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SIDE- EFFECTS OF siRNA- INDUCED GENE SILENCING

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Hradec Králové 2007

Acknowledgments:

I would like to thank my supervisor – specialist Dr. Šárka Holasová PhD and Assoc. Prof. PharmDr. František Štaud PhD, for advice, suggestions and revision of the thesis. Thanks Generi Biotech s.r.o. for providing space and means for the experiment.

Also thanks to Daniel, who pushed me to go on when I did not feel like doing so, to the Feřtek family and to Barbir family (especially my brother and sister) for their support.

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List of abbreviations

2-5A	2',5' oligoadenylates
A	adenine
AAV	adeno-associated virus
AGO	argonaute gene
ATP	adenosine triphosphate
AV	adenovirus
bp	base pairs
C	cytosine
CMV	cytomegalovirus
CP	crossing point deviation
C_t	threshold cycle
D	Dalton
dsDNA	double-stranded DNA
DRAF	dsRNA-activated factor
dsRNA	double-stranded RNA
E	efficiency
ECACC	European Collection of Cell Cultures
eIF	eukaryotic initiation factor
ego-1	enhancer of GLP-1
FDA	food and Drug Administration
FMCA	fluorescence melting curve analysis
FRET	Förster resonance energy transfer
G	guanine
GADPH	glyceraldehyde-3-phosphate dehydrogenase
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HRMA	high-resolution melting analysis
HSV	herpes simplex virus
IFIT	interferon-induced transmembrane protein
IFN	interferon
IL	interleukin
IPCR	real-time immuno PCR

IRF	interleukin regulatory factor
kb	kilobase
LATE PCR	linear-after the-exponential PCR
LTR	long, terminal repeats
LUX	light upon extension
MDS	myelodisplastic syndrome
MMLV	Moloney murine leukemia virus
mRNA	messenger RNA
MW	molecular weight
nt	nucleotides
NUP	nucleoporin
OAS	2',5' oligoadenylate synthase
PAZ	Piwi, Argonaute, Zwille/Pinhead
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEI	polyethyleneimine
PKR	protein kinase
PNA	peptide nucleic acid
poly (I:C)	polyinosinic acid:polycitidylic acid
PTGS	post-transcriptional gene silencing
qde	quelling deficient gene
QRT PCR	quantitative real-time polymerase chain reaction
R	relative expression ratio
rde	RNA-deficient gene
RdRP	RNA-dependent RNA polymerase
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNL	Ribonuclease L
rRNA	ribosomal RNA
SGS	suppressor of gene silencing
siRNA	small interfering RNA
T	thymine
TGS	transcriptional gene silencing

TLR	Toll-like receptors
Tm	melting temperature
VSV	vesicular stomatitis virus
X-SCID	X-linked severe combined immunodeficiency

1. INTRODUCTION

Gene therapy is a new possibility of treating inherited diseases, such as cancer or various metabolic disorders. It involves inserting the therapeutic gene into the organism by using a viral vector, or silencing the impaired gene by using triplex-forming oligonucleotides, antisense technology, or RNA interference (RNAi).

The principle of the triplex-forming oligonucleotides and antisense technology is either inhibition of transcription by formation of the DNA-oligonucleotide triplex, or inhibition of translation by binding a specific antisense oligonucleotide to the target mRNA [3].

RNAi is rather newly discovered and not yet perfectly understood natural phenomenon. It involves restriction of the double-stranded RNA into 20-25 nt long small interfering RNAs (siRNAs) by enzyme Dicer. These siRNAs react with an enzymatic complex called RNA-induced silencing complex (RISC). Finally, the RISC uses the antisense chain of the siRNA to find and restrict the target mRNA [11].

Unfortunately, none of the above mentioned methods proved to be reliable enough to be used safely in wide population. The reason for this is various side-effects, the most serious being the immune response of the body to the viral vectors and, especially in case of RNAi, possible up-regulation of other genes. Therefore, gene therapy remains mostly on the level of *in vitro* testing and phase I clinical trials.

The aim of this work is to test the side-effects of siRNA on cells after successful silencing of cyclooxygenase I expression. Cell line used for testing is Hep2, which are immortalized human larynx cancer cells. Genes which were tested for the possible up-regulation are interferon-induced genes. Up-regulation of these genes would lead to biochemical pathways which would result in the activation of interferons and immune response.

The technique used for testing the interferon-induced genes was quantitative real-time PCR (QRT PCR). This technique allows monitoring of the product amplification after each PCR cycle, and thus, calculating the initial amount of the product in each sample.

2. THEORETICAL PART

2.1 Gene therapy

Genes are specific nucleotide sequences that code proteins, essential units of cellular structure and functions. When genes are altered so that the encoded protein cannot carry out its function, genetic disorder can result [1].

Gene therapy is a technique for correcting impaired genes responsible for the disorder development [1]. It involves introduction of the genetic material (DNA of RNA) into cells to fight or prevent the disorder [2].

Inserting of the genes can be done either *in vitro* or *in vivo* and it requires use of vectors which are highly specific for the target cells and which would not affect the healthy cells. Since it is very difficult to find such vectors, the contemporary gene therapy is focused on *in vitro* gene insertion [3].

2.1.1 Gene therapy strategies

So far, four major strategies on gene therapy have been developed [3]:

1. Gene augmentation strategy is used for diseases characterized by the loss of functional gene, so there is a deficiency in the production of the protein coded by this gene. The treatment of such disorders involves the introduction of the normal gene into the cells, so that the production of the resulting protein increases to normal levels. This type of treatment is suitable for recessive disorders, only.

2. Direct and indirect targeted killing of the specific cells are methods used widely in the treatment of tumors. The principle of such treatment is the introduction of genes, the expression of which would lead to the cell's death. Direct killing involves the expression of genes which code some lethal toxin. Indirect killing involves the inserting of the immunostimulatory genes followed by the death of the target cells as a consequence of the activity of the immunity system.

3. Targeted repair of mutations is suitable for dominant hereditary diseases, but difficult to achieve in practice.

4. Targeted inhibition of gene expression is gene silencing that can be achieved on the level of DNA, RNA or protein.

2.1.2 Vectors used in gene therapy

In order for the gene to be delivered into the target cell, a vector (carrier molecule) must be used [1]. Scientists have used three major approaches to deliver genes: viral vectors, non-viral vectors and physical gene transfer [3].

Viral vectors

Viruses used for gene delivery include adenoviruses, adeno-associated viruses, retroviruses, lentiviruses, herpes simplex viruses and vaccinia viruses [3]. All viruses have evolved the way of encapsulating and delivering their genes to human cells in pathogenic manner. Scientists have tried to take advantage of such behavior and manipulate the viral genome to remove the disease-causing genes and insert therapeutic genes. Target cells are infected with such virus and new protein product from therapeutic genes restores the cells' normal function [1].

Adenoviruses (AV). They are widely used in gene therapy because they exhibit high transfection efficiencies both *in vivo* and *ex vivo*. They replicate in dividing as well as in non-dividing cells in high titers so that sufficient quantities of the vector can be prepared for the clinical trials. Adenoviruses are linear, double-stranded DNA viruses with a genome of 36kb. The viral DNA is encapsulated within the protein coat. First-generation adenoviruses were designed for the gene transfer by deletion of the essential E1 gene. This deletion resulted in replication-defective virus because the E1 gene is required for the induction of promoters for other early genes. Such defective virus was then grown in cell lines which provide the E1 protein.

Adenovirus does not integrate into host chromosomal DNA and remains an episome in the cell. The first-generation adenovirus can often trigger a cytotoxic T-lymphocyte response directed at the transferred cells expressing adenoviral protein. These two

characteristics of adenovirus vector permit only transient expression of the therapeutic genes. For this reasons, these vectors are administered repeatedly to prolong the duration of the gene expression.

For clinical trials, high titer preparation of adenovirus must be free of helper virus proteins and replication-competent adenovirus. Recombination could potentially occur between the viral sequence and the chromosome of a producer cell line to create a replication-competent virus. These concerns may be partially alleviated by the use of the third-generation "gutless" adenoviruses that contain no viral gene. These vectors trigger low immune response and can receive up to 38kb of foreign DNA. Gutless adenovirus vectors are replication deficient and require a helper virus for the propagation. One advantage of the gutless vectors is that using helper viruses derived from another adenovirus can alter vector serotype and therefore, the production of host antibodies against adenovirus coat proteins may not be triggered during the repeated administration of a therapeutic gene with a gutless vector [4].

Adeno-associated virus vectors (AAV). They are small parvoviruses with 4,7kb single-stranded DNA genome surrounded by a protein coat. In order to cause an infection, they need a helper virus such as adenovirus or herpesvirus. Adeno-associated virus can infect a large variety of cells, both dividing and non-dividing ones. They integrate into host genome and the wild-type virus also integrates on chromosome 19. The AAV proteins are not toxic to cell and do not trigger a strong host immune response. For gene therapy, the AAV vectors are stripped of most wild-type genes and they retain only two 145bp inverted terminal repeats that are required for viral packaging and integration. Due to its small genome size and packaging constraints, cDNA greater than 4.5 kb cannot be inserted into AAV. Another concern is a virus production; it depends on multiple helper functions from other viruses for both replication and infection. Thus, the helper virus proteins must be completely removed from the stock so that a host immune response is not triggered during the clinical administration. One way of overcoming this problem is cloning of the required helper genes into plasmids. The cotransfection with the AAV helper plasmid allowed helper-free AAV production and increased the AAV titers [4].

Retrovirus vectors. They are derived from Moloney murine leukemia virus (MMLV) and they are the most widely used viral vectors in gene therapy. MMLV has

a genome of 8500 nucleotides surrounded by a lipid bilayer. Retroviruses infect only dividing cells because the resulting provirus cannot cross an intact nuclear membrane. However, retroviruses do integrate into chromosome of the dividing cells and may provide a sustained gene expression. The ability of the virus to infect only dividing cells may be beneficial in treatment of tumors. Retroviruses require three structural proteins (Gag, Pol and Env) for viral replication, receptor binding, reverse transcription and viral encapsulation. These viral proteins are provided in *trans* by a stable cell line so that the defective retrovirus needs only *cis* acting elements for the assembly. Retrovirus vector contains the two viral long terminal repeats (LTR) flanking the therapeutic gene. The LTR includes promoter/enhancer functions and sequences required for the integration. Vector also must contain the psi packaging signal. These replication-defective viruses utilize the endogenous retrovirus promoter or promoters from cytomegalovirus to drive expression of therapeutic genes.

Retroviruses generally accept 8kb of foreign DNA. They do not trigger the immune response; however, the expression of therapeutic genes has led to cytotoxic T-cell responses that can selectively destroy the transformed cells. Retroviruses have the potential of producing the replication-competent virus. For clinical studies, packaging cell lines that are free of provirus contamination are required for the vector production [4].

Lentivirus vectors. They are a subclass of retrovirus vectors that express a preintegration complex that controls the infected cell's nuclear import functions. Lentiviruses can replicate in both dividing and non-dividing cells. They infect a variety of cells including hematopoietic stem cells. Thus, they show promise in treating genetic disorders in blood cells.

Because the earliest Lentivirus vectors were derived from HIV-1, a replication-deficient vector is crucial for the successful gene therapy. First generation of vectors used an envelope protein of the vesicular stomatitis virus (VSV) that was cloned into separate plasmid. This protein core inhibited the wild-type viral replication and widened the range of target tissue. Third-generation lentiviruses construct use up to four transcriptional units for preparation of the recombinant virus. These constructs use a conditional viral packaging system, which further decreases the chance of replication-competent vector.

Several retrovirus gene products are toxic when constitutively expressed in the cells. A high-titer packaging cell line developed for Lentivirus vector was engineered by stably transfecting several viral genes and the VSV envelope gene under the control of the tetracycline-inducible promoter. Other safety feature of the lentivirus vectors includes the removal of the HIV accessory genes [4].

Herpes simplex virus (HSV). HSV types 1 and 2 are linear, double-stranded DNA viruses with genomes of approximately 150kb and an outer lipid membrane. Such large genome allows insertion of up to 30 kb long sequences of therapeutic DNA. HSV is made replication-defective by removing several non-essential immediate-early genes that can be supplied in *trans*. As several immediate-early genes are toxic to the cells, their removal is essential for the successful gene therapy [4].

Vaccinia virus vectors. Vaccinia is a double-stranded DNA virus with a genome of approximately 200kb and a lipid outer membrane. Vaccinia virus remains in the cytoplasm of the infected cell and it uses polymerases for the replication and transcription. It infects nearly all mammalian cell types.

Vaccinia vectors can accept sequences up to 25kb long. However, it induces strong cytotoxic T-cell response in tissue. Thus, vaccinia vectors are not suitable for treating chronic diseases, but show promise in treatment of solid tumors [4].

Non-viral vectors

Cationic lipids and liposomes. When plasmid DNA is mixed with small, unilamelar liposomes containing cationic lipids, a lipid-plasmid complexes form most probably due to electrostatic forces between negatively charged phosphate group of the DNA and positively charged lipid residue. Cationic lipids enter the cell by endocytosis.

One major advantage of the cationic lipid vectors is that they appear to protect DNA from degradation by cellular endonucleases. Cationic lipids seem to be promising as a delivering mechanism for *in vivo* delivery of genes to cells, and appear to work well when administered regionally to specific organs. Furthermore, cationic lipids do not contain any proteins so there is no risk of immune response. Also, synthetic lipid

technology eliminates risks of biological contamination. Finally, cationic lipids can be modified through covalent addition of a target ligand.

A current drawback of cationic lipids is the low transfection efficiency, probably due to inactivation in the blood. Most DNA transfected into cells by cationic lipids remains in endosomes, leading to lower efficiencies than those of viral vector systems. For gene therapy, manufacture of cationic lipid-DNA complexes can be difficult due to aggregation of lipids and DNA during production and storage. Systemic administration of protein-free cationic lipids requires a targeting mechanism for efficient gene delivery.

Recently, sterically stable liposomes have been prepared that do not aggregate, but circulate in the bloodstream for extended times. Adding polyethylene glycol derivatives to phospholipids at the liposome surface produces "stealth" liposomes. These findings should further the development of liposomes and their derivatives for the DNA delivery to target cells [4].

Other non-viral vectors often used for gene insertion include cationic polyethyleneimines (PEI) which are polymers characterized by high cationic charge density. This feature appears to be valuable for binding anionic DNA in physiological pH range and forcing the DNA to form condensates small enough to be effectively endocytosed into the cell. The PEI/DNA complexes travel to nucleus via endosomal compartment. Another property of PEI that makes it a suitable vector is its structure, in which every third atom is protonable amino nitrogen that allows the polymer to function as a buffering system for the sudden decrease in pH from the extracellular environment into endosomal/lysosomal compartment. This feature is important for the protection of the genetic material as it travels to the nucleus [27].

2.1.3 Problems associated with gene therapy

Gene therapy is still experimental and has not proven very successful in clinical trials (Fig. 1).

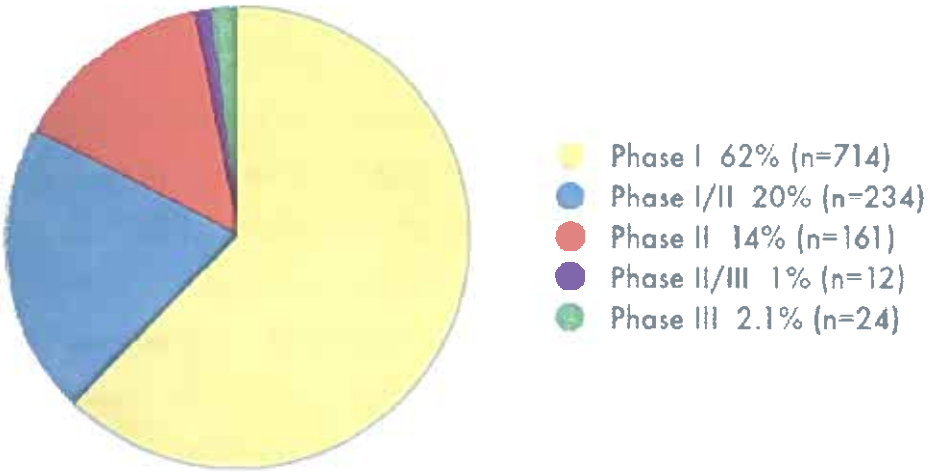

Fig.1: Phases of gene therapy clinical trials (the Journal of Gene Medicine, © 2006 John Wiley and Sons Ltd)

There are several important factors which have kept gene therapy from becoming successful [1]:

1. Short-lived nature of gene therapy. Before gene therapy can become a cure for any condition, the therapeutic DNA introduced into the target cells must remain functional and the cells containing such DNA must be stable and long-lived. Problems with the integration of the therapeutic gene in the genome and rapidly dividing nature of cells have prevented the gene therapy from achieving long-term benefits. Patients would have to undergo multiple rounds of gene therapy.
2. Immune response. The risk of stimulating the immune response in a way to reduce the gene therapy effectiveness is always the potential risk. Furthermore, the immune system has strong reactions to foreign matter that has already invaded the organism in the past. For this reason, repeated rounds of gene therapy in patients can be very difficult.
3. Viral vectors. Viruses, as the carriers of choice for many therapeutic genes, present a number of problems- toxicity, immune response, gene control and targeting issues. There is also danger that the virus, once inside the organism, can regain its pathological properties.
4. Multigene disorders. Disorders caused by the defect of only one gene are ideal candidates for the gene therapy. Unfortunately, most common diseases (e.g.

Alzheimer's disease or diabetes) are multifactorial, thus, very difficult to treat with gene therapy.

For the gene therapy to be successful, it must fulfill certain conditions. Some of them are: availability of the target cells (easy access, manipulation, assurance that the new gene will be transferred to the daughter cells), appropriate system for gene transfer (e.g. retroviral systems), assurance that the therapy is safe (recombination of retrovirus with endogenous virus together with uncontrolled spreading of foreign DNA must be prevented) [3].

S6 far, the only commercially available gene therapy product is Vitravene® (fomivirsen sodium) from Isis Pharmaceutical. Fomivirsen sodium is a phosphorothioate oligonucleotide used to treat ocular CMV infections in AIDS patients. Its mechanism of action is inhibition of CMV replication through an antisense mechanism. The product was authorized by FDA in 1998 and by EMEA in 1999; in 2002 marketing authorization holder voluntarily withdrew the product from the European market (except Switzerland) for commercial reasons [28, 29].

