RNA interference as a tool to prevent acute and chronic enterovirus myocarditis

der Fakultät für Biologie
der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines Doktors
der Naturwissenschaften

von
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aus Triest, Italien
vorgelegte
Dissertation

2007

Tag der mündlichen Prüfung:	12. 06. 2007
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TO MY PARENTS

''This deserves to be inquired into''

Bell C. 1844

Acknowledgements

I thank the Department of Molecular Pathology, University Hospital of Tuebingen, for giving me hospitality and allowing me to carry out this interesting Ph. D research.
In the first place I would like to recall my gratitude to Dr. Gabriele Grassi for his supervision, outstanding advice, constant help and moral support from the very early stage of this research and throughout these years.
In addition I want to thank for valuable contribution Dr. R. Kandolf for taking care of my situation and Dr. K. Klingel for finding financial support, thus giving me the possibility to complete the Ph. D. program, and for using her time to read the thesis and giving her critical comments.
Many thanks to Dr. N. Blin for showing me a new critical point of view and for the patience and helpfullness without which I wouldn' t have been able to finish in time.
I' ve benefited by advices and guidance from Dr. G. Szalay who always kindly granted me her time for answering many questions.
Many thanks to Dr. Bock, for offering me his time and following my work progresses during these three years.

I want to thank H. Keiser and S. Bundschuh for sharing with me their experience and for giving me pratical and important advices.

Thanks to my colleagues A. Duchting and B. Koberland for the difficult moments in the lab, for giving me help and support and for being friends.

A special thank to K. Petersen, for being always present in difficult moments and being a very important friend in my whole abroad experience.

I am deeply grateful to my friends Dr. Susan Aberle and Dr. Birgit Haack, those long hours at the computer in the lab will not be forgotten. I am thankful that in the mid of all their activity they offered me their advices and comments. Their patience and helpfulness have no prize.

Finally and from the deep of my heart an immeasurable thank to my parents, who permitted me to study and to work in such an interesting field. I am profoundly grateful for the encouragement they gave me throughout my entire life.
A special thank to my sister Sandra who never left me alone during these period in Germany, giving me help and support; and to my brother Marco for being always there for me and for his wise life advices.

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Abbreviations

AAV	adeno-associated virus
bp	basepair
°C	degree Centigrade
cDNA	complementary DNA
CTGF	connective tissue growth factor
CVB3	coxsackievirus B3
DNA	deoxyribonucleic acid
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	dimethylsulfoxide
dNTP	deoxyribonucleoside triphosphate
ds	double stranded
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
et al.	and others
EtBr	ethidiumbromide
EtOH	ethanol
EV	enterovirus
FAM	6-carboxy-fluorescein
FCS	fetal calf sera
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
µg	microgram
h	hour
H_2O_{dd}	double-destilled water
IRES	internal ribosome entry site
kb	kilo base
kDa	kilo dalton
µl	microlitre
M	Mol/l
min	minute
ml	millilitre
µM	micromolar

mM	millimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaAc	natrium acetate
NaCl	natrium chloride
ng	nanogram
nm	nanometer
PBS	phosphate-buffered saline
PFU	plaque forming unit
PCR	polymerase chain reaction
p.i.	post infection
pmol	picomol
qPCR	quantitative PCR
RNA	ribonucleic acid
RNAi	RNA interference
rpm	rotations per minute
RT	room temperature
RT-PCR	reverse transcription ploymerase chain reaction
s	second
SDS	sodium dodecyl sulfate
siRNA	small interfering RNA
shRNA	short hairpin RNA
ss	single stranded
TAMRA	6-carboxy-tetramethylrhodamine
Taq polymerase	thermostable polymerase isolated from *Thermophilus aquaticus*
TEMED	N,N,N'N'-tetramethylenethylendiamine
TGF-β	transforming growth factor-beta
T_m	annealing temperature
Tris	Tris(hydroxymethyl)aminomethane
U	unit
V	volt
VP1	viral protein 1
W	watt

1. Introduction

1.1 Enteroviral myocarditis and dilated cardiomyopathy.

Myocarditis is clinically and pathologically defined as “inflammation of the myocardium”. Used in this broad term, any inflammatory process affecting the myocardium may be caused by several different causes, such as viruses, bacteria, funghi, worms and drugs.

Infectious and noninfectious myocarditis are the major causes of sudden unexpected death in patients younger than 40 years of age and contribute substantially to dilated cardiomyopathy, one of the leading causes of heart failure worldwide (Baboonian et al., 1997).

There is a consensus that viruses are an important cause of myocarditis in North America and in Europe (Feldman and McNamara, 2000). Studies on murine models infected with cardiotropic viruses focusing on the sequence of events and the molecular basis of the pathogenesis of myocarditis suggest that sustained viral infection of the heart is an important etiological fact in the progression of dilated cardiomyopathy (Martino, 1995).

Many viruses have been associated with myocarditis and, due to positive PCR results in endomyocardial biopsies, enteroviruses, adenoviruses, parvovirus B19, herpesviruses and hepatitis C virus have been suggested as causative agents of the disease.

Enteroviruses can cause various clinical symptoms ranging from mild rash, fever, infection of the respiratory tract, Hand-Foot-Mouth disease to encephalitis, meningitis, hepatitis and myocarditis. The severity of symptoms can vary tremendously. In case of enteroviral myocarditis the disease can manifest in a subacute, acute or chronic myocarditis (Wodruff, 1980). Generally, enterovirus infections are considered to be acute events with symptoms and virus titers peaking within a few days post infection, followed by the clearence of the virus mediated by the adaptive immune response (Mena et al., 1999; Opavsky et al., 1999). However, in susceptible patients the myocardial inflammation may persist and progress to a chronic inflammatory heart disease (Kandolf et al., 1993; Martino, 1994), leading to heart transplantation or death.

Enteroviruses belong to the *Picornaviridae* family and are classified, based on molecular and biological properties, into five species: poliovirus (PV), human enterovirus A (HEV-A), human enterovirus B (HEV-B, including coxsackieviruses of group B (CVB)), human enterovirus C (HEV-C) and human enterovirus D (HEV-D), as shown in Table 1. Several new serotypes were recently described (Hyypia, 1997).

Table 1. Current classification of the human Picornaviruses

Genus	Species	Serotypes
Enterovirus	Poliovirus	PV serotypes, 3
	Human enterovirus A (HEV-A)	CVA serotypes, 11; EV-71
	Human enterovirus B (HEV-B)	CVB serotypes, 6; CVA9
		Echovirus (E), 28 ; EV-69 ; SVDV
	Human enterovirus C (HEV-C)	CVA serotypes, 11
	Human enterovirus D (HEV-D)	EV-68 ; EV-70
Rhinovirus	Human rhinovirus A (HRV-A)	HRV serotypes, 18
	Human rhinovirus B (HRV-B)	HRV serotypes, 3
Cardiovirus	Encephalomyocarditis virus (EMCV)	EMCV
	Theilovirus (Th V)	TMEV, VHEV
Aphtovirus	Foot and mouth disease virus (FMDV)	FMDV serotypes, 7
Hepatovirus	Hepatitis A virus	HAV
Parechovirus	Human parechovirus (HPeV)	HPeV-1 ; HPeV-2

From B. Semler and E. Wimmer (Molecular Biology of Picornaviruses, 2002).

The coxsackieviruses, belonging to group B enteroviruses, have been well established as causative agents of viral myocarditis since they have been detected serologically or by molecular techniques such as polymerase chain reaction (PCR) (Jin et al., 1990; Wee et al., 1992) or *in situ* hybridization (Kandolf et al., 1987; Klingel et al., 1992) in patients with myocarditis.

The progression from viral myocarditis to dilated cardiomyopathy follows mechanisms which remain unclear. Analogous to many other viral illnesses both direct viral injury and the immune response of the host play an important role in the pathogenesis of viral heart disease (Feldman and McNamara, 2000).

The first stage of myocarditis is characterized by the viral replication in myocytes. After the recognition of the cellular receptors and viral uptake into the cells, viral replication takes place and can directly damage the heart by destroying cardiac myocytes. In most cases an appropriate immune reponse is able to clear the virus and eliminate the infected cells, so that the myocarditis can resolve completely without residual damage.

When the immune response and the recruitment of cell infiltrates are inappropriate, the inflammatory processes are unable to effectively clear the virus, leading to chronic inflammation (Liu and Mason, 2001). The presence of additional viral proteins and damaged host tissue expose the immune system to additional sources of antigens and stimulate

continued proliferation of inflammatory cells. Finally, the inflammatory process alters the structure of the myocardium to such an extent that dilated cardiomyopathy eventually results. The ongoing inflammatory processes can also induce the deposition of matrix proteins which results in cardiac remodeling processes and in cardiac fibrosis (Spinale, 2002).
An additional cause of heart damage may involve an autoimmune respose, a process that concerns the recognition of host peptides that resemble pathogens epitopes, thus leading to abnormal processing of normal host proteins (Rose, 2000).

Until now, relatively little is known regarding factors that determine susceptibility to enteroviral infection but it is well known that chronic CVB3 infection of the myocardium is associated with a restricted virus replication and a reduced capsid protein synthesis (Klingel et al., 1992). The ability of a cytolytic virus to have a long term persistence within the cells, and the corresponding cell damage, is thought to be a consequence of several factors. The balance between the virus replication and the host immune response, as well as age-related and immune system conditions, are playing a foundamental role in the outcome of the disease.

1.2 The coxsackieviruses

The coxsackieviruses, genus enterovirus, belong to the *Picornaviridae* family. The name is derived from “pico” meaning small, and “RNA” referring to the ribonucleic acid genome, so "picornavirus" literally means small RNA virus. The five major genera of this large family are distinguished by their physical property, clinical manifestation and RNA sequence.
The coxsackieviruses are divided into A and B subgroups on the basis of the disease originally observed in newborn mice: CVAs cause flaccid paralysis while CVBs cause spastic paralysis. The 6 serotypes identified for the coxsackieviruses of group B have been proven to cause a wide variety of human illnesses, including inflammatory heart disease, pancreatitis, aseptic meningitis and type 1 diabetes (Pallansch, 1997), with the coxsackievirus B3 being one of the most frequent causes of human myocarditis (Huber et al., 1998).

1.2.1 The coxsackievirus B3

The first reported isolate of the coxsackieviruses occurred in the upstate New York town of Coxsackie (Dalldorf G 1948). Soon after its first isolation, the coxsackievirus B3 (CVB3) serotype was recognized as an agent of human myocarditis (Gear et al., 1956).

CVB3 is a non-enveloped, single-strand RNA virus of positive polarity. The viral genome is about 7,4 kb in length and contains an untranslated region at the 5´ and at the 3´termini. The presence of the so called VPg, a protein of just over 20 aminoacids covalently attached to the 5´ terminus of the RNA genome, is an important characteristic of the picornaviruses and serves as a primer for the RNA polymerase in the initiation of the RNA synthesis (Wimmer, 1982) (Nomoto et al., 1977). A poly(A) tail is linked at the 3´terminus.

The virus entry occurs through the recognition of proteins associated with the plasma membrane, which represent virus receptors, that facilitate viral attachment (Kuhn, 1997; Rossmann et al., 2000), virus-induced host cell surface rearrangement (Crowell, Krah et al. 1983) and internalization. In a recent study, Coyne and Bergelson (Coyne and Bergelson, 2006) presented a strategy for coxsackieviruses to invade the organism through the intestinal epithelium. This strategy involves binding of the CVB3 to the Decay Accelerating Factor (DAF), a glycosylphosphatidylinositol (GPI)-anchored protein located at the apical surface of intestinal cells. The binding, clustering several DAF molecules, activates the downstream tyrosine kinase Abl which is known to mediate actin remodeling. This binding of CVB3 to DAF facilitates the driving of DAF-bound-CVB3 to its primary receptor CAR (coxsackie-adenovirus-receptor), an integral transmembrane protein which is a structural component of thight junctions (TJ). The binding of CVB3 to the TJ is a necessary step to induce capsid destabilization.

After viral entry and uncoting, the genomic RNA on one hand acts as a template for translation and subsequent transcription to synthesize more copies of positive genomic RNA through a negative-strand intermediate and on the other hand can also be directly employed as an mRNA template for translation. The translation process yields one long polyprotein, which will be subsequently processed into 11 structural and non-structural proteins. The capsid proteins yielded by cleavage of the precursor polyprotein associate with the positive-strand RNA molecules to form progeny virions, that will be released from the cell by lysis.

1.2.1.1 The CVB3 capsid

The capsid, which is approximately 30 nm in diameter, has an icosahedral symmetry. It is composed of 12 pentamers of proteins, each containing four major subunits, VP1, VP2, VP3 and VP4. The first three of these proteins have molecular weights of around 30 kDa and form the external face of the icosahedral shell. The small VP4, in conjunction with the amino terminus of VP1 and VP2, forms an interface between the capsid and the internal RNA genome.

The structure shows an eight-stranded antiparallel β-sandwich of VP1, VP2 and VP3, while the VP4 protein is completely in the interior of the capsid (Muckelbauer et al., 1995).

A prominent feature of enteroviruses is a narrow depression formed roughly at the junction of VP1 and VP3, which provides a binding site for cellular receptors. On account that this "canyon" is inaccessible to larger antibodies, there is an hypothesis that it is protected from immune surveillance (Rossmann et al., 1985)(Figure 1 B).

Furthermore, in the β-barrel of VP1 there is a hydrophobic pocket to which a variety of small organic compounds can bind, thus inducing conformational changes in the virion and suggesting that this site may be required to trigger uncoating during viral entry (Rossmann, 1994).

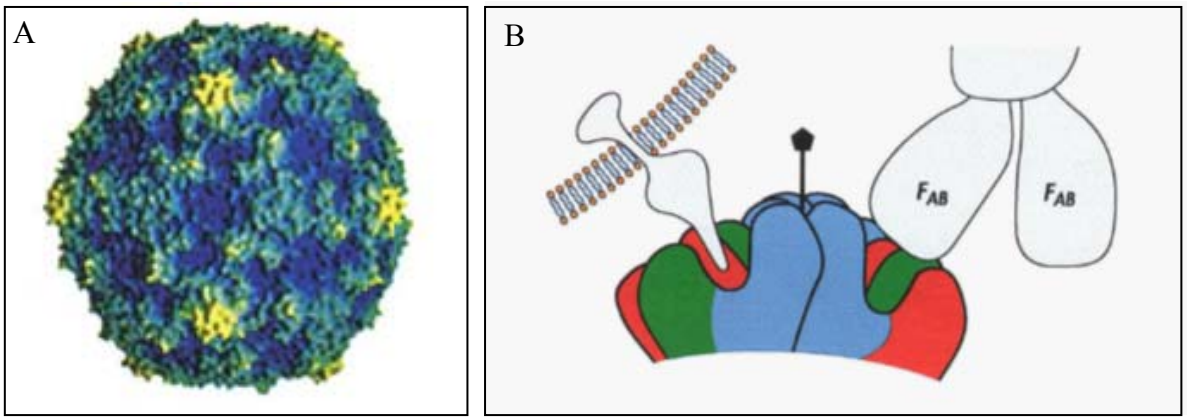

Figure 1: Structure of CVB3

A) Overall structure of CVB3. Regions of dark blue color represent surface depressions while regions of yellow color represent surface protrusions (from Muckelbauer et al. 1995) .

B) Schematic representation of the "canyon" which dimensions hinder antibody´s recognition of the residues at the bas of the site while allowing recognition and binding by a smaller cellular receptor (from Rossmann et al. 1985).

1.2.1.2 The CVB3 genome

The CVB3 genome is composed of two functionally and distinct segments: a *cis* element ensuring and controlling translation initiation (i.e. IRES) and the coding sequence.

The regulatory *cis* element contains a highly structured region which folds to form an internal ribosome entry site (IRES) and is located within the 5´ end of the viral genome (Trono et al., 1988). The elements of the IRES region are recognized by cellular translation factors, ribosomes, and other cellular proteins to assemble an initiation complex directly on the downstream AUG, thus leading to the initiation of transcription in a cap-independent manner.

The coding sequence of CVB3 has only one functional open reading frame (ORF), which directs the synthesis of the polyprotein, precursor of all the viral proteins. Picornavirus genomes can be subgrouped in three coding sequences, the P1, P2 an P3 (Figure 2). The P1 subregion codes for capsid polypeptides, VP1, VP2, VP3 and VP4 that build up the capsid of the mature virion; the P2 encodes three non-structural proteins and, especially for the 2B and 2C, an involvement in rearrangement of intracellular membrane network was described (Koch, 1985; Doedens et al., 1997; van Kuppeveld et al., 1997). The P3 encodes four non-structural polypeptides, from 3A to 3D, involved in modification of host cell metabolism and pathways (Huber et al., 1999; Wessels et al., 2005) and RNA replication (Cornell and Semler, 2002).

