), which has a intracellular signaling moiety.

In accordance with our microarray data, we found a significant change of the apoptotic rate between the CEA-reduced cells and the cells with normal CEA levels. But the cell cycle and proliferation rate did not differ between CEA-reduced and normal CEA-expressing cells.

Our hypothesis is, that CEA-expressing colon cancer cells may have a growth advantage in patients because of the protective function of CEA, which can help

colon cancer cells to survive the hostile condition (external stress) they are exposed to during progression.

We demonstrated an influence of CEA on the apoptotic rate in HT29 colon cancer cells. We hypothesized, that the protective effect of CEA, would result in a growth advantage for cells with higher CEA levels. We performed a proliferation assay, in order to see a probable difference in the proliferation rate, but the difference in the apoptotic rate did not seem to affect the proliferation rate of HT29 cells. After 72 hours there was no difference in the proliferation rate between CEA-depleted cells and cells with normal CEA levels. The next step was to further analyze if the difference of apoptotic rate between the CEA-depleted cells and the cells expressing normal levels of CEA would influence the colony formation of HT29 cells. We found out, that when we grew the HT29/Rz4 cells in soft agar and counted the number of colonies after two weeks the imbalance of proliferation and apoptosis resulted in a significant higher number of colony formation in cells with normal CEA levels. This would suggest the importance and the influence of CEA in tumor growth. Cells with normal CEA levels having a lower apoptotic rate than CEA-depleted cells would result in a growth advantage. Additionally, it is possible, that CEA could function besides as a protective factor also as an autocrine growth factor, which is released from tumor cells into the surrounding microenvironment.

To compare the impact of CEA-dependent aggregation and apoptosis in vivo we analyzed the metastatic growth of HT29 cells in nude mice. We assumed that differences in aggregate formation would primarily alter the phase of tumor seeding which would occur within the first hours of tumor cell injection. Interestingly, one hour after tail vein injection of HT29/Rz4 tumor cells with high and low CEA levels, we found almost identical numbers of single cells and tumor cell aggregates in the lung. 24 hours later virtually all cells were eliminated in both groups. This finding could suggests that differences in aggregate formation *in vitro* had no significant impact on tumor cell seeding *in vivo*. In contrary, another very interesting theory is the “seed and soil”-hypothesis. Already over 100 years ago Paget noted a highly non-random spread of cancer to specific target organs. From these observations , he formulated the “seed” (primary cancer) and “soil” (target organ)-hypothesis of metastasis (PAGET, S. 1889). The “seed and soil”-hypothesis is about tumors, which can have enormously different characteristic metastatic

rates. In contrast to the variability in rates, there is a surprising degree of specificity in the target organs colonized by metastases from particular type of tumor. In the case of colorectal cancer they are known to most often target the liver, but they also do metastasize to the lung. In an animal study from Kuo, T.-H. et al, human colon tumors were transplanted into the nude mouse colon as intact tissue blocks by surgical orthotopic implantation. They used cells taken from a metastatic and non-metastatic tumor. Both classes of tumors were equally invasive locally into tissues and blood vessels. However, the cells from each class of tumor behave very differently when injected directly into the mouse liver. Only cells from metastasizing tumors were competent to colonize after direct intrahepatic injection (KUO, T.-H. et al, 1995).

However, 6 weeks after tumor cell injection, we detected in all mice which were treated with doxycycline enriched food to continuously block ribozyme expression, numerous lung metastases, while cells with ribozyme reduced CEA levels did not develop metastatic lesions in four out of five mice. These data suggest that CEA has its major effect when tumor cells have already seeded in their metastatic target organ. The question still is how CEA is involved in the pathway of metastasis. It has been shown, that CEA induces the production of cytokines, especially IL-6 and TNF-α (EDMINSTON, K. H. et al, 1997, GANGOPADHYAY, A. et al, 1998 and MINAMI, S. et al, 2001). The production of cytokines might result in a microenvironment, which is favorable for tumor cell growing. Previously there was a study that demonstrated in animal models, that human cancer cells find optimal growth conditions when placed orthotopically in their organ of origin, but show a higher apoptotic rate when placed as metastatic lesions (ENGLER, S. et al 2001). This would speak again for the importance of the microenvironment for tumor cell growing. Taking this all together with our *in vitro* data, i.e. increased apoptotic rate under external stress and reduced colony formation in CEA-depleted cells, it seems obvious that CEA plays a major role in colon cancer progression.