2.1.4 Recent developments of gene therapy

The Food and Drug Administration (FDA) in 2003 placed a temporary halt on all gene therapy trials using retroviral vectors after it had learned that a child in French clinical trial developed leukemia after gene therapy for X-SCID (severe combined immunodeficiency) [1]. Despite these problems, scientists are coming up with new ways of gene delivery into the living organism.

Genes inside liposomes can pass the blood-brain barrier. Liposomes are coated with polyethylene glycol (PEG) so that the antibodies that latch to some of the brain-capillary receptors get tethered to a few of the PEG strands. The antibodies trick the receptors into letting the liposomes pass into the brain. So far the method has proven successful in rats and rhesus monkeys. Disadvantage is that the genes are delivered into other organs, too. But, by choosing the right switch to turn on the gene, the gene will be active only in desired tissue. Such method can be used to treat Parkinson's, epilepsy, and many other brain disorders. Liposomes can be used to deliver cargo other than genes, e.g. antisense oligonucleotides or drugs [6].

Another approach is to repair the messenger RNA instead of trying to replace the damaged gene. This method shows promising results for the treatment of

thalassemia, hemophilia A, or cystic fibrosis. The principle is the usage of the antisense oligonucleotides which block the splice sites of the extra coding sequences that cause the production of the malfunctioning protein [7]. Antisense oligonucleotides are synthetic sequences which are complementary to the target mRNA and by binding to such mRNA, they are capable of blocking the process of association of mRNA with ribosomes and the translation process. In this way, the production of the defective protein can be prevented [3].

Using gene therapy to silence genes instead adding new ones could help treat a wide range of inherited diseases. One of these gene silencing methods is called the RNA interference. Small interfering RNA (siRNA) triggers the degradation of any other RNA in the cell with a matching sequence [8].

2.2 RNA interference

2.2.1 Discovery and development

At the beginning of the 1990s, a surprising observation was made in petunias. While trying to deepen the purple color of these flowers, Rich Jorgensen and colleagues introduced a pigment –producing gene under the control of a powerful promoter. Instead of the deep purple color, many of the flowers appeared white. Jorgensen named the observed phenomenon "cosuppression", since the expression of both the introduced gene and the homologous endogenous gene was suppressed.

The question raised was what caused this gene silencing effect. Although transgene-induced silencing in some plants appears to involve gene-specific methylation (transcriptional gene silencing or TGS), in others, silencing occurs in post-transcriptional manner (post-transcriptional gene silencing or PTGS). Nuclear run-on experiments in the latter case show that the homologous transcript is made, but it rapidly degraded in the cytoplasm [9].

Silencing can also be triggered by introduction of certain viruses. Once triggered, PTGS is mediated by a *trans*-acting molecule. Now it is known from the further research that this *trans*-acting molecule is the double-stranded RNA (dsRNA) [9].

The first evidence that the dsRNA could lead to gene silencing came from the work with the nematode *Caenorhabditis elegans*. Series of experiments shown that the

injection of only small amounts of the dsRNA into the worm caused gene silencing not only throughout the worm, but also in its first generation offspring [9].

RNAi was also observed in *Drosophila*. Microinjecting the *Drosophila* embryos with the dsRNA resulted in gene silencing. Since then, many research groups have been trying to answer the question of how these microinjections of dsRNA lead to gene silencing [9]. A key finding by Baulcombe and Hamilton provided the first clue. They identified RNAs of about 25nucleotides in plants undergoing cosuppression that were absent in non-silent plants. These RNAs were complementary to both sense and antisense strands of the gene being silenced [9, 37].

Further work in *Drosophila* shown that the dsRNA added to *Drosophila* embryo lysates was processed to 21-23 nucleotide species. It was also found that the homologous, endogenous mRNA was cleaved only in the region corresponding to the introduced dsRNA and that cleavage occurred at 21-23 nucleotide intervals. Rapidly, the mechanism of RNAi was becoming clear [9].

2.2.2 Mechanism of RNA interference

Since its discovery, much progress has been made towards identification and characterization of the genes implicated in the RNAi events in *C. elegans, Arabidopsis, Drosophila* and mammals. RNAi has emerged as a more complex mechanism than expected since it involves several different proteins and small RNAs. So far, it is known that the process of RNA silencing involves the initiator step in which long, dsRNA is cleaved into short fragments, and an effector step in which these fragments are incorporated into protein complex, unwound and used as a guide sequence to recognize homologous mRNA that is subsequently cleaved [9]. Mechanism of RNA interference is shown on Fig.2.

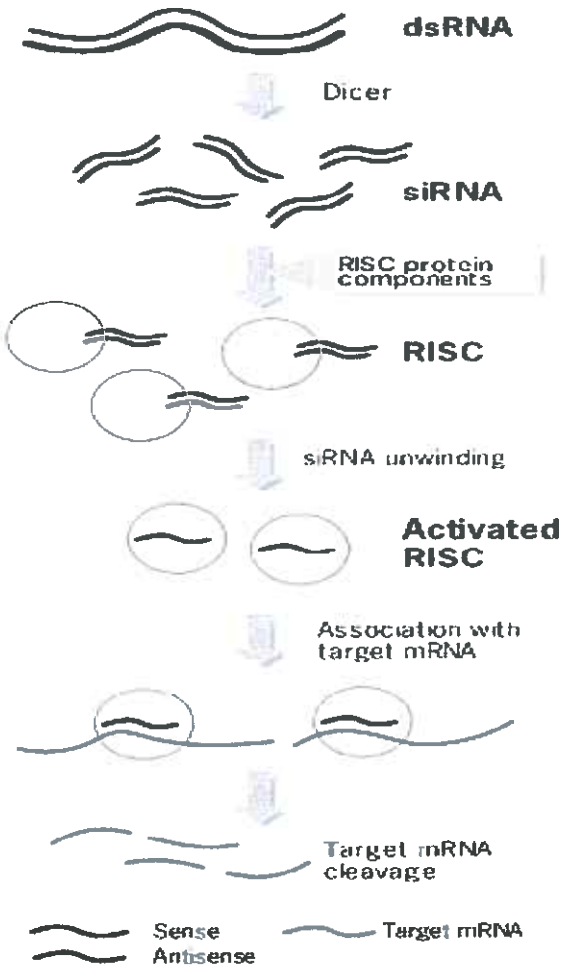

Fig.2: Mechanism of RNA interference (Ambion, the RNA Company)

In the initiation step, the input dsRNA is digested into 21-23 nucleotide siRNAs which have also been called "guide RNAs". Evidence indicates that siRNAs are produced when the enzyme Dicer, a member of RNase III family of dsRNA-specific endonucleases, processively cleaves dsRNA (introduced via e.g. virus) in an ATP-dependent manner [9]. Dicer contains an N-terminal RNA helicases domain, a Piwi, Argonaute, Zwille/Pinhead (PAZ) domain, two RNase III domains, and a C-terminal dsRNA-binding motif [39]. Successive cleavage events degrade the RNA into 19-21 bp duplexes (siRNAs), each with 2-nucleotide 3' overhangs [9]. These siRNAs contain 5' phosphates and 3' hydroxyl groups [39].

In the effector step, the siRNA duplexes bind to a nuclease complex to form the RNA-induced silencing complex (RISC) [9]. An ATP-dependent unwinding of the siRNA duplex is required for the activation of the RISC [9,40]. The active RISC then targets the homologous transcript by base pairing interactions and cleaves the mRNA

at about 10-12 nucleotides from the 3' terminus of the siRNA [9,38]. This suggests that the point-of-target RNA cleavage can be determined by the end of the dsRNA and implies that processing to 21-23mers starts from the ends of the duplex [38]. Each RISC contains a single siRNA and an RNase which appears to be distinct from Dicer [9].

Because of the remarkable potency of RNAi in some organisms, an amplification step within the RNAi pathway has been proposed. Amplification could occur by copying of the input dsRNA, which would generate more siRNAs, or by replication of siRNAs themselves. Alternatively or in addition, the amplification could be achieved by multiple turnover events of the RISC [9].

2.2.3 Possible role of RNA-dependent RNA polymerases in RNAi

Genetic screenings in *Neurospora, Arabidopsis* and *C.elegans* have identified several genes that appear to be crucial for the post transcriptional gene silencing and RNA interference. Several of these, including *Neurospora qde-1*, *Arabidopsis SDE-1/SGS-2* and *C. elegans ego-1,* appear to encode RNA-dependent RNA polymerases (RdRPs). This may appear to be the proof that RdRP is necessary for RNA interference. The existence of the RdRP may explain the potency of dsRNA-induced gene silencing if it amplified either dsRNAs prior to cleavage or siRNAs directly. But mutants of these genes have varying phenotypes which makes the role of RdRP in RNA interference difficult to discern [9].

In *C. elegans ego-1* mutants, RNAi functions normally in somatic cells, but it is defective in germline cells where *ego-1* is primarily expressed. In *Arabidopsis SDE-1/SGS-2* mutants, siRNAs are produced when dsRNA are introduced via endogenously replicating virus but not when introduced by a transgene. It has been proposed that the viral RdRP is substituting for the *Arabidopsis* enzyme in these mutants [9].

Although no homologue of RdRP has been found in flies or humans, RdRP activity has been recently reported in *Drosophila* embryo lysates. One model of amplification, termed the "random degradative PCR" model, suggests that RdRP uses guide strand of siRNA as a primer for the target mRNA, generating dsRNA substrates for Dicer, and thus, more siRNAs [9].

2.2.4 RNAi initiators and effectors

Two *C. elegans* genes, *rde-1* and *rde-4* are believed to be involved in the initiation step of RNAi. *Rde-1* gene is a member of a large family of genes and is homologous to the *Neurospora qde-2* and *Arabidopsis AGO-1* gene which is involved in *Arabidopsis* development. Although the function of these genes in PTGS is not clear, a mammalian member of the *Rde-1* family has been identified as a translation initiation factor [9]. In *D. Melanogaster*, AGO1 and AGO2 proteins appear to be important for forming the RISC complex [39].

Important genes for the effector step include the *C. elegans rde-2* and *mut-7* genes. Worms with mutated *rde-2* and *mut-7* genes exhibit defective RNAi, but have increased levels of transposon activity. Thus, silencing of transposons appears to occur by mechanisms similar to those of RNAi. Although the rde-2 gene product has not yet been identified, the *mut-7* gene encodes a protein with homology to the nuclease domain of RNase D and a protein implicated in Werner syndrome (a rapid ageing disease) in humans [9].

2.2.5 RNAi in mammalian cells

The natural function of RNAi appears to be the protection of the genome against invasion by mobile genetic elements such as transposons and viruses, which produce aberrant RNA or dsRNA in the host cell when they become active. Specific mRNA degradation prevents transposon and virus replication, although some viruses are able to overcome this process by expressing proteins that suppress PTGS [38]. Transfection of long, dsRNAs more than 30nt long into most mammalian cells may cause non-specific gene silencing, as opposed to the specific gene suppression in other organisms [9]. This suppression has been attributed to the antiviral response, which occurs through one of the following pathways [9].

In one pathway, the dsRNA activate protein kinase (PKR). Activated PKR, in turn, phosphorylates and deactivates the translation initiation factor eIF2a, leading to repression of translation. In the other pathway, dsRNAs activate RNaseL which leads to non-specific RNA degradation [9].

dsRNAs less than 30nt do not activate the PKR pathway. This observation, as well as knowledge that dsRNAs are cleaved to form siRNAs in worms and flies and that siRNAs can induce RNAi in *Drosophila* lysates prompted researchers to test whether the introduction of siRNAs can induce gene-specific silencing in mammalian cells. It was found that siRNAs induce sequence-specific gene silencing in mammalian cells with various efficiencies [9].

Exogenous synthetic siRNAs induce gene suppression that lasts only about ten cell cycles. In order to induce a long-term gene silencing, it is necessary to ensure the intracellular transcription of siRNA. For this reason, siRNA-coding templates were inserted into the transcriptional units which normally code a small nuclear RNA U6. In these transcriptional units, siRNAs can be directed in two ways: as sense (positive) or antisense (negative) chains which form siRNA duplex. These chains arise by transcription of two independent promoters. The second possibility is the transcription of both chains from a single promoter, the result being the hairpin RNA which is then processed into siRNA duplex [11].

2.2.6 Small interfering RNA design and development

Much research has been done on what determines siRNA functionality. Several important characteristics of siRNA have been identified: low G/C content, a bias towards low internal stability on the 3' terminus, lack of inverted repeats and sense strand base preferences [12].

It is known that the low G/C content in the target RNA is connected with the extent of gene silencing. It was also discovered that the most efficient siRNA had the G/C content between 36 and 52%. Those siRNAs containing higher or lower number of G/C bases than the reference range were less functional [12].

Low internal stability of siRNA at the 5' antisense end is a prerequisite for effective silencing and probably important for duplex unwinding and entry of antisense into the RISC. The A/U content is relatively simple measure of internal stability. This parameter can be quantified by measuring the percentage of A/U base pairs on the terminal positions of the duplex. Duplexes which do not have any A/U base pairs on the 5' terminus on the antisense strand (positions 15-19 on the sense strand) were found to be nonfunctional. However, the presence of one or more A/U base pairs on these positions correlated to an increasing siRNA activity. The optimal number of A/U

base pairs is three or more. If the internal stability of the siRNA duplex is too high, the unwinding cannot proceed. If, on the other hand, is too low, the affinity of siRNA for the target RNA and the subsequent cleavage by RISC cannot proceed [12].

Presence of palindromes in siRNA can cause the formation of the fold-back structures similar to the hairpins. Such structures can reduce the amount of the duplex siRNAs, and thus dramatically decrease the gene-silencing potential. One way how to determine the percentage of these hairpin-like structures is to perform the measurement of the melting temperature (Tm). High Tm values refer to high ratio of the hairpin structures. Duplexes which have no palindromic sequences were found to be better silencers [12].

Further criterion for effective gene silencing and successful siRNA design is the presence of one, specific base in a certain position. It was found that A in positions 3 and 19, U at position 10 contribute to the siRNA functionality. Absence of G or C in position 19 and G in position 13 have the same effect. It is believed that such choice of bases in these positions positively affects the siRNA-protein assembly [12].

These parameters, taken individually, are not sufficient for effective gene silencing. However, their algorithmic combination substantially increases functionality of siRNA [12].

2.2.7 Off-target and non-specific effects of small interfering RNAs

In mammalian cells, the use of RNAi for gene silencing is limited due to non-specific side-effects induced by dsRNAs, which result in interferon (IFN) activation [13].

siRNAs have the potential to elicit immune response via Toll-like receptor 3 (TLR3) and trigger interferon response like dsRNA and its analogues such as Polyinosinic acid:polycitidylic acid (poly (I:C)) [14].

In cultured cells, there are reports of non-specific gene target effects which include off-target gene suppression and up-regulation of type I interferons α and β. Hypotheses for the IFN response include the recognition of the siRNA by Toll-like receptors 3 (TLR3) and 9 (TLR9) and/or induction of protein kinase PKR pathway [14].

Toll-like receptors play a critical role in detection of microbial infection in mammals by recognizing conserved microbial structures. The intracellular signal transduction

pathways of TLR3 and TLR9 promote the transcription of genes regulated by NFκB transcriptional activator that include cytokines such as interleukin-12 (IL-12). Both ssRNA and dsRNA are molecular structures associated with viral infection; while ssRNA has been shown to interact with TLR7 and TLR8, dsRNA is the ligand for TLR3 [14].

Besides the activation of the PKR pathway, there are numerous reports showing that the dsRNAs can activate several protein kinases such as p38, J2 and IKK. Induction of these signaling pathways can alter gene expression by regulating the activity of transcription factors such as NFκB, IRF3 and ATF1.

2.2.8 Interferon-induced genes

Ribonuclease L (RNase L, RNL). It is a component of the interferon-regulated 5'-triphosphorylated, 2'5'-oligoadenylates (2-5A) system that functions in the antiviral and antiproliferative roles of interferons [15]. Besides RNase L, the 2-5A system consists also of 2-5A synthetases and 2-5A phosphodiesterase. 2-5A synthetases are a family of four IFN-inducible enzymes which, upon the activation by dsRNA, convert ATP into 2-5A oligomers. 2-5A phosphodiesterase might be involved in catabolism of the 2-5A from its 2'3' end. RNase L is the effector enzyme of this system. Its activation by 2-5A leads to cleavage of mRNA and inhibition of protein synthesis [16].

RNase L gene is assigned to 1q25 gene map locus. Several congenital and neoplastic disorders map to that region of the long arm of chromosome 1 [17].

Protein kinase (PKR). Interferon-induced, dsRNA-activated protein kinase is a serine-threonine kinase of 68.000 D in human cells [17]. It is involved in regulation of transcription, mRNA splicing and control of translation as a result of cells' response to stress [18].

Cells respond to environmental stress by rapidly changing their capacity to translate specific mRNAs. Translational control occurs primarily at the initiation step. Stress induces reversible phosphorylation of α chain of the eukaryotic initiation factor 2 (eIF2α), blocking GDP/GTP exchange needed for recycling of eIF2 between rounds of translation. A prominent eIF2α kinase is PKR which is expressed in latent form in most cells [18].

Besides these functions, PKR also plays an important role in signal transduction, apoptosis, cell growth and differentiation [18].

PKR is an important part of the interferon-mediated antiviral response. The action of interferons (including IFNγ) involves the induction of the PKR. Expressed mainly in activated T-cells and natural killer cells, IFNγ is immunomodulator important for the protective immunity. Phosphorylation of eIF2α by PKR leads to inhibition of translation, blocking virus spread and inducing apoptosis of the infected cells [18].

Activation of PKR requires *trans*-autophosphorylation and depends on the dsRNA produced during viral replication. The activation also requires highly ordered dsRNA structures rather than the specific sequences [18].

Human IFNγ mRNA uses local activation of the PKR in the cell to control its own translation yield. The activation occurs through a pseudoknot in the 5' untranslated region. Mutations that impaired the pseudoknot stability reduced the ability to activate PKR and strongly increased the translation efficiency of the IFNγ mRNA [17].

Interferon regulatory factor 1 (IRF1). IRF1 is a nuclear factor which binds to the upstream *cis* elements of both IFNα and IFNβ genes and functions as a transcriptional activator for type I IFN genes. IRF1 effect is antagonized by IRF2 which competes for the same element. To assess the possible role of IRF1 in cell growth and differentiation, scientists have generated transgenic mice which carry human IRF1 gene. It was found that these mice had a dramatic reduction in B lymphocytes [17].

IRF1 is located in position 5q31.1 on the gene map. An interstitial deletion of the 5q or loss of entirety of chromosome 5 is among the most frequent cytogenetic abnormalities in human leukemia and preleukemic myelodysplastic syndromes (MDS). Among the genes in 5q31.1 region, only IRF1 was constantly deleted in cases of leukemia or myelodysplasia with aberrations in 5q31 [17].

