SPARC-like 1 (SPARCL1), a gene downregulated in non-small cell lung cancer

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Introduction

Cancer: a general introduction

In 2001 cancers were the third prevalent cause of death (12.6%) in all WHO regions (according to the annex table 2 of the world health report 2002, http://www.who.int/whr/en). Only cardiovascular diseases (29.3%) and infectious & parasitic diseases (19.3%) were causing more deaths.

Cell growth is a carefully regulated process that responds to specific needs of the body. Very occasionally, the exquisite controls that regulate cell multiplication break down and a cell begins to grow and divide, although the body has no need for further cells of its type. When the descendants of such a cell inherit the propensity to grow without responding to regulation, the result is a clone of cells able to expand indefinitely. Ultimately, a mass called a tumor may be formed by this clone of unwanted cells (Lodish et al., 1995). As long as these cells remain clustered together the tumor is said to be benign and a complete cure can usually be achieved by removing the mass surgically. A tumor is counted as a cancer only if it is malignant, meaning that its cells have the ability to invade surrounding tissue and eventually metastasize to distant organs (Alberts et al., 1994). The incidence and the mortality rate differ for each kind of cancer. For example, liver cancer is often lethal, whereas prostate cancer can frequently be cured (Friedmann et al., 1984). Carcinomas are by far the most common forms of cancer; they originate from epithelial cells, which line the body cavities and form the outer layer of the skin (Weinberg, 1996).

The tumor development occurs in different stages: First a resting cell within a normal population undergoes a genetic mutation that increases its ability to proliferate (genetically altered cell). The altered cell and its descendants look normal, but they have grown to a cell cluster. Years later, one in a million of these cells acquires another mutation that further loosens controls on cell growth (hyperplasia). Dysplastic cells still show signs of differentiation, but the extend is incomplete, and the proliferating cells are seen abnormally far above the basal lamina (dysplasia). The carcinoma in situ cells are proliferating and apparently undifferentiated in all the layers that are bordered by the basal lamina (carcinoma in situ).

If the genetic changes allow the tumor to cross the basal lamina to invade the underlying tissue and shed cells into the blood or lymph fluid, the cancer is regarded as invasive. The renegade cells are likely to establish new tumors (metastases) throughout the body leading to death by disrupting vital organ function (invasive cancer) (Alberts et al., 1994; Weinberg, 1996).

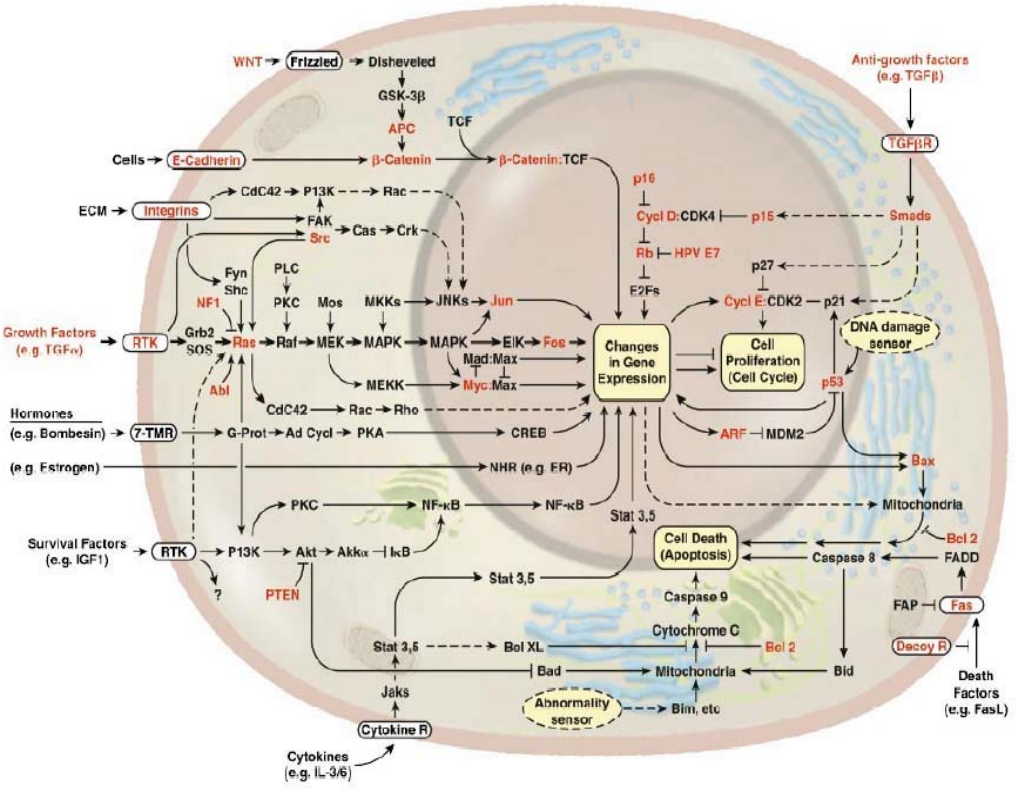
Hanahan and Weinberg suggest that six essential alterations in cell physiology have to occur for malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). All these properties reflect alterations in the cellular signaling pathways that in normal cells control cell proliferation, motility, and survival (Martin, 2003).

- Self-sufficiency in growth signals: Normal cells require mitogenic growth signals before they can move from a quiescent state into an active proliferative state. These signals are transmitted into the cell by transmembrane receptors that bind distinctive classes of signaling molecules: diffusible growth factors, extracellular matrix components, and cell-to-cell adhesion/interaction molecules. No type of normal cell can proliferate in the absence of such stimulatory signals. Many of the oncogenes act by mimicking normal growth signaling in one way or another (Hanahan and Weinberg, 2000).
- Insensitivity to growth-inhibitory signals: Within a normal tissue, multiple antiproliferative signals operate to maintain cellular quiescence and tissue homeostasis; these signals include both soluble growth inhibitors and immobilized inhibitors embedded in the extracellular matrix and on the surfaces of nearby cells. Cancer cells must evade these antiproliferative signals if they want to succeed. At the molecular level, many and perhaps all antiproliferative signals are funneled through the retinoblastoma protein and its two relatives, p107 and p130 (Hanahan and Weinberg, 2000).
- Evasion of programmed cell death: Apoptosis, a physiological cell death program that controls normal cell numbers during development and disease, is disabled in tumors. Altered expression or mutation of genes encoding key apoptotic proteins can provide cancer cells with both an intrinsic survival advantage and inherent resistance to chemotherapeutic drugs (Johnstone et al., 2002).

- Limitless replicative potential: Telomeres control the total number of replicative generations of cells. The initiation of senescence and crisis depends upon correct telomere length. In the cancerous cell, however, the telomere-based counting system has completely failed and therefore the excessive proliferation cannot be aborted anymore (Weinberg, 1996).
- Sustained angiogenesis: Angiogenesis is a fundamental process by which new blood vessels are formed and is therefore essential in reproduction, development, and wound repair. Folkman et al. found that tumor growth and metastasis are angiogenesis dependent (Folkman and Shing, 1992).
- Tissue invasion and metastasis: Whereas normal cells are anchorage dependent that is mediated by cell-surface molecules, cancer cells are anchorage independent. To metastasize successfully, cancer cells have to detach from their original location, invade a blood or lymphatic vessel, arrest at the distant vascular bed, extravasate into the target organ interstitium and parenchyma, and proliferate as a secondary colony. Cancer cells do invade by releasing degradative enzymes (metalloproteinases) that dissolve basement membranes and other extracellular matrices. Patterns of metastasis can be explained in part by the architecture of the circulatory system (Ruoslahti, 1996).

Many growth factors and their receptors, as well as their cytoplasmic and nuclear downstream effectors have been identified as oncogenes or tumor suppressor genes (Malumbres and Barbacid, 2001).

Proto-oncogenes regulate the cascade of events that maintains the ordered progression through the cell cycle, cell division, and differentiation. A mutation within a proto-oncogene makes a stimulatory gene hyperactive. The altered gene is called oncogene and has a dominant effect: only one allele needs to undergo the change. The oncogenic effect is generated by point mutation, chromosomal rearrangement, or gene amplification of the proto-oncogene sequence. In general, tumor suppressor genes function in growth regulatory or differentiation pathways and loss of their function contributes directly to the altered phenotype of cancer cells (Vogelstein and Kinzler, 1998). In contrast to proto-oncogenes, both alleles of a tumor suppressor gene need to have loss-of-function (inactivating) mutations in cancer cells (Fearon, 1997). DNA methylation of tumor suppressor gene is also a frequent mechanism of transcriptional silencing in cancer.



Signaling pathways involved in cancer (from D. Hanahan and R. A. Weinberg, Cell, Vol. 100, 57–70):
In this scheme genes, known to be altered in cancer, are highlighted in red.

Cancer cells present global hypomethylation of the genome and hypermethylation of islands of CpG dinucleotide clusters within specific DNA regions (Di Croce et al., 2002).

In individuals with inactivating germline mutations in a tumor suppressor allele, inactivation of the remaining allele often occurs in the cancer via loss of heterozygosity (LOH) (Fearon, 1997).

The functions of some proto-oncogenes and tumor suppressor genes (Vogelstein and Kinzler, 1998):

function:	examples:
proto-oncogenes:	
tyrosine-specific protein kinases (receptor)	PDGF receptor, EGF receptor
tyrosine-specific protein kinases (non-receptor)	fes, src, BCR/ABL
serine/threonine-specific protein kinases	raf
GTP-binding proteins	ras proteins
growth factors	PDGF, EGF, FGF
nuclear proteins	myc, fos, jun, ets
tumor suppressor genes:	
transcription factor	p53
transcriptional repressor	Rb, WT-1
regulation of β-catenin degradation	APC
cyclin-dependent kinase inhibitor	p16

A relatively small subset of mutations (in proto-oncogenes, tumor suppressor genes, or DNA repair genes) is present in the germline of individuals and predisposes them to cancer. The vast majority of mutations that contribute to the development of cancer cells are somatic and only present in the neoplastic cells of the patient (Vogelstein and Kinzler, 1998).

A link between DNA repair systems and carcinogenesis is suggested by the finding that humans with inherited genetic defects that make specific repair systems non-functional have an enormously increased probability of developing certain cancers (e.g. Xeroderma pigmentosum, hereditary non-polyposis colon carcinoma) (Lodish et al., 1995).

The mutation rate within a cell is affected by mutagens in the environment and/or by defects in the cellular DNA repair and replication machinery. A correlation between mutagenesis (the production of a change in the DNA sequence) and carcinogenesis (the generation of cancer) has been clarified for the following three classes of agents:

- Chemical carcinogens such as dimethylbenzanthracene (DMBA), which typically cause changes in the nucleotide sequence. Some of the carcinogens act directly on the target cell; many others take effect only after they have been changed to a more reactive form by metabolic processes – notably by a set of intracellular enzymes known as the cytochrome P-450 oxidases.
- Ionizing radiation such as X-rays which typically cause chromosome breaks and translocations.
- Viruses, which introduce foreign oncogenes into the cell. There are two ways in which a proto-oncogene can be converted into an oncogene upon incorporation into a retrovirus: the gene sequence may be altered or truncated so that it codes for a protein with abnormal activity, or the gene may be brought under the control of powerful promoters and enhancers in the viral genome that cause its product to be made in excess or in inappropriate circumstances. (Alberts et al., 1994).

Lung Cancer

Lung cancer is the leading cause of cancer related death for both men and women in the United States and the solid tumors with the most defined relationship to a known environmental cause (Parker et al., 1996). 90% of all patients with lung tumors develop their disease because of exposure to tobacco products, and in most instances through cigarette smoking. Fortunately only 10% of all smokers at risk develop lung carcinomas despite of the fact that virtually all of these individuals have a degree of preneoplastic changes in their bronchial epithelium. These data and the average for diagnosis of 60 years suggest that the development of lung cancer occurs over a period of 20-30 years and involves multiple changes.

The clinical and biological aspects of this disease are complex as four major histological cancer types, derived from the bronchial epithelium, can be distinguished:

- Squamous cell carcinoma
- Adenocarcinoma
- Large cell undifferentiated carcinoma
- Small cell carcinoma

Large cell undifferentiated carcinomas, squamous cell carcinomas, and adenocarcinomas are collectively known as non-small cell lung cancer (NSCLC). NSCLCs comprise approximately 75% of all lung tumors. They generally metastasize later than small cell lung cancers (SCLCs) and often can be cured by surgery at an early tumor stage. Alterations in dominantly acting oncogenes (e.g. ras, myc, and cyclin D) and tumor suppressor genes (e.g. p53, p16, Rb, and FHIT-1) occur in lung carcinogenesis (Vogelstein and Kinzler, 1998).

The proteins encoded by normal **ras** genes transmit stimulatory signals from growth factor receptors to other proteins downstream in the signaling cascade. Proteins encoded by mutant ras genes, however, fire continuously, even when growth factor receptors are not prompting them. Hyperactive Ras proteins are found in about a quarter of all human tumors. Mutations in ras family genes occur frequently in lung cancer (K-ras and H-ras). In NSCLC, the presence of ras mutations has been reported to be a negative prognostic factor, especially in patients with adenocarcinomas (Vogelstein and Kinzler, 1998).

Oncogenes, such as those of the **myc** family, alter the activity of transcription factors in the nucleus. In many types of cancer, especially malignancies of the blood-forming tissues, Myc levels are kept constantly high even in the absence of growth factors (Vogelstein and Kinzler, 1998). Members of the myc family of oncogenes (c-myc, N-myc, and L-myc) can be activated in lung cancer, usually by gene amplification (Little et al., 1983).

Cyclin D is involved in traversing the G_1 cell cycle checkpoint for entry into S phase, at least in part by inactivating the Rb tumor suppressor protein. Thus cyclin D can act as an oncogene. Cyclin D1 is overexpressed in most cases of NSCLC (Vogelstein and Kinzler, 1998).