The processing of the polyprotein is a step which requires both, virus encoded proteinases and cellular proteins (Macejak and Sarnow, 1992). Most of the proteolytic events to yield capsid proteins are carried out by the 3C (or its precursor 3CD) and 2A proteinases .

Like the processing of the polyprotein, the replication of the viral RNA takes place in the cytoplasm of infected cells (Bienz et al., 1994; Rust et al., 2001) and two proteins of the P3 region, the RNA polymerase 3D ($3D^{pol}$) and VPg (3B), are directely involved in the synthesis of viral RNA.

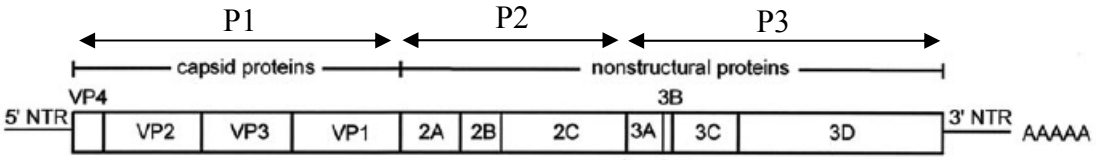

Figure 2. CVB3 genome and its organization (from Wessels et al., 2005)

1.2.1.3 The proteinase 2A of CVB3

The proteinase 2A ($2A^{pro}$) is a cysteine proteinase (Koenig, 1988) with proteolytic activity. This proteinase catalyzes the first cleaving step in processing of the polyprotein. It is able to cleave between the C terminus of VP1 and its own N terminus, thus separating the P1 capsid precursor from the precursor of the non structural proteins, the P2-P3.

The proteinase 2A has also been shown to be involved in other cellular functions. The $2A^{pro}$ indirectly induces cleavage of two cellular proteins. The first target is the eukaryotic initiation factor 4G (eIF-4G) (Lamphear et al., 1993), which is thought to contribute to the specific inhibition of host cell protein synthesis. The eIF-4 components are polypeptides that collectively recognize the m^7GTP-containing cap of cellular mRNAs, thus facilitating the binding of the 40 S ribosomal subunit, a necessary step in the eukaryotic protein synthesis. The poly(A)-binding protein, an RNA-binding protein essential for protein translation, is the second target of $2A^{pro}$ (Joachims et al., 1999). The proteolysis of host cell proteins mediate the interference of the RNA replication with the cellular metabolism, thus leading to the "shutoff" of the host protein synthesis (Lamphear et al., 1993; Kerekatte et al., 1999).

Moreover, Badorff et al. showed a direct involvement of the $2A^{pro}$ activity in the inactivation of proteins essential to myocyte function (Badorff et al., 1999)(Figure 3).

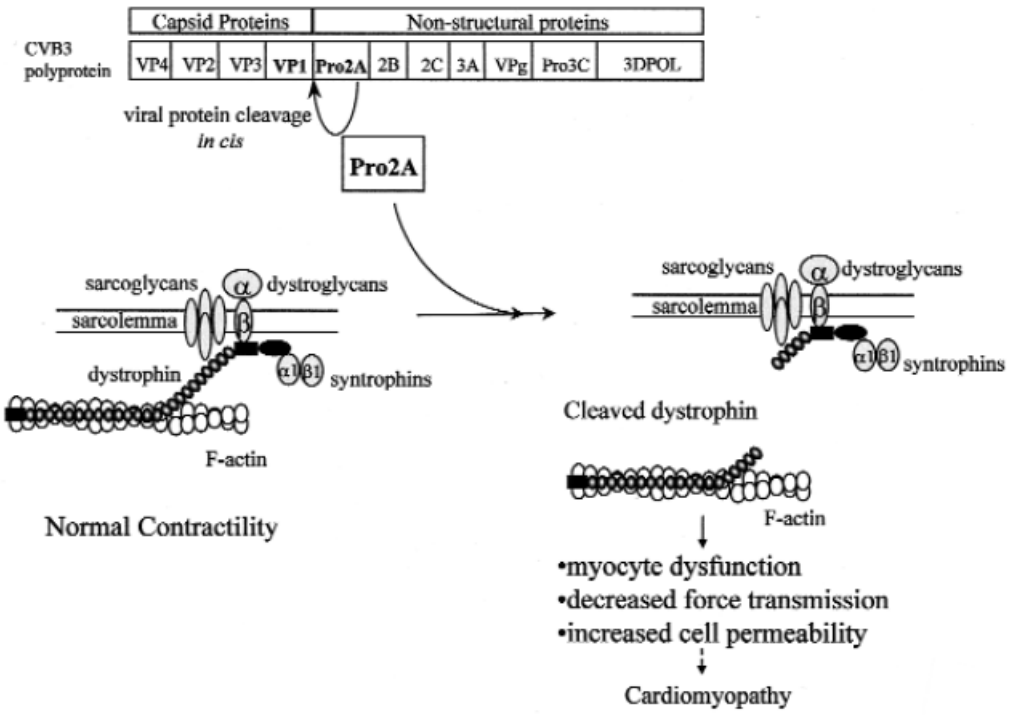

Figure 3. The role of dystrophin in coxsackievirus-induced dilated cardiomyopathy (from Badorff et al., 1999)

$2A^{pro}$ cleaves dystrophin, a large cytoplasmic protein that connects the cytoskeleton of a muscle fiber to the surrounding extracellular matrix through the cell membrane. Once the cleavage has occurred, the defective transmission of force through abnormal cytoskeletal structures, or the loss of the sarcolemmal integrity, initiates a cascade of events that may lead to dilated cardiomyopathy.

1.3 The connective tissue growth factor

The connective tissue growth factor (CTGF) is a member of the recently described CCN family of early immediate genes. In humans the CCN family contains six members: CTGF, cysteine-rich61 (Cyr61), nephroblastoma overexpressed (Nov), Wnt-induced secreted protein-1 (WISP-1), WISP-2, WISP-3 (Moussad and Brigstock, 2000).

The CCN proteins are extracellular matrix (ECM)-associated proteins that are involved in normal cellular processes such as adhesion, migration, mitogenesis, differentiation and development (Brigstock, 2003) as well as in processes related to tissue pathology, including wound healing and fibrotic disorders.

Damage to tissues can result from various acute or chronic stimuli, including infections, autoimmune reactions and mechanical injury. When a tissue damage occurs, repair processes eventually result by triggering an inflammatory process to heal the injured tissue. The repair processes involve two distinct phases: a regenerative phase, where the injured cells are replaced by cells of the same type, leaving no lasting evidence of damage, and a phase known as fibrosis, where connective tissue replaces normal tissue, resulting in substantial remodeling of the ECM and formation of permanent scar tissue.

The activation and proliferation of fibroblasts is a key point of the repair process as fibroblasts activation is involved in the *de novo* production and deposition of ECM proteins. A good resolution of tissue injury is the result of the well balanced ECM deposition and ECM degradation, the last carried out by recruited leukocytes during the inflammatory reactions. Nevertheless, the presence of a persistent irritant (i.e. viruses) at the site of injury, is known to be a major promoter of fibrosis in many organs, since it represents a continued source of tissue injury and chronic inflammation. In the heart, the cardiomyocytes are sorrounded by an interstitial matrix, that is crucial in maintaining cardiac geometry and function and in assuring well coordinated myocyte contractions (Tyagi, 1998). When chronic inflammation develops in the heart, the injury induced by inflammation reaction results in reparative fibrosis and progressive dilatation.

The expression of CTGF is strongly upregulated by mechanical stress and by several factors, including the transforming growth factor-β (TBG-β) (Chen et al., 2000; Leask and Abraham, 2004), a cytokine which mediates many processes, the wound healing process included. There is evidence that CTGF can promote fibroblast proliferation and extracellular matrix production in connective tissues (Frazier, Williams et al. 1996; Moussad and Brigstock 2000), thus suggesting an important role in wound repair and fibrotic disorders (Igarashi et al., 1993). It has been shown that CTGF is upregulated in many fibrotic diseases, like cardiac fibrosis (Matsui and Sadoshima, 2004; Dean et al., 2005; Vallon et al., 2006), lung fibrosis (Lasky et al., 1998) and diabetic nephropathy (Wang et al., 2001).

1.4 RNA interference

RNA interference (also called "RNA-mediated interference", abbreviated RNAi) is a mechanism for RNA-guided regulation of gene expression in which double-stranded RNA (dsRNA) inhibits the expression of genes with complementary nucleotide sequences. Conserved in most eukaryotic organisms, the RNAi pathway is thought to have evolved as a form of innate immunity against viruses (Gitlin and Andino, 2003) and also plays a major role in regulating development and genome maintenance.

The RNAi pathway has been particularly well studied in certain model organisms. It was first described as a post-transcriptional gene silencing (PTGS) event in plants (Napoli al., 1990; van der Krol et al., 1990). Later in 1998, Fire and Mello described and characterized the RNAi phenomenon in the nematode worm system *Caenorhabditis elegans*, thus showing the importance of the mechanism and creating the link to the animal kingdom (Fire et al., 1998). RNAi was further elucidated in an *in vitro* system based on embryo extracts of the fruit fly *Drosophila melanogaster* (Tuschl et al., 1999).

In initial experiments with mammalian cultured cells it was not possible to elicit a specific RNAi interference without stimulating a general and non-specific innate immunity, because of a predominant type 1 interferon response, which blocks the translation and cause mRNA degradation in a sequence independent manner. Nevertheless, in response to double-stranded small interfering RNAs (siRNAs) of ~21 nt in length, RNAi was shown to occur in mammalian cells (Elbashir et al., 2001).

In addition to siRNAs, different types of RNA molecules can trigger the RNAi machinery, including RNA viruses, transposons and small non-coding but endogenously encoded RNAs

(Figure 4). The latter, called micro RNAs (miRNAs), are transcribed in the nucleus to form a well structured hairpin like RNA molecule (Lee et al., 2004). After processing steps in the nucleus, a precursor miRNA is formed, which contains 5´phosphate groups and 2-nt overhangs at its 3´end. These precursors are transported by exportin 5, a recently described tRNA export factor, to the cytoplasm where they are processed by an RNAse, called Dicer, into mature ~22-nt miRNAs (Kim, 2005). miRNAs are then subsequentely loaded onto RISC, a ribonucleoprotein complex, for gene silencing (Preall and Sontheimer 2005). Unlike siRNAs, miRNAs do not cleave the target mRNA but instead suppress mRNA translation. A partial non-complementarity sequence between the miRNA and the target creates a bulged structure that prevent RISC from cleaving the target but, instead, leads to translation suppression.

Recently, an involvement of the RNAi-related processes in regulation at the genomic level has been described. Naturally occurring siRNAs originate from transposons (Lippman et al., 2003; Zilberman et al., 2003), bidirectionally transcribed repetetive sequences (Volpe et al., 2002) and genes (Ambros et al., 2003; Xie et al., 2004). As RNA is able to form base pairs not only with mRNA but also with DNA, new rules for the RNAi machinery in epigenetic processes, such as DNA methylation and heterochromatin modification have been described (Wassenegger et al., 1994; Volpe et al., 2002; Chan et al., 2004; Fukagawa et al., 2004; Kawasaki and Taira, 2004; Lippman et al., 2004) .

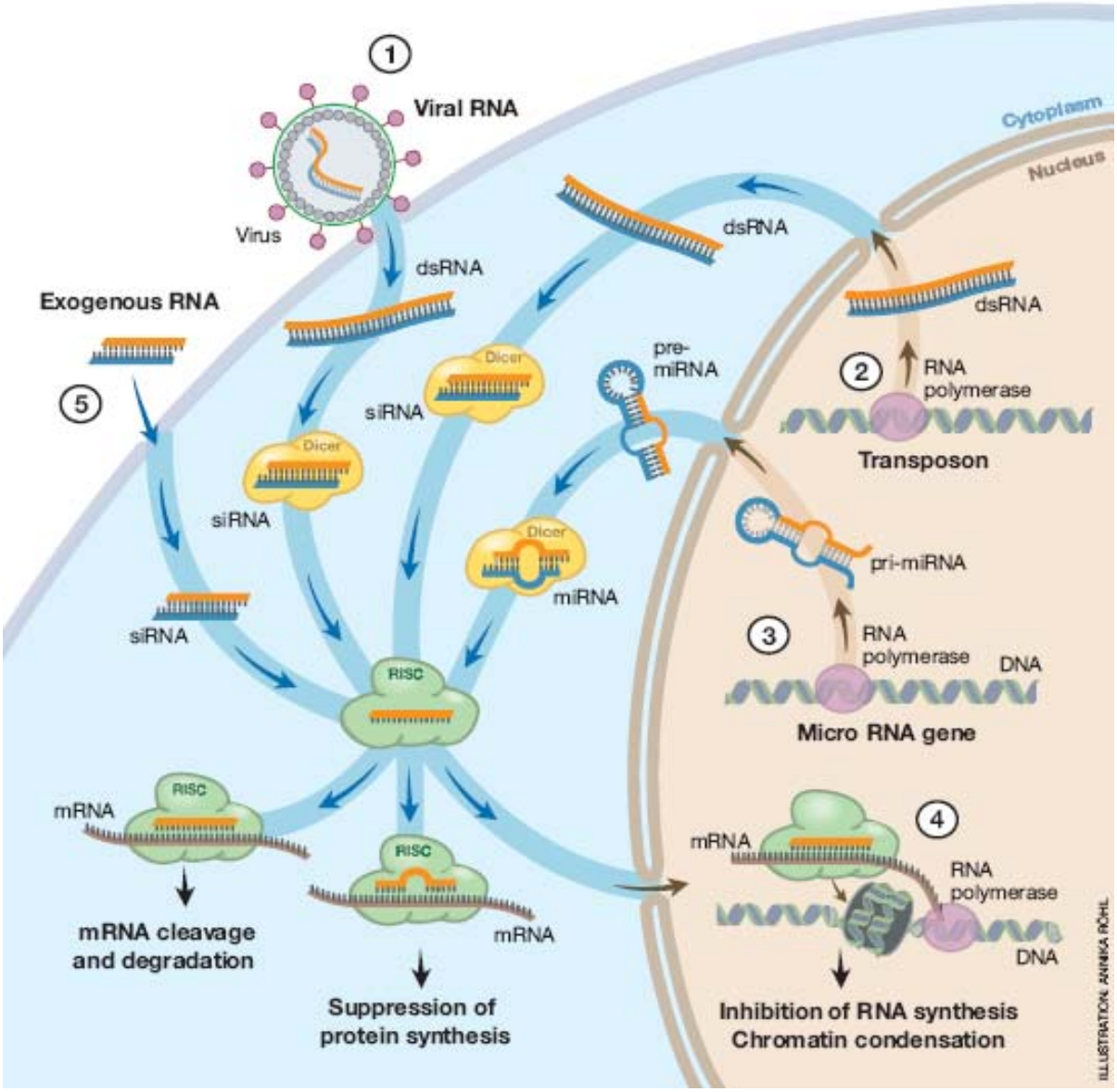

Figure 4. Involvement of the RNA interference in 1) destruction of invading viral RNA, 2) elimination of transcripts from transposons and repetetive DNA, 3) block of protein synthesis, 4) RNA-mediated suppression of transcription, 5) inhibition of activity of specific genes from artificial siRNAs. From www.nobelprize.org/nobel_prizes/medicine/laureates/2006/adv.html

1.4.1 Post-transcriptional gene silencing by siRNAs

Exogenous long dsRNAs are initially processed by the cytoplasmatic RNAse III-family endonuclease called Dicer (Bernstein et al., 2001; MacRae and Doudna, 2007) and converted into smaller 21-23 nt dsRNA molecules with 2-nt overhangs at their 3'ends and a phosphate group at the 5'ends (siRNAs) (Blaszczyk et al., 2001; Elbashir et al., 2001; Nykanen et al., 2001). The Dicer is able to cleave both dsRNAs or hairpin-like RNA molecules, the so called short hairpin RNAs (shRNAs). The cleaved products are subsequently assembled into an

RNA-Induced Silencing Complex, called RISC (Schwarz et al., 2003; Liu et al., 2004; Meister and Tuschl, 2004), which is an multicomponent nuclease that drives the siRNAs to the target mRNA and directs its cleavage. A core component of RISC, Argonaute 2 (AGO-2), is a member of a gene family conserved in most eukaryotic and several prokaryotic genomes (Hammond et al., 2001). The endonuclease AGO-2 is thought to mediate the cleavage of the sense strand of siRNA (Matranga et al., 2005; Rand et al., 2005), thus enabling the antisense strand siRNA to guide the RISC to complementary sequences in target mRNA. An effective post transcriptional gene silencing (PTGS) requires perfect or near-perfect Watson-Crick base pairing between the guide strand of siRNA and the target mRNA.