Loss of heterozygosity at the IRF1 locus occurs frequently in human gastric cancer. A point mutation in human gastric cancer cell line was identified that changed methionine at codon 8 to leucine and produced an IRF1 protein with reduced transcriptional activity but unaltered DNA-binding activity. In addition, alternative splicing of IRF1 mRNA was observed. Such splicing produced a non-functional IRF1 protein in patients with myelodysplastic syndrome and acute myelogenous leukemia [17].

Interferon regulatory factor 3 (IRF3). IRF3 is a component of the dsRNA-activated factor 1 (DRAF1), a positive regulator of the IFN-stimulated gene transcription that functions as a direct response to viral infection. It resides in the cytoplasm of cells and translocates to the nucleus following the viral infection. The translocation is accompanied by increased serine and threonine phosphorylation [19].Using microarray technology to compare gene expression profiles of mouse B lymphocytes stimulated with CD40LG or lipopolysaccharide, scientists have identified IRF3 as a factor specifically induced by stimulation of the TLR3 or TLR4. The primary response genes induced by this regulation were co-regulated by the NFκB pathway, common for TLR, TNFRs and IRF3 pathway. Additional secondary response genes were activated by autocrine and paracrine secretion of IFNβ. Selective TLR3/TLR4/IRF3 pathway activation potently inhibited the viral replication.

Interferon-induced transmembrane protein (IFITM). The 1-8 gene family which includes IFITM1, IFITM2 and IFITM3 is highly inducible by both type I (IFNα and IFNβ) and type II (IFNγ) interferons.

2'5' oligoadenylate synthase (OAS). Three forms of OAS corresponding to 40/46, 69/71 and 100 kD have been described. These OAS share a homologous region of about 350 amino acids that could represent their functional domain [20].

OAS represent a family of IFN-induced proteins implicated in the mechanism of antiviral action of IFNs. When activated by dsRNA, these proteins polymerize ATP into 2-5As. 2-5As bind to and activate RNL which degrades viral and cellular RNA and impairment of the viral replication [17].

2.3 Methods of molecular biology

2.3.1 Quantitative real-time polymerase chain reaction

Quantitative real-time PCR (QRT PCR) is a method of simultaneous quantification and amplification of the DNA [21]. QRT PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle [22].

Advantages of the QRT PCR in comparison to the end-point PCR are that the post-amplification processing of the PCR product is eliminated; this helps to increase the throughput and reduce the chance of the carryover contamination. QRT PCR also offers much wider dynamic range of up to 10^{7}-fold (compared to 1000-fold in end-point PCR). The dynamic range is the measurement of how much target concentration can vary and still be quantified. A wide dynamic range means that the wide range of ratios of the target and the normaliser can be assayed with equal sensitivity and specificity. It follows that the broader the dynamic range, the more accurate the quantitation [22].

QRT PCR is based on the detection and quantitation of the fluorescent reporter [22]. At the start of the reaction, reagents are in excess; the template and the product are at concentration low enough that the product renaturation does not compete with the primer binding [23]. During the PCR reaction, the signal increases in direct proportion to the amount of the PCR product in the reaction. By recording the amount of the fluorescent emission in each cycle, it is possible to monitor the PCR reaction in its exponential phase where the first significant increase in the amount of the PCR product correlates to the initial amount of the target template. The higher the starting copy number of the nucleic acid target, the sooner the significant increase in fluorescence is observed [22]. The point at which the reaction rate ceases to be exponential and enters the linear phase of amplification is extremely variable, even among replicate samples, but it appears to be primarily due to product renaturation competing with the primer binding (since adding more reagents or enzyme has little effect). At some later cycle, the amplification rate drops to zero and enters the plateau phase and little product is made [23].

2.3.2 QRT PCR detection systems

There are three main fluorescence-monitoring systems for the DNA amplification: hydrolysis probes, hybridizing probes and DNA-binding agents. Hydrolysis and hybridizing probes depend on the Förster or fluorescence resonance energy transfer (FRET) to generate the fluorescent signal via the coupling of a fluorogenic dye molecule and a quencher molecule to the same or different oligonucleotide substrates [23].

TaqMan probes. TaqMan probes depend on the 5' nuclease activity of the DNA polymerase used for PCR to hydrolyze the oligonucleotide that is hybridized to the target amplicon. TaqMan probes are oligonucleotides that have a fluorescent reporter dye attached to the 5' end and a quencher moiety (e.g. TAMRA or fluorescein) coupled to the 3' end. These probes are designed to hybridize to the internal region of the PCR product. In the unhybridized state, the proximity of the reporter and the quencher molecule prevents the detection of the fluorescent signal from the probe. During the PCR, when polymerase replicates the template which the probe is bound to, the 5' nuclease activity of the enzyme cleaves the probe. This uncouples quenching and fluorescent dye and FRET no longer occurs. Thus, the fluorescence increases in each cycle proportionally to the amount of the probe cleaved [23].

Molecular beacons. Molecular beacons also use FRET and have a reporter on the 5' end and a quencher on the 3' end of an oligonucleotide substrate. Unlike TaqMan probes, molecular beacons are designed to stay intact during the amplification reaction and must rebind to target in each cycle for the signal measurement. Molecular beacons form a stem-loop structure when they are free in solution. Thus, the close proximity of the reporter (FAM, TET, ROX, TAMRA) and the quencher (typically DABCYL) prevents the probe from fluorescing. When the probe hybridizes to the target, the reporter and the quencher separate, FRET does not occur, and the fluorescence is emitted [23].

Scorpions. The scorpion probe maintains the stem-loop structure in solution. The reporter is attached to the 5' end and the quencher to the 3' end. The 3' portion of a stem also has a sequence that is complementary to the extension product of a primer. This sequence is linked to the 5' end of a specific primer via non-amplifiable monomer. After the extension of the scorpion primer, the specific probe sequence is able to bind its complement within the extended amplicon, thus, opening a stem-loop structure. This prevents the fluorescence from being quenched, and the signal is observed [23].

Sunrise primers. They are similar to scorpions in that they combine both the PCR primer and the detection mechanism in the same molecule. These probes consist of a dual-labeled hairpin loop on the 5' end and the 3' end acting as the PCR primer.

When unbound, the hairpin is intact, causing the reporter quenching via FRET. Upon integration within newly formed PCR product, the reporter and the quencher are held far enough apart to allow reporter emission [24].

LUX fluorogenic primers. Light upon extension (LUX) primers are self-quenched, single-fluorophore labeled primers almost identical to Sunrise primers. However, rather than using a quencher fluorophore, the secondary structure of the 3' end reduces initial fluorescence to minimal amount. Important step for LUX primers, as well as for scorpions and Sunrise primers, is running the PCR product on agarose gel. The reason for this step is that PCR priming and probe binding are not independent in these chemistries [24]. The alternative is to run a melting curve to examine any erroneous amplification since the fluorogenic dye remains attached to the PCR product.

SYBR green. SYBR green is a fluorogenic, minor groove-binding dye that exhibits little fluorescence when free in solution, but emits strong signal when bound to the dsDNA [22]. Disadvantage of the SYBR green is that it will bind to any dsDNA in the reaction, including primer-dimers and other non-specific reaction products. This may result in the overestimation of the target concentration. Since the dye cannot distinguish between specific and non-specific products, follow-up assays are necessary to validate results [23].

TaqMan probes, molecular beacons and scorpions allow measurement of several DNA species in the same sample (multiplex PCR) since fluorescent dyes with different emission spectra may be attached to different probes. Multiplex PCR allows internal controls to be co-amplified and permits allele discrimination in single-tube, homogenous assays [23].

2.3.3 Theory of the QRT PCR

QRT PCR is the technique of collecting data throughout the PCR process as it occurs, thus combining the amplification and detection in a single step. Reactions are characterized by the point in time (or cycle) where the target amplification is first detected. This value is usually referred to as the threshold cycle (C_t), the point at

which the fluorescence intensity is greater than the baseline fluorescence. Consequently, the greater the amount of the DNA in the starting material, the lower is the C_t. C_t is a representative of the starting copy number of the template material and it is used for the calculation of the experimental results [24].

QRT PCR can be divided into four major phases: the linear ground phase, early exponential phase, log-linear phase and plateau phase (Fig.3) [24].

During the linear ground phase (usually 10-15 cycles), the PCR is beginning and the fluorescence has not yet risen above the background. At the early exponential phase, the amount of fluorescence has reached a threshold where it is significantly higher than the background levels. The cycle at which this occurs is C_t. During the log-linear phase, PCR reaches its optimal amplification period during which the PCR product doubles with every cycle in ideal reaction conditions. Finally the plateau phase is reached when the reaction components become limited and the fluorescence intensity is no longer useful for data analysis [24].

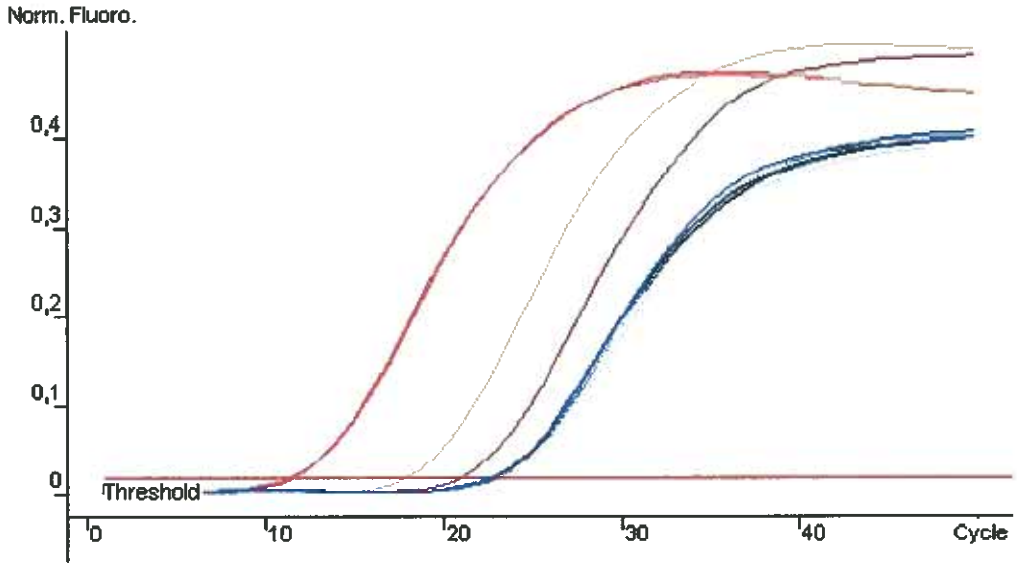

Fig.3: QRT PCR graph (Generi Biotech)

2.3.4 Quantitation of results

There are two types of the Quantitation of results obtained by QRT PCR: absolute and relative Quantitation [24].

Absolute Quantitation uses serially diluted standards of known concentration to generate a standard curve. This curve produces a linear relationship between the C_t

and the initial amounts of the total cDNA or RNA allowing the calculation of the concentration of unknowns based on their C_t values. This method assumes that all standards and samples have approximately equal amplification efficiencies [24].

Relative Quantitation measures changes in the sample gene expression in comparison with either an external standard or a reference sample also known as calibrator. When using a calibrator, the results are expressed as target/reference ratio. There are numerous mathematical models available to calculate the mean normalized gene expression from relative quantitation assays. Depending on the method employed, these can yield different results and thus different measures of the standard error [24].

When performing relative quantitation, reaction efficiency is very important consideration. Past methods for calculating gene expression have assumed that the efficiency is ideal (i.e. equals 1) which means that the PCR product concentration doubles with every cycle within the exponential phase. However, many PCR reactions do not have ideal amplification efficiencies and calculations without the appropriate correction factor may lead to overestimated concentration of the starting material [24].

Traditionally, amplification efficiency is calculated using data collected from the standard curve with the following formula:

$$\textbf{Exponential amplification} = 10^{(-1/\text{slope})}$$

$$\textbf{Efficiency} = [10^{(-1/\text{slope})}] - 1$$

The efficiency varies throughout the reaction from being rather stable in early exponential phase to declining gradually to zero. This decline occur due to depletion of the PCR components, decrease in polymerase activity and competition with the PCR product [24].

There are several other methods for the calculation of the amplification efficiency based on the raw data collected during the reaction. During the exponential phase, the absolute fluorescence increase at each PCR cycle for every sample reflects the true reaction kinetics of that sample. Consequently, data collected during exponential phase can be log-transformed and plotted with the slope of the regression line representing the sample's amplification efficiency. In another method, researcher

decides which cycles have exponential character or uses statistical analysis to define the period of exponential growth [24].

Standard curve method for relative quantitation. The quantity of each sample is first determined using the standard curve and then expressed relative to the calibrator. The calibrator is designated as 1-fold and the sample as n-fold difference relative to the calibrator. Standard curve method is often applied when the amplification efficiencies of the sample and the calibrator are unequal [24].

Comparative C_t ($2^{-\Delta\Delta Ct}$) method. This method calculates the differences in gene expression as a relative fold difference between the experimental and the calibrator sample [24]. It involves the comparison of the C_t values of the experimental and calibrator sample. These C_t values must be normalized to an appropriate endogenous housekeeping gene. For the calculation of the gene expression difference, the following formula is used:

$$\Delta\Delta C_t = \Delta C_{t,sample} - \Delta C_{t,reference}$$

Here, $\Delta C_{t,sample}$ is the C_t value for any sample normalized to the endogenous housekeeping gene and $\Delta C_{t,reference}$ is the C_t value of the calibrator also normalized to the housekeeping gene [23].

For this method to be valid, the amplification efficiencies of the target and the reference gene must be approximately equal [23]. Consequently, a validation assay must be preformed where serial dilutions are assayed for the target and reference gene and the results are plotted with the log input concentration for each dilution on the x-axis and the difference in C_t (target-reference) for each dilution on the y-axis. If the absolute value of the slope of the line is less than 0.1, the comparative C_t method may be used. Because the method does not require a standard curve, it is useful when assaying a large number of samples [24].

The Pfaffl method. The Pfaffl model combines gene quantification and normalization into a single calculation. This model incorporates the amplification efficiencies of the target and reference (normalization) genes for the correction of differences between two assays [24]. The relative expression ratio (R) of the target

gene is calculated on the basis of efficiency (E) and the crossing point (CP) deviation of an unknown sample versus control, and expressed in comparison to a reference gene. The formula is as follows:

$$\text{Ratio} = (E_{target})^{\Delta CP_{target}(\text{control-sample})} / (E_{ref})^{\Delta CP_{ref}(\text{control-sample})}$$

E_{target} is the real-time PCR efficiency of the target gene transcript; E_{ref} is the real-time PCR efficiency of the reference gene transcript; ΔCP_{target} is the CP deviation of control-sample of the target gene transcript; ΔCP_{ref} is the CP deviation of control-sample of the reference gene transcript. For the calculation of this ratio, the individual real-time PCR efficiencies and the CP deviation of the investigated transcripts must be known [25].

2.3.5 Housekeeping (internal standard) genes

QRT PCR-specific errors in the gene quantification easily arise by any variation in the amount of the starting material between the samples. This is especially important when these samples are obtained from different sources, and will result in misinterpretation of the expression profiles of the target genes. Consequently, the question of what constitutes an appropriate standard arises and makes an important part of experimental design [26].

The accepted method for minimizing these errors and correcting sample-to-sample variations, is to amplify, simultaneously with the target, a cellular RNA that serves as an internal reference against which other RNA values can be normalized. The ideal internal standard should be expressed at a constant level among different tissues of an organism, at all stages of development, and should be unaffected by experimental treatment. In addition, an endogenous control should be expressed at roughly the same levels as the sample RNA. In the absence of any single RNA with a constant level of expression in all these situations, three mRNAs are mostly used to normalize patterns of gene expression: the mRNAs specifying the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GADPH) and β-actin and ribosomal RNAs (rRNA). Other RNAs which can be used occasionally include cyclophilin, histone H3 and many others [26].

β-actin. β-actin mRNA is expressed at moderately abundant levels in all cell types and encodes a ubiquitous cytoskeleton protein. It was one of the first RNAs to be used as an internal standard in QRT PCR assays. This is in spite the fact that its levels of transcription can vary widely in response to experimental manipulation in various tissues. In addition, the presence of pseudogenes interferes with the interpretation of results and primers not designed optimally can also amplify DNA [26].

GADPH. The RNA encoding GADPH is ubiquitously expressed at moderate levels. It is used for real-time PCR analysis because, in some expression systems, its expression is constant in different conditions. However, there is numerous evidence that its use is inappropriate. GADPH levels vary significantly between individuals, during pregnancy, in developmental stage, during the cell cycle and after the addition of dexamethasone, carbon tetrachloride and tumor promoter 12-O-tetradecanoyl-phorbol-13acetate. Growth hormone, vitamin D, hypoxia, manganese, and the tumor suppressor TP53 have all been shown to activate GADPH transcription. Food deprivation and retinoic acid downregulate its transcription in the gut and in adipocytes, respectively. All these examples suggest that GADPH is a subject to complex transcriptional regulation and it is not appropriate internal standard [26].

rRNA. rRNAs, which constitute 85-90% of total RNA in the cell, are useful internal standards, as the various rRNA transcripts are generated by a distinct polymerase and their levels are less likely to vary under conditions that affect the expression of mRNAs. They have been shown to be reliable in number of different tissues. However, rRNA transcription can be influenced by biological factors and drugs and the variations in the level of transcription between samples taken from different individuals have not been quantified. In addition, there are two other drawbacks to its use: rRNA cannot be used for normalization when quantitating targets that have been enriched for mRNA because it is lost during purification and rRNA is expressed at much higher levels than the target mRNA [26].

