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APPLICATION OF RNAi TO SILENCE TARTRATE-RESISTANT ACID PHOSPHATASE:

Unexpected Effects on the Monocyte-Macrophage Lineage

by

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ABSTRACT

RNA interference (RNAi) was originally discovered in plants, and in 2000, RNAi was also applied as a gene silencing tool in mammalian cells. It is a mechanism in which short double stranded RNA molecules (siRNAs) are incorporated into a special protein complex further catalysing the complementary RNA degradation. Proteins are thus not translated after RNA degradation. In this study, a new siRNA design algorithm ***siRNA_profile*** was developed to improve the selection of potential siRNA candidate sequences in order to facilitate efficient and specific gene silencing by RNAi. By using optimally designed siRNA molecules it might be possible to obtain long-term gene silencing and specific knock down of the target protein in cells. Different modifications, such as the incorporation of Fluoro-substitution in the 2´-position of the siRNA riboses, were tried to increase their stability in plasma and to enhance their efficacy. These are important properties of siRNA molecules when applying RNAi for therapeutical purposes.

Tartrate-resistant acid phosphatase (TRACP) is an enzyme expressed in bone resorbing osteoclasts, in antigen presenting dendritic cells as well as in various tissue macrophages, which all are phagocytosing cells. The biological function of TRACP is still unknown, however, it has been suggested that TRACP´s capacity to generate reactive oxygen species (ROS) is involved in bone matrix degradation by osteoclasts and in the antigen presenting route of dendritic cells. Macrophages overexpressing TRACP have also increased intracellular ROS generating capacity and enhanced bacterial killing activity. siRNA and antisense DNA molecules specifically designed to silence the TRACP gene in monocyte-macrophage lineage originated cell cultures revealed an unexpected increase of TRACP expression. The effects of DNA and siRNA molecules on TRACP expression were further studied in monocyte-macrophage lineage originated from Toll-like receptor 9 (TLR9) knock-out mice. Induction of TRACP expression was confirmed to be a sequence and a TLR9 independent response against exogenous DNA and RNA molecules. This increased TRACP expression suggests a new function for TRACP as a part of the innate immunity system.

Keywords: RNA interference, siRNA, antisense DNA, tartrate-resistant acid phosphatase, Toll-like receptor 9, monocyte-macrophage lineage, innate immunity

Muhonen, Pirkko: Tartraatti-resistentin happaman fosfataasin hiljentäminen RNAi menetelmällä: odottamaton vaikutus monosyytti-makrofagi linjan soluissa
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YHTEENVETO

RNA interferenssi (RNAi) eli RNA:n hiljentyminen löydettiin ensimmäisenä kasveissa, ja 2000-luvulla RNAi menetelmä on otettu käyttöön myös nisäkässoluissa. RNAi on mekanismi, jossa lyhyet kaksi juosteiset RNA molekyylit eli siRNA:t sitoutuvat proteiinikompleksiin ja sitoutuvat komplementaarisesti proteiinia koodaavaan lähetti RNA:han katalysoiden lähetti RNA:n hajoamisen. Tällöin RNA:n koodaamaa proteiinia ei solussa tuoteta. Tässä työssä on RNA interferenssi menetelmän avuksi kehitetty uusi siRNA molekyylien suunnittelualgoritmi ***siRNA_profile***, joka etsii lähetti RNA:sta geenin hiljentämiseen sopivia kohdealueita. Optimaalisesti suunnitellulla siRNA molekyylillä voi olla mahdollista saavuttaa pitkäaikainen geenin hiljeneminen ja spesifinen kohdeproteiinin määrän aleneminen solussa. Erilaiset kemialliset modifikaatiot, mm. 2´-Fluoro-modifikaatio, siRNA molekyylin riboosirenkaassa lisäsivät siRNA molekyylin stabiilisuutta veren plasmassa sekä siRNA molekyylin tehokkuutta. Nämä ovat tärkeitä siRNA molekyylien ominaisuuksia kun RNAi menetelmää sovelletaan lääketieteellisiin tarkoituksiin.

