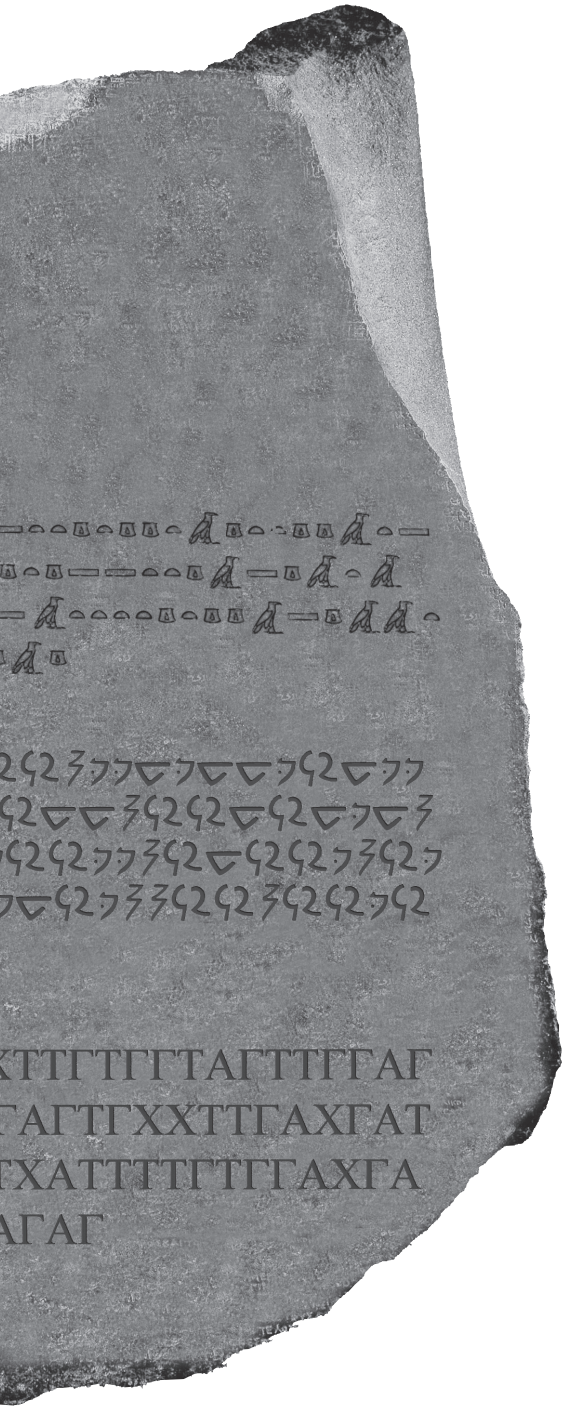
Towards KRAS-directed therapy



Dependency of metastatic colorectal cancer cells on mutant KRAS

N. Smakman

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The imitation of the Rosetta Stone on the cover describes the genetic code of the first exon of the KRAS gene with the mutational hot spot codons 12 and 13 encircled. It represents the decipherment of the genetic alterations in metastatic colorectal cancer. Soldiers in Napoleon's army discovered the Rosetta Stone in 1799 near the town of el-Rashid (Rosetta). The inscription on the Rosetta Stone (dated 196 BC) is a decree passed by a council of priests. The decree is inscribed on the stone three times, in hieroglyphic (suitable for a priestly decree), demotic (the native script used for daily purposes), and Greek (the language of the administration). The Greek inscription on the stone led to the decipherment of the hieroglyphs. The Rosetta Stone is property of the British Museum in London, UK.

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Dependency of metastatic colorectal cancer cells on mutant KRAS

Op weg naar therapie gericht tegen KRAS

Afhankelijkheid van darmkankercellen van mutant KRAS

(met een samenvatting in het Nederlands)

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Voor Nicole

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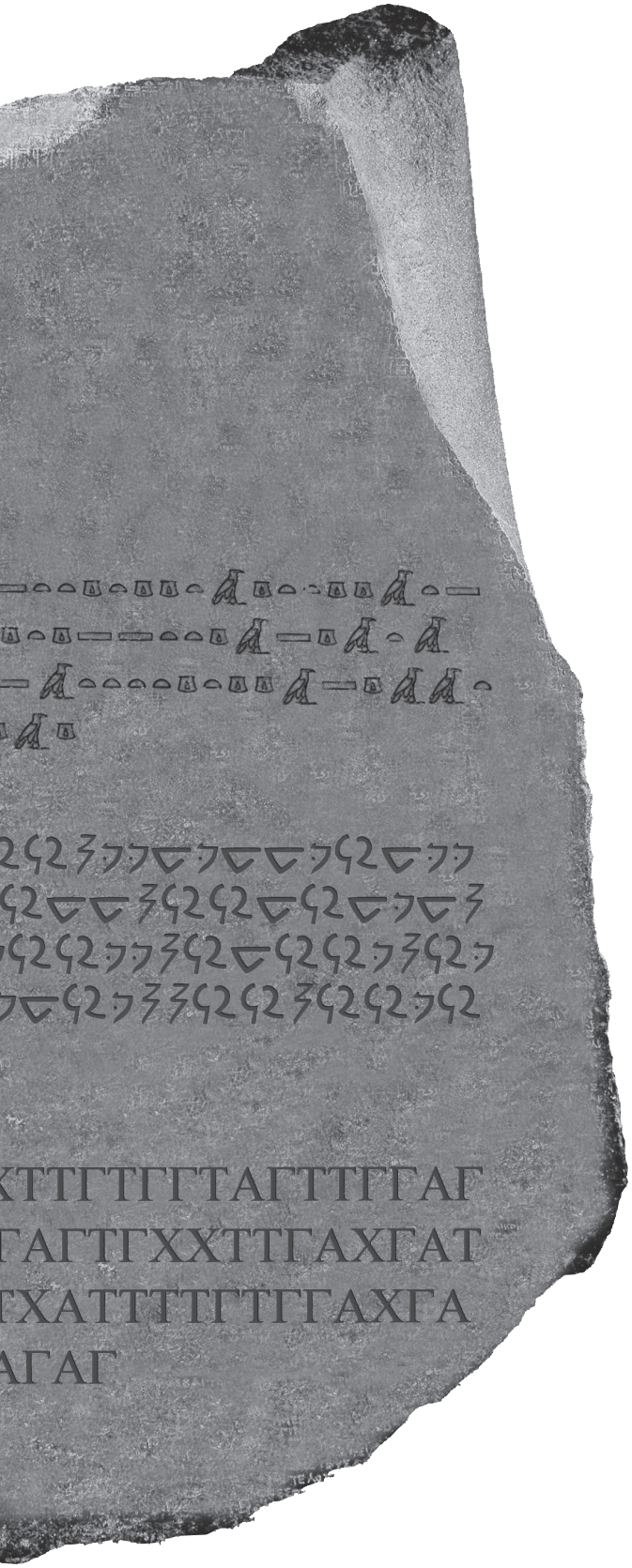

Chapter 1

General introduction and outline of the thesis

Targeted therapeutics in the treatment of cancer

Cancer ranks second behind cardiovascular disease as cause of death worldwide. The Central Bureau for Statistics in the Netherlands (CBS) has recently predicted that from the year 2010 cancer will be the number 1 cause of death in the Netherlands (http://www.cbs.nl). Traditional anticancer agents were developed prior to many of the landmark studies in cancer biology, and were consequently employed without insight into the molecular intricacies of the tumor cell. Conventional chemotherapeutics elicit cytotoxic effects by interfering with DNA synthesis and repair. This may be achieved by DNA alkylation, by incorporation of the drug into DNA, by direct binding to DNA, by inhibiting DNA topoisomerases or by binding to microtubules. Because DNA synthesis and repair occur in all dividing cells, conventional chemotherapeutics have a narrow therapeutic index and are frequently accompanied by significant adverse side effects. In addition, the accumulation of multiple mutations leads to drug resistance in many cancer cells. Molecular medicine paved the way for the development of drugs that specifically target tumor-associated oncogenically activated cell surface receptors and aberrantly activated signaling molecules. These agents have a much greater selectivity for tumor tissue and decreased risk of side effects.

One of the success stories in modern oncology is the discovery and development of imatinib, a potent tyrosine kinase inhibitor of the Bcr-Abl protein that is present in chronic myeloid leukemia (CML). A complete cytogenetic response (CCR) can be seen in more than 80% of newly diagnosed CML patients [1]. This has revolutionized the treatment of CML. Importantly, the success of imatinib has also opened a new era of molecularly targeted therapy as a basic concept in cancer treatment.

Colorectal carcinoma; epidemiology and treatment

Colorectal carcinoma (CRC) is the second most common form of cancer in the Western world and therefore poses a serious threat to public health. Death from this disease is usually associated with the formation of liver metastases. They develop in approximately 60% of CRC patients. Despite different new chemotherapeutic approaches, surgical resection is the only hope for cure. Unfortunately only 10% of the patients with liver metastases is eligible for resection [2]. This highlights the need for new therapeutic approaches.

From the late 1950s fluorouracil (FU) was the only drug approved for the treatment of colorectal cancer. However, response rates of 10% to 15% and a median survival of 10 months, resulted in only a minimal improvement over supportive care. The addition of leucovorin (LV) improved the response rate to nearly 25% at the cost of a small increase in toxicity. FU/LV became the standard first-line therapy for metastatic colorectal cancer from the late 1980s to 2002 [3,4].

Recently, new chemotherapeutic agents have been added to the standard therapeutic regimens against colorectal cancer. When incorporated into FU/LV-based regimens, both irinotecan and oxaliplatin show improved response rates and survival over FU/LV alone[5,6]. These combinations have prolonged the survival for patients with metastatic colorectal cancer to around 20 months.

Molecular targeted therapies against CRC

Molecular targeted therapies have also been introduced in the treatment of CRC. Bevacizumab, a monoclonal antibody directed against vascular endothelial growth factor (VEGF) improves the response rate and survival of patients with metastatic colorectal cancer when used with FU/LV regimens as initial therapy [7-9]. Cetuximab is a monoclonal antibody that specifically blocks the epidermal growth factor receptor (EGFR). The EGFR activates signaling pathways that are commonly deregulated in CRC cells. Cetuximab doubles the response rate and decreases the risk of cancer progression in irinotecan-refractory CRC patients [10,11].

Although both bevacizumab and cetuximab improve the outcome of CRC patients these drugs are far from cancer-specific. Dissecting the molecular mechanisms underlying CRC progression will not only accelerate the development of novel cancer-selective drugs but will also enable the therapeutic regimen to be personalized according to the molecular features of individual patients and tumors. One of the proteins that plays an important role in colorectal carcinogenesis is KRAS (see below). Given its crucial role in cancer development and possibly maintenance, KRAS may be an ideal molecular target in the treatment of (a subset of) CRC patients.

RAS proteins

RAS proteins (HRAS, KRAS and NRAS) are small (M_r~21,000 Da) membrane-bound guanine-nucleotide binding proteins which are regulated by a GDP/GTP cycle, being inactive when bound to GDP (RAS.GDP) and active when bound to GTP (RAS.GTP). RAS acts as an on/off switch that transduces extracellular signals (e.g. activated growth factor receptors) to intracellular effector pathways [12]. Approximately 30% of all human malignancies contain an oncogenic point mutation in codons 12, 13 or 61 of one of the RAS genes [13]. These mutations interfere with the intrinsic GTPase activity, rendering RAS constitutively active (RAS.GTP). This leads to growth factor-independent signaling. Activating point mutations in the three human RAS oncogenes have been detected in a wide variety of tumors. The frequency of mutations and the RAS isoform that is predominantly mutated displays tissue specificity. KRAS is by far the most frequently mutated isoform (~80%),

followed by NRAS (~20%) and HRAS (<1%). The highest incidence is found in adenocarcinomas of the pancreas (KRAS; 90%) [13]. In sporadic colorectal carcinomas (CRC), oncogenic RAS mutations are selectively observed in KRAS in approximately 40% of all patients [14-16]. KRAS mutations are already acquired at the very early premalignant stages of CRC formation. Targeted expression of KRAS ^{G12V} or KRAS ^{G12D} in intestinal epithelial crypt cells causes intestinal hyperplasia in the mouse [17-19]. Clearly, KRAS mutations are important as initiators of tumorigenesis. It is well established that signaling by activated RAS oncogenes also contributes to the metastatic phenotype of tumor cells (for a detailed review see chapter 1). Nevertheless, mutational activation of KRAS is in itself not sufficient to confer metastatic capacity on intestinal epithelial cells [17-19]. In order to progress to a metastatic carcinoma different genetic mutations have to accumulate. For CRC these molecular changes are relatively well defined and were originally described in the adenoma carcinoma sequence by Vogelstein et al [20]. If mutant KRAS is to be used as a target for specific molecular-based therapies (RAS-directed therapies), tumor growth should depend on its continued presence in the latest stages of CRC. Presently, this issue has not been addressed for human tumors. Wild-type RAS has important biological functions in normal cellular homeostasis. Ideally, RAS directed therapies should therefore only affect cells with oncogenic RAS or an aberrantly activated RAS pathway. In the following sections several approaches that meet some or all of these criteria are discussed.

RAS as a therapeutic target

If RAS is to be used as a therapeutic target, drugs can be developed that specifically a) inhibit RAS protein synthesis, b) inhibit RAS protein function, c) use mutant RAS as a basis of immunotherapy d) inhibit RAS effectors, or e) use mutant RAS in the concept of synthetic lethality.

a) Inhibition of RAS protein expression

Antisense oligonucleotide (ASO)

An antisense oligonucleotide (ASO) is a single-stranded, chemically modified DNA-like molecule that is 17–22 nucleotides in length and designed to be complementary to a specific mRNA. Specific binding of antisense oligonucleotides to target mRNA molecules by base pairing results in inhibition of mRNA translation by sterical hindrance or RNase H-mediated cleavage of the target mRNA [21]. The specificity of the antisense approach is based on the fact that a particular sequence of 17 bases in DNA occurs only once within the human genome. Several chemical modifications such as the development of β-D-Locked nucleic acids (LNAs) and ASOs with a phosphorothioate backbone have increased the resistance to nuclease digestion and prolonged tissue half-lives [22]. Currently, one HRAS targeting ASO (ISIS

2503) is under clinical investigation. ISIS 2503 is a 20-mer phosphorothioate oligodeoxynucleotide that hybridizes to the 5'-untranslated region of human HRAS mRNA. A phase I and II trial have reported several (partial) responses [23,24].

RNA interference (RNAi)

A technique that can be used to differentiate between mutated and wild-type transcripts is RNA interference (RNAi). RNAi is a sequence-specific post-transcriptional gene silencing mechanism induced by double-stranded RNA (dsRNA) molecules [25]. By reducing the length of the dsRNA molecules to <30 nucleotides (short interfering RNAs (siRNAs)) toxic non-specific effects are prevented [26]. Double-stranded siRNA blocks gene expression by sequence-specific base pairing to the target mRNA, resulting in post-transcriptional gene silencing. The dsRNA molecules are processed into siRNA fragments of about 22 nucleotides by the RNase III enzyme Dicer. These siRNAs are then incorporated into a RNA-induced silencing complex (RISC). This complex unwinds the dsRNA and uses the single stranded siRNA to recognize the substrate [27,28]. This leads to specific degradation of the target mRNA. One point mutation in the target gene significantly reduces the siRNA induced mRNA degradation. In contrast to other RAS directed therapies, this high specificity makes it possible to specifically target oncogenic RAS that harbors a single point mutation [29].

siRNA can be introduced into mammalian cells by several methods. First, siRNA molecules can be transfected into the target cells as a chemically synthesized product. Alternatively, siRNA molecules can be produced by the target cell itself after incorporation of the transcript-encoding genetic material. A crucial step forward for the use of RNAi as a therapeutic tool was made by the construction of expression vectors that mediate the continuous synthesis of siRNA-like molecules [30,31]. The use of RNAi in cancer therapy requires the development of a suitable delivery system. Several factors potentially limit the *in vivo* efficacy of RNAi therapy such as target cell selectivity, difficulties in getting the siRNA molecules across the target cell membrane, *in vivo* siRNA instability and side effects on non-target cells.

b) Inhibition of RAS protein function

Inhibition of RAS membrane localization

The RAS protein undergoes several post-translational modifications to become fully active. The first of these modifications is the covalent attachment of a hydrophobic farnesyl group to a conserved carboxy-terminal cysteine residue of the protein. The addition of hydrophobic molecules (i.e. prenylation) is necessary to anchor RAS to the inner side of the cell membrane. It is catalyzed by farnesyl protein transferase, which makes this enzyme an attractive target for intervention. Several farnesyltransferase inhibitors (FTIs) have recently been developed.

Unfortunately, the promising results in preclinical and *in vitro* models were not observed in the clinic [32]. Several factors can explain the observations in clinical

trials. It appeared that both KRAS and NRAS can also be modified by geranylgeranyl protein transferase as an alternative way of prenylation, particularly when cells were treated with FTIs [33]. This geranylgeranyl modification enables KRAS and NRAS to remain associated with the cell membrane and constitutes an escape mechanism for FTI-treated tumors. Furthermore, it has been shown that upon treatment with FTIs tumors displayed regression while the prenylation of RAS was not affected [34]. In addition, it has become clear that cell types without a RAS mutation are sensitive to FTIs as well [35]. These observations could indicate that farnesylated proteins other than RAS may be targets of FTIs. At the moment, approximately 30 proteins are known to be farnesylated and additional proteins are still being discovered. Clearly, the effects seen by FTIs are not limited to the inhibition of the function of RAS proteins. Therefore it is at least questionable whether treatment with FTIs should be seen as a "RAS-directed therapy".

Mutant RAS peptide inhibitors

Pincus et al. designed a series of peptides that correspond to RAS (effector) domains that display a changed conformation in mutant RAS [36]. These peptides, presumably acting in a dominant-negative fashion, selectively inhibited oncogenic but not insulin-activated wild-type RAS-induced oocyte maturation. None of these peptides have been tested for their anti-tumor effects in humans.

c) Mutant RAS-immunotherapy

Mutant RAS-immunotherapy is based on the fact that RAS proteins become tumor-specific by oncogenic point mutations. Mutant RAS is structurally and immunologically different from wild-type RAS and contains novel T-cell epitopes for recognition by the cellular immune system [37,38].

d) Inhibition of RAS effectors

RAS proteins regulate many different intracellular functions by acting on several different downstream targets. RAS activity is now linked to diverse biological responses, including cell proliferation, growth arrest, senescence, differentiation, apoptosis and survival. The importance of the RAF/MEK/ERK pathway in RAS oncogenic signaling has been firmly established. But during the last 30 years of RAS research, the list of RAS effectors has continued to grow. Understanding the relative contributions of the multiple RAS effector pathways to oncogenic transformation is not only a challenging issue today, but is also a prerequisite for the rational design of therapeutic strategies for specific cancer types.

Oncogenic RAS aberrantly activates its downstream effectors. Targeting these effectors could potentially be a way to target RAS-induced cellular transformation. Specific inhibitors have been designed against some of these targets and have been tested in clinical trials.

The RAF/MEK/ERK pathway

Since the discovery that activating mutations in the RAS oncogenes are found in 30% or more of human cancers, the RAF/MEK/ERK pathway has been the focus of intense drug discovery efforts. The development of molecularly targeted anti-cancer agents has resulted in some compounds specifically designed to target players of this pathway.

Sorafenib (BAY 43-9006), designed as a Raf kinase inhibitor, showed some anti-tumor activity in a phase I trial in combination with oxaliplatin in patients with refractory solid tumors, including colorectal cancer [39].

The oral mitogen-activated extracellular signal regulated kinase kinase (MEK) inhibitor, CI-1040 was generally well tolerated but demonstrated insufficient antitumor activity in a recent phase II trial in patients with advanced non-small-cell lung, breast, colon, and pancreatic cancer [40]. PD 0325901 and ARRY-142886, both second generation MEK inhibitors with significantly improved pharmacologic and pharmaceutical properties, have recently entered clinical development[41].

The PI3K/AKT pathway

In addition to the RAF/MEK/ERK pathway, drugs have been tested against other signaling pathways regulated by RAS. It has recently been shown that the PI3-K/AKT pathway is important for the maintenance of tumorigenic properties in RAS-mutated cells [42]. Several inhibitors of the PI3K downstream target mTOR (mammalian target of rapamycin) are currently being tested in clinical trials, including CCI-779, RAD001 and AP23573 [43].

COX-2

One of the downstream targets of RAS that has been implicated in tumor development and progression is cyclooxygenases-2 (COX-2) [44,45]. Overexpression of activated RAS isoforms stimulates COX-2 expression [46,47]. Nevertheless, it remains unclear how endogenous mutant KRAS relates to COX-2 expression and activity in CRC cells. Although epidemiological studies have shown that COX-2 inhibitors are active in the prevention of CRC development, it is unclear whether COX-2 is an attractive therapeutic target in the treatment of established or metastatic cancers that highly express COX-2.

e) Synthetic lethality / RAS context-driven therapeutics

Two genes ('*A*' and '*B*') are said to be synthetic lethal if "mutation of either gene alone is compatible with viability but simultaneous mutation of both genes causes death" [48]. In the context of cancer therapy this concept may result in the design of therapeutic compounds that are only lethal in the context of a cancer cell compared with normal cells. This phenomenon is called 'context-driven therapeutic index'. This cancer cell specific context can for example be (epi)genetic changes that are intrinsic to the cancer cell. Dolma et al. used a synthetic lethal high-throughput

screening to interrogate 23,550 compounds for their ability to kill RAS transformed cells but not their isogenic normal cell counterparts [49]. Camptothecin and a novel compound from a combinatorial library, named erastin (*e*radicator of RAS and ST-expressing cells) showed synthetic lethality with mutant RAS.

Reovirus T3D

In the field of synthetic lethality and RAS context-driven therapeutics reovirus T3D has great potential, as it specifically targets tumor cells with an activated RAS pathway [50,51]. Reovirus T3D is a double-stranded RNA-containing virus that belongs to the Reoviridae family. Reovirus was originally isolated from the respiratory and enteric tracts and lacked association with a disease. Therefore the virus was designated as an orphan virus an was named reovirus (respiratory enteric orphan virus) [52]. As a replication competent virus, reovirus has the advantage that progeny virus production at the tumor site increases the therapeutic effect. The exact mechanism that underlies the specific oncolysis of cells containing endogenous mutant RAS is not established. Furthermore, the ultimate targets of RAS signaling that promote reoviral oncolysis have yet to be defined. Apoptosis is the major mechanism of cell death induced by reoviruses [53,54]. It is well-established that under specific conditions oncogenic RAS deregulates processes that control apoptosis [55]. However, it is unknown if activated RAS signaling also affects reovirus-induced apoptosis.

Animal studies have demonstrated that reovirus exerts anti-tumor effects *in vivo* against numerous tumor types including CRC [56-61]. Nevertheless, it remains unclear whether human metastatic CRC is susceptible to reovirus T3D.

In addition to its oncolytic capacity, reovirus is known for its non-pathogenic nature in immunocompetent adults. Infections are subclinical and most adults have evidence of a past infection (up to 100% of adults are seropositive [62]). This makes reovirus an interesting oncolytic agent. However, the effect of the immune response on viral oncolysis is a relatively controversial topic. It has been suggested to reduce [63,64] or increase [65,66] the therapeutic response. Clearly, the effect of the host immune system on the therapeutic efficacy of reovirus T3D warrants further investigation.

Outline of the thesis

The aims of this thesis were 1) to assess the dependency of late-stage CRC cells on mutant KRAS and 2) to test the potential of reovirus T3D and COX-2 inhibitors as RAS-targeted therapeutics in experimental models of CRC and colorectal liver metastases.

The role of oncogenic RAS in the formation of colorectal liver metastases is evaluated in **chapter 2**. We give an overview of the existing literature of both experimental (*in vitro* and *in vivo*) and clinical studies that address this issue and we focus on the niches and questions that still exist and need to be answered.

Imaging of tumor growth, tumor characteristics and the effect of therapeutic interventions in living animals (intravital imaging) has been made possible by new molecular and optical techniques. In **chapter 3** we validate bioluminescence imaging as a minimally invasive tool to monitor tumor growth in the liver.

KRAS is important as an initiator of CRC tumorigenesis. In **chapters 4 and 5** we asses the dependency of late stage CRC cells on mutant KRAS. We analyze this with a highly aggressive CRC cell line that harbors an endogenous KRAS mutation. In **chapter 4** we assess the effect of endogenous mutant KRAS on the interplay between CRC cells and the immune system. In **chapter 5** we analyze which distinct stages in the process of liver colonization are affected by endogenous mutant KRAS.

RAS proteins signal through a number of distinct signaling cascades. COX-2 is important in the development of multiple cancers, including CRC and bladder carcinoma. The exact relationship between endogenous mutant RAS and COX-2 is unclear. In **chapter 6** we investigate this relationship in CRC cells. Furthermore, we investigate the potential therapeutic effects of selective COX-2 inhibitors in a mouse model of established CRC liver metastasis in **chapter 6** and in a mouse model of bladder carcinomas in **chapter 7**.

In the ensuing chapters we focus on reovirus, one of the most promising RAS-directed therapeutics. We investigate the mechanism underlying the RAS-specificity of tumor cell killing in **chapters 8 and 9**. Next, in **chapter 10** we assess the potential of reovirus as a therapeutic agent against experimental CRC liver metastases and investigate the role of the immune system on the therapeutic effect. In **chapter 11** we investigate the susceptibility of freshly resected human liver metastases to reovirus T3D infection.

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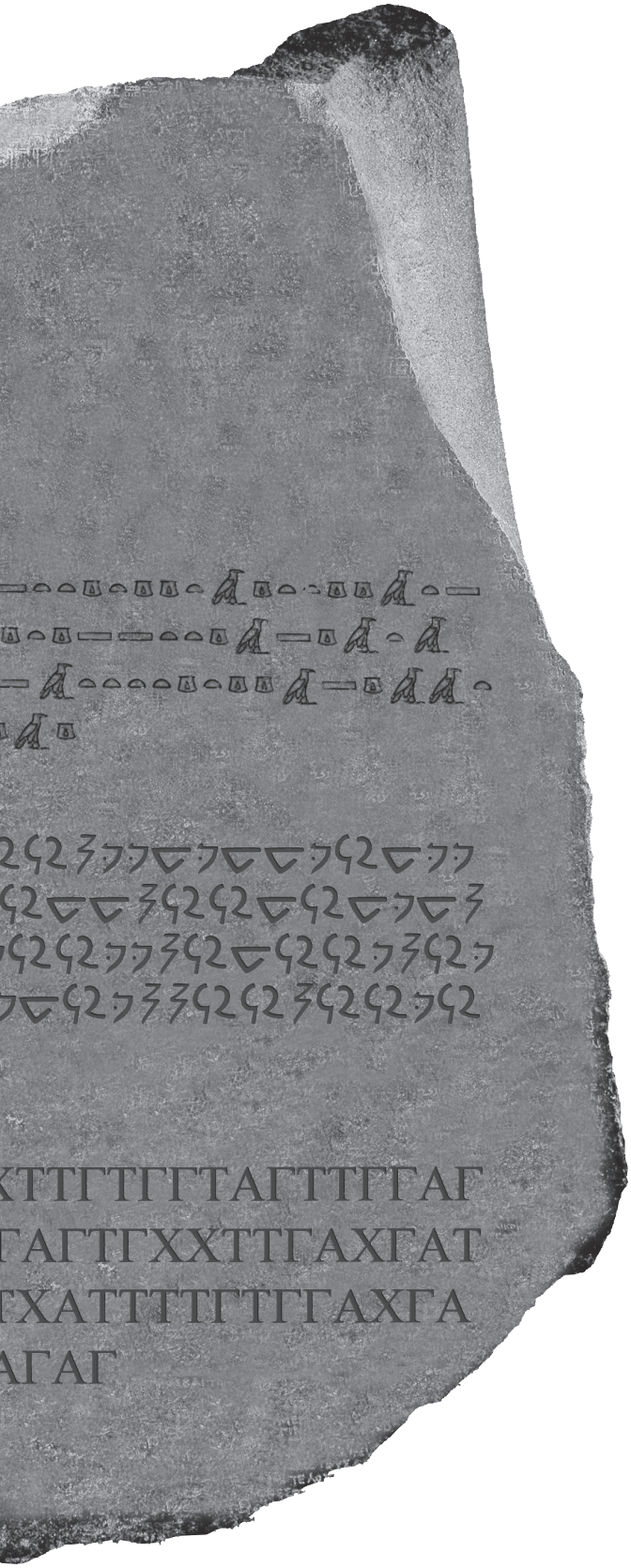

Chapter 2

Control of colorectal metastasis formation by KRAS

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Abstract

Mutational activation of the KRAS proto-oncogene is frequently observed during the very early stages of colorectal cancer (CRC) development. The mutant alleles are preserved during the progression from pre-malignant lesions to invasive carcinomas and distant metastases. Activated KRAS may therefore not only promote tumor initiation, but also tumor progression and metastasis formation. Metastasis formation is a very complex and inefficient process: Tumor cells have to disseminate from the primary tumor, invade the local stroma to gain access to the vasculature (intravasation), survive in the hostile environment of the circulation and the distant microvascular beds, gain access to the distant parenchyma (extravasation) and survive and grow out in this new environment. In this review we discuss the potential influence of mutant KRAS on each of these phases. Furthermore, we have evaluated the clinical evidence that suggests a role for KRAS in the formation of colorectal metastases.

Introduction

Activating mutations in the KRAS oncogene are observed in approximately 35% of all sporadic colorectal carcinomas (CRC) [1-3], and are already acquired at the very early pre-malignant stages of tumor formation. Furthermore, targeted expression of $KRAS^{G12V}$ or $KRAS^{G12D}$ in intestinal epithelial crypt cells causes intestinal hyperplasia in the mouse [4-6]. Thus, mutant KRAS acts as an initiator of tumorigenesis.

Mortality in CRC is primarily due to the consequences of tumor spread to the liver where metastases develop in the majority (~60%) of CRC patients. Mutational activation of KRAS is in itself not sufficient to confer metastatic capacity on intestinal epithelial cells [4-6]. Nevertheless, signaling by activated RAS oncogenes may contribute to the metastatic potential of tumor cells (for a recent review see [7]).

The formation of distant metastases is a multistage process that requires dramatic changes in epithelial cell behavior: First, the homotypic interaction between tumor cells within a solid tumor mass needs to be disrupted. Detached cells encounter a tumor-surrounding basement membrane which they have to degrade to disseminate from the primary tumor. Migration through the tumor-surrounding stromal tissue allows tumor cells to encounter blood and/or lymphatic vessels. Following breakdown of the vessel-surrounding basement membrane and migration through the endothelial cell layer, tumor cells enter the circulation (intravasation). During the hematogenous phase tumor cells are devoid of attachment to extracellular matrices and are exposed to cells of the immune system. Tumor cells disseminated from colorectal tumors travel through the gut-draining mesenteric veins and enter the liver via the portal system. Circulating tumor cells that arrive in the liver may adhere to the endothelium of larger vessels via specific receptor-ligand interactions. Alternatively, they get trapped in the sinusoidal microvascular bed which acts as a cellular sieve because the diameter of sinusoids is smaller than that of most tumor cells. Within the sinusoids, tumor cells encounter cells of the local immune system, notably liver-specific NK cells ('pit cells'), macrophages (Kupffer cells) and γδT cells which together form a very potent first line of defense against circulating tumor cells. Tumor cells that survive the hematogenous phase have to pass through the sinusoidal endothelial layer and degrade the basement membrane to reach the liver parenchyma. Extravasated tumor cells, still being attacked by activated and extravasated immune cells, have to adapt to the new microenvironment. Tumor cells may stay dormant either as single cells or as micrometastases in which apoptosis and proliferation are balanced. Further outgrowth is finally allowed by the formation of new blood vessels that provides the developing metastases with nutrients and oxygen.

In this review we will discuss the potential influence of mutant KRAS on overcoming each of the distinct barriers that protect an organism against metastatic tumor growth. Furthermore, we will evaluate the clinical evidence that suggests a role for KRAS in the formation of colorectal metastases.

KRAS regulates epithelial cell polarity

Epithelial cells are characterized by their polarized morphology with an apical plasma membrane organized into microvilli and a basolateral membrane that interacts with the matrix components of the basement membrane (BM), including laminin and collagen IV (Fig. 1). Polarization of epithelial cells is governed by cadherin-based cell-cell contacts (adherens junctions) and integrin-mediated adhesion to the extracellular matrix (ECM) [8]. Recently, it has been shown that colorectal cancer cells may also polarize in a cell-autonomous fashion [9].

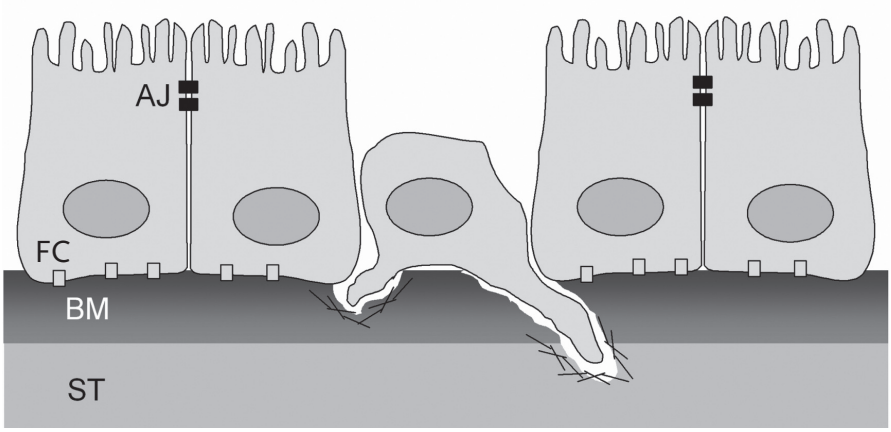

Figure 1. KRAS regulation of epithelial cell behavior. An acquired mutation in KRAS reduces adherens junction (AJ)-mediated cell-cell contacts. Cell-matrix interactions (focal contacts, FC) are reduced by the modulation of integrin expression, maturation and activity. Increased pericellular proteolysis by deregulated matrix metalloprotease-7 (MMP-7), urokinase and cathepsin B and L activities allows breakdown of the basement membrane (BM) and invasion of the stromal tissue (ST). Stimulation of cell motility by RAS involves modulation of Rho-family GTPase activities and cell-matrix interactions. Some of the protease-generated BM fragments could further stimulate cell motility. External factors like TGFβ are presumably required for the stable loss of epithelial characteristics. See text for details and references.

During the development of CRC, epithelial cells may loose their polarity and acquire a 'de-differentiated' spindle-shaped fibroblastoid morphology. This process is generally referred to as epithelial-mesenchymal transition (EMT). However, the degree of CRC de-differentiation varies greatly between tumors. Furthermore, the differentiation state of tumor cells within a single tumor may not be homogeneous: De-differentiated cells that have lost their polarization and their contact with other tumor cells are usually only observed at the invasive front of carcinomas where

single tumor cells detach from the primary tumor [10]. Such 'budding' tumor cell clusters identify tumors with a high propensity to form haematogenous and regional metastases in CRC patients [11,12].

Expression of $KRAS^{G12V}$ disrupts polarity of epithelial Madin-Darby canine kidney (MDCK) cells [13] and interferes with maturation of the integrin $\beta 1$ chain in human CRC cells [14]. Furthermore, deletion of endogenous $KRAS^{G12V}$ from CRC cells increased their adhesion to collagens I and IV, to laminin and to fibronectin, due to increased expression of the integrin subunits $\alpha 1$ and $\alpha 5$ [15]. Similarly, deletion of $KRAS^{G13D}$ from human HCT-116 colorectal carcinoma cells increased their adhesion to collagen [16]. Thus, mutant KRAS may interfere with epithelial cell polarity by interfering with integrin-mediated adhesion to the ECM.

Epithelial cell polarity also depends on the Ca-dependent cadherin-based adherens junctions [8]. Overexpression of $HRAS^{G12V}$ in rat intestinal epithelial cells or in MDCK cells leads to reduced cell-cell adhesion and adherens junction formation, which is accompanied by reduced expression and mislocalization of E-cadherin [17-20]. Overexpression of the RAS target c-Raf1 also causes MDCK cell depolarization and loss of intercellular contacts [21], whereas inhibition of MEK prevents it [17,21,22]. Thus, activation of the classical RAS-Raf-MEK-MAPK cascade controls epithelial cell polarity. Interestingly, a mutation in the RAS target B-Raf that causes hyperactivation of the MAPK cascade (V599E) is found in approximately 10% of all colorectal cancers [23], but whether this B-Raf mutant affects epithelial cell polarity has not been reported. In contrast to these studies, it was found that deletion of the endogenous $KRAS^{G13D}$ oncogene from HCT-116 CRC cells did not affect E-cadherin expression or adherens junction formation [16].

Carcino-embryonic antigen (CEA) is a cell surface glycoprotein that mediates homotypic Ca-independent cell-cell interactions [24]. In adult palisaded colonocyte monolayers CEA localization is tightly regulated and is only present at the apical luminal membrane. This localization is essential for maintaining basolateral polarity. $KRAS^{G12V}$, but not $HRAS^{G12V}$, up-regulates CEA expression in CRC cells, with improper membrane localization, increased CEA-mediated cell-cell adhesion and loss of basolateral polarity [25].

Taken together, mutant KRAS may disrupt epithelial cell polarity both by destabilizing adherens junctions and by reducing cell-matrix interactions through modulation of integrin expression [15], maturation [14] and activity [26] (Fig. 1). To what extent these phenomena are associated with the occurrence of KRAS mutations in human CRC remains to be established. It is important to note that the epithelial cells in aberrant crypt foci (ACF), the earliest recognizable precursor lesions in CRC, usually retain their polarization and intercellular adhesion, yet frequently contain mutant KRAS (reviewed in [27]). This suggests that an acquired mutation in KRAS during CRC development is not sufficient for a complete loss of epithelial cell polarity and for cell detachment.

Cooperation between KRAS and TGFβ

Local de-differentiation of tumor cells in the primary carcinoma is often reversed in distant metastases. This suggests that microenvironmental cues are important determinants of carcinoma cell differentiation and that mutational activation or overexpression of RAS oncogenes may not be sufficient to induce de-differentiation of epithelial cells. Signaling by transforming growth factor-*β* (TGF*β*) can cooperate with activated RAS to induce epithelial de-differentiation [21,28-31]. Most of these studies were based on the use of overexpressed $HRAS^{G12V}$ in mammary epithelial cells. In the absence of TGF*β* however, $HRAS^{G12V}$ promotes proliferation of fully polarized mammary epithelial cells [32]. In colonic epithelial cells however, $KRAS^{G12V}$, but not $HRAS^{G12V}$, induces TGF*β*-independent epithelial de-differentiation [25]. Therefore, it remains unclear to what extent the de-differentiation of colonic epithelial cells during CRC development requires the cooperative action of TGF*β* and oncogenic KRAS. We have recently found that the murine C26 colorectal carcinoma cell line contains an endogenous mutant allele. Stable RNA interference-mediated knockdown of $Kras^{G12D}$ resulted in reversion of the transformed spindle-like mesenchymal morphology and to a complete loss of metastatic potential (N.S., O.K., unpublished data). Inhibition of TGF*β* signaling in C26 cells by expression of a dominant-negative TGF*β* receptor also produced loss of the mesenchymal phenotype and strongly reduced metastatic potential [30]. Taken together, signaling by both endogenous $Kras^{G12D}$ and TGF*β* receptors appears to be essential for maintenance of the de-differentiated metastatic phenotype of C26 CRC cells.

The effects of TGF*β* signaling on colorectal cancer development are complex. In addition to the de-differentiation/metastasis-promoting effects outlined above, TGF*β* signaling can also suppress the initiation of colorectal tumor development. First, inactivating mutations in TGF*β*-R2 or the TGF*β* signal transducers Smad2 and Smad4 are found in a subset of human colorectal tumors [33]. Second, loss of Smad4 or Smad3, or expression of a dominant negative TGF*β*-R2 promotes colorectal tumorigenesis in mice [33,34]. Third, TGF*β* administration to azoxymethane-treated rats reduced the formation of ACF [35], whereas expression of a dominant negative TGF*β*-R2 promoted ACF formation [34]. The inhibitory effect of TGF*β* signaling on initiation of intestinal tumor growth and the stimulating effect of TGF*β* on epithelial cell de-differentiation and metastasis formation possibly reflects the differential effect of TGF*β* on pre-malignant epithelial cells and on established carcinoma cells [36]. Whereas KRAS and TGF*β* may cooperate to induce de-differentiation and metastasis-forming potential in carcinoma cells, it remains to be established whether TGF*β* differentially affects the pre-malignant lesions (ACF, adenomas) with and without KRAS mutations.

KRAS regulation of local tumor cell invasion.

Tumor cells that have lost their polarity and their contact with neighboring cells are usually found at the front of invading carcinomas. To disseminate from the primary tumor and to invade the local stroma, these cells have to pass through the epithelial basement membrane. Laminin and collagen IV are the major constituents of epithelial basement membranes and are readily degraded by a variety of proteolytic enzymes. Invading CRC cells produce matrix metalloproteases (MMP's) [37], cysteine proteases [38] (notably cathepsins B, H and L) and serine proteases [39] (notably urokinase plasminogen activator (uPA)) that promote BM breakdown and facilitate migration through the stromal extracellular matrix. Degradation of the BM not only removes a physical barrier for tumor cell dissemination, but also leads to the generation of laminin and collagen IV fragments that actively stimulate tumor cell migration and angiogenesis [40,41]. In addition, MMP's cleave a plethora of non-ECM substrates, including cell surface- and matrix-bound growth factors and cytokines, growth factor receptors, proteases and their inhibitors, and cell adhesion molecules [42,43], thus creating a microenvironment that is conducive to local tumor outgrowth and angiogenesis.

BMs in carcinomas are often discontinuous [44-53], which is indicative of local BM destruction. Disruption of the BM appears to be a late event in CRC development as it is associated with areas of local tumor invasion ('budding') involving poorly differentiated tumor cell islets, and with distant metastases [44-53]. As mentioned above, KRAS mutations are found already in pre-malignant lesions (ACF and adenomas) that usually contain intact BM's [27]. Thus, an acquired mutation in KRAS alone is apparently not sufficient for the loss of BM integrity. Nevertheless, mutant KRAS may contribute to BM breakdown by stimulating the expression and/or activity of several classes of proteases, including MMP's, cathepsins and uPA (see below).

It was recently reported that tumor cells may also migrate through the ECM in a protease-independent manner, by drastically changing their cell shape to an 'amoeboid' phenotype which allows them to traverse the ECM without destroying it [54]. It is presently unknown whether this is a relevant mechanism for disseminating CRC cells and whether KRAS modulates this mode of cell movement.

KRAS regulation of metalloproteases

MMP-7 (Matrilysin-1) is expressed early during colorectal cancer development [55] and its expression is correlated with metastatic potential [56,57]. Mutant KRAS can stimulate MMP-7 expression in colorectal carcinoma cells [58] and is essential for high level MMP-7 expression in a pancreatic carcinoma cell line [59]. Furthermore, MMP-7 expression correlates with the presence of KRAS mutations in pancreatic carcinomas [60], but not in colorectal carcinomas, where it correlates with nuclear β-catenin [61]. MMP-7 is also highly expressed in APC-deficient polyps (with active β-catenin) in the mouse intestine, and promotes intestinal polyp formation [62]. Thus,

MMP-7 expression is a determinant of metastatic potential that is controlled by both KRAS and β-catenin signaling. The early detection of MMP-7 in pre-malignant lesions during CRC development may therefore be the direct result of APC loss and/or mutational activation of KRAS.

MMP-2 and MMP-9 constitute the subclass of gelatinases. MMP-2 expression in mouse fibroblasts critically depends on the expression of endogenous KRAS [63]. Although it is clear that increased gelatinase expression and activity contributes to CRC development [64], it is less clear to what extent this is regulated by KRAS. In contrast to MMP-7, an epithelial-specific MMP, expression of MMP-2 and MMP-9 is usually confined to tumor-associated stromal cells [37]. Nevertheless, a screen for protease-activities in colorectal cancer biopsies identified MMP-1, MMP-2 and MMP-9 as the major tumor-cell-produced MMP's [65]. Thus far, it remains unclear to what extent KRAS contributes to enhanced MMP-2 and MMP-9 expression in colorectal cancer tissue, be it produced by tumor cells themselves or, via the release of diffusible factors, by stromal cells.

KRAS regulation of uPA and uPA-receptor

Urokinase and tissue-type plasminogen activators (uPA and tPA) are serine proteases with a highly selective substrate preference for plasminogen. PA-mediated cleavage of plasminogen results in the formation of the active protease plasmin. Plasmin has broad substrate specificity and degrades fibrin and extracellular matrix components, but it can also activate MMP's resulting in further ECM breakdown. uPA expression is associated with the progression of CRC and is associated with poor prognosis and metastasis formation [39,66-68]. Tumor-associated uPA is produced both by stromal cells [69,70] and by tumor cells [71]. On tumor cells the uPA receptor (uPAR) localizes uPA to the cell surface, thereby promoting pericellular proteolysis and tumor cell migration through basement membranes and extracellular matrices [39,67]. Furthermore, uPA binding to the uPAR stimulates cell proliferation and migration in a manner that is independent of uPA proteolytic activity [72].

It is well established that the overexpression of mutant RAS genes results in elevated uPA expression in several cell types [73,74]. Expression of $KRAS^{G12V}$ in fibroblasts strongly stimulated uPA release and cell surface binding [75]. Furthermore, $KRAS^{G13D}$ is required for high level expression of the uPA receptor in a human CRC cell line [76]. In the same cells, $KRAS^{G13D}$ is also required to maintain co-localization of uPA with cathepsin B in caveolae at the cell surface [77,78]. Cathepsin B, itself deregulated during CRC progression (see below), is a cysteine protease that can act as an activator of pro-uPA [79]. Thus, mutant KRAS orchestrates a proteolytic cascade by promoting uPA and uPAR expression and by stimulating uPA activation at the plasmamembrane [77,78].

In human CRC the expression of uPA and uPAR are often associated with areas of local invasion [80] indicating that local micro-environmental factors may influence uPA expression in invading tumor cells. These areas are also characterized by nuclear

β-catenin [81], which, in addition to KRAS, promotes uPA expression [82]. It is presently unknown whether KRAS mutations in CRC correlate with increased uPA activity.