The tumor suppressor protein **p53** monitors the health of the cell, the integrity of its chromosomal DNA, and the successful completion of different steps in the cell cycle (Weinberg, 1996). Mutation of this gene is probably the best defined tumor suppressor gene change in lung cancer. The loss of gene function appears to correlate to the very frequent LOH that occurs on chromosome 17p13.1 in all lung cancer types. p53 mutations are obviously one of the most common genetic changes in all types of human cancer, and they have been found in 50 percent of NSCLC and 90 percent of SCLC tumors (Chiba et al., 1990).

By blocking the activity of the cyclin-dependent kinase partners of cyclin D **p16** prevents the advance of the cell from G_1 to S phase during the cell cycle (Weinberg, 1996). Alterations in p16 occur frequently in lung cancers, as they do in most common forms of human cancer (Kamb et al., 1994). This gene is a strong tumor suppressor candidate to account for LOH and homozygous deletions, which occur at chromosome region 9p21 in lung and other tumor types (Cairns et al., 1994). Loss of p16 function also occurs frequently via transcriptional silencing associated with abnormal DNA methylation of the transcription start site region (Merlo et al., 1995).

The tumor suppressor gene **Rb**, which plays a critical role in the cyclin D pathway for cell cycle control, is altered in nearly all SCLC and in many NSCLC tumors (Hensel et al., 1990; Xu et al., 1991).

Altered transcription splice products for the **FHIT-1** gene, located in a frequent region of homozygous deletion at 3p14, have recently been described as a frequent characteristic of lung cancers (Sozzi et al., 1996).

Hereditary aspects of lung cancer are less well understood than for other forms of solid tumors in humans. Unlike for breast, colon, or renal cancers, no distinct familial forms of the common types of lung cancer have been defined (Vogelstein and Kinzler, 1998). There are at least three genetic syndromes (Li-Fraumeni, retinoblastoma, and Bloom syndrome) that predispose to other forms of cancer in which, however, lung cancer may also occur:

The **Li-Fraumeni syndrome (LFS)** is caused by a germline mutation in the p53 tumor suppressor gene and causes tumor formation at multiple sites. A substantial fraction of lung cancers in LFS appear in non-smokers and young (< 45 years) patients (Lavigueur and Bernstein, 1991).

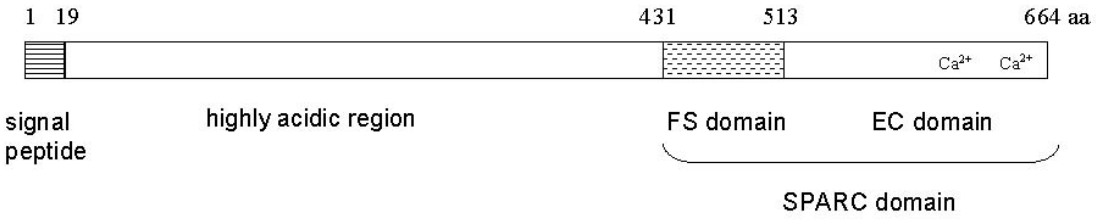
Patients harbouring an inactivating mutation in the retinoblastoma (**Rb**) gene on chromosome 13q commonly develop retinoblastoma and osteosarcoma. Primary relatives of bilateral retinoblastoma patients, many of whom are carriers of the mutation, have been reported to develop a variety of secondary cancers, including lung cancer, at relatively high frequency (Goodrich and Lee, 1990).

Bloom syndrome is an exceedingly rare recessive genetic disorder that is associated with defects in DNA repair. Leukemias and other cancers are quite common in this syndrome (German, 1993).

MAST9/hevin: a new tumor suppressor gene?

To identify new putative tumor suppressor genes Schraml et al. developed a magnet-assisted subtraction technique (MAST). They were looking for genes that were expressed in normal lung tissue but not in the corresponding NSCLC tissue of the same patient (Schraml et al., 1993). Several cDNA clones were found to be absent or decreased in NSCLC tissue, among them genes whose products act in diverse cellular processes including Ca^{2+} dependent reactions (calmodulin-like protein), cytoskeletal organization (β-actin), metabolism (glutamine synthetase), homeostasis in lung (SP-B), and cell surface interactions (RAGE) (Schraml et al., 1994). One clone, termed MAST9, encoded a so far unknown cDNA fragment and was therefore chosen for further studies. At the same time, Girard et al. isolated the identical gene from high endothelial venules (HEV) of human tonsils and called it hevin (Girard and Springer, 1995).

The MAST9/hevin protein has a hydrophobic domain at its amino terminus, which most likely represents a signal for secretion, followed by an extensive acidic region, and a SPARC-like domain at the carboxy terminus.



Schematic drawing of the SPARCL1 protein domains:

The protein domains are numbered according to their amino acid (aa) position. The cysteine rich follistatin-like domain (FS domain) and the extracellular calcium binding domain (EC domain) are marked using the abbreviations. Each Ca^{2+} represents an EF hand.

This SPARC-like domain exhibits high homology (62%) to the extracellular matrix protein SPARC (secreted protein acidic and rich in cysteine). Therefore, MAST9/hevin is a member of the SPARC protein family of extracellular multidomain glycoproteins, which includes SPARC, SC1, QR1, testican, and TSC36/FRP (Yan and Sage, 1999). Because of its high homology to SPARC, MAST9/hevin has been renamed to SPARC-like 1 (SPARCL1) (Isler et al., 2001). SPARC has only a short acidic N-terminal extension (72 residues), whereas SPARCL1, SC1, and QR1 have considerably larger acidic N-terminal domains (403-445 residues), which are more closely related to each other than to SPARC. Due to the high homology of human SPARCL1 and mouse SC1 protein, the similar gene structure, and the homologous intron/exon boundaries, we suggested that SPARCL1 is the species homologue (orthologue) of SC1 (Isler et al., 2001).

SC1 (synaptic complex components) was found during a screen of a rat brain cDNA library using a mixed polyclonal antibody directed against synaptic junction glycoproteins (Johnston et al., 1990).

SPARCL1/SC1

Cloning, sequencing, and characterization of the full-length SPARCL1 cDNA revealed an open reading frame of 1'992 nucleotides (SC1: 1'902 nucleotides) encoding a protein of 75 kD (SC1: 70.6 kD). By Western Blot analysis of total human lung homogenate SPARCL1 was detected as a protein doublet with an approximate molecular weight of 75/150 kD (SC1: 116/120 kD). Whereas Girard & Springer predicted seven (Girard and Springer, 1995), our group found six potential N-linked glycosylation sites within the SPARCL1 protein (SC1: four) (Bendik et al., 1998; Mendis et al., 1994). The SPARC-like domain of SPARCL1 consists of a conserved cysteine rich follistatin-like domain (FS domain) and an extracellular Ca^{2+} binding module (EC domain) with two calcium binding EF hands (SC1: one) (Bendik et al., 1998; Mendis et al., 1994). The EF hand is a highly conserved helix-loop-helix Ca^{2+} binding motif found in cytosolic proteins, whose structure and function are modulated by Ca^{2+} ions (Heizmann and Hunziker, 1991; Kretsinger, 1980; Maurer et al., 1996).

The amino acid sequence of the SPARC-like domain of SPARCL1 shows highest homology to the mouse/rat matrix glycoprotein SC1 (91% identity) followed by the quail QR1 protein (73% identity).

The 2.8 kb transcript of the SPARCL1 gene is expressed in many human tissues including brain, heart, lung, placenta, skeletal muscle, kidney, prostate, ovary, small intestine, colon, stomach, thyroid, spinal cord, trachea, adrenal gland, bone marrow, pancreas, testis, spleen, thymus, and lymph node (Bendik et al., 1998; Claeskens et al., 2000; Girard and Springer, 1995).

The 3.2 kb SC1 mRNA is expressed in similar tissues as SPARCL1. Soderling et al. found SC1 mRNA in mouse heart, adrenal gland, and lung and at lower levels in kidney, eye, spleen, and testis (1997). In situ hybridization revealed that the SC1 mRNA is expressed widely in the brain and is present in many types of neurons (Soderling et al., 1997). SC1 is expressed during development of the rat cerebellum, but also throughout postnatal development of the brain (Johnston et al., 1990). Therefore, Mendis et al. looked carefully at the expression of SC1 during the development of the rat cerebellum at both protein and mRNA level and found indications that SC1 was both temporally and spatially regulated during this process (Mendis et al., 1994). It was proposed that SC1 might participate, not only in the developing nervous system, but also in the functioning of the adult brain (Johnston et al., 1990). Soderling et al. showed by in situ hybridization that SC1 and SPARC transcripts were not expressed in the same regions of a particular organ or by the same cell type (Soderling et al., 1997).

Interestingly, parallel to the finding that SPARCL1 is downregulated on mRNA and protein level in lung tumors (Bendik et al., 1998) Nelson et al. showed that SPARCL1 is downregulated in transformed prostate epithelial cell lines and in metastatic prostate adenocarcinomas (Nelson et al., 1998). The SPARCL1 transcript is also downregulated in colorectal (Claeskens et al., 2000; Notterman et al., 2001) and bladder carcinomas (Dr. P. Schraml, personal communication). Claeskens et al. found that SPARCL1 is equally expressed in normal and tumorous kidney tissue (Claeskens et al., 2000), whereas Gerritsen et al. found a twofold upregulation in renal cell carcinomas (Gerritsen et al., 2002). The downregulation of SPARCL1 in different tumor tissues prompted us to propose that SPARCL1 might be a tumor suppressor gene.

By fluorescence in situ hybridization (FISH) and comparative genome hybridization (CGH) techniques SPARCL1 was assigned to chromosome 4q22-25 (Isler et al., 2001), a region, which has been speculated to contain a so far unidentified tumor suppressor gene (Mitra et al., 1994; Rumpel et al., 1999). In the meantime, SPARCL1 was located to chromosome 4q22.1 by the Human Genome Project (http://genome.ucsc.edu). Using fluorescence in situ hybridization (FISH) analysis SC1 was localized to band 5E4 of mouse chromosome 5 (McKinnon et al., 1996).

The physiological function of SPARCL1 is still unknown. SPARCL1 inhibits adhesion of endothelial cells indicating that SPARCL1 belongs to the family of adhesion modulating proteins. Girard et al. claimed therefore that SPARCL1 might modulate HEVEC (high endothelial venules endothelial cell) adhesiveness to facilitate abundant lymphocyte extravasation (Girard and Springer, 1996). As SPARCL1 ESTs were found at high frequency in inflammatory diseased tissues in artery and uterus Claeskens et el. claimed that SPARCL1 might be involved in the inflammatory process (Claeskens et al., 2000). SPARCL1 is also overexpressed in intracranial aneurysms, which are the site of a strong immune/inflammatory response (Peters et al., 2001). Overexpression of SPARCL1 in HeLa 3S cells inhibits the progression from G_1 to S phase or prolonged G_1 phase indicating that SPARCL1 is a negative regulator of cell proliferation (Claeskens et al., 2000). This result additionally supported our hypothesis that SPARCL1 might be a tumor suppressor gene.

SC1, the mouse homologue of SPARCL1, is an astrocyte marker and may play an important role in reactive astrocytosis subsequent to a wide variety of neural trauma, including neurodegenerative diseases such as Alzheimer and acute neural damage (McKinnon and Margolskee, 1996). By in situ hybridization it was demonstrated that SC1 mRNA was induced in astrocytes surrounding a wound, reaching maximal levels at 10 days post-lesion (Mendis et al., 1996a). Oritani et al. suggested that SC1 could contribute to the nurturing environment for B- lymphocyte precursors (Oritani et al., 1997). Surprisingly, the SC1-null mice showed no obvious defects in any organs and were also fertile (McKinnon et al., 2000). SC1 plays an important role in the developing central nervous system, whereas SPARC participates in events associated with skeletal development (Mothe and Brown, 2001). Mothe et al. also found that SC1 mRNA transport was diminished at 10 and 15 hours post-hyperthermia, but returned to control levels by 24 hours after heat shock.

As heat shock on cells grown in tissue culture induces a collapse of the cytoskeletal network this result can be interpreted as a blocked mRNA transport due to the lack of the network (Mothe and Brown, 2002).

QR1

The quail cDNA clone, named QR1, was isolated during a search for genes that might be involved in terminal mitosis or differentiation of neuroretina cells. QR1 encodes a 676 amino acid protein whose carboxy-terminal portion showed significant similarity to those of the extracellular glycoproteins SC1, SPARCL1, and SPARC. QR1 has only one EF hand to bind calcium at the carboxy-terminal region. The presence of a putative signal peptide and the lack of an internal hydrophobic transmembrane domain suggest that the QR1 protein is secreted.

The avian neuroretina (NR) is a part of the central nervous system and is composed of photoreceptors, neuronal cells, and glial cells. Transcription of QR1 takes place only during the late phase of retinal development and is shut off sharply at hatching (Guermah et al., 1991). Its expression coincides with the stage of withdrawal from the cell cycle and establishment of differentiation, therefore a role for QR1 gene product is suggested in the process of growth arrest and establishment of photoreceptor differentiation (Casado et al., 1996). QR1 mRNA is detected in the neuroretina, but not in other embryonic tissues examined, whereas SPARCL1 and SC1 are expressed in many different tissues. Interestingly, in vitro the levels of this mRNA are markedly reduced when non-dividing NR cells are induced to proliferate by the v-src oncogene (Guermah et al., 1991). Therefore, the expression of QR1 is regulated indirectly through this oncogene.

Aim of the thesis

The aim of this thesis was to find out whether SPARCL1 is a tumor suppressor gene with mutation or deletion in its genomic region and to reveal the mechanism leading to its downregulation. Furthermore, we wanted to see whether SC1 mRNA is also downregulated in mouse lung tumors. Using a new anti-SPARCL1/SC1 antibody we planned to investigate the protein expression pattern in different healthy tissues as well as in tumorous tissues of humans and mice.