The cleavage of the target mRNA occurs between bases 10 and 11 relative to 5' end of the siRNA guide strand (Elbashir et al., 2001), leading to subsequent degradation of the cleaved mRNA transcript by cellular exonucleases (Orban and Izaurralde, 2005).

Once activated by the siRNA guide strand, RISC can undergo multiple rounds of mRNA cleavage to mediate a robust PTGS response against the target gene (Hutvagner and Zamore, 2002).

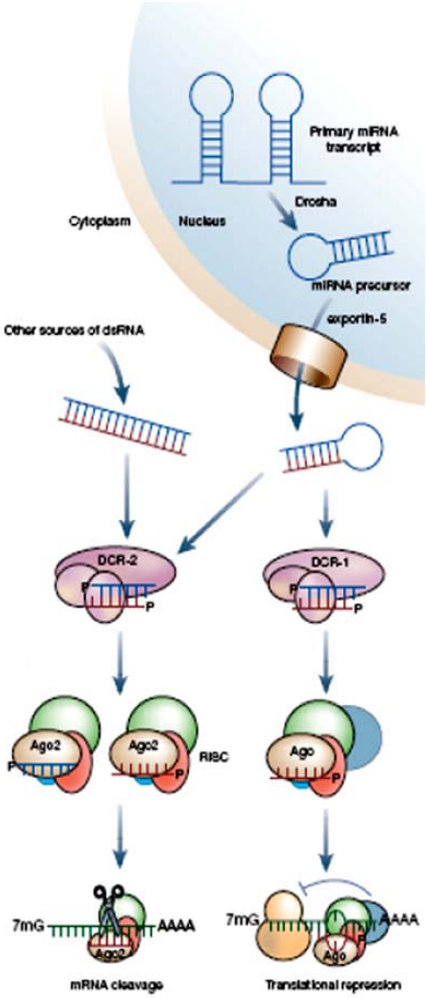

Figure 5. Mechanisms of RNA interference in mammalian cells (from Meister et al., 2004)

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1.4.2 Design of siRNAs

To knockdown the expression of a gene by artificial siRNAs, many factors in siRNA design must be taken into account, in order to ensure an efficient gene silencing. These factors include sequence characteristics of siRNAs, secondary structures of the target mRNA, siRNA duplex end-stabilities and the specificity of siRNA to its target.

In two independent experiments it has been proven that certain characteristics in the sequence of siRNAs are of basic importance for their efficiency (Reynolds et al., 2004).

The siRNA efficiency based on secondary structure of taget mRNA has been considered by Luo et al., using a prediction method based on free energy target mRNA values (Luo and Chang, 2004). They state that the more accessible a target is, the more efficiently an siRNA can cleave the target sequence. This result has been confirmed by other working groups (Overhoff et al., 2005; Yiu et al., 2005).

Also, the specificity of siRNAs, considered as missing off-target effects, is an important parameter to assess their efficiency. In order to avoid off-target effects, many methods and programs have been developed (Saxena et al., 2003; Snove et al., 2004; Qiu et al, 2005; Yamada and Morishita, 2005). Since it is not yet clear how many bases of the antisense siRNA are needed to be complementary to potential sequences of non-target mRNAs, thus triggering off-target effects, the criteria for determining siRNA specificity need to be further elucidated. Therefore, most programs to predict siRNA efficiencies are using algorithms for sequence characteristics and secondary structure of target mRNAs (Poliseno et al., 2004).

1.4.3 Delivery of siRNAs

There are two different systems for delivering small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs): the endogenous delivery system, and the exogenous system which is carrier-mediated. The choice of the delivering system to be used is based on which kind of expression of siRNAs/shRNAs is required. To gain a stable transfection the endogenous delivery system is suggested. One possibility to achieve endogenous synthesis of siRNAs/shRNAs is the transfection of cells with plasmids. These DNA vectors carry the sequence of the designed shRNA, under the control of the RNA polymerase III or the RNA polymerase II promoters. The promoters are used to drive the expression of shRNA molecules, which are then incorporated into the RISC complex, thus exerting the desired silencing effect. Another way to express siRNAs/shRNAs within the cells considers the use of

viruses as vectors. In this context adenoviruses, adeno-associated viruses and lentiviruses are generally employed.

For the exogenous delivery, chemically or *in vitro* synthetized siRNAs are needed. SiRNAs may be transfected with cationic lipids, by electroporation or with carrier peptides or proteins (Dykxhoorn et al., 2003; Puebla et al., 2003; Simeoni et al., 2003). Duplex siRNAs are negatively charged polymers and cannot easily penetrate hydrophobic cellular membranes without assisting carriers. Mixing cationic lipids with negatively charged oligonucleotides results in their absorption onto the liposome surface. The exogenous siRNA delivery always results in a transient RNAi effect, since the effector molecules undergo dilution through cell division and degradation over time.

Exogenous, as well as endogenous siRNA delivery, has been successfully applied *in vivo.* Injection of a large volume of a solution containing siRNA into the tail vein without any transfection agent resulted in a profound inhibition of gene expression in a variety of murine tissues (McCaffrey et al., 2002; Song et al., 2003). Intraperitoneal injection of complexes consisting of cationic lipids and siRNAs against tumor necrosis factor-α reduced its expression and protected mice against septic shock (Sorensen et al., 2003).

1.5 Aim of the project

The aim of this study was to contribute to a better understanding of factors involved in progression of coxsackievirus B3-mediated myocarditis to fibrotic heart diseases and to evaluate the potential of RNA interference in preventing CVB3 replication in susceptible host cells.

For these experiments, the first step was to exclude secondary effects of siRNAs on transfected cells by examination of cell toxicity and cell viability assays. The efficacy of specific siRNAs in inhibiting virus replication was planned to be evaluated on three levels: by quantification of the viral RNA, of viral capsid proteins and of virus particles released by infected cells.

As CTGF has found to be relevant in cardiac fibrosis, siRNAs specific for CTGF were used to elucidate the role of this factor in developing fibrosis in the course of CVB3 myocarditis. Data from microchip analysis from CVB3-infected mice indicate that there is an increase in the CTGF expression after infection. A crucial point in understanding the interrelations between CVB3 replication and CTGF expression is to clarify if CTGF up-regulation is a

consequence of the activation of cellular pathways induced by the virus and if CTGF is necessary for the virus replication itself. This question was intended to be addressed by stimulation and/or silencing of CTGF after CVB3 infection in a cell culture model.

2. Materials and Methods

All the chemicals were from Sigma Aldrich Chemie GmbH (Taufkirchen, Germany) and Merk (Darmstadt, Germany), unless specified.

All buffers and solutions were prepared using distilled water (dH_2O, Millipore) and have been autoclaved before use. Kits have been used in accordance with manufacturer' s instructions.

2.1. Materials

2.1.1 Buffers and solutions

2.1.1.1 General buffers and solutions

10 x PBS

NaCl	80 g/l
KCl	2 g/l
Na_2HPO_4	11,5 g/l
KH_2PO_4	2 g/l

pH 7,4 to adjust with HCl, followed by autoclavation

10 x TBS-buffer

Tris	200 mM
NaCl	0,9 %

pH 7,6 to adjust with HCl

2.1.1.2 Buffers and solutions for SDS-PAGE and Western Blot

Lämmli-buffer (buffer B)

Tris-HCl	250 mM
SDS	8%
Glycerin	40%
ß-Mercaptoethanol	20%
Bromophenol blue	0,008%

pH 6,8 to adjust with HCl

Lysis buffer (buffer A)

Tris-HCl	470 mM
SDS	10%

Pepstatin	10 mM
Natrium bisulfite	37%

Stacking buffer

Tris	470 mM

pH 6,7 adjust with HCl

Running buffer

Tris	3 M

pH 8,9 adjust with HCl

10 x Electrophoresis-buffer (SDS-PAGE)

Tris	250 mM
Glycin	2 M

Shortly before use, add SDS to have 0,1 % final concentration

Coomassie staining

Coomassie R250	0,5 g/l
Methanol	30%
Acetic acid	10%

Transfer buffer

Glycin	39 mM
Tris-HCl (pH 8,3)	48 mM
SDS	0,037%
Methanol	20%

10 x Red Ponceau staining

Ponceau S	2%
Trichloroacetic acid	30%

Stripping-buffer

Glycin	200 mM
Tween 20	0,05%

pH 1,8 adjust with HCl

ECL

Before using, mix 1 to 1 in volume the ECL1 and ECL2 solutions

ECL1:

dH_2O	17,7 ml
Luminol	200 µl (2,75 mM)
Cumaric acid	88 µl (0,43 mM)
Tris-HCl (pH 8,5)	2 ml

ECL2:

dH_2O	18 ml
H_2O_2	12 µl
Tris-HCl (pH 8,5)	2 ml

Running gel	12,5 %	Stacking gel	4%
d H_2O	4,75 ml	d H_2O	6 ml
Running buffer	3,75 ml	Running buffer	2,5 ml
10 % SDS	150 ul	10 % SDS	100 ul
Acrylamide	6,25 ml	Acrylamide	1,3 ml
10 % APS	130 ul	10 % APS	100 ul
TEMED	13 ul	TEMED	15 ul

2.1.1.3 Buffer and solutions for RT-PCR

The kit for RNA extraction was from peQLab Biotechnologies (Erlagen, Germany), the kit for running RT-PCR was from Applied Biosystems (Weiterstadt, Germany). Primers and probe were purchased from MWG Biotech AG (Ebersberg, Germany) and TIB MOLBIOL (Berlin, Germany).

All buffer and solutions were prepared using distilled water (dH_2O, Millipore) and have been autoclaved before use. Kits have been used in accordance with manufacturer' s instructions.

2.1.2 Transfection reagents

Lipofectamine 2000, Lipofectin, Oligofectamine and Cellfectine were from Invitrogen (Karlsruhe, Germany), Fugene was from Roche (Mannheim, Germany).

2.2 Methods

2.2.1 siRNAs

siRNAs duplexes with 3'-UU overhangs were purchased from Eurogentec S.A (Seraing, Belgium). The target sequences of siRNA 35 and siRNA 36 are GAGGUCCAAGAGAGU (nucleotides 3541-3559 of coxsackievirus B3 genome) and GCGGUAUCCUAAGGUG (nucleotides 3626-3644) respectively. The target sequences of siRNA CTGF1 and siRNA CTGF2 are GACCUGGAAGAGAACA (nucleotides 876-897 in the mRNA of CTGF) and GGCGAGGUCAUGAAGA (nucleotides 1071-1090) respectively.

A negative siRNA control, the siRNA GL2, has the target sequence CGUACGCGGAAUACUU of the luciferase gene. The siRNA control has no matches either in the viral or in the human genome.

To study the distribution patterns of siRNA into the cells and the efficiency of the transfection, siRNA GL2 sense strand was annealed with the siRNA GL2 antisense strand labelled with FITC molecule (Eurogentec S.A, Seraing, Belgium).

2.2.2 Cell culture

2.2.2.1 Cell lines

HeLa	Human cervical cancer cell line	ATCC
Vero	African monkey kidney cells	ATCC

2.2.2.2 Culture of adherent cells

Cell lines were cultivated in an humidified incubator at 37°C in 5% CO_2. HeLa and Vero cells were maintained in Dulbecco´s modified Eagle´s medium (DMEM)(Invitrogen, Karlsruhe, Germany) supplemented with 10% heat inactivated fetal calf serum (FCS) and 1% penicillin/streptomycin.

Confluent cells were washed with 2 ml 1x phosphate-buffered saline (PBS) and incubated with 2 ml 1x Trypsin-EDTA for 2 minutes at 37°C in 5% CO_2. Detached cells were then centrifugated at 700 rpm for 5 minutes and the pellet was washed with 1 x PBS and re-centrifugated. The cells were then diluted in complete medium and seeded at a 1:3 or 1:4 dilution.

2.2.2.3 Freezing of cells

Cell pellet was resuspended in 800 ul FCS and 200 ul DMSO in cryovials. Then, the cells were frozen in -80°C overnight. The day after frozen cells were put in nitrogen liquid.

2.2.2.4 Defrosting of cells

The cryovials containing the cells were quickly put in 40°C water bath until the cell solution was almost defrosted. Soon after 10 ml 1 x PBS were added, cells were resuspended and centrifugated at 800 rpm for 5 minutes. Cell pellets were then resuspended with complete medium and grown in an incubator at 37°C in 5 % CO_2.

2.2.3 Cell morphology analysis

Cellular morphology was observed under a phase contrast microscope (x100) (IMT-2; Phase contrast KLWCD 0.30, Olympus).

2.2.4 Histology

For the detection of the FITC labelled siRNA, transfected cells were fixed in 2% paraformaldehyde (Merk, Darmstadt, Germany) in PBS for 20 minutes, washed two times with PBS for 5 minutes and observed under the fluorescence microscope (Axiophot, Zeiss) after embedding with the mounting medium (Pro Textura, Berlin, Germany).

2.2.5 FACS analysis

For the quantification of the up-take of FITC-labelled siRNA GL2 into HeLa cells, transfected cells were harvested, washed two times with 1 x PBS and the cell pellets resuspended in 1% BSA in PBS prior to analysis by flow cytometry (FACSCalibur, Becton Dickinson).

2.2.6 Assessment of cell viability and cytotoxicity

2.2.6.1 LDH assay

Cell cytotoxicity was assessed by measuring the release of the lactate dehydrogenase into the supernatant of injured cells. LDH was quantified using the LDH-Cytotoxicity Assay Kit (Biovision, Mountain View, California) by a colorimetric assay and the absorbance was measured at 550 nm using an enzyme-linked immunosorbent assay (ELISA) reader (PR 2100 Sanofi).

2.2.6.2 MTT assay

Cell viability was measured using the Vybriant^{R} (MTT) Cell Proliferation Assay Kit (Invitrogen, Molecular Probes, Karlsruhe, Germany) according to manufacturer' s instructions. Cells were incubated with MTT solution for 4 h and the absorbance was measured at 550 nm using an enzyme-linked immunosorbent assay (ELISA) reader (PR 2100 Sanofi).

2.2.7 Transfection with siRNAs

5x 10^5 Hela cells were grown in 35-mm dishes (Nunc, Roskilde, Denmark) in 5% CO_2 at 37°C overnight. Upon reaching 60 to 70% confluency, cells were washed with 1 x PBS and overlayed for 2 h with transfection complexes containing siRNAs and Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany), in a 1:1 ratio. The transfection mixtures were prepared as follows. In a sterile tube, 5 ul Lipofectamine 2000 was added to 200 ul DMEM, mixed well gently and kept at room temperature (RT) for 5 minutes. In another sterile tube 5 ul of siRNA at the appropriate concentration was added to 200 ul DMEM and the sample was mixed well gently and kept at RT for 5 minutes. The two tubes were combined, mixed well, and kept at RT for another 30 minutes. The final volume for the transfection reaction was 1,25 ml in DMEM. HeLa cells were overlaid with the final transfection mixture of Lipofectamine 2000-siRNA (5 ul/well) and incubated for 2 h at 37°C. The transfection mixture was then removed and cells were washed with 1 x PBS.

2.2.8 Infection with CVB3

cDNA generated CVB3 (Nancy strain) was grown and propagated in Vero cells as described previously (Kandolf and Hofschneider 1985) and titers were routinely redetermined by plaque assay at the beginning of all individual experiments.

For viral infection HeLa cells were infected at a multiplicity of infection (MOI) of 0,01 for cells transfected with siRNAs against CVB3. Infection was performed in 200 ul final volume for 1 h. HeLa were then washed with 1x PBS and cultured in complete DMEM at 37°C for 24 h. For infection of HeLa cells transfected with siRNA against CTGF, cells were infected with 10 MOI CVB3 for 1 h and, five h post infection, cells were washed and harvested for RNA and protein extraction.

2.2.9 RNA analysis

For RNA quantification, total RNA was extracted from HeLa cells using TriFast (peQLab Biotechnologies GmbH, Erlangen, Germany) reagent, according to the manufacturer' s instructions. The amount of RNA samples was determined at the spectrophotometer

(SmartSpec 3000, Bio-Rad) and RNA was quantified by real-time quantitative RT-PCR or stored at -80°C.