KRAS regulation of cathepsins

The cysteine cathepsins are a class of lysosomal proteases involved in intracellular protein degradation. However, cathepsins are also found outside the lysosomes, notably in the cytoplasm, in the nucleus and at the cell surface. Furthermore, cathepsins may be secreted into the extracellular space. Secreted and cell surface-localized cathepsins B, L and H control CRC progression [38], presumably by degrading basement membrane components like laminin and collagen IV [83-85].

Early studies have shown that in fibroblasts expressing either $HRAS^{G12V}$ or $KRAS^{G12V}$ cathepsin L mRNA and protein is most abundantly induced [86,87]. Furthermore, KRAS mutations in colorectal cancer are associated with increased cathepsin L expression [88]. As mentioned above, $KRAS^{G13D}$ promotes association of cathepsin B with caveolae in human CRC cells, where it may control uPA activity and pericellular proteolysis [77,78]. In line with this, cathepsin B is found at the basal membrane of colorectal adenomas and carcinomas [89] where it is ideally positioned to degrade BM components. In addition, cathepsin B expression has been closely associated with local colorectal tumor invasion ('budding') and areas of BM disruption, and may therefore be a critical factor in controlling metastasis formation [49,90,91]. It is presently unknown whether cathepsin H is also regulated by KRAS. The modulation of cathepsin B/L expression and localization by mutant KRAS is likely to contribute to the metastatic phenotype of colorectal cancer cells (Fig. 2).

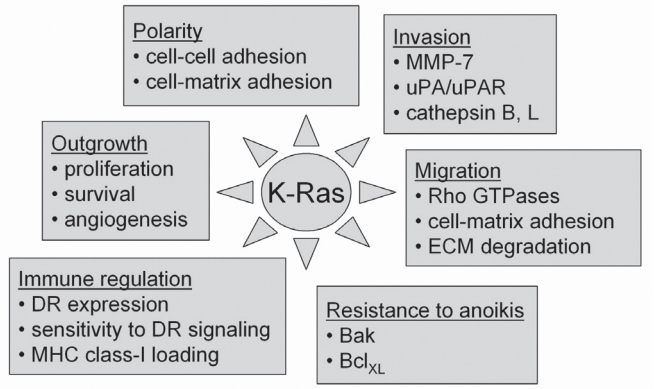

Figure 2. KRAS modulation of the metastatic cascade. KRAS promotes the initial de-polarization of epithelial cells within a tumor mass and stimulates their invasive and migratory capacity. In addition, KRAS promotes survival of non-adherent tumor cells and their resistance to immune-mediated clearance. Finally, KRAS promotes the outgrowth of micrometastases by stimulating proliferation, survival and angiogenesis. See text for details and references. ECM: extracellular matrix. DR: death receptor.

KRAS regulation of tumor cell migration

Following breakdown of the BM and release from the primary tumor, the detached tumor cells migrate through the stromal ECM towards nearby vascular beds. Tumor cell locomotion requires the coordinated attachment and detachment of cells to components of the ECM. This process is governed by composition of the ECM, by the repertoire of ECM-degrading enzymes produced by tumor cells and stromal cells, and by the presence of motility promoting growth factors such as PDGF, EGF and TGFβ. Furthermore, tumor cells themselves may change the local composition of the ECM by aberrantly producing ECM components. For instance, expression of the laminin-5 γ2 chain is especially high in budding CRC cells [92,93].

It is well established that the overexpression of mutant RAS isoforms promotes the motility of several cell types [94,95]. Interestingly, KRAS appears to be more effective than H- and NRAS in promoting cell migration [96,97]. *Vice versa*, deletion of the endogenous $KRAS^{G13D}$ oncogene from HCT-116 CRC cells markedly reduced cell motility [98]. Distinct mechanisms may underlie modulation of cell migration by activated KRAS. First, RAS controls the activity of the Rho family GTPases Rac and RhoA, key regulators of cell migration and adhesion [94,95,99]. Second, as mentioned in section 2, RAS signaling affects the expression, maturation and activity of several integrins [14,15,26]. Third, RAS can stimulate the expression of ECM components. For instance, antisense inhibition of the endogenous $KRAS^{G12D}$ oncogene in a pancreas carcinoma cell line decreased expression of the laminin-5 chain β3 [59]. In addition to stimulating laminin 5 expression, KRAS also stimulates the production of laminin-degrading proteases in CRC cells (see above). This could lead to the local overproduction of laminin 5 fragments with migration-stimulating potential [40].

Intravasation

Migrating tumor cells that encounter blood vessels need to pass through the BM to reach the vasculature. Although the composition of epithelial- and endothelial- associated BM's may vary, it seems likely that the KRAS-induced changes in pericellular proteolysis, as outlined in section 4, promotes the degradation of both tumor- and endothelium-associated BM. However, evidence for an intravasation-promoting effect of mutant KRAS during CRC development is currently lacking. In contrast to blood vessels, lymphatic vessels are devoid of a continuous BM and display large gaps between the endothelial cells [100]. Presumably, this facilitates the intravasation of tumor cell into the lymphatic system and the formation of lymph node metastases. Lymphatic vessels are closely associated with developing adenomas and invasive carcinomas during CRC development [101]. Interestingly, a large population-based study involving 1413 CRC patients showed that $KRAS^{G12}$ mutations were more frequently detected in advanced stage CRC with regional lymph node and distant metastases than in non-metastatic tumors [102].

KRAS prevents anoikis

Intravasated tumor cells are carried via the mesenteric veins into the portal system of the liver. During this hematogenous phase, tumor cells are devoid of contact with extracellular matrices, experience shear stress and encounter cytotoxic immune cells. Most adherent cells including intestinal epithelial cells undergo programmed cell death in response to loss of cell-matrix adhesion (anoikis). Therefore, metastasis formation requires the development of anoikis-resistance in circulating tumor cells. RAS oncogenes can induce both pro- and anti-apoptotic signaling. In carcinoma cells anti-apoptotic signaling prevails. It has long been known that overexpression of an exogenous RAS oncogene in MDCK epithelial cells protects these cells against anoikis [103]. Furthermore, ribozyme-mediated suppression of endogenous $KRAS^{G12V}$ in lung carcinoma cells or deletion of $KRAS^{G13D}$ in CRC cells sensitizes these cells to anoikis [104,105]. Several RAS-activated signaling pathways may mediate protection against apoptosis [106]. Central to RAS-induced resistance to anoikis is suppression of the pro-apoptotic Bak gene [107]. In addition, RAS prevents downregulation of the anti-apoptotic Bcl_{XL} protein in detached cells [105].

KRAS regulation of the anti-tumor immune response

The anti-tumor immune response in the liver typically involves cytotoxic $CD8^+$-T cells, $NK1^+$-T cells, $\gamma\delta$T cells, macrophages (Kupffer cells) and natural killer (pit) cells [108-110]. Metastasis-competent tumor cells have acquired mechanisms that enable them to survive these attacks either by preventing their recognition by immune cells, and/or by defending themselves against the cytotoxic compounds that are secreted to kill them.

Tumor cell survival in the sinusoids

As circulating tumor cells are arrested in the liver sinusoids, either by size restriction or by active adhesion, they encounter the sinusoid-resident immune cells. In particular, liver NK cells ('pit' cells) and $NK1^+$-T cells are potent suppressors of liver metastasis formation [110-112]. Can KRAS signaling modulate the susceptibility of CRC cells to cell killing by these cytotoxic lymphocytes? Overexpression of activated RAS isoforms may promote cellular sensitivity to cytolysis by NK cells [113-116]. However, overexpression of $HRAS^{G12V}$ had no effect or decreased cytolysis of human CRC cells [117,118]. These studies have used peripheral or splenic NK cell populations. It should be noted that pit cells are a specialized subset of NK cells with properties of chronic activation [112]. Therefore, tumor cells may show differential sensitivity to NK cells isolated from peripheral blood or the spleen when compared to those isolated from the liver. At present it remains unclear whether the presence of KRAS in human CRC cells affects their sensitivity to cytolysis by pit cells.

KRAS suppression of antigen processing and presentation

CD8+ cytotoxic T-lymphocytes (CTLs) recognize antigenic peptides buried in the antigen-presenting groove of MHC class I molecules and are designed to kill cells that display non-self antigens [119]. By lowering MHC class I expression, tumors may prevent the presentation of tumor-specific antigens and thereby prevent effective recognition and clearance by CTLs [120]. Transfection of NIH-3T3 fibroblasts with $HRAS^{G12V}$ resulted in strongly decreased MHC class I expression and a reduced CTL response [121-123]. $HRAS^{V12}$-transformed NIH_3T_3 cells expressed strongly reduced levels of TAP and LMP, two essential components of the antigen processing machinery (APM). This resulted in reduced peptide-loading and unstable cell surface expression of MHC class I molecules [123]. Immunohistochemical analysis of CRC samples indicated that the presence of KRAS mutations in CRC correlated with a deficiency of the APM components TAP1, LMP, tapasin and HLA class I [124]. Moreover, loss of APM components was more pronounced in lymph node metastases than in the corresponding primary lesions [124]. Thus, modulation of antigen processing and MHC class I loading by KRAS may contribute to immune evasion of CRC cells and, thereby, to metastasis formation.

KRAS modulation of tumor cell sensitivity to death receptor ligands

Cytotoxic lymphocytes, including NK cells and CTLs kill their target cells via two distinct pathways. First, perforin promotes 'perforation' of the target cell membrane which enables transfer of apoptosis-inducing granzymes to the target cell. It is unknown whether RAS signaling interferes with tumor cell killing by this pathway. Second, cytokines like TNFα, TNF-related apoptosis-inducing ligand (TRAIL) and Fas-ligand bind to death receptors on their target cells to induce apoptosis. Overexpression of RAS oncogenes can cause downregulation of Fas and TNF-R in several cell systems [125-128], thus rendering cells insensitive to FasL and TNFα. Alternatively, overexpressed $KRAS^{G12V}$ interfered with FasL-induced caspase-3 activation [129], possibly due to modulation of Bax and Bcl_{XL} expression (See above). Taken together, overexpression of mutant RAS appears to protect cells against FasL- and TNF-induced apoptosis. In contrast, TRAIL-induced apoptosis is facilitated in cells overexpressing either $HRAS^{G12V}$ or $KRAS^{G12V}$, possibly by up-regulating the TRAIL receptor death receptor 5 (DR5), and/or by promoting caspase-8 recruitment to DR5 [130,131].

To what extent CRC sensitivity to death receptor activation is regulated by endogenous mutant KRAS is not clear. A characteristic trait of CRC cells is their relative resistance to apoptosis-inducing ligands. In fact, overexpression of FasL is commonly observed in CRC liver metastases [132], which may cause tumor-induced killing of infiltrating lymphocytes ('counterattack') and surrounding hepatocytes [133,134].

Extravasation, survival, and proliferation

Once circulating tumors cells reach the liver they may arrest in the sinusoidal capillary bed. This can occur by two distinct mechanisms. First, tumor cells may attach to the endothelium in a process resembling the adherence of leucocytes to the sinusoidal endothelium. This interaction involves binding of tumor cell surface carbohydrates to selectins expressed on the surface of endothelial cells [135]. KRAS in colorectal cancer cells may modulate the cell surface carbohydrate repertoire [136,137] and may thereby modulate the interaction between tumor cells and the sinusoids. Second, tumor cells may simply arrest by size restriction, if the tumor cell diameter is larger than the diameter of the sinusoids [138,139].

Following attachment to the liver sinusoids, tumor cells pass through the endothelial cell layer and encounter the low-density BM which allows easy diffusion and extravasation [140]. Laminin and collagen IV are major components of the sinusoidal BM. It seems likely that tumor cells use the same repertoire of proteolytic enzymes for extravasation that allowed them to disseminate and intravasate. As outlined above, the expression of some of these enzymes is controlled by mutant KRAS. Ectopic expression of $HRAS^{G12V}$ in fibroblasts promotes the proliferation and survival of growing micrometastases, but not extravasation [141]. Whether this is also true for endogenous mutant KRAS in CRC cells has not been tested.

Once extravasated, tumor cells need to adapt to their new microenvironment. Under continuous attack of liver-associated immune cells they have to survive and start proliferating to form new tumors. Infiltration of primary and metastatic colorectal tumors by lymphocytes (tumor-infiltrating lymphocytes, TIL) is strongly correlated with a favorable prognosis [142,143]. When compared to primary CRC tumors, liver metastases show a marked reduction in the presence of TIL [132]. Signaling by overexpressed RAS oncogenes can stimulate the production of immune suppressing molecules such as Il-6, COX-2 and TGFβ [74,144-149]. Mutant KRAS in liver metastases may thus contribute to reducing the recruitment and activation of lymphocytes.

The effect of mutant KRAS on (epithelial) cell proliferation is well documented and has been the subject of many reviews (See for instance [150]). In CRC this is probably best illustrated by the high frequency of KRAS mutations in hyperplastic ACF which are characterized by an increased number of cytologically normal epithelial cells [27]. No other (known) genetic alterations are associated with the formation of hyperplastic ACF. In addition, hyperplastic ACF are induced in transgenic mice expressing KRAS in intestinal epithelial cells [4]. Whether mutant KRAS is still required for CRC proliferation in liver metastases has not been formally shown and is difficult to assess. However, the persistence of mutant KRAS in liver metastases (having undergone a stringent selection process) suggests that it is (see below).

Finally, developing tumors are dependent on and stimulate the formation of a new vascular network (angiogenesis) to supply it with nutrients and oxygen. We have recently reviewed the stimulating effect of RAS on angiogenesis [74].

Clinical evidence that KRAS promotes metastasis formation

In the above sections we have reviewed the literature describing how KRAS may influence the distinct phases of metastasis formation, based on *in vitro* studies and on pre-clinical models of metastasis formation. In this section we will review the available clinical data implying mutant KRAS in metastasis formation in human CRC.

Table 1. Association of KRAS mutations with tumor stage and disease free survival (DFS).

					Association with	
Reference	*patient #*	*Detection method*	*Codon*	*% mutated*	*stage*	*DFS*
2	3439	Diverse	12,13	NR	NR	+
1	2721	Diverse	12,13	38	-	+
3	1413	direct sequencing	12,13	32	+	NR
151	345	SSCP, sequencing	12,13	39	NA	NR
152	247	Sequencing	12,13	37	+	-
153	245	IHC	NA	59	+	NR
154	220	SSCP, sequencing	12,13	41	-	NR
155	210	ASH, sequencing, SSCP	12,13	30	+	NR
156	191	TTGE	12,13	32	-	NR
157	160	SSCP, sequencing	12,13	46	+	+
158	138	IHC	NA	59	+	NR
159	135	ASH	12,13	40	NR	NR
160	132	RFLP, sequencing	12,13	41	-	-
161	122	SSCP	12,13	38	NA	NR
162	118	IHC	NA	69	+	NR
163	117	SSCP, sequencing	12,13	65	-	NR
164	115	RFLP, SSCP	12,13	38	-	NR
165	114	ASH	12,13,61	29	-	NR
166	112	direct sequencing	12,13	27	-	-
167	109	IHC	NA	32	-	NR
168	100	SSCP, sequencing	12,13	30	-	NR
169	100	RFLP, sequencing	12,13	40	-	NR
170	100	RFLP	12	35	-	NR
171	100	RFLP	12	24	-	NR

The table only includes studies with ≥100 patients. DFS: disease free survival, NR: not reported, NA: not applicable, SSCP: single strand conformational polymorphism, IHC: immunohistochemistry, ASH: allele specific hybridization, TTGE: transient tem-perature gradient gel electrophoresis, RFLP: restriction fragment length polymorphism.

Do KRAS mutations predispose to the metastatic behavior of CRC tumors? If that is indeed the case KRAS mutations should be associated i) with increased tumor stage, and ii) with decreased disease-free survival (DFS), defined as the time from treatment to the recurrence of local and/or distant disease. Many studies have addressed this question and have come to markedly divergent conclusions (table 1). Several inter-study variables may affect the outcome of such studies. First, various methods have been used to detect mutations in KRAS, including 'restriction fragment length polymorphism' (RFLP), 'single strand conformational polymorphism' (SSCP), allele-specific hybridization (ASH), and direct sequencing of PCR-amplified genomic DNA. Second, whereas activating mutations are generally found in codons 12, 13 and 61, many studies have only focused on codon 12 and 13, and some have only looked at specific mutations in one of these codons. Third, the tumor material used may vary from freshly isolated specimens to paraffin-embedded archival material. Fourth, tumors can be heterogeneous and may contain multiple tumor clones with different genetic aberrancies and different metastatic potential. In addition, the presence of intra-tumoral stromal tissue may vary. Thus, the outcome of mutation analyses may depend on the selection of specific tumor area(s). Fifth, the type of mutation within a single codon may influence protein function and tumor aggressiveness, but is not always analyzed. Sixth, racial or topographic differences play a role in oncogenesis. Therefore, homogeneity of the study population is preferable.

In the RASCAL (I and II) studies the association of KRAS mutations with CRC clinicopathological parameters was evaluated, using data on 3439 CRC patients that were gathered from institutes from around the world [1,2]. There was no association between the presence of KRAS mutations and tumor stage (i.e. metastasis formation) [1,2]. However, multivariate analyses showed that the specific G12V amino acid substitution, caused by G>T mutation, was associated with reduced DFS in Dukes C tumors which are characterized by regional metastases [1,2]. The RASCAL study was based on data obtained from many different institutes with unavoidable inter-institutional variations in experimental setup that influence the statistical analyses. Analysis of a patient group from a single institution would reduce such undesirable variables. By far the largest single institution population-based study (1413 patients) showed that tumors diagnosed at an advanced disease stage, with distant and/or regional metastases, were significantly more likely to harbor a KRAS mutation than those diagnosed at an early stage [3]. This association was significant for mutations in codon 12, but not for mutations in codon 13 [3]. In contrast to the RASCAL studies, an association of KRAS mutations with DFS was not found [3].

If KRAS mutations predispose to metastasis formation, one would expect the mutation frequency in metastases to be higher than that in the primary tumors. Relatively few studies have evaluated the KRAS mutation rate in CRC metastases (Table 2).

Table 2. Association of KRAS mutations with metastasis formation in colorectal cancer patients

reference	*Detection method*	*Codon*	*primary*	*R+D Mets.*	*Liver Mets.*
172	ASH	12, 13	34 (26/78)	36 (21/58)	38 (10/26)
173	Sequencing	12, 13	37 (92/247)	43 (69/161)	59 (20/34)
174	Sequencing	12, 13	35 (42/119)	65 (35/54)	68 (13/19)
175	SSCP, sequencing	12, 13	NR	NR	37 (7/19)
176	SSCP, sequencing	12, 13	56 (14/25)	50 (10/20)	NR
177	SSCP, sequencing	12, 13	NR	51 (20/39)	11 (2/19)
178	ASH	12, 13, 61	33 (31/93)	53 (23/43)	50 (10/20)
179	ASH	12	NR	NR	15 (6/41)
180	ASH	12, 13	NR	46 (11/24)	39 (7/18)
181	SSCP, sequencing	12, 13	NR	NR	43 (15/35)
182	ASH	12	21 (23/109)	NR	26 (15/58)
Total			34 (228/671)	47 (189/399)	36 (105/289)

The table shows the percentages of tumors (primary versus regional (R) and distant (D) metastases versus liver metastases) that are positive for any mutation in KRAS. The numbers of positive tumors per total number of tumors analyzed is shown in parentheses. NR: not reported, SSCP: single strand conformational polymorphism, ASH: allele specific hybridization.

We have compiled the data from these studies and evaluated the KRAS mutation rate i) in primary tumors, ii) in metastases occurring at any regional or distant location (lymphatic and hematogenous), and iii) in liver metastases specifically. The KRAS mutation frequency in primary CRC tumors (34%) correlates well with the published frequencies from larger studies [1-3]. The KRAS mutation frequency in liver metastases is similar (36%). However, the KRAS mutation frequency in all regional and distant metastases appears to be considerably higher (47%) than that in the primary tumors. This suggests that primary CRC tumors harboring KRAS mutations could be more prone to metastasize than tumors carrying wild type KRAS, although not selectively to the liver. Well-designed prospective studies are required to definitively assess whether (which) mutations in KRAS predispose to metastasis formation. Taken together, the available data suggest that the presence of activating KRAS mutations, in particular in codon 12, is associated with increased tumor stage, with decreased disease-free survival, and, possibly, with the formation of metastases.

Conclusions and perspectives

Through its effects on cell polarity, invasiveness, migratory potential, proliferation and survival activated RAS may increase the efficiency with which tumor cells proceed through the distinct phases of the metastatic cascade (Fig. 2). Indeed, overexpression of activated RAS in tumor cells or in fibroblasts promotes their metastasis forming potential in mouse model systems. Furthermore, the presence of $KRAS^{V12}$ in human CRC appears to be associated with an increased propensity to form metastases. Nevertheless, it remains unclear how mutant KRAS facilitates metastasis formation during the progression of CRC in man.

The majority of studies on oncogenic RAS have been carried out by overexpressing mutant H-, K- or NRAS, often in non-epithelial cell types. Although this has immensely increased our understanding of RAS signal transduction and protein function, it is important to note that the changes in cell behavior induced by activated RAS are critically dependent on the level of expression, on the RAS isoform used, on the specific activating mutation, and on the cellular context. Thus, conclusions based on the use of $HRAS^{V12}$-overexpressing fibroblasts may not always apply to CRC cells carrying an endogenous mutant KRAS allele. This is illustrated by the finding that $KRAS^{V12}$ expression in intestinal epithelial cells fails to generate metastatic tumors, whereas fibroblasts overexpressing activated $HRAS^{V12}$ can be highly metastatic.

To further increase our understanding of mutant KRAS function in colorectal metastasis formation it is desirable to assess how suppression of endogenous mutant KRAS alleles affects the distinct phases of metastasis formation *in vivo*. In addition, cooperative signals like those elicited by TGFβ are likely to be essential for KRAS-facilitated metastasis formation and require further identification.

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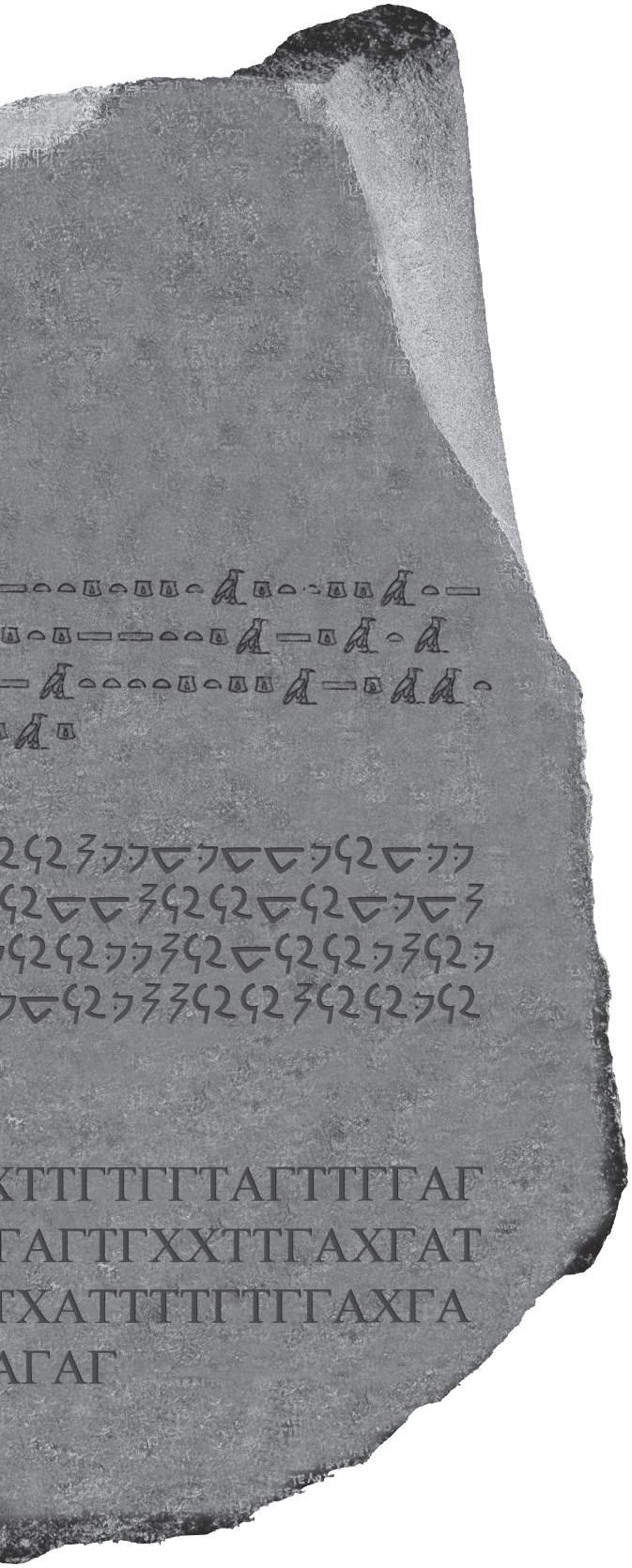

Chapter 3

Validation of bioluminescence imaging of colorectal liver metastases in the mouse

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Abstract

Background

In mouse models for metastatic growth of colorectal carcinoma (CRC) cells in the liver, tumor growth is routinely measured by determining the area of liver tissue that has been replaced by tumor tissue (HRA: hepatic replacement area). This technique has several major disadvantages. Modern visualization techniques make it possible to image tumor growth non-invasively. In the present report we have validated bioluminescence imaging of liver metastases by comparing it to standard HRA measurements and liver weight.

Materials and Methods

BALB/c mice received an intrasplenic injection of luciferase-expressing C26 CRC cells and the spleen was subsequently removed. On days 5, 7, 9 and 11 post-injection, luciferase activity was measured. After imaging, the mice were sacrificed and the liver was removed, weighed and fixed. HRA was determined by analyzing liver tissue sections. Comparative trend analyses between luciferase activity, wet liver weight and HRA were then performed.

Results

Luciferase activity, wet liver weight, and HRA all increased over time. Statistical analyses showed that all three types of measurements display a highly significant degree of correlation.

Conclusions

The measurement of tumor growth in the liver by imaging luciferase activity correlates well with the standard method of determining the HRA and with the increase in liver weight that results from tumor growth. Given the great advantages of measuring luciferase activity over measuring HRA, we conclude that bioluminescent imaging is a reliable and superior method for measuring experimental CRC growth in the liver.

Introduction

Colorectal cancer (CRC) is the second most common form of cancer in Europe. Death from this disease is usually associated with the formation of liver metastases. Surgical resection is as yet the only hope for cure, but only 10% of the patients with liver metastases is eligible for resection [1]. Mouse models have become invaluable tools for testing novel approaches in the treatment of liver metastases. Metastatic CRC cell lines are commonly used to study CRC tumor growth in the liver. Tumor growth is usually quantified on histological tissue sections by morphometric determination of the area of liver tissue that has been replaced by tumor tissue (HRA: hepatic replacement area) [2,3]. This technique has several major disadvantages. First, tumor growth can only be measured *post-mortem*. Second, prior to the start of therapy the mice cannot be randomized into groups with comparable tumor outgrowth. Third, analysis of the HRA is a laborious and time-consuming technique. Modern visualization techniques such as positron emission tomography, magnetic resonance imaging, single photon emission tomography, fluorescence imaging and bioluminescence imaging (BLI) make it possible to image tumor growth non-invasively [4]. In humans tomography and MRI are accurate methods for non-invasive determination of tumor size. For experiments in small laboratory animals fluorescence and bioluminescence imaging are ideal imaging techniques since they are comparatively cheap and the memory size of the generated data sets is small, allowing rapid analysis [4-7]. The growth of tumor cells expressing the firefly luciferase gene can be measured by BLI without the disadvantages of conventional assessment of tumor growth outlined above. BLI has been successfully used for imaging tumor growth and/or therapeutic efficacy in brain, lung, heart, prostate, lymph nodes, bone marrow, liver and subcutaneous tissue [8-16]. However, a thorough validation for this technique in quantifying the growth of multiple isolated liver metastases in the mouse is lacking. This is especially relevant since the liver contains high levels of hemoglobin that may interfere with the detection of emitted light [17]. Furthermore, as tumors grow larger the efficiency of light emission may decline [18]. Therefore, we set out to validate BLI for measuring CRC liver metastasis growth by comparing it to standard HRA measurements and liver weight.

Materials and Methods

Cell lines and culture conditions

The murine colon carcinoma cell line C26 was obtained from the American Type Tissue Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Dulbecco, ICN Pharmaceuticals, Costa Mesa, CA, USA) supplemented with 5% (v/v) fetal calf serum, 2mM glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin. All cells were kept at 37°C

in a humidified atmosphere containing 5% CO_2. Prior to injection, semi-confluent cultures were harvested by brief trypsinization (0.05% trypsin in 0.02% EDTA), and resuspended in phosphate-buffered saline (PBS) to a final concentration of 1.0×10^6 cells/ml.

Lentiviral Transduction

C26 mouse colon carcinoma cells were transduced with a lentiviral construct harboring the firefly luciferase gene under control of the CMV promoter. Lentiviral particles were generated in 293T cells by cotransfection (using fuGENE 6 Transfection Reagent (Roche Diagnostics, Mannheim, Germany)) of plasmids encoding the lentiviral gag and pol elements (pMDLgpRRE), the rev protein (pRSV_Rev), the viral envelope (pMD2G; all kindly provided by Prof. D. Trono) and the luciferase construct (pRRL-CMV-Luc, kindly provided by Prof. R.C. Hoeben). After 48 hours the supernatant was collected and C26 cells (1.0×10^4 cells) were transduced in the presence of hexadimethrine bromide (7μg/ml; Aldrich, Steinheim, Germany). Prior to injection approximately 1.0×10^5 cells were lysed to confirm expression of luciferase with a commercially available Luciferase Assay System (Promega Benelux BV, Leiden, The Netherlands).

Animals and Surgery

Male BALB/c mice, aged 8-10 weeks, purchased from Harlan (Leicestershire, UK), were housed under standard conditions and received food and water *ad libitum*. Colorectal liver metastases were induced in all mice as follows. Mice were anaesthetized with an intramuscular injection of ketamine hydrochloride (100 mg/kg; Vétoquinol BV, Den Bosch, The Netherlands) plus xylazine (10 mg/kg; Eurovet Animal Health BV, Bladel, The Netherlands) and atropine (0.05 mg/kg; Pharmachemie BV, Haarlem, The Netherlands) (KXA). Through a left lateral flank incision C26 cells (1.0×10^5 in 100 μl) were injected into the spleen parenchyma. To avoid intrasplenic tumor growth, the spleen was removed after 10 minutes. The incision was closed in two layers, using vicryl 5/0 for the abdominal wall and vicryl 4/0 for the skin. All experiments were performed in accordance with the guidelines of the University's Animal Experimental Committee, University Medical Center Utrecht, the Netherlands.

***In vivo* bioluminescent imaging (BLI)**

On days 5, 7, 9 and 11 post-injection hepatic tumor growth was assessed by *in vivo* bioluminescent imaging with a highly sensitive, cooled charge-coupled device (CCCD) camera (VersArray 1300B, Roper Scientific Inc., Vianen, The Netherlands) mounted in a light-tight imaging chamber (Roper Scientific Inc., Vianen, The Netherlands). Imaging and quantification of signals were controlled by the acquisition software MetaVue (Universal Imaging Corporation, Downingtown, USA). Prior to imaging mice were anaesthetized with an intramuscular injection of KXA. The sub-

substrate D-luciferin sodium salt (Synchem Laborgemeinschaft OHG, Kassel, Germany) dissolved in phosphate-buffered saline (PBS) was injected i.p. at a dose of 125 mg/kg [19]. Mice were then placed onto the stage inside the light-tight camera box. Approximately 5 minutes after the intraperitoneal injection of D-luciferin, the bioluminescent signal has reached maximum intensity and remains fairly constant for over 15 minutes (See also: [19]). Therefore, all mice were imaged with an integration time of 5 minutes, exactly 10 minutes after the i.p. injection of D-luciferin. Three to four mice were imaged simultaneously. Total photon counts were quantified with MetaMorph software measuring the same delineated abdominal region in each mouse, large enough to fit the biggest tumor-bearing liver.

Hepatic Replacement Area (HRA)

Directly after bioluminescence imaging the mice were sacrificed and their livers were harvested, fixed with formaldehyde, and embedded in paraffin. Intrahepatic tumor load was scored as the percentage of hepatic tissue replaced by metastatic tumor cells [2]. HRA was assessed on 3 haematoxylin- and eosin-stained sections obtained from 3 non-sequential levels of the embedded liver, separated at least 2mm from each other. At a magnification of 10x40, 100 fields per slide were scored using a four points grid overlay [3]. All analyses were performed in a blinded manner.

Statistical Analyses

The mean bioluminescence counts, wet liver weight, HRA and the corresponding standard errors were determined for all four time points. Correlation plots were used to describe the relationship between bioluminescence and HRA, between bioluminescence and wet liver weight and between HRA and wet liver weight; The Pearson's correlation coefficient (r) was determined to assess the degree of correlation. A P-value < 0.05 was considered to be statistically significant.

Results

In the present study we chose to validate the quantification of C26 tumor growth in the liver by BLI. This was performed by comparing the results obtained with BLI with those obtained by the golden standard, determination of the hepatic replacement area (HRA). To this end, we generated C26 cells expressing the firefly luciferase gene, by lentiviral gene transduction. This method allows us to transduce 100% of the cells as assessed by using a lentivirus encoding the enhanced green fluorescent protein (EGFP) (not shown).

Prior to using these cells in the liver metastasis model, luciferase expression was assessed luminometrically. We obtained cells producing $3x10^6$ light units per 40 μg of protein (C26-luc). In addition, we compared the proliferation rate and cell cycle distribution of C26-luc cells to that of the original C26 cells *in vitro*. Both

parameters were unaffected by expression of the luciferase gene (not shown).

Next, we used the C26-luc cells to induce liver metastases in BALB/c mice. The cells were injected into the spleen, the spleen was removed (to prevent intrasplenic tumor growth) and tumor growth was allowed for 11 days. On post-operative days 5, 7, 9 and 11, bioluminescence was assessed in each of the mice and increased from a mean $1x10^5$ light units on pod 5 to a mean $5.5x10^6$ light units on pod 11 (Fig. 1). After imaging, the livers were removed and weighed. Liver weight increased over time from a mean 1.5 grams on pod 5 to a mean 5.1 grams on pod 11 (Fig. 2). The livers were subsequently fixed overnight in formalin to allow assessment of the HRA on paraffin-embedded tissue sections. HRA increased from a mean 2% on pod 5 to a mean 45% on pod 11 (Fig. 3). We then performed statistical analyses to assess the degree of correlation between the three different modes of analysis in each single mouse. Figure 4 shows that the measurement of tumor load by BLI correlates extremely well with the standard method of HRA measurements (r=0.9829), as well as with liver weight (r=0.9889). Liver weight and HRA measurements are also highly correlated (r=0.9896).

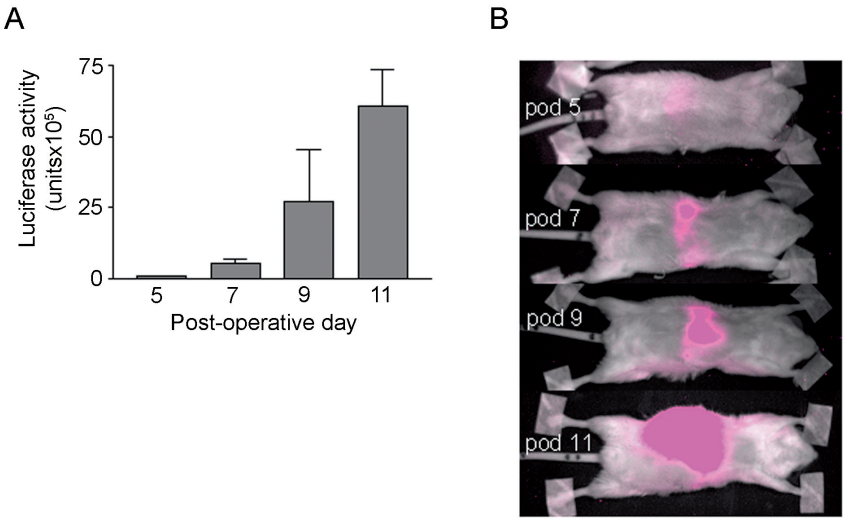

Figure 1. Analysis of intrahepatic tumor growth by bioluminescence imaging. C26 colorectal carcinoma cells expressing the firefly luciferase gene were injected into the spleen to induce liver metastases. After 10 minutes the spleen was removed. (A) On post-operative days (pod) 5, 7, 9, and 11 luciferin (125 mg/kg) was injected intraperitoneally and bioluminescence was measured before sacrificing (error bar = SEM; n=3 (pod5,7,9); n=4 (pod11)). (B) Representative bioluminescence images of mice bearing luciferase-expressing liver metastases on pod 5, 7, 9, and 11.

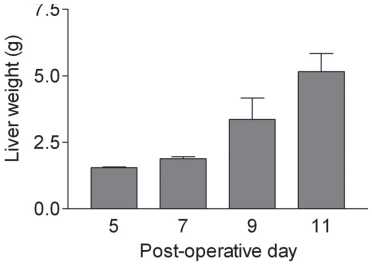

Figure 2. Analysis of hepatic tumor burden by wet liver weight. After bioluminescence imaging on post-operative days (pod) 5, 7, 9, and 11, the mice were sacrificed and their livers were harvested. Wet liver weight (in grams) was determined (error bar = SEM).

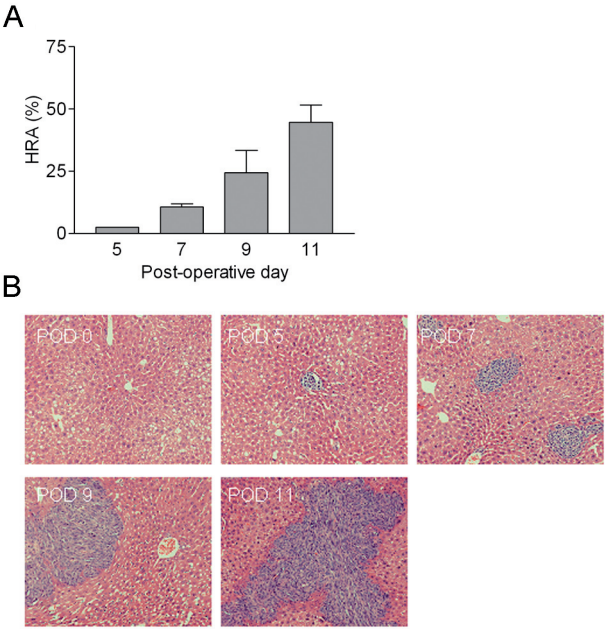

Figure 3. Analysis of hepatic tumor growth by Hepatic Replacement Area (HRA). (A) Intrahepatic tumor growth was assessed by measuring the percentage of hepatic tissue that was replaced by metastatic tumor cells (HRA) as described in the Materials and Methods section. On days 5, 7, 9, and 11 after induction of liver metastases HRA was determined on 3 haematoxylin- and eosin-stained (H&E) sections from 3 non-sequential levels of each liver (error bar = SEM). (B) Representative H&E-stained sections on post-operative days 5, 7, 9, and 11 are shown (magnification 400x).

Discussion

The C26 cell line was originally established from a colon tumor that was induced by N-nitroso-N-methylurethane (NMU) in BALB/c mice [20]. The tumor was characterized as a highly metastatic undifferentiated carcinoma. Since its isolation the C26 cell line has been widely used in model systems for intrahepatic metastatic growth of CRC cells.

Several methods are frequently used for determining tumor growth in the liver. These include i) incidence (i.e. livers either with or without tumortake), ii) number of tumor nodules on the liver surface, iii) wet liver weight, iv) hepatic replacement area (area of liver tissue that has been replaced by tumor tissue). A major drawback of all these methods is that measurement of tumor growth can only be performed once, *post-mortem*. In the present study we have validated the non-invasive technique of bioluminescence imaging (BLI) by comparing the results obtained with this method with those obtained by measuring HRA and wet liver weight. BLI was very sensitive as signals from livers with as low as 2% HRA were readily detected. The correlation between BLI and HRA measurements is surprisingly good over a large range of tumor loads (2-50% HRA). Apparently, absorption of light by hemoglobin [17] or by the tumor mass itself [18] does not significantly affect the measurement of tumor size by BLI in our C26/BALB/c model. On post-operative day 9 the variance in tumor take was rather large and mice with intermediate tumor growth were not detected in this group. This accounts for the apparent gap in tumor loads (2.5<>6x10^6 light units, 3.1<>4.9 grams, 25<>42% HRA) that is observed in the correlation analyses depicted in figure 4. Nevertheless, correlation analyses between all three parameters in all mice examined clearly shows that the degree of correlation between the three methods is very high, despite the fact that intermediate values were not detected.

When compared to measuring HRA, BLI is a fast and easy to perform method for analyzing intrahepatic tumor growth *in vivo*. An additional advantage is that multiple consecutive measurements can be performed in a single mouse, so that tumor growth can be assessed over time. The use of BLI in therapeutic studies allows the effect on tumor growth to be assessed at any given point in time. Furthermore, prior to the start of therapy tumor-bearing mice can be randomized into treatment groups on the basis of their tumor load. It may therefore be expected that this will reduce the number of mice that are required to reach statistical significance between treatment groups.

Finally, two potential drawbacks are associated with the use of BLI. First, the behavior of tumor cells may be affected by the expression of luciferase. Therefore, a thorough analysis of the potential effects of luciferase expression on tumor cell proliferation, cell cycle profile and viability is essential prior to testing the cells in tumor models. Second, immunecompetent mice may develop a cytotoxic T-lymphocyte (CTL)-response against luciferase-expressing tumor cells [21]. This undesired effect

may be prevented by using a recently developed non-immunogenic 'stealth' variant of luciferase that escapes detection by the immune system [21]. In our experiments we have so far not encountered immune-mediated anti-tumor effects.

In conclusion, our results show that the non-invasive detection of intrahepatic tumor load by BLI yields results that correlate extremely well with those obtained by widely accepted invasive techniques such as determination of wet liver weight and HRA. This finding, taken together with the great advantages of measuring luciferase activity over measuring HRA (simplicity, reduced laboriousness, possibility to randomize, possibility to perform consecutive measurements), leads us to conclude that BLI is a reliable and superior method for measuring tumor growth in the liver.

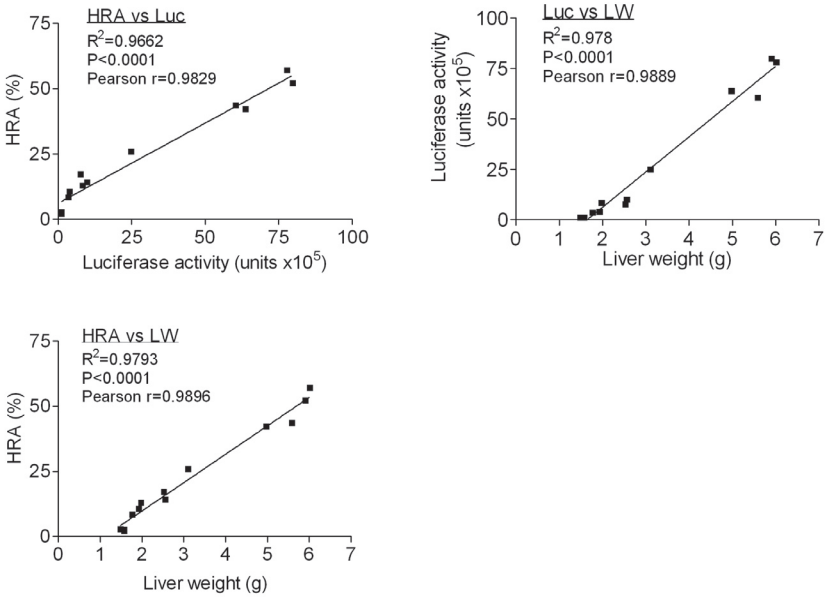

Figure 4. The degree of correlation between the three different methods of measuring intrahepatic tumor growth was assessed by using GraphPad software. The Pearson's correlation coefficient (r) shows highly significant degrees of correlation ($p<0.0001$) between the three methods. The results of all 13 mice (n=3 (pod5,7,9) n=4 (pod11)) are shown in all the graphs.