Section 1: Paper

Evidence for transcriptional repression of SPARC-like 1 (SPARCL1), a gene downregulated in human lung tumors

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Abstract

SPARCL1 mRNA was shown to be downregulated in NSCLC as well as in prostate, colon, and bladder carcinomas. Therefore, SPARCL1 was suggested to be a tumor suppressor gene. By microsatellite analysis, real-time quantitative PCR, and sequence analysis of all exons including the intron-exon junctions and a part of the putative promoter region, we could not find any deletion or mutation that might be responsible for the downregulation of SPARCL1 in NSCLC. We conclude that SPARCL1 is therefore not a classical tumor suppressor gene with a deletion or mutation in one allele and another mutation in the second allele.

To test whether SPARCL1 could be downregulated by repression of transcription we performed luciferase reporter gene assays with 10 different SPARCL1 promoter constructs. These experiments revealed that the presence of exon 1 is able to cause a reduction in luciferase activity. Furthermore, we show that the inhibitory activity of exon 1 can be transferred to a heterologous promoter. This indicates that SPARCL1 downregulation might be mediated (at least in part) through transacting factors that bind to exon 1.

Introduction

Lung cancer is the leading cause of cancer-related death for both men and women in the United States and the solid tumor with the best defined relationship to a known environmental cause, cigarette smoking. Four major histological lung cancer types exist: squamous cell, adeno-, large cell undifferentiated, and small cell lung carcinomas. The first three types, collectively known as non-small cell lung cancers (NSCLCs), comprise approximately 75% of all lung tumors. NSCLCs metastasize later than small cell lung cancers and can be cured by surgery if detected at an early stage (Vogelstein and Kinzler, 1998).

Searching for genes downregulated in human non-small cell lung carcinomas Schraml et al. (Schraml et al., 1994; Schraml et al., 1993) identified and sequenced a novel gene termed MAST9. Independently, Girard et al. (Girard and Springer, 1995) isolated an identical gene from high endothelial venules (HEV) of human tonsils and called it hevin. The MAST9/hevin gene encodes a glycoprotein, which exhibits 62% identity in its carboxy terminus to the extracellular matrix protein SPARC (secreted protein acidic and rich in cysteine) (Bendik et al., 1998; Girard and Springer, 1995). Therefore, MAST9/hevin was renamed to SPARC-like 1 (SPARCL1) (Isler et al., 2001). SPARCL1 is closely related to two other SPARC-like proteins called SC1 (mouse and rat, 92 and 91% identity) and QR1 (quail, 73% identity) (Bendik et al., 1998; Girard and Springer, 1995). We suggested that SC1 is the murine homologue of SPARCL1 as it has similar intron sizes as well as conserved intron/exon junctions (Isler et al., 2001).

SPARCL1 mRNA is expressed in many human tissues including brain, heart, lung, placenta, skeletal muscle, kidney, prostate, ovary, small intestine, colon, stomach, thyroid, spinal cord, trachea, adrenal gland, bone marrow, pancreas, testis, spleen, thymus, and lymph node (Bendik et al., 1998; Claeskens et al., 2000; Girard and Springer, 1995). Parallel to our finding that SPARCL1 is downregulated in lung tumors Nelson et al. (Nelson et al., 1998) showed that SPARCL1 is downregulated in transformed prostate epithelial cell lines and in metastatic prostate adenocarcinomas. In addition, the SPARCL1 transcript was found to be downregulated in colorectal (Claeskens et al., 2000; Notterman et al., 2001) and bladder carcinomas (Dr. P. Schraml, personal communication) but not in kidney tumors (Claeskens et al., 2000).

Gerritsen et al. (Gerritsen et al., 2002) reported a twofold upregulation of SPARCL1 in renal cell carcinomas. The downregulation of SPARCL1 in many different tumor types, however, prompted us to propose that SPARCL1 might be a tumor suppressor gene.

By fluorescence in situ hybridization (FISH) and comparative genome hybridization (CGH) techniques SPARCL1 was assigned to chromosome 4q22-25 (Isler et al., 2001), a region, which has been speculated to contain a so far unidentified tumor suppressor gene (Mitra et al., 1994; Rumpel et al., 1999).
In this report we evaluate whether SPARCL1 gene inactivation might be caused by a mutational event. We performed microsatellite analysis, real-time quantitative PCR, and a search for mutations by sequencing of matched normal/tumor samples from NSCLC patients. In addition, we performed luciferase reporter gene assays in order to identify regulatory elements in the SPARCL1 promoter that could be responsible for transcriptional repression of SPARCL1.

Materials and Methods

Extraction of genomic DNA

Genomic DNA was extracted from lung tissue of 54 NSCLC patients (43 men, 11 women; age 62.7±8.9 years). Normal lung and corresponding tumor tissues were snap frozen immediately after resection and stored in liquid nitrogen. Genomic DNA was extracted from lung tissue according to standard methods (Davis et al., 1986). The NSCLCs consisted of 29 squamous cell carcinomas (SCCs), 10 large cell carcinomas (LCCs), and 12 adenocarcinomas (ADCs). For two tumors the type of NSCLC was not defined and one tumor was a mixed form of ADC/LCC. The extraction was done for an earlier study, which has been approved by the ethic committee of the St. Claraspital (Basel, Switzerland) (Schenk et al., 2001).

Microsatellite analysis

Microsatellite analysis was performed on chromosome 4q21-24 using 7 highly polymorphic microsatellite markers. The PCR reactions were performed using the Hot Star Polymerase kit (Qiagen), fluorescent labeled forward primers (6-FAM, HEX or TET), and 50 ng genomic DNA. PCR products of intermediate intensity on the gel (~30ng/ µl sample) were diluted 1:20 for 6-FAM or TET labeled reactions and 1:10 for HEX labeled reactions. For each sample 1 µl of the diluted PCR reaction was separated with a size standard (0.5 µl Genescan-350 TAMRA size standard; Applied Biosystems) on the ABI Prism 310 Genetic Analyzer (Applied Biosystems) using the POP4 Performance Optimized Polymer (Applied Biosystems). The microsatellite patterns in normal and tumor tissues were determined with the GeneScan 3.1 Analysis software (Applied Biosystems).

Loss of heterozygosity (LOH) was scored if the peak height ratio of both alleles in the tumor DNA divided by the ratio of the corresponding alleles in the normal DNA of the same patient was more than 1.5 or less than 0.67.

Real-time quantitative PCR

Real-time quantitative PCR was performed on genomic DNA with primers and probe sets specific for SPARCL1 (forward primer 5'-ATT GAG CCT CAG GAG AAA AAA CTC T- 3', reverse primer 5'-TCT TGT TGG TTA GAA TCT GTG AAG GA- 3', and FAM labeled probe 5'-AGA GAA CAC TGA TTT TTT GGC TCC TGG TGT TAG- 3') and human acidic ribosomal phosphoprotein P0, also known as 36B4 and ARP, (forward primer 5'-CTC CAA GCA GAT GCA GCA G- 3', reverse primer 5'-GAA GGC TGT GGT GCT GAT G- 3', and VIC labeled probe 5'-TCC GCA TGT CCC TTC GCG- 3'). Primers and probes were selected using the Primer Express software (Applied Biosystems). The reaction mixtures (multiplex, final volume: 50 μl) contained 100 ng genomic DNA, 300 nM of each forward and reverse primer, 100 nM of each probe, and 1x TaqMan Universal PCR Master mix (Applied Biosystems). All reactions were performed in triplicates using the ABI PRISM 7700 Sequence Detector (Applied Biosystems).

The Q-gene software (Muller et al., 2002) was used to calculate the relative amount of SPARCL1 genomic DNA in normal and tumor tissue, normalized to human acidic ribosomal phosphoprotein P0. Finally, the amount of SPARCL1 in the tumor was divided by the amount of the corresponding normal tissue (<0.75 deletion, >1.5 amplification) and the standard error was calculated applying the Gauss equation for error propagation (Bevington and Robinson, 1992). Data with a standard error greater than 15% were excluded.

Sequencing of genomic DNA

Fragments of the SPARCL1 gene were amplified by PCR from genomic DNA. 40 ng of the PCR products were cycle sequenced using the BigDye Terminator ready reaction mix (Applied Biosystems) according to the instructions of the manufacturer. The samples were then analyzed on the ABI Prism 310 Genetic Analyzer (Applied Biosystems) using the POP6 Performance Optimized Polymer (Applied Biosystems) and the ABI PRISM 310 Collection software (Applied Biosystems). The same primers were used for the PCR reaction and for sequencing (listed in table I).

amplified region	primer sequences
promoter	F: 5'-CTT GAA AAG AAA CTC CAT GCT G-3'
	R: 5'-TAT GGG AAA CCT CCT TCT TAG-3'
promoter-exon1	F: 5'-CAG CAT GGA GTT TCT TTT CAA G-3'
	R: 5'-CAG ACG AGA ATT TGG AGG TT-3'
exon1-intron1	F: 5'-AGG GAA ATC CAG GAA TCT GCA-3'
	R: 5'-CAG CTT TAT CAG ATC ACG C-3'
intron1-exon2-intron2	F: 5'-CAA GCT CAG TAT TTG CTA AGA C-3'
	R: 5'-TCT GCA TTA AAT GTC AGG AG-3'
intron2-exon3-intron3	F: 5'-GAT GAC AAC AAG AGT AAC TAG C-3'
	R: 5'-GCA GTT GAG GTG ATC ATC ATG-3'
intron3-exon4	F: 5'-TGT GGC TAC AGA GTG TGA GG-3'
	R: 5'-GTT CTG CTG ACT GTT CAT-3'
exon4-intron4	F: 5'-AAT CCA TTG CCT ATC ACC TC-3'
	R: 5'-GAC ACT GTG AGA GCA CAG AG-3'
intron4-exon5-intron5	F: 5'-ATA CTC GTG AAA GTA TGC AC-3'
	R: 5'-CAA GCC CAA AGT GGC AGA TT-3'
intron5-exon6-intron6	F: 5'-TAA CTT TGT CCA TGC TTC CT-3'
	R: 5'-TGA TAA CCT AGG TAT CTA CC-3'
intron6-exon7-intron7	F: 5'-GCA GAG TGT CTT CCA GTG AG-3'
	R: 5'-GTG TTC AGA ACT ACA TCA GC-3'
intron7-exon8-intron8	F: 5'-ATT ATG AGG ACT TGC CAT AG-3'
	R: 5'-CAC CAC GTC TTT CTT CCT CA-3'
intron8-exon9-intron9	F: 5'-TGT TAC TGT CAC TCT TCA AG-3'
	R: 5'-AAT GGA ACT GGG AAA TGT CC-3'
intron9-exon10-intron10	F: 5'-TCC TTG GTG AGC CTG AGA GT-3'
	R: 5'-TGT GGC AGG ACC TCT CTA TG-3'
intron10	F: 5'-TAG GGA TTC TGA TTC TAG AG-3'
	R: 5'-GGA ATT CCT CAA AAC AAG AGA TTTTCA T-3'

Table I: List of primer pairs used for genomic SPARCL1 amplification and sequencing in 8 NSCLC patients
Primers binding within the exons were selected according to the published cDNA sequence of SPARCL1/MAST9 (GenBank accession nr X86693). For primers that bind to intron sequences own (unpublished) sequences were considered. All intron primers were selected to bind near to the intron/exon junctions.

Coding sequences were aligned to the MAST9 and the hevin cDNA sequences (GenBank accession nr X86693 and X82157), whereas promoter sequences were aligned to the SPARCL1 promoter sequence (AF321976).

Reporter gene assay

10 different luciferase reporter gene constructs were used in this study (see figure 4). The SPARCL1 promoter sequences were amplified by PCR or cut out from a previously characterized genomic SPARCL1 clone (GenBank accession nr AF321976) and were subsequently cloned into the pGL3-basic vector (Promega) as listed in table II.

For the exon 1-TK construct we cut out the thymidine kinase (TK) promoter from the pT81-luc vector (Nordeen, 1988) using the restriction enzymes XmaI/BglII and cloned it into the pGL3 basic vector (construct named TK) prior to cloning of the SPARCL1 exon 1 into this vector (construct named exon1-TK).

HEK293-T cells were maintained in DMEM medium supplemented with 10% FCS. For transient transfections HEK293-T cells were seeded in 6 well plates. After 12-16 hours they were cotransfected with 1 μg pSV-β-galactosidase control vector (Promega) and 1 μg luciferase reporter gene construct using the FuGENE 6 transfection reagent (6 μl per 2 μg vector; Roche). 24 hours later the cells were processed for measuring luciferase and β-galactosidase activity according to Bagutti et al. (Bagutti et al., 2003) using a modified luciferin substrate solution (22.5 mM MgSO4 and 37.5 mM glycine final pH 7.8, 0.3 mM luciferin in MgSO4/glycine, and 7.5 mM ATP pH 7.8 in water).

Construct	insert length	SPARCL1 clone
A	1753 bp	nt 771-2524
B	1943 bp	nt 774-2717
C	1389 bp	nt 1118-2507
D	1599 bp	nt 1118-2717
E	1040 bp	nt 1467-2507
F	1250 bp	nt 1467-2717
G	708 bp	nt 1816-2524
H	919 bp	nt 1798-2717
exon1-TK	294 bp	nt 2423-2717

Table II: Constructs used for luciferase assay

The sequence of the SPARCL1 clone can be accessed on the GenBank homepage (accession nr AF321976; exon 1 nt 2405-2714). For the exon 1 construct the TK promoter from the pT81-luc vector was cloned into the pGL3-basic vector (construct TK) before adding the exon 1. For all other constructs the original pGL3-basic vector was used.

Results

Microsatellite analysis

To determine the frequency of deletions within or nearby the SPARCL1 locus, microsatellite analysis was performed on genomic DNA of 54 matched normal/tumor NSCLC patients using 7 highly polymorphic microsatellite markers covering chromosome 4q21-24. Six markers contained CA dinucleotide repeats, one contained GATA repeats (D4S2634). A summary of the deletion mapping and the location of the 7 markers used in this study are shown in figure 1. LOH was observed in 34 of 54 NSCLC specimens (63%) at least at one of the loci tested. Ten of 54 tumors (19%) showed allelic loss with three or more markers. However, we could not observe any hot spots of deletion within chromosome 4q21-24. The lowest frequency of LOH was 7/35 informative cases (20%) at D4S2460 (65% informative) and the highest was 13/39 informative cases (33%) at D4S1534 (72% informative).