2.3.6 Applications of the quantitative real-time PCR

QRT PCR can be applied to traditional PCR applications as well as new applications that would have been less effective with the traditional PCR. With the ability to collect data in the exponential log phase, the power of QRT PCR has been expanded to the following applications [22]:

Quantitation of gene expression

Array verification

Biosafety and genetic stability testing

Drug therapy efficacy / drug monitoring

Real-Time Immuno-PCR (IPCR)

Viral quantitation

Pathogen detection

DNA damage measurement

Radiation exposure assessment

In vivo imaging of cellular processes

Mitochondrial DNA studies

Methylation detection

Detection of inactivation at X-chromosome

Determination of identity at highly polymorphic HLA loci

Monitoring post transplant solid organ graft outcome

Monitoring chimerism after haematopoietic stem cell transplantation

Monitoring minimal residual disease after haematopoietic stem cell transplantation

Genotyping by fluorescence melting-curve analysis (FMCA) or high-resolution melting analysis (HRMA) or specific probes/beacons

Linear-after-the-exponential (LATE)-PCR: a new method for real-time quantitative analysis of target numbers in small samples, which is adaptable to high throughput applications in clinical diagnostics, biodefense, forensics, and DNA sequencing

3. EXPERIMENTAL PART

3.1 Aim of experiment

After successful down-regulation of COX1 gene expression in Hep2 cell lines [36], the next aim was to explore the influence of the same siRNA used for COX1 silencing on IFN-induced genes RNL, PKR, IRF1, IRF3, IFITM, IFIT1, OAS1, OAS2, OAS3. The main focus was:

- to calculate the gene copy number/µg RNA of IFN-induced genes (absolute quantification);

-to compare the expression of IFN-induced genes in cell lines with inserted siRNA for COX1 down-regulation with the expression in non-influenced Hep2 cell line;

-to find out if the IFN-induced genes are up-regulated, i.e. if the siRNA inserted causes immune response in influenced cell lines.

3.2 Materials and methods

3.2.1 Cell line

Hep2 cell line – cells of human larynx cancer, obtained from European Collection of Cell Cultures (ECACC), cultivated in DMEM 1x high glucose growing medium.

3.2.2 Machines and accessories

Machines:

Freezer –70°C, Forma Scientific, USA

Freezers –20°C, Ardo, Zanussi

Apparatus for the preparation of the deionized water milli Q, Millipore, USA

Fume cupboard with laminar flow Fatran, Czech Republic

Vortex TK3S, TechnoKartell; Velp Scientifica, Italy; VX-100, Labnet International, USA

Centrifuge IEC Micromax, IEC, USA

Autoclave Chirana, Czech Republic

Ice maker, Brema Ice Makers, Italy

Real-time PCR cycler ROTOR GENE, Corbett Research, Australia

UV transilluminator, Ultra Lum, USA

Accessories:

Pipettes Kartell, Italy; Eppendorf, Germany

Pippette tips, Sarstedt, Germany

3.2.3 Chemicals, enzymes, software

Chemicals:

10x buffer for PCR, ABGene, UK

Magnesium chloride, Sigma, USA

Deoxynucleotide triphosphates, TaKaRa, Japan

Primers and real-time PCR probes, Generi Biotech, Czech republic

Enzymes:

Polymerase ThermoStart, ABGene, UK

M-MuLV reverse transcriptase Finnzyme, Finland

Software:

REST version 2 - relative expression software

Rotor Gene version 6

3.3 Laboratory methods

3.3.1 Description of plasmid, cell lines and siRNA

Hep2 cells are cells of human larynx cancer obtained by European Collection of Cell Cultures (ECACC). siRNA transfected into the cells was cloned into psiRNA plasmid. psiRNA is a plasmid which is used to insert siRNA in such a way that after the transcription from RNA polymerase III promoter, short, hairpin RNAs arise. Such RNAs have higher stability than synthetic ones, and they allow long-term gene silencing [30]. The plasmid has a blasticidin resistance encoded as a selectable marker that allows the selection in Hep2 cells. The plasmid also contains a U6 promoter which is snRNA-type promoter. snRNA U6 genes are transcribed by polymerase III [31].

Plasmid psiRNA was transfected in Hep2 cells by several different methods and following lines were derived:

1. Hep2+psiRNA_U6_COX1_3 (plasmid was transfected by Effectene Transfection Reagent)

2. Hep2+psiRNA_U6_COX1_3n (plasmid was transfected by nucleofection)

3. Hep2+psiRNA_U6_COX1_3 clone V9 (more homogenous line obtained from Hep2+psiRNA_U6_COX1_3)

4. Hep2 is a control cell line not influenced by siRNA

psiRNA plasmid for Hep2+psiRNA_U6_COX1_3, Hep2+psiRNA_U6_COX1_3n and Hep2+psiRNA_U6_COX1_3 clone V9 contained siRNA with the following sequence:
GATCCAGAACAGTGGCTCGTTCAAGAGACGAGCCACTGTTCTGGATCTTTTTTGGa

Target mRNA for siRNA is shown on Fig. 4.

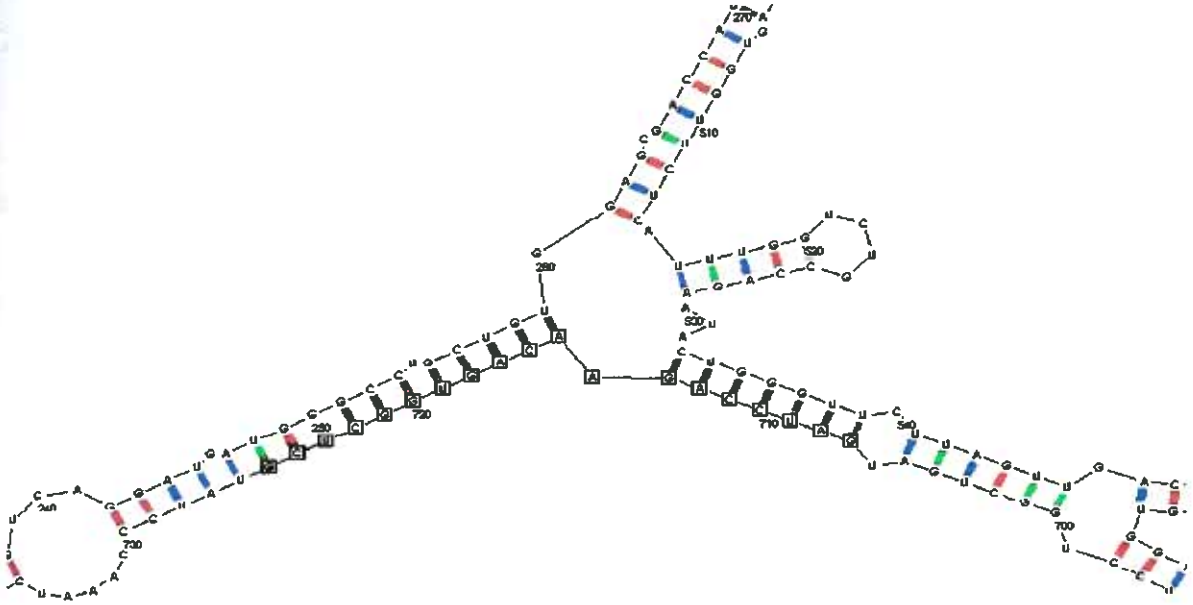

Fig. 4: predicted secondary structure of the target mRNA for siRNA by using Mfold program[35]

siRNA was expected to bind to the portion of the target RNA (squared bases on Fig. 4) with its loop portion (marked red in the sequence) and suppress the expression of COX1.

Before the transfection, the kill curve was done in order to obtain the minimal concentration of antibiotic blasticidin which kills the cells. The testing was performed in 24-well plate, and after seven days, it was found that the minimal concentration was 4µg blasticidin/ml medium.

Nucleofection (as a type of electroporation) of cells was performed in 6-well plate by using Nucleofector™ technology (Amaxa). $1x10^6$ cells/well and 2 µg psiRNA_U6 plasmid were used.

Cell line Hep2+psiRNA_U6_COX1_3 had plasmid transfected by using Effectene Transfection Reagent by Qiagen. 4µl of Effectene and 400ng plasmid was used. The transfection was done in 24-well plate with $6x10^4$ cells/well according to the instruction manual provided by Qiagen. Since Effectene is cytotoxic reagent, cells were washed in 1xPBS and new medium was added 8 hours post transfection. 36 hours post transfection, selective antibiotic blasticidin (4µg/ml) was added. In 14 days, the antibiotic was removed.

All cell lines are maintained in 75 cm^2 sterile tissue culture flasks with 13ml DMEM 1x high glucose growing medium. The flasks are kept in the incubator at 37°C with 5% CO_2.

Composition of DMEM 1x high glucose medium:

L-glutamine	584mg/l
Sodium pyruvate	110mg/l

Heat-inactivated FCS (10% in the medium) and gentamicin (50µg/ml medium) were added to DMEM 1x high glucose growing medium.

The DMEM growing medium was changed on regular basis according to the rate by which the cells metabolized it.

When the cell confluence reaches more than 75% of the flask bottom surface, trypsinisation would be performed to remove the excess of cells.

RNA from sample cell lines was isolated using Qiagen protocols for RNA isolation. cDNA was obtained by reverse transcription of the isolated RNA samples; the enzyme used in this method was M_MuLV reverse transcriptase (c=200U/µl). 1µg RNA in 15 µl reaction mixture was used.

3.3.2 Calculation of the copy number of the reference samples

Target genes: RNL, PKR, IRF1, IRF3, IFIT1, IFITM, OAS2, OAS3

Housekeeping gene: NUP gene which codes nucleoporin, a member of the nuclear pore complex. This complex is a large supramolecular assembly that spans the nuclear envelope and mediates molecular exchanges between the nucleus and cytoplasm [17].

Each target gene was cloned into plasmid, transformed into *E. Coli,* and isolated using Qiagen Plasmid Midi Kit. Concentration of the plasmid was measured and the exact copy number of the cloned genes was calculated with the use of the following formulas:

$(6{,}02 \times 10^{23}$ copies/mol) x (concentration in g/µl) / (MW in g/mol) = copies/µl

MW (g/mol) = (number of base pairs) x (660 Daltons/base pair)

The calculated copy number of each gene is shown in Table 1:

gene	plasmid (µl)	water (µl)	copy number (copies/ µl)
RNL	1,8	38,2	$5x10^9$
PKR	1,90	38,1	$5x10^9$
IRF1	1,80	38,2	$5x10^9$
IRF3	1,34	38,66	$5x10^9$
IFIT1	1,30	38,7	$5x10^8$
IFITM	1,28	38,72	$5x10^9$
OAS1	1,50	38,50	$5x10^9$
OAS3	1,18	38,82	$5x10^8$

Table 1: Sequence size and copy number of IFN-induced genes

Since the copy number of each gene was known, it was possible to make a dilution series as a reference for QRT PCR testing. In order to have a dilution series with an exponential decrease of the copy number, it was necessary to take such volume of each plasmid that would give a round copy number for real-time PCR reaction (e.g. $1,0x10^{10}$ copies/reaction volume). Starting volumes taken are shown in Table 2:

gene	size (bp)	copy no. (copies/ µl)
RNL	4092	$6,68x10^{11}$
PKR	4061	$1,05x10^{11}$
IRF1	4074	$2,2x10^{11}$
IRF3	4092	$7,36x10^{11}$
IFIT1	4094	$7,69x10^{10}$
IFITM	4122	$4,68x10^{11}$
OAS1	4129	$6,7x10^{11}$
OAS3	4176	$5,08x10^{10}$

Table 2: Starting volumes of plasmid and water for the dilution series

Dilution series of 1:10 were prepared from data in Table 2, until the final concentration of 5 copies/µl was achieved.

3.3.3 Real-time PCR

Real-time PCR was performed in the RotorGene cycler. The detection system used was fluorescein as a reporter dye, and BHQ1 as a quencher dye. They belong to the TaqMan class.

Before preparation of the reaction mixtures, all the equipment (test tubes, pipettes) were illuminated in the UV box for five minutes. This step was necessary in order to decrease the risk of contamination by foreign genetic material.

Reference for each gene tested was dilution series of pure plasmid with cloned gene. Target was cDNA from Hep2 cell lines transfected with siRNA vector and non-influenced Hep2 cell line. Doubles of each sample were prepared to minimize pipetting errors.

Sample input data for the QRT PCR measurements are described in Table 3-9. The data include the concentrations of the reaction components and volumes of the components for the PCR reaction and they are valid for all samples.

Table 3 lists the numbering of the test tubes and the content of each test tube. first ten samples are dilution series with known concentration; the rest are tested cell lines.

Test tube no.	Sample	Concentration (copies/reaction)
1-2	TESTED GENE IN PLASMID	10^7
3-4	TESTED GENE IN PLASMID	10^6
5-6	TESTED GENE IN PLASMID	10^5
7-8	TESTED GENE IN PLASMID	10^4
9-10	TESTED GENE IN PLASMID	10^3
11-12	Hep2	unknown
13-14	Hep2+psiRNA_U6_COX1_3	unknown
15-16	Hep2+psiRNA_U6_COX1_3n	unknown
17-18	Hep2+psiRNA_U6_COX1_3 clone V9	unknown

Table 3: Description and concentration of measured samples

Table 4 and Table 5 show the concentrations of primers and probes used in the PCR reaction mixture. Table 4 shows reference primer and probe concentration (i.e.

for NUP gene), and Table 5 target primer and probe concentration (i.e. for IFN-induced tested genes).

Name	Code	Reaction mixture conc. (nM)
REFERENCE sense	225K2	300
REFERENCE antisense	225K3	900
REFERENCE probe	225Z0	100

Table 4: Concentration of the reference primer and probe

Gene	TARGET sense		TARGET antisense		TARGET probe	
	Code	Reaction mixture conc. (nm)	Code	Reaction mixture conc. (nm)	Code	Reaction mixture conc. (nm)
RNL	225K8	900	225K9	300	238D0	150
PKR	225L0	900	225L1	300	238D1	150
IRF1	225L2	900	225L3	300	238D2	150
IRF3	225L4	900	225L5	300	238D3	100
IFITM	225L8	900	225L9	900	238D4	250
IFIT1	225L6	900	225L7	900	219D9	250
OAS3	225M4	900	225M5	300	238D7	50

Table 5: Concentrations of the target primers and probes

The stock solution concentration of each reference and target primer, as well as each reference and target probe was 0,1mM. These primers and probes were diluted with water in ratios described in Table 6 to the concentration necessary for the PCR reaction mixture.

reaction mixture conc (nm)	primer (µl)	water (µl)
50	1	99
100	2	98
150	3	97
250	5	95
300	6	94
900	18	82

Table 6: Dilution of the reference and target primers and probes

The actual composition of the reaction mixture for the QRT PCR is listed in Table 7 (the dilution series) and Table 8 (the target samples). The temperature and duration of each PCR phase, and the number of cycles are all listed in Table 9.

Component	Amount per test tube (µl)
deionized water	7,15
PCR buffer	2,00
$MgCl_2$ (25mM)	4,00
dNTP (2,5mM)	1,60
REFERENCE primer F (nM)	0,00
REFERENCE primer R (nM)	0,00
REFERENCE probe (nM)	0,00
TARGET primer F (nM)	1,00
TARGET primer R (nM)	1,00
TARGET probe (nM)	1,00
Taq polymerase (5,0 U/µl)	0,25
Template cDNA	2,00
Volume of the master mix per test tube	18,00
Total volume per test tube	20,00

Table 7: Composition of the reaction mixture for the dilution series (tube numbers 1-10)

Component	Amount per test tube (µl)
deionized water	4,15
PCR buffer	2,00
$MgCl_2$ (25mM)	4,00
dNTP (2,5mM)	1,60
REFERENCE primer F (nM)	1,00
REFERENCE primer R (nM)	1,00
REFERENCE probe (nM)	1,00
TARGET primer F (nM)	1,00
TARGET primer R (nM)	1,00
TARGET probe (nM)	1,00
Taq polymerase (5,0 U/µl)	0,25
Template cDNA	2,00
Volume of the master mix per test tube	18,00
Total volume per test tube	20,00

Table 8: Composition of the reaction mixture for the target samples (tube numbers 11-18)

Cycler: real-time ROTORGENE			
Operation	Temperature (°C)	Duration	Number of cycles
Pre-PCR	95	15 min	
Denaturation	95	30sec	
Annealing	60	60sec	
			50

Table 9: Thermocycler parameters

Composition of the PCR mixture, as well as the thermocycler parameters is valid for all tested samples.

4. RESULTS

Analysis of the real-time PCR results was performed using software RotorGene 6 and REST 2. Results of measurement are shown in Fig. 5A-11C.