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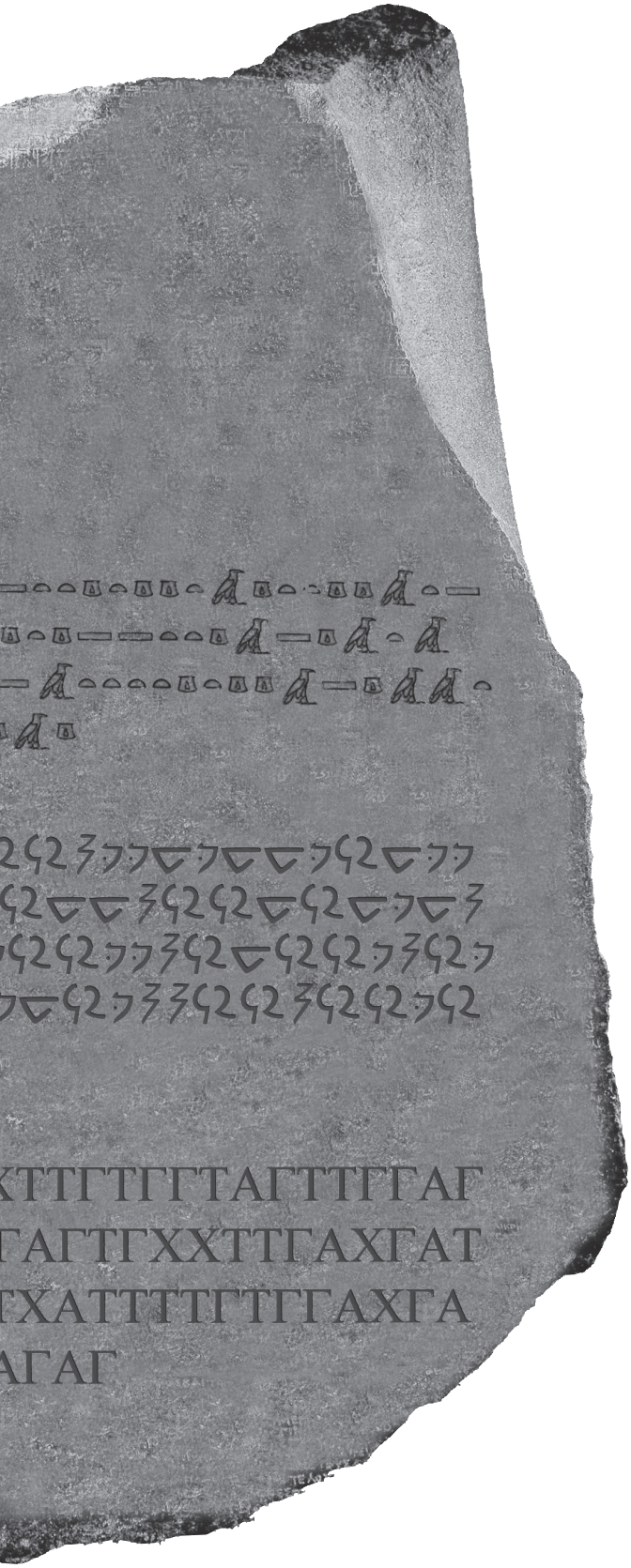

Chapter 4

Dual effect of Kras^{D12} knockdown on tumorigenesis: increased immune-mediated tumor clearance and abrogation of tumor malignancy

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Abstract

Activating mutations in the human KRAS proto-oncogene are acquired during the earliest stages of colorectal cancer development. If mutant KRAS is to be used as a target for therapy in colorectal cancer, tumor growth should depend on its continued presence. Here we report that stable knockdown of $Kras^{D12}$ in murine C26 colorectal cancer cells by RNA interference resulted in loss of transformed properties *in vitro*. The incidence of subcutaneous tumor formation was reduced by 60% and the lag time was increased 7-fold. $Kras^{D12}$-knockdown tumors grew non-invasively and did not cause morbidity. Remarkably, some of the $Kras^{D12}$-knockdown tumors regressed spontaneously, which rendered these mice resistant to parental C26 tumor growth. In immune-deficient hosts the incidence of tumor formation by $Kras^{D12}$-knockdown cells was 100%. None of these tumors regressed spontaneously. We conclude that the reduced incidence of tumor formation by $Kras^{D12}$-knockdown cells is due to tumor cell clearance by the host immune system, but not to an intrinsic inability of these cells to grow out as tumors. Interestingly, $Kras^{D12}$ knockdown resulted in increased production of interleukin 18 (IL-18), an immune-stimulatory cytokine that has been implicated in limiting colorectal tumor formation. Thus, mutant $Kras^{D12}$ suppresses IL-18 production in colorectal tumor cells, which may contribute to evasion of the local immune system during tumor development.

Main text

C26 is an aggressive murine colorectal cancer (CRC) cell line that is widely used for studying CRC growth and metastasis formation. The cell line was originally established from an N-nitroso-N-methylurea (NMU)-induced colorectal carcinoma in BALB/c mice [1]. The KRAS mutation status of many human but not murine CRC cell lines is known. Therefore, we analyzed the expression and activity of the three Ras isoforms (H-, K- and N-ras) in C26 cells. Hras was not expressed in C26 cells, nor in any of the other human and mouse CRC cell lines analyzed. Nras was highly expressed but was not active, and Kras was expressed and constitutively active (Fig. 1a). We next analyzed the C26 Kras gene for activating mutations by RT-PCR and sequence analysis. Whereas codons 13 and 61 showed the wild-type Kras sequence, codon 12 contained a point mutation (GGT>GAT) that results in a G12D amino acid substitution (Fig. 1b). Alkylating N-nitroso compounds primarily cause G>A point mutations [2,3]. Since activating mutations in Kras contribute to tumor initiation, it seems likely that NMU-induced mutational activation of the Kras gene has been a major causative event during development of the original C26 tumor. G12D is also the most frequently found activating KRAS mutation in human CRC [4-6].

To test the contribution of mutant Kras^{D12} to the transformed and tumorigenic properties of C26 cells, we targeted the Kras^{D12} allele by RNA interference using a lentiviral vector. We isolated a set of cell lines in which Kras, but not Nras, was stably suppressed (C26-KrasKD). As a control, we established cell lines using lentiviruses that were produced using the empty lentiviral pLL3.7 vector (C26-pLL) (Fig. 1c). C26 cells have a spindle-shaped morphology, characteristic for many transformed cells (Fig. 1d). Knockdown of Kras^{D12} resulted in loss of the spindle shape and cells appeared flattened and enlarged when compared to parental control cells or to cells transduced with control lentivirus (Fig. 1d). In the set of stable cell lines that were isolated we noted that the morphological reversion strictly correlated with successful Kras knockdown. The *in vitro* proliferation rate of C26-KrasKD cells was approximately 4-fold lower than that of the C26-pLL control cells (Fig. 1e). Analysis of the cell cycle profile showed that Kras^{D12} knockdown prolonged G1 relative to the S and G2/M phases of the cell cycle (Fig. 1f). Taken together, the results show that Kras^{D12} knockdown produced a dramatic reversion of the transformed phenotype in these aggressively growing CRC cells *in vitro*.

Next, we analyzed the effect of Kras^{D12} knockdown on the tumorigenic potential of C26 cells *in vivo*. To this end, we injected C26-pLL and C26-KrasKD cells subcutaneously into the flanks of syngenic BALB/c mice. C26-pLL control cells rapidly produced visible tumors with a lag time of about 6-7 days. Within 21 days the tumors had reached a volume of more than 1000 mm^3 with necrosis of the overlying skin and signs of local inflammation. Over time, the health of the mice deteriorated and eventually they had to be sacrificed within 24 days following tumor cell injection.

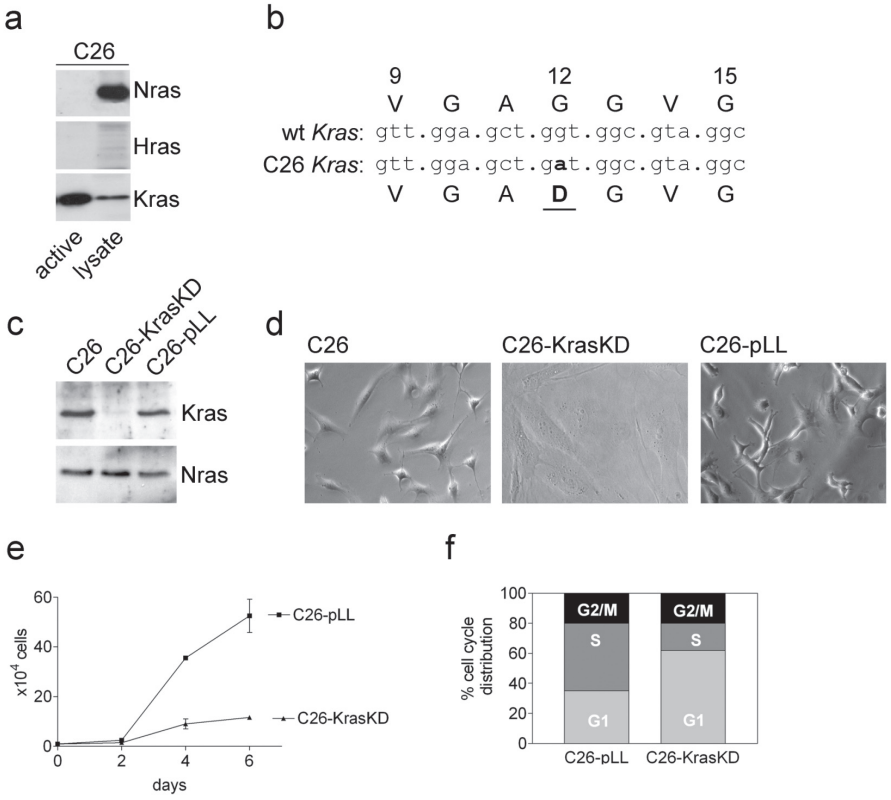

Figure 1. C26 cells loose their transformed characteristics upon $Kras^{D12}$ knockdown. (a) Exponentially growing C26 cells were serum-starved overnight and were lysed. The expression and activity of Hras, Nras and Kras was then assessed by the Ras activity assay and subsequent Western blotting, using isoform-specific antibodies (Santa Cruz Biotechnology, F235, F155 and F234) exactly as described [17]. Nras is expressed but inactive. Hras is not expressed. Kras is expressed and constitutively active. (b) Total RNA was isolated from C26 cells using RNAzol B. 1 μg total RNA was reverse transcribed with random hexamers to obtain cDNA for PCR with forward (5'-atgactgagtataaacttgtg) and reverse (5'-tcacataactgtacaccttgtcc) primers, yielding a 541 bp product. DNA was isolated from agarose gels (Zymoclean Gel DNA Recovery Kit; Zymo Research, Orange, CA) and was sequenced using primer 5'-gtattatttatggcaaatacac and the Big Dye terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington UK), according to the manufacturer's instructions. Analysis of the products was performed on an ABI Prism 377 DNA Sequencer (PE Biosystems) and revealed an activating point-mutation in codon 12 (GGT>GAT), resulting in a G>D amino acid substitution. (c) pLentiLox 3.7 (pLL3.7) (kindly provided by Prof. Van Parijs [18]), was digested with XhoI and HpaI and the annealed oligos 5′tGTTGGAGCTGATGGCGTAG-ttcaagaga-CTACGCCATCAGCTCCAAC-ttttttc3′ and 5′-tcgagaaaaaa-GTTGGAGCTGATGGCGTAG-tctcttgaa-CTACGCCATCAGCTCCAACa-3′ were ligated into pLL3.7 to yield a $Kras^{D12}$-directed shRNA-producing vector. The 19 nt $Kras^{D12}$ target

sequences are indicated in capitals in the oligonucleotide sequence; the G-A mutation that generates the Gly-Asp substitution in the twelfth amino acid of Kras is underlined. Lentiviruses were produced as described [19]. Exponentially growing C26 cells were transduced either by control lentivirus or by lentivirus targeting Kras^{D12} using plasmids encoding the lentiviral gag and pol elements (pMDLgpRRE), the rev protein (pRSV-Rev) and the viral envelope (pMD2G), all kindly provided by Prof. D. Trono. Following isolation of stable clonal cell lines, knockdown of mutant Kras (C26-KrasKD) was tested by Western blot analysis for Kras and Nras. Parental (C26) and control (C26-pLL) cells are shown as a reference. (d) Light microscopic images (10x40) of parental, control (C26-pLL) and Kras knockdown (C26-KrasKD) cells. C26-KrasKD cells are larger and flatter and have lost the characteristic spindle-shaped appearance of Ras-transformed cells. (e) Control (C26-pLL) and Kras knockdown (C26-KrasKD) cells were plated at a 10^4 cells/9.6 cm^2 density in 6-well plates, in medium containing 5% fetal calf serum. Cells were trypsinized and counted in a blinded manner 2, 4, and 6 days after plating. Results shown are from 2 independent experiments, performed in triplicate. (f) Exponentially growing C26-pLL and C26-KrasKD cells were labeled with BrdU (50μM) for 10 min. and were subsequently processed for FACScan analysis using FITC-labeled anti-BrdU (Boehringer, Mannheim) and propidium iodide. Fluorescence was measured on a FACSCalibur (Becton Dickinson) and the data were analyzed using CellQuest software (Becton Dickinson).

In contrast, 60% of mice (15 of 25) that were injected with C26-KrasKD cells failed to develop macroscopic tumors throughout the course of the experiment which was ended 100 days after tumor cell injection (Table 1). The remaining 40% of the mice (10/25) did develop tumors, but the mean lag time was extended to 44.4 days (Table 1). In contrast to the aggressively growing C26-pLL tumors, the tumors formed by C26-KrasKD cells grew slowly and indolently over time with the overlying skin remaining intact. Despite the fact that C26-KrasKD tumors grew to far larger volumes than C26-pLL control tumors, the mice remained in excellent condition and their health status remained unaffected (Fig. 2a, table 1). On cross sectioning and microscopic examination of Haematoxylin and Eosin (H&E)-stained tissue sections, C26-pLL tumors showed massive epidermal invasion, whereas the skin overlying C26-KrasKD tumors remained intact and was not invaded (Fig. 2b, upper panel, table 1). Towards the centre of C26-pLL tumors large necrotic areas were observed. In contrast, C26-KrasKD tumor tissue was healthy throughout the entire tumor (Fig. 2b, lower panel). C26-pLL tumors are characterized as poorly differentiated carcinomas with no signs of tubule formation or mucus production [7] (Fig. 2b). We noted that Kras knockdown had no apparent effect on the histological differentiation grade (Fig. 2b, lower panel).

Table 1. Tumor incidence in immune-competent and immune-deficient mice

Mouse strain	*Cell line*	*Number of mice*	*Tumor incidence n (%)*	*Lag time (days ± SEM)*	*Regression n (%)*	*Host morbidity*	*Dermal invasion*
Immune-competent	C26-pLL	9	9 (100)	6.4 ± 0.4	0 (0)	Yes	Yes
	C26-KrasKD	25	10 (40)	44.4 ± 5.0	3 (30)	No	No
Immune-deficient	C26-pLL	4	4 (100)	6.5 ± 0.5	0 (0)	Yes	Yes
	C26-KrasKD	12	12 (100)	33.8 ± 0. 9	0 (0)	No	No

C26-pLL and C26-KrasKD cells (10^6) were injected subcutaneously into immune-competent male BALB/c mice and into athymic immune-deficient male BALB/cAnNCrl-*Nu*BR mice, aged 10 weeks. Tumor growth was assessed thrice weekly until the end of the experiment on day 100 post-injection and the incidence of tumor formation was calculated. The lag time was defined as the time (in days) from the injection until the first signs of macroscopic tumor growth were detected. Regression of established macroscopic tumors was seen in 3 out of 10 mice. Host morbidity was classified 'Yes' when the health status of the host was compromised as described in figure 2a. Dermal invasion was analyzed on H&E-stained tissue sections. These experiments were performed in accordance with the guidelines of the Animal Experimental Committee, University Medical Center Utrecht, the Netherlands.

The effect of RAS deletion on the differentiation state, invasiveness, immunogenicity and morbidity of experimental tumors has so far not been investigated. It has previously been reported that deletion of the KRAS gene or mRNA from human tumor cells completely abolished tumor growth [8,9]. This is in apparent contrast to our results showing delayed non-malignant tumor growth of Kras-suppressed C26 cells. Our results are supported by another study showing that loss of NRAS from human HT1080 fibrosarcoma cells or deletion of KRAS from human DLD-1 colorectal carcinoma cells does not abrogate their tumorigenic potential in immune-deficient mice [10]. How may these discrepancies be reconciled? First, given the non-identical genetic background of the different cell lines they may differ in their dependency on endogenous mutant KRAS/Kras. A second explanation could be that tumor growth may have been missed in some of these studies due to termination of the experiments prior to a prolonged lag time.

C26 cells are poorly immunogenic and invariably produce tumors when injected into syngenic BALB/c mice without eliciting a detectable host CTL response [11,12] During the course of the experiments 3 out of 10 C26-KrasKD tumors underwent spontaneous regression after having reached a volume of approximately 400 mm^3 (table 1). We reasoned that, if tumor regression was due to an anti-tumor immune response, the mice may have become immunized against the parental C26 cells.

Indeed, we found that the C26-KrasKD 'regressor' mice failed to develop tumors following subcutaneous injection of wild-type C26 cells, while control mice developed large subcutaneous tumors within 15 days (Fig. 3a). Furthermore, depletion of CD8+ T-cells from the regressor mice by repeated injections of anti-CD8 antibody allowed subcutaneous C26 tumor growth following re-injection of tumor cells (Fig. 3b). Although the number of mice in these experiments (n=3) is too low to draw firm conclusions, the results suggested that the failure of Kras knockdown cells to efficiently develop tumors could be due, at least in part, to tumor cell clearance by the host immune system.

To test this hypothesis, we injected C26-pLL control cells and C26-KrasKD cells into wild-type and immune-deficient BALB/c mice. C26 control cells formed aggressively growing tumors in 100% of the wild-type and in 100% of the immune-deficient mice with a similar mean lag time of 6.4 and 6.5 days respectively (Table 1). This result is consistent with the notion that the parental C26 cells are poorly immunogenic. In both groups of mice tumor growth had a strong adverse effect on animal health. In contrast to C26-pLL cells, C26-KrasKD cells formed tumors in only 40% of immune-competent mice, but the incidence in immune-deficient mice was 100% (Table 1). Furthermore, the lag time of 33.8 days in immune-deficient mice was significantly ($p<0.05$) shorter than the lag time in immune-competent mice (44.4 days). Partial clearance of the injected C26-KrasKD cells by immune-competent hosts may explain this difference. The slower kinetics with which Kras-suppressed tumors grow out allows more time for the generation of a CTL response and T helper cell production. In addition, a 4-5 fold increase in cell surface expression of H-2K^{d} MHC class I molecules in Kras^{D12} knockdown cells (our unpublished results) may further facilitate tumor cell clearance [13]. We conclude that the reduced incidence of tumor formation as a result of Kras^{D12} knockdown is most likely due to tumor cell clearance by the host immune system and not to an intrinsic inability of these cells to grow out as tumors.

To gain more insight into a possible role for Kras in modulating tumor immunity we performed a comparative cDNA microarray analysis of the gene expression profiles of parental C26 and Kras^{D12} knockdown cells. cDNA preparations of both cell types were labeled with either Cy-3 or Cy-5 and were competitively hybridized on 20K Agilent Mouse Development Chips. Technical variation was compensated for by performing dye swaps. We found that one of the most strongly upregulated genes (11.7 fold) following Kras knockdown was the gene encoding interleukin-18 (IL-18). This result was confirmed by RT-PCR analysis of IL-18 mRNA levels (Fig. 4a). Furthermore, ELISA analysis of IL-18 levels in the conditioned media of both cell types indicated that Kras^{D12} knockdown resulted in a drastic increase in IL-18 secretion (Fig. 4b).

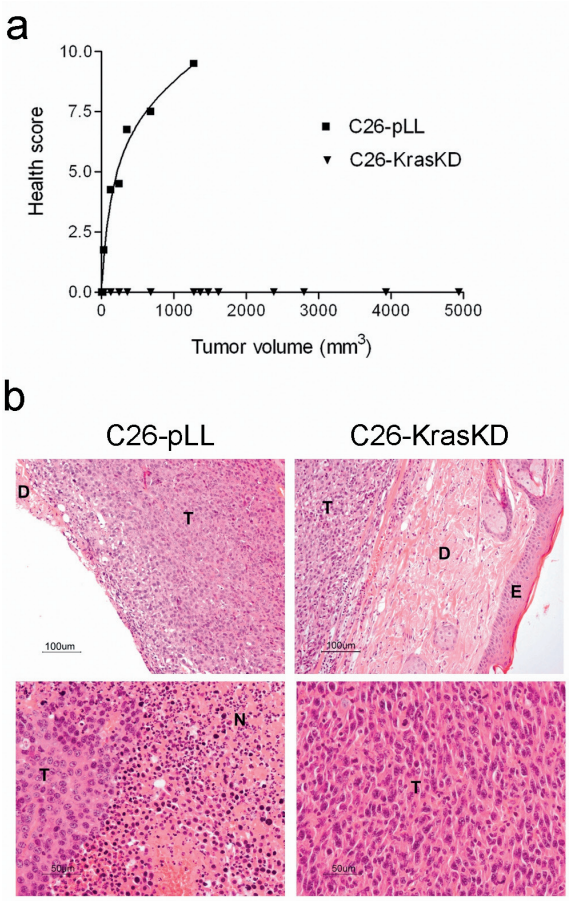

Figure 2. $Kras^{D12}$ knockdown delays subcutaneous tumor formation and prevents tumor-associated disease. **(a)** C26-pLL and C26-KrasKD cells (10^6) were injected subcutaneously and standard caliper measurements were performed thrice weekly. Tumor volumes were calculated using the following equation: volume (mm^3)= $AxB^2x0.5236$, where A is the largest dimension and B is the diameter perpendicular to A. The health status of all individual mice was assessed by using the 'clinical appearance scoring system' which analyses appearance, behavior, reactivity and body weight [20]. An increased score in this system signifies increased morbidity (i.e. 0=healthy). Health score is plotted as a function of C26-pLL or C26-KrasKD tumor volume. **(b)** C26-pLL tumors (day 21) and C26-KrasKD tumors (day 91) were excised, fixed in formalin and processed for standard H&E histochemistry. Excessive dermal invasion and large central necrotic areas were observed in C26-pLL tumors, but not in C26-KrasKD tumors. Kras knockdown had no overt effect on the differentiation grade. E: epidermis, D: dermis, T: tumor, N: necrotic area. Representative pictures are shown. Bars upper panel=100 μm; Bars lower panel=50 μm. These experiments were performed in accordance with the guidelines of the Animal Experimental Committee, University Medical Center Utrecht, the Netherlands.

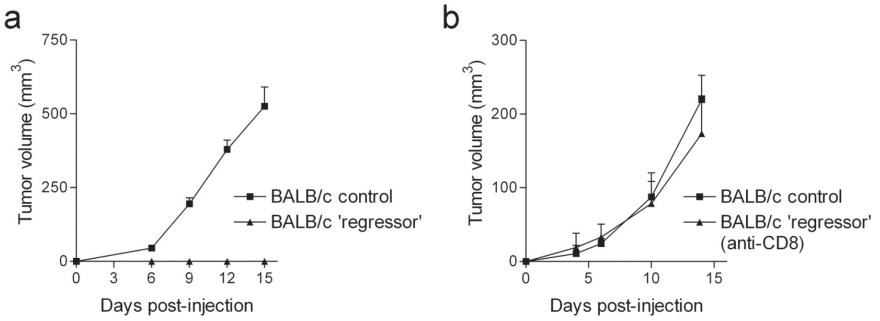

Figure 3. CD8-dependent anti-tumor immunity in C26-KrasKD regressor mice. **(a)** The regressor mice described above (n=3) and 2 control mice were challenged with 10^6 parental C26 cells by subcutaneous injection. Tumor growth was then followed over time by standard caliper measurements 6, 9, 12 and 15 days post injection. After 15 days the control mice had developed large subcutaneous tumors. The regressor mice did not develop tumors. **(b)** The regressor mice received intraperitoneal injections of anti-CD8 antibody (clone 2.43; 100 μg) on days –7, –3 and 4 hours prior to injection of 10^6 wt C26 tumor cells on day 0. The mice received two additional doses anti-CD8 antibody (100 μg) on days 3 and 7 after tumor cell injection. Tumor growth was measured by standard caliper measurements. These experiments were performed in accordance with the guidelines of the Animal Experimental Committee, University Medical Center Utrecht, the Netherlands.

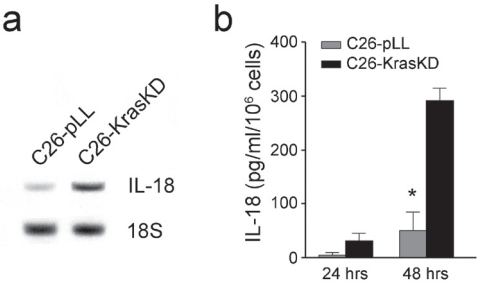

Figure 4. Kras^{D12} suppresses IL-18. (a) Total RNA was isolated (Trizol) and 10 μg aliquots were used for reverse transcription. PCR analysis (30 cycles) was performed using primer pairs designed to amplify mouse IL-18 (forward: 5'-actgtacaaccgcagtaatacg, reverse: 5'-agtgaacattacagatttatccc), and 18S rRNA (forward: 5'-agttggtggagcgatttgtc, reverse: 5'-tattgctcaatctcgggtgg) at 94°C (30 sec), 53°C (30 sec) and 72°C (1 min.). (b) C26-pLL and C26-KrasKD cells were grown at 10^6 cells/200 μl complete DMEM for either 24 h or 48 h. The medium was harvested and IL-18 levels were measured using the IL-18 enzyme linked immunosorbent assay (ELISA) kit (MBL, Naka-ku Nagoya, Japan). The cells were harvested and counted. The bar diagram shows the production of IL-18 per 10^6 cells after 24 and 48 h. The asterisk indicates statistical significance ($p<0.05$).

IL-18 is produced and released by activated macrophages, Kupffer cells, dendritic cells, Langerhans cells and B-cells [14,15]. Importantly, it is also produced by epithelial cells of the gastrointestinal tract, the skin and the airway, where it has been implicated in the host immune defense against tumor development [14,15]. In line with this, IL-18 mRNA and protein are strongly reduced during colorectal tumor formation [14,15]. Interestingly, C26 tumor growth in the liver can be strongly suppressed by IL-18-producing hepatocytes, which was associated with increased cytolytic activity of splenic CTLs towards C26 cells [16]. This demonstrates the dramatic impact of local IL-18 production on C26 tumor development.

Our results indicate that endogenous mutant Kras, although acquired at the initial stage of C26 tumor development, remains essential for the malignant properties of this highly aggressive CRC cell line, but not for tumor growth *per se*. Furthermore, the Kras oncogene controls initiation and maintenance of tumor growth by evasion of the host immune system. The strong suppression of IL-18 by mutant Kras in epithelial tumor cells may contribute to immune evasion.

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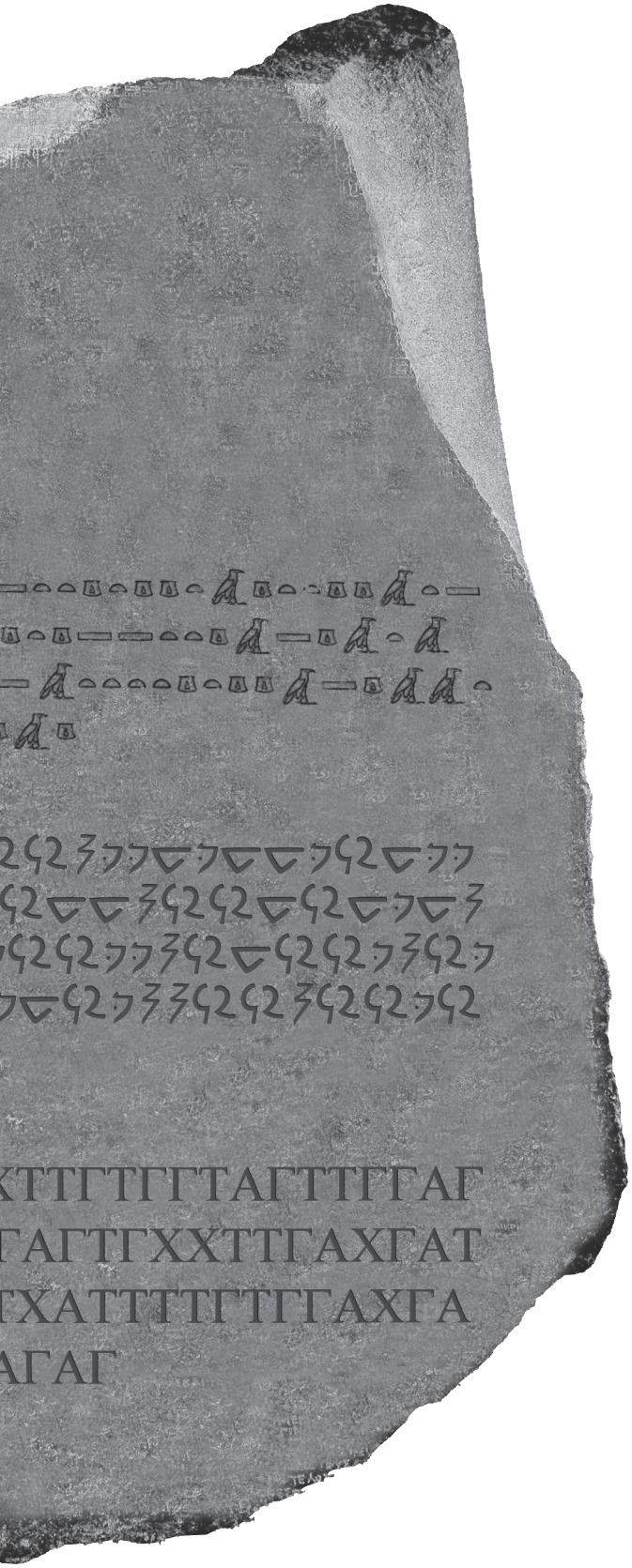

Chapter 5

Suppression of mutant $Kras^{D12}$ promotes clearance of colorectal tumor cells from the liver and abrogates metastasis formation

Submitted.

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Abstract

Activating mutations in the KRAS oncogene are acquired during the very early pre-malignant stages of tumor growth and are preserved during tumor progression. Approximately 40% of primary colorectal tumors and colorectal metastases harbor activating mutations in KRAS. Colorectal metastases primarily develop in the liver. Here we have investigated the contribution of endogenous mutant Kras to the formation of liver metastases by circulating colorectal tumor cells. Stable suppression of $Kras^{D12}$ by RNA interference completely abrogated liver metastasis formation. Tumor cells lacking $Kras^{D12}$ failed to extravasate and were rapidly cleared from the liver sinusoids. Likewise, $Kras^{D12}$-suppressed cells showed a drastic reduction in the ability to traverse a laminin-rich basement membrane *in vitro*. Surprisingly, loss of the invasive and metastatic phenotype was not accompanied by epithelial re-differentiation. Bypassing the need for extravasation by direct injection of tumor cells into the liver parenchyma allowed tumor formation in the livers of immunodeficient, but not immunocompetent mice. We conclude that endogenous mutant $Kras^{D12}$ in circulating tumor cells stimulates metastasis formation by promoting extravasation and by protecting cells against clearance by cytotoxic immune cells.

Introduction

The mortality of patients with colorectal carcinoma (CRC) is primarily due to the consequences of metastatic tumor growth in the liver. The formation of liver metastases is dependent on the successful progression of tumor cells through multiple distinct stages. Tumor cells have to detach from the primary tumor, migrate through the tumor-surrounding stroma and break down the vessel-surrounding basement membrane to enter the bloodstream (intravasation). To reach the liver, tumor cells have to survive the hostile environment of the blood or the lymphatic system and cope with the lack of cell-matrix adhesion. Tumor cells that reach the liver are entrapped in the sinusoidal microvasculature either by receptor-ligand-mediated tumor cell adhesion to the vessel walls, or by passive entrapment due to size restriction. Tumor cells may then pass the endothelial cell lining of the vessel wall and degrade the vessel-surrounding basement membrane (extravasation). Within the liver parenchyma tumor cells have to adapt to the new microenvironment, withstand the continuous attack of host immune cells and start proliferating to form micrometastases. Finally, the outgrowth of established micrometastases requires the formation of new blood vessels (angiogenesis) to provide the developing tumor with nutrients and oxygen [1].

Activating mutations in the KRAS oncogene are found in 38% of all sporadic CRCs [2-4] and are acquired at the very early pre-malignant stages of tumor formation [5]. There is no doubt that acquired mutations in Kras/KRAS are initiating events in the development of pancreas, lung and colorectal carcinomas. However, it remains unclear whether mutant Kras plays an additional role during metastasis formation, a process occurring much later during disease progression, often decade(s) after initial tumor development and in a background of many additional genetic changes. *In vitro* studies have shown that endogenous mutant KRAS controls colorectal tumor cell adhesion and motility [6,7]. In addition, overexpression studies using activated RAS isoforms suggest a role for RAS in tumor cell survival [8] and differentiation [1] and in tumor maintenance [9,10]. To what extent these diverse functions play a role in controlling metastasis formation by tumor cells expressing endogenous activated Kras is unknown. To our knowledge, only one study has examined how activated RAS/Ras controls the development of (micro-)metastases in the liver: Varghese et al. used NIH3T3 fibroblasts overexpressing activated HRAS^{V12} and concluded that HRAS^{V12} promotes proliferation and reduces apoptosis in developing micrometastases in the liver, without affecting early cell survival and extravasation [11].

In the present study we have chosen a different approach. We used colorectal carcinoma cells harboring an endogenous activated Kras allele (Kras^{D12}) and assessed how suppression of this allele affected the various phases of liver metastasis development.

Materials and Methods

Cell lines and culture conditions

The murine colon carcinoma cell line C26 was obtained from the American Type Tissue Culture Collection (ATCC, Rockville, MD). We previously established C26 cell lines in which the endogenous $Kras^{D12}$ allele is stably suppressed by mutant-specific RNA interference, using a lentiviral vector (C26-KrasKD)[12]. As a control, we established cell lines transduced with the empty lentiviral pLL3.7 vector (C26-pLL). C26 cells expressing the firefly luciferase gene (C26-luc) were described previously [13]. C26-pLL and C26-KrasKD cells were transduced with a lentiviral vector encoding enhanced green fluorescent protein (EGFP) (pWPT-GFP, kindly provided by Professor Didier Trono). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Dulbecco, ICN Pharmaceuticals, Costa Mesa, CA) supplemented with 5% (v/v) fetal calf serum, 2 mM glutamine, 0.1 mg/ml streptomycin, and 100 U/ml penicillin. All cells were kept at 37°C in a humidified atmosphere containing 5% CO_2.

In vitro invasion assay

To measure the invasive properties of C26-pLL and C26-KrasKD cells, we used 24-well BioCoat Matrigel invasion chambers (cat. # 354480, BD Biosciences, Alphen aan den Rijn, The Netherlands) with an 8-μm pore PET membrane coated with Matrigel basement membrane matrix according to the manufacturer's protocol. Control membranes without the Matrigel coating were used as a reference. In the upper compartment, 5 X 10^4 cells/well were plated in 0.50 ml serum-free medium. The lower compartment contained 0.75 ml medium with 10% FCS. The invasion chambers were incubated for 16 hrs at 37°C in a humidified incubator with 5% CO2. The remaining cells in the upper container were carefully removed by a cotton swab. The transmigrated cells were then fixed in 3.7% formaldehyde and were stained with hematoxylin and eosin and were counted by analyzing microscopic images. Data are expressed as percentage invasion through Matrigel-coated membranes, relative to migration through control uncoated membranes. All assays were performed in duplicate and were repeated twice.

Antibodies

For western blot analysis the following antibodies were used: anti-β-catenin (BD biosciences, cat # 610153, clone 14), anti-active-beta-catenin (Upstate, catalog# 05-665, clone 8E7), anti-alpha smooth muscle actin (αSMA) (Sigma-Aldrich, catalog# A2547, clone 1A4), anti-fibronectin (Sigma-Aldrich, catalog# F3648), anti-N-cadherin (BD biosciences, catalog# 610920, clone 32) and anti-E-cadherin (BD biosciences, catalog# 610181, clone 36).

Animals and surgery

Immunocompetent male BALB/c mice and athymic immunodeficient male BALB/cAnNCrl-*Nu*BR mice, aged 8-10 weeks, were purchased from Charles River Laboratories (Maastricht, The Netherlands). Colorectal liver metastases were induced as follows. Mice were anaesthetized and through a left lateral flank incision C26-pLL-luc or C26-KrasKD-luc cells (1.0 x 10^5 in 100 μL) were injected into the spleen parenchyma. To avoid intrasplenic tumor growth, the spleen was removed after 10 minutes. Alternatively, 10^5 C26-pLL-luc or C26-KrasKD-luc cells in 50 μl PBS were injected just under the capsule of the left liver lobe after a midline abdominal incision. All experiments were performed in accordance with the guidelines of the University's Animal Experimental Committee, University Medical Center Utrecht, the Netherlands.

Bioluminescence imaging (BLI)

Tumor outgrowth was measured non-invasively by bioluminescence imaging (BLI), exactly as described previously [13]. In brief, on days 7, 11 and thereafter weekly after tumor cell injection, hepatic tumor growth was assessed by *in vivo* BLI with a highly sensitive, cooled charge-coupled device (CCCD) camera (VersArray 1300B, Roper Scientific Inc., Vianen, The Netherlands) mounted in a light-tight imaging chamber (Roper Scientific Inc., Vianen, The Netherlands). Before imaging mice were anesthetized and the substrate D-luciferin sodium salt (Synchem Laborgemeinschaft OHG, Kassel, Germany) dissolved in PBS was injected i.p. at a dose of 125 mg/kg. All mice were imaged with an integration time of 5 minutes, exactly 10 minutes after the i.p. injection of D-luciferin. Imaging and quantification of signals were controlled by the acquisition software MetaVue (Universal Imaging Corporation, Downingtown, PA). Total photon counts were quantified with MetaMorph software.

Intravital Microscopy

The earliest phases of the metastatic process were analyzed by intravital microscopy on POD 0-4, and 7 (n=3/day/group). Liver metastases were induced by intrasplenic injection of EGFP-expressing C26-pLL or C26-KrasKD cells as described above. At the indicated time points after tumor cell injection, the liver was exposed by a midline abdominal incision. Intravital fluorescence microscopy was performed using a Nikon TE-300 inverted microscope (Uvikon, The Netherlands) equipped with a fluorescence filter for fluorescein isothiocyanate (FITC) (excitation 450-490, emission >515 nm). 10 random fields (magnification 100X) per animal were recorded digitally with a charge coupled device camera (Exwave HAD, Sony, the Netherlands) and stored for off-line data analysis.

Statistical Analyses

Differences between the groups were statistically evaluated using the Mann-Whitney test. Results are presented as means ± SE. All *P* values were two tailed. $P < 0.05$ was considered statistically significant.

Results

Stable suppression of $Kras^{D12}$ abrogates metastatic potential

C26 cells are highly metastatic murine colorectal carcinoma cells expressing endogenous mutant $Kras^{D12}$ [14]. Therefore, these cells are ideally suited to analyze the contribution of endogenous activated Kras to the metastatic phenotype. To this end, we injected C26 control cells (C26-pLL) and C26 cells in which $Kras^{D12}$ was stably suppressed (C26-KrasKD) into the spleens of syngenic BALB/c mice.

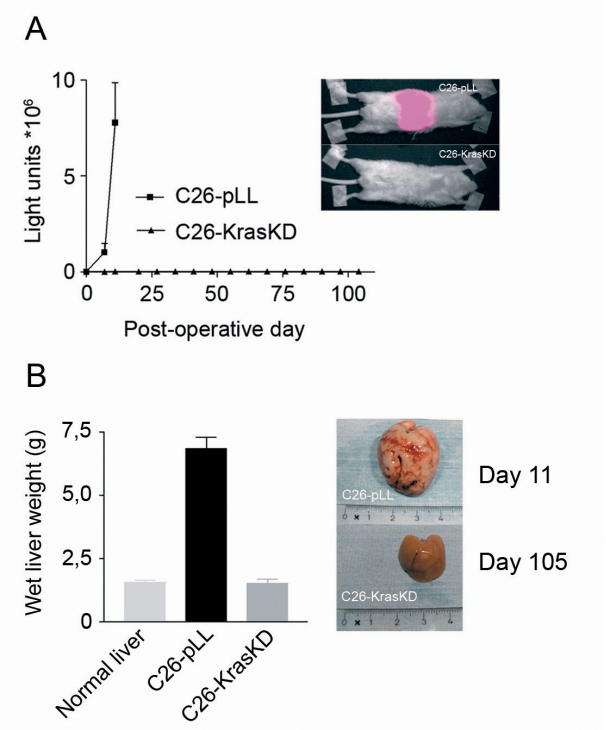

Figure 1. $Kras^{D12}$ is essential for liver metastasis formation. (A) Luciferase-expressing C26-pLL and C26-KrasKD cells were injected into the spleens of BALB/c mice. Following splenectomy, tumor growth in the liver was followed by bioluminescence imaging over time at the indicated post-operative days. (B) All livers were excised and weighed after 11 days (C26-pLL-injected mice) or after 105 days (C26-KrasKD-injected mice).

C26-pLL cells rapidly formed massive liver metastases within 11 days after injection. By that time the mice had to be sacrificed due to the tumor burden. In contrast, none of the mice challenged with C26-KrasKD cells developed liver metastases over a period of 105 days (Fig. 1A). After sacrificing the mice, the livers were excised, weighed, fixed in formalin, and processed for H&E histochemistry. C26-pLL tumor growth induced a 3.5-fold increase in wet liver weight (day 11). In contrast, wet liver weight was not affected by C26-KrasKD cells (day 105; Fig. 1B). Microscopic examination of H&E-stained sections of liver tissue from C26-KrasKD-injected mice showed that the liver architecture was completely normal with no evidence for the formation of (micro)metastases in any of the livers examined.

We have previously shown that C26-KrasKD cells can form benign subcutaneous tumors when grown in immunodeficient but not when grown in immunocompetent mice [12]. Therefore, we analyzed whether C26-KrasKD cells could form liver metastases in immunodeficient mice. C26-KrasKD cells still failed to form liver metastases in immunodeficient mice (Table 1). C26-pLL cells formed massive liver metastases in both immunocompetent and immunodeficient mice, as expected (Table 1).

Table 1. Liver metastases formation by C26-pLL and C26-KrasKD cells in immunocompetent and athymic immunodeficient mice

Mouse strain	*Cells*	*Method*	*Number of mice*	*Incidence n (%)*
Immunocompetent BALB/c	C26-pLL	intrasplenic	12	12 (100%)
	C26-pLL	intrahepatic	12	12 (100%)
	C26-KrasKD	intrasplenic	6	0 (0%)
	C26-KrasKD	intrahepatic	6	0 (0%)
Athymic BALB/c	C26-pLL	intrasplenic	6	6 (100%)
	C26-pLL	intrahepatic	4	4 (100%)
	C26-KrasKD	intrasplenic	6	0 (0%)
	C26-KrasKD	intrahepatic	4	3 (75%)

Table 1. Liver metastases were induced by injecting C26-pLL and C26-KrasKD cells (10^6) into immunocompetent and athymic immunodeficient BALB/c mice. Tumor cells were injected in the spleen to target the liver by the portal vasculature and directly in the hepatic parenchyma to bypass the need for tumor cell extravasation. Tumor growth was assessed weekly until the mice had to be sacrificed due to tumor burden or otherwise until the end of the experiment on day 187 post-injection and the incidence of tumor formation was calculated.

Kras^{D12} knockdown is not associated with re-differentiation to an epithelial phenotype

Overexpression of mutant Ras isoforms can induce mesenchymal properties in epithelial cell types [1]. In general, the transition from an epithelial to a more mesenchymal phenotype (EMT) has been implicated in the acquisition of metastatic potential [14]. C26 cells have a spindle-shaped transformed morphology and fail to form E-cadherin and ZO-1 based cell junctions [15]. Since Kras^{D12} knockdown was associated with loss of metastatic potential, we analyzed whether this was associated with a transition of the mesenchymal phenotype to a more epithelial phenotype (MET) by analyzing the expression of several epithelial and mesenchymal markers. The epithelial markers E-cadherin, beta-catenin (and activated beta-catenin) did not dramatically change upon Kras knockdown (Fig. 2). In line with this, neither C26-pLL nor C26-KrasKD cells formed E-cadherin-based cell-cell junctions, neither when grown on plastic nor on a reconstituted basement membrane (Matrigel). Furthermore, neither C26-pLL nor C26-KrasKD cells displayed apical-basolateral polarity, as evidenced by a failure to organize their f-actin cytoskeleton into apical brush borders (not shown).

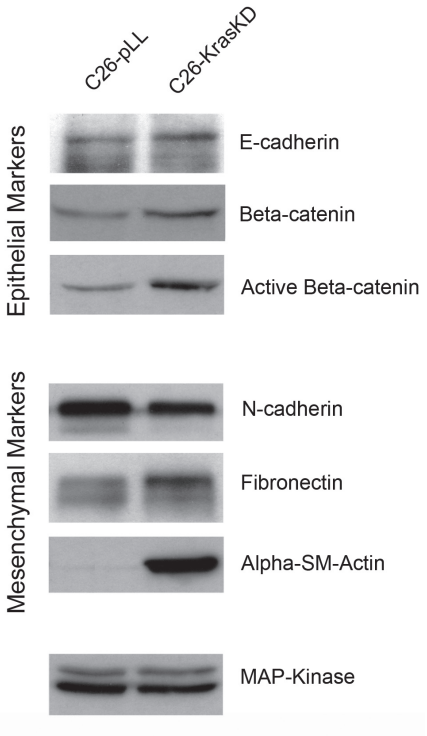

Figure 2. Kras knockdown does not induce mesenchymal-to-epithelial transition. Cell extracts of actively growing C26-pLL and C26-KrasKD cells were prepared and mesenchymal and epithelial differentiation markers were analyzed by western blotting using the indicated antibodies. MAPK was used as a loading control.

Expression of the mesenchymal markers N-cadherin and fibronectin remained unaffected by Kras^{D12} knockdown. In contrast, expression of α-smooth-muscle-actin (αSMA), a marker for mesenchymal myofibroblast-like differentiation, was drastically increased following Kras^{D12} knockdown (Fig 2). We have recently shown that subcutaneous C26-KrasKD tumors, like C26-pLL tumors, are morphologically classified as poorly differentiated carcinomas with no evidence of tubule formation or mucus production [12]. Taken together, we conclude that suppression of Kras^{D12} in C26 cells abrogates metastatic potential without restoring a typical epithelial phenotype.

Kras^{D12} knockdown prevents extravasation and cell division of intra-sinusoidal tumor cells

The loss of metastatic potential following suppression of Kras^{D12} may be due to reduced seeding efficiency, to reduced extravasation and/or to reduced outgrowth of micrometastases. To distinguish between these possibilities, we performed intravital microscopy (IVM) analysis of C26-pLL and C26-KrasKD cells expressing enhanced green fluorescent protein (EGFP).

Following intrasplenic injection, the fate of the injected cells was analyzed immediately after injection (day 0) and on days 1-4 and 7 after injection. Immediately after injection the fluorescent tumor cells were clearly visible as single rounded cells trapped in the liver microvasculature. C26-pLL and C26-KrasKD cells displayed equal seeding efficiency and neither cell type showed any sign of movement, 'rolling' or cell flattening. Within 2 days the vast majority (90%) of C26-KrasKD cells was cleared from the liver whereas half of the C26-pLL cells remained detectable as single cells (Fig. 3). From day 2 onwards, the majority of C26-pLL cells underwent clear shape changes with extensive flattening of the cell body and development of dendrite-like extensions, indicative of active cell adhesion and migration. This was not observed in any of the C26-KrasKD cells. Over time, the majority (approximately 83%) of C26-pLL cells grew out to form small (2-5 cells) and eventually larger (>5 cells) micrometastases. In contrast, the few C26-KrasKD cells that were still detectable after 2 days remained trapped in the sinusoids, retained their small rounded morphology, and were eventually cleared from the liver without undergoing cell division.