69% of the squamous cell carcinoma cases showed LOH in at least one of the microsatellite markers tested, whereas in adenocarcinoma and large cell carcinoma cases 55% and respectively 50% showed LOH. However, we could not detect any correlation of number of LOHs per patient with NSCLC type, tumor stage, number of lymph nodes affected, age, or gender.

Real-time quantitative PCR

Since the region covered by our microsatellite markers was around 15.2 million basepairs long we wanted to check for deletions of the SPARCL1 gene itself. Therefore, the relative amount of SPARCL1 genomic DNA was determined for the normal and tumor tissue by real-time quantitative PCR and by using the Q-gene software (Muller et al., 2002). Genomic DNA of 50 NSCLC tumors was used to amplify a part of SPARCL1 together with a part of the reference gene human acidic ribosomal phosphoprotein P0, also known as 36B4 and ARP (GenBank accession nr M17885).

We found that 88% of the NSCLC patients tested retained both alleles of SPARCL1 in the tumor tissue; in 6% of the patients we found a deletion and in another 6% of the patients we even found an amplification (see figure 2).

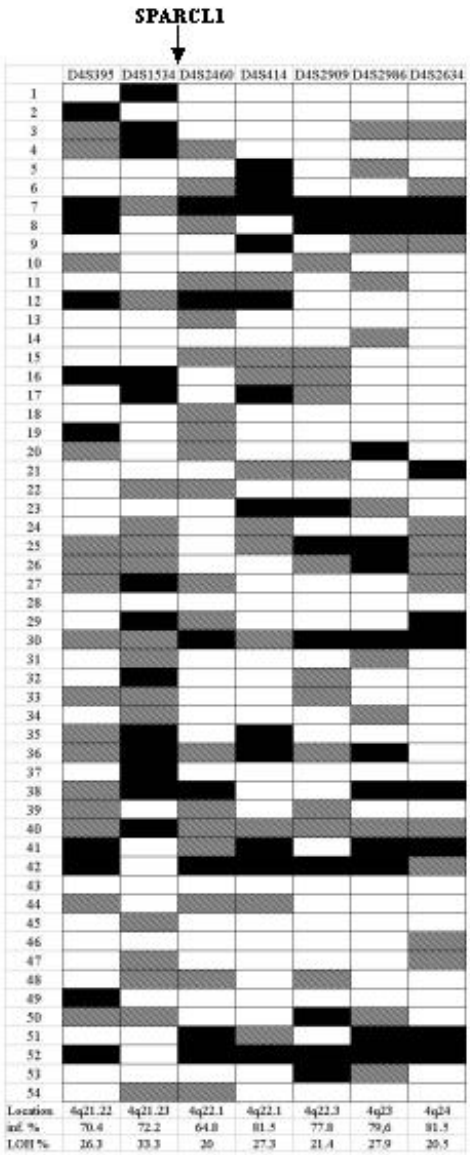

Figure 1: Microsatellite analysis on chromosome 4q21-24

Patterns of LOH in 54 NSCLC patients using 7 different microsatellite markers. On the left the sample number and at the top the name of the marker is noted. As listed below the markers are placed in the predicted order. According to the Human Genome Project (http://genome.ucsc.edu) SPARCL1 is located in-between D4S1534 and D4S2460. The percentage of informative cases (inf) for each marker and the percentage of LOH per informative cases are noted at the bottom. Filled boxes stand for LOH, empty boxes for heterozygosity, and shaded boxes for homo- or hemizygosity.

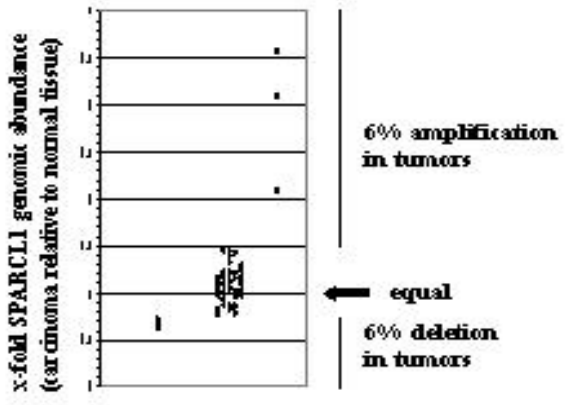

Figure 2: Real-time quantitative PCR of SPARCL1

Genomic abundance of SPARCL1 in 50 NSCLC patients found with an exon 4 specific probe and primer set. The value 1, indicating equal amount of genomic DNA in normal and tumor tissue, was marked by a block arrow whereas the vertical line is indicating deletions or amplifications in the tumor tissue. The patients have been grouped into three groups (from left to the right: deletion of one allele in tumor tissue, retention of both alleles, amplification in tumor tissue).

Sequence analysis

To detect mutations that might lead to the downregulation of SPARCL1 in NSCLC we sequenced the SPARCL1 gene of 8 matched normal/tumor samples. All 8 tumors were previously shown to have a downregulation of SPARCL1 on mRNA and protein level (Bendik et al., 1998). The analysis included all exons, the intron/exon boundaries, and a part of the putative promoter region (530 nucleotides 5' of the exon 1 containing a putative TATA and CAAT box).

We could not detect any mutations that were restricted to the tumor samples. Instead we found 6 polymorphisms in the exons of SPARCL1 from which 3 result in an amino acid change (see figure 3). All polymorphisms were detected in the normal and the corresponding tumor tissue and only in some of the 8 NSCLC patients. For all except the last one we found homo- and heterozygous patients, whereas for the exon 5 polymorphism only heterozygous patients were examined. Two polymorphisms were already published by our group (Bendik et al., 1998) and two by other groups (Claeskens et al., 2000; Girard and Springer, 1995). We also detected 8 sequencing errors within the MAST9/SPARCL1 sequence (GenBank accession number X86693).

In summary, we could conclude from microsatellite analysis, real-time quantitative PCR, and sequencing that SPARCL1 is not a classical tumor suppressor gene and therefore the SPARCL1 mRNA and protein must be downregulated in tumors by an alternative mechanism.

Promoter analysis

We next considered the possibility that the SPARCL1 gene is intact but its expression is impaired. Transcriptional repression by hypermethylation of promoter sequences is a mechanism often involved in inactivation of tumor suppressor genes in cancer (Santini et al., 2001). In preliminary experiments we tested whether forced DNA demethylation could restore SPARCL1 expression *in vitro*. Five different SPARCL1 negative lung cancer cell lines (A549, H1299, Hotz, Calu1, and Calu6) were treated with the methylation inhibitor 5-deoxyazacytidine. However, none of the tested cell lines could (re-) express SPARCL1 suggesting that promoter hypermethylation does not play a role in SPARCL1 downregulation in these cell lines (data not shown).

Since promoter hypermethylation occurs at CpG islands, we tried to map CpG islands within the putative SPARCL1 promoter (GenBank accession nr AF321976) using the CpGpromoter program (http://cgsigma.cshl.org/CpG_promoter). In agreement with our preliminary data we could not detect any CpG islands within this sequence, indicating that promoter hypermethylation is unlikely to be the cause of SPARCL1 downregulation in NSCLC. Therefore, deregulation of transacting factors might be responsible for SPARCL1 downregulation in NSCLC.

	nt (cDNA)	variation	aa change	correct seq	
exon 1	2	no G	-	no G	
	139	A/T	-	T	
	161	G/A	-	G or A	★
	273	T/TT	-	TT	
	291	G/A	-	G or A	★
exon 2	336	C/T	P/L	T (L)	
exon 3	433	T/C	-	T or C	
	458	T/A	W/R	A (R)	
	468	C/A	A/D	C or A	★
exon 4	638	C/G	H/D	C or G	★
	702	T/G	I/S	G (S)	
exon 5	1577	A/G	T/A	A or G	★
exon 8	1957	T/C	-	C	
exon 9	2047	T/C	-	C	

Figure 3: Mutational analysis by sequencing

Polymorphisms (★) and sequencing errors found in 8 NSCLC patients. All exons, part of the putative promoter, and the intron/exon boundaries of SPARCL1 were sequenced. The nucleotide positions (nt) of the polymorphisms are according to the MAST9 cDNA sequence (GenBank accession number X86693). Amino acid (aa) changes and the correct sequence (seq) are listed.

To analyze whether SPARCL1 expression can be downregulated on the level of transcription, we performed luciferase reporter gene assays. Aligning the 5' flanking region of exons 1 of SPARCL1 and the mouse orthologue SC1 two local stretches of high homology were found, which were called box 1 and 2 (Isler et al., 2001). We assume that this upstream region of exon 1 contains the core promoter of SPARCL1 since it contains a putative TATA and CAAT box. Eight different promoter constructs of varying lengths including these two boxes were constructed from a genomic SPARCL1 clone (GenBank accession nr AF321976).

Then HEK293-T cells were cotransfected with the promoter constructs and β-galactosidase control vector for normalization of the transfection efficiencies. As displayed in figure 4 this promoter region was indeed functional and led to a significant increase in luciferase activity by all SPARCL1 constructs. We realized that the inclusion of a longer stretch of exon 1 in front of the luciferase coding region lead to a significant reduction in promoter activity in all constructs tested. To find out whether this inhibitory activity can be transferred to a heterologous promoter exon 1 was cloned 5' of a thymidine kinase promoter (construct called exon1-TK). Luciferase assays with this construct in comparison to the TK vector alone revealed that the addition of exon 1 was able to reduce the luciferase activity driven by the thymidine promoter, confirming that this DNA segment confers transcriptional repression.

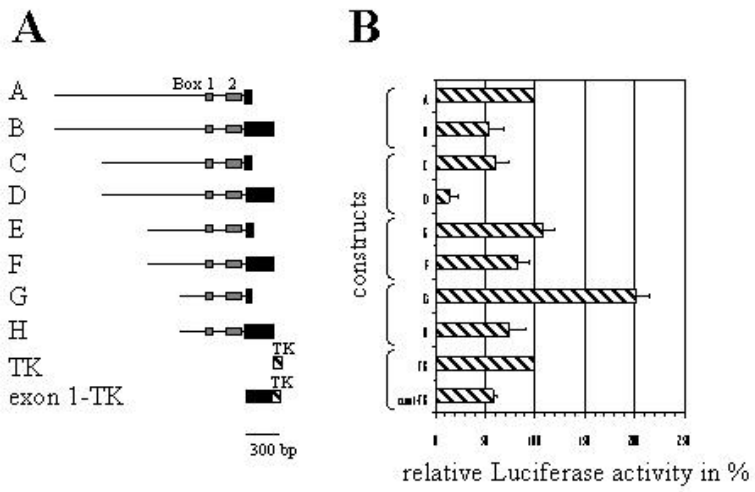

Figure 4: Luciferase reporter gene assay of the SPARCL1 promoter

Relative luciferase activity measured 24 hours after transfecting HEK293-T cells with 10 different promoter constructs. A: The inserts of the constructs used for luciferase assay are depicted 5' to 3' (black box: exon 1 or part of exon 1, striped box: thymidine kinase promoter, grey boxes: box 1+2). On the left the names of all constructs are indicated. B: Relative luciferase activity (normalized with β-galactosidase) in % is noted on the X axis whereas on the Y axis the different constructs are listed. Using brackets the corresponding constructs (with and without exon 1) are marked. In every series of measurements the mean value of construct A was set as 100% for the first 8 constructs (A→H), whereas the mean value of construct TK was set as 100% for the exon 1-TK construct.

Discussion

SPARCL1 mRNA is downregulated in many epithelial tumors (Bendik et al., 1998; Claeskens et al., 2000; Nelson et al., 1998; Notterman et al., 2001). In addition, SPARCL1 can act as a negative regulator of cell proliferation when overexpressed in HeLa 3S cells (Claeskens et al., 2000), therefore we hypothesized that SPARCL1 might be a novel tumor suppressor gene. Tumor suppressor genes normally regulate cell growth and differentiation in a negative fashion and their downregulation or functional inactivation in malignant cells allows survival and cell cycle progression. Classical tumor suppressor genes are known to be inactivated by a deletion or mutation in one allele and a mutation in the second allele. Therefore, we considered that gene inactivation of SPARCL1 in NSCLC may be a consequence of gene deletions and/or point mutations leading to frameshifts, premature translation stop, or to amino acid changes that result in a non-functional SPARCL1 protein. Mutations that lead to incorrect splicing of the pre-mRNA could lead to rapid degradation or sequence variations in the promoter might alter the transcription rate of SPARCL1.

To test whether SPARCL1 is a tumor suppressor gene, we initiated a LOH study nearby the SPARCL1 locus (in the meantime assigned to chromosome 4q22.1; http://genome.ucsc.edu) as well as a search for mutations by direct sequencing. So far we could not find a hot spot region of gene deletion within chromosome 4q21-24 by microsatellite analysis. We also could not detect any tumor-associated mutations in the SPARCL1 gene (promoter and coding regions). This indicates that SPARCL1 downregulation is not regulated in the “classical tumor suppressor way” and that the SPARCL1 mRNA is downregulated using different mechanisms.

Since preliminary result exclude promoter hypermethylation as a cause of downregulation and since so far little is known about the transcriptional regulation of SPARCL1 we performed luciferase reporter gene assays with different SPARCL1 promoter constructs (with and without exon 1). We showed that the presence of exon 1 consistently reduced luciferase activity of all our constructs. This attenuation of promoter activity by exon 1 was also transferable to a heterologous thymidine kinase promoter. Additional supportive evidence for a regulatory role of exon 1 comes from studies of the quail homologue QR1.

Exon 1 of SPARCL1 shows sequence homology to a promoter region of QR1 and this conserved region in QR1 was shown to contain a regulatory element termed A box (Pierani et al., 1993; Pierani et al., 1995; Pouponnot et al., 1995).