2.2.9.1 Quantitative real-time RT-PCR for CVB3 detection

Quantitative real-time PCR was performed using the ABI Prism™ 7700 Sequence Detection System (PE Applied Biosystem, Foster City, CA, USA). This system is based on the ability of the 5' nuclease activity of Taq polymerase to cleave a dual-labeled fluorogenic hybridization probe during DNA chain extension. The probe is labeled with a reporter fluorescent dye FAM at the 5' end and a quencher fluorescent dye TAMRA (6-carboxy-tetramethyl-rhodamine) at the 3' end. During the extension phase of PCR, the nucleolytic activity of the DNA polymerase cleaves the hybridization probe and releases the reporter dye from the probe with a concomitant increase in reporter fluorescence. The following sequence-specific primers and probe were designed using Primer Express software (PE Applied Biosystems): coxsackievirus B3 forward primer: 5' ACC TTT GTA CGC CTG TT 3', reverse primer: 5' CAC GGA CAC CCA AAG TA 3', probe: 5'FAM-CGG AAC CGA CTA CTT TGG GTG WCC GT-3'TAMRA for the reverse transcription; coxsackievirus B3 forward primer: 5' AAG CAC TTC TGT TTC CC 3', coxsackievirus B3 reverse primer: 5' ATT CAG GGG CCG GAG GA 3', probe: 5'-FAM CGG AAC CGA CTA CTT TGG GTG TCC GT TAMRA-3' for the amplification.

Primers were used at a final concentration of 100 nM and the probe at 100 nM in each reaction. QIAGEN One-Step reverse transcriptase-polymerase chain reaction (RT-PCR) kit (QIAGEN Inc, Hilden, Germany) was used in all RT-PCR reactions using the following parameters: 48°C for 30 min (reverse transcriprion), 95°C for 10 min (Taq polymerase activation), 45 cycles of 94°C for 15 s and 58°C for 1 min. Relative quantification of viral RNA was calculated using standard curves of *in vitro* transcribed CVB3 RNA.

2.2.9.2 Quantitative real-time RT-PCR for CTGF and TGF-β mRNA detection

Sequence specific primers and probe for CTGF mRNA were: 5' GAG GAA AAC ATT AAG AAG GGC AAA 3' forward primer, 5' CGG CAC AGG TCT TGA TGA 3' reverse primer, 5'FAM TTT GAG CTT TCT GGC TGC ACC AGT GT 3'TAMRA probe. The reaction was run for 45 cycles with the following parameters: 48 °C for 30 min, 95 °C for 10 min, 94 °C for 1 min and 60 °C for 1 min. Relative quantification was calculated to HPRT gene expression, 5' GGC AGT ATA ATC CAA AGA TGG TCA A 3' forward primer, 5' GTC TGG CTT ATA TCC AAC ACT TCG T 3' reverse primer, 5'FAM CAA GCT TGC TGG TGA AAA GGA CCC C 3' TAMRA probe.

Sequence specific primers and probe for TGF-β mRNA were: 5' GAG CCC AAG GGC TAC CAT 3' forward primer, 5' GGG TTA TGC TGG TTG TAC AGG 3' reverse primer, probe used was the Universal Probe Library, Probe n° 29 (Roche, Mannheim, Germany). Relative quantification was calculated to HPRT gene expression.

Primers were used at a final concentration of 100 nM and the probe at 100 nM in each reaction. QIAGEN One-Step reverse transcriptase-polymerase chain reaction (RT-PCR) kit (QIAGEN GmbH, Hilden, Germany) was used in all RT-PCR reactions using the following parameters: 48°C for 30 min (reverse transcriprion), 95°C for 10 min (Taq polymerase activation), 45 cycles of 94°C for 15 s and 60°C for 1 min.

2.2.10 Protein analysis

2.2.10.1 Protein extraction

Treated cells were harvested with 1 x Trypsin-EDTA and washed with 1 x PBS. Cell pellets were resuspended in 80 ul buffer A and incubated at 95°C for 10 minutes or until the pellet was dissolved. Each sample was diluted 1:50 against buffer A and the absorbance at 235 and 280 nm read with the spectrophotometer (SmartSpec 3000, Bio-Rad). The samples were then mixed with buffer B at a ratio of 1:1 with buffer A, loaded into the gel or stored at -20°C.

2.2.10.2 SDS-PAGE

Twenty micrograms of protein extract from each siRNA treatment were denatured at 95°C for 3 minutes and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on a 12,5% acrylamide gel. Samples in the stacking gel were run at 35 mA for 30 minutes, followed by run at 55 mA for 1,5 h in the running gel.

2.2.10.3 Western blot

After the SDS-PAGE the samples were transfected to a PVDF membrane via semi-dry blotting (Trans-Blot SD Semi-Dry Transfer Cell, Bio-Rad). The PVDF membrane was incubated for 1 minute with methanol, washed with deionized water and assembled with the gel and the Whatman paper together with the transfer buffer. The transfer was run at 2,5 mA/cm^2 membrane for 1 h and 15 minutes at 4°C. Efficient transfer onto the membrane was confirmed with the staining of transferred protein with Red Ponceau.

2.2.10.4 Immune reaction

Before incubation with the primary antibody, the membrane was blocked in 5% skim milk in PBST for 1 h at RT. The membrane was then washed with 1 x PBS and incubated with first antibody.

For viral protein detection, 4°C overnight incubation with the polyclonal antibody against the capsid protein VP1 (Werner et al., 1988) (1:300, vol/vol in 1% BSA in PBS) was followed by three washing steps with 1 x PBS and subsequent incubation for 1,5 h with a goat anti rabbit IgG secondary antibody (Vector, Burlingame, Calif. USA) (1:5000, vol/vol of 1% BSA in PBS). To washing steps with 1 x PSB were done and VP1 protein was visualized with enhanced chemiluminescence (ECL) according to manufacturer' s instruction (Amersham, Freiburg, Germany).

For detection of CTGF protein, the membrane was incubated at 4°C overnight with the polyclonal antibody directed against CTGF (Santa Cruz Biotechnology, Santa Cruz, California) (1:400, vol/vol in 5% milk in PBST), then washed 6 times for 10 minutes each with 0,15 % Tween 20 in PBS followed by 1 h washing at RT. Two h incubation with the donkey anti-goat secondary antibody (Santa Cruz Biotechnology, Santa Cruz, California)(1:2000, vol/vol in 5% milk in PBST) followed and washing was done as above. The CTGF signal detection was conduced with the ECL reagents as described above.

2.2.10.5 Stripping of the membrane

Prior to stripping, the membrane was incubated for 1 minute with methanol and washed two times with deionized water. PVDF membrane stripping was carried out with incubation with stripping buffer for 30 minutes at 50°C. Two 1 x PBS washing steps were then perfomed and then the membrane was incubated with the GAPDH antibody conjugated with the horseradish peroxidase (Santa Cruz Biotechnology) (1:1000 vol/vol in 5% milk in PBS) for 1 h at RT. After 2 washing steps with 1 x PBS the glyceraldehyde-phosphate dehydrogenase (GAPDH) signal was developed by chemiluminescence method (ECL).

2.2.11 Plaque assay

CVB3 titers in culture medium were determined from monolayers of Vero cells by an agar overlay plaque assay in triplicate. Briefly, samples were serially diluted 10-fold, overlaid on 80% confluent monolayers of Vero cells in six-well plates, and incubated for 45 min. The medium was removed, and 2 ml of complete DMEM containing 1% soft Bacto Agar-Mem was overlaid in each well. Cells were then incubated at 37°C for 48 h, followed by fixation with 5% trichloroacetic acid for 1 h at 4°C. The agar was gently removed and cells were stained with 1% crystal violet for 15 min. Plaques were counted, and concentration was calculated as PFU per milliliter. Supernatants from HeLa monolayer non treated but CVB3 infected were used as controls.

2.2.12 Statistical analysis

All values are expressed as means ± SD. Statistical analyses were performed with unpaired Student' s *t* test. *P* values $< 0,05$ were considered to be statistically significant.

3. Results

3.1 Inhibition of coxsackievirus B3 replication by RNA interference

3.1.1 Design of siRNA sequences specific for CVB3 proteinase 2A

The aim of this work was to explore the possibility to inhibit CVB3 replication, i.e. viral load, in susceptible cells by means of siRNAs. The siRNAs used here have been selected according to a previously established mathematical algorithm (Poliseno et al., 2004). Two different siRNAs, both directed against the viral proteinase 2A ($2A^{pro}$), were selected (table 1). A luciferase siRNA (siRNA GL2) was used as a sequence-unrelated control siRNA.

The CVB3 region encoding for the $2A^{pro}$ was chosen as siRNA target for the following reasons. The proteinase is of fundamental importance for virus replication (Rueckert, 1996) as it processes the viral polyprotein into individual structural and non-structural proteins, it cleaves the eukaryotic translation initiation factor 4G (eIF4G) (Kräusslich et al., 1987), resulting in the host cap-dependent translation inhibition, and it cleaves (Kerekatte al., 1999) the poly(A)-binding protein (PABP), an RNA binding protein essential for protein translation. Additionally, both in cultured cardiomyocytes and in murine hearts infected by CVB3 (Badorff et al., 1999), $2A^{pro}$ proteolytically cleaves dystrophin and the dystrophin-associated glycoproteins, subverting cardiac tissue architecture, thus contributing to the pathogenesis of dilated cardiomyopathy, a late sequelae of CVB3 infection (Liu and Mason, 2001; Xiong et al., 2002). Finally, reduced dystrophin levels in the cardiac tissue was associated with higher viral replication rates (Xiong et al., 2002), suggesting a negative regulation function of dystrophin. Thus, the reduction of $2A^{pro}$ amount may potentially attenuate virion production, the host protein synthesis shut off, and the modification of cardiac tissue architecture.

Table 1. Sequences of siRNAs used to target the CVB3 RNA.

Name	5'-3' sequence	Target gene in CVB3 RNA and relative nucleotide position
siRNA 35	GAGGUCCAAGAGAGUGAAU	2A proteinase, 3541-3560
siRNA 36	GCGGUAUCCUAAGGUGUGA	2A proteinase, 3626-3645
siRNA GL2	CGUACGCGGAAUACUUCGA	----------

All molecules consist additionally of dTdT overhangs.

3.1.2 Transfection optimization and evaluation of transfection effects on HeLa cell viability

All the data reported in this work have been generated in human cervical carcinoma (HeLa) cells. The reason to perform the investigations in HeLa cells instead of cultured cardiomyocytes, the natural CVB3 target, is that HeLa cells can be cultivated and manipulated much easier than cardiomyocytes, thus rendering the determination of the activity of the mathematically selected siRNA easier and faster. Additionally, the validity of HeLa cells as a model to study CVB3 infection has been established in a significant number of works of different groups (Carthy et al., 1998; Kerekatte et al., 1999; Badorff et al., 2000; Roberts et al., 2000; Zhang et al., 2002; Luo et al., 2003; Yanagawa et al., 2003). A confirmation of the data reported here has to be performed in the mouse model of CVB3-induced myocarditis to evaluate the effects of the siRNA molecules on the course of the disease.

3.1.2.1 Transfection reagents and transfection efficiency

To evaluate the activity of the selected siRNAs in reducing intracellular CVB3 genome amounts, it has been decided to deliver the siRNAs to the target HeLa cells by means of liposomes (exogenous delivery strategy). The exogenous delivery was preferred over the endogenous delivery system (viral mediated). This choice allowed to circumvent the long and time consuming work dealing with the preparation of viral constructs, the selection of the proper promoter and the cloning of the siRNA into the viral vector backbone. Also, the possible interference between the virus of the delivering system and the CVB3 can be avoided.

To optimize HeLa cell transfection, different liposomes have been tested: Lipofectamine 2000, Lipofectin, Oligofectamine, Fugene and Cellfectine. Two aspects have been considered: the number of transfected cells and the intracellular distribution. The siRNA GL2 conjugated with a fluorescein molecule (FITC) was used for the uptake studies. This allowed to determine the intracellular distribution of the transfected siRNA by fluorescence microscopy and to count the number of fluorescein positive cells by flow cytometry (FACS). Beside the obvious importance of determining the amounts of transfected cells, the cellular distribution was also considered as it may influence the activity of the siRNA molecule. This has been shown for other nucleic acid based molecules, such as hammerhead ribozymes, where an homogenous but not a spotted distribution of the nucleic acid molecules within the cells resulted in a significant biological effect (Grassi et al., 2001).

A more homogeneous cellular distribution was observed for siRNA-GL2-FITC transfected by Lipofectamine 2000 (Figure 1), Oligofectamine and Lipofectin but not for Cellfectin and Fugene.

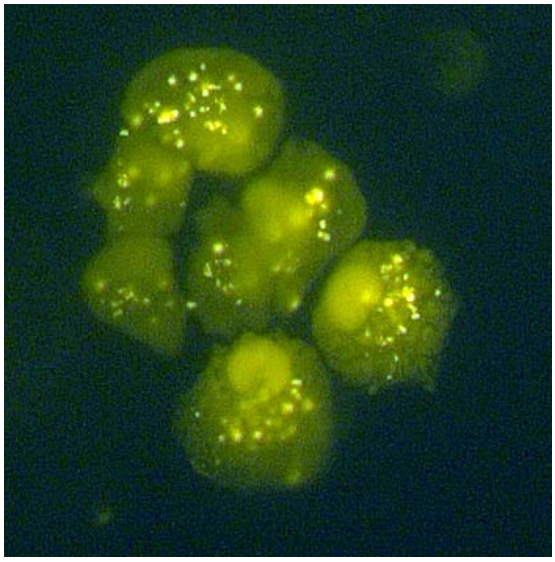

Figure 1.
Cellular distribution of Lipofectamine 2000 in HeLa cells. FITC labelled siRNA GL2 molecules were transfected at a final concentration of 400 nM for 2 h. The cells were then fixed in 2% paraformaldheyde and investigated using a fluorescence microscope.

Based on these results, the use of the transfection reagents Cellfectin and Fugene was discontinued.

As the weight ratio of transfection reagent/siRNA is known to be relevant in terms of transfection efficiency, this variable was investigated for the remaining three transfection reagents. Immediately after transfection, the number of stained cells was calculated by FACS analysis. The data, reported in Figure 2, indicate that both, Lipofectamine 2000 and Lipofectin transfection resulted in an up-take of about 80%, almost at all ratios transfection reagent/siRNA tested. Whereas higher ratios did not result in any improvements in transfection efficiency, ratios lower than 1:1 considerably reduced transfection efficiency. Compared to Lipofectamine 2000 and Lipofectin, Oligofectamine showed a significant lower ($p<0.05$) transfection efficiency, around 25%. Also in this case, the increase of the ratio did not result in any improvements in transfection efficiency.

Optimal transfection time was found to be for 2 h. Whereas a shorter transfection time resulted in a reduction of the efficiency of transfection, a longer time resulted in a significant increase of cell toxicity, as evaluated by optical microscopy inspection. Moreover, an siRNA concentration of 400 nM was found to give the highest transfection efficiency (data not shown).

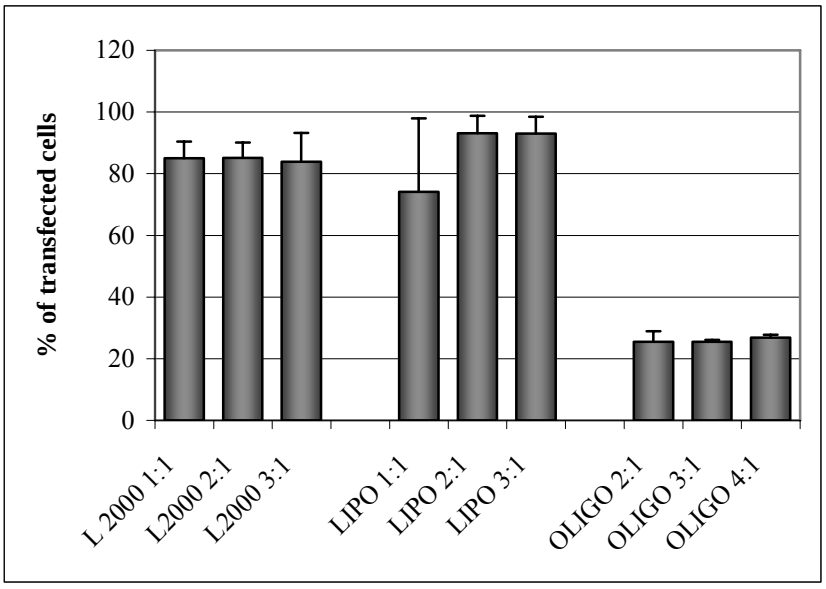

Figure 2.

FACS analysis. HeLa, 60% confluent, were transfected with different liposomes and siRNA GL2 FITC labelled (400 nM) for 2 h. Soon after transfection the quantification of stained cells was performed by FACS. L2000=cells treated with Lipofectamine 2000, Lipo=cells treated with Lipofectin, Oligo=cells treated with Oligofectamine. Data are expressed as mean ± SEM, n=3.