Stable suppression of Kras^{D12} decreases tumor cell invasion *in vitro*

One of the major components of the basement membrane (BM) of liver sinusoids is laminin [16]. The lack of extravasation observed in the IVM experiments could be due to a reduced ability to invade the sinusoidal BM. Therefore, we analyzed whether Kras knockdown affected the invasion of a laminin-rich BM (Matrigel). To this end C26-pLL and C26-KrasKD cells were plated onto Matrigel-coated and uncoated porous membranes in transwell chambers and invasion was evaluated over time.

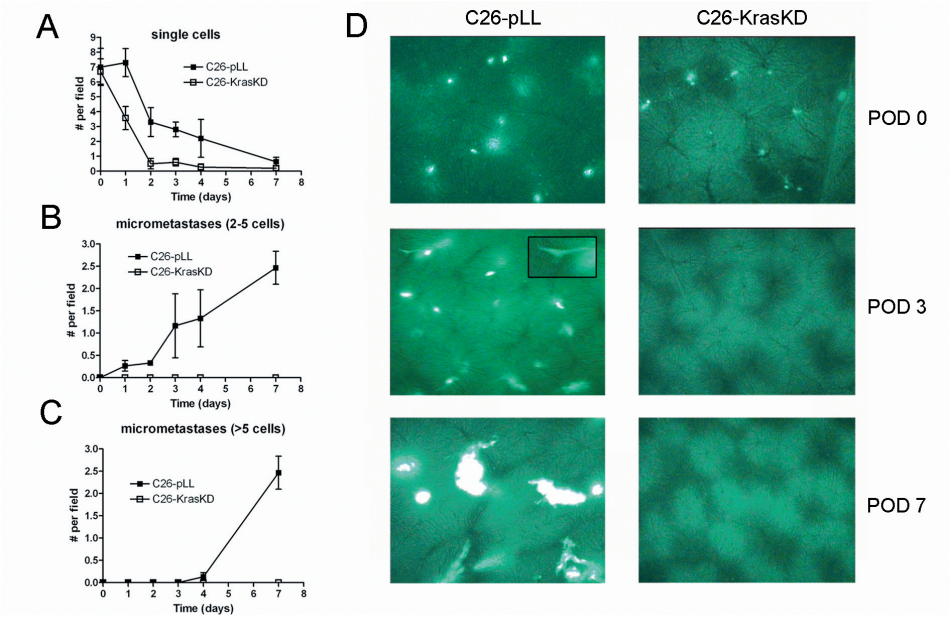

Figure 3. $Kras^{D12}$ knockdown promotes tumor cell clearance from the liver sinusoids. EGFP-expressing C26-pLL or C26-KrasKD cells were injected into the spleens of BALB/c mice. Immediately after injection the liver was exteriorized and fluorescent tumor cells were visualized by intravital microscopy. The number of single cells (A), micrometastases of 2-5 cells (B) and micrometastases larger than 5 cells (C) were analyzed at the indicated timepoints. (D) Representative stills from movies recorded immediately following tumor cell injection (POD 0), and 3 and 7 days following injection. The inset in the C26-pLL POD3 image shows an enlargement of a single tumor cell displaying flattened morphology with dendrite-like extensions.

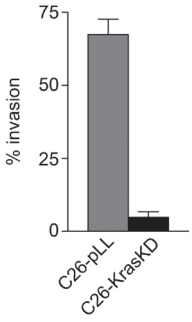

Figure 4. Kras knockdown abrogates tumor cell invasion. C26-pLL and C26-KrasKD cells were plated onto Matrigel-coated or control membranes in transwell chambers. After 16 hours the percentage invasion was assessed as described in the Materials and Methods section.

Figure 4 shows that C26-pLL cells rapidly traversed the Matrigel-coated membrane with approximately 67% invasion after only 16 hours. Invasion of the Matrigel BM by C26-KrasKD cells was reduced to 5% invasion (93% inhibition). Thus, Kras^{D12} knockdown has a dramatic inhibitory effect on the invasive properties of C26 cells *in vitro*, which may explain the lack of extravasation from the sinusoids *in vivo*.

Direct intrahepatic injection allows tumor growth by C26-KrasKD cells

The above results suggest that reduced extravasation and enhanced clearance from the sinusoids prevented intrahepatic outgrowth of C26-KrasKD metastases. Therefore, we tested whether tumor growth by C26-KrasKD cells could be restored if the need for extravasation was by-passed. To this end, we injected C26-pLL or C26-KrasKD cells directly into the liver parenchyma of immunocompetent or immunodeficient mice. C26-pLL cells rapidly formed aggressively growing tumors in both backgrounds and all mice had to be sacrificed after approximately 2 weeks (Table 1). In contrast, none of the immunocompetent mice developed intrahepatic C26-KrasKD tumors and the mice were eventually sacrificed 187 days following tumor cell injection. Intrahepatic injection of C26-KrasKD cells into immunodeficient nude mice however, did result in the formation of large intrahepatic tumors in 75% of the mice which developed slowly over time without causing morbidity (Table 1), similar to what we observed for subcutaneous tumors [12]. Thus, Kras knockdown abrogates the capacity of C26 tumor cells to establish (micro-)metastases in the liver, but not the intrinsic ability of these cells to grow out as liver tumors.

Discussion

Signaling by RAS oncogenes affects multiple aspects of cell behavior. Some of these changes control the metastatic capacity of tumor cells. These include changes in cell polarity, invasion, proliferation and migration, but also altered sensitivity to apoptosis induction by either cell detachment (anoikis) or by cytotoxic ligands secreted by immune cells [1]. Although expression of oncogenic Kras alone causes intestinal tumor development, it is insufficient for the formation of regional and distant metastases [17-19]. This raises the question whether mutant KRAS/Kras in metastatic colorectal carcinomas is essential for liver metastasis formation. Our results, using Kras^{D12}-suppressed C26 CRC cells, show that endogenous Kras^{D12} is essential for liver metastasis formation by promoting the survival of single tumor cells in the liver sinusoids and by stimulating their extravasation and subsequent outgrowth in the liver parenchyma. Kras^{D12}-suppressed cells did not make it beyond the intrasinusoidal single cell stage. Our previous work has shown that interleukin-18 (IL-18), an essential activator of natural killer (NK) cells, is one of the most strongly upregulated genes following Kras^{D12} knockdown[12]. Possibly, C26-KrasKD

cells stimulate their own clearance by activating the sinusoid-resident population of NK cells through IL-18 production. IL-18 also plays a role in the development of T-helper cell responses, but the clearance of C26-KrasKD cells is too rapid (>90% within 2 days) for CTL-mediated tumor cell clearance to play a major role.

At first sight our results appear to be in conflict with a recent report demonstrating that overexpression of $HRAS^{V12}$ in NIH3T3 fibroblasts promotes the outgrowth of metastases in the liver by shifting the proliferation:apoptosis balance in developing micrometastases without affecting seeding and extravasation [11]. However, changes in cell behavior induced by activated RAS are critically dependent on the level of RAS expression, on the RAS isoform used, on the specific activating mutation and on the cellular context. For instance, NIH3T3 fibroblasts already display an invasive phenotype and extravasate efficiently in the absence of mutant $HRAS^{V12}$ [11]. Furthermore, mutations in KRAS, but not HRAS, are associated with colorectal tumor development. In general, conclusions obtained by studying overexpressed $HRAS^{V12}$ in fibroblasts may not always apply to epithelial tumor cells carrying an endogenous activated Kras/KRAS allele.

The acquisition of metastatic potential is associated with the loss of epithelial and the gain of mesenchymal characteristics. However, whether or not metastatic tumor cells undergo true epithelial-to-mesenchymal transdifferentiation is under debate [20,21]. It is clear that if such a phenomenon occurs it must be transient given the epithelial phenotype of most liver metastases [14]. Several studies have shown that signaling by activated RAS in epithelial cells can lead to EMT-like phenomena, including loss of epithelial polarity and reduced cell-cell and cell-matrix interactions [1,6,7]. However, loss of the epithelial phenotype in colorectal cancer cells is not only governed by mutant Kras but also by additional genetic changes such as those in the Wnt and LKB signaling cascades, and by extracellular cytokines and growth factors [14,22]. The effect of suppressing or deleting mutant Kras/KRAS on cellular (trans-)differentiation is therefore most likely dependent on the specific genetic background of the carcinoma cell, as well as on microenvironmental cues. One of the strongest EMT-inducing cytokines is TGFβ [23]. Interestingly, expression of a dominant-negative TGFβ-type II receptor in C26 cells induced reversion of the spindle-like cell shape to a cobblestone epithelial-like morphology [15]. This was accompanied by re-expression of epithelial markers, and the formation of hemicysts *in vitro* [15]. Importantly, none of these MET-like phenomena were observed in $Kras^{D12}$-suppressed C26 cells. Thus, at least in C26 cells, the loss of metastatic capacity can be attained either by interfering with TGFβ signaling and subsequent induction of MET, or by interfering with RAS signaling, independently of trans-differentiation.

The frequency of mutant KRAS in human colorectal liver metastases is similar to that in primary carcinomas (~40%) [1]. Preservation of activated KRAS during tumor progression indicates that colorectal tumors may be continuously dependent on active KRAS. Our results are in line with this notion and show that activated $Kras^{D12}$ in highly metastatic C26 cells is essential for the formation of liver metastases.

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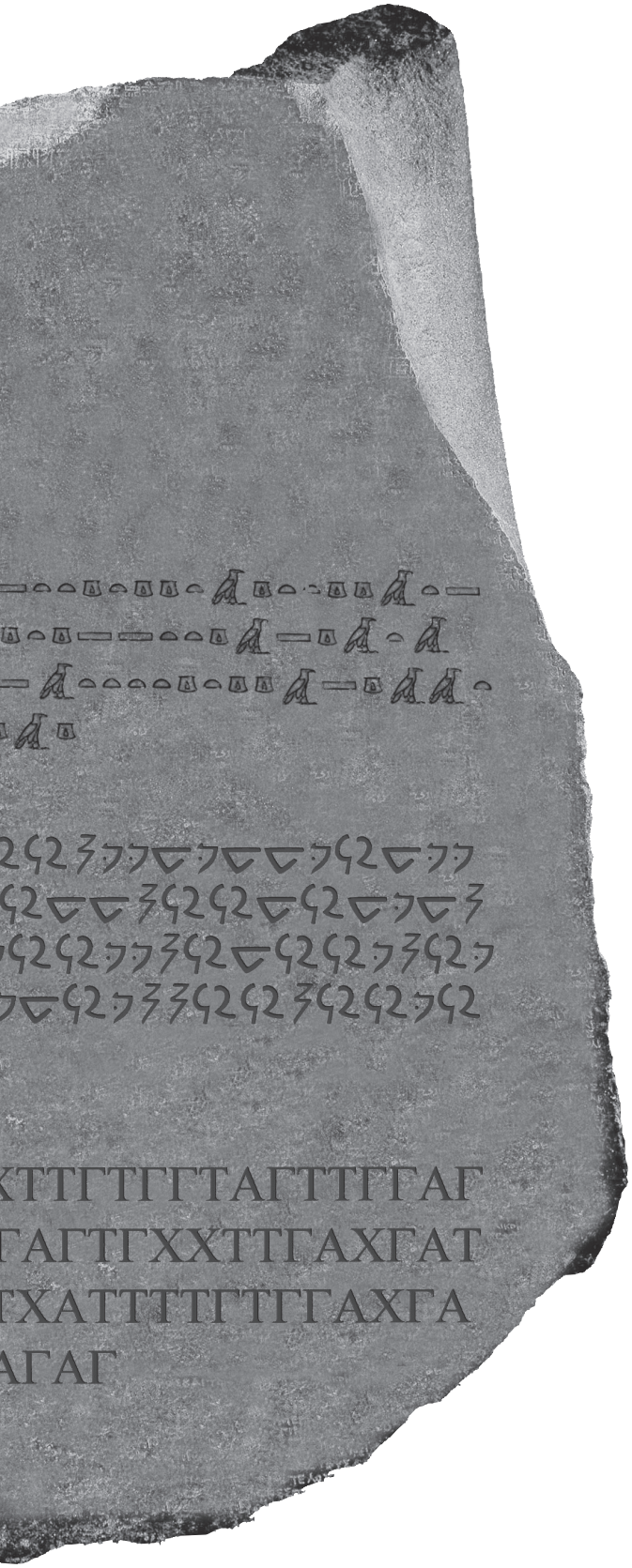

Chapter 6

Cyclooxygenase-2 is a target of $Kras^{D12}$ which facilitates the outgrowth of murine C26 colorectal liver metastases

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Abstract

Purpose

Mutational activation of the KRAS oncogene and overexpression of cyclooxygenase-2 (COX-2) contribute to colorectal carcinoma (CRC) development, but the relationship between these two events is unclear. This study was designed to clarify that relationship and to assess the contribution of KRAS-dependent COX-2 to the seeding of CRC cells in the liver and to their outgrowth as liver metastases in an experimental mouse model.

Experimental Design

The effect of RNA interference-mediated Kras knockdown on COX-2 expression and activity was tested in murine C26 CRC cells. The contribution of Kras-dependent COX-2 to early metastatic tumor cell seeding (by intravital microscopy) and outgrowth of metastases in the liver (by bioluminescence imaging) was studied by using Parecoxib, a novel and highly selective liver-activated COX-2 inhibitor. Intratumoral cell proliferation, apoptosis, and tumor-associated angiogenesis were assessed by immunohistochemistry on liver tissue sections.

Results

Stable knockdown of mutant $Kras^{D12}$ in murine C26 CRC cells by RNA interference lead to a dramatic reduction of COX-2 synthesis and prostaglandin-E_2 production. Inhibition of host or tumor cell COX-2 activity had no effect on early metastatic cell seeding in the liver, but greatly reduced intrahepatic tumor cell proliferation and the rate of liver metastasis outgrowth. COX-2 inhibition had no effect on early tumor vascularisation or on tumor cell apoptosis.

Conclusions

The high levels of COX-2 enzyme and prostaglandin production in C26 CRC cells are primarily caused by the presence of endogenous mutant $Kras^{D12}$. Furthermore, COX-2 inhibition affects the tumoral rather than the vascular compartment during the early stages of C26 liver metastasis outgrowth.

Introduction

Mutations in KRAS are detected at the early stages of adenoma development and contribute to oncogenic transformation of intestinal epithelial cells [1-8]. In addition, tumor cells containing mutant KRAS stimulate endothelial cells in nearby vascular beds to grow out and form new blood vessels that supply the developing tumor with nutrients and oxygen [9]. The mechanism underlying this phenomenon is complex and involves RAS-stimulated production of several pro-angiogenic factors, including cyclooxygenase-2 (COX-2) [9]. COX-2 is an interesting RAS target as it is highly expressed during the development of many tumor types, including CRC [10]. The presence of mutant KRAS and high level COX-2 expression both correlate with tumor recurrence after surgery, with metastatic spread to the liver and with reduced survival [11-23]. Conversely, COX-2-inhibitors reduce the risk of developing CRC, both in FAP patients and in sporadic cases [10].

Whereas the contribution of COX-2 to the early stages of intestinal tumorigenesis is well established, relatively few reports have concentrated on the role of COX-2 during the late metastatic stages of the disease. Clinical trials in which the therapeutic effects of COX-2 inhibitors on metastasized CRC were studied have shown variable results [24,25]. In an experimental model, enforced overexpression of COX-2 in CRC cells promoted the incidence of experimental liver metastasis formation [26], whereas COX-2 inhibitors reduced intrahepatic tumor growth [13,26-29]. However, there appears to be no consensus as to how and on what stage of metastasis formation these inhibitors sort their effect [13,26-29].

While overexpression of activated RAS isoforms can stimulate COX-2 expression [30,31], it remains unclear how KRAS mutation status in CRC cells relates to COX-2 expression and activity. Therefore, we have studied the cause and effect relationship between the presence of mutant Kras^{D12} and COX-2 expression and activity in C26 CRC cells. In addition, we have investigated whether inhibition of Kras^{D12}-dependent COX-2 by a novel and highly selective liver-activated COX-2 inhibitor (Parecoxib) affected early tumor cell seeding in the liver, intrahepatic tumor cell proliferation, apoptosis, angiogenesis and overall outgrowth of experimental CRC liver metastases.

Materials and Methods

Cell lines and culture conditions

C26, HT-29, and T24 cell lines were all obtained from the American Type Tissue Culture Collection (ATCC, Rockville, MD, USA). The C26 cell line stably expressing the firefly luciferase gene (C26-Luc) was described previously [32]. The C26-KrasKD and C26-pLL control cells were described elsewhere [33]. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Dulbecco, ICN Pharmaceuticals, Costa

Mesa, CA, USA) supplemented with 5% (v/v) fetal calf serum, 2mM glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin. All cells were kept at 37°C in a humidified atmosphere containing 5% CO2.

Chemicals

Indomethacin (1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3-acetic acid) and NS-398 (N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Parecoxib was purchased from Pfizer (New York, USA).

Antibodies

Primary antibodies were anti-COX-2 (#33, BD biosciences, Alphen aan den Rijn, The Netherlands), anti-KRAS and NRAS, anti-actin (F234, F155, C11 Santa Cruz Biotech., Heidelberg, Germany), anti-vWF (Dako, Amsterdam, The Netherlands), anti-Ki67 (MM1; Novocastra, Newcastle UK) and anti-active caspase-3 (C92-605; BD biosciences PharMingen). Secondary antibodies were rabbit anti-mouse-HRP (Pierce, Rockford, IL, USA) or swine anti-rabbit-HRP (DAKO, Glostrup, Denmark).

RAS assay

The RAS activity assay was performed by using the RAS-binding domain of Raf fused to glutathione-S-transferase immobilised on glutathione-sepharose as an affinity matrix for activated RAS exactly as described [34].

PGE_2 assay

PGE_2 levels were measured in the culture medium using a commercial EIA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. The corresponding cells were lysed and protein concentration was determined to normalize the PGE_2 levels measured in the medium samples.

Immunohistochemistry and image analysis

Immunohistochemistry on paraffin-embedded tissue sections was performed as described [35]. Randomly chosen tumors were scored as angiogenic, peri- or intra-vascular, or non-vascularized. The percentage of Ki-67-positivity was determined by counting all positive and negative tumor cells in 5 randomly chosen tumors in each specimen. Microvessel densities were determined in all angiogenic tumors. The percentage of tumor area that was occupied by vWF-positive cells was determined by analyzing digital tumor images using Leica QWIN software. All analyses were performed in a blinded manner. The percentage of apoptotic cells was determined by counting all activated caspase-3-positive and negative tumor cells in 5 randomly chosen tumors in each specimen. At least 600 cells were counted in each specimen. In all cases the observer was blinded to treatment.

Animals and Surgery

Male BALB/c mice aged 8-10 weeks (Harlan; Leicestershire, UK) were housed under standard conditions and received food and water ad libitum. Liver metastases were induced exactly as described [36]. All experiments were performed in accordance with the guidelines of the University's Animal Experimental Committee, University Medical Center Utrecht, the Netherlands.

Hepatic Replacement Area (HRA)

Intrahepatic tumor load was scored as the percentage of hepatic tissue replaced by metastatic tumor cells as described [36].

Analysis of tumor cell seeding in the liver

C26 cells were labeled (15 min., 37°C) with 4% carboxyfluorescein succinyl ester (CFSE; Molecular Probes, Leiden, the Netherlands) and were resuspended in PBS to a concentration of 10^5 cells/100 ml. Red fluorescent polystyrene microspheres (ø10 μm Molecular probes, Leiden, the Netherlands) were included at a 5:1 cell:bead ratio. Following anesthesia with fentanyl citrate/fluanisone (0.3 mg/mouse; Janssen-Cilag, Brussels, Belgium) and midazolamchloride (12.5 mg/mouse; Roche, Brussels, Belgium) the cell/bead mixture (100 μl) was injected into the spleen parenchyma. Mice were then placed in heated cages until microscopy.

Mice were pre-treated with Parecoxib (5 mg/kg i.p. or saline 0.9%) 38, 26, 14 and 2 hrs prior to tumor cell injection. Alternatively, tumor cells were pre-treated *in vitro* with indomethacin or NS-398 (20μM) or vehicle (0.05%DMSO) 24 hrs prior to injection. Cell viability was assessed by Trypan Blue staining.

Intravital microscopy

Intravital microscopy was performed as described [37]. Using a 20x lens, 30 randomly selected hepatic fields were chosen in each animal. Images were recorded and were analyzed off-line. The effect on tumor cell seeding was measured as the ratio of tumor cells per beads per high power field. Data are average counts of two procedures performed by independent observers blinded to treatment.

In vivo bioluminescent imaging (BLI)

Intrahepatic tumor growth was assessed by BLI 7, 9 and 11 days after intrasplenic injection of luciferase-expressing C26 cells exactly as described [32]. Data are presented as total photon counts as obtained by acquisition (MetaVue) and analysis (MetaMorph) software.

Statistical Analyses

Differences between the groups were statistically evaluated using the Mann-Whitney test. The results are presented as means ± s.e. All P-values were two-tailed, and a P-value < 0.05 was considered to be statistically significant.

Results

Knockdown of endogenous Kras^{D12} reduces COX-2 expression and PGE_2 secretion in murine C26 CRC cells

To assess how KRAS mutation status is related to COX-2 (over)-expression we have used murine C26 CRC cells. We recently found that C26 cells contain constitutively active Kras, due to an activating mutation in codon 12 (G12D) [33]. Whereas Kras in C26 cells is constitutively active, it is wild type in human HT-29 CRC and in T24 bladder carcinoma cells (Fig. 1A).

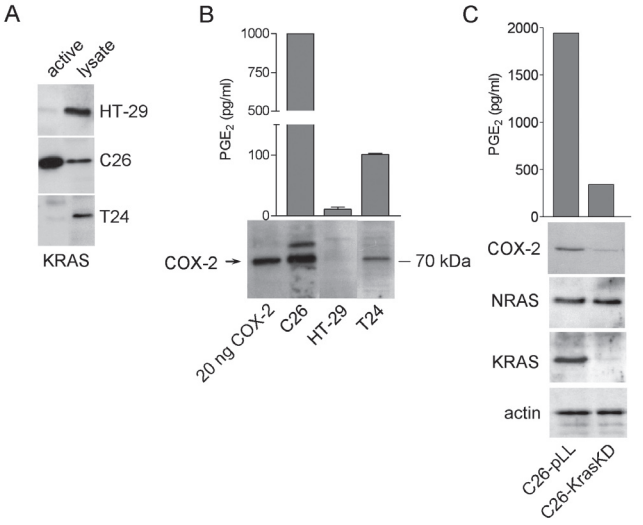

Figure 1. Suppression of Kras^{D12} by RNA interference reduces COX-2 expression and PGE2 secretion in C26 cells. (A) Exponentially growing murine C26 CRC, human HT-29 CRC and human T24 bladder carcinoma cells were lysed and the activity of KRAS was determined by the RAS activity assay, followed by Western blotting using an isoform-specific anti-KRAS antibody. Total lysates are shown as a reference. (B) The same lysates were then used for determination of COX-2 expression by western blotting, using an anti-COX-2 antibody. Medium conditioned for 48 hours by the same set of cell lines was used for determination of PGE_2 secretion by an Enzyme Immuno Assay (EIA). The values were normalized to protein content in the corresponding lysates. T24 human bladder carcinoma cells served as a positive control. (C) C26-KrasKD cells were derived from C26 cells and express short hairpin RNA's that stably suppress mutant Kras^{D12}. C26-pLL (empty vector) and C26-KrasKD cells were grown in complete tissue culture medium for 48 hours. The medium was then harvested and the cells were lysed. PGE_2 levels were determined in the medium by EIA as above and values were normalized to protein content in the corresponding lysates. Cell lysates were analyzed by Western blotting using anti-KRAS, anti-NRAS, anti-COX-2 and anti-actin antibodies. The figure shows that Kras knockdown results in a dramatic reduction of COX-2 protein levels and PGE_2 secretion.

Figure 1B shows that C26 cells, but not HT-29, express high levels of COX-2 enzyme and secrete prostaglandin E_2, one of the major COX-2-produced inflammatory mediators. The human T24 bladder carcinoma cell line (expressing $HRAS^{V12}$) is shown as a positive control for COX-2 expression and activity. To test whether the high levels of COX-2 in C26 cells are related to the presence of mutant $Kras^{D12}$ we generated a C26-derived cell line in which mutant Kras, but not Nras, is suppressed by RNA interference (C26KrasKD) (Fig. 1C). Kras knockdown resulted in strongly reduced COX-2 expression and PGE_2 secretion (Fig. 1C). We conclude that activated $Kras^{D12}$ is, to a large extent (~80%), responsible for the high levels of COX-2 synthesis and activity in C26 cells.

COX-2 inhibition reduces C26 liver metastasis formation

We previously found that stable suppression of $Kras^{D12}$ in C26 cells abrogates intrahepatic C26 tumor growth [33]. Since high level COX-2 expression in C26 cells depends on the presence of $Kras^{D12}$ (Fig. 1C), we investigated whether COX-2 contributes to $Kras^{D12}$-dependent liver metastasis formation. To this end we used Parecoxib, a highly specific water soluble pro-drug that is activated in the liver, the organ in which COX-2 inhibitory activity was required. It is among the most potent ($IC_{50}^{COX\text{-}2}$ = 0.005 μM) and selective ($IC_{50}^{COX\text{-}1}$= 140 μM) COX-2 inhibitors that have been identified to date [38,39].

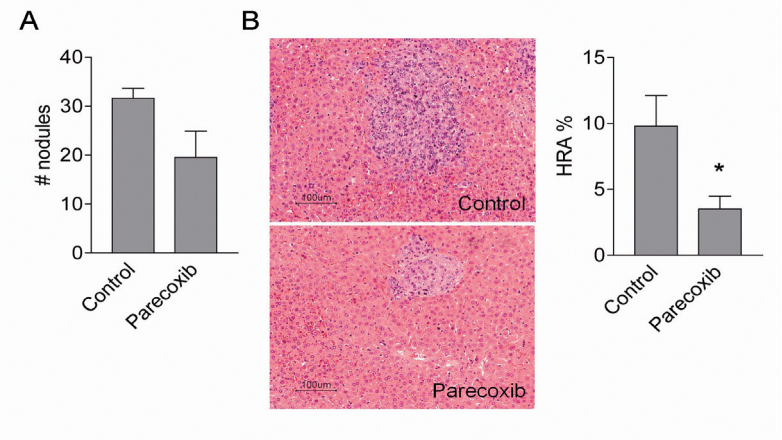

Figure 2. Parecoxib reduces intrahepatic tumour growth. Liver metastases were induced in BALB/c mice as described in the Materials and Methods section. The mice were treated either with Parecoxib (5 mg/kg; twice daily; n=8) or vehicle (n=5) by intraperitoneal injections. 7 days after the injection of tumor cells, intrahepatic tumor growth was assessed (A) by counting macroscopic tumor nodules on the liver surface, and (B) by measuring the percentage of hepatic tissue that was replaced by metastatic tumor cells (HRA) on haematoxylin- and eosin-stained (H&E) sections from non-sequential levels of each liver (300 fields/liver; error bar = SEM). Both assays were performed by observers blinded to treatment. * denotes statistical significance ($p<0.05$). Representative H&E-stained sections are shown (magnification 400x).

C26 liver metastases were induced by injection of C26 cells into the spleen. Treatment was then started by intraperitoneal injections of Parecoxib (5 mg/kg; twice daily). 7 Days after tumor cell injection, intrahepatic tumor growth was assessed by counting macroscopic tumor nodules on the liver surface as well as by histochemical determination of the area of liver tissue that had been replaced by tumor tissue (hepatic replacement area (HRA)). Parecoxib reduced the number of macroscopic tumor nodules by approximately 30% (Fig. 2A) and reduced the HRA by a dramatic 63% (Fig. 2B).

COX-2 inhibition does not affect tumor cell seeding in the liver

Inhibition of COX-2 activity may affect liver metastasis formation at distinct stages of the metastatic process. To discriminate between the early and late stages we studied the effects of COX-2 inhibition on tumor cell seeding and on the rate of tumor outgrowth. First, intravital microscopy (IVM) was used to assess the seeding of CFSE-labelled (green) fluorescent C26 cells in the liver. Red fluorescent polystyrene beads which are trapped in the liver microvasculature served as an internal reference for determining the efficiency of cell seeding. PGE_2 is produced by the tumor cells (Fig. 1B) but also by sinusoidal cells in the liver (i.e. Kupffer, endothelial and stellate cells [40-42]. Therefore, we investigated the effects of inhibiting either host or tumor cell COX-2. Host COX-2 was inhibited by pre-treating the mice with intraperitoneal injections of Parecoxib 38, 26, 14, and 2 hrs prior to tumor cell injection. IVM was then performed one hour after intrasplenic tumor cell injection.

We found no differences in the efficiency of tumor cell seeding in the control versus the Parecoxib-treated groups (Fig. 3A). Next, we assessed the effect of inhibiting COX-2 in the tumor cells. Both a selective (NS-398) and a non-selective (Indomethacin) COX-2 inhibitor abrogated PGE_2 synthesis as expected without affecting COX-2 expression (Fig. 3B) or cell viability (Fig. 3C). The use of Parecoxib in these *in vitro* experiments was excluded as it is an inactive pro-drug. IVM analysis one hour after intrasplenic injection of C26 cells revealed no differences in the seeding of untreated, Indomethacin-treated, or NS-398-treated cells (Fig. 3D). Taken together, the results show that inhibition of either host COX-2 or tumor cell COX-2 has no effect on early C26 tumor cell seeding in the liver.

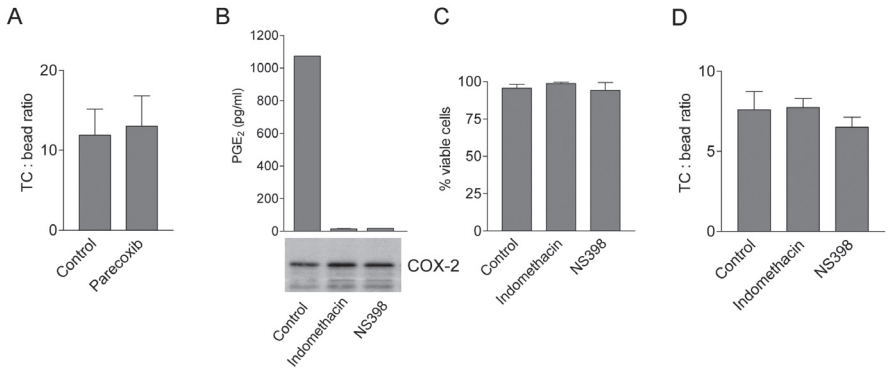

Figure 3. Effect of COX inhibitors on tumor cell seeding in the liver by Intravital Microscopy (IVM). (A) BALB/c mice received intraperitoneal injections of Parecoxib (5 mg/kg) (n=7) or saline (0.9%) (n=6) at 38, 26, 14 and 2 hrs prior to injection of a mixture of CFSE-labeled C26 cells (10^5) and red fluorescent latex beads ($2x10^4$) in 100 μl PBS into the spleen. One hour after injection IVM images of the liver parenchyma were registered with a charge coupled device camera. Using a high magnification lens (20x), 30 randomly selected hepatic fields were chosen in each animal. Images were recorded and analyzed off-line. Data are expressed as the ratio between tumor cell number and the number of beads per high power field. Data are average counts of two procedures performed by independent observers blinded to treatment. (B) C26 cells were grown for 24 hrs in culture medium containing Indomethacin (20 μM), NS-398 (20 μM), or vehicle (0.05%DMSO). The culture medium was collected and PGE2 levels were analysed by EIA. The corresponding lysates were analysed for COX-2 expression. (C) C26 cells were grown and treated as in (B) and cell viability was assessed by the standard Trypan Blue exclusion method. The values shown are means of triplicates. (D) C26 cells were grown and treated as in (B) and were subsequently injected into the spleen. (vehicle, n=9; indomethacin, n=8; NS-398, n=8). Tumor cell seeding in the liver was then assessed as in (A).

COX-2 inhibition by Parecoxib slows down the outgrowth of liver metastases by reducing tumor cell proliferation

We next studied whether COX-2 inhibition affected the rate of tumor outgrowth in the liver. Following intrasplenic injection of luciferase-expressing C26 cells (day 0), mice received daily intraperitoneal injections of Parecoxib or vehicle. Intrahepatic tumor growth was subsequently measured by BLI on days 7, 9 and 11 post-injection. The results presented in Figure 4A show that Parecoxib treatment greatly reduced the rate of C26 tumor growth in the liver. To get insight into the underlying mechanism we studied the effects of Parecoxib on tumor cell proliferation and apoptosis and on tumor-associated angiogenesis. To this end, we performed immunohistochemistry using Ki67 as an indicator of cell proliferation, active caspase-3 as an indicator of apoptotic cells, and factor VIII (von Willebrand Factor; vWF) as an indicator of newly-formed blood vessels.

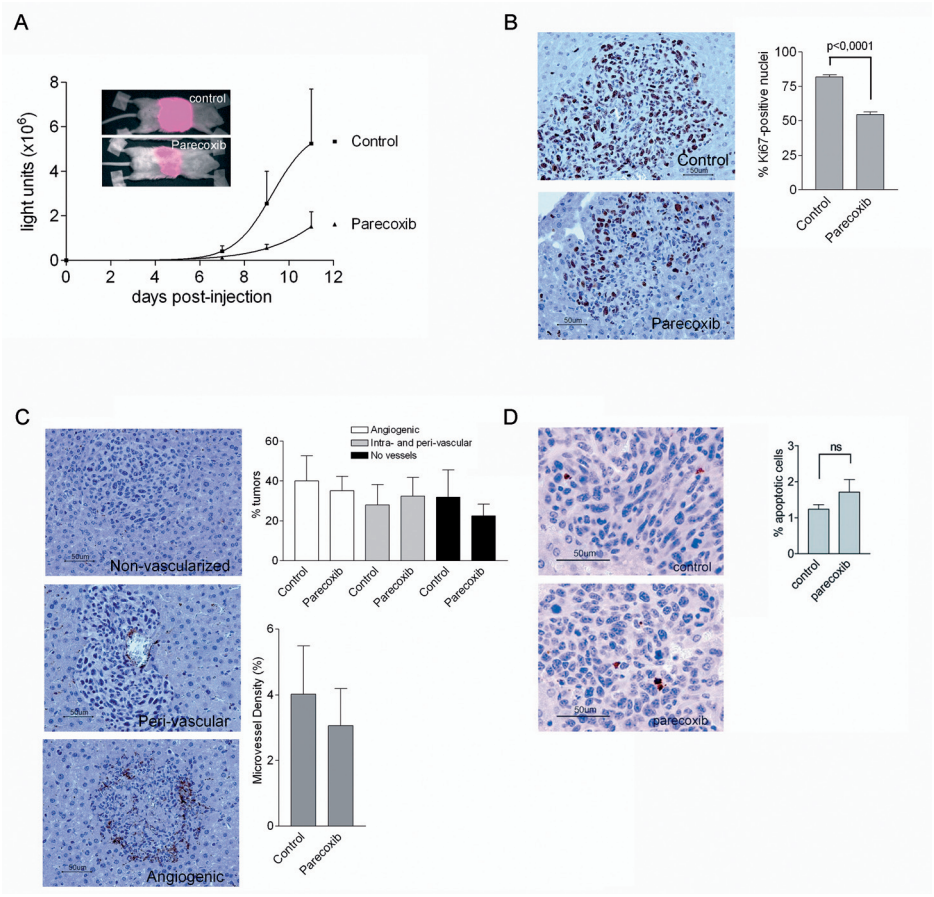

Figure 4. Parecoxib slows down the outgrowth of C26 liver metastases. (A) C26-luc cells (10^5) were injected into the spleens of BALB/c mice, followed by splenectomy. Mice received i.p,. injections of either Parecoxib (10 mg/kg/day) (n=3) or vehicle (n=3). Bioluminescence was measured for 5 minutes on post-operative days (pod) 7, 9, and 11. (Error bar = SEM). * denotes statistical significance ($p<0.05$). Representative bioluminescence images on pod 11 are shown in the inset. Following BLI on day 11 the livers were removed, and processed for immunohistochemistry using anti-Ki67 (B), anti-von Willebrand factor (C) or anti-active caspase-3 (D). Images of 5 tumors per section (of each individual liver) were recorded and analysed in a blinded manner. The number of Ki67-positive tumor cells, the vascular profile and the microvessel densities (expressed as % vWF-positive tumor-area) and the apoptotic indices are shown in the bar diagrams. NS indicates non-significant differences. Representative anti-Ki67- and anti-active caspase-3-stained sections are shown. Examples of vascularised, avascular, and peri-vascular growing tumors are also shown.

We found that the percentage of proliferating C26 cells in the liver metastases was dramatically reduced from a mean 82% in the control group to 54% in the Parecoxib-treated group (Fig. 4B), a reduction by 37%. The angiogenic profile of the liver metastases was remarkably heterogeneous in both groups. Many lesions had not yet undergone the angiogenic switch and grew either peri- or intra-vascularly (Fig. 4C). We found no significant differences between the angiogenic profiles of liver metastases from Parecoxib-treated and control mice (Fig. 4C). In addition, the microvessel densities in the vascularised tumor subgroups were not significantly different (Fig. 4C). Finally, the apoptotic indices in tumors isolated from control and Parecoxib-treated mice were not significantly different (Fig. 4D).

Discussion

Activated KRAS affects the proliferation, survival and invasiveness of tumor cells but also affects the behavior of stromal cells by stimulating the secretion of soluble factors like VEGF and prostaglandins [9]. The ectopic overexpression of mutant HRAS results in elevated expression of COX-2 [30,31], the major prostaglandin-synthesizing enzyme in neoplastic tissue. Conversely, our results show that the removal of endogenous mutant $Kras^{D12}$ from C26 CRC cells was sufficient to reduce COX-2 expression and PGE_2 secretion by ~80%. Thus, the stimulation of COX-2 expression and activity was the direct result from an acquired activating mutation in Kras.

COX-2 has been implicated in a number of processes that affect tumor growth and metastasis formation. These include effects on tumor cell survival, proliferation and invasiveness as well as on early metastatic seeding and angiogenesis (reviewed in [43]. Moreover, COX-2 synthesizes inflammatory mediators that induce a state of chronic inflammation and may thereby promote tumor growth [44].

Our study shows that Parecoxib reduces the proliferation of extremely aggressive CRC cells in the liver by 37%, as determined by the Ki67 proliferation marker. This effect presumably accounts for the reduced outgrowth of CRC liver metastases. NS-398, another highly selective COX-2 inhibitor, reduces C26 cell proliferation *in vitro* [29]. Likewise, the proliferation rate of intestinal adenoma cells in COX-2-deficient mice is suppressed by 50% when compared to control mice [45]. Taken together, the data indicate that COX-2 is essential for efficient tumor cell proliferation in early developing intestinal adenomas, but also in liver metastases.

An inherent disadvantage of using COX-2 inhibitors is the possibility that enzymes/factors other than COX-2 may be inhibited. COX-independent effects have been reported for a number of non-steroidal anti-inflammatory drugs, including sulindac, celecoxib, NS-398 and indomethacin [46]. At present, the only Parecoxib target known is COX-2. Nevertheless, we cannot exclude the possibility that COX-2-independent effects may contribute to the observed reduction in tumor cell prolifer-

ation. Formal proof for the involvement of COX-2 in intrahepatic tumor cell proliferation and outgrowth of liver metastases should come from genetically modified tumor cells lacking COX-2. Unfortunately, our attempts to generate such cells by RNA interference have failed so far.

The interaction between human CRC cells and macrovascular human umbilical vein endothelial cells (HUVECs) *in vitro* can be reduced by celecoxib [26]. This may implicate COX-2 in the regulation of early metastatic seeding in the liver. Our study, based on the use of intravital microscopy, does not support a role for COX-2 in the adhesion of C26 tumor cells to the sinusoidal endothelium of the liver *in vivo*.

A series of elegant studies in genetically modified mice have unequivocally demonstrated that COX-2 and the PGE_2 receptor EP2 contribute to the development of intestinal papillomas through stimulation of angiogenesis [45,47-49]. In addition, COX-2 inhibitors act as anti-angiogenic compounds in several angiogenesis assays *in vitro*, in the chorioallantoic membrane assay, and in the cornea assay [50-54]. In marked contrast, it remains unclear whether the anti-tumorigenic effect of COX-2 inhibitors on tumorigenesis in general, and on liver metastasis formation in particular, is due to inhibition of angiogenesis. Studies that addressed this issue by analyzing microvessel density in surgically removed tumor specimens of patients that had been treated with COX-2 inhibitors have generated conflicting results [18,55]. In light of the above results, it was perhaps surprising that Parecoxib had no discernable effect on angiogenesis of C26 colorectal liver metastases. A possible explanation for this result could be that the majority (~60%) of the liver metastases that we have analyzed had not yet undergone the angiogenic switch, due to their still limited size and due to the fact that many of the tumors grew peri- and intra-vascularly. Therefore, the heterogeneous nature of the angiogenic profile of these tumors may have precluded the detection of anti-angiogenic effects in Parecoxib-treated mice.

In conclusion, our study shows that the inhibition of $Kras^{D12}$-stimulated COX-2 by Parecoxib reduces intrahepatic tumor cell proliferation and tumor growth during the early outgrowth of C26 liver metastases.

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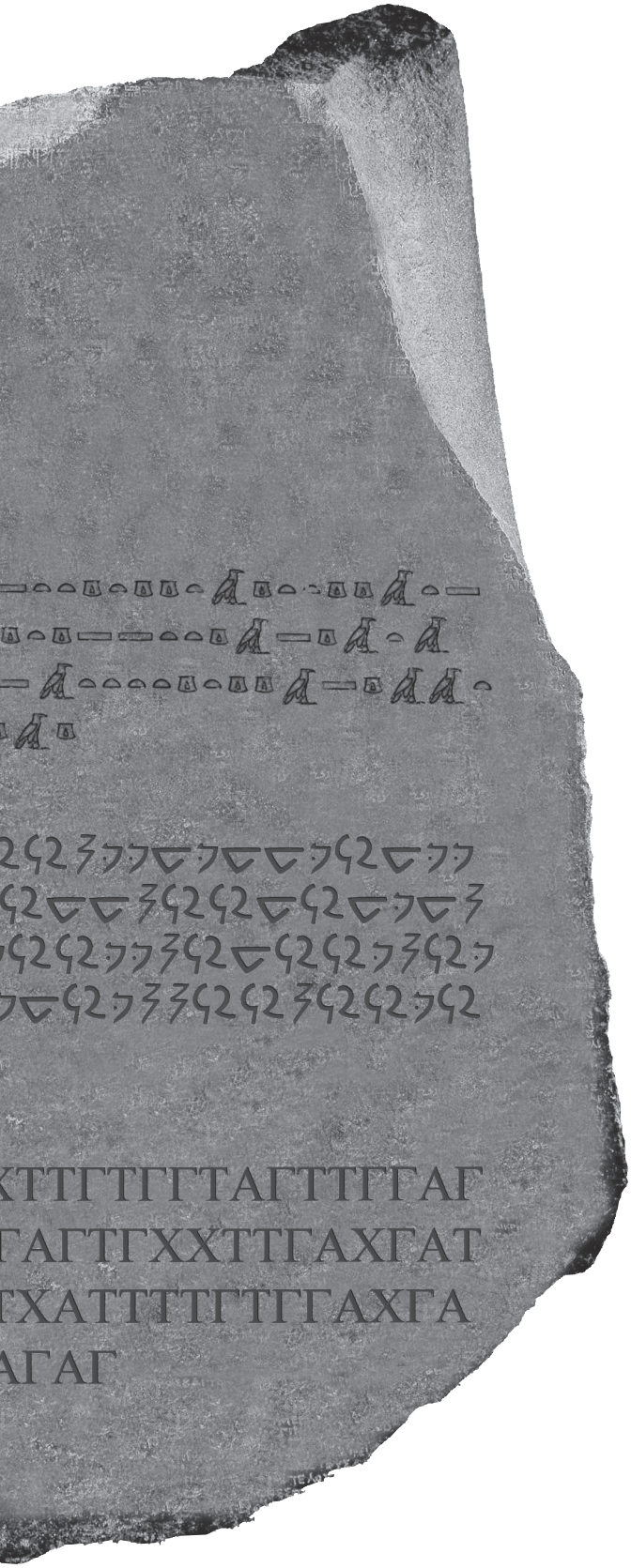

Chapter 7

NS-398, a selective cyclooxygenase-2 inhibitor, reduces experimental bladder carcinoma outgrowth by inhibiting tumour cell proliferation

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Abstract

Objectives

Cyclooxygenase-2 (COX-2) overexpression is frequently observed in bladder carcinomas and has been correlated with increased stage of disease and with reduced patient survival. The objectives of this study were to evaluate the efficacy of the selective COX-2 inhibitor NS-398 in treating experimental T24 bladder carcinomas and to assess its effect on tumour cell proliferation and survival and on tumour vascularisation.

Methods

NS-398 was used to selectively inhibit COX-2 in human T24 bladder carcinoma cells. Lentiviral transduction of the firefly luciferase gene allowed us to assess the effect of NS-398 on orthotopic bladder carcinoma growth over time in a non-invasive manner. Immunohistochemistry on bladder tissue sections was performed to determine the effect of NS-398 treatment on tumour cell proliferation (Ki67), apoptosis (cleaved caspase-3), and angiogenesis (von Willebrand factor; CD31).

Results

T24 cells expressed COX-2 and secreted PGE_2. Selective COX-2 inhibition using NS-398 abrogated PGE_2 secretion and inhibited cell proliferation *in vitro* in a dose dependent manner, without affecting cell viability. *In vivo* administration of NS-398 reduced the outgrowth of experimental orthotopic T24 bladder carcinomas. This was accompanied by a significant reduction in Ki-67-positive tumour cells but not by a reduction in tumour cell viability or tumour vascularisation.