According to the Ensembl homepage (http://www.ensembl.org/Homo sapiens; accession nr X86693) the intron 1 of SPARCL1 is 29.5 kb long and the whole gene encompasses 56.0 kb. We cannot exclude that intron 1 contains further regulatory sequences in addition to the 5' sequence of exon 1 and exon 1 itself.

It is likely that SPARCL1 expression is regulated in a complex fashion perhaps involving several transcription factors as it has been found for SPARC (Vial et al., 2000). To identify factors that bind to the promoter sequence of SPARCL1 it will be interesting to perform a yeast one-hybrid screen and to define the binding regions of the transcription factors using DNA footprint assays.

In summary, we propose that SPARCL1 is downregulated in cancer by transcriptional repression, possibly by a factor recognizing sequences within exon 1.

Abbreviations

aa	aminoacid
ADC	adenocarcinoma
FCS	fetal calf serum
HEK	human embryonic kidney
kb	kilobasepairs
LCC	large cell carcinoma
LOH	loss of heterozygosity
nr	number
nt	nucleotide
NSCLC	non-small cell lung cancer
SCC	squamous cell carcinoma
SPARC	secreted protein acidic and rich in cysteine
SPARCL1	SPARC-like 1
TK	thymidine kinase

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Section 2: Additional experiments

Materials and Methods

5' End labeling

For end labeling 20 pMol $(AC)_{15}$ primer (Microsynth), 25 µCi $\gamma^{32}P$ labeled ATP (Amersham), 1x forward reaction buffer (Invitrogen), and 10 units T4 Polynucleotide kinase (Invitrogen) were mixed in an Eppendorf tube and the volume was adjusted to 20 µl with deionized water. The tube was quickly centrifuged and afterwards incubated for 10 minutes at 37°C. The kinase was then heat-inactivated for 10 minutes at 65°C. The non-incorporated nucleotides were removed using a Sephadex G-25 column. For this column a 1 ml syringe was placed in a 15 ml Falcon tube before a little bit of glass wool was packed into the syringe. Afterwards Sephadex G-25 (in TE pH 8.0) was loaded and the tube was centrifuged for 5 minutes at room temperature with 1'200 rpm (300 g). First the syringe was placed into a new 15 ml Falcon tube, then the probe was loaded onto the Sephadex column, and finally the tube was centrifuged for 5 minutes at room temperature with 1'200 rpm (300 g). The probe was eluted with 50 µl TE pH 8.0. After scintillation counting, 100'000 counts per ml hybridization buffer were mixed with 100 µl water before boiling the probe for 10 minutes at 100°C.

Southern Blot

2.5 µg of the genomic P1 clone 24 (for construction and purification see Isler et al., 2001), 1x H-SuRE/Cut buffer (Roche), and 3 units of XbaI or EcoRI restriction enzyme (Roche) were mixed and the volume was adjusted to 20 µl with deionized water. After over night digestion at 37°C, the digests were loaded on a 1% agarose gel, which was run in a CHEF-DR II pulsed field gel electrophoresis apparatus (BioRad; settings: 1-6 seconds switch time, 6 V/cm voltage gradient, and 11 hours run time). The gel was blotted onto a Hybond N+ membrane (Amersham) by capillary transfer with 0.4 M NaOH at room temperature over night. Then the membrane was autocrosslinked with 120 mJoules in a UV Stratalinker 1'800 (Stratagene) and prehybridized in 20 ml Church Gilbert hybridization buffer (1% BSA, 1 mM EDTA pH 8.0, 0.14 M NaH_2PO_4, 0.36 M Na_2HPO_4, and 7% SDS) at 68°C.

After 1 hour prehybridization the denatured end labeled probe was added and the hybridization was performed at 68°C over night. The membrane was washed in 2x SSC/0.1% SDS for 15 minutes at 68°C, followed by 10 minutes at room temperature in 0.1% SSC/0.1% SDS and exposure to Kodak BioMax MR films (Kodak) for 3-7 days.

Filter lift

Hybridizing restriction fragments found by Southern Blot were subcloned into Bluescript KS+ vector (Stratagene). Afterwards calcium chloride treated XL-1 blue competent cells (Stratagene) were transformed with these constructs. The cells were plated on prewarmed LB plates containing 100 μg/ml ampicillin and incubated over night at 37°C. The next day a piece of Hybond N+ membrane was carefully placed onto the agar plate with the transformed cells. After 1 minute the membrane was removed and placed colony side up on a dry filter paper. Then the membrane was placed colony side up on a pad of filter paper soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH). After 7 minutes the membrane was placed colony side up on a pad of filter paper soaked in neutralizing solution (1.5 M NaCl, 0.5 M Tris pH 7.2, and 1 mM EDTA). After 3 minutes the membrane was once more placed colony side up on a pad of filter paper soaked in neutralizing solution for 3 minutes. Finally the membrane was washed in 2x SSC, air-dried, and autocrosslinked with 120 mJoules in a UV Stratalinker 1'800 (Stratagene). Then the membrane was prehybridized in 20 ml Church Gilbert hybridization buffer for 1 hour at 68°C. Afterwards the denatured end labeled probe was added and the hybridization was performed at 68°C over night. The membrane was washed in 2x SSC/0.1% SDS for 10 minutes at 68°C, followed by 10 minutes at room temperature in 2% SSC/0.1% SDS and was exposed to Kodak BioMax MR films (Kodak) for one day. Clones, which were hybridizing with the probe, were picked to start a culture. After QIAprep miniprep (Qiagen) DNA extraction the inserts of the clones were sequenced on the ABI Prism 310 Genetic Analyzer (Applied Biosystems) using the BigDye Terminator ready reaction mix (Applied Biosystems).

cDNA synthesis and riboprobe production

From 2 µg mouse brain total RNA cDNA was produced using Oligo(dT)$_{15}$ primer (Promega) and the Omniscript reverse transcriptase (Qiagen) according to the instructions of the manufacturer. Afterwards a SC1 specific fragment (nt 1385-1662; GenBank accession nr U64827) was amplified by PCR and cloned into the pBluescript II SK- vector (Stratagene) using the EcoRI and an artificial SalI restriction site. The insert was sequenced before linearizing the plasmid with EcoRI or SalI. DIG-labeled riboprobes were synthesized from 1 µg of the linearized plasmid using the DIG RNA labeling mix (Roche) according to the instructions of the manufacturer and T3 (sense: SalI) or T7 (antisense: EcoRI) RNA polymerase (Promega).

Northern Blot

For testing the riboprobes total RNA was extracted from adult mouse brain using the peqGOLD RNApure reagent (Peqlab Biotechnologie; according to the instructions of the manufacturer) followed by a lithium chloride praecipitation. 15 µg total RNA from mouse brain or 3 µg RNA ladder (New England BioLabs), 1x MOPS buffer (20 mM morpholinopropansulfonic acid, 5 mM NaAcetate, and 1 mM EDTA pH 8.0; final pH 7.0), 6.5% formaldehyde, 50% formamide, and 0.25 µg/µl ethidium bromide were mixed and the volume was adjusted with DEPC treated water to 30 µl. The samples were heated to 70°C for 5 minutes and cooled on ice for 5 minutes. Finally 5 µl Blue/Orange 6x loading dye (Promega) were added. 1 g agarose was mixed with 10 ml 10x MOPS buffer and 85 ml DEPC treated water and melted in the microwave. After cooling down to 60°C 5 ml 37% formaldehyde were added and the gel was poured. The gel was prerun 10 minutes in 1x MOPS buffer at 50 Volts, the samples were loaded and then the gel was run at 50 Volts for 2.5 hours. After electrophoresis the gel was rinsed for 10 minutes in 10x SSC before it was blotted onto a Hybond N+ membrane (Amersham) by capillary transfer with 10x SSC at room temperature. 4 hours later the membrane was autocrosslinked with 120 mJoules in a UV Stratalinker 1’800 (Stratagene), quickly rinsed in DEPC treated water, and air-dried. The membrane was then placed in a hybridization bottle with 10 ml hybridization buffer (5x SSC, 0.5% SDS, 50% deionized formamide, and 2% blocking reagent from Roche).

500 ng of the DIG labeled riboprobe were mixed with 100 µl hybridization buffer and heated to 80°C for 3 minutes. After 5 minutes cooling on ice the probe was added to the hybridization buffer and the membrane was incubated in this solution at 68°C over night. Then the membrane was washed quickly in 2x SSC/0.5% SDS followed by 2 times for 15 minutes in 200 ml 2x SSC/0.5% SDS with vigorous shaking at room temperature. The membrane was washed twice for 15 minutes in 50 ml 0.1x SSC/0.5% SDS with vigorous shaking at 68°C. The membrane was transferred to a tray with 100 ml DIG buffer 1 (0.1 M maleic acid and 150 mM NaCl, final pH 7.5) and incubated for 5 minutes at room temperature with gentle shaking. Next the membrane was blocked for 1 hour at room temperature in 100 ml DIG buffer/1% blocking reagent (Roche) with gentle shaking. The membrane was then incubated with anti-digoxigenin-AP antibody (Roche) diluted 1:20'000 in 10 ml DIG buffer/1% blocking reagent (Roche) for 30 minutes at room temperature with gentle shaking. Afterwards the membrane was washed 3 times for 15 minutes in 200 ml DIG buffer 2 (DIG buffer 1/0.3% Tween 20) with shaking. The membrane was equilibrated for 5 minutes in 50 ml freshly made DIG buffer 3 (0.1 M Tris pH 9.5/0.1 M NaCl) before incubation for 5 minutes with 5 ml CDP star (Roche) diluted 1:100 in DIG buffer 3 at room temperature. Excess buffer was blotted off before exposure of the membrane to Kodak BioMax MR films (Kodak).

In Situ Hybridization

12 weeks old A/J mice were injected intraperitoneal with 3.5 mg NNK per 25 g of body weight. 12 months later the mice were sacrified and the lungs were perfused with paraformaldehyde before embedding in paraffin. For in situ hybridization 16 µm lung paraffin sections from NNK treated A/J mice were used (tissue kindly provided by Dr. Karin Wertz, DSM Nutritional Products, VFHC, Basel).

A modified version of the in situ hybridization protocol from Henry et al. (Henry et al., 1996) was used. The sections were not additionally fixed after rehydration. After proteinase K digestion the slides were soaked for 10 minutes in 0.2% glycine/PBS solution. The prehybridization step was done at 68°C for two hours in 5x SSC, 50% formamide, 0.1% Tween 20. For hybridization 5x SSC, 50% formamide, 0.1% Tween 20, 1x Denhardt's solution, 50 µg/ml heparin, and 50 µg/ml type III baker's yeast RNA was used.

3 μg/ml denatured riboprobe were added and the hybridization was performed at 68°C for 2 nights. All washing steps were done without addition of CHAPS and at 68°C instead of 60°C. The 1:3 prehybridization solution/2x SSPE washing step was also performed at 68°C. RNAse A digestion was done for 20 minutes. The anti-digoxigenin-AP antibody (Boehringer Mannheim) was incubated with the slides over night at room temperature. Afterwards they were washed 3 times in TBST for 20 minutes before incubation in BM purple (Roche). This reaction was stopped by incubation in PBS/20 mM EDTA for 10 minutes before fixation for 45 minutes in 4% paraformaldehyde. Then the sections were embedded in DPX mountant for histology (Fluka).

Antibody production and purification

A rabbit antibody against a SPARCL1/SC1 peptide (DPNKDKHITLKEWGHCFG) was obtained from Neosystems S.A. (Strasbourg, France).

The serum of the rabbits was purified over a CNBr-activated Sepharose 4B (Amersham) column coupled with peptide according to the instructions of the manufacturer. In brief, 0.3 g of CNBr-activated Sepharose 4B (Amersham) was swollen in 3 ml 1 mM HCl for 15 minutes on a shaker at room temperature (in general, 1 g freeze dried material gives about 3.5 ml final gel volume). Then the gel was washed on a sintered glass filter with 200 ml 1 mM HCl before washing with 5 ml coupling buffer (0.1 M $NaHCO_3$ and 0.5 mM NaCl, pH 8.3). Afterwards the gel was immediately transferred to the peptide solution in a 15 ml Falcon tube (5 mg peptide in 2 ml coupling buffer; in general, 5-10 mg protein are coupled to 1 ml of gel). After incubation for two hours on a shaker at room temperature the mixture was centrifuged for 5 minutes at 1'500 rpm (≈ 350 g). The supernatant was removed, the gel was gently resuspended in 10 ml blocking buffer (1 M glycine in coupling buffer) and incubated on a shaker at 4°C over night. To remove excess of uncoupled peptide the gel was washed 5 times on a sintered glass filter alternately with 50 ml coupling buffer and with 50 ml wash buffer (0.1 M NaAcetate pH 4.2 and 0.5 M NaCl). The gel was washed with coupling buffer, then with PBS and finally it was stored in borate buffer (50 mM Na_3BO_3 pH 8.0, 150 mM NaCl) at 4°C. The gel was transferred to a column and washed with 0.1 M glycine pH 2.5. In the meantime, the serum was centrifuged in 30 ml Corex tubes for 10 minutes at 9'000 rpm (≈ 6'300 g).

The supernatant was transferred to a 50 ml Falcon tube. After washing the column with PBS the serum was twice loaded onto the column. Another washing step with PBS was performed before the anti-SPARCL1/SC1 antibody was eluted with 0.1 M glycine pH 2.5. 800 µl aliquots were collected in Eppendorf tubes containing 200 µl Tris pH 7.5 to neutralize the samples.
Aliquots containing antibodies were detected by SDS-PAGE (Gelcode Blue Stain Reagent from Pierce; according to the instructions of the manufacturer). The specificity of the antibody was tested by ELISA on wells coated with peptide and by Western Blot on transfected/untransfected HEK293-T cells.