Considering the homogenous distribution within HeLa cells and the high transfection efficiency, especially at the ratio transfection reagent/siRNA of 1:1, Lipofectamine 2000 was chosen for subsequent experiments. Unless otherwise indicated, all the below reported data have been performed using a weight ratio Lipofectamine 2000/siRNA of 1:1 and a final siRNA concentration of 400 nM.

3.1.2.2 Impact of siRNA transfection on HeLa cell viability

In parallel to the evaluation of siRNA effects on CVB3 load in infected HeLa cells, the impact of siRNAs was evaluated on HeLa biology, i.e. on cell viability, adopting the optimized transfection conditions above reported. Particular attention was paid to a potential cytotoxic effect and to cell metabolism alterations induced by siRNAs transfection, in the absence of CVB3 infection. This investigation allowed to exclude that substantial modification in HeLa cell viability could have contributed to determine the antiviral effects observed following treatment by selected siRNA as reported in paragraph 3.1.3.

To asses a possible cytotoxic effect, the release in the cell culture medium of the enzyme lactate dehydrogenase (LDH) was measured, whose amount is known to be proportional to the number of injured cells (Decker and Lohmann-Matthes, 1988). Transfection, performed

according to points 1-3 of the "transfection-infection procedure" described in the beginning of paragraph 4.1.3, revealed no significant increase in LDH amounts 24 h post transfection in any of the transfected cell group compared to non-transfected cells (Figure 3). Comparable results were obtained for shorter (16 h) and longer (36 h) time after transfection (data not shown).

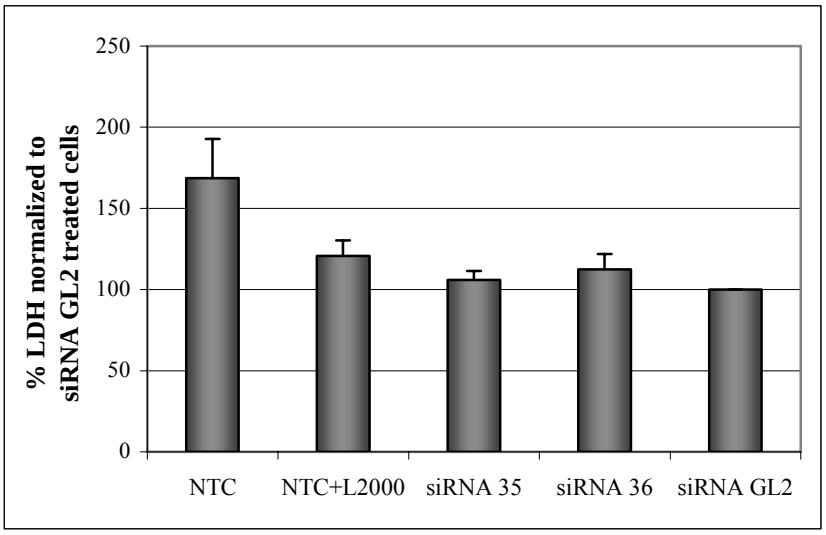

Figure 3.

LDH assay. Transfection of HeLa cells with siRNAs at a final concentration of 400 nM. At 24 h post transfection the LDH release was measured spectrophotometrically. NTC=non-transfected cells; NTC+L2000=cells treated by Lipofectamine 2000. Data are expressed as mean ± SEM, n=6

To quantify possible cell metabolism alterations, mitochondria activity was evaluated by means of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. By MTT the conversion of the water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to an insoluble formazan was evaluated, accomplished by mitochondria of viable cells (Mosmann, 1983). Thus, the conversion reaction is directly related to the number of living cells.

Values of MTT were found to be comparable for non-transfected cells and cells transfected either with the liposome alone or complexed with the siRNAs, indicating that the transfection procedure here adopted does not substantially impair HeLa viability (Figure 4), thus confirming the LDH test above reported. Comparable results were obtained for shorter (16 h) and longer (36 h) time after transfection (data not shown).

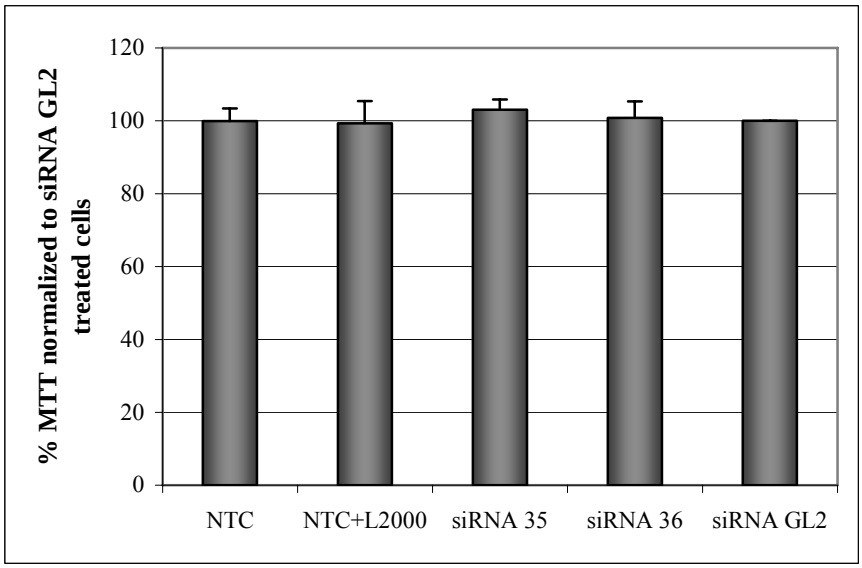

Figure 4.

MTT assay. HeLa cells were transfected with siRNAs at a final concentration of 400 nM for 2 h. At 24 h post transfection, the formazan absorbance was quantified by a spectrophotometer. NTC=non-transfected cells; NTC+L2000=cells treated by Lipofectamine 2000. Data are expressed as mean ± SEM, n=6.

3.1.3 Antiviral effects of the selected siRNAs

In this paragraph the reported data regard the selected siRNA, delivered as exogenous molecules, and their ability to silence CVB3 genome expression. The effects of virus silencing were examined morphologically using the light microscope. Also, the levels of viral RNA and viral protein synthesis as well as the production of infectious viral particles were determined.

Finally, cell viability of siRNA transfected and CVB3-infected cells was evaluated.

The experimental scheme, which is used throughout the below reported data section, is defined as “transfection-infection procedure” and consists of:

1) seeding of HeLa cells,
2) transfection of 60-70% confluent HeLa cells,
3) transfection time of 2 h,
4) cell washing and CVB3 infection (0.01 MOI) for 1 h,
5) cell washing and evaluation of transfection effects at different time point after the end of CVB3 infection.

3.1.3.1 Analysis of cellular morphology

Since CVB3 is a cytolytic virus, a first insight on the possible reduction of virus load in infected HeLa cells was made by investigating morphological aspects of cells after transfection and infection. Cellular morphology of HeLa cells, following transfection-infection procedure, was observed at 24 h post infection (p.i.) using a phase contrast microscope. Whereas cells non-transfected but infected (NTC+CVB3) and cells transfected by the control siRNA GL2 and infected (siRNA GL2) displayed a severe cytopathic effects, a viable phenotype is maintained in siRNA 35 and siRNA 36 treated cells, in which only few cells showed the typical rounded shape of infected cells (arrows) (Figure 5).

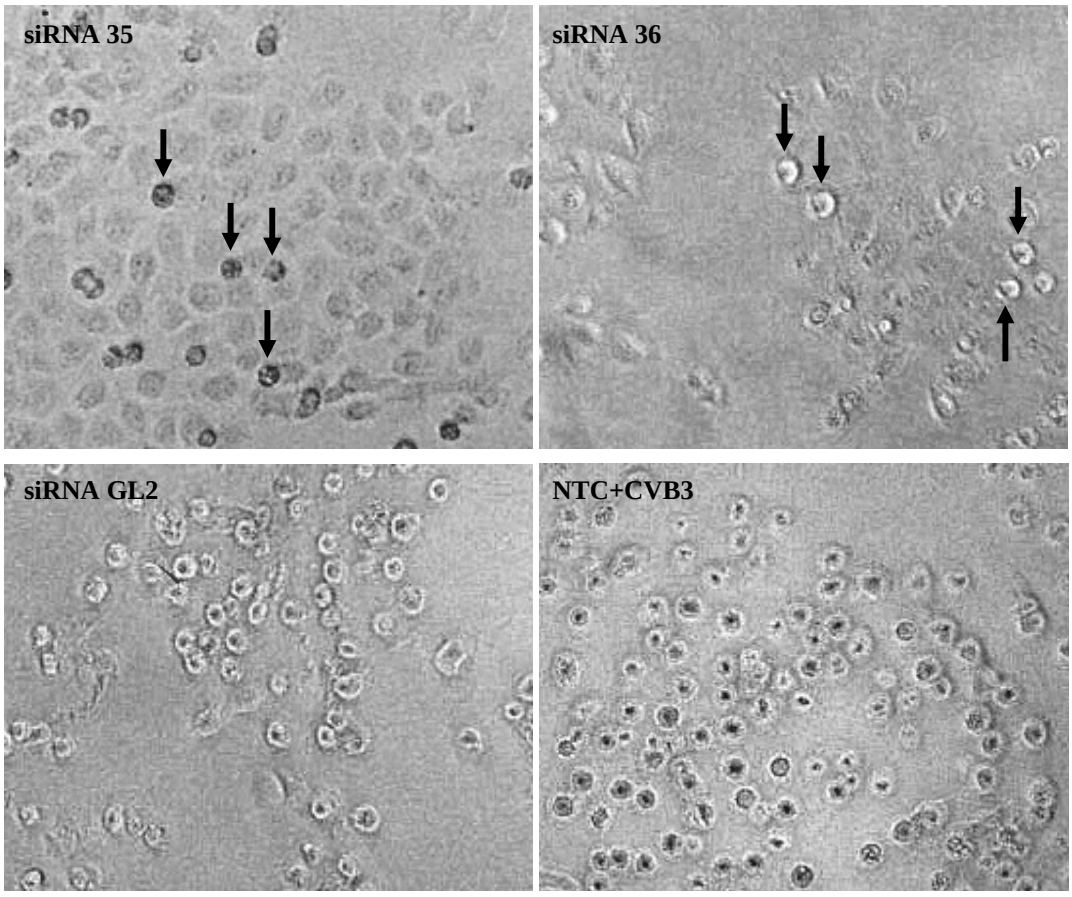

Figure 5.

Representative phase-contrast images of HeLa cells treated with 400 nM siRNA 35, siRNA 36 and siRNA GL2 and infected with 0,01 MOI CVB3. Positive controls are non-transfected and infected cells (NTC+CVB3).

3.1.3.2 Effects of siRNAs on the amount of CVB3 genome equivalents

To evaluate the effects of the selected siRNAs on CVB3 load, viral genomic RNA was quantified by quantitative RT-PCR following the transfection-infection procedure. Maximum reduction was observed 24 h p.i., and resulted in HeLa cells treated by the siRNA 35 and siRNA 36, in a decrease to 20% of genome equivalents of controls ($p<0.05$)(Figure 6).

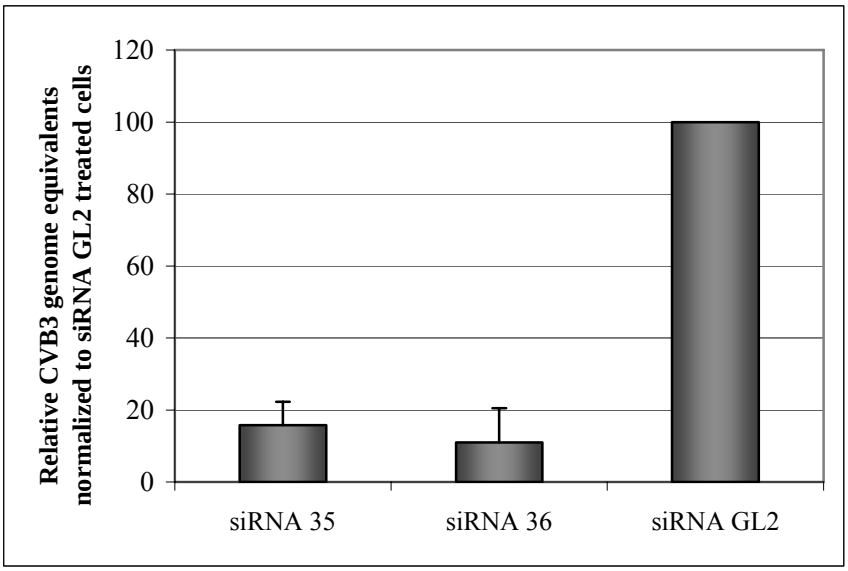

Figure 6.

Quantitative RT-PCR analysis of viral RNA. Transfection with Lipofectamine 2000 and siRNAs at a final concentration of 400 nM was performed for 2 h. Then, HeLa cells were infected with 0,01 MOI CVB3. At 24 h p.i. total RNA was extracted and quantitative RT-PCR, relative to HPRT, was performed. Data are expressed as mean ± SEM, n=3

3.1.3.3 Effects of siRNAs on viral protein amounts

After proving the reduction of morphological cytotoxicity and of CVB3 genome equivalents following siRNA 35 and siRNA 36 treatments, attention was focused on the evaluation of the amounts of viral proteins. After the transfection-infection procedure, the amount of the CVB3 capsid protein VP1 was evaluated 16 and 24 h p.i..

The data obtained 16 h p.i. are reported in a representative blot in Figure 7 and are summarized in Figure 8. The reduction of VP1 levels is evident in siRNA 35 and siRNA 36 treated cells compared to controls. In particular, a reduction down to 40 and 25% of control siRNA GL2 treated HeLa cells was observed for siRNA 35 and siRNA 36 treated cells, respectively ($p<0.05$).

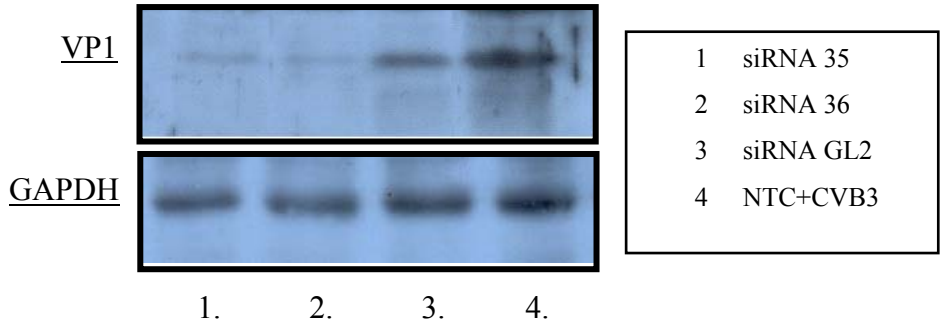

Figure 7.

Western blot analysis for the detection of CVB3 capsid protein VP1. HeLa cells were transfected with siRNAs at a final concentration of 400 nM using Lipofectamine 2000 and were then infected with 0,01 MOI CVB3 for 1 h. At 16 h p.i. cell lysates were collected for VP1 detection. GAPDH detection was used for normalization. NTC+CVB3=non-transfected but infected cells.

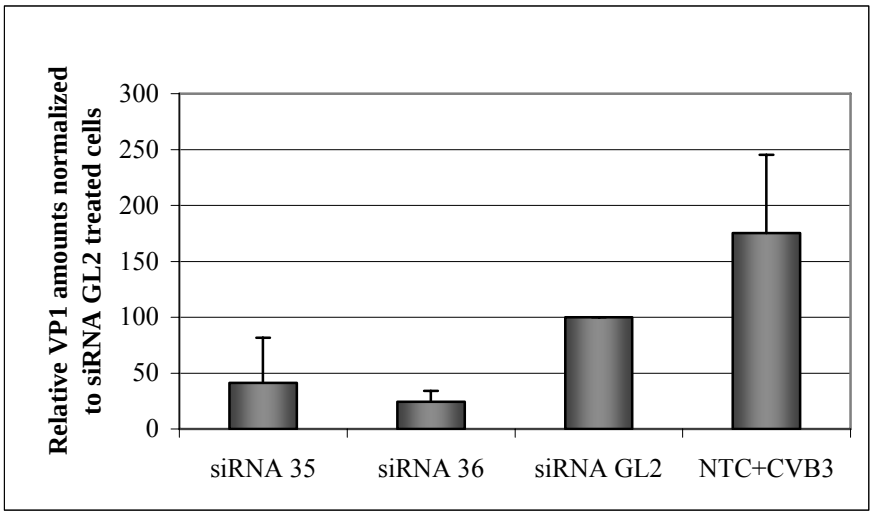

Figure 8.