4.1 Expression of RNL gene

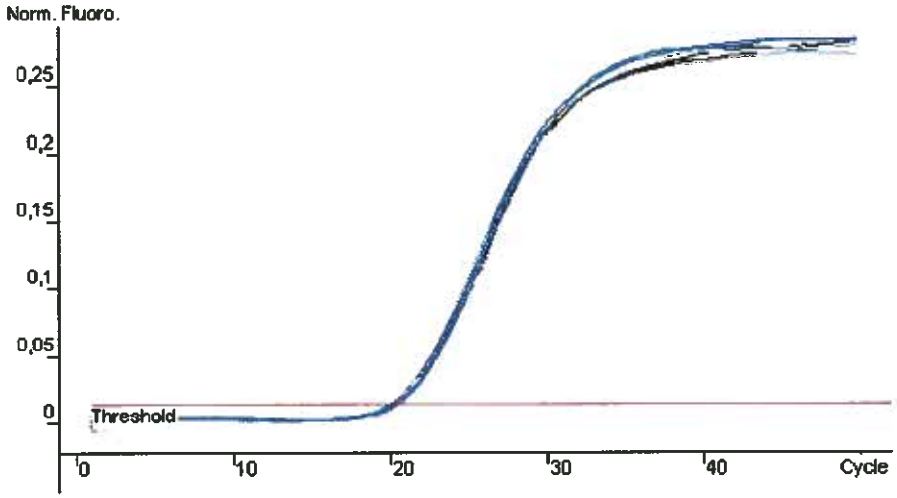

Fig.5A: housekeeping gene expression (channel JOE)

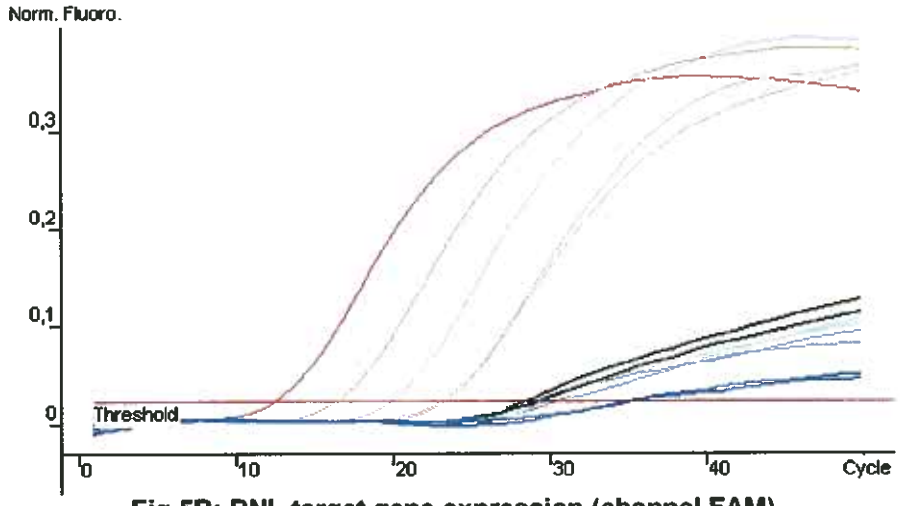

Fig.5B: RNL target gene expression (channel FAM)

R=0,99

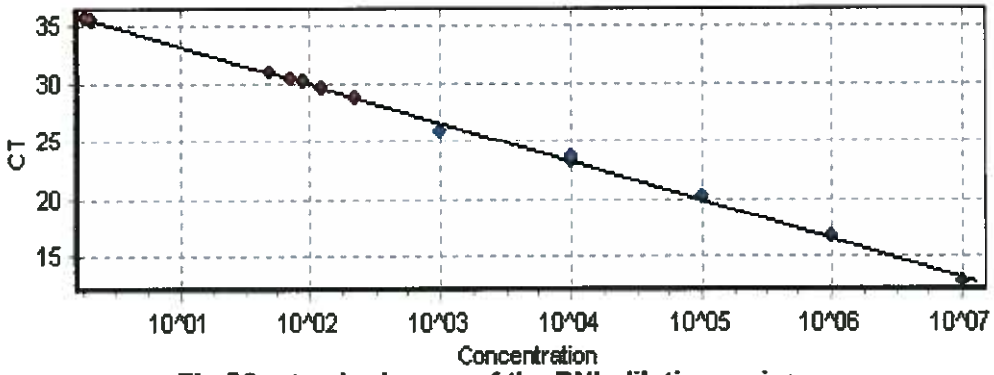

Fig.5C: standard curve of the RNL dilution series

Color	Name	Ct	Given Conc. (copies/reaction)	Calc Conc. (copies/reaction)
	10^7	12,78	10 000 000	12 961 838
	10^6	16,63	1 000 000	921 194
	10^5	20,15	100 000	81 660
	10^4	23,30	10 000	9 389
	10^4	23,68	10 000	7 239
	10^3	25,96	1 000	1 509
	Hep2	28,78		217
	Hep2	29,63		121
	Hep2+COX1_3eff	30,95		49
	Hep2+COX1_3eff	29,59		124
	Hep2+COX1_3n	30,08		89
	Hep2+COX1_3n	30,42		70
	Hep2+COX1_3cloneV9	35,70		2
	Hep2+COX1_3cloneV9	35,52		2

Legend to the Fig.5B

Color	Name	Ct
	Hep2	20,72
	Hep2	20,18
	Hep2+COX1_3eff	20,36
	Hep2+COX1_3eff	20,24
	Hep2+COX1_3n	20,81
	Hep2+COX1_3n	21,27
	Hep2+COX1_3cloneV9	20,44
	Hep2+COX1_3cloneV9	20,74

Legend to the Fig.5A

Fig. 5A shows the expression of a housekeeping gene NUP. It is visible from the graph, as well as from the Ct in the legend to the Fig.5A that the NUP expression was uniform. On Fig. 5B, the expression of RNL and dilution series is represented. Dilutions were in rather regular intervals, but the RNL gene expression was very low, even in non-influenced Hep2. From the Fig.5B and its legend, it is obvious that there is a significant difference in gene expression in Hep2. It is problematic to evaluate such results because it is impossible to determine which one is more accurate and true.

4.2 Expression of PKR gene

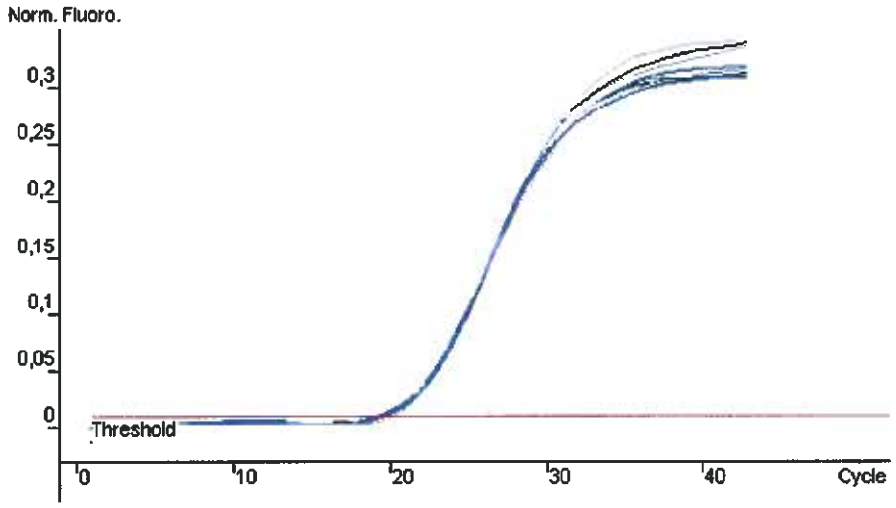

Fig.6A: expression of the housekeeping gene (channel JOE)

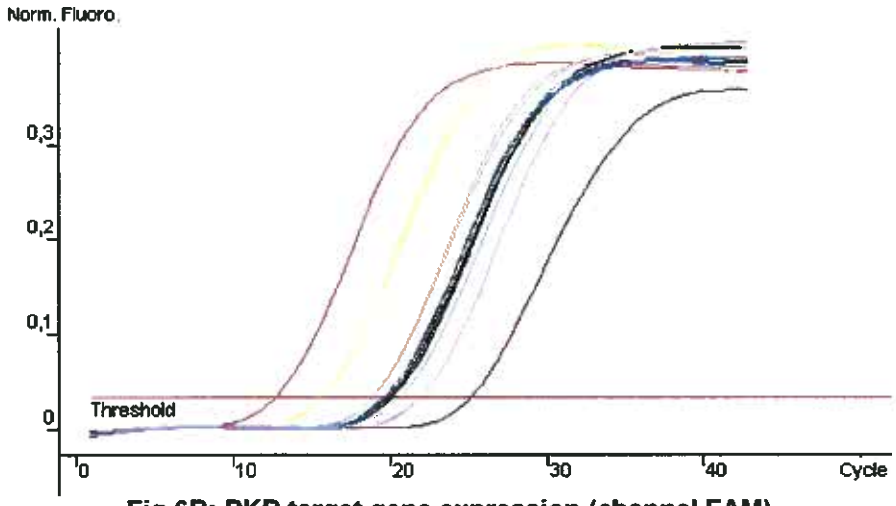

Fig.6B: PKR target gene expression (channel FAM)

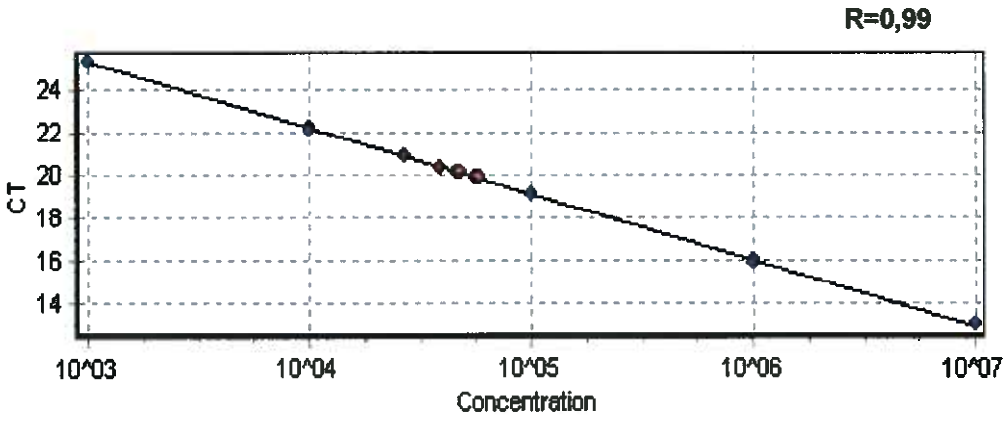

Fig.6C: standard curve of the PKR dilution series

Color	Name	Ct	Given Conc. (copies/reaction)	Calc Conc. (copies/reaction)
	10^7	12,93	10 000 000	9 575 335
	10^6	16,03	1 000 000	966 901
	10^6	15,83	1 000 000	1 121 326
	10^5	19,10	100 000	99 037
	10^5	19,16	100 000	95 074
	10^4	22,18	10 000	10 115
	10^4	22,14	10 000	10 408
	10^3	25,34	1 000	972
	Hep2	20,14		45 994
	Hep2	20,38		38 363
	Hep2+COX1_3eff	19,88		55 722
	Hep2+COX1_3eff	19,83		57 661
	Hep2+COX1_3n	20,86		26 829
	Hep2+COX1_3cloneV9	20,07		48 209
	Hep2+COX1_3cloneV9	19,83		57 551

Legend to the Fig 6B

Color	Name	Ct
	Hep2	19,93
	Hep2	20,03
	Hep2+COX1_eff	20,27
	Hep2+COX1_eff	19,69
	Hep2+COX1_3n	19,83
	Hep2+COX1_3n	20,53
	Hep2+COX1_3cloneV9	20,26
	Hep2+COX1_3cloneV9	19,94

Legend to the Fig.6A

NUP expression is uniform in all cell lines (Fig.6A). The dilution series show slightly greater variation between the given and calculated (measured) concentration. PKR gene in all cell lines except Hep2+COX1_3n was up-regulated (Fig.6B).

4.3 Expression of IRF1 gene

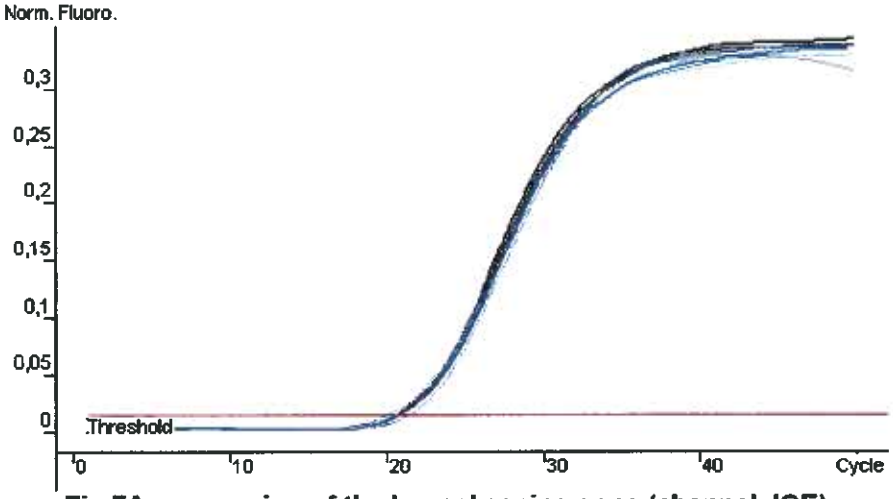

Fig.7A: expression of the housekeeping gene (channel JOE)

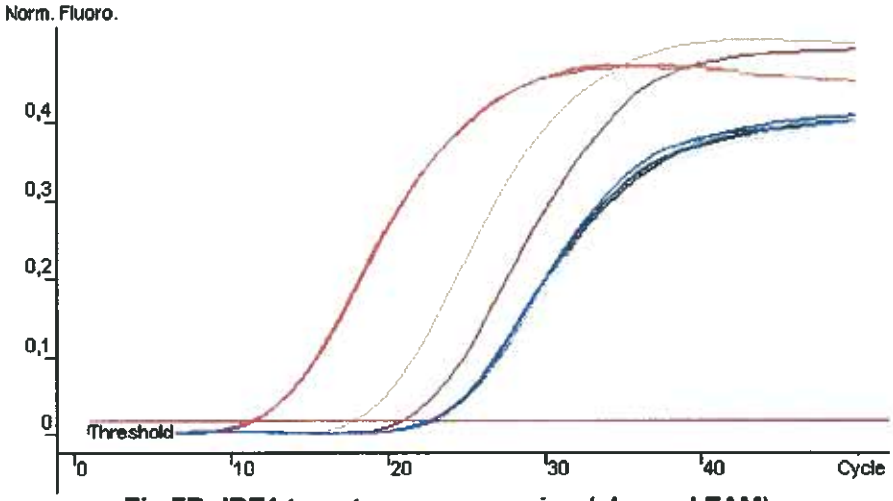

Fig.7B: IRF1 target gene expression (channel FAM)

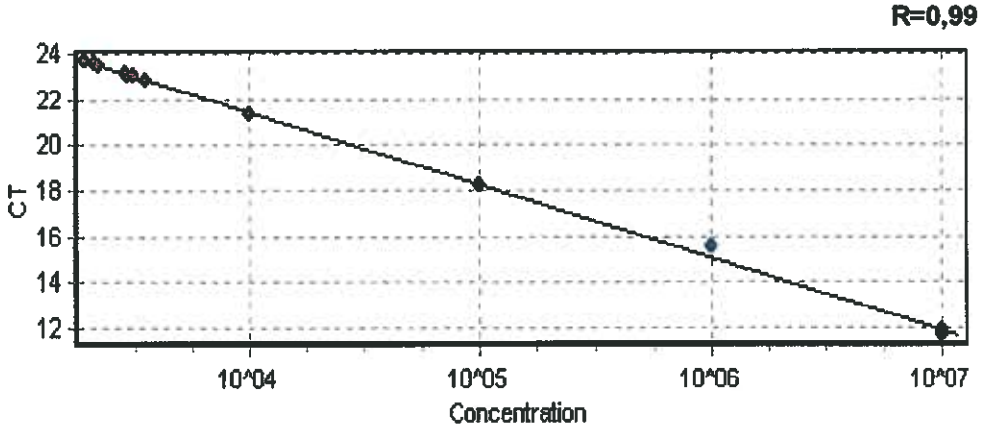

Fig.7C: standard curve of the IRF1 dilution series

Color	Name	Ct	Given Conc. (copies/reaction)	Calc Conc. (copies/reaction)
	10^7	11,88	10 000 000	10 325 239
	10^7	11,70	10 000 000	11 794 331
	10^6	15,50	1 000 000	739 357
	10^5	18,22	100 000	101 291
	10^4	21,27	10 000	10 965
	Hep2	23,00		3 119
	Hep2	23,07		2 953
	Hep2+COX1_3cloneV9	22,83		3 516
	Hep2+COX1_3cloneV9	23,47		2 210
	Hep2+COX1_3n	23,66		1 920
	Hep2+COX1_3n	23,52		2 124
	Hep2+COX1_3eff	23,07		2 961
	Hep2+COX1_3eff	23,10		2 890

Legend to the Fig.7B

Color	Name	Ct
	Hep2	21,04
	Hep2	20,87
	Hep2+COX1_3cloneV9	20,39
	Hep2+COX1_3cloneV9	21,63
	Hep2+COX1_3n	21,14
	Hep2+COX1_3n	21,32
	Hep2+COX1_3eff	21,13
	Hep2+COX1_3eff	21,14

Legend to the Fig.7A

NUP gene was uniformly expressed (Fig.7A). The dilution series were accurate and very close to the given concentration. In the tested cell lines, the IRF1 gene was also quite accurately measured in doublets (larger differences only in Hep2+COX1_3cloneV9). Taken as average, the IRF1 gene was down-regulated in all influenced cell lines (Fig.7B).

4.4 Expression of IRF3 gene

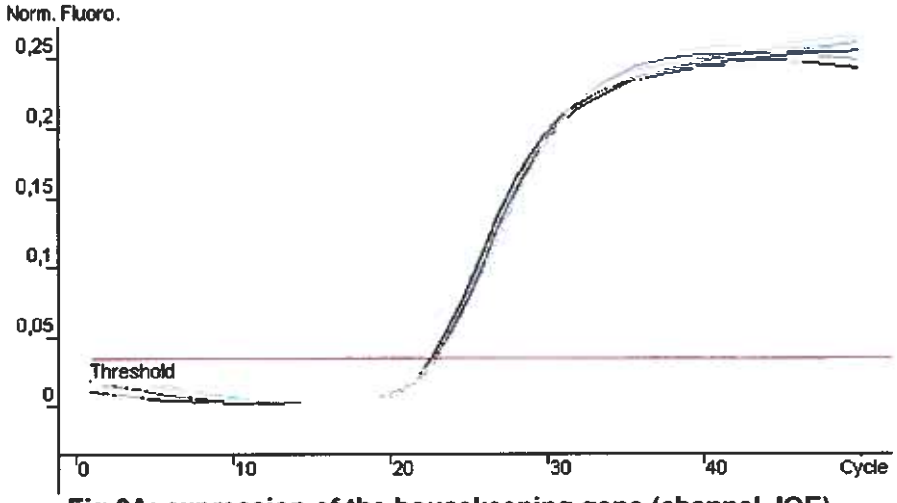

Fig.8A: expression of the housekeeping gene (channel JOE)

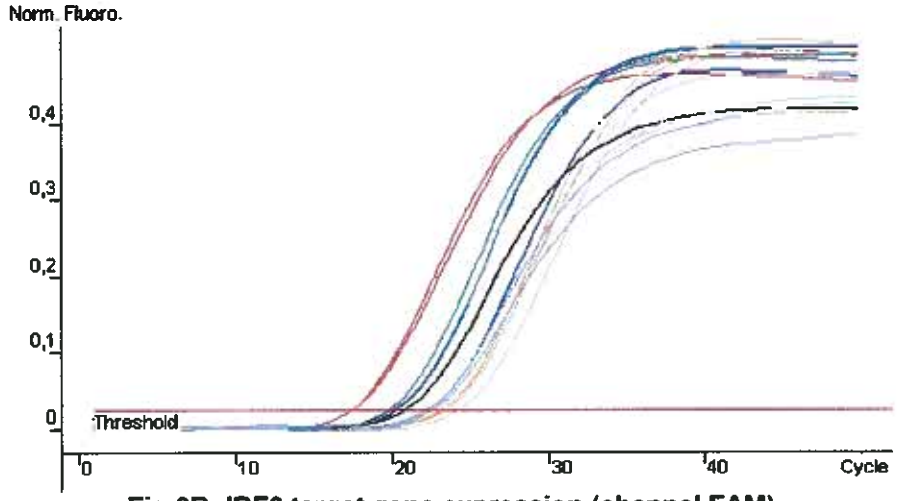

Fig.8B: IRF3 target gene expression (channel FAM)

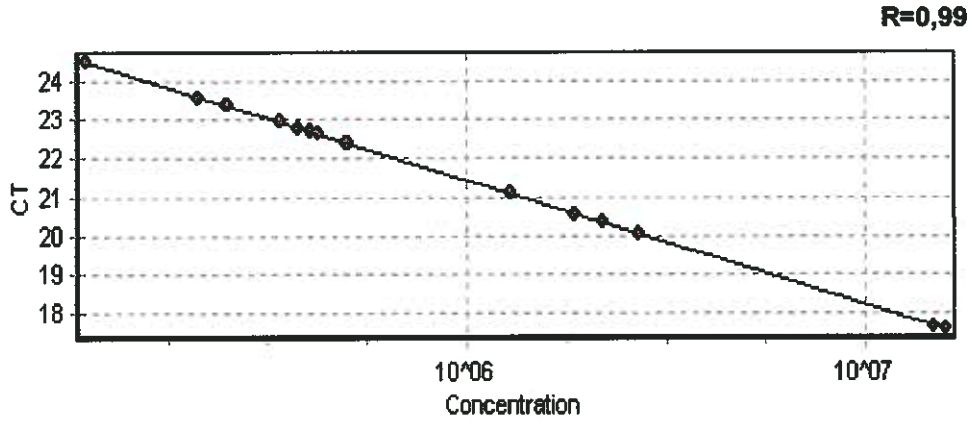

Fig.8C: standard curve of the IRF3 dilution series

Color	Name	Ct	Given Conc. (copies/reaction)	Calc Conc. (copies/reaction)
	10^7	17,59	10000000	15940225
	10^7	17,68	10000000	14953015
	10^6	20,57	1000000	1872665
	10^6	22,68	1000000	410062
	10^5	23,36	100000	251762
	10^5	24,49	100000	111739
	10^4	20,34	10000	2214408
	10^4	20,06	10000	2707206
	10^3	23,60	1000	212566
	10^3	23,35	1000	253254
	Hep2	21,08		1299568
	Hep2+COX1_3cloneV9	22,39		507551
	Hep2+COX1_3n	22,93		344180
	Hep2+COX1_3n	22,63		426464
	Hep2+COX1_3eff	22,41		499705
	Hep2+COX1_3eff	22,79		379330

Legend to the Fig.8B

Color	Name	Ct
	Hep2	22,85
	Hep2	23,11
	Hep2+COX1_3cloneV9	23,13
	Hep2+COX1_3clonev9	23,52
	Hep2+COX1_3n	23,36
	Hep2+COX1_3n	23,34
	Hep2+COX1_3eff	22,99
	Hep2+COX1_3eff	23,19

Legend to the Fig.8A

NUP is expressed uniformly (Fig.8A). The dilution series are not so accurate and the measured concentration does not fully respond to the given concentration. The gene in tested samples was down-regulated, but the differences in the expression between the doublets were significant (Fig.8B).