Conclusions

Selective COX-2 inhibition by NS-398 reduces the outgrowth of T24 human bladder carcinomas in an orthotopic mouse model. The therapeutic activity is most likely caused by inhibition of tumour cell proliferation rather than by inhibition of angiogenesis or tumour cell survival.

Introduction

Cyclooxygenases (COX) promote the conversion of arachidonic acid into tissue-specific prostaglandins (PGs). COX-1 is constitutively expressed in many tissues. It controls vasodilatation in the kidney and the stomach and is essential for thromboxane production in platelets [1]. In contrast, COX-2 expression is undetectable in most tissues but is strongly stimulated during tissue inflammation and during the formation of several types of tumours, including bladder carcinomas [1-3]. Whereas COX-2 expression is undetectable in normal urothelium [4], it is highly upregulated during bladder carcinogenesis and this is associated with increased muscle invasion, increased stage of disease and with reduced patient survival [4-13].

Non-steroidal anti-inflammatory drugs (NSAIDs) are compounds that inhibit the enzymatic activity of both COX isoforms and thereby exert anti-inflammatory, analgesic and antipyretic effects. Whereas these therapeutic benefits are mediated through COX-2 inhibition, the major side effects like erosion and ulceration of the gastric mucosa are caused by inhibition of COX-1 [14]. To prevent these side effects, selective COX-2 inhibitors have been developed. NS-398 is a highly selective COX-2 inhibitor which does not cause the adverse side effects that are associated with COX-1 inhibition [15].

A recent epidemiological study showed that the regular intake of NSAIDs is correlated with a reduced risk of developing bladder carcinomas [16]. Furthermore, NSAIDs reduce bladder carcinogenesis in experimental models in mice, rats and dogs (reviewed in [3]). However, it is not clear how these inhibitors sort their therapeutic effect. COX-2 has been implicated in a number of processes that affect tumour growth and metastasis formation. These include effects on tumour cell survival, proliferation and invasiveness as well as on early metastatic seeding and tumour angiogenesis (reviewed in [2,3,17]).

Materials and Methods

Chemicals and antibodies

NS-398 (N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Primary antibodies were anti-COX-2 (#33, BD biosciences, Alphen aan den Rijn, The Netherlands), anti-Ki67 (MM1; Novocastra, Newcastle UK), anti-vWF (Dako, Amsterdam, The Netherlands), anti-CD31 (JC/70A; Biogenex), anti-cleaved caspase-3 (#9661; Cell Signalling Technology Inc.). Secondary antibodies were rabbit anti-mouse-HRP (Pierce, Rockford, IL, USA) or swine anti-rabbit-HRP (DAKO, Glostrup, Denmark).

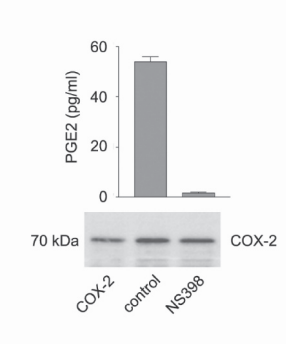

Figure 1. COX-2 expression in human T24 TCC cells and inhibition of PGE_2 production by NS-398. T24 cells were cultured in medium containing 5% FCS and either received NS-398 (40μM) or vehicle (control) as indicated for 48 hours. The expression of COX-2 was then assessed by Western-Blot analysis of cell extracts using an anti-COX-2 antibody. The tissue culture supernatant was harvested and PGE_2 levels were determined by an Enzyme Immune Assay (EIA). Values were normalized to protein content in the corresponding lysates. NS-398 treatment abolished PGE_2 secretion.

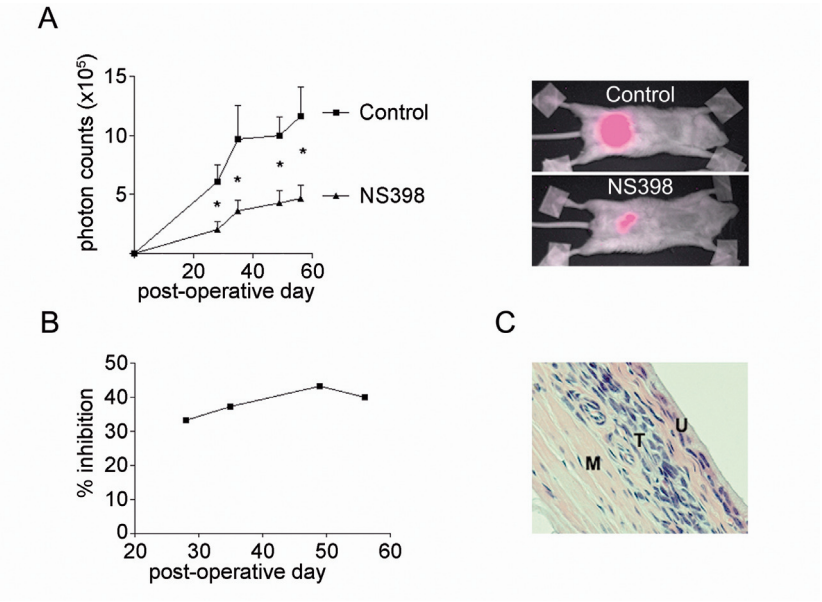

Figure 2. NS-398 reduces the outgrowth of orthotopic T24 bladder carcinomas. (A) T24-luc cells ($6x10^5$) were injected into the dome of the bladder of male C.B-17/IcrCrl-scid mice (n=10/group). After tumour induction mice were treated with intraperitoneal injections of NS-398 (3mg/kg) or vehicle three times a week. Bioluminescence was measured for 5 minutes on post-operative days 28, 35, 49 and 56. (Error bar = SEM). * denotes statistical significance ($p<0.05$). Representative bioluminescence images on pod 35 are shown in the right panel. (B) By using the mean photon counts as measured in (A), the effect of NS-398 was calculated as % inhibition relative to control over time. (C) Following BLI on day 56 the bladders were removed and fixed in formalin. Haematoxylin and eosin-stained sections demonstrate the orthotopic location of tumour growth (T) between the urothelium (U) and underlying smooth muscle layers (M). Magnification 200x.

Cell lines and culture conditions

The human bladder carcinoma cell line T24 was obtained from the American Type Tissue Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Dulbecco, ICN Pharmaceuticals, Costa Mesa, CA, USA) supplemented with 5% (v/v) fetal calf serum, 2mM glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin. All cells were kept at 37°C in a humidified atmosphere containing 5% CO_2.

Western blotting

Western blotting was performed exactly as described [18].

PGE_2 assay

PGE_2 levels were measured in the culture medium using a commercial EIA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. The corresponding cells were lysed and protein concentration was determined to normalize the PGE_2 levels measured in the medium samples.

Cell proliferation

T24 cells were seeded in a 96-well plate (5000 cells/well) in medium containing 10, 20, 40 or 100 μM NS-398. Control cells were treated with vehicle (DMSO). After 96 hours 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide-thiazolyl blue (MTT; Sigma Chemical Co., St. Louis, MO) was added to the culture medium at a concentration of 7.5 mg/ml for 2 hours at 37°C. Subsequently, cells were washed with PBS and the purple formazan was solubilized in 100% DMSO. Absorbance was read at 570 nm.

Lentiviral transduction

For bioluminescence imaging T24 cells were transduced with a lentivirus harbouring the firefly luciferase gene under control of the CMV promoter (pRRL-CMV-Luc, kindly provided by Prof. R.C. Hoeben) exactly as described [19].

Animals and Surgery

Male C.B-17/IcrCrl-scid mice aged 8-10 weeks (Charles River Laboratories, Maastricht, The Netherlands) were housed under standard conditions and received food and water ad libitum. Bladder tumours were induced as follows. Mice were anaesthetized with an intramuscular injection of ketamine hydrochloride (100 mg/kg; Vétoquinol BV, Den Bosch, The Netherlands) plus xylazine (10 mg/kg; Eurovet Animal Health BV, Bladel, The Netherlands) and atropine (0.05 mg/kg; Pharmachemie BV, Haarlem, The Netherlands) (KXA). Through a lower abdominal incision the bladder was exposed and luciferase expressing T24 carcinoma cells ($6x10^5$/30 μl) were injected into the dome of the bladder through a 30G needle. After tumour induction (day 0), mice received an intraperitoneal injection of NS-398 (3mg/kg)

and thrice weekly after that for the duration of the experiment. NS-398 was dissolved in 100% DMSO to 12.5 mg/ml. The calculated dose was diluted to a volume of 750 μl with PBS. Control mice received the same amount of DMSO in 750 μl PBS.

All experiments were performed in accordance with the guidelines of the University's Animal Experimental Committee, University Medical Center Utrecht, the Netherlands.

In vivo bioluminescent imaging (BLI)

Bioluminescence imaging was performed exactly as described [19].

Immunohistochemistry and image analysis

Immunohistochemistry was performed by using anti-Ki-67, anti-von Willebrand factor (vWF), anti-CD31, and anti-cleaved-caspase-3 antibodies. The number of Ki-67-positive cells was determined by counting all positive tumour cells in 3 randomly chosen high power fields completely filled with tumour cells (hpf, 200X) in each specimen. Microvessel densities were determined by identifying and counting vWF-positive vessels in 5 randomly chosen high power fields completely filled with tumour cells (hpf, 400X) in each specimen. All analyses were performed by observers blinded to treatment.

Statistical Analyses

Differences between the groups were statistically evaluated using the Mann-Whitney test. The results are presented as means ± SEM. All P-values were two-tailed, and a P-value < 0.05 was considered to be statistically significant.

Results

NS-398 inhibits PGE_2 production of T24 human TCC cells *in vitro*

The human transitional cell carcinoma (TCC) cell line T24 expressed COX-2 and secreted PGE_2 (Fig. 1). Treatment with the COX-2 selective inhibitor NS-398 (40μM) for 24 hours abolished the secretion of PGE_2 without affecting COX-2 expression levels (Fig. 1). These results suggest that COX-2 but not COX-1 produces the bulk of PGE_2 in T24 cells.

NS-398 reduces the outgrowth of orthotopic T24 bladder carcinomas

We next analysed the effect of NS-398 on the outgrowth of T24 bladder carcinomas in an orthotopic mouse model. To this end we generated T24 cells expressing the firefly luciferase gene (T24-Luc), allowing non-invasive bioluminescence imaging (BLI) of tumour growth. Orthotopic bladder tumours were induced by a subcapsular injection of T24-Luc cells (day 0). From then on, mice received intraperitoneal injections of NS-398 (3mg/kg) or vehicle thrice weekly. NS-398 treatment

reduced the rate of T24 bladder carcinoma outgrowth by 33-43% (Fig. 2A). The extent of inhibition remained stable throughout the 8-week course of the experiment (Fig. 2B). After imaging on day 56, the mice were sacrificed and their bladders were isolated and fixed in formalin. Analysis of haematoxylin and eosin-stained sections demonstrated that in all bladders examined, tumour growth had occurred between the urothelium and the underlying smooth muscle layers at the correct orthotopic site (Fig. 2C).

NS-398 inhibits tumour cell proliferation but not angiogenesis or tumour cell viability

COX-2 has been implicated in the stimulation of bladder tumour angiogenesis [20]. Therefore, we analysed the vascularization of tumours isolated from control and NS-398-treated animals. To this end we performed immunohistochemistry on paraffin-embedded tissue sections using von Willebrand Factor (vWF) and CD31 to stain the endothelial cells that line the blood vessels. Surprisingly, we found that NS-398 had no effect on either the vWF or CD31 staining patterns (Fig. 3). Digital image analysis showed that the vascular densities (defined as the tumour area occupied by either vWF- or CD31-positive cells) were not significantly different between the treatment groups. Based on the use of these two endothelial cell markers we conclude that NS-398 did not affect T24 tumour vascularization.

Next, we analysed whether NS-398 affected tumour cell apoptosis. To this end we performed immunohistochemistry on paraffin-embedded tissue sections using an antibody that selectively recognizes activated (cleaved) caspase-3. We found that active-caspase-3-positive cells occur very infrequently (<0.5% of tumour cells) in tumours isolated from either control or NS-398-treated mice (Fig. 4A). Between the groups these values were not significantly different (Fig. 4B). In line with these results, we found that NS-398 (up to 100 μM) had no discernable effect on T24 cell viability *in vitro*, as judged by Trypan Blue exclusion and FACS analyses.

COX-2 may also control bladder carcinoma cell proliferation [21]. Therefore, we analysed the number of Ki67-positive cells in tumour tissue sections isolated from control and NS-398-treated animals. NS-398 treatment significantly reduced the number of proliferating T24 cells *in vivo* (Fig. 5A,B). Next, we tested whether this could be due to a direct effect of NS398 on the T24 tumour cells. Continuous treatment of T24 cells with NS-398 *in vitro* resulted in reduced cell proliferation (Fig. 5c). Taken together, our results show that NS-398 reduces the growth of orthotopic T24 bladder carcinomas, most likely by reducing T24 tumour cell proliferation.

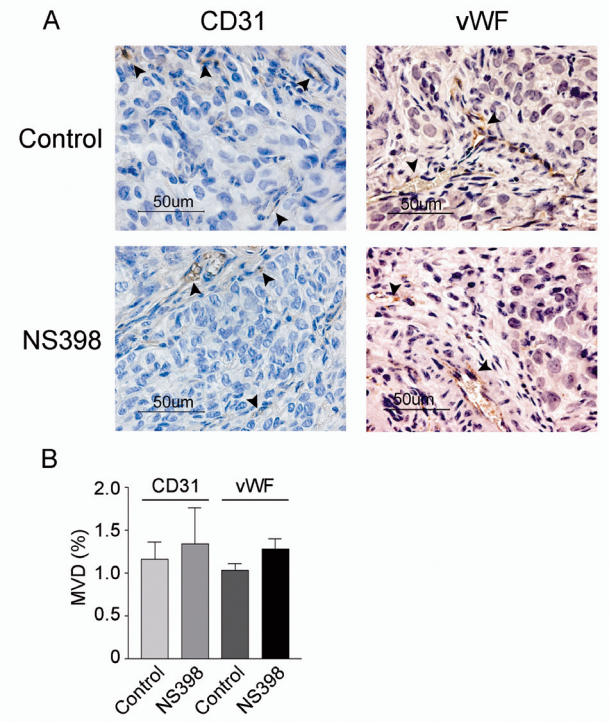

Figure 3. NS-398 does not affect T24 tumour vascularisation. (A) Formalin-fixed tumours were processed for immunohistochemistry using anti-von Willebrand factor (vWF) and anti-CD31 antibodies. A representative section is shown demonstrating vWF- and CD-31-positive vessels, indicated by arrowheads. (B) The % of tumour tissue area that was occupied by either vWF- or CD31-positive cells was determined by unbiased (blinded) digital image analysis of 5 randomly chosen high power fields (hpf) in each specimen.

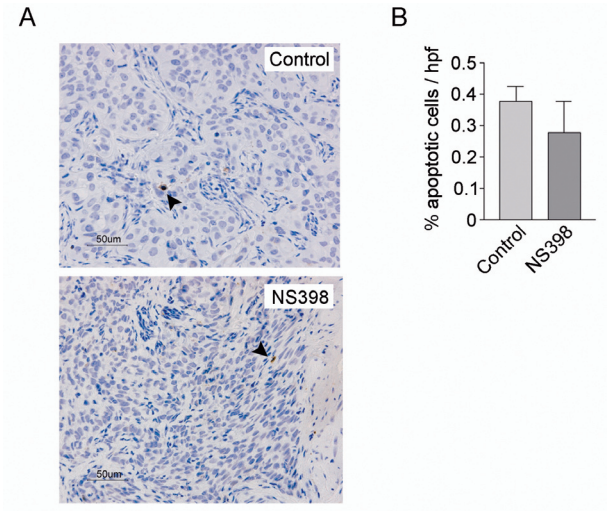

Figure 4. NS-398 does not affect tumour cell apoptosis. (A) Formalin-fixed tumours were processed for immunohistochemistry using an anti-cleaved-caspase-3 antibody. Representative sections are shown. Arrow heads indicate positive cells. (B) The % of cleaved-caspase-3-positive cells per high power field (hpf) was determined by counting all positive tumour cells in 3 randomly chosen hpf's (200X) in all tumours analysed (n=10).

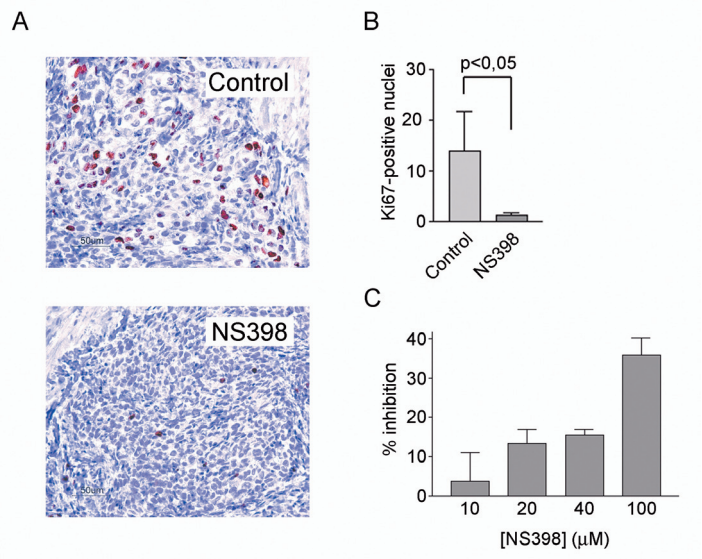

Figure 5. NS-398 reduces T24 tumour cell proliferation. (A) Formalin-fixed tumours were processed for immunohistochemistry using an anti-Ki-67 antibody. Representative anti-Ki67-stained sections are shown. (B) The number of Ki-67-positive cells per high power field was determined by counting all positive tumour cells in 3 randomly chosen fields (hpf, 200X) in all tumours analysed (n=10). (C) T24 cells were cultured in the absence or the presence of NS-398 at the indicated concentrations and the relative number of viable cells was analysed by a standard MTT assay. The data are presented as % inhibition compared to control and are means from two independent experiments performed in triplicates. NS-398 reduces T24 cell proliferation in a dose-dependent manner.

Comment

We analysed orthotopic bladder tumour progression non-invasively with BLI. To our knowledge this is the first report that uses BLI to image bladder carcinoma growth. BLI proved to be a fast, easy and reliable technique that abolishes the need for cumbersome morphometric analyses of tissue sections to quantify tumour load in the bladder. Furthermore, BLI measurements can be performed consecutively in the same mice, thereby greatly facilitating the assessment of tumour growth and therapeutic responses over time.

The expression of COX-2 is up-regulated during bladder carcinogenesis. It may affect tumour growth and metastasis formation by modulating a number of distinct processes, including tumour cell proliferation, invasion and survival as well as tumour angiogenesis [1,2,17,22]. High COX-2 expression is associated with increased microvessel density (MVD) in human TCC [20]. In addition, selective and non-selec-

tive COX-2-inhibitors have anti-angiogenic effects in a variety of tumour types, including bladder carcinomas [17,23]. However, in the present study NS-398 treatment had no effect on the vascular density of T24 bladder carcinomas.

In addition to modulating tumour cell proliferation and angiogenesis COX-2 may also affect tumour cell apoptosis [17]. Analysis of the apoptotic indices revealed very low rates of tumour cell apoptosis (<1%) in our experiments, both in control and in NS-398-treated mice. Apoptosis may be stimulated through a direct effect on the tumour cells themselves, or through inhibition of angiogenesis. In the latter case the reduced availability of oxygen and nutrients may indirectly stimulate tumour cell apoptosis. NS-398 did not affect T24 viability *in vitro*, nor affected tumour angiogenesis *in vivo*. These results may explain why also the apoptotic indices were not changed.

COX-2 has also been implicated in the regulation of tumour(cell) invasiveness [12,17]. During the 8-week course of our tumour experiments we did not observe invasion of the muscle layer by T24 tumour cells. This precluded the detection of a possible anti-invasive effect of NS-398 in our model.

In the present report we have found that a reduction in tumour cell proliferation (rather than survival, invasion or angiogenesis) most likely accounts for the therapeutic effect of NS-398 on T24 bladder carcinoma development. This is in line with the finding that increased tumour cell proliferation correlates with increased expression of the PGE_2-synthesising machinery in human TCC specimens [21]. The anti-proliferative effect of NS-398 and other selective COX-2 inhibitors may prove to be of therapeutic benefit in the management of human bladder carcinomas. This is especially relevant since a high proliferative index in these tumours correlates with worse prognosis [24,25].

Conclusions

Bioluminescence Imaging is a powerful tool for non-invasive monitoring of experimental tumour growth in the bladder. BLI was used to demonstrate that NS-398, a selective COX-2 inhibitor, stably reduced the rate of human T24 bladder carcinoma growth in an orthotopic mouse model over a period of 8 weeks. A direct inhibitory effect of NS-398 on tumour cell proliferation but not on survival or on tumour angiogenesis presumably accounts for the therapeutic effect. Therefore, NS-398 may have therapeutic potential in the management of established bladder carcinomas.

Acknowledgements

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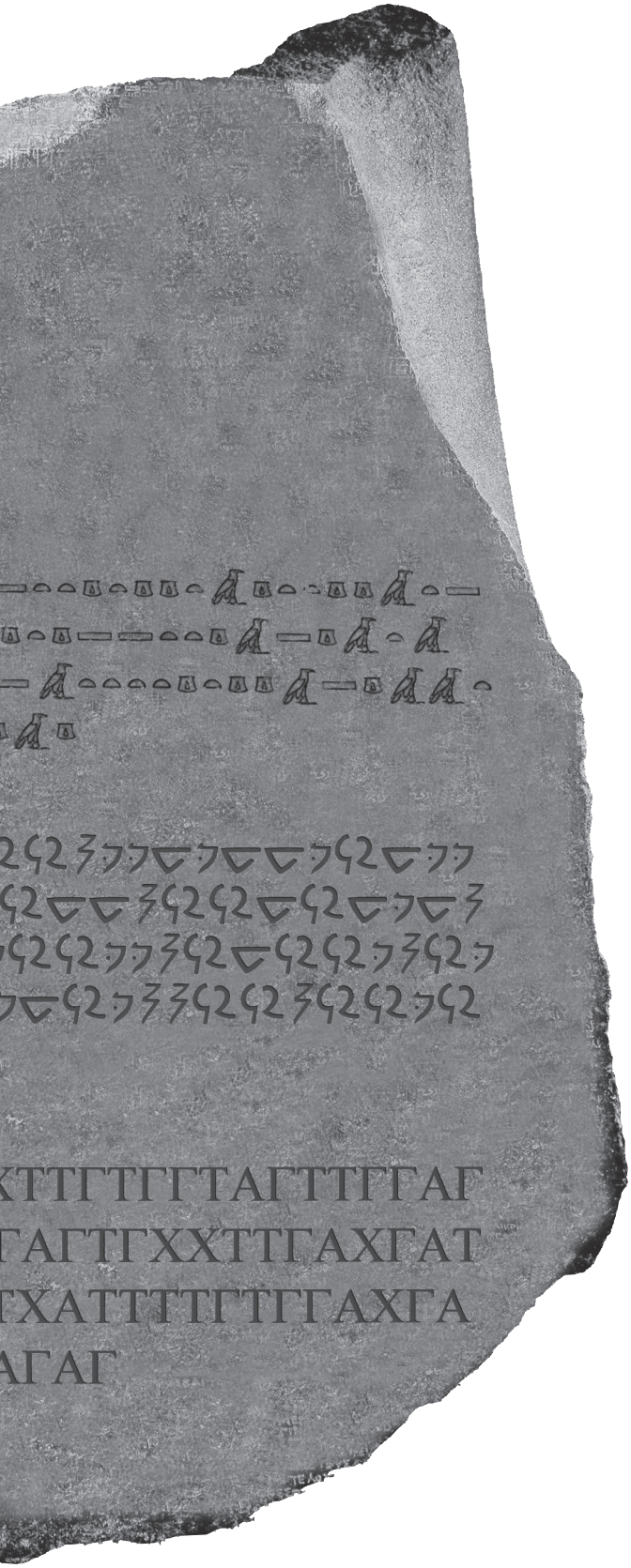

Chapter 8

Sensitization to apoptosis underlies $Kras^{D12}$-dependent oncolysis of murine C26 colorectal carcinoma cells by reovirus T3D

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Abstract

Reovirus T3D is an oncolytic agent that preferentially targets tumor cells expressing an activated RAS oncogene. RAS signaling interferes with the cellular stress response that inhibits translation of reovirus RNAs. Murine C26 colorectal carcinoma cells express a mutant $Kras^{D12}$ gene. Reovirus T3D efficiently kills C26 cells, but not C26 cells in which the $Kras^{D12}$ mRNA is stably repressed by expression of $Kras^{D12}$-directed short-hairpin RNAs. Surprisingly, neither reovirus T3D protein synthesis, nor T3D virus yields were suppressed by deletion of $Kras^{D12}$. Rather, reovirus-induced tumor-cell apoptosis was completely abrogated as a result of Kras knockdown. We conclude that sensitization of C26 tumor cells to reovirus-induced apoptosis underlies the RAS-dependency of reovirus T3D oncolysis.

Text

Reovirus T3D is a double-stranded RNA-containing virus belonging to the Reoviridae family. Fibroblasts transformed by an activated HRAS oncogene are highly sensitive to reoviral oncolysis [1-3]. In untransformed cells, viral RNA's stimulate a cellular defense mechanism by activation of double-stranded RNA-dependent protein kinase (PKR) [3]. Activated PKR prevents the translation of transcripts by inactivation of translation initiation factor 2α (eIF2α) through phosphorylation on ser51 [4,5]. RAS signaling interferes with this defense mechanism by inhibiting virus-induced PKR activation, thereby allowing reovirus replication [3]. Reovirus infections are non-pathogenic in immunocompetent adults, which makes this virus an interesting candidate for exploitation as an oncolytic agent [3,6-9].

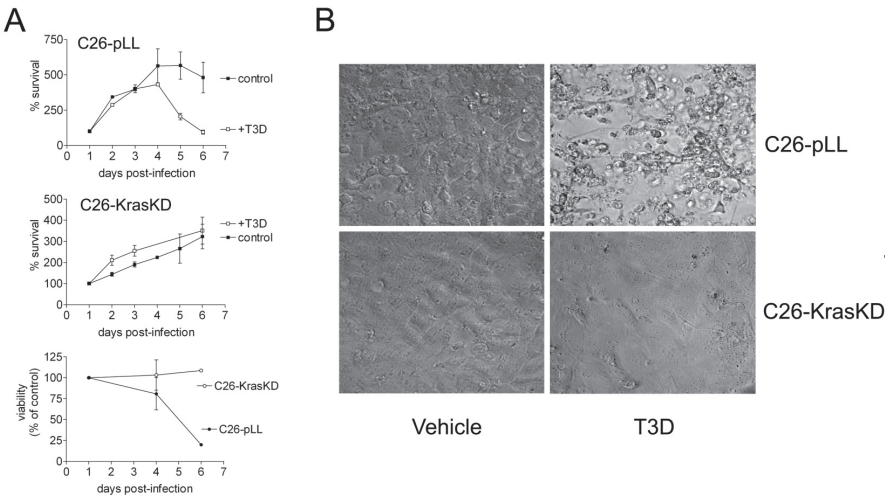

Figure 1. Kras^{D12} sensitizes C26 colorectal carcinoma cells to reovirus T3D. (A) Cell viability of C26 and C26-KrasKD cells was measured on 6 consecutive days following infection with reovirus T3D, using the MTT assay. (B) Light-microscopy images of C26 and C26-KrasKD cells infected with reovirus T3D, 5 days post infection.

C26 is an aggressive colorectal cancer (CRC) cell line that contains constitutively activated Kras due to an activating point mutation in codon 12 (G12D) [10]. The Nras and Hras genes in this cell line do not contain activating mutations. We previously established cell lines in which the endogenous Kras^{D12} allele is stably suppressed by mutant-specific RNA interference, using a lentiviral vector (C26-KrasKD). As a control, we established cell lines transduced with the empty lentiviral pLL3.7 vector (C26-pLL). Efficient and specific knockdown of Kras was demonstrated by Western blot analysis for Kras and, as control, Nras [10]. These cell lines were used to

analyze the effect of Kras on C26 sensitivity to reovirus-induced oncolysis. Tumor cells (5000/well) were plated in a 96-wells plate and were immediately infected with reovirus T3D (25 pfu/cell). Tumor-cell viability was then analyzed for 6 consecutive days with standard 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assays. After an initial growth phase (1-3 days), reovirus-infected C26-pLL cells rapidly lost viability, resulting in near-complete cell death by day 6 (Fig. 1A). In contrast, C26-KrasKD cells were completely refractory to reovirus-induced cell death (Fig. 1A). Evaluation of cellular morphology by light microscopy clearly showed the cytopathic effect of reovirus T3D on C26-pLL cells, but not on C26-KrasKD cells (Fig. 1B).

We expected that C26-KrasKD cells were refractory to reovirus T3D infection due to inhibition of viral protein synthesis and replication [3]. To test this, $5x10^4$ C26-pLL and C26-KrasKD cells/well were plated in a 24-well plate and infected with reovirus T3D (25 pfu/cell) or control vehicle and were labeled for 4 hours with [^{35}S]-methionine 5-days post-infection. Surprisingly, the production of reoviral proteins could be clearly demonstrated in both C26-pLL and C26-KrasKD cells (Fig. 2A). However, cellular protein synthesis was detectable only in C26-KrasKD cells, and had been completely shut off in C26-pLL cells (Fig. 2A). PKR-mediated inhibition of cellular protein synthesis is part of the immediate integrated stress response by which cells respond to virus infections, oxidative stress, ER stress or amino acid deprivation [3,11]. We have not been able to detect an inhibition of cellular or viral protein synthesis in C26 cells nor in C26-KrasKD cells at 4, 8, 24 and 48 hours post-infection, by performing 1-hour [^{35}S]-methionine-labeling experiments (not shown). Rather, inhibition of cellular protein synthesis in reovirus-infected C26-pLL cells is evident only 5 and 6 days post infection when primarily viral RNA's are transcribed (Fig. 2A). Total abrogation of protein synthesis (both viral and cellular) reflecting massive cell death usually occurs 1-2 days later. Our study is in apparent contradiction with published studies showing that ectopic expression of exogenous $HRAS^{V12}$ in fibroblasts promotes reovirus protein synthesis [3,7,12]. A possible explanation for this discrepancy may be that the strength and the selection of signaling pathways, which are activated by endogenous *versus* overexpressed exogenous RAS genes, may be different. Indeed, the expression level of $KRAS^{V12}$ is a highly critical parameter in determining cellular responses to RAS expression [13]. In this respect it is worth mentioning that our results show for the first time that the knockdown of an *endogenous* Kras oncogene abrogates cellular sensitivity to reovirus T3D.

Recently, it was published that the facilitation of reovirus replication by overexpressed $HRAS^{V12}$ in fibroblasts depends on the activity of p38 MAPK [12]. However, several studies show that cancer cell lines harboring an endogenous mutant KRAS allele display very low to undetectable levels of constitutive p38 activity [14-16]. Furthermore, p38 activity in human colorectal cancer cells was not affected by $KRAS^{D13}$ deletion or by $HRAS^{V12}$ overexpression [14]. Therefore, differential signaling to p38 by overexpressed $HRAS^{V12}$ in fibroblasts and by endogenous $Kras^{D12}$ in tumor cells may explain the differential effects of these RAS genes on reovirus replication.

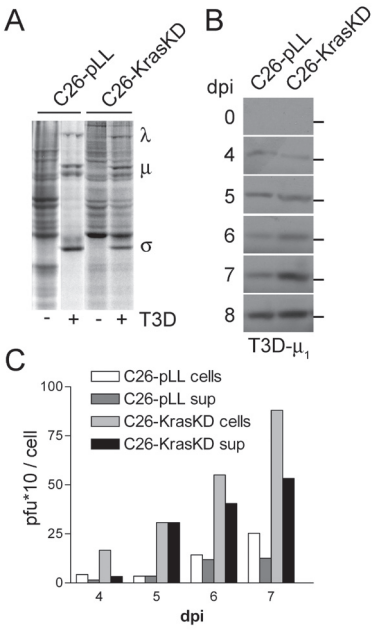

Figure 2. Kras^{D12} knockdown does not affect reovirus protein synthesis or virus propagation. (A) C26 and C26-KrasKD cells were infected with reovirus T3D and were labeled with [^{35}S]-methionine for 4hrs, 5 days post infection. Total cell extracts were analyzed by gel electrophoresis and subsequent autoradiography. (B) C26 and C26-KrasKD cells were infected with reovirus T3D. Cell extracts were prepared on the indicated days post infection (dpi) and were tested for the presence of the reovirus μ_1 protein by Western blot analysis. – indicates the 75 kDa marker. (C) C26 and C26-KrasKD cells were infected with reovirus T3D. Medium samples (sup) were subsequently analyzed on dpi 4, 5, 6 and 7. The cells were counted and cell extracts were prepared by freeze-thawing. The number of infectious virus particles (plaque-forming units (pfu)) in both fractions was determined using 911 cells [25].

Next, we assessed whether Kras knockdown affects reovirus T3D propagation in C26 cells. To this end we infected C26-pLL and C26-KrasKD cells with reovirus T3D (25 pfu/cell) and analyzed reovirus protein synthesis as well as the number of infectious particles produced by both cell types over time. We made use of a polyclonal antibody raised against UV-inactivated reovirus T3D in rabbits. This antibody primarily recognizes the major structural μ_1 protein with an apparent molecular weight of 76 kDa. Figure 2B shows that both C26-pLL and C26-KrasKD cells synthesize μ_1 following infection with T3D. In addition, infectious particles were assayed both in the medium and in freeze-thawed lysates from cell populations. To determine the amount of reovirus released by the cells, the medium was carefully harvested from the cultures. The cell debris was pelleted by low-speed centrifugation

(2 min., 250g, at room temperature). The reovirus in the supernatant was quantified by plaque assays on 911 cells. To determine the quantity of cell-associated reovirus, the medium was carefully removed. Subsequently, the remaining adherent cells were detached in 100 μl fresh medium by tapping the dish and by triturating using a small-volume pipette. The cell suspension was removed from the dish and added to the pellet fraction obtained after centrifugation of the conditioned medium. The cells were resuspended and lysed by three cycles of freeze-thawing. Subsequently the lysate was added to a new tube and spun at 1600 g for 10 min. The amount of infectious T3D particles in the supernatant was then assessed by plaque assays on 911 cells. Figure 2C shows that reovirus T3D is efficiently propagated in and released from C26-pLL as well as C26-KrasKD cells. In fact, virus propagation was more pronounced in the T3D-resistant C26-KrasKD cells (Fig. 2B,C). Taken together, the results show that C26-KrasKD cells can support reovirus T3D replication and release without overt cytopathic effects.

Reovirus T3D causes cell death by inducing apoptosis [17]. As RAS may control apoptosis signaling either positively or negatively [18], we hypothesized that resistance to reovirus-induced apoptosis may underlie the differential sensitivity of C26-pLL and C26-KrasKD cells to reovirus-induced cell death. To test this, C26-pLL and C26-KrasKD cells were exposed to control vehicle or reovirus T3D (25 pfu/cell), and after 5 days the induction of apoptosis was analyzed by FACScan analysis of propidium iodide-stained cells, as well as by indirect immunofluorescence and Western analysis for activated caspase-3. The fraction of apoptotic C26-pLL cells (with sub-G1 DNA content) increased to 31% 5 days post-infection. In contrast, reovirus T3D infection had no effect on the fraction of C26-KrasKD cells with sub-G1 DNA content (Fig. 3A, right panel). In addition, immunofluorescence analyses showed that 18.2% of C26-pLL cells were positive for activated caspase-3, whereas only 2.6% of C26-KrasKD cells were positive for activated caspase-3 (Fig. 3A, left panel; C92-605, BD Biosciences PharMingen, and Fig. 3B). Furthermore, Western blot analysis showed that activated caspase-3 was readily detected in lysates of C26-pLL cells, but not in lysates of C26-KrasKD cells (Fig. 3C). Genomic DNA is cleaved during apoptosis, yielding single- and double-stranded DNA breaks with free 3'-OH termini that can be labeled with the TUNEL reaction. Figure 3B shows that 5 days following infection with reovirus T3D, 21% of the C26-pLL cells were TUNEL-positive whereas C26-KrasKD cells remained negative (*in situ* cell death detection kit; Roche Applied Science). The baculovirus protein p35 inhibits virus-induced apoptosis [19]. To determine the contribution of reovirus-induced apoptosis to the cytopathic effect in C26 cells we transduced C26 cells with a lentiviral vector carrying the p35-coding region, resulting in cell line C26-p35. C26-p35 and, as control, C26-pLL cells were subsequently infected with reovirus T3D (25 pfu/cell) and 5 days after infection cell viability was assessed by MTT assays. Whereas reovirus T3D infection reduced the viability of C26-pLL cells by 52%, the viability of p35-expressing C26 cells was unaffected (Fig. 3D). Reovirus T3D-induced apoptosis requires virus binding to sialic acid via the σ1 protein [20].

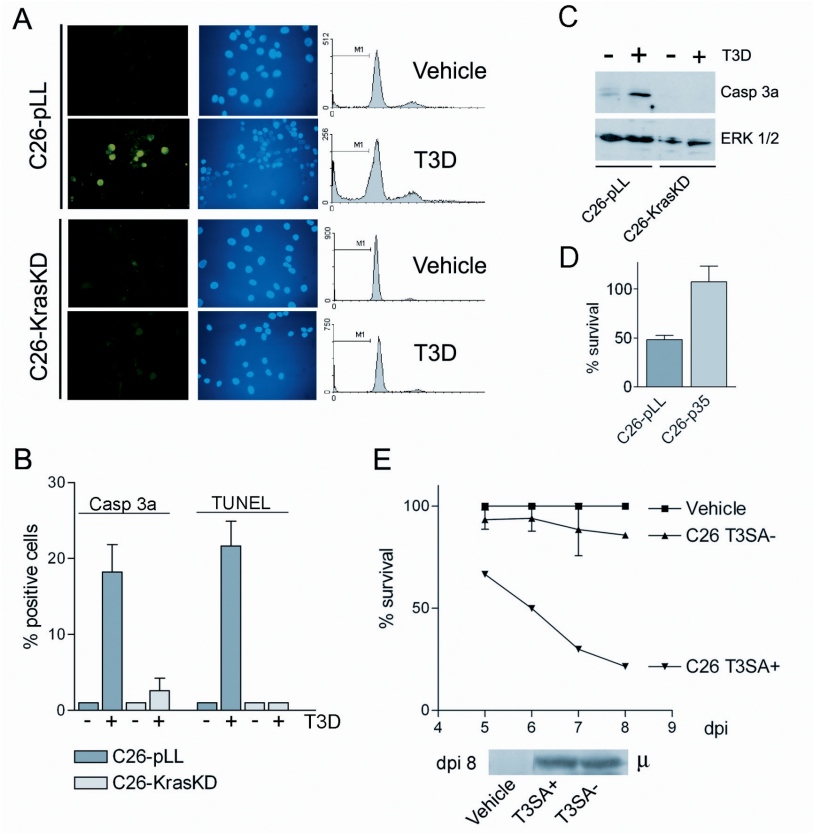

Figure 3. Kras^{D12} knockdown prevents apoptosis induction by reovirus. (A) C26-pLL and C26-KrasKD cells were infected with reovirus T3D (25 pfu/cell) and 5dpi they were analyzed by anti-active caspase-3 immunofluorescence (A, left panel and B), by FACScan analysis of propidium iodide-stained cells (A, right panel), by the TUNEL reaction (B), or by Western blot analysis for activated caspase-3 (C). (D) Baculovirus–p35-expressing cells and control cells were infected with reovirus T3D (25 pfu/cell) and cell viability was determined 5 dpi by the standard MTT assay. (E) C26-pLL cells were infected with reovirus T3SA- and T3SA+ (25 pfu/cell) and cell viability was measured at the indicated time points using the MTT assay. Synthesis of the viral μ_1 protein was analyzed by Western blotting 8 dpi as a measure for virus production.

Type 1 Lang (T1L) reovirus does not bind to sialic acid and does not induce apoptosis [21]. A reassortant virus strain carrying the T3D S1 gene in a T1L background (T3SA+) has gained the potential to bind sialic acid and to induce apoptosis [20]. In contrast, a reassortant T1L virus carrying a mutated (L204P) T3D S1 gene (T3SA-) fails to do so [20].

We compared T3SA+ and T3SA- for their capacity i) to induce oncolysis and ii) to replicate in C26 cells. We found that T3SA+ induced oncolysis with kinetics similar to that induced by T3D (Fig. 3E). The apoptosis-defective T3SA- mutant however, did not induce oncolysis of C26-pLL cells (Fig. 3E). Importantly, the amount of reovirus μ_1 protein produced at the end of the experiment (8 dpi) was similar for both T3SA+ and T3SA-, indicating comparable virus production (Fig. 3E). C26-KrasKD cells were completely resistant to either T3SA+ or T3SA- as they are to T3D (not shown and Fig. 1A).

Taken together, our results demonstrate an alternative mechanism for the selective sensitivity of tumor cells carrying a Kras oncogene to reovirus T3D: sensitization to reovirus-induced apoptosis.

There has been an increasing awareness that RAS, depending on the cell type and context, can induce either pro- or anti-apoptotic signaling [18]. Our results clearly show that the presence of $Kras^{D12}$ facilitates the induction of apoptosis by reovirus T3D. Reovirus-induced apoptosis in HEK293T and Hela cells is mediated by TNF-related apoptosis-inducing ligand (TRAIL) [22]. Furthermore, over-expression of $HRAS^{V12}$ sensitizes normal human fibroblasts and human embryonic kidney cells to TRAIL-induced apoptosis [23]. Therefore, RAS-dependent sensitization to TRAIL may underlie the differential susceptibility of C26 and C26-KrasKD cells to reovirus-induced apoptosis. However, treatment of C26-pLL and C26-KrasKD cells with recombinant TRAIL (up to 500 ng/ml; 24 hours) did not induce apoptosis in either cell line, whereas HCT 116 cells were efficiently killed (Fig. 4). The TRAIL batch used was highly active, as it induced HCT-116 apoptosis already at 100 ng/ml (not shown). Although TRAIL did not induce apoptosis in C26 or C26-KrasKD cells, it caused a marked increase in the %G1 in both C26 cells (from 40 to 58%) and in C26-KrasKD cells (from 52 to 68%). Recently, it was found that TRAIL reduced the proliferation of human T cell lines, possibly by suppressing cdk4 levels [24]. It is presently unknown whether this is a general phenomenon in TRAIL-resistant tumor cells. In conclusion, the ras-dependent susceptibility of C26 cells to reovirus-induced apoptosis is not accompanied by susceptibility to TRAIL. This suggests that TRAIL is not the only critical factor in reovirus-induced apoptosis, at least in the C26 cells studied here.

Whether an activated RAS pathway sensitizes transformed cells to reoviral oncolysis by interfering with cellular antiviral stress signaling or by facilitating apoptosis may be cell type-dependent. It is even conceivable that these pathways act in concert to allow tumor-specific oncolysis, although this was not observed in the present study. Taken together, our results support the feasibility of using reovirus T3D as an oncolytic agent for tumor cells carrying mutant Kras. Nonetheless, the mechanism underlying oncolytic selectivity may involve RAS-dependent sensitization of tumor cells to reovirus-induced apoptosis, rather than to facilitation of reovirus replication. This may have ramifications for the design of combination therapies involving reovirus T3D.

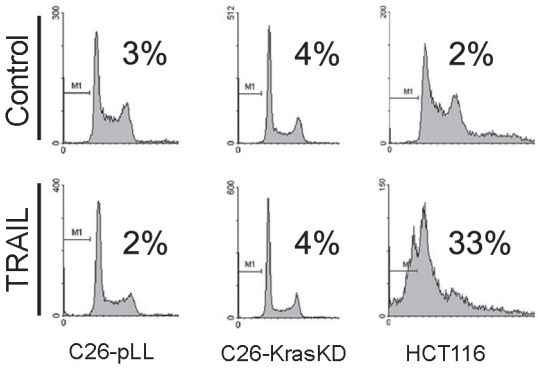

Figure 4. Resistance to TRAIL-induced apoptosis. Exponentially growing C26-pLL, C26-KrasKD and HCT116 cells were treated with recombinant TRAIL (500 ng/ml) for 24 hours. All detached and adherent cells were collected, and processed for FACScan analysis of DNA content using propidium iodide. WinMDI software was used to determine the % of apoptotic cells displaying subG1 content. Values represent means of triplicates.

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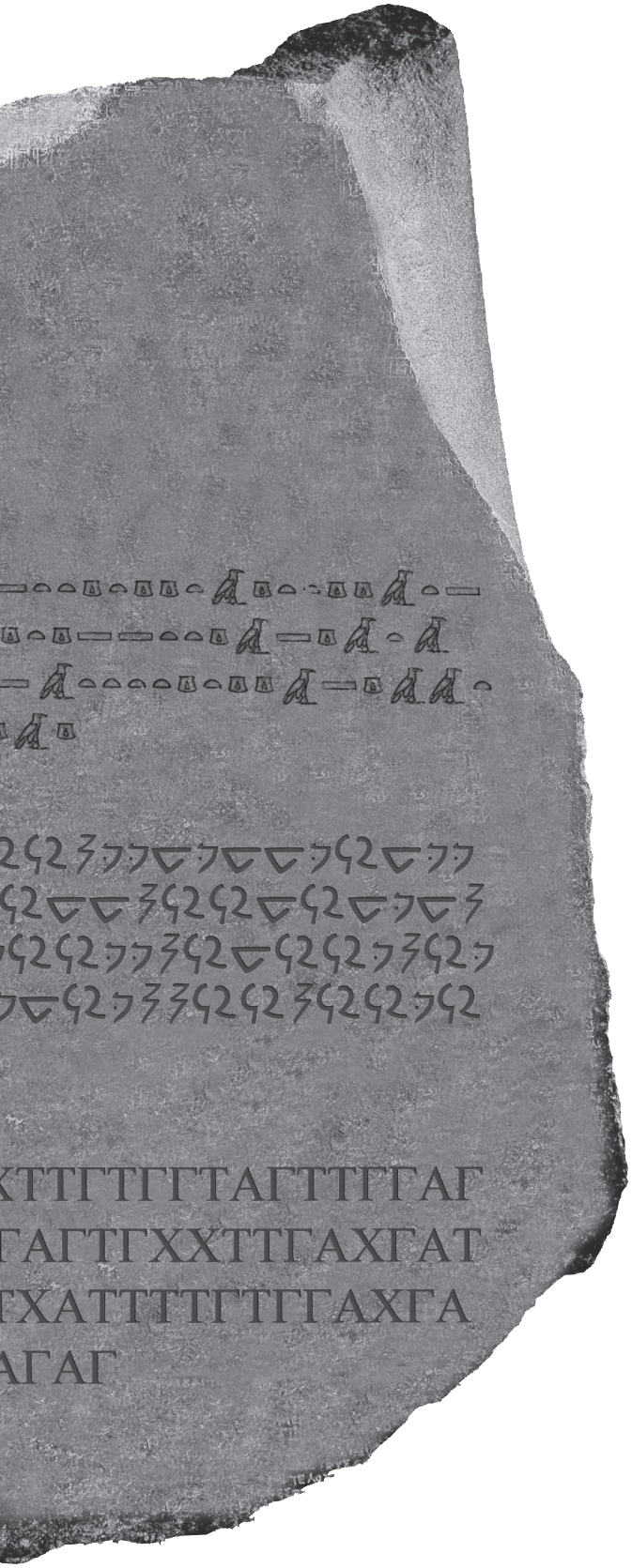

Chapter 9

KRAS^{D13} promotes apoptosis of human colorectal tumor cells by reovirus T3D and oxaliplatin, but not by TRAIL

Cancer Research, in press.