At this point it is worth to mention, that all these results are not the result of unspecific binding, or cleavage of the ribozyme. Studies have been done to document the specificity of ribozymes. Werner, M. et al studied the effect of base mismatches in the substrate recognition helices of hammerhead ribozymes on binding and catalysis. They demonstrated that ribozymes lack already cleavage

activity if there is a mismatch of two or more nucleotides (WERNER, M. et al 1995). In addition, the use of a highly specific target sequence, which is unique for the human carcinoembryonic antigen strongly emphasizes that the observed changes are ribozyme related and CEA specific.

As mentioned earlier, CEA does not have an intracellular signaling moiety. Nevertheless, our finding, that CEA has a protective function against apoptosis, suggests that CEA has to operate indirectly through something else.
The idea of CEA interacting with other molecules led us to the idea of screening for CEA and it's binding to other proteins by using the phage display analysis.
Phage display is based on the ability of bacteriophage to present protein fragments on their surface coat. Diverse libraries of proteins, for example cDNAs, may be displayed. Interactions between phage displayed proteins and target antigens can therefore be identified. The power of phage display is that it enables identification of candidate epitopes without knowledge of the protein interaction *a priori*. The basis of phage selection is an affinity interaction between the displayed protein and its target. Phage are prepared from the library, which is in our case a human colon cancer cell line (LS174T) and are selected against the antigen, or target, which is in our case CEA.
One important issue in performing phage display analysis is, that the stringency of affinity selection is more or less opposed to yield, which is controllable in some degree by the choice of conditions. Yield is all-important in the first round of selection, but in later rounds yield can be sacrificed in the interest of stringency. There is a limit to stringency, however. The reason is that there is always a background yield of non-specifically bound phage; if stringency is set too high, the yield of specifically captured phage will fall far below the background of non-specifically bound phage, and all power of discrimination in favor of high affinity is lost. In practice, because the relationship between selection conditions and stringency is unknown in advance, there might be the risk of loosing proteins which might be of interest.
As a result of the phage display, we were able to demonstrate that specific binding between peptides (displayed as a fusion protein on phages) and CEA occurred. Furthermore, we were able to select these clones, which demonstrated specific binding to CEA. The results of the phage display suggests that CEA is able to

interact with different proteins, and therefore might be involved also in many different pathways, unknown before. Of course, the fact that there is specific binding of phages to CEA, does not automatically lead to the conclusion, that this specific binding has relevance *in vitro* or *in vivo*. Proteins might be in reality in different compartments of the cells and therefore never be able to interact with each other. Another critical factor is peptide scaffolding. Three dimensional structures might be formed which might lead to binding of your peptide to your target protein (RODI, D.J. and MAKOWSKI, L. 1999). This phenomenon has been described already and it is known as "mimotopes".
Even though phage display is a very powerful method the selection of proteins from a phage display analysis is just the first step of a big process. Whether binding to a specific protein is happening under normal circumstances would be the second step. And if there is binding, what is the result of it ?

CEA seems to be involved in many different processes. In this study it was demonstrated to function as an adhesion molecule. In addition, it was demonstrated that CEA has protective function against apoptosis and that there is a CEA dependent gene expression in HT29 colon cancer cells. Tumor cells (with normal CEA levels), injected into the tail vain of nude mice, were able to form more metastatic lesions in the lung than cells with reduced CEA levels.

In conclusion, this study demonstrates a multifunctional role of CEA in colon cancer cells, such as tumor cell aggregate formation and protection against apoptosis. We showed a significant interaction between CEA and apoptosis-related gene expression. The animal experiment suggests that the growth regulating effect of CEA is of importance for metastatic growth while aggregate formation plays a less significant role in tumor progression. Reduced apoptosis in cells with high levels of CEA after exposure to γ-interferon, or 5-FU suggests that inhibition of CEA expression may provide a novel therapeutic strategy to circumvent drug resistance. Further studies warranted which determine the signal transduction pathway of CEA and, thus, the therapeutic potential of treatment modalities targeting CEA and its related signaling pathway in cancer cells.

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# APPENDIXES

# Curriculum Vitae

## Education

1999 Master's degree in pharmacy,
Department of Pharmacology and Toxicology
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## Publications

1. Lehtolainen, P., Taskinen, A.K., Wirth, T., Kannasto, J., Lappalainen, M., Lehenkari, P., Pulkkanen, K., Marttila, A., Marjomäki, V., Airenne, K.J., Kulomaa M.S., and Ylä-Herttuala, S. Cloning and characterization of Lavivedins, low density lipoprotein receptor-avidin fusion proteins for targeted delivery of biotinylated compounds. Gene Therapy (submitted).

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## Abstracts

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