ELISA (Enzyme-Linked ImmunoSorbent Assay)

For testing whether the antibody recognizes the peptide the wells of a 96 well plate were coated with 50 µl 1 mM SPARCL1/SC1 peptide (see antibody production) at 4°C over night. Unbound peptides were removed by aspiration and the wells were rinsed with PBS. Blocking was then performed with 100 µl 4% skim milk/PBS for one hour at 37°C. After three washing steps with PBS 50 µl of the anti-SPARCL1/ SC1 antibody diluted differently in 1% skim milk/PBS was added to the wells. Five washing steps with PBS were done before 50 µl horseradish peroxidase-conjugated goat anti-rabbit antibody (Socochim) was added (1:1'000 in 1% skim milk/PBS) and incubated for 1 hour at 37°C. Five washing steps with PBS were performed and then 100 µl OPD (1,2-phenylendiamine-dihydrochloride) solution (20 mg OPD, 24 ml 0.1 M citric acid, 26 ml 0.2 M Na_2H-phosphate, and 40 µl 30% H_2O_2) were added for 10 minutes at room temperature. The reaction was stopped by addition of 50 µl 2.5 M H_2SO_4 before reading absorbance at 490 nm (and 630 nm as reference) in an ELISA reader.

Cell extract (HEK293-T)

HEK293-T cells were maintained in DMEM medium supplemented with 10% FCS and grown in a CO_2-incubator at 37°C (6% CO_2). For transient transfections $1.5x10^5$ HEK293-T cells were seeded in 6 well plates. After 24 hours the cells were transfected with 2 µg human SPARCL1 construct using the FuGENE 6 Transfection Reagent (Roche; according to the instructions of the manufacturer).

After incubation for 48 hours the cells were quickly rinsed with ice-cold PBS and then scraped with 200 µl RIPA buffer containing proteinase inhibitors (120 mM NaCl, 50 mM Tris pH 8.0, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS, and complete mini proteinase inhibitor; Roche, concentration according to the instructions of the manufacturer). The scraped cells were transferred to a 1.5 ml Eppendorf tube before incubation on ice for 30 minutes. The tubes were centrifuged at 12'500 rpm for 10 minutes at 4°C in a tabletop centrifuge. The supernatant was frozen at -80°C. As negative control untransfected cells were used.

To express human SPARCL1 a construct based on the pcDNA3.1/Zeo+ vector (Invitrogen) was produced. Using the NheI-EcoRI restriction sites, a Kozak sequence, an ATG start site, a human influenza A haemaglutinin signal peptide for secretion, and a 5x myc tag were added to the pcDNA3.1/Zeo+ vector. The human SPARCL1 sequence lacking its signal peptide was ligated into the modified vector (3' of the myc tag) using the EcoRI-ApaI restriction sites.

Mouse tissue extract

Fresh tissue (cortex, cerebellum, kidney, liver, lung, heart, and small intestine) from 2.5 months old nude mice was frozen directly after dissection on dry ice. The frozen tissue was grinded with liquid nitrogen in a mortar. The pulverized tissue was transferred to an Eppendorf tube and 750 µl lysis buffer containing proteinase inhibitors (25 mM Tris pH 7.5, 50 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 5mM DTT, and complete mini proteinase inhibitor; Roche, concentration according to the instructions of the manufacturer) were added. The dissolved tissue was Dounce homogenized and then centrifuged at 5'000 rpm for 10 minutes at 4°C in a tabletop centrifuge.

The concentration of the supernatant (containing soluble, non nuclear proteins) was determined using the Bio-Rad Protein Assay (Bio-Rad) and the mouse tissue extract was stored at -80°C.

Human tissue extract

Fresh human tissue (colon epithelium and muscle) was frozen directly after surgery on dry ice. The frozen tissue was basically processed in the same way as the mouse tissue, however, with a different lysis buffer (120 mM NaCl, 50 mM Tris pH 8.0, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS, and complete mini proteinase inhibitor; Roche, concentration according to the instructions of the manufacturer).

Western Blot

To 100 µg mouse/human tissue extract or 22.5 µl of cell extract 4x SDS-PAGE sample buffer (1.6x stacking gel buffer, 4% SDS, 17.4% glycerol, 20% β-mercapto-ethanol, and 0.01% bromphenolblue) was added to a final 1x concentration. The samples were boiled for 5 minutes at 95°C and the denatured protein extracts were loaded on a 7.5% SDS-PAGE Minigel (BioRad). After gel electrophoresis proteins were transferred electrophoretically to Immobilion P membrane (Millipore) for two hours at 45 mA per gel. The membrane was washed 3 times for 5 minutes in TBST with vigorous shaking before blocking in 3% skim milk/TBST for one hour at room temperature on a shaker. Then it was transferred to a new tray with anti-SPARCL1/SC1 antibody (final concentration 300 ng/ml) in 3% skim milk/TBST. After incubation over night at 4°C on a shaker the membrane was washed 3 times for 5 minutes in TBST with vigorous shaking. The membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (Socochim) in 3% skim milk/TBST (1:10'000) for one hour at room temperature on a shaker. After washing 3 times for 5 minutes in TBST with vigorous shaking immunoreactive bands were identified by incubation for 5 minutes with ECL Western Blotting detection reagents (Amersham) and exposure to Kodak BioMax MR films (Kodak). As protein molecular weight standard Benchmark pre-stained marker or Benchmark marker (Invitrogen) were used.

Immunofluorescence

Cos-7 cells (african green monkey) were maintained in DMEM medium supplemented with 10% FCS and grown in a CO_2-incubator at 37°C (6% CO_2).

For transient transfections $1x10^5$ Cos-7 cells were seeded in a 35 mm dish with 4 compartments (Greiner Bio-One). After 24 hours each compartment was transfected with 0.25 µg human SPARCL1 construct (see cell extract) using the FuGENE 6 Transfection Reagent (Roche; according to the instructions of the manufacturer). After 24 hours the cells were fixed for 15 minutes with 4% paraformaldehyde before they were washed 3 times for 5 minutes with 1% BSA/PBS. Then the cells were permeabilized for 5 minutes with 0.1% Triton X-100 in PBS and washed again 3 times for 5 minutes with 1% BSA/PBS. After blocking in 2% BSA/PBS for one hour at room temperature, the anti-SPARCL1/SC1 antibody (final concentration 6 µg/ml) was added in 1% BSA/PBS and the cells were incubated at 4°C over night. The next day the cells were washed 3 times for 5 minutes with 1% BSA/PBS before adding the goat anti-rabbit rhodamine antibody (Santa Cruz) diluted 1:1'000 in 1% BSA/PBS for 1 hour at room temperature. Then the cells were washed 3 times for 5 minutes with 1% BSA/PBS and finally they were embedded in Mowiol (Calbiochem).

Immunohistochemistry (mouse)

5 µm thick paraffin sections were dried on Super Frost Plus slides (Menzel-Glaeser) over night at room temperature before two deparaffinization steps of 10 minutes in xylene were performed. Afterwards the sections were rehydrated by incubating 5 minutes in 100, 80, 70% ethanol, and finally PBS. To quench the endogenous peroxidase activity the slides were soaked for 30 minutes in 3% H_2O_2/ 10% methanol followed by 5 minutes washing in PBS. Blocking was performed in 10% normal goat serum/0.3% Triton X-100 in TBS/4 drops Avidin D per ml (Blocking kit; Vector Laboratories) for 30 minutes at room temperature. The sections were rinsed for 5 minutes in TBS, then the anti-SPARCL1/SC1 antibody (final concentration 10 µg/ml) was added in 10% normal goat serum/0.3% Triton X-100 in TBS/4 drops Biotin per ml (Blocking kit; Vector Laboratories). After incubation at 4°C over night the slides were washed 3 times for 5 minutes with TBS. Then the sections were incubated with a biotinylated goat anti-rabbit antibody (Vectastain ABC kit; Vector laboratories) diluted 1:200 in 10% normal goat serum/0.3% Triton X-100 in TBS for 30 minutes at room temperature.

After washing 3 times for 5 minutes with TBS the sections were incubated for 30 minutes with ABC reagent (prepared 30 minutes before use: 1 drop reagent A in 2.5 ml TBS and 1 drop reagent B; Vectastain ABC kit; Vector laboratories). The slides were soaked another 5 minutes in TBS and then they were incubated for 20 minutes in 50 mM Tris pH 7.6 at room temperature. Afterwards DAB substrate (DAB substrate kit for peroxidase: 2.5 ml ddH_2O, 1 drop buffer stock solution, 2 drops DAB stock solution, and 1 drop hydrogen peroxide solution; Vector Laboratories) was added to the sections. After 10 minutes incubation the slides were rinsed for 5 minutes in tap water and then they were counterstained for 10 seconds in Mayer's haematoxylin solution (Sigma diagnostics). The slides were dehydrated and after incubation in xylene embedded in DPX mountant for histology (Fluka).

Immunohistochemistry (human)

Immunohistochemistry on 5 μm thick human colon paraffin sections was performed according to the instructions of the Discovery system (Ventana). The anti-SPARCL1/ SC1 antibody was used at a final concentration of 12 μg/ml.

Results

Search for CA repeats close to the SPARCL1 gene

Unfortunately, we did not find any hot spot of deletion by microsatellite analysis using 7 different highly polymorphic microsatellite markers (see section 1 of this thesis). At the time the study was performed, we only had a rough chromosomal localization of SPARCL1 (4q22-25) from FISH and CGH (Isler et al., 2001) and the region encompassed by our microsatellite markers was about 15.2 million basepairs long. Therefore, we wanted to know if some so far unidentified CA repeats could be detected near by the genomic region of SPARCL1 and if these potential CA repeats could be used for further microsatellite analysis experiments. By Southern Blot analysis we were looking for CA repeats on the genomic P1 clone 24 (91.6 kb encompassing the 5' promoter region and exon 1 to intron 6 of human SPARCL1 gene; for construction and purification see Isler et al., 2001). With an end labeled $(AC)_{15}$ primer we detected several bands in the EcoRI or XbaI digests of the P1 clone 24 and these bands were subcloned in Bluescript KS+ vector. After filter lift assay and hybridization of the subcloned fragments with the end labeled $(AC)_{15}$ primer all colonies with CA repeats were used to start bacteria cultures. The inserts of the clones were sequenced to find the CA repeats. Unfortunately, the longest stretch found (at the 3' end of the intron 1) was only 4 CA repeats long and was therefore much too short for microsatellite analysis.

Validation of riboprobes: Northern Blot analysis and in situ hybridization on mouse brain sections

SC1 specific riboprobes were tested for specificity using Northern Blot analysis. Total RNA from mouse brain was separated on a gel and transferred electrophoretically to Hybond N+ membrane. After hybridization we detected a single band in brain with the antisense probe, whereas the sense probe did not detect any band (see figure 1). The size of the band corresponds to the published mRNA size of SC1 (3.2 kb). Additionally this band seems to be specific as it is not detected with the sense probe.

By in situ hybridization the riboprobes were also tested on 10 µm adult mouse brain paraffin sections. The staining found (dentate gyrus, pyramidal cells) was in agreement with published data (Mendis et al., 1996b).

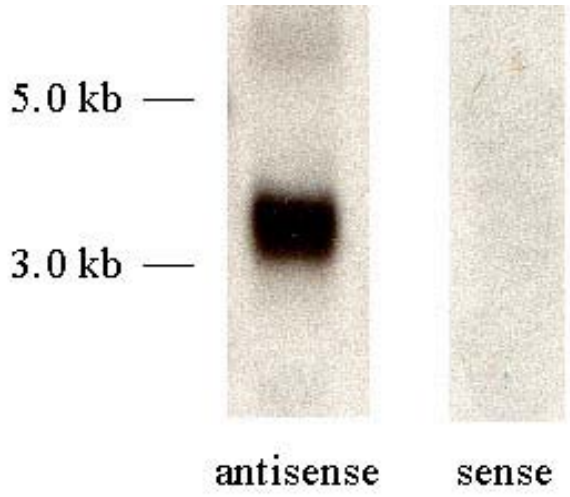

Figure 1: Northern Blot analysis with mouse brain RNA
To test the specificity of the SC1 riboprobes used for in situ hybridization we performed Northern Blot analysis with mouse brain RNA. On the left the antisense, on the right the sense probe was used for hybridization.

In situ hybridization on lung sections of A/J mice

To find out whether SC1 is also downregulated in mouse lung tumors, in situ hybridization was performed on lung paraffin sections of A/J mice (tissue kindly provided by Dr. Karin Wertz, DSM Nutritional Products, VFHC, Basel). This study included three A/J mice, which were treated with the tobacco specific nitrosamine NNK (=4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) to enhance the induction of lung tumors. The resulting lesions have a similar histology as human lung adenomas and evolve over a distinct progression from hyperplasia, to benign appearing adenomas, and then to frank carcinomas. By in situ hybridization we could show that SC1 is also downregulated in mouse lung tumors as it is seen in human lung cancers (see figure 2). Only two of the three mice developed lung tumors, but all tumors lacked SC1 mRNA. This result suggests that the downregulation of SPARCL1/SC1 might be important for lung tumor formation.