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Abstract

Colorectal tumors frequently contain activating mutations in KRAS. Reovirus T3D is an oncolytic virus that preferentially kills tumor cells with an activated RAS pathway. Here we have assessed the contribution of endogenous mutant KRAS in human colorectal cancer (CRC) cell lines to reovirus T3D replication and to tumor cell oncolysis. In addition, treatment combinations involving reovirus T3D, oxaliplatin and TNF-related apoptosis-inducing ligand (TRAIL) were tested for their efficacy in tumor cell killing. The mutation status of KRAS did not predict the sensitivity of a panel of human CRC cell lines to reovirus T3D. Virus replication was observed in all cell lines tested regardless of KRAS status and was not affected by deletion of endogenous mutant $KRAS^{D13}$. However, deletion of $KRAS^{D13}$ or p53 did reduce apoptosis induction by reovirus T3D, whereas deletion of β-$catenin^{\Delta S45}$ had no effect. Likewise, $KRAS^{D13}$- or p53-deficient cells display reduced sensitivity to oxaliplatin, but not to death receptor activation by TRAIL. Finally, the treatment of CRC cells with reovirus T3D combined with either oxaliplatin or TRAIL resulted in an additive, non-synergistic increase in tumor cell killing. We conclude that oncolysis of human tumor cells by reovirus T3D is not determined by the extent of virus replication but by their sensitivity to apoptosis induction. Oncogenic $KRAS^{D13}$ increases tumor cell sensitivity to activation of the cell-intrinsic apoptosis pathway without affecting reovirus T3D replication.

Introduction

In the search for alternative effective anti-cancer drugs that selectively target tumor cells over normal cells, an arsenal of oncolytic viruses has been identified [1]. Whereas most of these viruses have been genetically modified to attain tumor selectivity, some display inherent selectivity for transformed or tumor cells. One such virus is reovirus T3D that exploits signaling pathways activated by oncogenic RAS [2], the most frequently activated oncogene family in human cancer. Reovirus T3D-induced cell killing is greatly enhanced in cells overexpressing mutant HRAS^{V12} [2,3]. One of the tumor types that frequently (~35%) harbor activating mutations in the KRAS gene are colorectal carcinomas [4,5]. Colorectal tumors are the second leading cause of cancer-related deaths world-wide and response rates to conventional chemotherapeutics are low. Thus, reovirus T3D may be an attractive additive therapeutic in the treatment of CRC tumors.

Reovirus-infected cells contain double stranded viral RNA's that activate the cellular protein kinase R (PKR). Activated PKR phosphorylates the translation elongation factor 2α and thereby inhibits cellular and viral protein synthesis. Overexpression of HRAS^{V12} interferes with this anti-viral cellular defense mechanism and allows reovirus replication in otherwise non-permissive cells [3]. It has been suggested that RAS-mediated suppression of this defense mechanism underlies the selective oncolysis of human tumor cells harboring mutant KRAS or an 'activated RAS pathway' [3]. However, we have recently shown that suppression of endogenous Kras^{D12} in mouse colorectal carcinoma cells had no effect on reovirus protein synthesis or on virus replication but abrogated reovirus T3D-induced tumor cell apoptosis [6]. Activation of the apoptotic program in virus-infected cells is a natural defense mechanism that limits viral spread in the infected host [7]. Alternatively, apoptosis of infected cells may facilitate the release of virus progeny and subsequent dissemination [7]. From a therapeutic point of view, the apoptosis-inducing strains of reovirus may be regarded as self-amplifying inducers of tumor cell apoptosis with limited pathogenic side effects. If reovirus T3D is to be used as an additional therapeutic agent in the treatment of human colorectal cancer, it is imperative that the mechanisms underlying KRAS-dependent human tumor cell killing are fully understood. In the current report we have investigated how endogenous mutant KRAS in human colorectal carcinoma cells affects reovirus T3D protein synthesis, reovirus T3D replication and cellular sensitivity to reovirus T3D-induced oncolysis.

Materials and Methods

Cell lines and culture conditions

The colorectal cancer cell lines HT29, DLD1, SW480, HCT15, and LS174T were purchased from ATTC. The HCT116 cell line and its isogeneic derivatives lacking β-catenin$^{\Delta S45}$ or p53 were kindly provided by Prof. Bert Vogelstein and were described before [8,9]. The HCT116 cells lacking KRAS^{D13} (Hkh2) with their own HCT116 control cells were obtained from Dr. Shirasawa and were also described before [10]. All cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and antibiotics in a 5% CO_2-humidified atmosphere.

Materials

Oxaliplatin was obtained from Sanofi-Aventis (Bridgewater, NJ, USA). A bacterial expression vector encoding His-tagged TRAIL (pETdwHisTRAIL114-281) was kindly provided by Dr. Dai-Wu Seol. TRAIL was produced in bacteria and was purified on a Ni^{2+}-agarose column.

Reovirus T3D stock

Reovirus T3D was purchased from ATCC (VR-824). The virus stock was prepared using 911 cells [11]. Cells were infected with reovirus T3D (5 pfu/cell) in DMEM/2% FCS. After initial infection (2 hr at 37°C, 5 % CO2) the medium was replaced by normal DMEM containing 10% FCS. The virus was harvested after 48h by resuspending the cells in PBS with 2% FCS at a density of approx. 10^9 cells/ml and subsequent freeze-thawing for three cycles. The lysates were cleared by centrifugation at 2000 rpm in a tablecentrifuge for 10 minutes. The virus was purified by layering the supernatant on a CsCl gradient as described for purification of adenovirus particles [11]. To remove the CsCl, reovirus was dialysed against reovirus T3D storage buffer (10 mM Tris-HCl, 150 mM NaCl, 10 mM $MgCl_2$.6H2O). The final dialysis was performed in reovirus T3D storage buffer containing 5% sucrose. Virusstocks were stored at -80°C and the concentration was determined by performing standard plaque assays on 911 cells [11].

^{35}S methionine labeling

Infected or mock-infected cells were labeled with Redivue ^{35}S-methionine Pro-mix (200 μCi/ml; Amersham) for 4 hours 3 days post-infection. Cells were washed with PBS once, and total cell extracts were prepared in sample buffer and were run out on long 45 cm SDS-polyacrylamide gels. Gels were then dried and exposed to radiographic film.

RAS assay

The RAS-binding domain of Raf fused to glutathione-S-transferase (GST) and coupled to glutathione-sepharose was used as an affinity matrix for activated RAS. The assay was performed exactly as described [12].

Viability assay

Cells were plated at a density of 5000 cells/well in 96-well plates. Cell viability of infected and mock-infected cells was then analyzed for 6 consecutive days by standard 3-(4,5dimethylthiazolyl-2)-2,5-diphenyltetrazoleumbromide (MTT) assays (Roche Diagnostics) according to the manufacturer's instructions.

Results

The sensitivity of colorectal cancer cells to reovirus T3D does not correlate with the presence of active KRAS

The notion that reovirus replication and oncolysis of tumor cells depends on RAS signaling is largely based on the use of non-epithelial cells overexpressing mutant RAS or growth factor receptors [3,13]. Therefore, we first assessed whether the sensitivity of human colorectal carcinoma cells to reovirus T3D correlates with the presence of endogenous active KRAS. The mutation status of the KRAS and BRAF genes in a panel of human colorectal cancer cell lines was analyzed by PCR and sequence analysis and was in line with previous analyses in all cases. Next, KRAS activity was measured in the same panel of serum-starved cell lines. High levels of constitutive KRAS activity were detected in LS174T (KRAS^{G12D}), DLD1 (KRAS^{G13D}), SW480 (KRAS^{G12V}) and HCT116 (KRAS^{G13D}) cells (Fig. 1*A*). HCT15 cells also harbor a KRAS^{G13D} allele, but constitutive KRAS activity was markedly lower than in the other cell lines expressing mutant KRAS (Fig. 1*A*). HT29 cells, with 2 wild type KRAS alleles (but carrying BRAF^{V600E}) displayed the lowest level of constitutive KRAS activity, as expected (Fig. 1*A*). None of the cell lines harbored mutations in the NRAS or HRAS genes at codons 12, 13 or 61 (not shown) and mutant BRAF was only found in HT29.

Next, we assessed whether constitutive KRAS activity would correlate with cellular sensitivity to oncolysis by reovirus T3D. All cell lines were infected with reovirus T3D (20 pfu/cell) and the relative number of viable cells was assessed over a period of 6 consecutive days. All cell lines, except LS174T, showed a reduction in cell viability following infection with reovirus T3D. However, the sensitivity to oncolysis varied considerably between the cell lines tested, from LS174T cells being completely resistant, to HT29, HCT15, DLD1 and SW480 showing intermediate sensitivity, and HCT116 cells being extremely sensitive (Fig. 1*BC*). Surprisingly, neither the level of active KRAS protein nor the presence of mutant KRAS alleles correlated with tumor cell sensitivity to reovirus T3D-induced oncolysis (Fig. 1*A-C*).

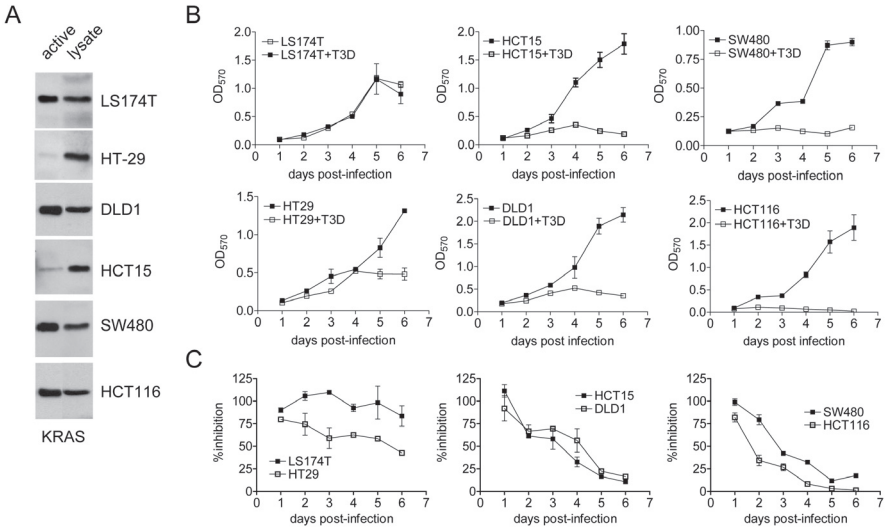

Figure 1. KRAS activity does not correlate with cellular sensitivity to reovirus T3D. A, LS174T, HT29, DLD1, HCT15, SW480 and HCT116 cells were serum-starved overnight and KRAS activity was assessed by the RAS activity assay. B, The same cell lines were seeded in 96-well plates (5000/well) and were subsequently infected with reovirus T3D at an moi of 20 pfu/cell, or were mock infected using reovirus T3D storage buffer. The relative number of viable cells was then assessed over time by standard MTT assays. C. The reduction in cell viability induced by reovirus T3D over time is plotted relative to untreated control cells.

The sensitivity of colorectal cancer cells to reovirus T3D does not correlate with virus replication

We next tested whether reovirus replication correlated with either the presence of mutant KRAS or with tumor cell killing. Cells were infected with reovirus T3D and were labeled with [^{35}S]-methionine for 4 hours, 3 days following infection. Cell extracts of infected and uninfected cells were then analyzed by SDS-PAGE. Reovirus T3D replicated in all cell lines examined regardless of KRAS activity and regardless of their sensitivity to oncolysis. The most extreme example is the cell line LS174T which was completely resistant to reovirus-induced cell killing while supporting extensive virus replication (Fig. 2). Thus, reovirus T3D replication can occur in the absence of active KRAS (HT29) and does not necessarily result in the induction of an overt cytopathic effect (LS174T).

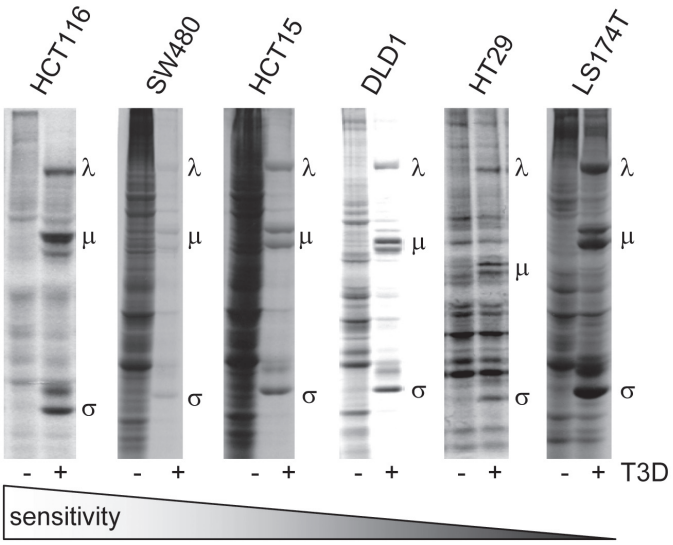

Figure 2. Reovirus T3D replication does not correlate with cellular sensitivity to reovirus T3D. A, HCT116, SW480, HCT15, DLD1, HT29 and LS174T cells were seeded in 24 well plates and were infected with reovirus T3D (20pfu/cell), or were mock-infected using reovirus T3D storage buffer. Three days after infection, cells were labeled with 35S-methionine and cell lysates were analyzed for reovirus T3D replication. The positions of the reovirus λ, μ and σ proteins are indicated. All cell lines sustained virus replication, irrespective of KRAS mutation status or KRAS protein activity.

KRAS^{D13} deletion does not affect T3D replication but reduces cellular sensitivity to oncolysis

To further assess the correlation between mutant KRAS, reovirus T3D replication, and sensitivity to oncolysis, we used the HCT116 cell line and its isogeneic derivative Hkh2 from which the KRAS^{G13D} allele has been deleted through homologous recombination [10]. We first tested KRAS activity in HCT116 and Hkh2 cells and found that KRAS was constitutively active in HCT116 but not in Hkh2 cells, as expected (Fig. 3*A*). HCT116 and Hkh2 cells were then infected with reovirus T3D and viral protein synthesis was examined 24 and 48 hours post infection. Figure 3*B* shows that KRAS^{D13} deletion had no effect on reovirus T3D protein synthesis. We next assessed whether KRAS deletion would affect reovirus T3D propagation. HCT116 and Hkh2 cells were infected and viral progeny was quantified 4 days post infection. Hkh2 cells produced approximately 50-fold more virus than HCT116 cells (Fig. 3*C*). Finally, we assessed the capacity of reovirus T3D to induce oncolysis in both cell lines. Cell viability was assessed for 4 consecutive days following infection. We found, as above, that HCT116 cells were very sensitive to oncolysis by reovirus

T3D as viability rapidly dropped between 2 and 3 days post infection (Fig. 3*D*). Hkh2 cells however, were more resistant and viability dropped approximately 24 hours later, between 3 and 4 days post-infection. Importantly, the delay in oncolysis was highly reproducible and was not the result of delayed viral protein synthesis or virus production (Fig. 3*B,C*). The delay in oncolysis provides a possible explanation for the fact that KRAS-deleted cells produce 50-fold more infectious reovirus T3D particles. By inference, KRAS-deleted Hkh2 cells loose their viability when exposed to a much higher dose of reovirus T3D than the HCT116 cells as infectious virus particles are continuously being produced during the course of the experiment.

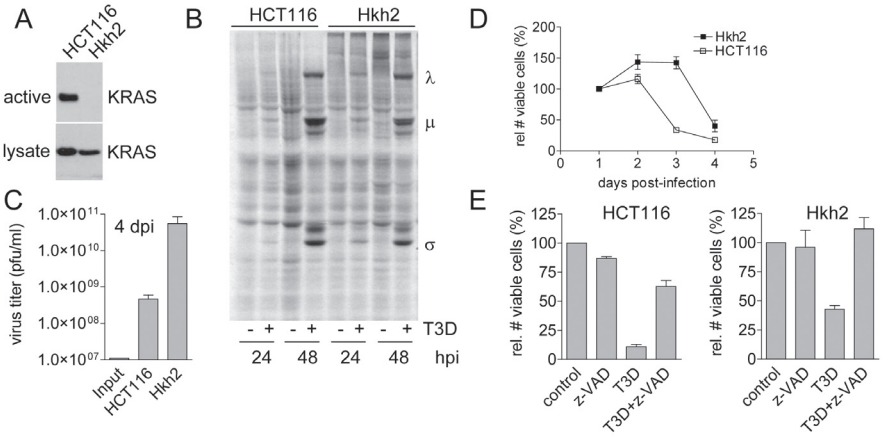

Figure 3. Deletion of KRAS^{D13} reduces oncolysis but not virus replication. A, HCT116 cells and Hkh2 cells were serum-starved and KRAS activity was assessed by the RAS activity assay. B, HCT116 cells and Hkh2 cells were infected with reovirus T3D, or were mock-infected and viral protein synthesis was assessed 24 and 48 hours after infection. C, HCT116 cells and Hkh2 cells were infected with reovirus T3D, or were mock-infected. Four days after infection, when most cells in both cultures have died, the virus yield was assessed by plaque assays on 911 cells. D, HCT116 cells and Hkh2 cells were infected with reovirus T3D or were mock-infected, and the relative number of viable cells was then assessed over time by standard MTT assays. E, HCT116 cells and Hkh2 cells were pre-treated for 2 hours with z-VAD (100μM) or solvent (DMSO) for 2 hours prior to infection with reovirus T3D or mock-infection. Two days after infection cell viability was assessed as above and this is expressed as % viability relative to mock-infected, DMSO-treated control cells.

The cytopathic effect of reovirus on susceptible cells and tissues is due to apoptosis induction [7]. Therefore, we ascertained the apoptotic nature of reovirus T3D-induced HCT116 and Hkh2 cell death. To this end HCT116 and Hkh2 cells were incubated with the pan-caspase-inhibitor z-VAD, a widely used inhibitor of apoptotic but not necrotic cell death or virus replication. Figure 3*E* shows that, as expected, z-VAD prevented oncolysis of both HCT116 and Hkh2 cells by reovirus.

From the above experiments we conclude that KRAS promotes reovirus T3D-induced tumor cell apoptosis, but does not facilitate virus replication. These results argue against a model in which activated KRAS stimulates human tumor cell oncolysis by facilitating virus replication.

KRAS^{D13} and p53 but not β-catenin$^{\Delta S45}$ modulate tumor cell sensitivity to reovirus T3D oncolysis

Multiple genetic changes underlie the progressive development of colorectal carcinomas. These include activating mutations in KRAS, deregulated Wnt signaling (either by inactivation of the tumor suppressor APC, through oncogenic activation of β-catenin, or through epigenetic silencing of SFRP genes) and loss of the tumor suppressor p53 [14]. HCT116 cells not only express oncogenic KRAS^{D13}, but also oncogenic β-catenin$^{\Delta S45}$ and wild type p53 [8,9]. To assess the possible contribution of p53 and mutant β-catenin to CRC cell killing by reovirus T3D we used HCT116-derived cell lines in which either mutant β-catenin$^{\Delta S45}$ or p53 were deleted through homologous recombination [8,9]. Each mutant cell line was compared to the control HCT116 cell line obtained from the same laboratory. All cell lines were infected with reovirus T3D and viral protein synthesis and sensitivity to oncolysis were assessed as above. Virus replication was not affected in any of the mutant cell lines (Fig. 4*A*). However, deletion of p53, like deletion of KRAS^{D13}, but not deletion of β-catenin$^{\Delta S45}$ delayed tumor cell killing by reovirus T3D by approximately 24 hours (Fig. 4*B*). Thus, neither KRAS^{D13}, nor β-catenin$^{\Delta S45}$, nor p53 affect reovirus T3D replication in human HCT116 CRC cells. Tumor cell oncolysis through apoptosis induction however, is stimulated by KRAS^{D13} and p53 but not by β-catenin$^{\Delta S45}$.

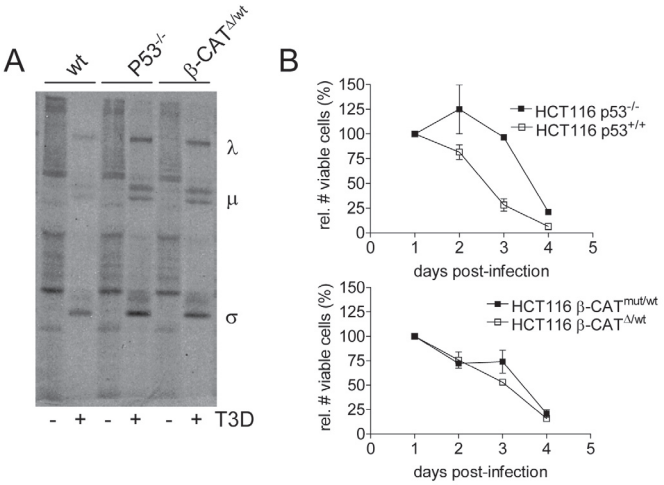

Figure 4. Deletion of p53, but not *β*-catenin$^{\Delta S45}$ reduces reovirus T3D-induced oncolysis but not virus replication. A, HCT116 cells and isogeneic derivatives lacking either p53 or *β*-catenin$^{\Delta S45}$ were infected with reovirus T3D or mock-infected and viral protein synthesis was assessed 48 hours after infection. All cell lines sustained virus replication, irrespective of p53 or *β*-catenin status. B, HCT116 cells and isogeneic derivatives lacking either p53 or *β*-catenin$^{\Delta S45}$ were infected with reovirus T3D or mock-infected and the relative number of viable cells was then assessed over time by standard MTT assays as above.

KRAS^{D13} deletion reduces CRC cell sensitivity to oxaliplatin, but not to TRAIL.

p53-deleted HCT116 cells are not only relatively resistant to reovirus T3D (this report), but also to standard chemotherapeutics used in the adjuvant treatment of colorectal cancer: 5-FU and oxaliplatin [8,15]. Furthermore, it has recently been shown that KRAS-deleted HCT116 cells are relatively resistant to 5-FU [16]. Therefore, we tested whether KRAS^{D13} deletion affected HCT116 cell sensitivity to oxaliplatin. Figure 5*A* (upper panel) shows that KRAS-deleted HCT116 cells show reduced sensitivity to oxaliplatin when compared to HCT116 cells. Thus, KRAS-deletion reduces cellular sensitivity to genotoxic stress induced by both 5-FU and oxaliplatin. Apoptosis may ensue from activation of the intrinsic cascade (in response to genotoxic stress) or via activation of death receptor ligands (extrinsic cascade). Reovirus-induced apoptosis may require both pathways [17-19]. We tested whether KRAS deletion affected apoptosis induction by TNF-related apoptosis-inducing ligand (TRAIL) a ligand for death receptor 4/5 that induces apoptosis in several human cancer cell lines. Figure 5*A* (lower panel) shows that KRAS-deleted cells are not resistant to TRAIL-induced apoptosis. In fact, we consistently observed that KRAS-deleted cells were slightly more sensitive to TRAIL-induced apoptosis than the control HCT116 cells.

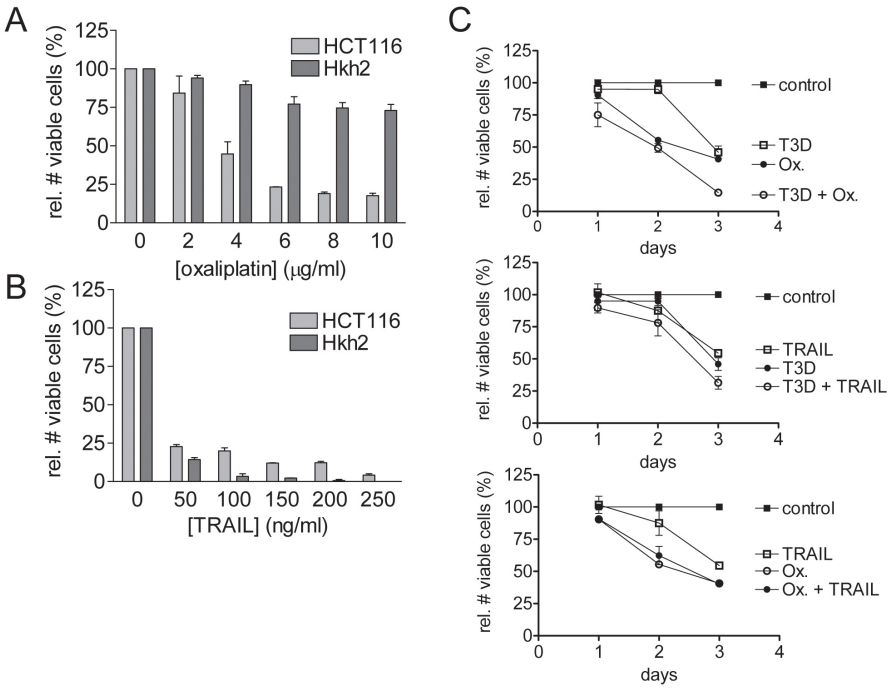

Figure 5. Deletion of KRAS^{D13} reduces oncolysis by oxaliplatin but not by TRAIL. HCT116 cells and Hkh2 cells (lacking KRAS^{D13}) were infected with reovirus T3D or mock-infected and were treated with A, oxaliplatin or B, TRAIL at the indicated concentrations for 2 days. Cell viability was then assessed by standard MTT assays after 2 days as above. C, HCT116 cells were treated with combinations of reovirus T3D (10pfu/cell), oxaliplatin (2 μg/ml) and TRAIL (25 ng/ml) and cell viability was assessed over time.

Simultaneous activation of the intrinsic and extrinsic pathways of apoptosis induction may lead to synergistic tumor cell killing. Therefore, we investigated how combinations of reovirus T3D, oxaliplatin and TRAIL at suboptimal concentrations would affect HCT116 cell viability. Figure 5*C* shows that the combination of reovirus T3D with either oxaliplatin or with TRAIL was more effective in killing HCT116 cells than either compound alone. However, in both cases this effect was additive rather than synergistic. Similarly, HCT116 cell treatment with oxaliplatin and TRAIL failed to kill HCT116 cells in a synergistic fashion. It was recently reported that oxaliplatin also fails to synergize with FasL in the induction of apoptosis in HCT116 cells [20].

Taken together, we propose that mutant KRAS promotes the induction of apoptosis in reovirus T3D-infected tumor cells by sensitizing these cells to activation of the intrinsic apoptosis cascade that is known to be associated with reovirus T3D infection [18,19]. Our results do not support a role for KRAS in promoting reovirus T3D replication.

Discussion

It has been proposed that RAS-stimulated reovirus T3D replication underlies the KRAS-dependency of human tumor cell killing by reovirus [3]. Although our work subscribes the view that reovirus T3D oncolysis of tumor cells is facilitated by endogenous mutant KRAS, we propose that a different mechanism underlies this selectivity. We have recently shown that suppression of endogenous mutant $Kras^{G12D}$ in murine colorectal cancer cells does not affect reovirus T3D replication but abrogates reovirus T3D-induced apoptosis [6]. In the present report we show that reovirus T3D can infect and replicate in all human CRC cell lines tested, but that this does not correlate with either RAS/BRAF status or with tumor cell killing. The relative importance of mutant KRAS in determining the sensitivity of human tumor cells to reovirus T3D is very much dependent on the specific tumor cell under study. We find that although endogenous mutant KRAS can modulate the sensitivity of human tumor cells to reovirus T3D-induced apoptosis it is certainly not the only and prime determinant. Additional factors, including p53, clearly play a role in determining cellular sensitivity to oncolysis. It may therefore be expected that the response of individual colorectal tumors to reovirus T3D as a therapeutic drug will show considerable variation, regardless of KRAS status. The safety and efficacy of reovirus T3D therapy in the treatment of human cancer is currently being evaluated in clinical trials [21].

Oxaliplatin and 5-FU are standard chemotherapeutics in the treatment of metastatic colorectal cancer [22-25]. The sensitization of human colorectal tumor cells to reovirus T3D by mutant KRAS was accompanied by a similar sensitization to oxaliplatin, which kills tumor cells by inducing genotoxic stress and subsequent apoptosis induction. Apoptosis induction by oxaliplatin is accompanied by activation of p53 and is reduced in HCT116 cells from which the genes encoding either p53 or the pro-apoptotic bcl2 family member Bax are deleted [15,26]. Thus, oxaliplatin-induced apoptosis occurs through activation of the intrinsic mitochondria-dependent apoptosis pathway. Likewise, reovirus T3D-induced apoptosis involves activation of the mitochondrial pathway [19,27], and is reduced in HCT116 cells from which p53 is deleted (this study). Reovirus T3D-induced apoptosis also requires activation of a death receptor-dependent (extrinsic) pathway that is activated by TRAIL [17,18]. In the present study the reduction in reovirus T3D-induced apoptosis in KRAS-deleted cells was accompanied by a similar reduction in oxaliplatin- but not TRAIL-induced apoptosis. Therefore, a general sensitization to apoptosis induction via the intrinsic apoptosis pathway may underlie the KRAS-dependency of tumor cell oncolysis by reovirus T3D. Interestingly, reovirus T3D-induced apoptosis is associated with decreased expression of DNA repair enzymes, which may promote apoptosis induction through accumulation of DNA damage and activation of the intrinsic pathway of apoptosis induction [28].

Combination of compounds that induce genotoxic stress (such as oxaliplatin) with those that activate death receptors (such as TRAIL) could theoretically generate synergistic anti-tumor responses. It has previously been demonstrated that reovirus T3D can synergize with TRAIL in the induction of apoptosis in adenovirus-transformed human embryonic kidney (293) cells [17,29]. In the present study we have not been able to demonstrate synergistic colorectal tumor cell killing by combining reovirus T3D with either oxaliplatin or with TRAIL. A possible explanation for the lack of synergy could be that reovirus T3D alone already activates both the intrinsic and the extrinsic pathways that lead to apoptosis induction. Future studies should assess whether or not reovirus T3D therapy has added value in the combinatorial treatment of colorectal tumors and whether tumor responses depend on the presence of mutant KRAS.

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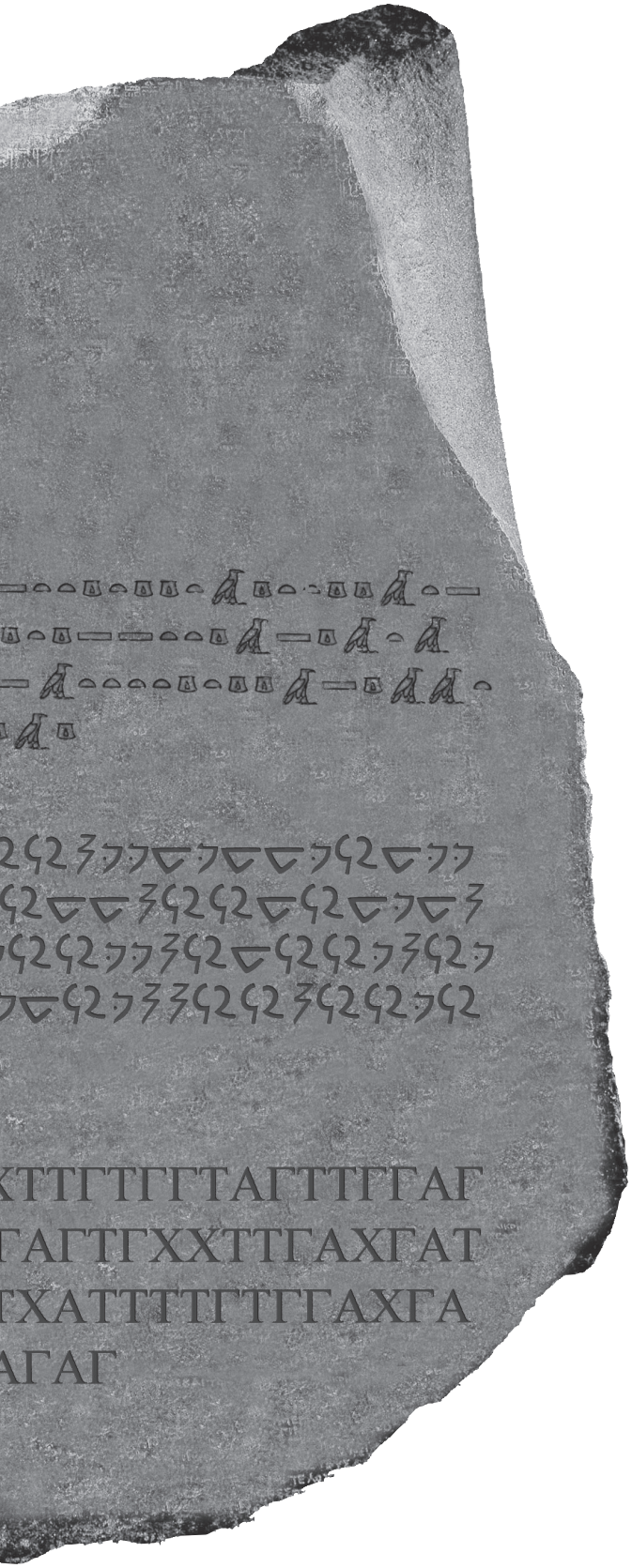

Chapter 10

Immunosuppression promotes reovirus therapy of colorectal liver metastases

Cancer Gene Therapy, in press.

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Abstract

Mortality due to colorectal cancer (CRC) is high and is associated with the development of liver metastases. Approximately 40% of human CRCs harbor an activating mutation in the KRAS oncogene. Tumor cells with activated KRAS are particularly sensitive to reovirus T3D, a non-pathogenic oncolytic virus. The efficacy of virus-based therapies may be positively or negatively modulated by the host immune system. This study was designed to assess the effect of immunosuppression on reovirus T3D oncolysis of established colorectal micrometastases in the liver. Mouse C26 CRC cells harbor a mutant Kras gene and are susceptible to Kras-dependent oncolysis by reovirus T3D *in vitro*. Isolated C26 liver tumors were established in syngenic immunocompetent BALB/c mice by intrahepatic injection. reovirus T3D therapy was given as a single intratumoral injection in control mice and in cyclosporin A-treated immunosuppressed mice. Tumor growth was analyzed over time by non-invasive bioluminescence imaging. The outgrowth of established CRC liver metastases in immunocompetent mice was efficiently but temporarily inhibited with a single injection of reovirus T3D. Immunosuppression with cyclosporin A markedly increased and prolonged the therapeutic effect and allowed complete reovirus T3D-induced tumor eradication in a subpopulation of the mice. We conclude that reovirus T3D is an effective therapeutic agent against established C26 colorectal liver metastases and that immunosuppression enhances treatment efficacy.

Introduction

Colorectal carcinoma (CRC) is the second most common cancer in the western world. Despite multiple therapeutic options, mortality is still high and is mainly due to the consequences of metastatic tumor growth in the liver. Therefore, effective novel therapeutics are urgently needed. The idea to use viruses as tumoricidal agents is relatively old, but clinical applications have been limited. Renewed interest was sparked by the finding that genetic defects within tumor cells can allow viruses to preferentially kill tumor cells over normal cells [1]. Reovirus T3D is a double-stranded RNA-containing virus that belongs to the Reoviridae family [2]. Reovirus T3D specifically kills (tumor) cells with activated RAS which may facilitate virus replication and stimulates virus-induced tumor cell apoptosis [2-4]. As approximately 40% of human CRCs harbor an activating mutation in the KRAS oncogene [5], reovirus T3D may be an attractive therapeutic agent against (metastatic) CRC. Reovirus has an excellent safety profile as infections in adults usually occur asymptomatically. Recently, several animal studies have demonstrated the efficacy of reovirus as an oncolytic agent for colon and ovarian tumors, intracranial gliomas, breast cancer and lymphoid malignancies [1,6]. Reovirus therapy in nonhuman primates is safe and well tolerated [6]. Furthermore, a phase I study demonstrated that reovirus therapy in patients with cutaneous metastases from systemic cancer was safe and several tumor responses were recorded [7].

When using oncolytic viruses in immunocompetent hosts, the role of the host immune system has to be taken into consideration. Immune-mediated virus clearance may reduce the therapeutic response. Indeed, immunosuppression by cyclosporin A or by T-cell depletion improved systemic reovirus therapy of experimental lung metastases in mice and prolonged survival [8]. Conversely, viral infection of established tumors may also lead to the generation of an anti-tumor immune response and to increased tumor clearance [1]. In addition, reovirus infections produce a robust inflammatory response in the liver [2] which may affect the therapeutic anti-tumor response.

Here we have analyzed the efficacy of reovirus T3D as an oncolytic agent against colorectal metastases in the liver and we assessed how immunosuppression affected the therapeutic response.

Materials and Methods

Cell lines and culture conditions

The murine colon carcinoma cell line C26 was obtained from the American Type Tissue Culture Collection (ATCC, Rockville, MD). C26 cells expressing the firefly luciferase gene (C26-luc) were described previously [9]. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Dulbecco, ICN Pharmaceuticals, Costa Mesa, CA) supplemented with 5% (v/v) fetal calf serum, 2 mM glutamine, 0.1 mg/ml streptomycin, and 100 U/ml penicillin. All cells were kept at 37°C in a humidified atmosphere containing 5% CO_2.

In vivo experiments

Isolated liver metastases were induced in BALB/c mice by injecting 10^5 C26-luc cells in 50 μl PBS into the parenchyma of the left liver lobe. 24 hours after injection of tumor cells the liver was exteriorized and at the exact sites of identifiable tumor growth, the tumor-bearing liver lobe was injected with 10^{11} pfu reovirus in 50 μl PBS or PBS alone as a control, and the abdomen was closed (n=9/group without CyA, n=16/group with CyA). All experiments were performed in accordance with the guidelines of the University's Animal Experimental Committee, University Medical Center Utrecht, the Netherlands.

Bioluminescence imaging (BLI)

Tumor outgrowth was measured non-invasively by bioluminescence imaging (BLI), exactly as described previously [9]. In brief, on days 5, 7, 9, 11 and 13 after tumor cell injection, hepatic tumor growth was assessed by *in vivo* BLI with a highly sensitive, cooled charge-coupled device (CCCD) camera (VersArray 1300B, Roper Scientific Inc., Vianen, The Netherlands) mounted in a light-tight imaging chamber (Roper Scientific Inc., Vianen, The Netherlands). Before imaging, mice were anesthetized and the substrate D-luciferin sodium salt (Synchem Laborgemeinschaft OHG, Kassel, Germany) dissolved in PBS was injected i.p. at a dose of 125 mg/kg. All mice were imaged with an integration time of 5 minutes, exactly 10 minutes after the i.p. injection of D-luciferin. Imaging and quantification of signals were controlled by the acquisition software MetaVue (Universal Imaging Corporation, Downingtown, PA). Total photon counts were quantified with MetaMorph software.

Immunosuppression

The host immune system was suppressed preoperatively and during the course of the experiment by i.p. injections of cyclosporin A (Novartis, Arnhem, The Netherlands) at 50mg/kg on pod -2, 0, 2, 4, 6, 8, 10 and 12.

Statistical Analyses

Differences between the groups were statistically evaluated using the Mann-Whitney test. Results are presented as means ± SE. All *P* values were two tailed. $P < 0.05$ was considered statistically significant.

Results and discussion

Reovirus T3D selectively kills tumor cells with an activated RAS pathway [3]. This has raised considerable interest in using reovirus T3D as an oncolytic agent in the treatment of a variety of tumor types both in pre-clinical and in clinical studies. C26 is an aggressive colorectal cancer (CRC) cell line that contains constitutively activated Kras due to an activating point mutation in codon 12 (G12D) [4]. We have recently shown that knockdown of $Kras^{D12}$ by RNA interference prevented apoptosis induction by reovirus T3D [4]. Therefore, we chose to assess the therapeutic effect of reovirus T3D on the outgrowth of pre-established C26 liver tumors.

Liver tumors were induced by direct intrahepatic injection of 10^5 C26 cells. After 24 hours the abdomen was re-opened and small macroscopic lesions (approximately 1 mm in diameter) were visible in all mice. Reovirus (10^{11} pfu) was injected directly into the liver, at the sites of these lesions.

Injection of reovirus T3D strongly inhibited tumor growth (by 76%) 5 days after tumor induction, when compared to injection of PBS ($p=0.07$). However, the anti-tumor effect rapidly diminished from day 7 onwards to only 11% inhibition on day 13 (Fig. 1a and b). We hypothesized that the decreased anti-tumor effect was due to an anti-viral immune response raised by the immunocompetent hosts. Therefore, the mice were (pre)treated with intraperitoneal injections of cyclosporin A (CyA; 50mg/kg) every second day, beginning 2 days before tumor cell injection. Again, tumor growth was strongly inhibited by reovirus T3D when measured 5 days after tumor induction (by 77%). However, CyA treatment markedly enhanced and prolonged the therapeutic effect of reovirus T3D: 11 and 13 days following tumor induction, tumor growth was still inhibited by 81% and 67% respectively (Fig. 1a and b). Importantly, CyA treatment did not significantly affect tumor growth in control mice, which excludes a potential direct inhibitory effect of CyA on C26 tumor growth (Fig. 1c). Remarkably, reovirus T3D therapy resulted in complete tumor regression in two CyA-treated mice (Fig. 1d). This was not observed in any of the mice in the reovirus T3D-treated group without CyA nor in the control groups. In fact, we have never observed regression of C26 tumors in any of our previous studies using several chemo- and anti-angiogenic therapeutics. The only other therapy that completely cures mice of C26 liver tumors is local destruction of the liver tumor by laser coagulation, combined with chemotherapy (our unpublished results).

Our finding that reovirus T3D can cause regression of C26 liver tumors as a single treatment modality in immunosuppressed mice highlights its potential as an oncolytic agent. Immunosuppression increased and prolonged the anti-tumor effect of a single reovirus T3D injection This is in line with the findings by Hirasawa et al. who reported that immunosuppression increased the anti-tumor response after systemic application of reovirus [8]. Conversely, immunosuppression had no effect on reovirus oncolysis of gliomas in a model in rats [6]. Reovirus therapy in immunocompromised SCID mice is associated with severe morbidity [10] but we have not observed this in the CyA-treated mice used in the present study. Apparently, CyA treatment sufficed to prevent reovirus T3D clearance from the liver, without inducing a profound systemic infection and morbidity. Acceleration of tumor growth is a potential risk factor of immunosuppression for cancer patients. This was not observed in our experiments in mice. Whether immunosuppression is applicable and/or beneficiary in the treatment of cancer patients remains to be determined.

Acknowledgements

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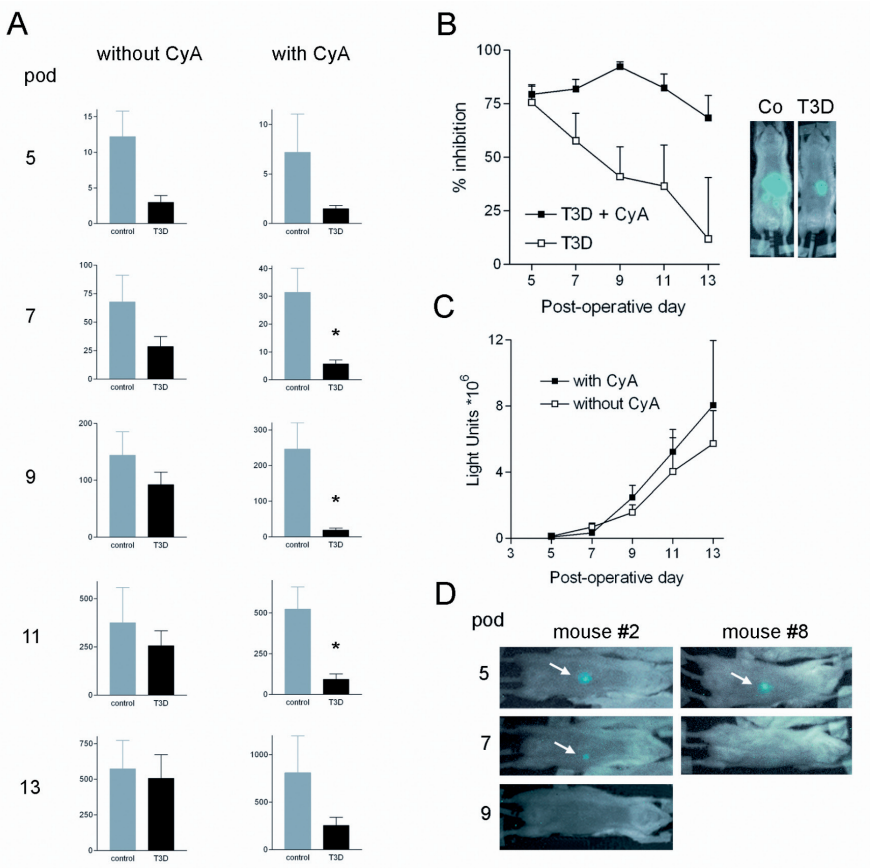

Figure 1. Reovirus T3D therapy inhibits the outgrowth of C26 liver tumors. (a) Isolated liver tumors were established by intrahepatic injection of 10^5 C26 cells expressing firefly luciferase. After 24 hours the tumor-containing liver areas were injected with reovirus (10^{11} pfu) or PBS and tumor growth was assessed non-invasively by bioluminescence imaging on post-operative day 5, 7, 9, 11 and 13 (left panel, n=9 mice/group). Immunosuppression by CyA (pre)treatment (50mg/kg, every second day, from two days before tumor induction) increased and prolonged the anti-tumor effect of reovirus (right panel, n=16 mice/group). On the y-axis the relative light units $*10^4$ are shown. *denotes statistical significance $p<0.01$. (b) The depicted graphs show the inhibition of tumor growth by reovirus T3D treatment relative to control (PBS) treatment (open squares) and the inhibition of tumor growth by reovirus T3D+CyA treatment relative to CyA treatment alone (filled squares). The inset shows representative bioluminescence images of control- and reovirus T3D-treated mice on post-operative day 11. (c) Tumor growth curves in control-treated (PBS; open squares) and CyA-treated mice (filled squares). Data are expressed as relative light units obtained by BLI over time. None of the differences were statistically significant. (d) A single reovirus T3D injection results in complete regression of established C26 colorectal liver tumors. The arrow indicates the BLI signal produced by the two intrahepatic tumors that regressed completely during the course of the experiment.