4.5 Expression of IFIT1 gene

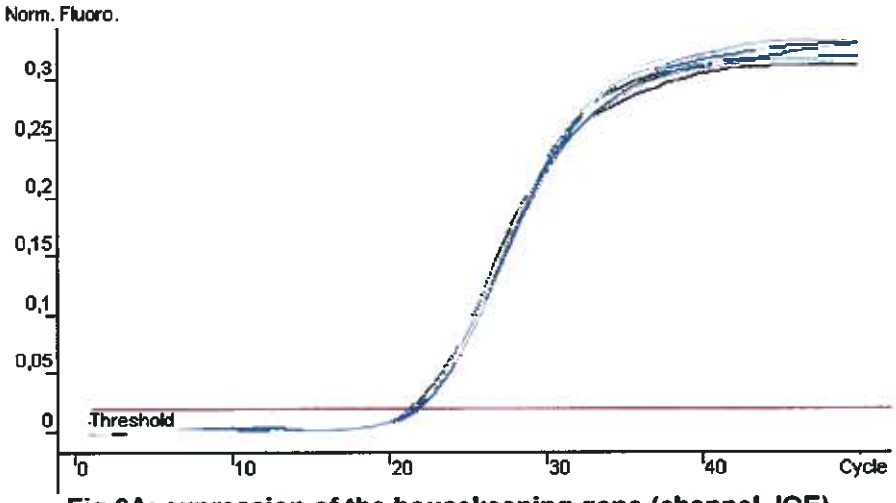

Fig.9A: expression of the housekeeping gene (channel JOE)

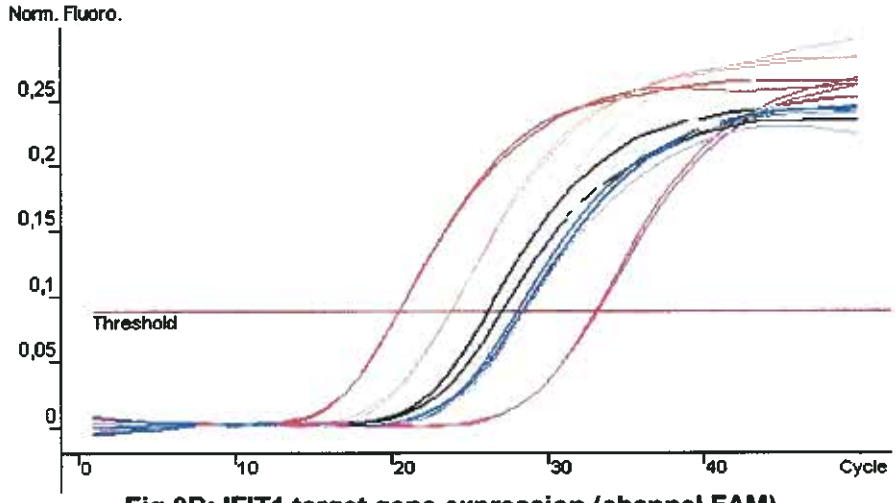

Fig.9B: IFIT1 target gene expression (channel FAM)

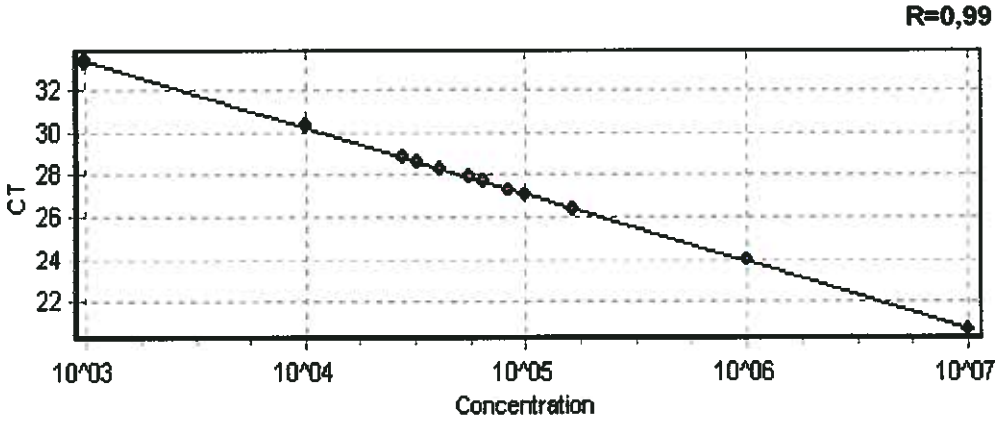

Fig.9C: standard curve of IFIT1 dilution series

Color	Name	Ct	Given Conc. (copies/reaction)	Calc Conc. (copies/reaction)
	10^7	20,70	10 000 000	10 350 407
	10^7	20,69	10 000 000	10 455 751
	10^6	23,93	1 000 000	975 169
	10^6	23,98	1 000 000	940 054
	10^5	27,01	100 000	102 505
	10^4	30,33	10 000	9 007
	10^3	33,33	1 000	1 002
	10^3	33,21	1 000	1 090
	Hep2	27,29		83 484
	Hep2	26,37		163 010
	Hep2+COX1_3cloneV9	28,26		41 024
	Hep2+COX1_3cloneV9	28,60		31 963
	Hep2+COX1_3n	27,86		54 982
	Hep2+COX1_3n	27,65		64 124
	Hep2+COX1_3eff	28,78		27 982
	Hep2+COX1_3eff	28,80		27 617

Legend to the Fig.9B

Color	Name	Ct
	Hep2	21,53
	Hep2	21,78
	Hep2+COX1_3cloneV9	22,14
	Hep2+COX1_3cloneV9	21,98
	Hep2+COX1_3n	21,80
	Hep2+COX1_3n	21,59
	Hep2+COX1_3eff	21,96
	Hep2+COX1_3eff	21,76

Legend to the Fig.9A

NUP gene was uniformly expressed (Fig.9A). Although the down-regulation was achieved, the differences in the IFIT1 expression between the doublets were large (Fig.9B).

4.6 Expression of IFITM gene

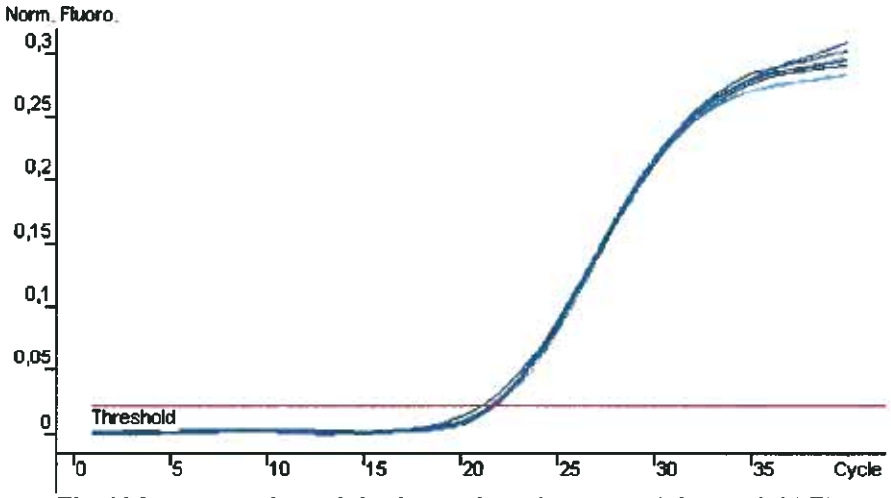

Fig.10A: expression of the housekeeping gene (channel JOE)

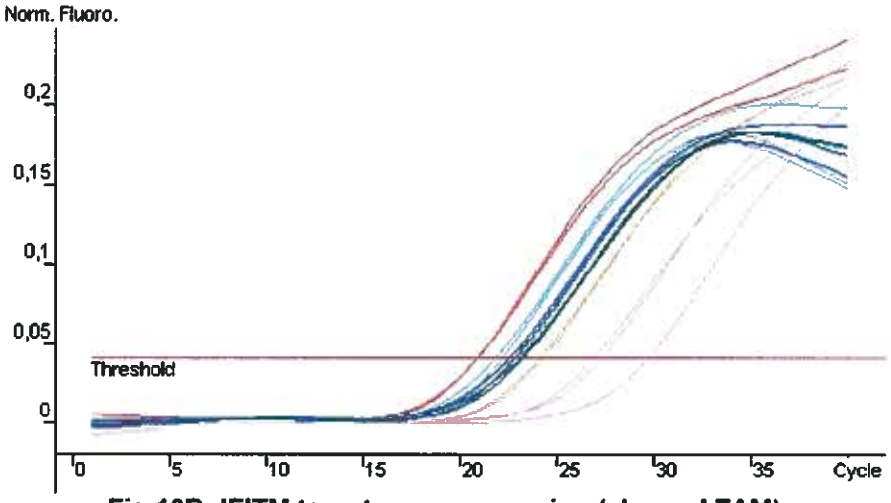

Fig.10B: IFITM target gene expression (channel FAM)

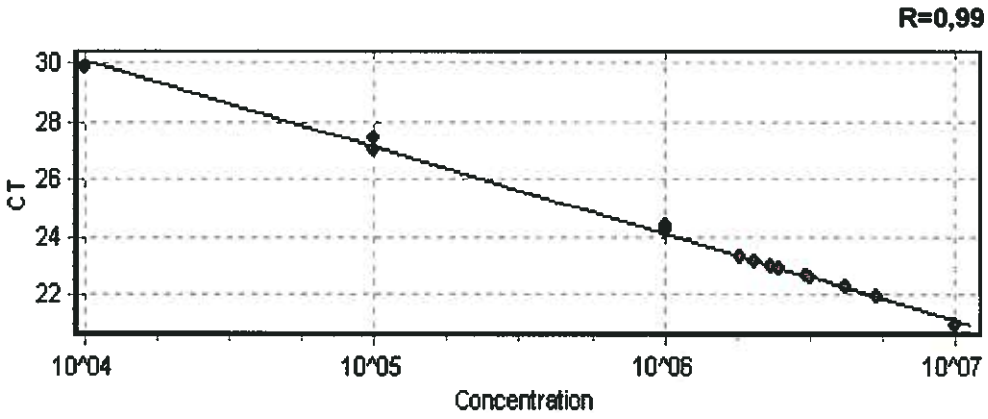

Fig.10C: standard curve of IFITM dilution series

Color	Name	Ct	Given Conc. (copies/reaction)	Calc Conc. (copies/reaction)
	10^7	20,99	10 000 000	11 254 934
	10^7	20,97	10 000 000	11 379 897
	10^6	24,41	1 000 000	801 026
	10^6	24,21	1 000 000	932 788
	10^5	27,02	100 000	106 192
	10^5	27,38	100 000	80 280
	10^4	29,81	10 000	12 257
	Hep2	22,27		4 174 045
	Hep2	21,94		5 385 806
	Hep2+COX1_3cloneV9	22,67		3 069 626
	Hep2+COX1_3cloneV9	23,04		2 302 531
	Hep2+COX1_3n	22,94		2 493 287
	Hep2+COX1_3n	22,63		3 151 190
	Hep2+COX1_3eff	23,21		2 022 366
	Hep2+COX1_3eff	23,35		1 810 947

Legend to the Fig.10B

Color	Name	Ct
	Hep2	21,86
	Hep2	21,75
	Hep2+COX1_3cloneV9	21,70
	Hep2+COX1_3cloneV9	22,02
	Hep2+COX1_3n	21,85
	Hep2+COX1_3n	21,67
	Hep2+COX1_3eff	21,20
	Hep2+COX1_3eff	21,66

Legend to the Fig.10A

NUP gene was uniformly expressed (Fig.10A). The dilution series were not very accurate. The down-regulation was achieved in the samples, but with large differences between the doublets (Fig.10B).

4.7 Expression of OAS3 gene

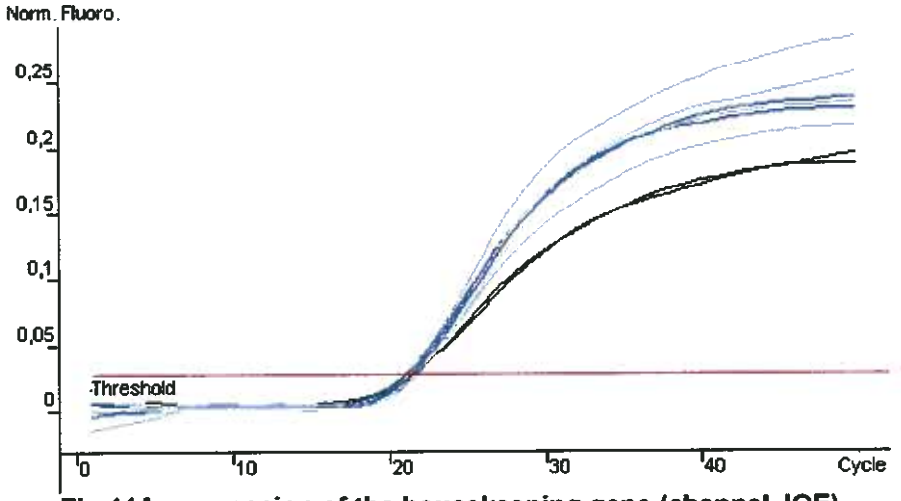

Fig.11A: expression of the housekeeping gene (channel JOE)

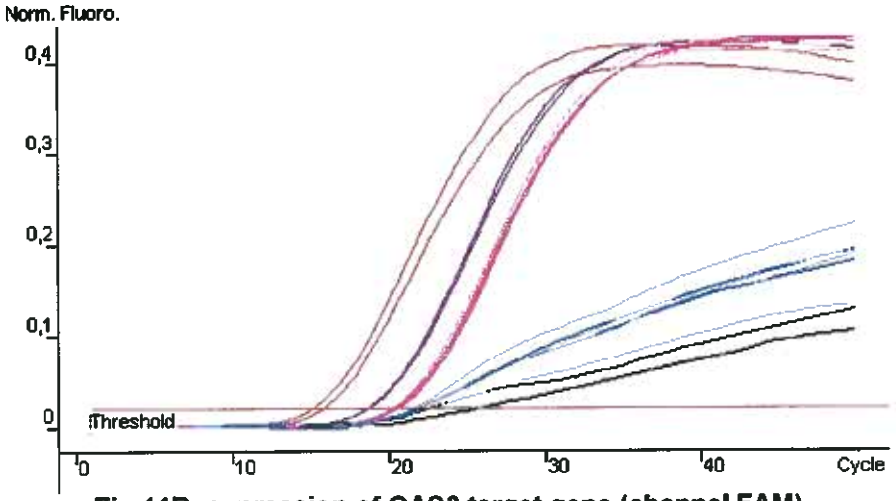

Fig.11B: expression of OAS3 target gene (channel FAM)

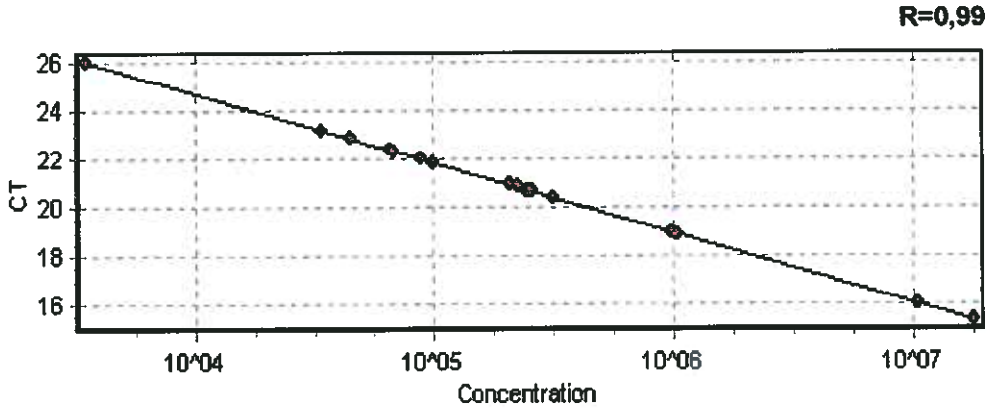

Fig.11C: standard curve of OAS3 dilution series

Color	Name	Ct	Given Conc. (copies/reaction)	Calc Conc. (copies/reaction)
	10^7	15,31	10 000 000	17 934 989
	10^7	15,98	10 000 000	10 505 051
	10^6	18,95	1 000 000	976 545
	10^6	18,88	1 000 000	1 031 870
	10^5	20,36	100 000	315 931
	10^5	20,62	100 000	256 640
	10^4	20,89	10 000	206 736
	10^4	20,67	10 000	246 250
	10^3	20,69	1 000	242 154
	10^3	20,79	1 000	223 477
	Hep2+COX1_3eff	22,79		45 043
	Hep2+COX1_3n	22,27		68 259
	Hep2+COX1_3n	22,31		65 928
	Hep2+COX1_3cloneV9	23,15		33 707
	Hep2+COX1_3cloneV9	22,33		64 987
	Hep2	21,80		99 671
	Hep2	21,95		87 890

Legend to the Fig.11B

Color	Name	Ct
	Hep2+COX1_3eff	21,76
	Hep2+COX1_3eff	21,82
	Hep2+COX1_3n	21,40
	Hep2+COX1_3n	21,71
	Hep2+COX1_3cloneV9	21,92
	Hep2+COX1_3cloneV9	22,09
	Hep2	21,24
	Hep2	21,23

Legend to the Fig.11A

NUP gene was less uniformly expressed in comparison to the other tested genes (fig.11A). There is a large variability in the dilution series concentration and almost no differences in the calculated copy number at lower given concentration - 10^4 and 10^3 copies/reaction (Fig.11B). As for the expression of the gene in tested samples, down-regulation was achieved.