Densitometric analysis of the VP1 Western blots. Data are expressed as ratio VP1/GAPDH protein levels normalized to siRNA GL2 treated HeLa cells. NTC+CVB3=non-transfected and CVB3-infected cells. Data are expressed as mean ± SEM, n=3.

Comparable results were obtained for siRNA 36, 24 h after the end of infection as shown in a representative Western blot in Figure 9.

Finally, a dose dependent effect of the selected siRNA was observed in relation to the amount of VP1 levels (data not shown).

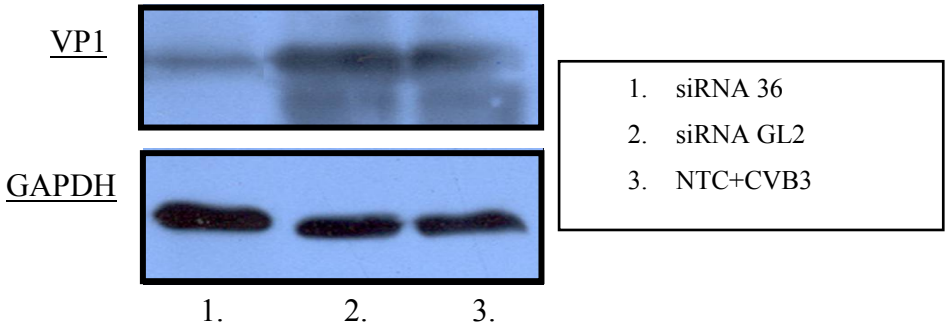

Figure 9.

Western blot analysis for the detection of CVB3 capsid protein VP1. HeLa cells were transfected with siRNAs at a final concentration of 400 nM using Lipofectamine 2000 and then were infected with 0,01 MOI CVB3 for 1 h. At 24 h p.i. cell lysates were collected for VP1 detection. GAPDH detection was used for normalization.

As an enhanced antiviral effect of multiple siRNAs targeting the HIV-1 genome has been reported (Ji et al., 2003), a cotransfection of cells with a combination of the two specific siRNAs targeting different regions of the viral proteinase 2A was performed. The transfection-infection procedure was followed using either the siRNA 35 and 36 at 400 nM each or a combination of both of them each at 200 nM. At 24 h p.i. the VP1 protein levels were determined by Western blot. As shown in Figure 10, the combined administration at an equimolar amount of a combination of the two siRNAs did not result in a more pronounced reduction in VP1 levels compared to the single administration of each of them. These data were also confirmed by evaluating the CVB3 genome equivalents by quantitative RT-PCR (data not shown).

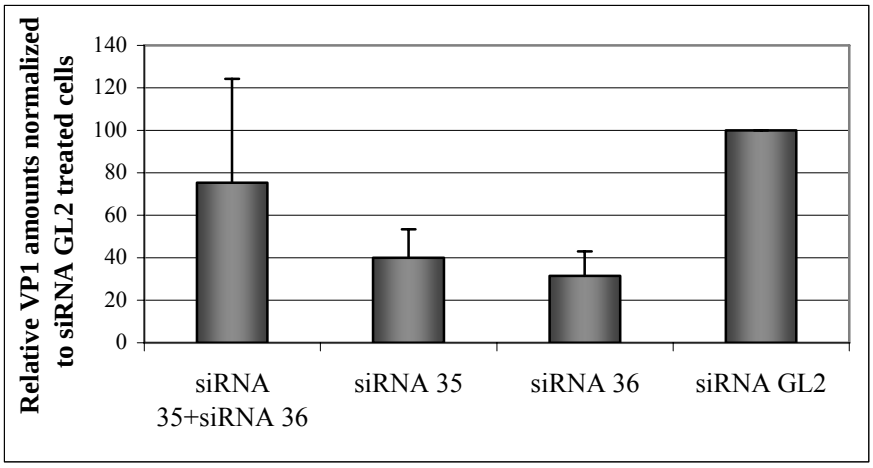

Figure 10.

Densitometric analysis of VP1 Western blots. HeLa cells were transfected with siRNA 35 (400 nM), siRNA 36 (400 nM) and siRNA 35+siRNA 36 (200 nM+200 nM), and then infected for 1 h by CVB3 at a MOI of 0,01. Data are expressed as ratio VP1/GAPDH protein level normalized to siRNA GL2 treated cells. Data are expressed as mean ± SEM, n=3.

3.1.3.4 Plaque assay

To complete the analysis of the antiviral effects of the selected siRNAs, the amount of infectious viruses in the supernatant of treated HeLa cells was evaluated by plaque assay (Figure 11). After subjecting HeLa cells to the "transfection-infection procedure", supernatants were collected 24 h later and subjected to plaque assay. Viral plaques were counted 48 h after overlaying soft agar on Vero cell monolayers and the numbers of PFU/ml supernatants were calculated. Figure 12 demonstrates that siRNA 36, as well as siRNA 35, strongly inhibited the production of infectious CVB3 particles causing a significant ($p < 0.05$) decrease compared to controls. This result is consistent with the data obtained by morphological inspection of the cells, by the quantification of CVB3 genome equivalents (RT-PCR) and by the quantification of the CVB3 capsid protein VP1 (Western blot) above reported. At a shorter time point (16 h) the trend was similar although the extent of PFU reduction in siRNA 35 and 36 treated cells was somewhat less pronounced (data not shown).

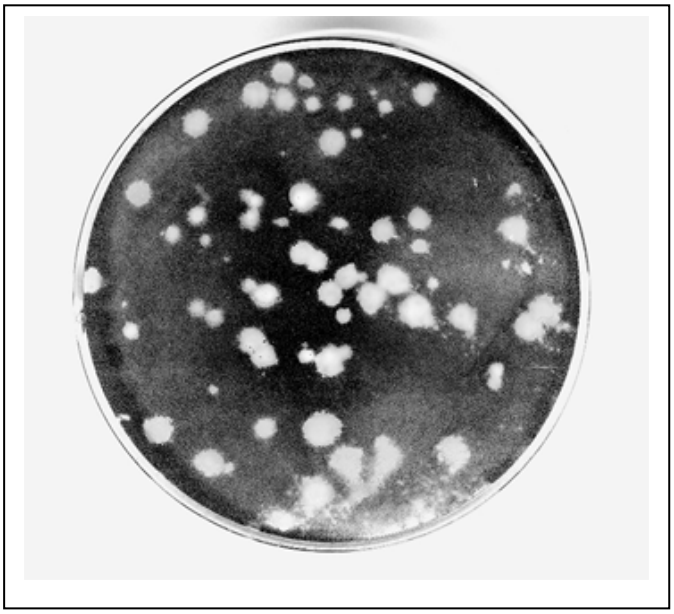

Figure 11.
Representative plaque assay for the detection of infectious virions of CVB3.

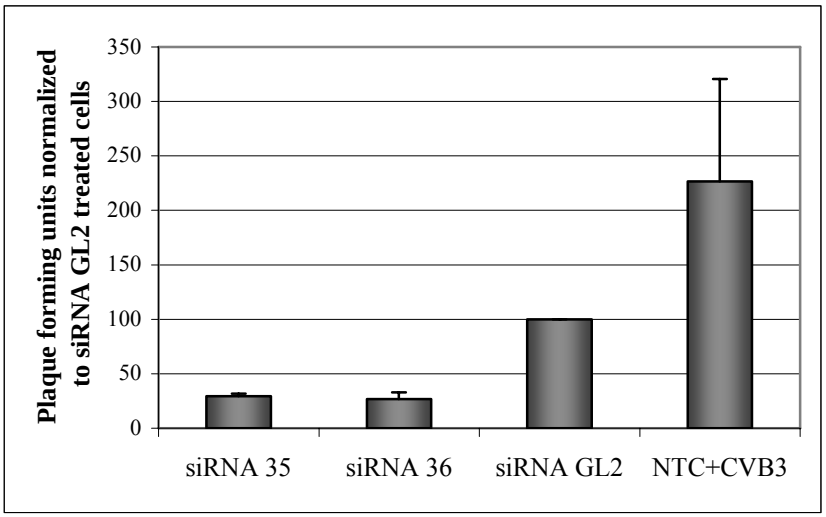

Figure 12.

Plaque assay for the detection of infectious viral particles. HeLa cells were transfected with 400 nM siRNAs for 2 h and infected with 0,01MOI CVB3 for 1 h. At 24 h p.i. supernatants were collected and plaque assays were performed. NTC+CVB3=HeLa cells non-transfected and infected by CVB3. Data are expressed as mean ± SEM, n=3.

3.1.3.5 siRNA effects on HeLa cell viability following CVB3 infection

To evaluate the efficacy of RNA interference in attenuating CVB3-mediated cytotoxicity, selected siRNAs were transfected into HeLa cells and their viability was evaluated. HeLa cells were treated according to the "transfection-infection procedure" and 24 h p.i., both LDH and MTT tests were performed as described in paragraph 3.1.2.2. LDH levels, proportional to the amount of virions inducing cytolysis, were clearly reduced ($p<0.05$) in siRNA 35 (not shown) and siRNA 36 (Figure 13) treated cells compared to controls, further confirming the protective antiviral effects of the selected siRNAs. Finally MTT tests, proportional to mitochondria activity, i.e. to cell viability, clearly indicated ($p<0.05$) higher viability (higher conversion of the water soluble MTT to the insoluble formazan) in siRNA 36 (Figure 14) treated cells compared to controls. These findings match the results of LDH tests and the morphological analysis reported in Figure 5.

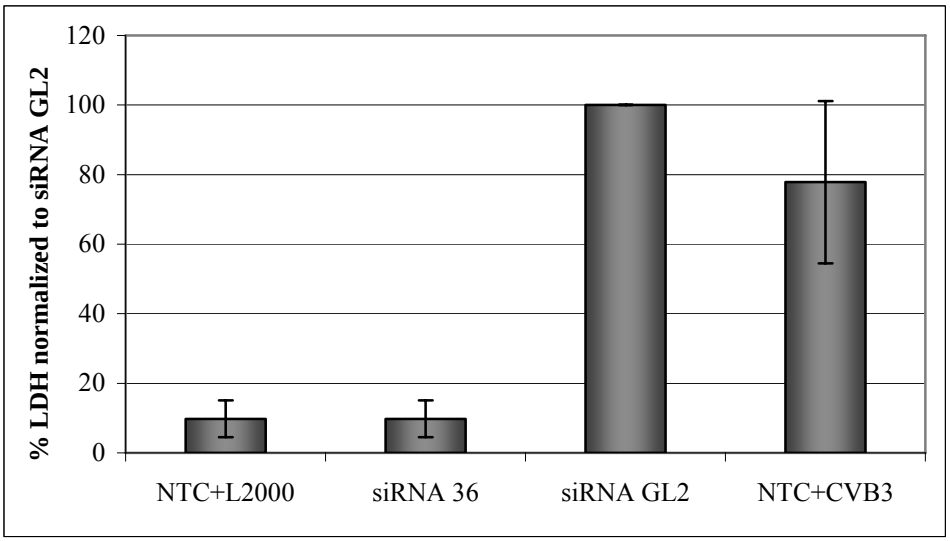

Figure 13.

LDH assay. HeLa cells were transfected with siRNA 36 at 400 nM and infected with 0,01 MOI CVB3 for 1 h. At 24 h p.i. LDH levels were measured spectrophotometrically. NTC+L2000=cell transfected by Lipofectamine 2000 but not infected; siRNA GL2=cell transfected by siRNA GL2 and infected; NTC CVB3=cells non-transfected and infected by CVB3. Data are expressed as mean ± SEM, n=3.

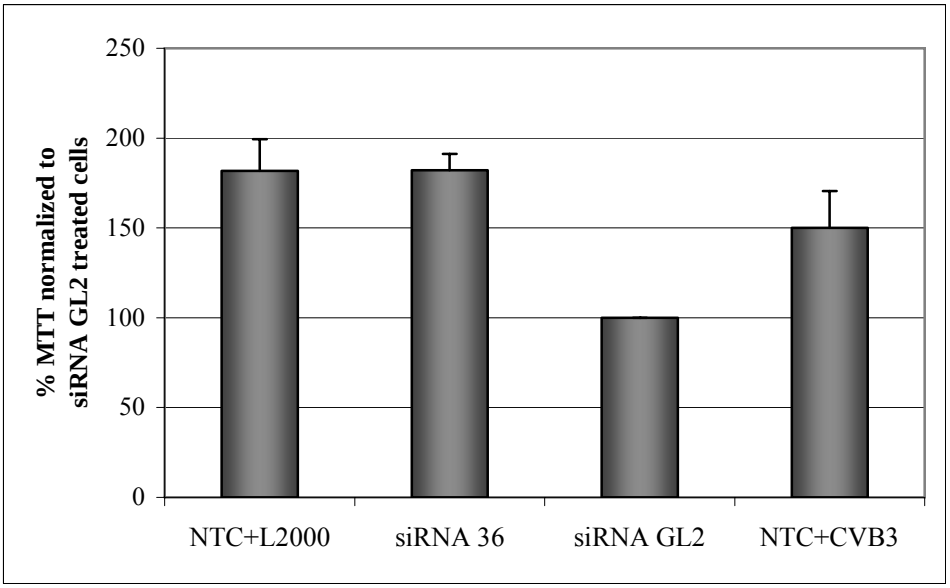

Figure 14.

MTT assay. HeLa cells were transfected with siRNA 36 at 400 nM and infected with 0,01 MOI CVB3 for 1 h. At 24 h p.i. MTT levels were measured spectrophotometrically. NTC+L2000=cell transfected by Lipofectamine 2000 but not infected; siRNA GL2=cell transfected by siRNA GL2 and infected; NTC CVB3=cells non-transfected and infected by CVB3. Data are expressed as mean ± SEM, n=3.

3.2 Relationship between CVB3 infection and CTGF expression

Microarray data from our laboratory conducted in a murine model of chronic CVB3-myocarditis, showed connective tissue growth factor as a novel factor highly expressed in infected hearts and possibly involved in the late sequelae of CVB3 induced myocarditis (Figure 15)(Lang et al., J Mol Med, in revision). In an attempt to deeper investigate any possible relationships between CTGF expression and CVB3 infection, siRNAs against CTGF were generated and tested in cultured HeLa cells together with the above characterized anti-CVB3 siRNAs. The inhibition of the expression of either CTGF or CVB3 in infected HeLa cells allowed to improve the comprehension of the interrelationship between these molecules.

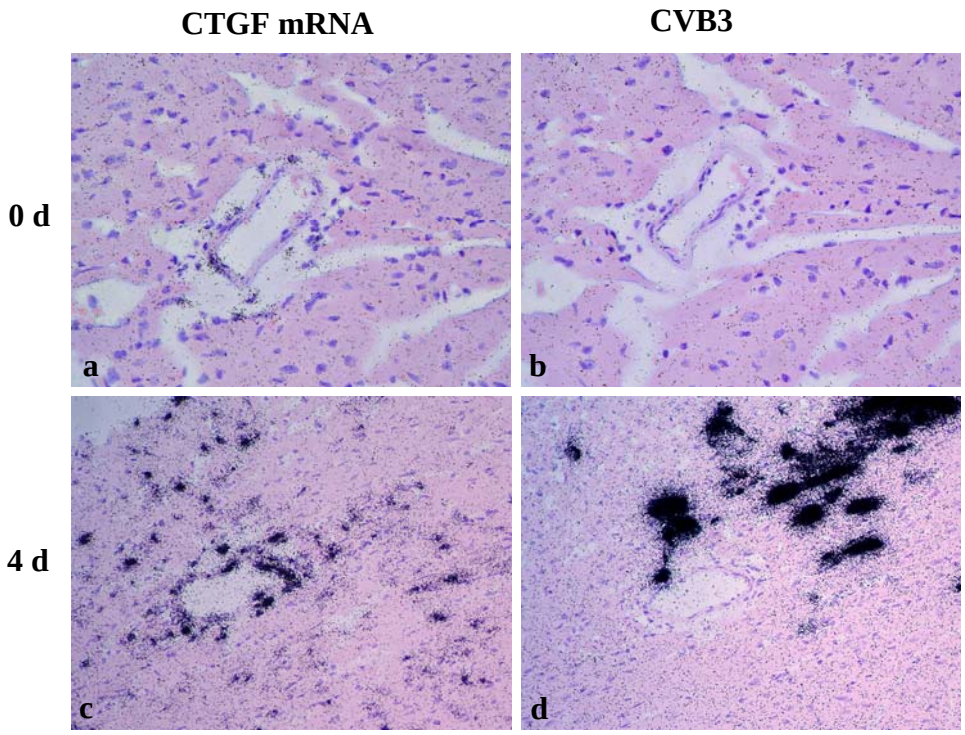

Figure 15.
Expression of CTGF mRNA and CVB3 infection in consecutive heart tissue sections at day 0 and day 4 p.i. in susceptible ABY/SnJ mice. Radioactive *in situ* hybridization. From: C. Lang et al., J Mol Med, in revision.