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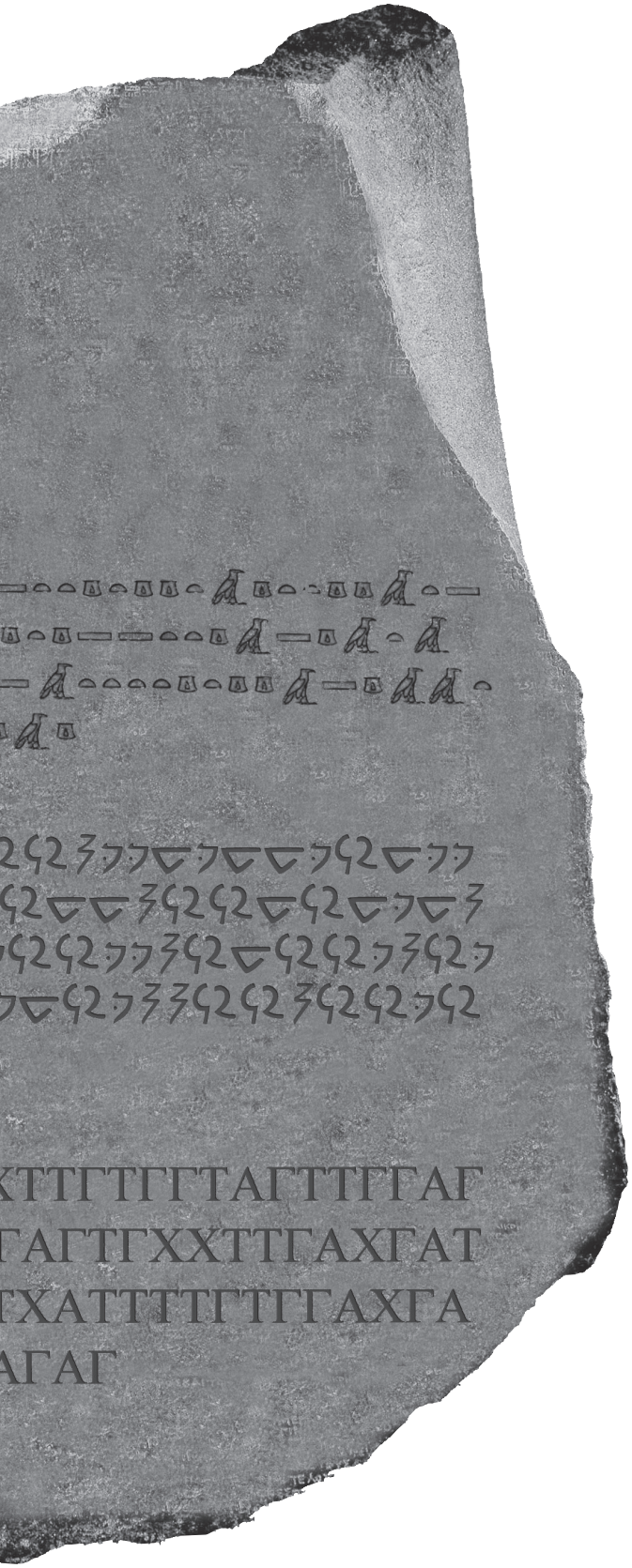

Chapter 11

Human colorectal liver metastases are resistant to reovirus T3D and display aberrant localization of the reovirus receptor JAM-1

Submitted.

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Abstract

Introduction

Liver metastases from colorectal cancer (CRC) are the major cause of death in CRC patients. Activating muations in the KRAS oncogene are found in approximately 40% of all cases. Reovirus T3D might be an attractive therapeutic as it specifically kills tumor cells with an activated RAS pathway and side effects are marginal. This study was designed to analyze the susceptibility of freshly isolated CRC liver metastasis fragments to reovirus T3D replication and oncolysis.

Methods

Directly after surgical resection, , tumor fragments of CRC liver metastasis (~1mm^3) were prepared and infected with reovirus T3D (5*10^7 pfu/fragment). Viral protein synthesis and replication was tested for 8 consecutive days by 24hrs [^{35}S]-methionine-labeling experiments. Maintenance of epithelial (tumor) cell viability was tested by analyzing the expression of cytokeratine 20 (CK20) by immunoblotting of tissue fragment lysates as well as by standard H&E light microscopy. The HRAS, KRAS, NRAS and BRAF genes were analyzed for activating mutations by PCR and sequence analysis. JAM-1 expression and localization was analyzed on a tissue microarray of 61 patients of whom material from normal colon, primary CRC and corresponding CRC liver metastases was available.

Results

Cultured tumor fragments harbored viable CRC cells during the entire course of the experiment. Nevertheless, none of the 13 tested specimens were susceptible to reovirus T3D infection. Six of 11 (55%) samples harbored an activating mutation in one of the RAS genes; 5 in KRAS and 1 in NRAS. All samples of normal colonic epithelium displayed membranous and cytoplasmic JAM-1 expression. In contrast, both primary CRC and CRC liver metastasis samples showed cytoplasmic but completely lacked membranous JAM-1 expression.

Conclusion

Freshly isolated CRC liver metastasis specimens are not susceptible to reovirus T3D replication and oncolysis. This is associated with a complete absence of the reovirus receptor JAM-1 at the cell membrane in neoplastic CRC tissue. This may be an important hindrance for the application of reovirus T3D as a therapeutic modality in the treatment of CRC liver metastases.

Introduction

Colorectal carcinoma (CRC) is the second most common cancer in the western world. Despite multiple therapeutic options, mortality is high and is associated with the development of liver metastases. Liver metastases develop in approximately 60% of CRC patients but only a minority of these patients is eligible for surgical resection with curative intent. This highlights the need for new therapeutic-interventions.

Reovirus T3D is a double-stranded RNA virus that belongs to the Reoviridae family. JAM-1 serves as a serotype-independent reovirus receptor capable of mediating virus attachment, infection and intracellular signaling [1]. JAM-1 contains a plasma localization signal peptide, is specifically localized at the tight junctions of epithelial and endothelial cells and is involved in the regulation of junctional integrity and permeability [2]. The viral attachment protein $\sigma 1$ directly binds to JAM-1 [1]. Antibodies specific for JAM-1 are capable of inhibiting infections by all three reovirus serotypes (type 1 Lang, type 2 Jones and type 3 Dearing), demonstrating that these strains use JAM-1 as a receptor [3]. Furthermore, reovirus binding to both sialic acid and JAM-1 is required to induce maximal levels of virus-induced apoptosis [4].

Reovirus has oncolytic potential because it specifically kills tumor cells with an activated RAS pathway by facilitating reovirus replication and/or by stimulating reovirus-induced tumor cell apoptosis [5-7]. Activating mutations in one of the RAS proto-oncogenes (HRAS, KRAS, NRAS) are found in many different tumor types with varying frequencies [8]. KRAS is mutated in approximately 38% of human CRCs [9]. Animal studies have demonstrated that reovirus exerts anti-tumor effects *in vivo* against several tumor types including CRC and established CRC liver metastases [10-15] (and chapter 10). Furthermore, in nonhuman primates, reovirus therapy is safe and well-tolerated [13].

It is well established that reovirus T3D can replicate in and kill human and mouse CRC cell lines *in vitro* and cell line-derived tumors grown in mice. However, it remains unclear whether human metastatic CRC tumors are susceptible to reovirus T3D. In the present report we investigated the potential of reovirus T3D to replicate in and cause oncolysis of freshly resected human CRC liver metastases. Furthermore, we evaluated JAM-1 expression in normal and neoplastic colorectal tissue.

Materials and methods

Clinical samples

Patients who underwent a resection for colorectal liver metastases from January 2004 to August 2005 were included in the study. Directly after resection, the specimen was taken to the pathology department where a part of the tumor was excised for experimentation. This biopsy was cut into small fragments (~1 mm^3), that were cultured in a 96 wells plate in Dulbecco's Modified Eagle's Medium (DMEM; Dulbecco, ICN Pharmaceuticals, Costa Mesa, CA, USA) supplemented with 10% (v/v) fetal calf serum, 2mM glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin. All fragments were kept at 37°C in a humidified atmosphere containing 5% CO_2.

Reovirus infection and replication

Actively metabolizing fragments were infected with reovirus T3D ($5*10^7$ pfu/fragment) or control vehicle. Viral protein synthesis and replication was tested daily from post-operative day (pod) 1-8 by 24hrs [^{35}S]-methionine-labeling experiments.

Assessment of CRC cell viability in tumor fragments

The presence of viable epithelial CRC cells in the cultured tumor fragments was assessed over time by preparing lysates of tissue fragments daily until pod 8. The expression of the epithelial marker protein cytokeratine 20 (CK20) was assessed by standard Western blotting, using an anti-CK20 antibody (DakoCytomation, M 7019, Clone Ks 20.8). Simultaneously, 3 tumor fragments were harvested each day, fixed with formaldehyde, and embedded in paraffin. CRC cells were identified microscopically on haematoxylin- and eosin-stained sections.

DNA extraction

Genomic DNA was extracted from formalin-fixed, paraffin-embedded tumor specimens corresponding to the tissue fragments that were successfully analyzed by [^{35}S]-methionine-labeling experiments. Sections (10 μm) were cut, deparaffinized and rehydrated. The indicated (tumor) areas were collected by microdissection with minimal stroma contamination. The dissected tissue was suspended in extraction buffer (1M Tris, 0.5M EDTA, 10% sodium dodecyl sulphate) containing proteinase K (1mg/ml) and incubated at 56°C for 48 hours. Proteinase K was freshly added every 12 hours. The solution was extracted twice with a 25:24:1 mixture of phenol-chloroform-isoamylalcohol. Genomic DNA was precipitated with ethanol, pelleted and resuspended in TE (Tris-HCl 10mM, EDTA 1mM). The concentration of DNA was measured by optical densitometry.

PCR and sequence analysis

Target sequences encompassing codons 12, 13 and 61 of the HRAS, KRAS and NRAS genes and codon 600 of the BRAF gene were amplified by nested PCR in 384-well plates. Sequencing was performed using the Big Dye terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington UK), according to the manufacturer's instructions. Analysis of the products was performed on an ABI Prism 377 DNA Sequencer (PE Biosystems). All target sequences were amplified in duplicate by independent PCR reactions and sequencing was done with both the forward and reverse sequencing primer. Only if a mutation was found in both independently amplified fragments, it was considered a real mutation and not a PCR artifact. All primer sequences will be supplied upon request.

Tissue microarray (TMA)

A tissue microarray (TMA) was prepared to analyze the expression of JAM-1 in normal colon epithelium and in CRC cells in the primary tumors and the corresponding liver metastases. We collected representative paraffin-embedded tissue blocks from neoplastic and non-neoplastic regions and the TMA was constructed as described [16]. In brief, samples from surgical resections of 61 patients were selected for preparing a TMA. Only those patients were selected of whom normal colon, primary CRC, and the corresponding CRC liver metastasis were available. For each patient, nine cylindrical tissue cores were included in the TMA; three from the normal colon, three from the primary CRC and three from the corresponding liver metastases. Immunostaining was performed using standard procedures. After incubation with 3% hydrogen peroxide, antigen retrieval was achieved by boiling in 10 mM citrate buffer pH 6.0. Sections were blocked with 5% goat serum in TBS and incubated with an anti-JAM-1 antibody (H-80, sc-25629 Santa Cruz Biotech., Heidelberg, Germany) at 4°C overnight. HRP-conjugated secondary antibodies (DPVM-55HRP, Immunologic, Duiven, The Netherlands) were detected with 3.3'-diaminobenzidine substrate (D4418, Sigma, Saint Louis, USA). Slides were counterstained with haematoxylin and rinsed with water, dehydrated in ethanol, cleared in xylene and coverslipped. The localization, intensity and extent of JAM-1 positivity was determined in each tissue core by two independent experienced observers. Membranous staining of JAM-1 was scored as positive (1) or negative (0). When a tissue core displayed weak and focal positivity in less than 5% of the total number of tumor cells, it was classified as negative. Cytoplasmic staining was scored as 0: no staining, 1: weak staining, 2: moderate staining and 3: strong staining.

Results

Resected human CRC liver metastases do not sustain reovirus T3D replication

Freshly isolated CRC liver metastases were mechanically disrupted to yield fragments of less than 1mm^3. Subsequently, these fragments were cultured for 8 days and the persistence of viable tumor cells in the tissue fragments was assessed over time. Expression of the epithelial marker CK20 remained clearly detectable during the 8-day culture period (Fig. 1A). Next, the presence of CRC cells was assessed microscopically on haematoxylin- and eosin-stained sections from daily isolated fragments. Viable tumor cells were detected until the end of the experiment (Fig. 1B). In conclusion, the CRC cells in the 1mm^3 tissue fragments remain viable for at least 8 days after resection. Simultaneous with the above experiments, actively metabolizing tissue fragments were infected with reovirus T3D (5*10^7 pfu/fragment) and cellular and viral protein synthesis was tested by daily 24hrs [^{35}S]-methionine-labeling experiments until day 8 after resection. Complete time courses could be successfully obtained for 13 liver metastases (Fig. 2).

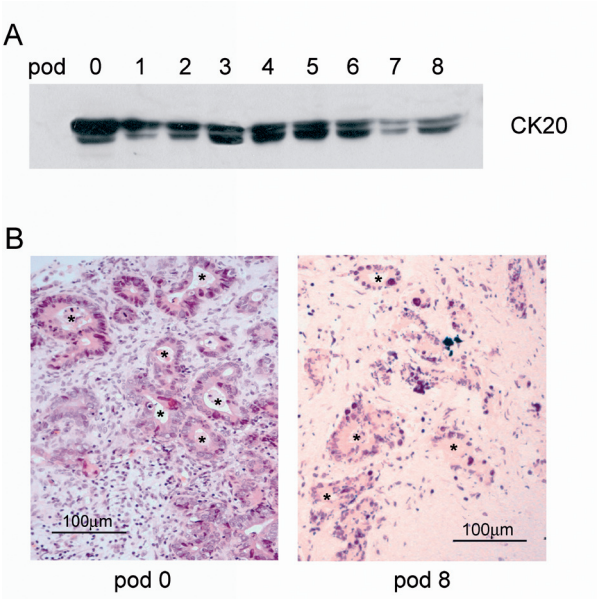

Figure 1. CRC cells remain viable in cultured tissue fragments. Viability of CRC cells in the cultured tumor fragments was analyzed over time. A) Western-blot analysis demonstrating the expression of the epithelial marker cytokeratin 20 (CK20) in lysates of tumor fragments from post-operative day (pod) 0-8. B) The presence of CRC cells was assessed by standard light microscopy on haematoxylin- and eosin-stained sections from daily isolated fragments. Representative images directly after resection (pod 0, left panel) and at the end of the experiment (pod 8, right panel) demonstrate viable CRC cells surrounded by tumor stroma (indicated by *).

Table 1. RAS/BRAF mutations and reovirus T3D replication

Tumor	*RAS/BRAF*	*T3D replication*
1	wt	-
2	wt	-
3	$KRAS^{G13D}$	-
4	$KRAS^{G13D}$	-
5	wt	-
6	$KRAS^{G12D}$	-
7	nd	-
8	wt	-
9	$KRAS^{G13D}$	-
10	$KRAS^{G12V}$	-
11	nd	-
12	$NRAS^{Q61R}$	-
13	wt	-

Wt: wild-type, nd: not determined

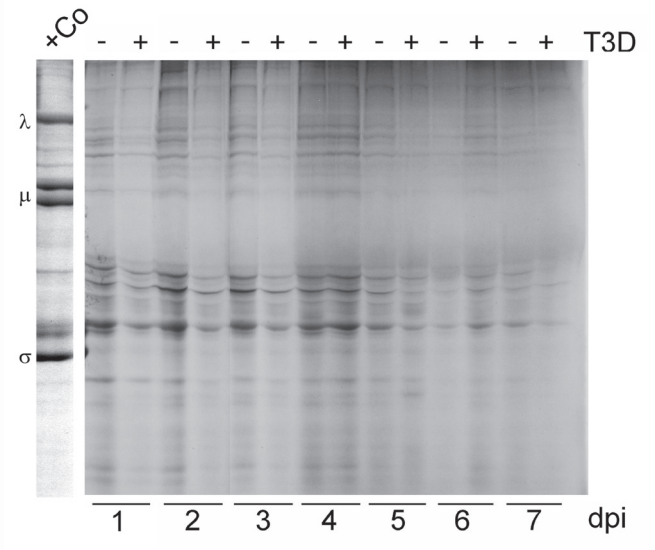

Figure 2. Time course of protein synthesis by 24hrs [35S]-methionine-labeling experiments. Actively metabolizing tissue fragments were infected with reovirus T3D ($5*10^7$ pfu/fragment) on post-operative day 1 (=days post infection 0 (dpi 0)). Viral and cellular protein synthesis was tested by daily 24hrs [35S]-methionine-labeling experiments until day 8 after resection (dpi 7). Lysates were prepared in sample buffer and were run out on long 45 cm SDS-polyacrylamide gels. Gels were dried and exposed to radiographic film. The positive control HCT116 (+Co) demonstrates the λ, *μ* and *σ* reoviral proteins. T3D; reovirus T3D.

Normal cellular protein synthesis was readily detectable from pod 1-8 in the non-infected samples. None of the reovirus-treated fragments showed a consistent reduction in cellular protein synthesis, indicating that reovirus T3D infection failed to induce a cytopathic effect. Furthermore, reoviral proteins were not detected in any of the labeled fragments (Table 1). Using the same protocol, reoviral protein synthesis was clearly detected in a single resected human bladder carcinoma on days 3 and 4 after infection (not shown). The human CRC cell line HCT116 served as a positive control in each of these experiments.

RAS/BRAF mutations in 55% of resected specimens

Reovirus preferentially kills RAS-transformed cells. Therefore, we analyzed whether the lack of virus replication and cytopathic effect was due to a lack of mutations in one of the RAS or BRAF genes. Sequence analysis of KRAS, HRAS, NRAS or BRAF in eleven of the resected CRC liver metastases revealed a KRAS mutation in 5 cases and one specimen had an NRAS mutation (Table 1). Thus, all 13 human CRC specimens were resistant to reovirus T3D, despite the fact that at least 6/13 contained activated KRAS or NRAS.

CRC and colorectal liver metastases display aberrant localization of the reovirus receptor JAM-1

JAM-1 is the major receptor for reovirus and is localized at the tight junctions of epithelial and endothelial cell-cell contacts [1]. Upon binding to JAM-1, reovirus is internalized and virus replication ensues [1]. We hypothesized that the lack of reovirus replication in CRC tumor tissue could be due to downregulation of the reovirus receptor JAM-1. To test this, we performed a JAM-1 immunohistochemical staining on a tissue microarray of 61 patients containing normal colonic epithelium, primary CRC and the corresponding CRC liver metastases from each individual patient. A total of 52 normal colonic epithelium, 61 primary CRC and 53 liver metastases were available for analysis. We found that JAM-1 in normal colonic epithelium is localized at the cell membrane as expected. In contrast, in both primary CRC and CRC liver metastases JAM-1 staining was completely absent at the cell membrane in almost all tumors. Only four primary CRC and two liver metastases displayed focal weak JAM-1 positivity at the cell membrane (Fig. 3A and C, page 157). Cytoplasmic staining however, was detected in the majority of primary CRC and metastasis specimens, but the staining intensity varied considerably between tumor samples (Fig. 3B, page 157) Thus, the resistance of human CRC specimens to reovirus T3D infection is associated with mislocalization of the major reovirus receptor JAM-1.

Discussion

Reovirus T3D is non-pathogenic in immunocompetent adults and specifically kills tumor cells with an activated RAS pathway [6]. We previously showed that the outgrowth of established CRC liver metastases in mice was efficiently inhibited by reovirus T3D (chapter 10). This effect was maximized by concomitant immunosuppression of the host. In the present report, we evaluated the susceptibility of freshly isolated human CRC liver metastases to reovirus T3D infection. None of the tested specimens sustained reovirus replication or demonstrated loss of viability.

Several mechanical and biological factors may contribute to the lack of susceptibility of CRC specimens to reovirus T3D. Virus spread in the tissue fragments may be hampered by physical constraints. Isolated tumor fragments of approximately 1 mm^3 consist of tumor cells and stroma. The cells at the outer surface of the fragments are readily accessible to the virus. Reovirus infection of the tumor cells in the inner part of the tissue fragments depends either on successive cycles of infection and virus release into the tissue or on passive diffusion of the virus. It has been suggested that the extracellular matrix (ECM) in a tumor acts as a physical barrier that limits the interaction between adenovirus and tumor cells [17]. We readily demonstrated reovirus T3D replication in tumor fragments of a freshly resected bladder carcinoma by using the same experimental protocol.

Although this result suggests that the protocol allows tissue infection by reovirus T3D, it should be noted that the tissue structure of bladder and colorectal carcinomas may be considerably different. In addition to mechanical constraints, the lack of reovirus replication may also be caused by tumor cell intrinsic properties. First, CRC cells might selectively loose viability in the cultured tissue fragments, whereas unsusceptible stroma cells might survive. However, our results show that the CRC cells in the tissue fragments remain viable for at least the entire course of the experiments (8 days). Second, it is known that reovirus T3D selectively replicates in and kills tumor cells with an activated RAS pathway. At least 6/13 samples harbored an activating RAS mutation, ruling out the possibility that reovirus failed to kil the CRC cells due to insufficient RAS activity. This does not take into account the possibility that the RAS pathway may also be activated by upstream activators of RAS such as overexpression of the EGF receptor. Finally, the lack of reovirus replication might be attributed to a failure of reovirus to bind to the fragments. In the present report, we show a complete absence of JAM-1 at the cell membrane in primary and metastatic CRC. We propose that aberrant localization of JAM-1 is likely to contribute to the observed lack of reovirus replication and oncolytic efficacy. To our knowledge this is the first study to report aberrant localization of JAM-1 in neoplastic tissue.

The cellular localization of JAM proteins may depend on specific signals from the extracellular environment. For instance, it has previously been shown that cytokine activation can induce redistribution of JAM-1 away from lateral junctions [18]. The exact pathways that mediate such changes have not been identified yet, but phosphorylation of the cytoplasmic tail of JAM proteins may control their localization [19]. Whether this mechanism underlies the aberrant cytoplasmic localization in primary CRC and CRC liver metastases is presently unclear. *In vitro* studies demonstrate high susceptibility of epithelial tumor cell lines, including CRC cell lines, to reovirus infection. For instance, the epithelial tumor cell lines Hela (cervix carcinoma), A549 (lung carcinoma), MDA231 (breast carcinoma), T-47D (breast carcinoma) and HT29 (colon carcinoma) are highly susceptible to reovirus induced cell killing [14,20] (and our own unpublished results). In these cell lines JAM-1 is highly expressed at the cell membrane [21,22]. Possibly, JAM-1 may re-localize to the membrane in the process of cell line establishment.

The cytoplasmic localization and absence of JAM-1 at the cell membrane in intact CRC tissue is a plausible explanation for a) the lack of viral replication, b) the lack of virus induced cell death and c) the lack of a "KRAS-effect" in the CRC fragments. Taken together, our results suggest that the aberrant localization of JAM-1 may be an important hindrance for reovirus therapy against CRC and CRC liver metastases.

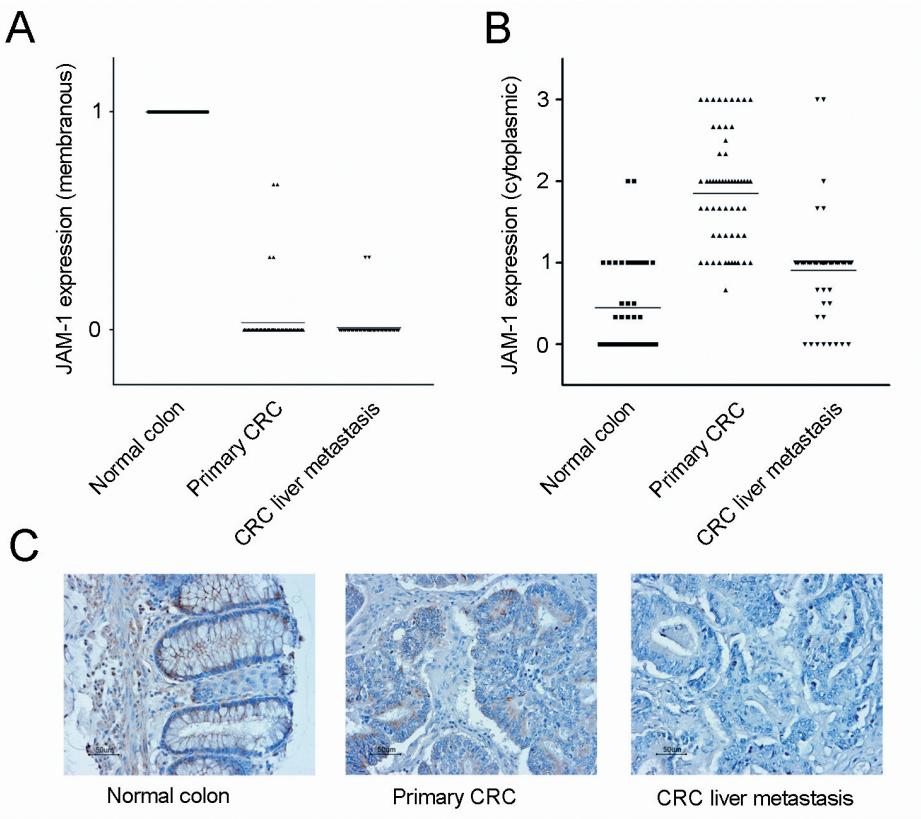

Figure 3. Neoplastic CRC tumors display absence of JAM-1 expression at the cell membrane. The expression of JAM-1 at the cell membrane and in the cytoplasm was analyzed on a tissue micro array of 61 CRC patients with normal colonic epithelium, the primary CRC and the corresponding liver metastasis of each patient. Membrane bound JAM-1 expression was scored on each tissue core as present (1) or absent (0). Cytoplasmic staining was scored as 0: no staining, 1: weak staining, 2: moderate staining and 3: strong staining. Scatter diagram showing the average count of A) membrane bound and B) cytoplasmic JAM-1 expression of 3 tissue cores per tissue type in normal colonic epithelium (n=52 patients), primary CRC (n=61 patients) and CRC liver metastasis (n=53 patients). C) Representative images of immunohistochemical staining for JAM-1 in normal colonic epithelium (left panel), primary CRC (middle panel) and CRC liver metastasis (right panel).

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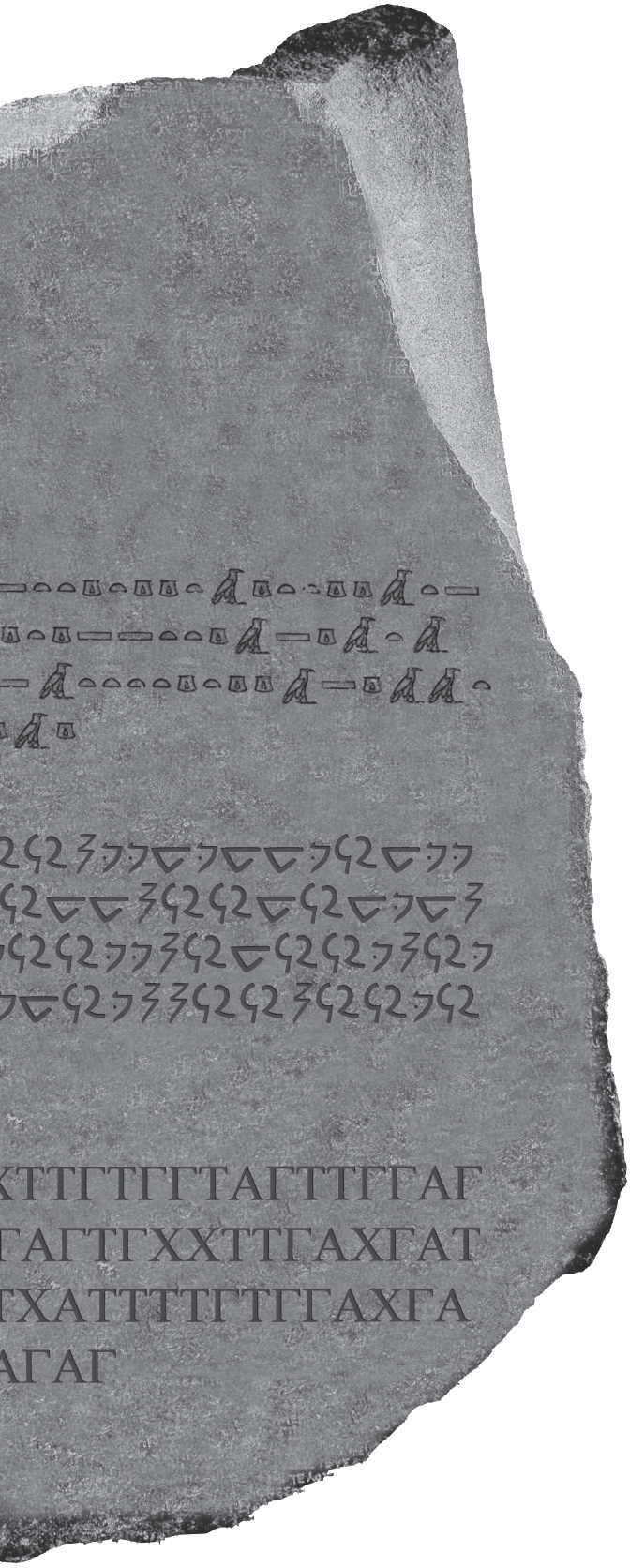

Chapter 12

General discussion

Animal studies in cancer research

Animal studies and especially mouse models are extremely important in cancer research. Many mouse models have been developed to evaluate the various features of CRC and liver metastasis formation in humans. Since none of the existing mouse models mimic all the characteristics of human CRC, it is of crucial importance that the optimal model is chosen for each experiment [1]. During the last decade, new molecular and optical techniques have made it possible to analyze tumor growth and characteristics in living animals (intravital imaging). One of these techniques is bioluminescence imaging (BLI). In **chapters 3 and 7** we have shown that BLI is an easy to perform, fast and reliable method to determine intrahepatic (**chapter 3**) and intravesical (**chapter 7**) tumor growth. Multiple consecutive measurements in a single mouse make it is possible to analyze tumor growth over time. This facilitates to determine a) when an experimental treatment reaches its maximal effect and b) the optimal moment to terminate an experiment and perform post-mortem analyses. Taken together, the use of BLI makes it more likely that a therapeutic effect will not be missed.

For researchers, animal-rights activists and the general public, animal welfare is an important topic in animal reseach [2,3]. The three R's described by Russell and Burchof (Replacement, Reduction and Refinement) are the hallmark of laboratory animal science, a multidisciplinary branch of science on the quality of animal experiments and the welfare of laboratory animals. Because repetitive measurements can be made in a single mouse, BLI can significantly reduce the number of mice necessary in preclinical cancer research. BLI can therefore make an important contribution to one of three R's (Reduction).

BLI also has some disadvantages. First, the exact location of tumor growth cannot be determined (poor spatial resolution), e.g. it is not possible to distinguish intrahepatic tumor growth in separate liver lobes or to distinguish separate liver metastases in a single lobe. Second, it has been suggested that the absorption of light by hemoglobin in tumor tissue can interfere with a proper assessment of the level of reporter gene expression [4]. However, our results suggest that this does not significantly affect the measurement of tumor size by BLI.

Several other non-invasive imaging methods are presently available. Compared to BLI, fluorescence-enhanced imaging has the advantage that injection of a substrate is not required. However, the sensitivity of fluorescence-enhanced imaging is much lower than BLI due to scattering and the absorption of light by surrounding tissue [5]. Other non-invasive imaging modalities such as micro-computed tomography (micro-CT) and magnetic resonance imaging (MRI) offer increased spatial resolution but are far more time-consuming, expensive and less sensitive than BLI [6,7]. Therefore, as a highly sensitive, inexpensive and easy to perform imag-

ing modality, we strongly recommend the use of BLI for experiments that are designed to evaluate the effect of (therapeutic) interventions on intrahepatic tumor growth. The applicability of BLI in other tumor models and (larger) animals needs further investigation.

Dependency of CRC tumors on mutant KRAS

It is well-established that mutations in the KRAS oncogene are acquired during the earliest stages of CRC formation. When KRAS is to be used as a therapeutic target, tumor growth should be dependent, even at the latest stages, on the continued presence of mutant KRAS. We have clearly demonstrated that in the highly aggressive C26 mouse CRC cell line, Kras plays an important role in the maintenance of tumor growth in immunocompetent hosts. Furthermore, we have shown that upon Kras knockdown these cells are rapidly cleared by the immune system. In **chapter 4** we have demonstrated that the induction of an anti-tumor immune response upon Kras knockdown is associated with increased H-2K^{d} MHC class I molecules at the cell surface and increased (de-repressed) IL-18 production. However, the causal relationship between these molecular changes and the induction of the observed anti-tumor immune response needs to be further clarified.

Strikingly, the anti-tumor immune response was directed against the Kras-suppressed as well as the parental cell line (**chapter 4**). This underscores the common origin of these cells and indicates that the immunogenic epitope is expressed in both Kras-suppressed and parental cells. Furthermore, these observations suggest that it may not be necessary to target all RAS mutated cells to evoke an anti-tumor immune response that may ultimately lead to immune-mediated clearance of targeted and non-targeted cells.

This might have important implications for the clinic particularly for the therapeutic application of KRAS-directed gene therapeutic approaches such as RNA interference, as it remains a challenge to target all tumor cells.

Besides the effects on the immune system, we have shown in **chapter 5** that upon Kras knockdown the invasive capacity and metastatic potential of CRC cells is dramatically reduced. This suggests that KRAS-directed therapies may be applicable in the adjuvant setting to prevent locoregional recurrences and metachronous metastases. Furthermore, it is well-established that tumor cells are shed in the circulation during surgical manipulation [8-10]. However, the fate of these circulating tumor cells is uncertain. If these cells contribute to recurrence, KRAS-directed therapies may also be applicable in the neoadjuvant setting.

One of the challenging but necessary steps is to confirm the dependency of human tumor growth and invasiveness on mutant KRAS in freshly resected CRC specimens. Furthermore, it is essential to determine the critical downstream targets that mediate the requirement for Kras in tumor maintenance. Giving the diversity of downstream targets, it seems likely that multiple downstream elements act in concert to sustain tumorigenic properties. Recently however, it has been suggested that the RAS effector pathways involving MAPK, RalGEF and PI3K are required to initiate tumor growth, whereas only the PI3K/AKT pathway is necessary for tumor maintenance [11]. This implies that it should be possible to identify therapeutics that target these specific downstream effectors, thereby reducing potential non-specific side effects.

Many studies analyzing RAS function and biology have used $HRAS^{V12}$ overexpression experiments. However, in most tumor types, including CRC, mutations in HRAS are rarely found. Furthermore, it is clear that the RAS isoforms HRAS, KRAS and NRAS differentially activate downstream targets and control different cellular functions such as cell motility and basolateral polarity [12-15]. It is therefore of the utmost importance to analyze RAS function in the correct context; preferentially with cell lines carrying endogenous mutant RAS alleles. In addition, it will be important to understand the individual roles of HRAS, KRAS and NRAS and the interplay between these proteins.

Application of RNA interference in cancer therapy

Given its important role in normal cellular homeostasis, inhibition of wild-type RAS might result in a plethora of side-effects. Gastro-intestinal toxicity, myelosuppression, neurotoxicity, acute pancreatitis and renal failure have all been described as side-effects of farnesyl transferase inhibitors, that act on both wild-type and mutant RAS [16]. RNAi is highly specific. A single base change dramatically reduces the suppressing effect on the target mRNA. Because single point mutations in codons 12, 13 and 61 activate the RAS oncogenes, RNAi makes it possible to target only the mutant allele [17]. This will be an important step forward in making cancer therapy more specific and avoiding the possible drawbacks of inhibiting wild-type RAS. As with any gene therapy, a suitable delivery system remains the main obstacle for successful application in humans. Recently, several groups reported successful use of RNAi *in vivo* in mammals. Systemic application of siRNA in a cationic formulation [18] and a cationic lipid-based formulation [19] successfully silenced target gene expression *in vivo* in mice. This was also seen after hydrodynamic pressure-assisted delivery of siRNA molecules in an unformulated form. This consists of rapid intravenous administration of siRNA molecules in a large volume of physiological saline (approx. 2 ml in mice) [20]. Although successful in mice, it is at least questionable

whether high-pressure hydrodynamic transfection can be applied in humans. More promising is the delivery of siRNA via vector-mediated gene therapy with e.g. a lentivirus, adenovirus or delivery vectors that are used in other gene therapies. A recombinant adenoviral mediated siRNA delivery system resulted in gene specific silencing *in vivo* [21]. However, problems with integrating vectors have recently arisen from gene therapy trials [22]. Therefore, the biggest challenge for RNAi as a therapeutic tool will be a way to specifically deliver the effective molecules into the target (tumor) cells in the body.

COX-2 as a downstream therapeutic target of KRAS

We have demonstrated that the expression of COX-2 is regulated by endogenous mutant Kras (**chapter 6**) and that selective COX-2 inhibitors significantly reduce the outgrowth of COX-2 expressing CRC liver metastases (**chapter 6**) and bladder carcinomas (**chapter 7**) by inhibiting tumor cell proliferation. These data suggest that there might be a role for COX-2 inhibitors in the (adjuvant) treatment of these tumor types. Currently, a phase I study (RPCI-RP-0224) of irinotecan and celecoxib in patients with unresectable or metastatic colorectal cancer is being performed. Rofecoxib did not appear to increase antitumor activity for patients with metastatic colorectal cancer but resulted in increased gastrointestinal toxicity when combined with 5-FU/LV [23]. Furthermore, it has become clear that the regular use of selective COX-2 inhibitors, especially rofecoxib is associated with an increased risk for severe cardiovascular events including stroke and myocardial infarction [24,25]. This led to the withdrawal of rofecoxib from the market by Merck. These new data regarding the side-effects of the selective COX-2 inhibitors raise important concerns about their use as a mode for cancer prevention and treatment.

Nevertheless, the observation that COX-2, as a downstream target of mutant Kras, plays an important role in the tumorigenicity of CRC and bladder carcinomas and that COX-2 inhibition may have an anticancer effect is important as it further unravels the molecular effectors in the development of these tumors. Whether or not the COX-2 inhibitors will be used for cancer prevention and treatment in the future will depend on the analysis of the balance between their anticancer effect and their safety. This can only be evaluated by large prospective studies. In a Phase I study, the addition of escalating doses of the selective cyclooxygenase-2 (COX-2) inhibitor celecoxib to 5-fluorouracil (FU) and leucovorin (LV) did not increase toxicities expected from the chemotherapy alone [26]. In addition, continuous drug development may yield other selective COX-2 inhibitors without major cardiovascular side effects.

Reovirus T3D in the treatment of CRC and liver metastases

As an oncolytic virus that specifically targets tumor cells with an "activated RAS pathway", reovirus T3D has promising therapeutic potential for the future. In **chapters 8 and 9** we have clearly demonstrated that reovirus replication does not depend on the presence of endogenous mutant KRAS, but that mutant KRAS sensitizes CRC cells to reovirus-induced apoptosis. These observations might have important consequences for its implication in the clinic, especially for the rational design of combination strategies. The potential use of reovirus in combinatorial regimens with current cancer therapies will be an important next step in the development of reovirus application as an oncolytic therapeutic.

Theoretically, some agents are not suitable for their use in combination strategies with reovirus. Reovirus T3D oncolysis is facilitated in cells with an activated RAS pathway. The combination of reovirus with therapeutics that inhibit RAS activation such as FTIs and EGF-receptor antagonists may interfere with reovirus oncolysis and antagonistic effects can be anticipated. In **chapter 9** we have not been able to demonstrate therapeutic synergy *in vitro* when reovirus is combined with oxaliplatin or TRAIL. We suggest that this may be explained by the fact that reovirus T3D alone already activates both the intrinsic and extrinsic pathways that lead to apoptosis induction [27]. Therapeutic synergy may be achieved when reovirus T3D is combined with compounds that either target the tumor vasculature (anti-angiogenic compounds) or (tumor-associated) inflammatory cells, rather than the tumor cells themselves. Recently, it has been reported that anti-angiogenic agents can transiently "normalize" the abnormal tumor microvasculature which results in improved delivery of therapeutics to solid tumors [31]. Combination of reovirus with such agents may result in increased tumor accessibility and more efficient reovirus-induced oncolysis.

In addition, we have shown that the therapeutic effect of reovirus T3D against established liver metastases can be maximized by concomitant immunosuppression with cyclosporin A (CyA) (**chapter 10**). Whereas reovirus therapy in immunocompromised SCID mice is associated with severe morbidity [32], reovirus therapy in CyA treated mice did not lead to reovirus-induced side effects. Apparently, CyA treatment sufficed to prevent reovirus clearance by the immune system, without inducing a profound systemic infection and morbidity. Many malignancies are associated with a state of host immunodeficiency [33]. Furthermore, during disease progression, immunodeficiency frequently develops and is often compounded by chemotherapy. This suggests that the immune system in cancer patients may be sufficiently suppressed to facilitate reovirus therapy without the need for concomitant immunosuppression. Whether immunosuppressive drugs are applicable and/or beneficiary in the treatment of cancer patients with reovirus remains to be determined.

In **chapter 11** we have demonstrated that reovirus T3D does not infect freshly isolated CRC specimens. Our results suggest that the aberrant localization of the reovirus receptor JAM-1 may be an important hindrance for reovirus therapy against CRC and CRC liver metastases in the clinic. A possible solution is to genetically modify reovirus to enhance (tumor) cell attachment to other cell surface receptors. Several oncolytic viruses have been genetically engineered to promote cell killing, augment specificity and stimulate lasting anti-tumoral immunity [34,35]. Genetic manipulation of reovirus is challenging due to its double-stranded RNA genome and the tight packaging constraints imposed on progeny virus. Roner et al successfully modified reovirus using a reverse genetics system [36]. However, whether the application of this technology will lead to improvement of reovirus cancer therapy needs to be explored.

In conclusion, we have demonstrated that late stage CRC cells are dependent on mutant KRAS for their invasiveness, metastatic potential and for immune evasion. This makes mutant KRAS an interesting therapeutic target with great potential against metastatic colorectal carcinomas. COX-2 is a direct target of mutant KRAS and selective COX-2 inhibitors have therapeutic potential against colorectal liver metastases and bladder carcinomas.

Mutant KRAS sensitizes CRC cells to reovirus T3D induced apoptosis. The therapeutic efficacy of reovirus T3D against established liver metastases is hampered by the host immune system but can be increased by concomitant immunosuppression. The mislocalization of the reovirus receptor JAM-1 in human colorectal liver metastases may be an obstacle for its use as a oncolytic agent in the clinic.

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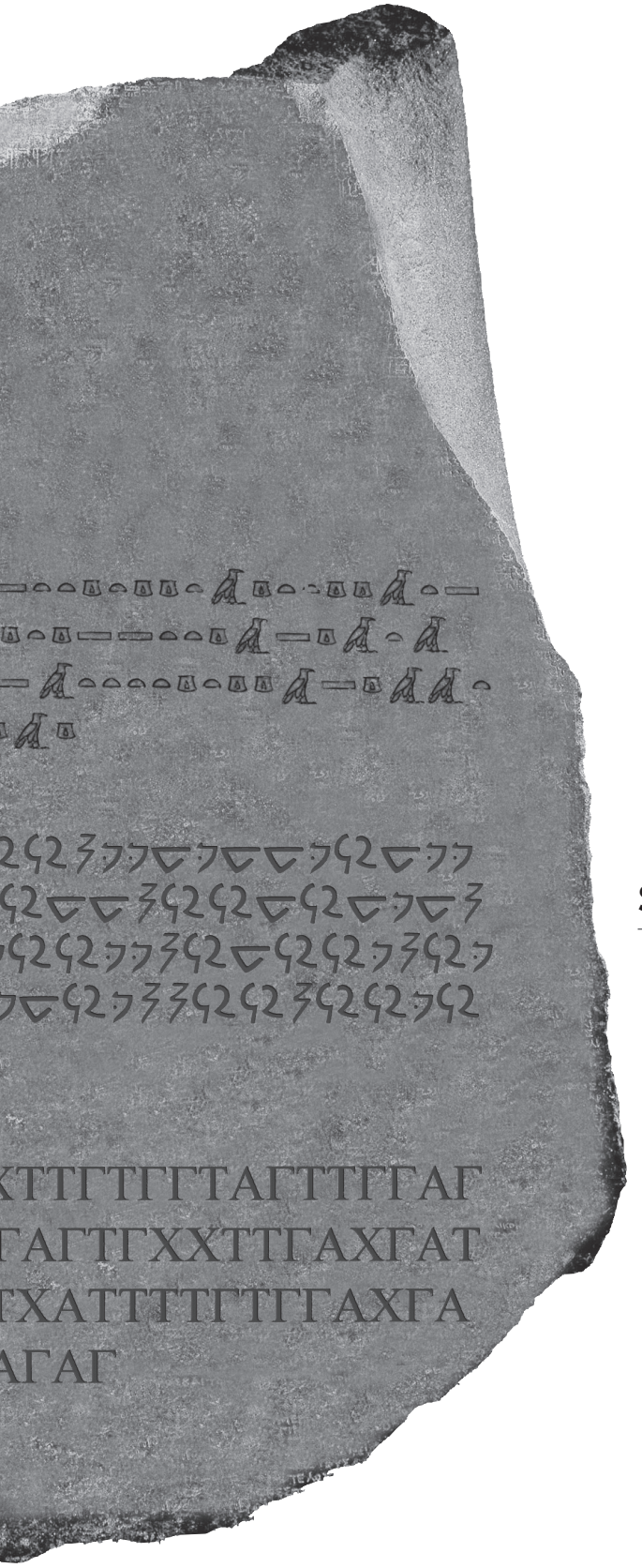


Summary

Chapter 1 gives a short introduction on the development of molecular targeted therapeutics in cancer treatment, the epidemiology and treatment of colorectal carcinoma (CRC), the biology of RAS proteins and the development of RAS-directed therapies. At the end of **chapter 1**, the outline of this thesis is described.