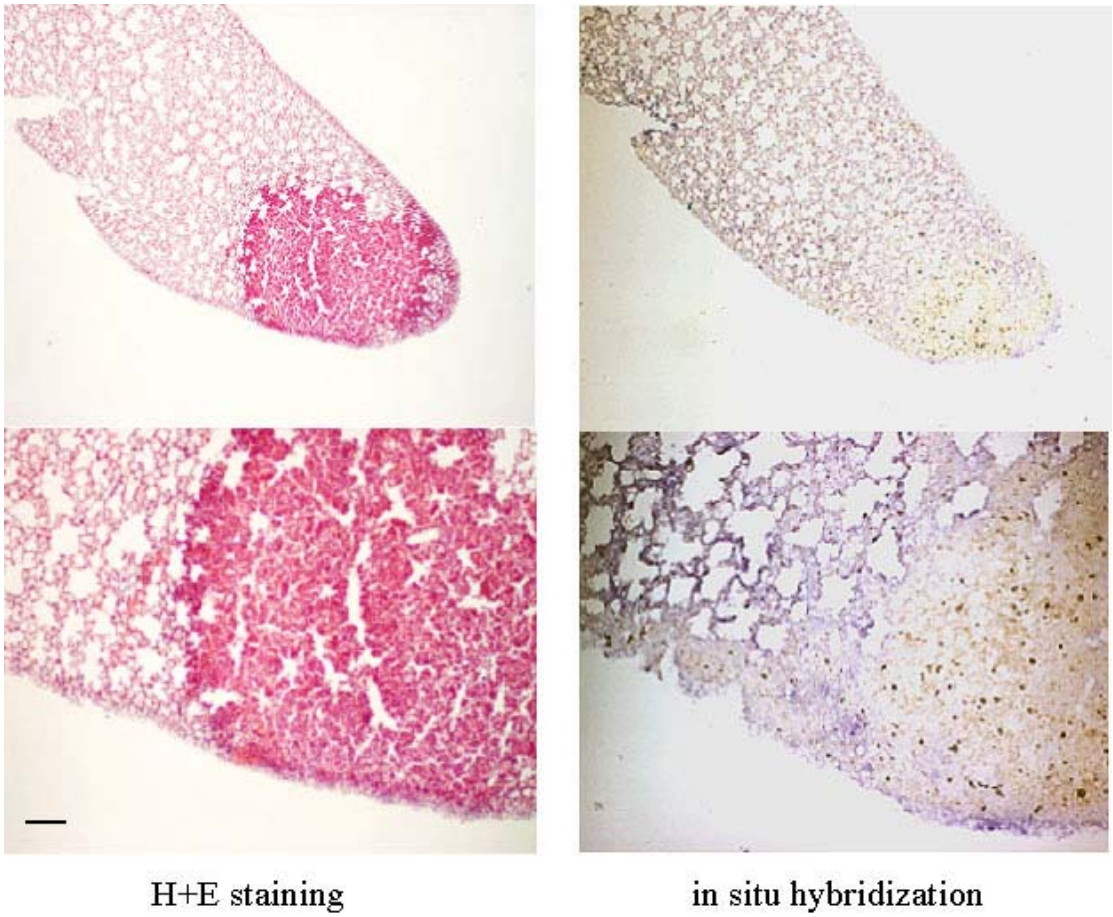

Figure 2: In situ Hybridization of mouse lung sections

SC1 mRNA expression and downregulation found with an exon 4-6 specific riboprobe in lung of A/J mice treated with NNK. On the left H+E staining is shown whereas on the right in situ hybridization is shown (bar 100 μm).

Production of a polyclonal antibody and validation

SPARCL1 mRNA expression is downregulated in many human tumors as well as in murine lung tumors. Therefore, we were interested in the precise localization of the SPARCL1/SC1 protein in normal and neoplastic tissue sections.

An existing polyclonal chicken antibody against the acidic N-terminal part of the SPARCL1/MAST9 protein (anti-human MAST9Δ; (Bendik et al., 1998)) proved to work very specifically on Western Blots. However, we were not able to establish immunohistochemistry using this antibody, neither on paraffin sections nor on cryosections. Therefore, we decided to raise new antibodies against a different part of the SPARCL1 protein. We selected a C-terminal peptide that exhibited 100% identity between the human and mouse SPARCL1 sequence. To avoid cross-reactivity we took care to have minimal homology between SPARCL1 and the closely related SPARC protein (see figure 3).

Figure 3: Alignment of SPARCL1/SC1 peptide with mouse and human SPARC
For antibody production a peptide from the C-terminal end of SPARCL1 was chosen which is 100% identical to the mouse SC1 sequence. The SPARCL1/SC1 peptide is aligned to the corresponding sequence of SPARC (mouse: GenBank accession nr AAH04638, human: GenBank accession nr AAH08011).

The peptide was coupled to ovalbumin and two rabbits were immunized. As the final bleedings showed relative high background in Western Blots, we decided to affinity purify the antibodies over a Sepharose column with coupled peptide. The purified antibody was tested for specificity by ELISA and by Western Blot analysis.

Using ELISA on wells coated with uncoupled peptide we could show that the new anti-SPARCL1/SC1 antibody is recognizing the peptide. To test the specificity of the purified anti-SPARCL1/SC1 antibody on Western Blots we used RIPA extracts from HEK293-T cells that were transiently transfected to express the human SPARCL1 protein. As negative control non-transfected HEK293-T cells were used.

As expected, we detected two bands of 70/150 kD in SPARCL1 expressing cells (see figure 4A), which might represent mono- and dimers of the SPARCL1 protein (Bendik et al., 1998). Surprisingly, the 70 kD band was also present in non-transfected cell lysates. Preincubation of the anti-SPARCL1/SC1 antibody with the SPARCL1/SC1 specific peptide blocked the 150 kD band but not the 70 kD band (4B). The unspecific band could be blocked by changing the blocking and antibody dilution solutions from 3% skim milk to 10% goat serum (4C). In subsequent immunohistochemical experiments all blocking and antibody dilution steps were performed using 10% goat serum.

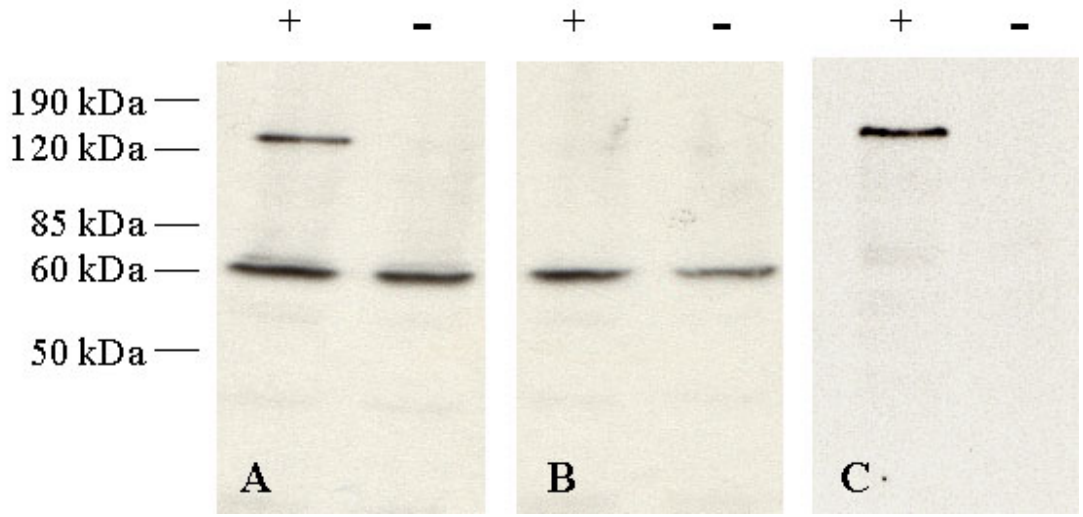

Figure 4: Western Blot analysis with HEK 293-T cell extracts

To test the specificity of the new anti-SPARCL1/SC1 antibody Western Blot analysis with transfected (+) and untransfected (-) HEK 293-T cells was performed. For transfection a human SPARCL1 construct was used.

A: standard Western Blot procedure using 3% skim milk for blocking and antibody dilutions. B: the antibody was preincubated with the corresponding peptide. C: blocking was performed with 10% goat serum instead of 3% skim milk.

Immunofluorescence on Cos-7 cells transfected with the human SPARCL1 construct showed a nice Golgi/ER staining (see figure 5) as expected from the fact that this construct contains a signal peptide for secretion.

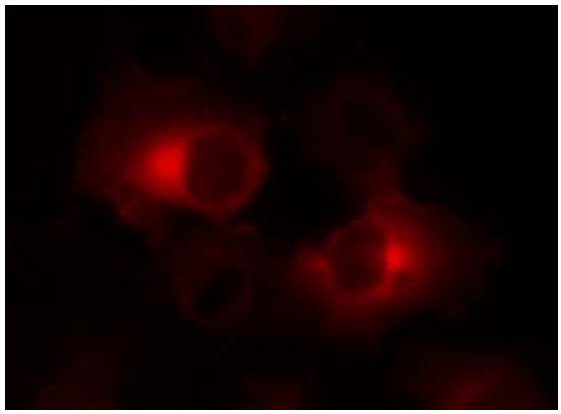

Figure 5: Immunofluorescence of SPARCL1 transfected Cos-7 cells
Cos-7 cells were transfected with a human SPARCL1 construct and immunofluorescence was performed with the anti-SPARCL1/SC1 antibody. The Golgi/ER staining showed here was not obtained when the cells were not transfected or when the antibody was preincubated with the corresponding peptide.

SC1 expression in murine tissue

To find out more about the SC1 protein expression in mouse we conducted Western Blot analysis with extracts from different mouse tissues (cortex, cerebellum, kidney, liver, lung, heart, and small intestine). We detected a single band of about 70 kD in all tissue extracts which corresponded to the predicted molecular weight of SC1 monomers but no bands that could correspond to SC1 dimers (see figure 6). The 70 kD band was specific as it could be blocked by preincubation of the antibody with the peptide.

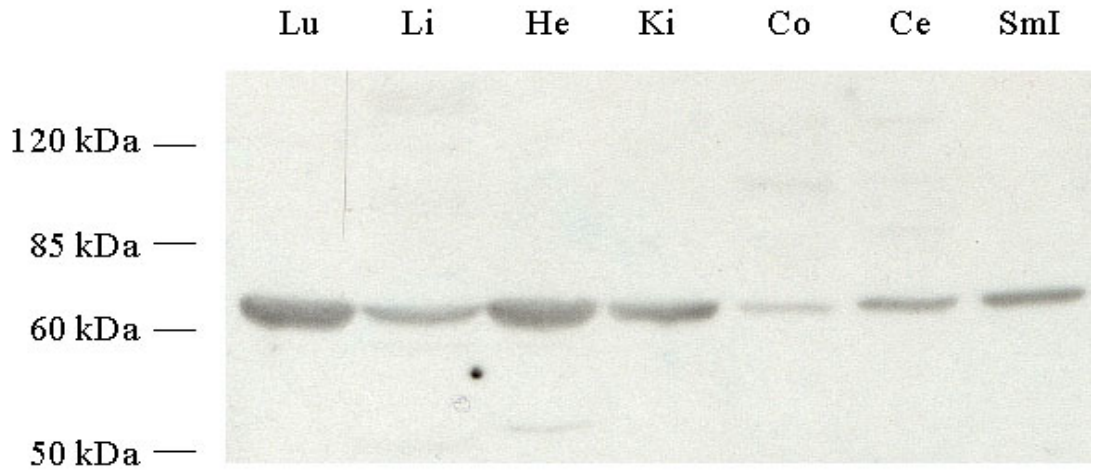

Figure 6: Western Blot analysis with mouse tissue extracts

To find out more about the protein expression in different mouse tissues Western Blot analysis with the anti-SPARCL1/SC1 antibody was performed. Lu=lung, Li=liver, He=heart, Ki=kidney, Co=cortex, Ce=cerebellum, and SmI=small intestine. Equal amounts were loaded except for small intestine where only half of the amount was loaded.

In 15.5 days old mice we found staining in muscle, lung, heart, liver, gastric mucosa, and skin, whereas in head sections of 17.5 days old mouse the vibrissae, the cornea, and the retina were stained. These results are in accordance to the staining of lung in 16 days old mice and of surrounding connective tissue of vibrissae in 18 days old mice found by in situ hybridization (Soderling et al., 1997). Immunohistochemical stainings of the vibrissae could be blocked by preincubation of the antibody with the peptide (see figure 7 A+B). Performing immunohistochemistry on mouse paraffin sections with the anti-SPARCL1/SC1 antibody we could show that the pattern in adult brain (dentate gyrus, pyramidal cells, astrocytes, and purkinje layer) is in accordance to published data: Mendis et al. found the same pattern using immunohistochemistry and in situ hybridization (Mendis et al., 1996a; Mendis et al., 1994; Mendis et al., 1996b).

Additionally we found staining of the basal lamina of the lung bronchioles (see figure 7 C), the muscle in different tissues (7 D), and the blood vessels in heart (7 E).
These results are in accordance to the staining of lung bronchioles and heart blood vessels found by in situ hybridization (Soderling et al., 1997). SC1 mRNA expression in muscle was so far only proven by Northern Blot analysis (McKinnon et al., 2000).