3.2.1 Design of siRNAs against CTGF

Two different siRNAs were designed to silence CTGF expression. The sequences and the locations in the CTGF target gene are indiated in Table 2. As for CVB3 specific siRNAs, siRNAs against CTGF were designed based on a mathematical algorithm (Poliseno et al., 2004). As a control, the siRNA GL2 directed against the luciferase gene was used.

Table 2. Sequences of siRNAs used to target the CTGF mRNA

Name	5'-3' sequence	CTGF mRNA target gene: relative nucleotide position
siRNA CTGF1	GACCUGGAAGAGAACAUUA	1071-1090
siRNA CTGF2	GGCGAGGUCAUGAAGAAGA	876-895
siRNA GL2	CGUACGCGGAAUACUUCGA	-----

All molecules consist additionally of dTdT overhangs.

3.2.2 Effects of siRNA targeting CTGF mRNA expression

To evaluate the effects of the selected siRNAs on CTGF expression, both siRNAs against CTGF (siRNA CTGF1 and siRNA CTGF2) and the siRNA GL2 control were transfected into HeLa cells for 2 h. At 24 h post transfection total RNA was extracted and quantitative RT-PCR for CTGF mRNA was performed. Compared to controls, i.e. non-treated HeLa cells, the maximum reduction on CTGF expression is exerted by the siRNA CTGF1 (Figure 16), which was chosen for further experiments.

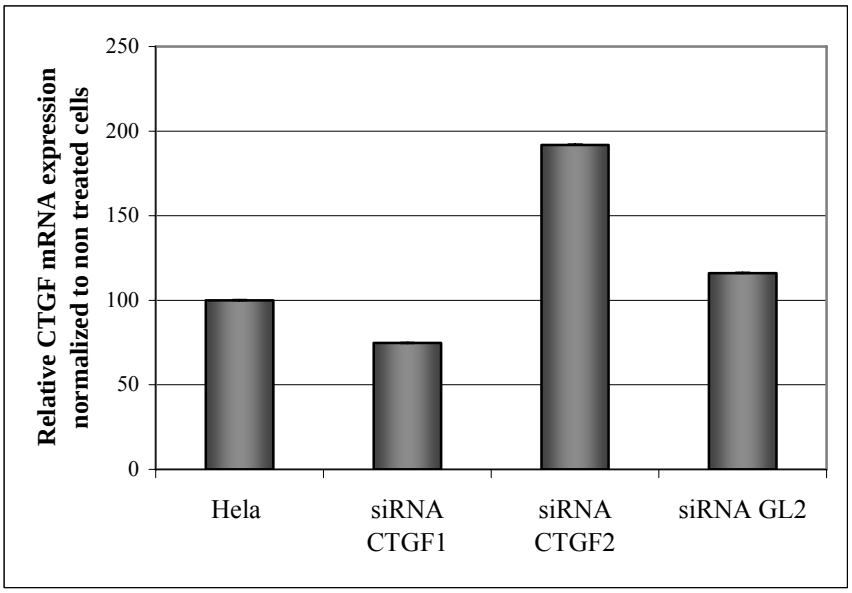

Figure 16.

Quantitative RT-PCR analysis for the detection of CTGF mRNA. HeLa, transfected with 400 nM siRNA CTGF1, siRNA CTGF2 and siRNA GL2 for 2 h were then washed and incubated with complete medium. At 24 h post transfection total RNA was extracted and quantitative RT-PCR, relative to HPRT, was performed. HeLa=non-treated cells. Data are expressed as mean ± SEM, n=3 .

3.2.3 Expression analysis of CTGF after CVB3 infection

Despite our observation of the *in vivo* induction of CTGF upon CVB3 infection in murine hearts (Lang et al., J Mol Med, in revision), it was necessary to prove this observation also in the *in vitro* model of HeLa cells. For this purpose HeLa cells, 60-70% confluent, were infected with 10 MOI CVB3 for 1 h and then incubated with complete medium for 5 h. Total proteins were then extracted and Western blot for the detection of CTGF was performed (Figure 17). As shown in a representative blot in Figure 15a and summarized in Figure 15b, a clear increase (p=0.035) in CTGF protein amounts of about 2,5 fold in infected HeLa cells compared to uninfected cells was observed.

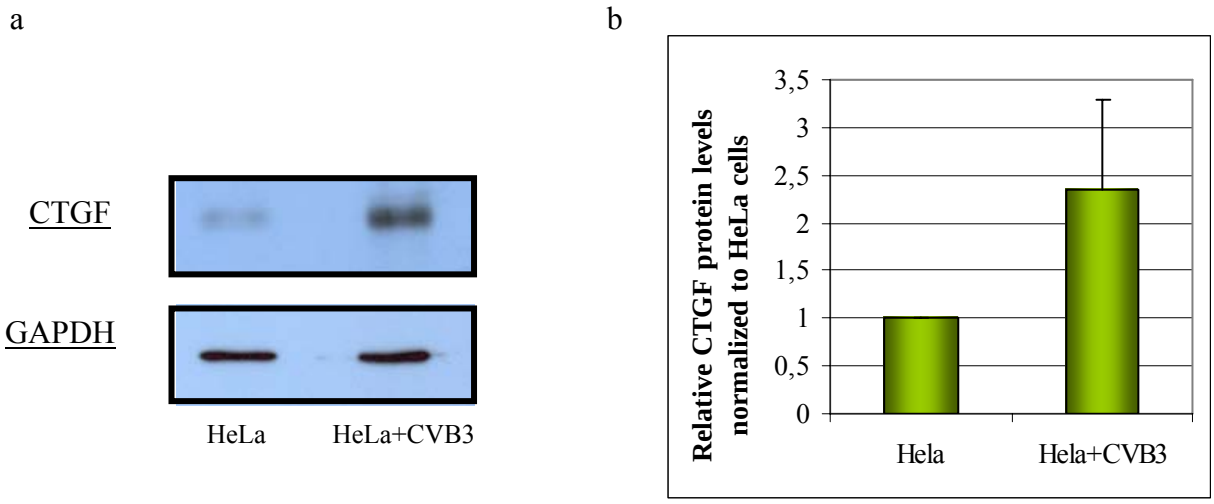

Figure 17.

a. Western blot for the detection of CTGF. HeLa cells were infected with 10 MOI CVB3 for 1 h. Then cells were washed and overlaid with complete medium for 5 h. Total proteins were then extracted and Western blot visualizing CTGF was performed. GAPDH detection was used for normalization b. Densitometric analysis of the Western blots. Data are expressed as ratio CTGF/GAPDH protein levels normalized to non-infected HeLa cells. HeLa+CVB3=infected HeLa cells. Data are expressed as mean ± SEM, n=3.

The results obtained at the protein level were also confirmed at the mRNA levels as reported in Figure 18, where CTGF mRNA amounts in infected HeLa cells showed an increase of about 3 fold compared to uninfected cells ($p<0.01$).

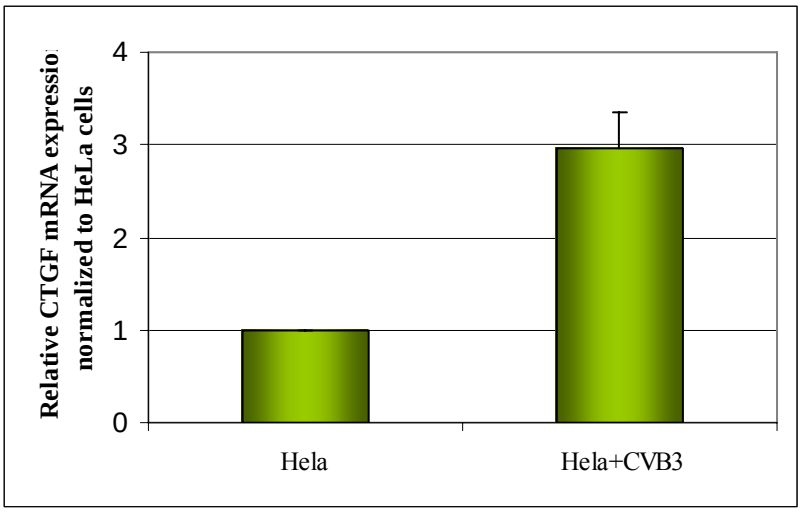

Figure 18.

Quantitative RT-PCR analysis for the detection of CTGF mRNA. HeLa cells were infected with 10 MOI CVB3 for 1 h. Soon after infection, cells were washed, overlaid with complete medium for 5 h, then total RNA was extracted. Data are expressed as ratio CTGF/HPRT mRNA levels normalized to non-infected HeLa cells. Data are expressed as mean ± SEM, n=3.

3.2.4 Effects of CVB3 inhibition on CTGF expression

After having shown the increase of CTGF expression upon CVB3 infection also in HeLa cells, additional experiments were conducted to explore this relationship. It was thus evaluated the CTGF expression levels in HeLa cells transfected by siRNA 36 and subsequently infected with CVB3 according to the "transfection-infection procedure". 24 h p.i., total RNA was extracted and analyzed by quantitative RT-PCR. As shown in Figure 19, the CTGF levels in siRNA 36 treated samples were significantly reduced as well as in samples treated with siRNA CTGF1 ($p<0.05$). These data show that the down-regulation of CVB3 replication as well the selected siRNA CTGF1 can reduce CTGF mRNA levels.

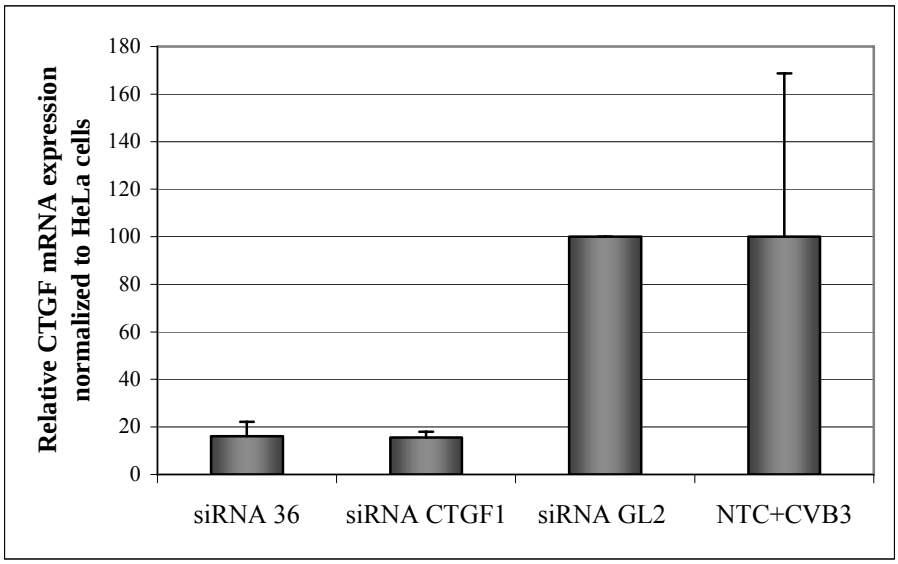

Figure 19.

Quantitative RT-PCR analysis for the detection of CTGF mRNA. HeLa cells were transfected with 400 nM siRNAs and infected with 0,01 MOI CVB3 for 1 h. At 24 h p.i. cells were harvested, total RNA extracted and the CTGF mRNA levels were evaluated. Data are expressed as ratio CTGF/HPRT mRNA levels normalized to siRNA GL2 transfected and CVB3-infected HeLa cells. NTC+CVB3= non-transfected and CVB3-infected HeLa cells. Data are expressed as mean ± SEM, n=3.

3.2.5 Effects of CTGF inhibition on CVB3 replication

Subsequently, it was tested whether CTGF up-regulation is essential for the CVB3 life cycle. For this purpose, HeLa cells were subjected to the "transfection-infection procedure" using the siRNA 36, siRNA CTGF1 and the control siRNA GL2. Quantitative RT-PCR analysis for the detection of viral genomes showed that, whereas siRNA 36 strongly inhibited the CVB3 replication (Figure 20), siRNA CTGF1 had a non significant effect on the amounts of CVB3 genome equivalents ($p=0.07$). These data were also confirmed at the protein levels by measuring CVB3 VP1 capsid proteins (data not shown). Together, these findings suggest that CTGF is not required for CVB3 replication.

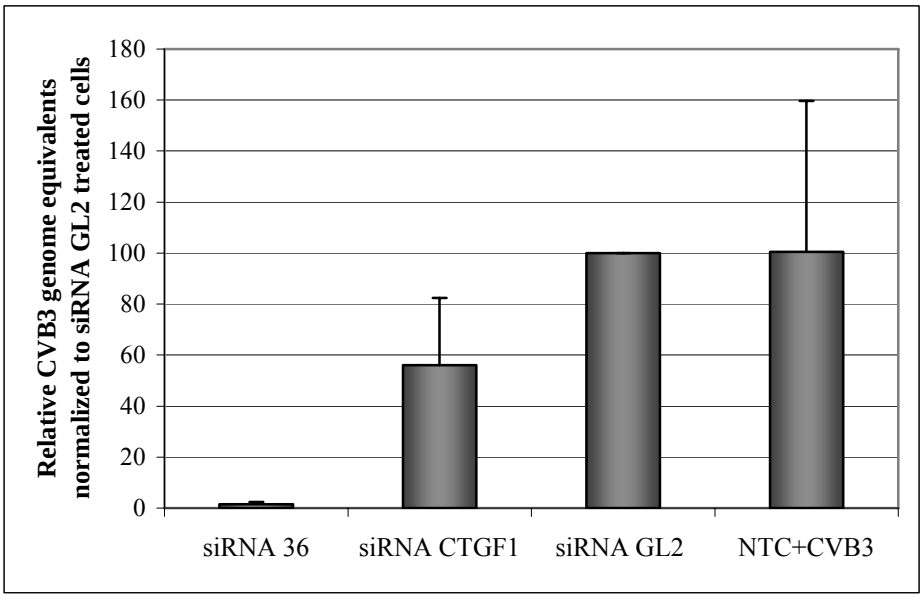

Figure 20.

Quantitative RT-PCR analysis for the quantification of CVB3 genome equivalents. HeLa cells were transfected with 400 nM siRNAs and infected with 0,01 MOI CVB3 for 1 h. At 24 h p.i. cells were harvested, total RNA extracted and the CVB3 genome levels evaluated. Data are normalized to siRNA GL2 transfected and CVB3-infected HeLa cells. NTC+CVB3=non-transfected and CVB3-infected HeLa cells. Data are expressed as mean ± SEM, n=3.

3.2.6 Involvement of TGF-β in CTGF downregulation by siRNA36

After having shown that siRNA 36 reduces CTGF levels, it was asked whether this effect may be mediated by a downmodulation of TGF-β, a well known stimulator of CTGF. For this purpose, the amount of TGF-β was evaluated in HeLa cells subjected to the transfection-infection procedure. At 24 h p.i., the mRNA levels of TGF-β were measured by quantitative RT-PCR. The data reported in Figure 21 indicate that siRNA 36 does not significantly ($p=0,21$) reduce TGF-β mRNA levels compared to controls. Thus, it was concluded that the downregulation of CTGF by the virus-specific siRNA 36 is probably not dependent on TGF-β.

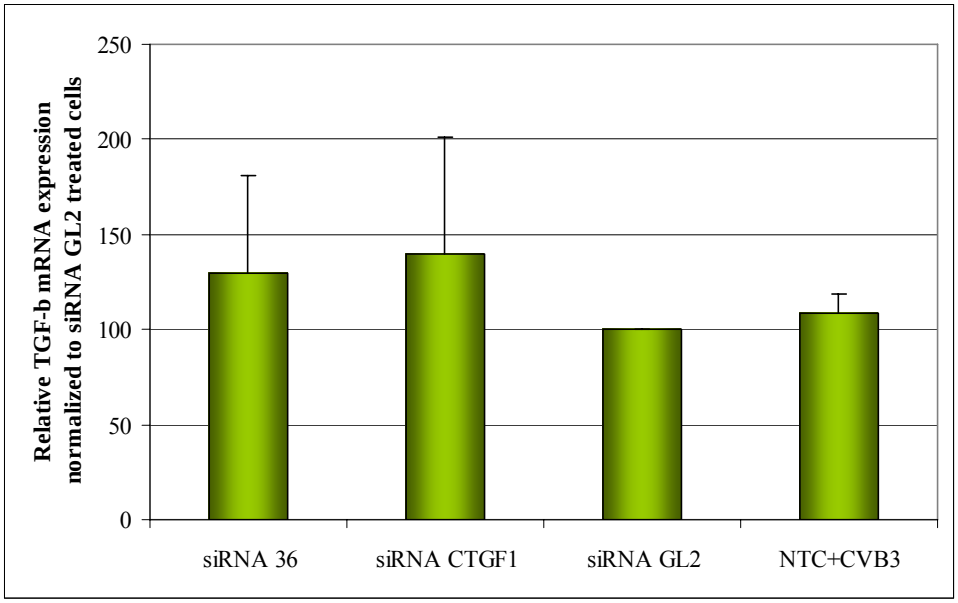

Figure 21.