Chapter 2 gives an overview of the current literature on oncogenic RAS proteins and their potential role in metastasis formation. The formation of metastases is a very complex and inefficient process with several distinct but intertwined stages. *In vitro* studies suggest that oncogenic RAS can stimulate progression through these stages, but it remains unclear whether oncogenic RAS in human tumors exerts the same effects. Several clinical studies have addressed the question whether mutant KRAS predisposes to CRC liver metastasis formation. Unfortunately, the results from these studies are not uniform. In addition, the difficulties and pitfalls that accompany clinical research on the seemingly simple question of KRAS involvement in CRC liver metastasis formation are also discussed in **chapter 2**.

Animal studies are extremely important in cancer research. Many mouse models have been developed to evaluate the various features of CRC and liver metastasis formation in humans. Since none of the existing mouse models mimics all the characteristics of human CRC, it is of crucial importance that the correct model is chosen for a given scientific question. During the last decade, new molecular and optical techniques have made it possible to analyze tumor characteristics in living animals (intravital imaging). One of these techniques is bioluminescence imaging (BLI). Once tumor cells are genetically engineered to express the firefly luciferase gene, BLI makes it possible to image tumor growth noninvasively. One of the advantages is that multiple consecutive measurements can be performed in a single mouse. This makes it possible to assess tumor growth over time. In **chapter 3** we have validated BLI of liver metastases by comparing it to standard post mortem analyses; HRA measurements and liver weight. We conclude that bioluminescent imaging is a reliable and superior method for measuring experimental CRC growth in the liver.

Oncogenic mutations in the KRAS gene are found in approximately 40% of human CRC. These mutations are acquired during the earliest stages of colorectal cancer development. Multiple additional changes are required for progression to malignant metastatic carcinoma. Mutant KRAS might have potential as a therapeutic target in the treatment of CRC, but tumor growth should then be still dependent on its continued presence in a background of many other genetic mutations. In **chapter 4**, we have analyzed the effect of suppression of the mutant KRAS allele by RNA interference in a highly aggressive mouse CRC cell line (C26). We have found that stable knockdown of the mutant $Kras^{D12}$ allele resulted in loss of transformed properties *in vitro*. The incidence of subcutaneous tumor formation was dramatical-

ly reduced and Kras-knockdown tumors grew noninvasively and did not cause morbidity. Remarkably, Kras-knockdown tumors elicited an anti-tumor immune response that resulted in spontaneous regression of some Kras-knockdown tumors. In addition, Kras-knockdown resulted in increased production of interleukin 18 (Il-18), a cytokine that has strong immune-stimulatory functions. Thus, mutant Kras suppresses Il-18 production in colorectal tumor cells. This may contribute to evasion of the local immune system during tumor development.

In **chapter 5** we have investigated the effect of mutant Kras on the potential of C26 cells to form liver metastases. Suppression of mutant Kras dramatically reduced the invasive capacity of C26 cells, *in vitro*. With both bioluminescence imaging and *in vivo* microscopy, we have demonstrated that the reduced capacity of Kras-suppressed cells to form liver metastases could be explained by a reduced capacity to extravasate the hepatic sinusoids. Once arrested in the liver, Kras-suppressed cells stayed in the hepatic microvasculature and were rapidly cleared by the immune system in immunocompetent hosts. We conclude that oncogenic Kras promotes the formation of liver metastases by stimulating tumor cell extravasation and immune evasion.

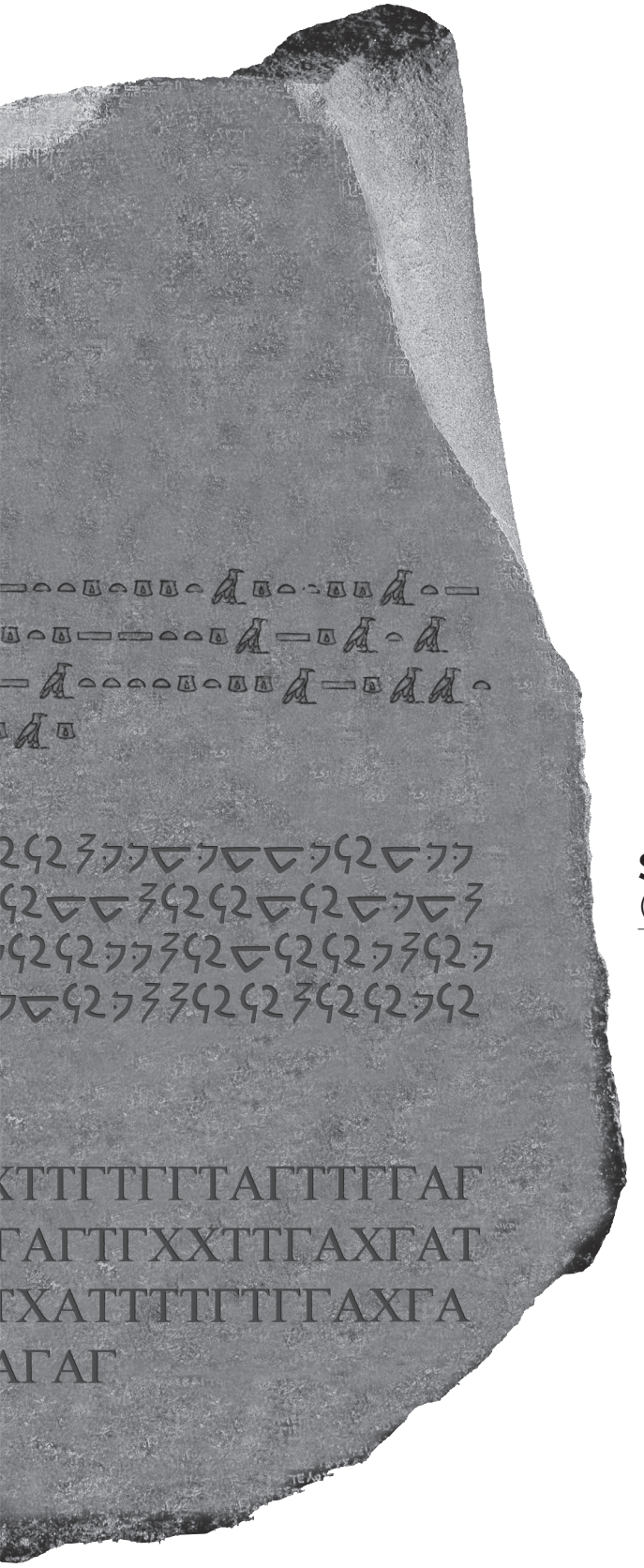
Besides mutational activation of the KRAS oncogene, overexpression of cyclooxygenase-2 (COX-2) contributes to CRC development. Whether these events are related was unclear. In **chapter 6** we have investigated the relationship between oncogenic KRAS mutations and COX-2 overexpression *in vitro*. COX-2 overexpression and prostaglandin E2 production in C26 cells was dependent on mutant KRAS. *In vivo*, we have used selective COX-2 inhibitors to assess the contribution of KRAS-dependent COX-2 to the seeding of CRC cells in the liver and to their outgrowth as liver metastases by *in vivo* microscopy and bioluminescence imaging, respectively. Inhibition of host or tumor cell COX-2 activity had no effect on early metastatic cell seeding in the liver but greatly reduced intrahepatic tumor cell proliferation and the rate of liver metastasis outgrowth. We have not found any effect of COX-2 inhibition on early tumor vascularization or on tumor cell apoptosis.

RAS mutations (especially HRAS) are also observed in bladder carcinomas. As in CRC, cyclooxygenase-2 (COX-2) overexpression is frequently observed in bladder carcinoma and has been correlated with an increased disease stage and with reduced patient survival. In **chapter 7** we have evaluated the efficacy of a selective COX-2 inhibitor as a treatment modality for bladder carcinoma. In an orthotopic mouse model for experimental bladder carcinoma we have used BLI to evaluate the therapeutic effect. We have demonstrated that selective COX-2 inhibition reduced the outgrowth of experimental human bladder carcinoma by inhibition of tumor cell proliferation, rather than by inhibition of angiogenesis or tumor cell survival.

In **chapters 4 and 5** we have demonstrated that late stage metastatic CRC cells are dependent on mutant KRAS. Therefore, KRAS may be an interesting therapeutic target in metastatic CRC. Several KRAS-directed therapeutics have been developed and are currently being evaluated for their efficacy. One of these therapies that has great potential is reovirus T3D. Reovirus T3D is a non-pathogenic oncolytic virus that preferentially targets tumor cells expressing an activated RAS oncogene. In **chapters 8 and 9** we have investigated the underlying mechanism of mutant RAS-specific reovirus-induced oncolysis in both mouse and human CRC cells. We have shown that reovirus T3D protein synthesis and virus yields in these cells were independent of KRAS activity. Rather, upon deletion of the mutant KRAS allele, reovirus T3D-induced tumor cell apoptosis was completely abrogated. We conclude that oncolysis of mouse and human CRC cells by reovirus T3D is not determined by the extent of virus replication but by their sensitivity to apoptosis induction. Oncogenic KRAS increases CRC cell sensitivity to reovirus T3D-induced apoptosis without affecting reovirus T3D replication.

In **chapter 10** we have investigated the therapeutic potential of reovirus T3D against established colorectal liver metastases *in vivo*. We have shown that the outgrowth of an established isolated CRC liver metastasis in syngenic immuno-competent mice was efficiently but temporarily inhibited with a single intralesional injection of reovirus T3D. The efficacy of virus-based therapies in immunocompetent hosts may be hampered by immune-mediated clearance of the therapeutic virus. Therefore we have also assessed the effect of immunosuppression with cyclosporin A on reovirus oncolysis in this model. We have shown that immunosuppression markedly increased and prolonged the effect of a single intratumoral reovirus T3D injection against established isolated liver metastases *in vivo*. Whether immunosuppression in conjunction with reovirus T3D therapy is applicable and will exert beneficiary effects in cancer patients in daily practice remains to be determined.

In **chapter 11** we have evaluated the susceptibility of freshly resected human CRC liver metastases to reovirus T3D infection. None of the 13 tested CRC specimens displayed reovirus T3D protein synthesis or oncolysis. The lack of infection could not be attributed to selective loss of tumor cell viability or to absence of oncogenic RAS mutations, but was associated with the absence of reovirus receptor JAM-1 on the tumor cell plasma membrane. JAM-1 mislocalization may be a significant hindrance for the therapeutic application of wild-type reovirus T3D in the treatment of metastatic CRC.



Summary in Dutch

(Nederlandse samenvatting voor leken)

Het menselijk lichaam bestaat uit een groot aantal organen en weefsels die zijn opgebouwd uit miljarden cellen. In de kern van deze cellen bevindt zich het erfelijk materiaal, het DNA. Het DNA bevat de code waarin alle erfelijke eigenschappen zijn vastgelegd. Een stukje DNA dat één zo'n erfelijke eigenschap bevat, wordt een gen genoemd. Elk gen bevat de code voor één van de vele eiwitten waaruit het lichaam is opgebouwd. Dit kunnen 'bouwstoffen' zijn, maar ook bijvoorbeeld hormonen en enzymen (deze maken de meeste chemische reacties in het lichaam mogelijk). Samen bepalen de genen alle erfelijke eigenschappen. In iedere cel treden er continu beschadigingen in het DNA op. Gelukkig heeft de cel vele verdedigings- en reparatiemechanismen om de beschadigingen te verhelpen. Als het herstelmechanisme faalt en als de betreffende mutatie ook nog op een zeer belangrijke plek in het DNA zit dan kan er kanker ontstaan.

Het RAS gen is zo'n belangrijke plek in het DNA. Het RAS gen bevat de genetische code voor een eiwit dat betrokken is bij de signaaloverdracht van groeistimulerende factoren. Als gevolg van een aantal specifieke mutaties in het RAS gen vindt er zelfs in de afwezigheid van deze groeistimulerende factoren een voortdurende overdracht van signalen plaats hetgeen resulteert in ontremde celding. Er zijn 3 verschillende RAS genen in het menselijk DNA: HRAS, KRAS en NRAS. In 30% van alle menselijke tumoren worden mutaties gevonden in een van de RAS genen. In welk RAS gen de mutatie wordt gevonden en in welk percentage hangt onder andere af van het tumor type. In blaaskanker worden bijvoorbeeld met name mutaties in HRAS gevonden en slechts 10% van alle kwaadaardige blaastumoren heeft een mutatie in HRAS. Bij alvleesklierkanker worden alleen mutaties in KRAS gevonden, in ongeveer 90% van alle alvleeskliertumoren. Bij dikkedarmkanker worden de mutaties ook alleen in KRAS gevonden, in ongeveer 40% van de gevallen.

De dikke darm is het laatste deel van het spijsverteringskanaal. Dikkedarmkanker is de nummer 2 doodsoorzaak onder alle kankerpatiënten. Per jaar wordt er in Nederland bij ongeveer 9.200 mensen dikkedarmkanker vastgesteld en 4.300 patiënten overlijden hieraan. Patiënten overlijden aan dikkedarmkanker over het algemeen nadat er uitzaaiingen zijn opgetreden. Dikkedarmkanker zaait met name uit naar de lever. Indien er leveruitzaaiingen aanwezig zijn, is tot op heden de enige mogelijkheid op genezing een operatie waarbij de uitzaaiing verwijderd wordt. Patiënten met veel uitzaaiingen of met een uitzaaiing welke gelegen is vlakbij een belangrijke structuur zoals een groot bloedvat, zijn ongeschikt voor operatie. Hierdoor komt uiteindelijk slechts 10% van alle patiënten in aanmerking voor een operatie. Het is duidelijk dat er grote behoefte is aan nieuwe therapieën.

Normale chemotherapie richt zich op het doden van snel delende cellen. Snel delende tumorcellen zijn daarom buitengewoon gevoelig voor chemotherapie. Maar ook normale cellen die snel delen, zoals bloedcellen en cellen in het maag/darmkanaal zullen doodgemaakt worden. Hierdoor ontstaan bijwerkingen zoals bloedarmoede en misselijkheid. Daarnaast is het probleem dat veelal een deel van de tumorcellen ongevoelig voor de behandeling is of dit wordt tijdens de behande-

ling. Deze resistente kankercellen zullen de therapie overleven en vervolgens weer uitgroeien tot nieuwe tumoren. Hierdoor komt de kanker vaak weer terug. Meer tumorspecifieke therapieën zouden dan ook een uitkomst kunnen bieden. Omdat normale cellen dan niet gedood zullen worden, zou een dergelijk tumor-specifiek geneesmiddel veel hoger gedoseerd kunnen worden. Door verschillende tumor-specifieke therapieën te combineren zou het uiteindelijke succespercentage veel hoger en de kans op resistentie veel kleiner worden. **Hoofdstuk 1** geeft een korte introductie over de recente ontwikkeling van tumorspecifieke therapieën, het voorkomen en de behandeling van dikkedarmkanker, de eigenschappen en functies van de RAS eiwitten en de ontwikkeling van RAS-specifieke therapieën.

Aangezien KRAS in vele menselijke dikkedarmtumoren gemuteerd is, lijkt het een voor de hand liggend aangrijpingspunt voor kankertherapie. KRAS mutaties ontstaan echter al in het voorstadium van dikkedarmkanker. Daarna treden nog vele mutaties in andere genen op voordat uiteindelijk dikkedarmkanker en eventuele uitzaaiingen kunnen ontstaan. Het is dan ook van belang om er achter te komen of, als de tumor ontdekt wordt (dit is per definitie in een "genetisch" laat stadium), tumorgroei nog steeds afhankelijk van het gemuteerde KRAS gen is. Het KRAS gen zou ook alleen belangrijk kunnen zijn bij het ontstaan van dikkedarmkanker, waarna het geen rol van betekenis meer zou kunnen spelen. In het eerste deel van dit proefschrift hebben we getracht deze vraag te beantwoorden (**hoofdstuk 2, 4 en 5**). Hiervoor hebben we gebruik gemaakt van een zeer agressieve muizen dikkedarmkanker cellijn welke uit een muizen dikkedarmtumor geïsoleerd is. Deze tumorcellen kunnen buiten de muis in het laboratorium in leven worden gehouden. Het DNA van deze cellen hebben we onderzocht en deze cellijn bleek een bij de mens veelvuldig voorkomende mutatie in het KRAS gen te hebben. Indien deze kankercellen vlak onder de huid van muizen gespoten worden, ontstaan er razendsnel (binnen 12 dagen) grote onderhuidse tumoren. Met een nieuwe moleculaire methode hebben we het gemuteerde KRAS gen uitgeschakeld in deze tumorcellijn en hebben we wederom deze cellen onderhuids bij de muizen ingespoten. Na het uitschakelen van het KRAS gen konden deze cellen nog in slechts 40% van de ingespoten muizen uitgroeien tot een zichtbare tumor. Daarnaast duurde het vele malen langer voordat deze tumoren begonnen te groeien en de muizen leken er geen enkele hinder van te ondervinden. Opmerkelijk genoeg verdwenen na enige tijd reeds ontstane tumoren in 30% van deze muizen. Dit bleek te berusten op het feit dat na het uitschakelen van het KRAS gen het immuunsysteem van de muis deze tumorcellen wist op te ruimen. Met een nieuwe methode genaamd bioluminescentie imaging (BLI) hebben we het effect van mutant KRAS op het ontstaan van leveruitzaaiingen onderzocht. Met BLI kunnen tumorcellen in de lever van een intacte muis zichtbaar worden gemaakt (zonder dat de muis hoeft te worden geopereerd). Deze methode hebben we eerst gevalideerd in **hoofdstuk 3**. In **hoofdstuk 5** hebben we aangetoond dat door het uitschakelen van het KRAS gen deze tumorcellen geen uitzaaiingen meer in de lever kunnen veroorzaken. Kortom door het uitschakelen van het KRAS

gen kunnen deze zeer agressieve tumorcellen nauwelijks nog uitgroeien, niet meer uitzaaien naar de lever en zijn ze in staat om het immuunsysteem te activeren. Het lijkt er dus op dat het KRAS gen vanuit therapeutisch oogpunt een aantrekkelijk aangrijpingspunt is om een therapie tegen te ontwikkelen. Een volgende stap moet echter zijn om in menselijke dikkedarmkanker en leveruitzaaiingen deze bevindingen te bevestigen.

Gemuteerd RAS zorgt voor ontremde celgroei (en dus tumorgroei) door het activeren van vele andere eiwitten en processen in de cel. Een van die eiwitten die door RAS geactiveerd wordt, is COX-2. Recent is aangetoond dat geneesmiddelen die COX-2 remmen, het ontstaan van dikkedarmtumoren kunnen verminderen bij mensen met een erfelijke aanleg voor dikkedarmkanker op jonge leeftijd. Het was echter onduidelijk of reeds gevormde tumoren en/of uitzaaiingen gevoelig zijn voor behandeling met deze zelfde COX-2 remmers. In **hoofdstuk 6 en 7** hebben we dit in een muizenmodel van leveruitzaaiingen van dikkedarmkanker en blaaskanker getest. Het blijkt dat de tumoren minder hard groeien indien de muizen behandeld worden met COX-2 remmers. Dit lijkt met name te berusten op het feit dat de tumorcellen zich minder snel delen. Of deze middelen ook in patiënten effectief zullen zijn, moet verder onderzocht worden.

Reovirus is een voor gezonde mensen onschuldig virus. Reovirus komt veelvuldig voor en bijna 100% van alle mensen hebben aantoonbaar ooit wel eens een infectie met reovirus doorgemaakt. Infecties met reovirus verlopen onopgemerkt aangezien het niet met ziekteverschijnselen gepaard gaat. Recent is ontdekt dat tumorcellen met een gemuteerd RAS gen buitengewoon gevoelig zijn voor reovirus. Aangezien gezonde cellen niet door reovirus gedood worden, zou reovirus als geneesmiddel tegen dikkedarmkanker veelbelovend kunnen zijn zonder belangrijke bijwerkingen. Reovirus treedt de cel binnen na binding aan de specifieke reovirus receptor JAM-1. Deze JAM-1 receptor staat als een vlaggenstok op de celmembraan, het omhulsel van iedere cel. In het tweede deel van het proefschrift hebben we onderzocht hoe reovirus dikkedarmkanker cellen met mutaties in het RAS gen specifiek kan doden. Vooralsnog werd verondersteld dat reovirus zich enkel in tumorcellen met mutant RAS kan vermenigvuldigen en daardoor deze cellen dood maakt. Wij tonen in **hoofdstuk 8 en 9** aan dat reovirus zich goed kan vermenigvuldigen in kankercellen onafhankelijk van de aanwezigheid van mutant RAS. De kankercellen met mutant KRAS bleken echter veel gevoeliger voor reovirus geïnduceerde celdood. Dit kan belangrijke consequenties hebben voor de eventuele ontwikkeling van combinatiestrategieën met andere anti-kanker middelen.

Een potentieel probleem voor het gebruik van reovirus als kankertherapie is het feit dat bijna iedereen ooit een infectie met reovirus heeft doorgemaakt en daardoor reeds antistoffen tegen dit virus heeft aangemaakt. Deze antistoffen zouden reovirus mogelijk uit kunnen schakelen voordat het zijn therapeutische effect kan uitoefenen. De rol van het immuunsysteem hebben we onderzocht in **hoofdstuk 10**. Hier hebben we onderzocht of leveruitzaaiingen van dikkedarmkanker in de muis

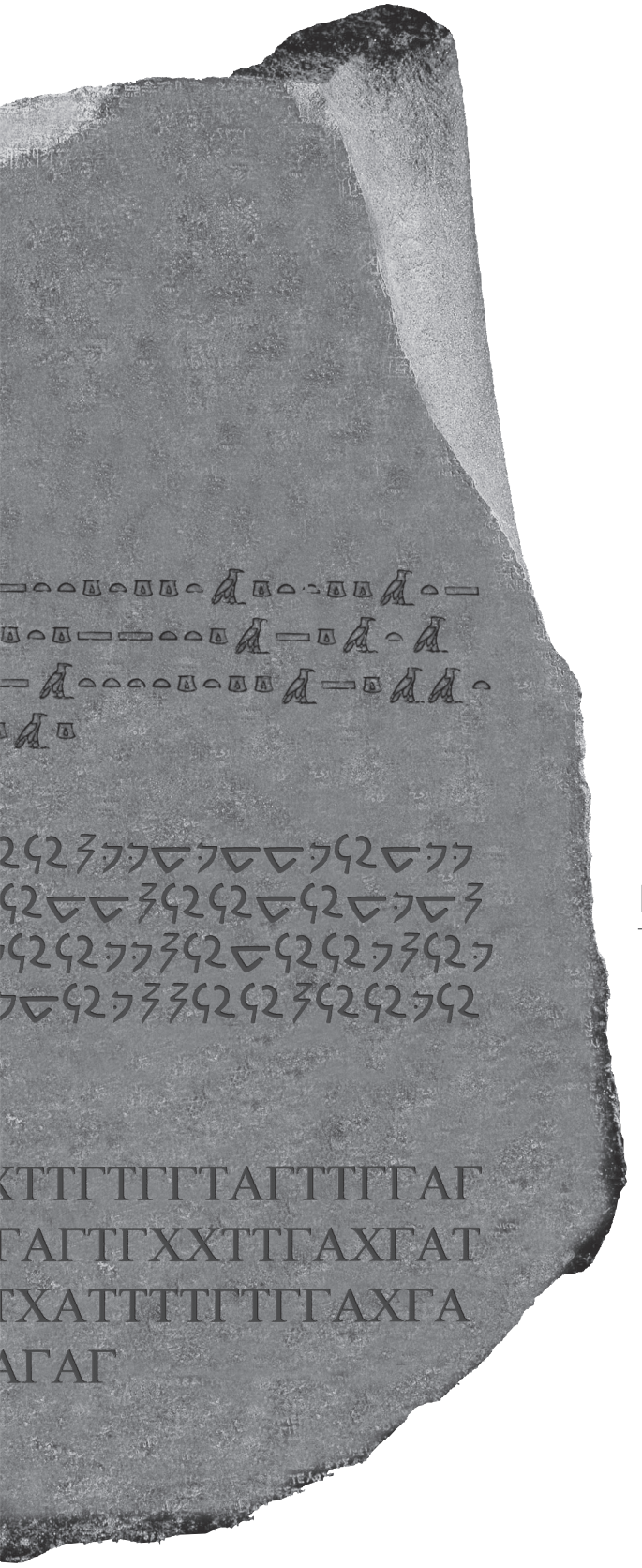
gevoelig zijn voor reovirus therapie. Reovirus bleek een sterk maar slechts tijdelijk anti-tumor effect te hebben. Omdat mogelijk een immuun respons tegen reovirus de reden was van de korte effectsduur, hebben we tevens onderzocht of het uitschakelen van het immuunsysteem de respons kan verbeteren. Het geneesmiddel cyclosporine A zorgt voor een (gedeeltelijke) uitschakeling van het immuunsysteem. Door toediening van cyclosporine A werd het anti-tumor effect van reovirus versterkt en hield het langer aan. Of deze bevindingen ook opgaan en toepasbaar zijn in patiënten dient nader onderzocht te worden.

In **hoofdstuk 11** hebben we onderzocht of leveruitzaaiingen van dikkedarmkanker van de mens infecteerbaar zijn met reovirus. Hiertoe hebben we direct nadat een leveruitzaaiing door de chirurg verwijderd was, enkele tumorfragmentjes in het laboratorium in leven gehouden en onderzocht op gevoeligheid voor reovirus infectie. Van de 13 onderzochte leveruitzaaiingen was geen van de tumoren infecteerbaar met reovirus, terwijl een blaastumor wel infecteerbaar bleek. De receptor voor reovirus, JAM-1, staat in normaal dikkedarmweefsel buiten op de celmembraan. Echter, in dikkedarmkanker en leveruitzaaiingen bleek JAM-1, niet meer buiten op de celmembraan te staan, maar was het aantoonbaar binnen in de cel waar het 'onzichtbaar' is voor reovirus. Het is waarschijnlijk dat de verkeerde localisatie van JAM-1 bijdraagt aan de geobserveerde ongevoeligheid van tumor weefsel voor reovirus. Bovendien vormt het een probleem voor de direkte toepasbaarheid van reovirus als therapie tegen deze tumoren. Om dit probleem op te lossen dient nader onderzoek verricht te worden om bijvoorbeeld het virus genetisch te modificeren waardoor het via een andere receptor of op een andere manier de tumorcellen binnen zal kunnen dringen.

Concluderend hebben we aangetoond dat dikkedarmkankercellen afhankelijk zijn van gemuteerd KRAS om uit te kunnen zaaien naar de lever en om te ontsnappen aan een anti-tumor immuun respons. Dit suggereert dat het KRAS gen een belangrijk en aantrekkelijk aangrijpingspunt is voor therapie tegen (uitgezaaide) dikkedarmkanker.

COX-2 wordt direct gereguleerd door mutant KRAS en selectieve COX-2 remmers kunnen belangrijk zijn in de behandeling van uitgezaaide dikkedarmkanker en blaaskanker.

De aanwezigheid van mutant KRAS maakt kankercellen gevoeliger voor reovirus geïnduceerde celdood. Het therapeutisch effect van reovirus tegen leveruitzaaiingen van dikkedarmkanker wordt beperkt door een immuun respons gericht tegen het virus. Onderdrukking van het immuun systeem kan het therapeutisch effect van reovirus versterken en verlengen. De mislokalisatie van de reovirus receptor JAM-1 in dikkedarmkanker van de mens kan een obstakel vormen voor direct gebruik van reovirus in de kliniek. Om dit probleem op te lossen dient nader onderzoek te worden verricht.

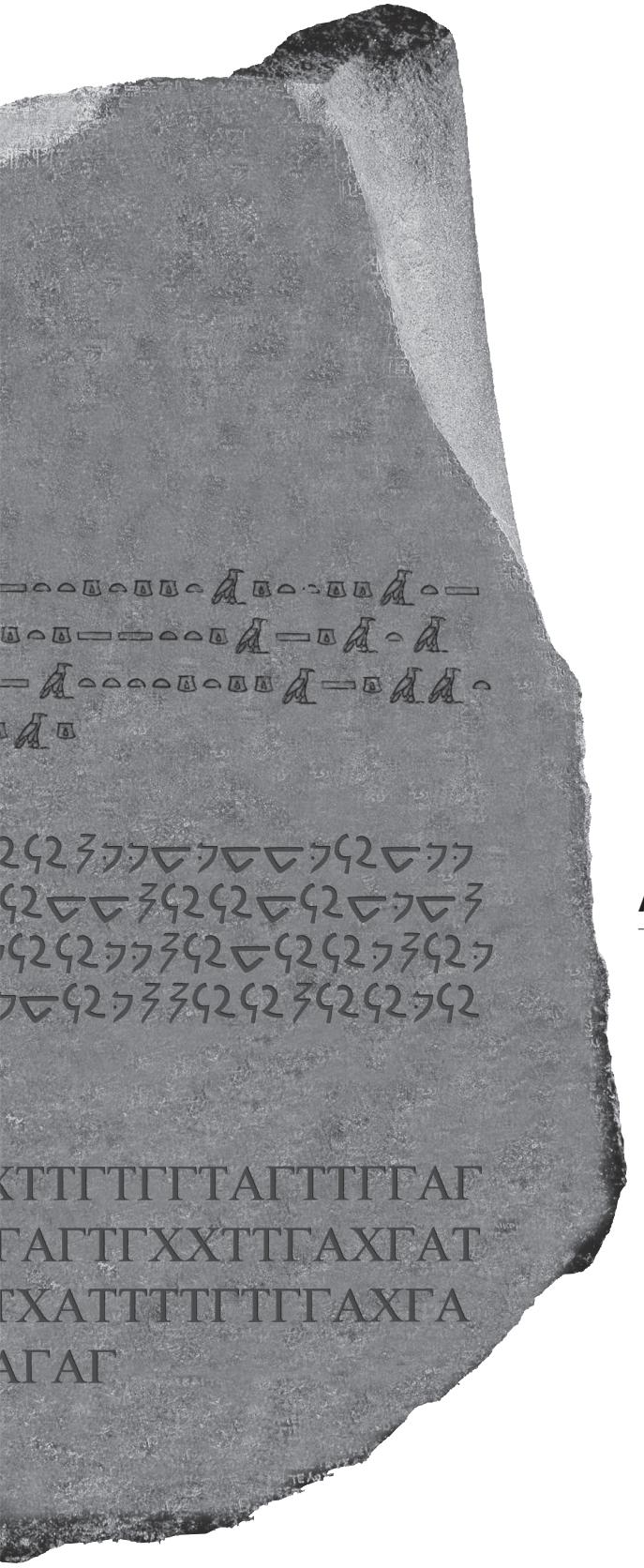


List of abbreviations

5-FU	5-fluorouracil
ACF	aberrant crypt foci
AJ	adherens junction
AKT	protein kinase B
APC	adenomatous polyposis coli
APM	antigen processing machinery
ASH	allele-specific hybridization
ASO	antisense oligonucleotide
BLI	bioluminescence imaging
BM	basement membrane
CCR	complete cytogenetic response
cdk4	cyclin-dependent kinase 4
cDNA	complementary DNA
CEA	carcino-embryonic antigen
CFSE	carboxyfluorescein succinyl ester
CK20	cytokeratine 20
CML	chronic myeloid leukemia
COX	cyclooxygenase
CRC	colorectal carcinoma
CsCl	caesium chloride
CTL	cytotoxic T-lymphocyte
CyA	cyclosporin A
DFS	disease-free survival
DMEM	dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid ribonucleic acid
DR5	death receptor 5
dsRNA	double-stranded RNA
ECM	extracellular matrix
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
EIA	enzyme immuno assay
ELISA	enzyme linked immunosorbent assay
EMT	epithelial-mesenchymal transition
FACS	fluorescence-activated cell sorter
FasL	Fas Ligand
FCS	fetal calf serum
FTI	farnesyltransferase inhibitor
GEF	guanine nucleotide exchange factor
GST	glutathione-S-transferase
H&E	haematoxylin and eosin

HLA	human leukocyte antigen
hpf	high power fields
HRA	hepatic replacement area
HRAS	Harvey RAS
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cell
IC50	inhibitory concentration 50%
IHC	immunohistochemistry
IL-6	interleukin 6
IVM	intravital microscopy
JAM-1	junctional adhesion molecule-1
KRAS	Kirsten RAS
KrasKD	Kras knockdown
LMP	latent membrane protein
LNA	β-D-locked nucleic acid
LV	leucovorin
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated extracellular signal regulated kinase kinase
MHC	major histocompatibility complex
MMP	matrix metalloprotease
MRI	magnetic resonance imaging
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide-thiazolyl blue
MVD	microvessel density
NK cell	natural killer cell
NMU	N-nitroso-N-methylurethane
NRAS	neuroblastoma RAS
NSAID	non-steroidal anti-inflammatory drugs
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PG	prostaglandin
PI3K	phosphatidyl inositol 3-kinase
PKB	protein kinase B
PKC	protein kinase C
PKR	double-stranded RNA-dependent protein kinase
RAS	rat sarcoma
RBD	RAS-binding domain
RFLP	restriction fragment length polymorphism

RISC	RNA-induced silencing complex
RNAi	RNA interference
RT-PCR	reverse transcriptase-polymerase chain reaction
SCID	severe combined immunodeficiency
SEM	standard error of the mean
SFRP	secreted frizzled related protein
shRNA	short hairpin RNA
siRNA	short interfering RNA
SSCP	single strand conformational polymorphism
T1L	type 1 Lang
T2J	type 2 Jones
T3D	type 3 Dearing
TAP	transporter associated with antigen processing
TCC	transitional cell carcinoma
TGFβ	transforming growth factor-beta
TIL	tumor-infiltrating lymphocytes,
TMA	tissue microarray
TNFα	tumor necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand
TTGE	transient temperature gradient gel electrophoresis
TUNEL	terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling
uPA	urokinase plasminogen activator
VEGF	vascular endothelial growth factor
vWF	von Willebrand Factor
z-VAD	Z-Val-Ala-Asp (pan-caspase inhibitor)



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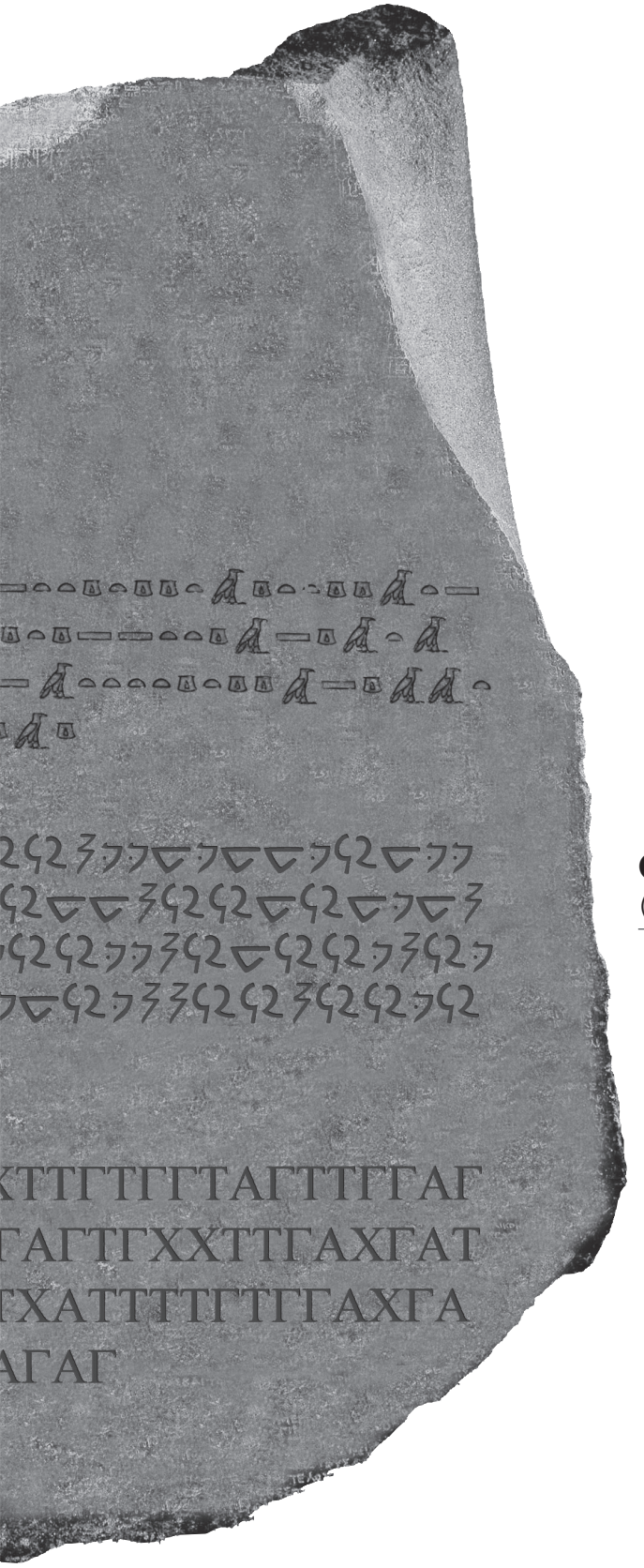
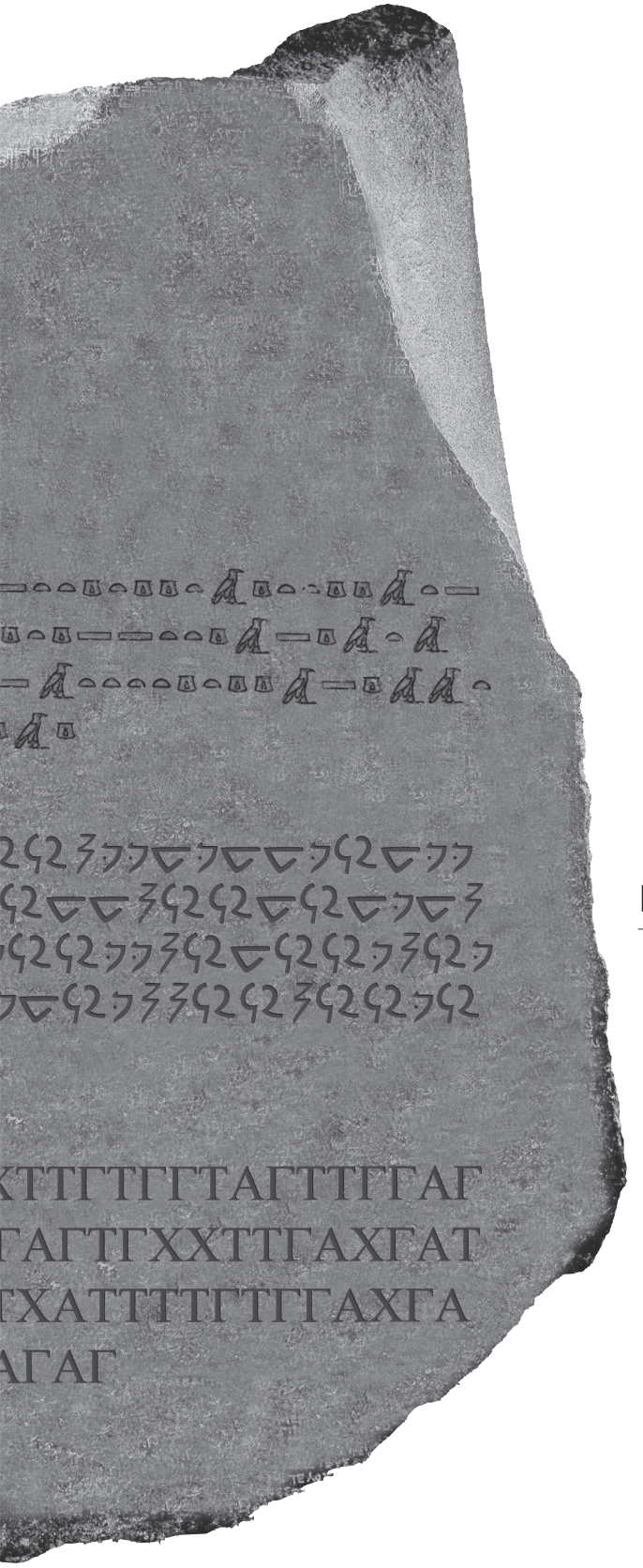


Curriculum Vitae

(auctoris)

Niels Smakman was born on October 7, 1975, in Amsterdam, the Netherlands. He graduated from the Utrecht Stedelijk Gymnasium in 1994. Next, he wanted to start medical school, but in the Netherlands, medical students are selected by means of a lottery. It took him two years to win this medical jackpot. During this two year period he studied Medical Biology at the University of Amsterdam for one year. In the second year he moved to Belgium, where he studied medical school for one year at the University of Antwerp. Then he took the hurdle of the numerus fixus in the Netherlands. He moved back to Utrecht and started medical school in 1996. As a student, he was the founder and manager of the "Medical Student Teams" at the University Medical Center Utrecht. With this in-hospital temping agency for medical students, he was the direct supervisor of 76 employees. He obtained his doctorate degree in medicine (*cum laude*) in 2000. He performed his research elective at the Department of Medical Genetics at the University Medical Center Utrecht, and his clinical elective at the trauma-unit at the Groote Schuur Hospital in Cape Town, South-Africa. He graduated from medical school (*cum laude*) in 2002.

He then started the research described in this thesis. This was made possible by financial support of the Wijnand M. Pon foundation. He and the work described in this thesis has been awarded with the Scholar-in-training Award at the 96th Annual Meeting of the American Association for Cancer Research in Anaheim CA, USA and with the best oral presentation at the 6th Congress of the European Hepato-Pancreato-Biliary Association Meeting in Heidelberg, Germany. In 2006 he started the residency program in general surgery, region Utrecht (Head: Prof. Dr. I.H.M. Borel Rinkes) at the Diakonessenhuis Hospital in Utrecht (Dr. G.J. Clevers).



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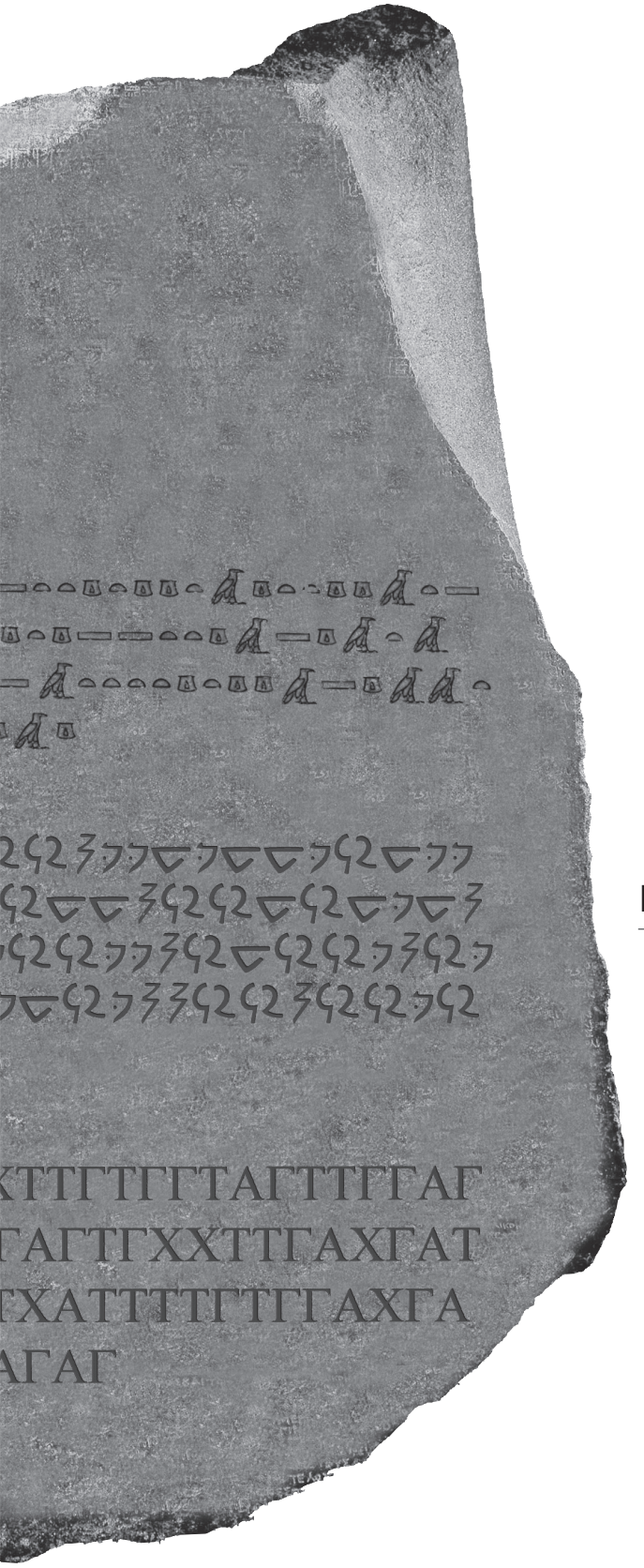

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