These are the results of gene expression measurement in RotorGene. The input parameter was the given concentration of the dilution series and the output was real concentration of the dilution series and Ct of the dilution series and samples.

Channel JOE measured the expression of the NUP housekeeping gene and it is obvious from the Fig. 5A-11A that the expression in all cell lines is very uniform and without any major differences in Ct. Channel FAM measured the expression of the IFN-induced genes and the dilution series (Fig. 5B-11B).

The graphs from the RotorGene show down-regulation of tested genes in comparison to the non-influenced Hep2 cell line. The least expression is of RNL and OAS3 genes, whilst PKR is slightly up-regulated. Differences among individual cell lines can be easily calculated on the basis of Ct and the copy number in the legends.

4.8 Absolute quantification of the target genes

Copy number of the target genes in each cell line was calculated per microgram total RNA used. Starting amount of RNA for the reverse transcription was 1µg RNA per 25µl reaction mixture. This means that the concentration of RNA in the mixture was 40ng/µl. For the QRT PCR reaction, 2 µl cDNA were used. This volume equals 80ng RNA. The average of the double samples was taken for the copy number/ µg RNA calculation.

RNL gene

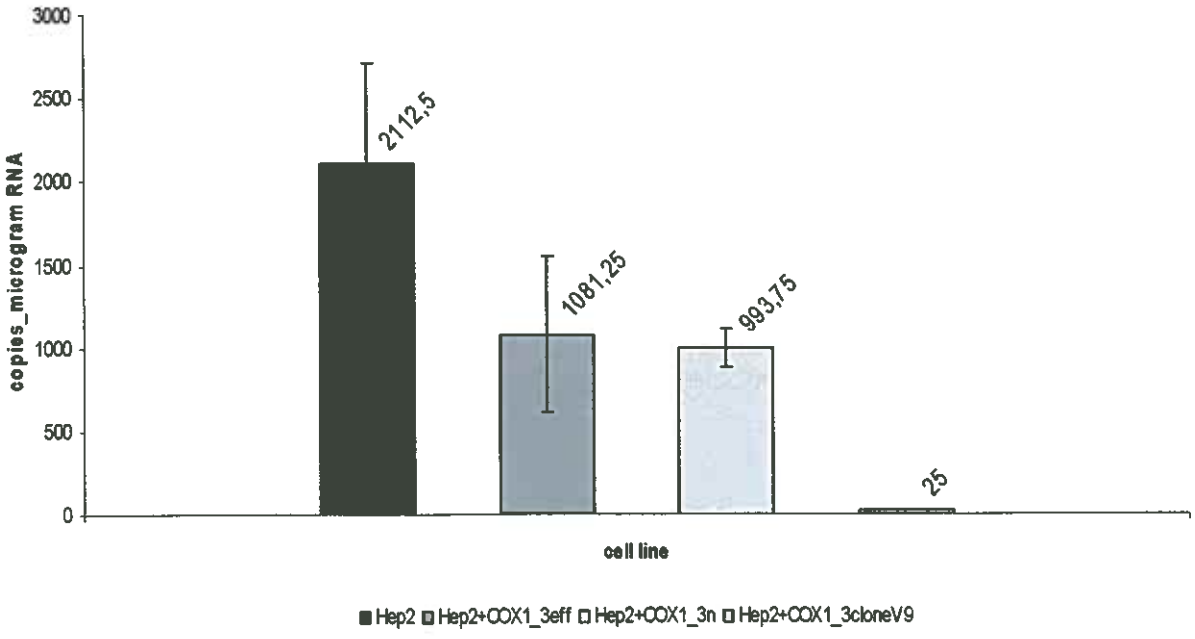

Fig.12: Absolute quantitation of RNL gene expressed in copies/microgram RNA

Although the expression of RNL in all tested cell lines was low (see Fig.5a), it was possible to measure the down-regulation of the gene in influenced cell lines. The extent of down-regulation was quite large, Hep2+COX1_3cloneV9 cell line to only 25copy numbers/µg RNA (see Fig.12).

PKR gene

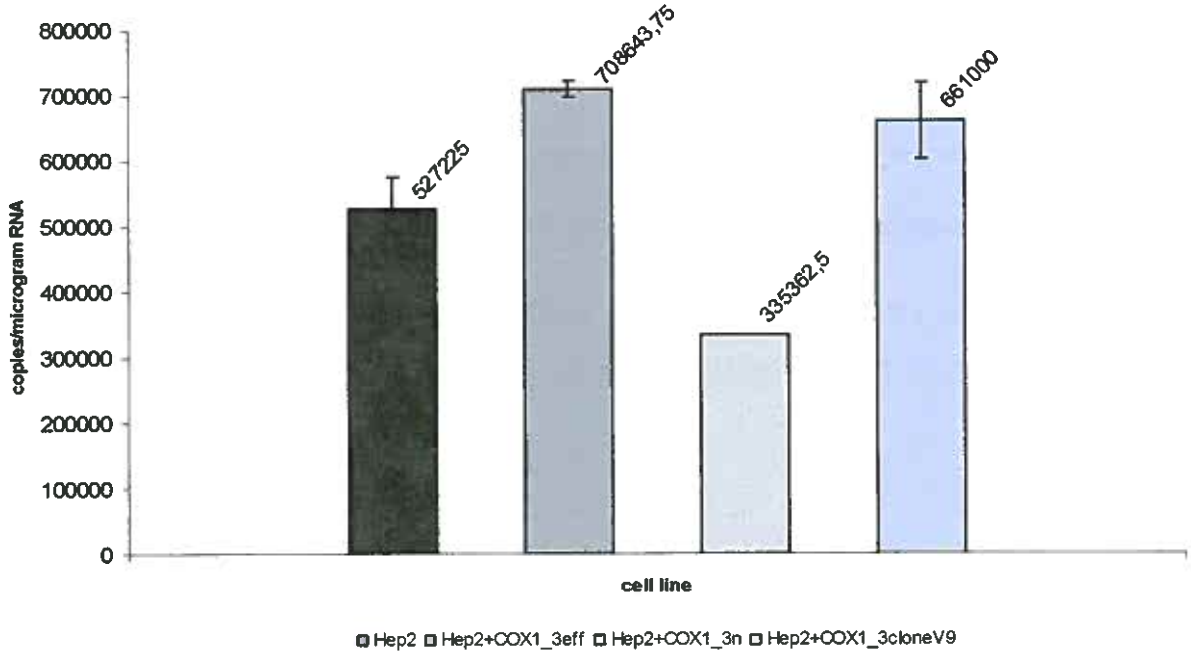

Fig.13: Absolute quantification of PKR gene expressed in copies /microgram RNA

PKR gene in two cell lines, Hep2+COX1_3eff and its derivative Hep2+COX1_3cloneV9 is up-regulated; in Hep2+COX1_3 it is down-regulated by almost half of Hep2 expression (Fig.13).

IRF1 gene

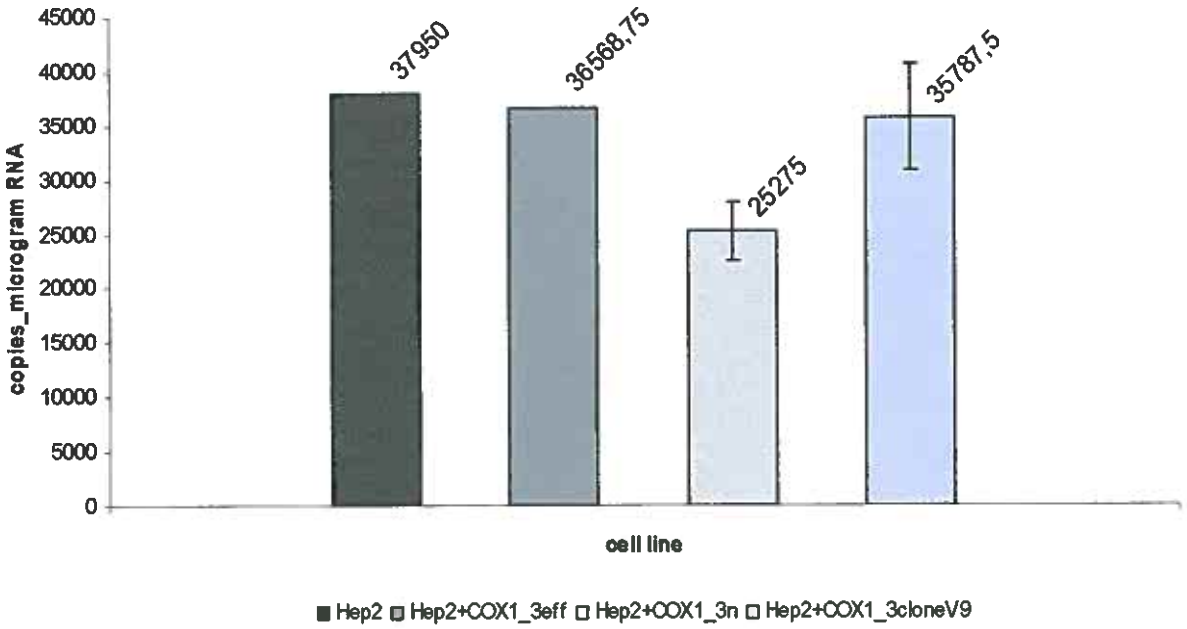

Fig.14: Absolute quantification of IRF1 gene expressed in copies/microgram RNA

Down-regulation of IRF1 in Hep2+COX1_3eff and Hep2+COX1_3cloneV9 is very little (only several hundred copy numbers/μg RNA). Hep2+COX1_3n down-regulation is more significant (see Fig.14).

IRF3 gene

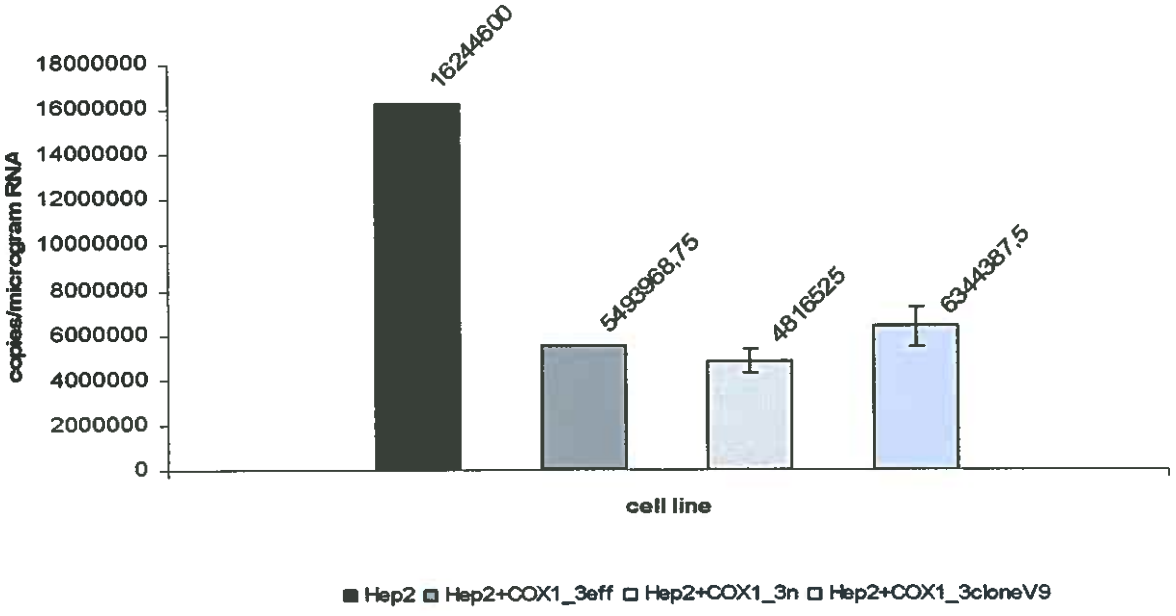

Fig.15: Absolute quantification of IRF3 gene

IRF3 gene showed quite large down-regulation of all genes, Hep2+COX1_3n again being the greatest. IRF3 gene in all influenced cell lines is expressed about 30% in comparison to Hep2 (estimated from the data in Fig.15).

IFIT1 gene

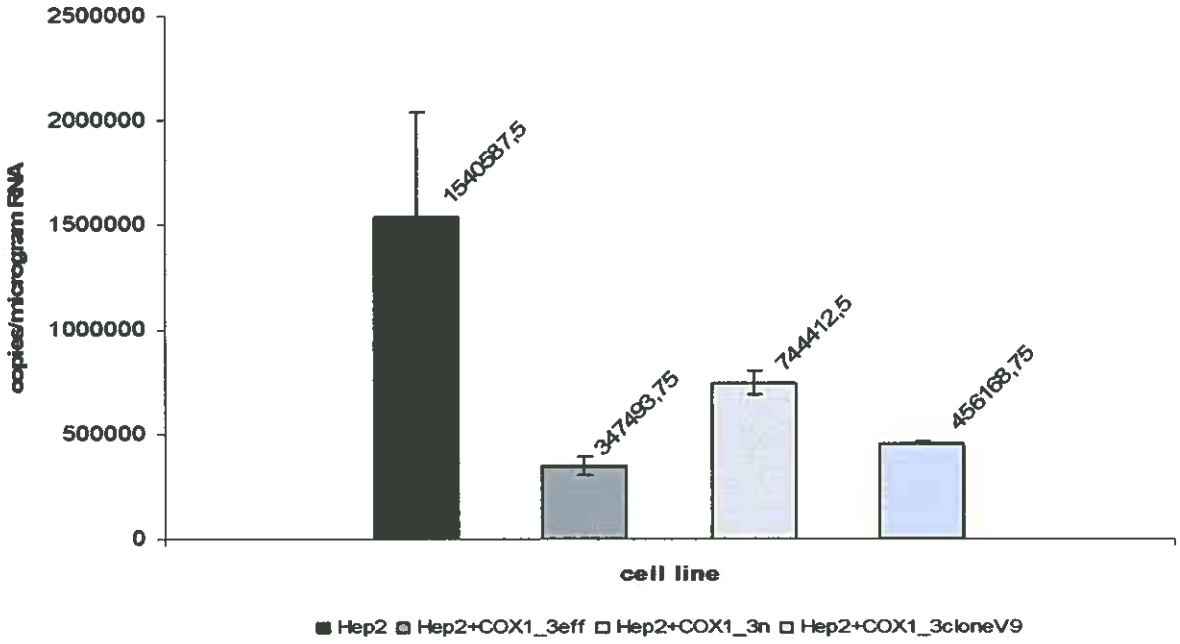

Fig.16: Absolute quantification of IFIT1 gene expressed in copy numer/microgram RNA

IFIT1 was well down-regulated as well, the highest being Hep2+COX1_3n (48% of IFIT1 expression in Hep2), the lowest Hep2+COX1_3eff (22% only). The data are calculated from the Fig.16.

IFITM gene

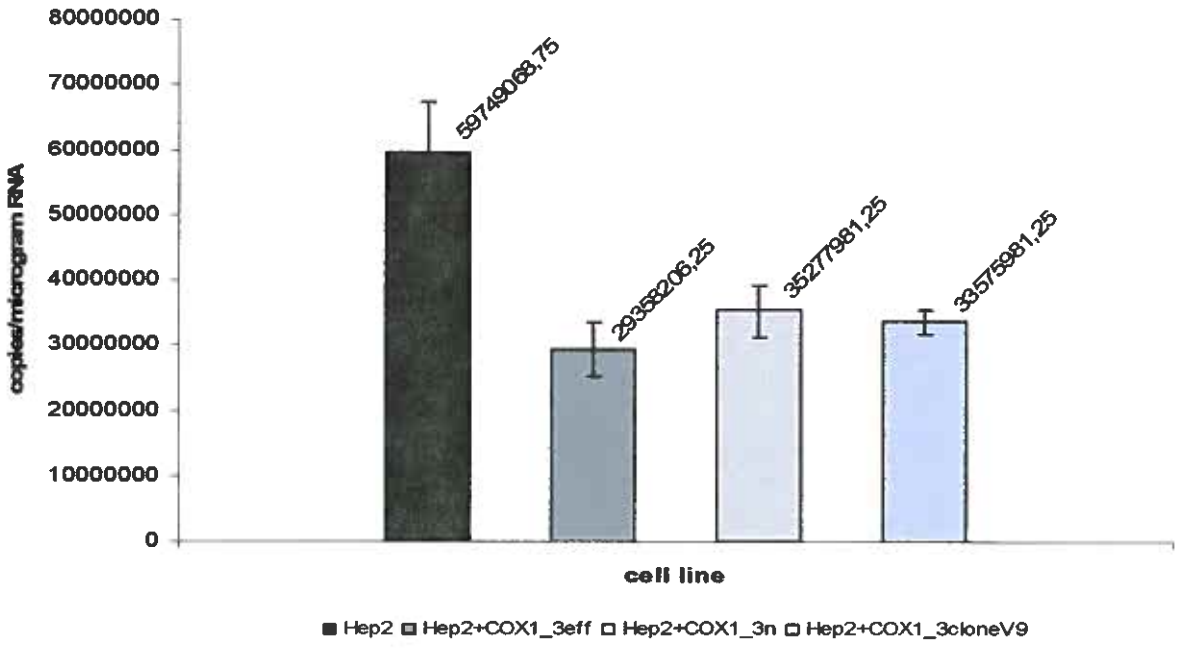

Fig.17: Absolute quantification of IFITM gene expressed in copy numer/microgram RNA

All influenced cell lines are down-regulated rather uniformly to about 50% (see Fig.17).