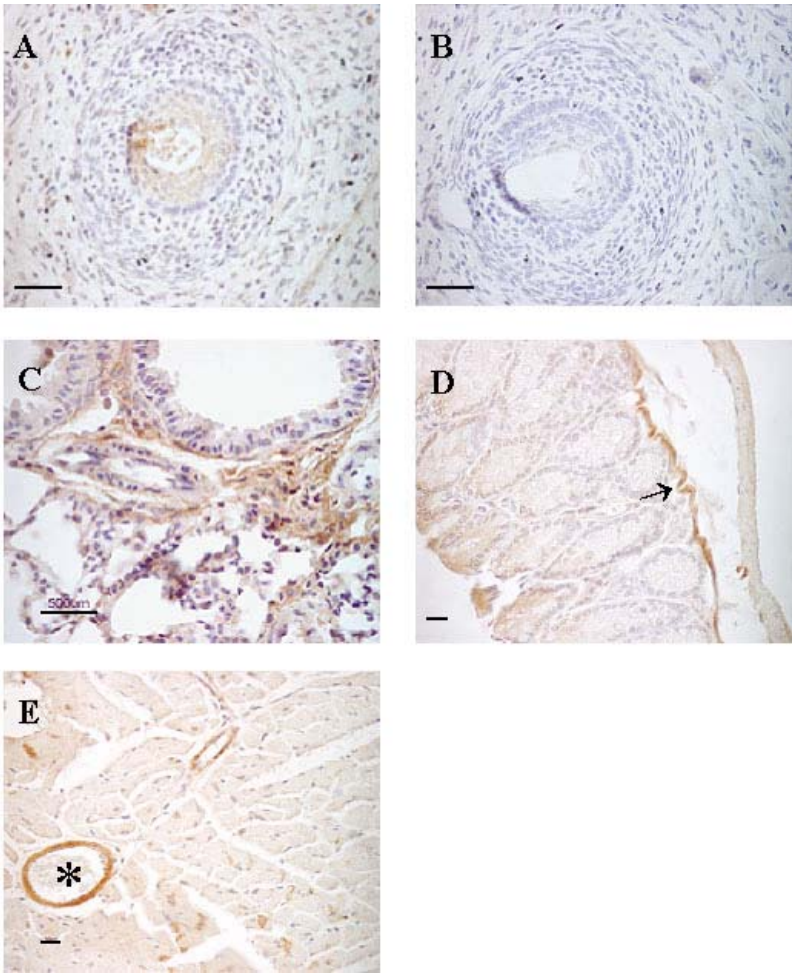

Figure 7: Selection of immunohistochemistry stainings on mouse paraffin sections
Immunohistochemistry on mouse paraffin sections was performed to confirm the specificity of the anti-SPARCL1/SC1 antibody. A: vibrissae staining of a 17.5 days old mouse embryo. B: vibrissae staining was blocked when the antibody was preincubated with the corresponding peptide. C: staining of the basal lamina of a bronchiole (adult mouse lung). D: muscular staining in adult mouse colon (marked with →). E: staining of a blood vessel (marked with ∗) in adult mouse heart. Counterstaining of the nuclei was performed using haematoxylin (blue). A, B, D, and E: bar 50 µm & C: bar 500 µm

SPARCL1 expression in human tissue

As SPARCL1 was found to be downregulated on mRNA and protein level in human NSCLCs and as its transcript is also downregulated in human colon carcinomas (Claeskens et al., 2000; Notterman et al., 2001) a role of SPARCL1 in colon cancer was expected. Therefore, we wanted to investigate the SPARCL1 protein expression pattern particularly in colon cancer. Additionally, investigation of SPARCL1 expression in colon adenoma could give insight whether the downregulation of SPARCL1 takes place in early stages of cancer development.

Immunohistochemistry on human tissue sections was not trivial and we could not directly adapt the mouse protocol for humans. Therefore, we tried to optimize the protocol using many different conditions and antigen retrievals such as citrate buffer heated in a microwave and 0.3% Triton followed by 0.75% glycine at room temperature. Finally we obtained specific staining patterns with the Discovery system (Ventana), whereas results obtained by conventional ("self-made") procedures were difficult to reproduce and often showed a patchy staining. The advantages of the Discovery system are: non-solvent based removal of paraffin, automated epitope unmasking, flexible primary antibody incubation options, flexible detection options, four available detection chemistries, and automated counterstaining. With this system we found a staining in colon muscle and blood vessels, whereas epithelium was not stained (see figure 8).

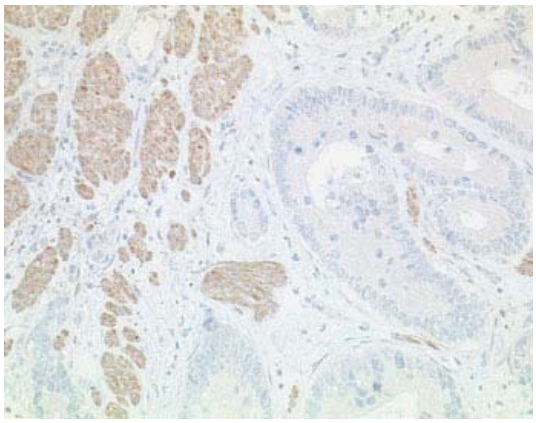

Figure 8: Immunohistochemistry on human paraffin sections

Using the Immunostainer (Ventana) we performed immunohistochemistry with the anti-SPARCL1/SC1 antibody on sections of human colon. The muscular staining showed here was found after an additional antigen retrieval step. Counterstaining of the nuclei was performed using haematoxylin (blue).

The staining of muscle but not epithelium was unexpected, however, our data could be confirmed by Western Blot analysis with human colon tissue extracts with carefully separated epithelium and muscle. On the blot we detected no signal in epithelium but two bands with a molecular weight of more than 190 kD in muscle (see figure 9). Both bands could be blocked by preincubation of the antibody with the SPARCL1/SC1 peptide and therefore seem to be specific.

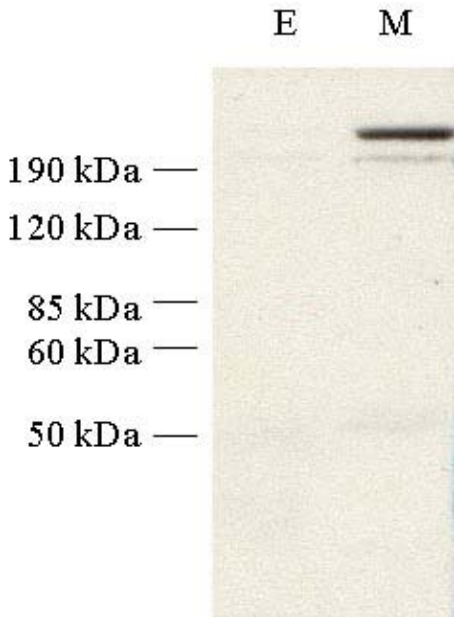

Figure 9: Western Blot analysis with human colon extracts

To define the protein expression pattern in human colon Western Blot analysis with the anti-SPARCL1/SC1 antibody was performed. E=epithelium and M=muscle.

Discussion

SPARCL1 is downregulated in human NSCLCs and expressed in similar tissue as SC1 (Bendik et al., 1998). Therefore, we wanted to know whether SPARCL1 downregulation is limited to human tumors or whether it also occurs in murine lung tumors.

The A/J mouse model is a good model for lung tumors as it has a high incidence of spontaneous tumors and tumors can be induced by carcinogen exposure (for example NNK). The lesions have a similar histology as human lung adenomas and evolve over a distinct progression from hyperplasia, to benign appearing adenomas, and then to frank carcinomas (Vogelstein and Kinzler, 1998). The major determinant of lung cancer predisposition in the A/J mouse strain was found to be the Pas1 locus (pulmonary adenoma susceptibility) (Manenti et al., 1997). Interestingly, the K-ras2 proto-oncogene, is located nearby this locus and has been studied in detail: this gene is mutated in 70 to over 90% of spontaneous and carcinogen-induced murine lung adenocarcinomas and is also mutated in a significant percentage of human lung adenocarcinoma (Vogelstein and Kinzler, 1998).

We had the opportunity to obtain lung tissue sections from A/J mice, which were treated with NNK to induce lung tumors. In our study we used a SC1 specific riboprobe to perform in situ hybridization on these sections. We found SC1 mRNA expression in normal mouse lung whereas it was absent in lung tumors. As SPARCL1 downregulation in lung tumors is conserved between human and mouse we suggest that SPARCL1 downregulation is, at least in lung tumors, an important step in tumor formation.

SPARCL1 is not only downregulated in lung tumors but also in colon (Claeskens et al., 2000; Notterman et al., 2001), bladder (Dr. P. Schraml, personal communication), and prostate tumors (Nelson et al., 1998). Colon cancer is of big importance as it is a very common cancer and as it can be detected at early stages. Additionally it is the cancer with the best known order of gene alterations leading to tumor formation. Using oligonucleotide arrays Notterman et al. found that SPARCL1 downregulation is an early event during colon cancer formation (Notterman et al., 2001).

This result is in accordance to unpublished data from our lab. By real-time quantitative PCR we found reduced SPARCL1 mRNA expression in colon adenomas (compared to normal tissue) in 10 out of 13 patients. However, in colon adenocarcinoma the result were not as clear (25% upregulated and 54% downregulated). To confirm this downregulation on the protein level and to learn more about the protein expression pattern in normal and tumor tissue we decided to produce a new polyclonal antibody against a SPARCL1/SC1 peptide in rabbits. This antibody was shown to detect specifically a human SPARCL1 construct by Western Blot analysis of transfected cell extracts and by immunofluorescence on transfected cells. Additionally, we showed that this antibody crossreacts with SC1 protein in Western Blot analysis of mouse tissue extracts and in immunohistochemistry on mouse paraffin sections.

Several attempts were needed to get positive immunohistochemistry staining on human paraffin sections. As we got patchy staining using conventional procedures we changed to the automated Discovery system (Ventana). To our surprise we could not detect any SPARCL1 protein in the normal colon epithelium (which is the origin of colon cancer), but it was present in the surrounding muscle. This was confirmed by Western Blot analysis of carefully separated human epithelium and muscle extracts. These data are in disagreement with the published data where SPARCL1 mRNA was detected in normal tissue. With our approach we show that SPARCL1 protein is not expressed in colon epithelium, but we cannot exclude that SPARCL1 mRNA is present and not translated. However, we have strong evidence that the mRNA detected in normal colon tissue extracts originates from muscle cells and not from epithelial cells. In summary, our results emphasize the necessity to confirm all mRNA data on protein level.

The predicted SPARCL1 monomer has a molecular weight of 75 kD, however, by Western Blot analysis of SPARCL1 in human colon we revealed two extraordinary high molecular weight SPARCL1 protein bands (>190 kD). These signals seem to be specific as we were able to block the reaction by preincubation of the antibody with the SPARCL1/SC1 peptide.

Interestingly, different molecular weights are published for SPARCL1: Claeskens et al. and Bendik et al. found two bands of 75 and 150 kD (Bendik et al., 1998; Claeskens et al., 2000), whereas Girard and Baekkevold only found one band (130, respectively 110 kD) (Baekkevold et al., 1999; Girard and Springer, 1996). Bendik et al. used lung extracts for Western Blot analysis. All the others were expressing the SPARCL1 protein in cells. Also for SC1 the published molecular weights differ a lot: Johnston et al. found two bands of 116 and 120 kD in brain extracts (Johnston et al., 1990), whereas Hambrock et al. found a single band of 94 kD when the protein was expressed by transfected cells, but two bands of 55 and 116 kD in tissue extracts (Hambrock et al., 2003). We found a single SC1 band of 70 kD in different tissue extracts.

The extraordinary high molecular weight of SPARCL1 in colon could be explained by strong covalent dimerization other than disulfide bridges (or even higher oligomerization), by N-glycosylations (high mannose and/or complex type), by O-glycosylations, and/or by anomalous electrophoretic behaviour due to atypical binding of SDS to the highly negative charged N-terminal domain. To clarify the proportion of N-glycosylations in the SPARCL1 protein, it would be necessary to digest the protein with endoglycosidase H or N-glycosidase F.

The putative function of SPARCL1/SC1 is still a matter of speculations. Purified SPARCL1/hevin inhibited adhesion of endothelial cells to a fibronectin substrate, indicating that SPARCL1 belongs to the family of adhesion modulating proteins, which includes SPARC, thrombospondin, and tenascins (Sage and Bornstein, 1991). These proteins are structurally unrelated modular glycoproteins. However, all of them antagonize the pro-adhesive activities of other matrix proteins, e.g. fibronectin, and are thought to regulate the ability of a cell to differentiate, migrate, or proliferate (Murphy-Ullrich, 2001). Soderling et al. investigated SC1 mRNA expression by in situ hybridization. In organs with SC1 expression, high levels of mRNA were found in cells that provide structural support, i.e., smooth muscle cells or fibroblasts (Soderling et al., 1997). However, cultured proliferating smooth muscle cells or fibroblasts had no detectable SC1 mRNA. Furthermore, it was shown that SPARCL1 could act as a negative regulator of cell proliferation in HeLa 3S cells (Claeskens et al., 2000). These data let us speculate that the main function of SPARCL1/SC1 may be to support cellular differentiation rather than promoting proliferation.

We hypothesize that SPARCL1 might interfere with cell adhesion and thereby might activate a signal that specifically leads to cell cycle arrest or apoptosis if it is expressed in inappropriate tissues (e.g. lung cancer).

It would be interesting to investigate the effects of purified SPARCL1 on cell adhesion and cell survival in different cell lines (including cancer cell lines) and to investigate the mechanism involved in the regulation of cell proliferation by SPARCL1.

Abbreviations

aa	aminoacid
ADC	adenocarcinoma
Ci	Curie
ELISA	Enzyme-Linked Immuno Sorbent Assay
ER	endoplasmatic reticulum
EST	expressed sequence tag
FCS	fetal calf serum
H+E	haemalaun + eosin staining
HEK	human embryonic kidney
kb	kilobasepairs
LB	Luria-Bertani medium
LCC	large cell carcinoma
LOH	loss of heterozygosity
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
nr	number
NSCLC	non-small cell lung cancer
nt	nucleotide
PAGE	polyacrylamide gel electrophoresis
SCC	squamous cell carcinoma
SPARC	secreted protein acidic and rich in cysteine
SPARCL1	SPARC-like 1
TK	thymidine kinase

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Genetic recombination (T. Bickle)
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Curriculum vitae

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S. G. Isler and C. U. Ludwig. SPARC-like 1 (SPARCL1), a gene downregulated in non-small cell lung cancer, Thesis, University of Basel, 2004

S. G. Isler, C. U. Ludwig, R. Chiquet-Ehrismann, and S. Schenk. Evidence for transcriptional repression of SPARC-like 1 (SPARCL1), a gene downregulated in human lung tumors (submitted to International Journal of Oncology)

Talks:

S. G. Isler, S. Schenk, and C. U. Ludwig. SPARC-like 1 (SPARCL1), a gene often deleted in NSCLC patients, 8th annual meeting of the European Cancer Center Basel – Freiburg – Strasbourg, 16th of march 2001, Strasbourg, France

Posters:

S. G. Isler, I. Bendik, S. Schenk, and C. U. Ludwig. Genomic characterization of the human SPARC-like protein 1 (SPARCL1), a putative lung tumor suppressor gene, annual meeting of the American Association for Cancer Research (AACR), 10th-14th of april 1999, Philadelphia, USA

S. Isler, S. Schenk, C. Ludwig, and R. Chiquet-Ehrismann. SPARC-like 1 (SPARCL1), a gene downregulated in human and murine lung tumors, annual meeting of the Friedrich Miescher Institute Basel, 19th-22th of september 2002, Lucerne, Switzerland