Quantitative RT-PCR analysis for the detection of TGF-β mRNA. HeLa cells were transfected with 400 nM siRNAs and infected with 0,01 MOI CVB3 for 1 h. At 24 h p.i. cells were harvested, total RNA extracted and the TGF-β mRNA levels were evaluated. Data are expressed as ratio CTGF/HPRT mRNA levels normalized to siRNA GL2 transfected and CVB3-infected HeLa cells. NTC+CVB3= non-transfected and CVB3-infected HeLa cells. Data are expressed as mean ± SEM, n=3.

4. Discussion

4.1 General consideration

Despite several current therapeutic approaches, myocarditis is still a major cause of morbidity and mortality worldwide (Dec and Fuster, 1994) and contributes significantly to the societal burden of heart failure. The affected individuals may require long-term medical therapy and in many cases heart transplantation. Significant evidence from animal models and clinical studies (Sole and Liu, 1993) indicates that viruses represent relevant etiological causes of myocarditis. In particular, coxsackieviruses of group B (CVB) are the most frequent cause of symptomatic viral myocarditis. Prominent cytopathic alterations in CVB3-infected cardiac cells as well as CVB persistence in the myocardium of affected individuals and mice have been well documented (Kandolf et al., 1987; Kandolf et al., 1991; Klingel et al., 1992; Klingel e al., 1998). Heart tissue damage depends not only on the direct viral cytolytic effects but also on the immune response elicited by the virus. In this regard, the contribution of the immune system to tissue injury relies on a complex balancing between protective and detrimental immunological pathways (Ayach et al., 2003). This implies that any interference with the immune response may have unpredictable, if not deleterious, effects on the course of the disease. Additionally, the observation that immunosuppressive therapy did not lead to an improvement in cardiac ejection fraction when compared with conventional therapy (Mason et al,. 1995), further suggests that targeting the immune system is not the optimal strategy for the treatment of CVB-myocarditis. Moreover, it was demonstrated in a murine model of CVB3-myocarditis that early limitation of the viral load is an important factor in the prevention of chronic myocarditis (Klingel et al., 2003). Thus, targeting the virus seems to be the more indicated approach to develop novel treatments for viral myocarditis.

The lack of available efficacious strategies to target CVB3, prompted us to explore the possibility to develop an antiviral approach based on the use of siRNAs. These molecules are particularly suited for this purpose because of their high efficiency to induce target RNA destruction. This ability, initially shown in 2001 (Elbashir et al., 2001) was then further confirmed in many other works (see for example Agostini et al., Drug of the future, in press). Moreover, the use of siRNAs become more and more attracting due to the relatively easy selection of active molecules. Nowadays, mathematical algorithms able to take into account the energy involved in the siRNA binding to the target RNA, the optimal base composition of the siRNA and target RNA folding can guarantee approximately 70-80% of success in selecting active siRNA (Poliseno et al., 2004). This percent of success is extremely more

elevated than that obtained for hammerhead ribozymes, a class of RNA molecules able to destroy the target RNA by chemically breaking a covalent bond within the target RNA chain (Grassi et al., 2004). The experience from our group suggests that the percent of success in selecting active hammerhead ribozymes by mathematical algorithms is around 25% (Grassi et al., 2001). This implies that a long and time consuming experimentally based work is required to find active molecules. Together all these observations strongly oriented our decision to consider siRNAs as antiviral agents.

4.2 Specific comments

4.2.1 Selection of siRNA and uptake optimization

As reported in paragraph 3.1.1 the selection of active siRNAs was greatly facilitated by the use of a previously described mathematical algorithms developed by some collaborators of us (Poliseno et al., 2004). Three out of four of the generated siRNAs (two against CVB3 and two against CTGF) resulted to be very active in destroying the target RNA, showing the potency of the followed approach.

The anti CVB3 siRNAs were targeted against the region of the viral genome encoding for the proteinase 2A. The reason for this choice depends on the fact that proteinase 2A is fundamental for virus replication (Rueckert, 1996) as it processes the viral polyprotein into individual structural and non-structural proteins, it cleaves the eukaryotic translation initiation factor 4G (eIF4G) (Krausslich et al., 1987), and it cleaves (Kerekatte et al., 1999) the poly(A)-binding protein (PABP), an RNA binding protein essential for protein translation. Moreover, proteinase 2A subverts (Badorff et al., 1999) cardiac tissue architecture, thus contributing to the pathogenesis of dilated cardiomyopathy, a late sequelae of CVB3 infection (Liu and Mason, 2001; Xiong et al., 2002). Taken together, these observations suggest that the reduction of proteinase 2A amounts may potentially attenuate virion production, the host protein synthesis shut off, and the modification of cardiac tissue architecture.

Before evaluating the antiviral activity of the selected siRNAs in the well established HeLa model of CVB3 infection (Carthy et al., 1998; Kerekatte et al., 1999; Badorff et al., 2000; Roberts et al., 2000; Zhang et al., 2002; Luo et al., 2003; Yanagawa et al., 2003), care was paid in optimising siRNAs uptake by liposomes and in evaluating possible toxic effects of the complexes siRNA/liposome to HeLa cells. The exogenous delivery of siRNAs was preferred over the endogenous delivery system (viral mediated) to minimize the set up of the delivery procedure. A viral mediated delivery would have required the preparation of viral constructs,

the selection of the proper promoter and the cloning of the siRNA into the viral vector backbone, without being sure to have successfully active siRNAs.

With regard to the uptake set up, optimal delivery of siRNAs to HeLa cells was obtained using the transfection reagent Lipofectamine 2000, a transfection time of 2 hours, a weight ratio Lipofectamine 2000/siRNA of 1:1 and a siRNA concentration of 400 nM. Whereas the value of 400 nM may appear to be somewhat elevated, it is in line with the results presented by other groups, aimed at the reduction of CVB3 genome by siRNAs experiments (Merl et al., 2005; Yuan, et al., 2005). Under these conditions, more than 80% of HeLa cells were transfected (Figure 2), thus ensuring excellent distribution of the therapeutic siRNAs to the cells. The 20% cells escaping transfection may account for the residual presence of virus observed in siRNA 35 and 36 treated cells (see for example Figure 6 and 12). Higher amounts of siRNAs were not tested as this would have implied also the increase of the amount of Lipofectamine 2000, leading to increased cell toxicity, a fact which was proven by optical microscopy inspection (data not shown).

With regard to the possible toxic effects of the transfection procedure to the cells, attention was paid to reduce toxicity as much as possible. This investigation allowed to exclude that substantial modification in HeLa cell viability could have contributed to determine non-optimal conditions for CVB3 replication, thus inducing a sort of "siRNA independent" reduction of viral load. The data reported in Figures 3 and 4 clearly show that no major toxic effects in transfected HeLa cells were detectable compared to non-transfected cells, further confirming the proper transfection conditions selected.

4.2.2 Antiviral effects of CVB3-specific siRNA 35 and 36

Both of the two siRNAs, siRNA 35 and siRNA 36 targeted against the CVB3 genome sequence coding for the proteinase 2A, were able to significantly ($p<0.05$) reduce the amounts of CVB3 genomes and of viral capsid protein VP1 as well as the number of infectious virions in treated cells compared to controls (Figure 6, 7, 8, 9 and 12). The effects of the selected siRNAs were not limited to the reduction of several markers of viral infection, they also allowed treated cells, compared to infected controls, to be definitively more viably as determined by microscopic inspection (Figure 5) and by LDH and MTT tests (Figures 13 and 14). Taken together, these data clearly indicate the potential of the selected siRNAs as anti-CVB3 drugs.

The data presented are consistent and quantitatively comparable to the *in vitro* results reported by Yuan et al. (Yuan et al., 2005) who designed siRNAs directed against the proteinase 2A,

but to a different region from those selected in this work. Interestingly, although Yuan et al. selected also siRNAs directed against different CVB3 regions such as the 5' UTR viral region, the capsid protein VP1 and the viral RNA polymerase 3D, highest reduction in viral load was achieved by targeting proteinase 2A. Beside being possible that the strongest CVB3 downmodulation displayed by the siRNA against proteinase 2A depends on an intrinsic higher efficacy of the siRNAs, ours and Yuan data favour the concept that proteinase 2A is one of the most indicated anti-CVB3 target. This hypothesis is also supported by the work of Merl et al. (Merl et al., 2005) who showed the potency of targeting proteinase 2A (a different region from those selected in this work) both *in vitro* and *in vivo*. In this last case, Merl et al administered the proteinase 2A siRNA by hydrodynamic transfection to lethally infected mice. Compared to controls, mice receiving the proteinase siRNA after viral infection significantly improved life span, with one out of eight animal surviving infection. In addition, it was shown that CVB3 titers in different organs such as the heart, liver, lung and kidney were markedly reduced in treated animals compared to controls.

In this work the analysis of the antiviral effects of the siRNAs 35 and 36 was extended also to the evaluation of the efficacy of their combined administration. The idea stems from the observation that in the virus infection by HIV-1, the combined administration of two anti-HIV siRNAs resulted in a more pronounced reduction of the virus load compared to the single equimolar administration of the two siRNAs (Yuan et al., 2005). Disappointingly, neither an additive nor a synergistic effect influencing CVB3 load could be observed in this work (Figure 10). Our data are in agreement with the work of Yuan et al. (Yuan et al., 2005) who, using a different couple of siRNAs, confirmed our results. Whether this effect depends on the siRNAs tested or on the specific biology of CVB3 remains to be clarified. In the case of HIV, the highest inhibitory effects exerted by the simultaneous administration of two siRNAs may be explained by the possibility to target different escape virus variants. It is known that the HIV genome has a high mutation rate which gives origin to progeny viruses which differ at the genomic level because of the presence of single point mutations, double point mutations, partial or complete deletion in the genome. Thus, mutations occurring in the regions targeted by the siRNA can abrogate siRNA action, i.e. target RNA destruction. It is convincible that targeting two distinct regions reduces the possibility that mutations occur in the regions targeted by the siRNA. Despite the lack of any enhancing effect in the case of CVB3, this approach remains potentially relevant for long-term treatment. Indeed, mutated virus variants may always be produced to escape from siRNA destruction, but the presence of multiple siRNAs against different regions of the genome may render the escape less likely to occur thus maintaining the efficacy of the siRNAs based approach.

4.2.3 siRNA against CTGF mRNA

The results reported by Merl et al (Merl et al., 2005) indicate that an siRNA targeted against CVB3 proteinase 2A can significantly improve the life span of CVB3 lethally infected mouse compared to controls. Despite this encouraging results, the majority of the siRNA treated animals died, most likely because of the persistence of low amounts of CVB3 which may have triggered immune-mediated remodelling processes of the infected organs. One possibility to circumvent the problem of uncompleted viral clearance consists of improving the *in vivo* delivery efficiency in order to increase the number of cells receiving the siRNAs and/or the amount of siRNAs/cells. Additionally, it is possible to think to a strategy aimed at the downmodulation of cardiac tissue remodelling. This is the approach we decided to explore, especially on the basis of the *in vivo* results our group recently obtained (Lang et al., J Mol Med, in revision). According to these results CTGF, a pro-fibrotic cytokine, has been shown to be a crucial molecule in the development of heart fibrosis in ongoing enteroviral myocarditis in mice. In particular, the expression of CTGF has been shown to be significantly up-regulated upon CVB3 infection in the heart. Thus, we reasoned that the downmodulation of CTGF could in principle be benefical as it would reduce the CVB3-associated heart fibrosis.

Before exploring the possibility to downregulate CTGF expression by siRNAs, it was necessary to verify that the CTGF up-regulation upon CVB3 infection occurs also in our cell model. As reported in Figures 17 and 18, an increased CTGF expression was observed upon HeLa infection by CVB3, thus proving the feasibility of the HeLa cell model to investigate the CVB3-CTGF relationship. It should be noted that these experiments were performed using 10 MOI of CVB3 to infect Hela cells instead of 0.01 MOI as done for the anti-proteinase 2A siRNA studies and subsequent anti CTGF siRNA investigations. The reason for this difference relies in the fact that by using a high MOI it was possible to elicit faster (few hours p.i.) the CTGF expression. Using 0.01 MOI, a comparable CTGF induction was obtained but at longer time points (24 hours) p.i. (data not shown).

The downregulation of CVB3 by means of the specific siRNA 36 significantly ($p<0.05$) reduced CTGF expression (Figure 19), in CVB3 infected HeLa cells compared to controls, proving the CVB3-CTGF interactions. To understand whether CTGF might be necessary for CVB3 life cycle, CTGF was downregulated by a specific siRNA in CVB3 infected HeLa cells and the virus levels were evaluated. Our data show that the downregulation of CTGF does not significantly reduce CVB3 load (Figure 20 and data not shown). This fact suggests that CTGF is not necessary for CVB3 life cycle and probably represents an unspecific response to the CVB3 infection. Thus, CTGF cannot be considered a host gene essential for CVB3 life cycle,

as it was shown for example for the CAR/DAF membrane receptor, required for virus internalization. The lack of a "helper" function for CTGF to CVB3 life cycle, however, does not exclude the possibility to think to an innovative therapeutic approach based on the simultaneous down-regulation of CVB3 replication and CTGF expression, especially in the light of the lack of treatments to prevent viral myocarditis and its sequelae, i.e. cardiac fibrosis. Obviously, CTGF expression should not be knocked down completely but the expression just reduced to the physiological level. The complete knock down of CTGF, a protein involved in many different biological function, might be detrimental for the heart tissue.

In addition, it should be noted that in HeLa cells transfected by the control siRNA GL2 and subsequently infected by CVB3, higher CTGF mRNA levels compared to HeLa cells non-transfected and infected were observed (Figure 19). It was hypothesized that the transfection procedure *per se* might be able to promote CTGF transcription. This idea was confirmed by observing a constant up-regulation of CTGF in HeLa cells undergoing transfection without infection (data not shown). This fact implies that the downregulation of CTGF observed upon siRNA CTGF1 treatment may be underestimated due to the positive stimulus to the expression of CTGF induced by transfection. Despite this possibility, siRNA CTGF1 significantly reduced CTGF mRNA (Figure 19).

It is well known that TGF-β is a stimulator of CTGF transcription. Thus, we asked whether the downregulation of CTGF following CVB3 load reduction by siRNA36 could have been mediated by a downregulation of TGF-β. The data reported in Figure 21 indicate that TGF-β mRNA levels are substantially not affected by siRNA 36 transfection (CVB3 load reduction). Thus the CVB3-CTGF relationship does not seem to be modulated by TGF-β but either requires others unknown mediators or is mediated by direct effects of CVB3 components on CTGF promoter. Obviously, the explanation of this relationship deserves further investigation.

5. Conclusions

Myocarditis is a major cause of morbidity and mortality worldwide and contributes significantly to the societal burden of heart failure. Given the lack of effective therapeutic approaches, novel strategies need to be identified. In this thesis it has been explored a novel approach based on the use of siRNAs to clear CVB3 in infected cells and to reduce the expression levels of CTGF, a known pro-fibrotic cytokine recently shown to be correlated to the CVB3-induced fibrosis. The presented data show the possibility to select active siRNAs, directed against the CVB3 region encoding for the proteinase 2A, able to remarkably reduce in the model cell line HeLa, the amounts of CVB3 genome equivalents, protein VP1 as well as the number of infectious virions in treated cells compared to controls, conferring higher viability of treated cells. Moreover, the data presented indicate that CVB3 infection triggers CTGF expression in HeLa cells and that this process does not involve TGF-β, a well known transcription regulator of CTGF. Finally, it is shown that CTGF is not necessary for CVB3 life cycle.

Together, the data presented suggest that it may be possible to think to a combined therapeutic approach to CVB3 myocarditis based on the simultaneous reduction of CVB3 and CTGF expression, thus clearing the virus and preventing cardiac fibrosis, a major sequelae of CVB3 myocarditis. Although confined to a cellular model, these investigations potentially open the way to an siRNA-based approach to treat acute and chronic viral myocarditis.

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Im Selbstverlag herausgegeben von:

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34135 Trieste, Italy