OAS3 gene

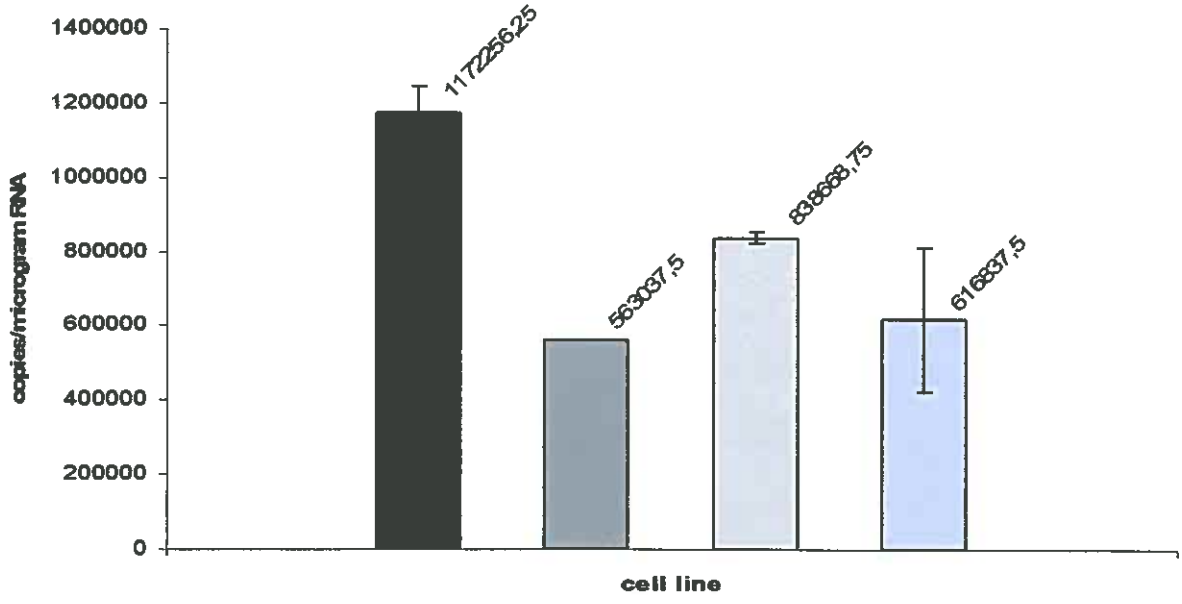

Fig.18: Absolute quantification of OAS3 gene expressed in copy numer /microgram RNA

It is obvious from the RotorGene graphs (Fig.11A – Fig.11B) that the expression of OAS3 in all cell lines was very low. Hep2+COX1_3cloneV9 also shows high standard mean deviation (see Fig 18). But generally, down-regulation was achieved here, too.

5. DISCUSSION

Small interfering RNAs (siRNAs) are one of the most important discoveries in functional genomics and valuable tool for analysis of gene function and creation of knock-out cell lines. Moreover, their potential as therapeutic agents has been recognized.

siRNAs are being intensively studied as therapeutic agents for treatment of various hereditary and infectious diseases such as Huntington's brain disorder, hepatitis C or type 2 diabetes [32]. A major problem in implementing siRNAs as common therapeutics is adverse effects in mammalian cells, mainly non-specific gene silencing and activation of the interferon system [13, 33]. It was thought that maintaining the length of the dsRNA below 30nt would avoid the activation of the interferon-induced response. However, several studies pointed that siRNAs as short as 19 nt can induce interferon response [34]. Activation of the response is triggered partly by dsRNA and the resulting signaling cascade is mediated by a variety of proteins, including the Janus family tyrosine kinases, Jak1 and Tyk2, the signal transducers and activators of transcription, Stats1 and 2, and IRF9 transcription factor – culminating in the induction of interferon-stimulated genes within the nucleus [33].

This work is the follow up of the diploma thesis [36] and its aim was to explore the extent of up-regulation of IFN-induced genes with siRNA used to silence cyclooxygenase genes. First, several siRNAs were tested for their silencing effect on COX gene in Hep2 cell lines. Sequences of the siRNAs are as follows:

siRNA1:

GAtCCAGAACAGtGGCtCGTTCAAGAGACGaGCCaCTGTTCTGGaTCTTTTTTGGa

siRNA2:

gatcCACTTCACCCACCAGTTCTTCATAGAGATAAGAACTGGTGGGTGAAGTGTTTTTTTGGa

siRNA3:

gATCCTTGCTGTTCCCACCCTTCAAGAGAGGGTGGGAACAGCAAGGATTTTTTTGGa

Each of these siRNAs was expected to bind the COX target mRNA with its loop portion (red bases of the sequence) and silence the expression of the COX gene to certain extent. The psiRNA plasmid containing those sequences was transfected into

Hep2 cells by methods described in chapter 3.2.1. Additional cell lines not mentioned in this work but which were influenced by siRNA2 and siRNA3 were Hep2+psiRNA_U6_COX1_6 and Hep2+psiRNA_U6_COX2_2x, respectively.

After performing the QRT PCR for all samples and calculating the extent of down-regulation by using the Pfaffl method [25], it was found that the best silencer was siRNA1. The calculated results are presented in Table 17.

Cell line	Down-regulation (%)
Hep2+psiRNA_U6_COX1_3	0,002
Hep2+psiRNA_U6_COX1_3n	0
Hep2+psiRNA_U6_COX1_3clone V9	0,227
Hep2+psiRNA_U6_COX1_6	80,65
Hep2+psiRNA_U6_COX2_2x	68,39

Table 10: COX1 down-regulation percentage

For this reason, it was decided to further work with cell lines influenced by siRNA1.

All cell lines were prepared and cultivated by Generi Biotech s.r.o. The siRNA for the experiment was designed in advance and the cDNA for the QRT PCR was also obtained prior to the experiment by reverse transcription.

The extent of up-regulation was measured by QRT PCR using NUP as a housekeeping gene of choice because its expression was constant in non-influenced Hep2 cell lines, as well as in those influenced by siRNA. Dilution series of each gene were prepared by inserting the tested sequence in the plasmid and making dilutions in range 10^7 – 10^3 copies/reaction. These dilutions served as a reference for calculating the absolute quantitation of the IFN-induced genes. The expression of the tested genes was calculated as the copy number per microgram RNA.

The QRT PCR graphs (Fig. 5A-11A) show uniform expression of the NUP housekeeping gene in all cell lines (OAS3 to a lesser extent) which means that all results are valid.

Doubles of each sample were prepared because pipetting errors may occur and some samples have to be omitted. Where possible, the average of the two was taken for the evaluation of results. The main criteria for the elimination of the sample were the visual evaluation of the real-time graph (Fig. 5B-11B) and the variance in Ct between the samples. First, dilution curves were evaluated. The evaluation was based on observing the shape of the curve; those that did not have the typical

phases (described in chapter 2.3.3) were eliminated. Dilution series curves should have the Ct in regular intervals; those that showed major deviation in Ct were eliminated. Similar criteria were applied on tested genes PKR, IRF1, IRF3, IFIT1 and IFITM because their expression was within the dilution range. Since it was impossible to apply this method on genes RNL and OAS3 because of very low expression, all the samples had to be taken into account.

RNL gene in all influenced cell lines was down-regulated in comparison to non-influenced Hep2. However, it is obvious from the real-time curve (see Fig. 5B) that the expression of the RNL in Hep2 was low as well. Even though the NUP housekeeping gene was uniformly expressed in all samples, there were quite large differences in the gene expression between the tested doublets. One possible reason for this is the properties of the PCR machine. The experience during the experiment was that the RotorGene would not precisely measure the concentration lower than 10^3 copies/reaction (in certain samples, as visible from the real-time graphs, the lowest dilution measured was 10^4 copies/reaction), and thus, it was not possible to precisely evaluate the expression of the gene.

Out of three OAS genes, only OAS3 was possible to measure. The expression of the NUP housekeeping gene was less uniform than in other measurements, and results for OAS3 were similar to those for RNL. OAS1 and OAS2 were not measured at all because of problematic cultivation and unsuccessful isolation from the plasmid. Isolation kit used was Qiagen MiniPrep. OAS3 expression could be related to the length of siRNA. In studies done with the interferon system, it was observed that the 19-mer siRNA does not induce OAS1 expression [41]. This could also apply to OAS3

Both RNL and OAS3 measurement results obtained are not reliable and objective so it would be advisable to repeat the measurements.

PKR gene expression was up-regulated in all cell lines except in Hep2+COX1_3n. The reason for this single cell line exception could be the pipeting error. Otherwise, the up-regulation could be connected to the length of siRNA [41].

All other genes measured were down-regulated to various extents in comparison to the non-influenced Hep2 cell line.

Even though the NUP housekeeping gene met all the requirements for the housekeeping gene (described in chapter 2.3.5), the results obtained for cyclooxygenase I expression show large variability and are not uniform. The fact that some of them had to be omitted from the analysis and non-objective standard mean

deviations (calculated either from only two samples or not calculated at all) put down the question of the reproducibility of the real-time PCR method. One problem in the evaluation was the variability in the expression of a same gene in Hep2 cell line doublet. Either expression could be taken as a standard, and in case the expression of a gene is not uniform in both samples, there is a risk of misinterpretation of results.

The unexpected result was the down-regulation of all genes except the PKR. There are many possible reasons for this, such as cultivation conditions, antibiotic selection of the transfected cell line and subsequent stress after transfection. Also, the cell line used in the experiment may also play role in the expression of the tested genes. So far, this aspect of RNAi has not been sufficiently explored and comparative experiments with other cell lines would be necessary [42].

Much more extensive screening with many more samples and more accurate statistical analysis is necessary in order to confirm or to challenge the results obtained in this series of measurements and give grounds for objective conclusion. However, these first results obtained indicate no major up-regulation of any of the tested genes. This can be considered as a positive signal for deeper analysis, and perhaps finding a reason for such results which may be used for further research in this field.

6. SUMMARY

Side-effects of siRNA were tested on cell lines after silencing the cyclooxygenase gene. The theoretical basis for this experiment was the observation that siRNAs may induce immune response. For this reason, IFN-induced genes expression was tested in non-influenced Hep2 cell line and three other Hep2 derivative cell lines in which the siRNA was inserted by different methods.

Cell lines were derived from Hep2 by using various methods for transfection of siRNA (i.e. nucleofection, Effectene technology and further cloning). RNA was isolated from these cell lines and cDNA was obtained by reverse transcription.

Also, dilution series (10^7 to 1 copy number/reaction) were prepared from the plasmid which contained the tested sequence.

Gene expression was measured by QRT PCR and quantified as copy number/ µg RNA. The degree of expression was compared to non-influenced Hep2 cell line. The housekeeping gene used was NUP. The results were evaluated using REST version 2 and Rotor Gene version 6 softwares.

Initially, nine IFN-induced genes, RNL, PKR, IRF1, IRF3, IFITM, IFIT1, OAS1, OAS2 and OAS3 were to be tested. Out of these, OAS1 and OAS2 were impossible to isolate and thus, impossible to measure. PKR expression was up-regulated, and all the other tested genes were down-regulated. There are many possible explanations for such result, but whatever the reason, much more extensive measurements are necessary.

7. LITERATURE

1. Human genome Project information: Gene therapy
November 2005
www.ornl.gov/sci/techresources/Human_Genome/medicine/genetherapy.shtml

2. National Cancer Institute: Gene Therapy for cancer: Questions and Answers
www.cancer.gov/cancertopics/factsheet/Therapy/gene#top

3. S. Rosypal: Úvod do molekulární biologie- dodatek
Brno, 1997

4. G. Smutzer: delivering the Goods
The Scientist 2000 14(19): 28

5. Gene Therapy Clinical Trials Worldwide
Provided by the Journal of Gene Medicine
January 2006
www.wiley.co.uk/genetherapy/clinical

6. A. Ananthaswamy: Undercover genes slip into the brain
The New Scientist 2003 14: 48

7. D. Penman: Subtle gene therapy tackles blood disorder
The New Scientist 2002 16: 26

8. B. Holmes: Gene therapy may switch off Huntington's
The New Scientist 2003 10: 32

9. RNA interference and gene silencing: History and Overview
Ambion, the RNA Company, 2006
www.ambion.com/techlib/hottopics/rnai/rnai_may2002_2.html

10. U. Schepers: RNA Interference in Practice
Weinheim, 2005

11. O. Taltynov: Molekulární biologie a genetika X
Ústav molekulární genetiky, Akademie Věd České Republiky, Praha 2002

12. A Reynolds, D Leake, Q Boese, S Scaringe, WS Marshal, A. Khvorova:
Rational siRNA design for RNA interference
Nature biotechnology 2004 22: 326-330

13. S.P. Persengiev, X. Zhu, M.R. Green: Non-specific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs)
RNA 2004 10: 12-18

14. J. D. Heidel, S. Hu, X. F. Liu, T.J. Triche, M.E. Davis: Lack of interferon response in animals to naked siRNAs
Nature biotechnology 2004 22: 1579-1582

15. B.A. Hassel, A. Zhou, C. Sotomayor, A. Maran, R.H. Silverman: A dominant negative mutant of 2-5A-dependent RNase suppresses antiproliferative and antiviral effects of interferon.
EMBO J 1993 12(8): 3297-304

16. C. Bisbal, C. Martinand, M. Silhol, B. Lebleu, T. Salehzada: cloning and characterization of a RNase L inhibitor
Journal of biological chemistry 1995 270(22): 13308-17

17. Online Mendelian inheritance in man (OMIM) database
www.ncbi.nlm.nih.gov/entrez

18. Y. Ben-Asouli, Y. Banai, Y. Pel-Or, A. Shir, R. Kaempfer: Human interferon-γ mRNA autoregulates its translation through a pseudoknot that activates the interferon-inducible protein kinase PKR
Cell 2002 108: 221-232

19. B.K. Weaver, K.P. Kumar, N.C. Reich: Interferon-regulatory factor 3 and CREB-binding protein/p300 are subunits of the double-stranded RNA-activated transcription factor DRAF1
Molecular Cell Biology 1998 18(3): 1359-1368

20. A. Hovnanian, D. Rebouillat, M.G. Mattei, E.R. Levy, I. Marie, A.P. Monaco, A.G. Hovanessian: The human 2'5' oligoadenylate synthase locus is composed of three distinct genes clustered on chromosome 12q24.2 encoding the 100-, 69- and 40-kDa forms
Genomics 1998 52(3): 267-77

21. Wikipedia, the free encyclopedia
http://en.wikipedia.org/wiki/Real-time_PCR

22. M. Tevfik Dorak: Real-time PCR
http://dorakmt.tripod.com/genetics/realtime.html

23. Ambion, the RNA Company: RT PCR- the basics
http://www.ambion.com/techlib/basics/rtpcr/index.html

24. M.L. Wong, J.F. Medrano: Real-time PCR for mRNA Quantitation
BioTechniques 2005 39(1): 75-85

25. M.W. Pfaffl: A new mathematical model for relative quantification in real-time PCR
Nucleic Acids Research 2001 29(9) e45

26. S.A. Bustin: Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays
Journal of molecular endocrinology 2000 25:169-193

27. C. Horbinski, M.K. Stachowiak, D. Higgins, S.G. Finnegan: Polyethyleneimine-mediated transfection of cultured postmitotic neurons from rat sympathetic ganglia and adult human retina
BMC Neuroscience 2001 2: 2

28. EMEA: Public statement for Vitravene (fomivirsen)
6. August 2002
www.emea.eu.int/pdfs/human/press/pus/1238202en.pdf

29. FDA: Vitravene injection-basis for approval
26. August 1998
www.fda.gov/cder/foi/label/1998/20961lbl.pdf

30. siRNAs and shRNAs
InvivoGen
http://www.invivogen.com/sscat.php?ID=14

31. C.M. Mach, B.W. Hargrove, G.R. Kunkel: The Small RNA Gene Activator Protein, *Sphl* Postoctamer Homology-bining Factor/Selenocysteine tRNA Gene Transcription Activating Factor, Stimulates Transcription of the Human Interferon Regulatory Factor-3 Gene
The Journal of Biological Chemistry 2002 277(7): 4853-4858

32. Sirna therapeutics
www.sirna.com

33. C.A. Sledz, M. Holko, M.J. de Veer, R.H. Silverman, B.R.G. Williams: activation of the interferon system by short-interfering RNAs
Nature Cell Biology 2003 5: 9

34. R.J. Fish, E.K.O. Kruithof: Short-term cytotoxic effects and long-term instability of RNAi delivered using lentiviral vector
BMC Molecular Biology 2004 5: 9

35. M. Zuker, D.H. Mathews, D.H. Turner: Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide
RNA Biochemistry and Biotechnology, J. Barciszewski and B.F.C. Clark, eds., NATO ASI Series, Kluwer Academic Publishers, 1999
http://molbio.info.nih.gov/molbio-nih/mfold.html

36. M. Feřtek: Small interfering RNA-mediated down-regulation of cyclooxygenase expression
Department of biochemistry, Pharmaceutical faculty in Hradec Králové, Charles University in Prague, 2005

37. A.J. Hamilton, D.C. Baulcombe: A species of small antisense RNA in posttranscriptional gene silencing in plants
Science 1999 286: 950-952

38. S.A. Elbashir, W. Lendeckel, T. Tuschl: RNA interference is mediated by 21- and 22-nucleotide RNAs
Genes and Development 2001 15: 188-200

39. T. Tuschl, A. Borkhardt: Small interfering RNAs: A revolutionary tool for the analysis of gene function and gene therapy
Molecular Interventions 2002 2: 146-157

40. A. Nykänen, B. Haley, P.D. Zamore: ATP requirements and small interfering RNA structure in the RNA interference pathway
Cell 2001 107: 309-321

41. R.J. Fish, E.K.O. Kruithof: Short-term cytotoxic effects and long-term instability of RNAi delivered using lentiviral vectors
BMC Molecular Biology 2004 5:9

42. I. Bantounas, L.A. Phylactou, J.B. Uney: RNA interference and use of small interfering RNA to study gene function in mammalian systems
Journal of Molecular Endocrinology 2004 33: 545-557