Tartraatti-resistentti hapan fosfataasi (TRACP) on entsyymi, joka esiintyy luunsyöjäsoluissa eli osteoklasteissa, antigeenejä esittelevissä dendiriittisissä soluissa sekä eri kudosten makrofageissa, jotka ovat syöjäsoluja. TRACP entsyymin biologista tehtävää ei ole saatu selville, mutta oletetaan että TRACP entsyymin kyvyllä tuottaa reaktiivisia happiradikaaleja on tehtävä sekä luuta hajoittavissa osteoklasteissa sekä antigeenia esittelevissä dendriittisissä soluissa. Makrofageilla, jotka yliekpressoivat TRACP entsyymiä, on myös solunsisäinen reaktiivisten happiradikaalien tuotanto sekä bakteerin tappokyky lisääntynyt. TRACP-geenin hiljentämiseen tarkoitetut spesifiset DNA ja siRNA molekyylit aiheuttivat monosyytti-makrofagilinjan soluviljelymallissa TRACP entsyymin tuoton lisääntymistä odotusten vastaisesti. DNA ja RNA molekyylien vaikutusta TRACP entsyymin tuoton lisääntymiseen tutkittiin myös Toll-like reseptori 9 (TLR9) poistogeenisestä hiirestä eristetyissä monosyytti-makrofaagisoluissa. TRACP entsyymin tuoton lisääntyminen todettiin sekvenssistä ja TLR9:stä riippumattomaksi vasteeksi solun ulkopuolisia DNA ja RNA molekyylejä vastaan. Havainto TRACP entsyymin tuoton lisääntymisestä viittaa siihen, että TRACP entsyymillä on tehtävä solun immuunipuolustusjärjestelmässä.

Avainsanat: RNA interferenssi, siRNA, antisense DNA, tartraatti-resistentti hapan fosfataasi, Toll-like reseptori 9, monosyytti-makrofagi linja, luontainen immuniteetti

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ABBREVIATIONS

AcP	acid phosphatase
AGO	Argonaute
ANOVA	analysis of variance
AP1	activating protein 1
BL	baseline
BLAST	basic local alignment search tool
BSP	bone sialoprotein
CHO	Chinese hamster ovary
DC	denritic cell
DNA	deoxyribonucleoside acid
dsRNA	double stranded RNA
EDTA	ethylene diamine tetra-acetate
EGTA	ethylene glycol tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EST	expressed sequence tag
FANA	2´-deoxy-2´-Fluoro-D-arabinonucleic acid
Fe^{2+}/Fe^{3+}	ferrous/ferric
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FSD	functional secretory domain
HEPES	N-(2-hydroxyethyl)-piperazine-N´-(2-ethanesulphonic acid)
hnRNA	heterogenous nuclear RNA
iFCS	heat-inactivated fetal calf serum
IFN	interferon
IFNR	interferon receptor
IL	interleukin
LAP	lysosomal acid phosphatase
LNA	locked nucleic acid
LPS	lipopolysaccharide
M-CSF	macrophage-colony stimulating factor
MEM	minimum essential medium
MHC	major histocompatibility complex
miRNA	micro RNA
MITF	microphthalmia transcription factor
mRNA	messenger RNA
NK cell	natural killer cell
4-NPP	4-nitrophenyl phosphate
nt	nucleotide
ODN	oligonucleotide
2´-*O*-Me	2´-*O*-methyl
2´-*O*-MOE	2´-*O*-methoxyethyl
OPN	osteopontin
OPG	osteoprotegerin

PAGE	polyacrylamide gel electrophoresis
PAP	purple acid phosphatase
PAZ	Piwi, Argonaute, Zwille/Pinhead
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pip	Pu.1 interacting protein
PMSF	phenylmethylsulfonylfluoride
PTGS	post-transcriptional gene silencing
RANKL	receptor activator of NF-κB ligand
RB	ruffled border
RdRP	RNA dependent RNA polymerase
RIG-1	retinoic acid inducible gene -1
RIPA buffer	radioimmunoprecipitation assay buffer
RISC	RNA induced silencing complex
RITS	RNA induced initiation of transcriptional gene silencing
RLC	RISC loading complex
RLR	RIG-1 like receptor
RNA	ribonucleoside acid
RNAi	RNA interference
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT	room temperature
RT-PCR	reverse transcriptase-polymerase chain reaction
shRNA	short hairpin RNA
siRNA	short interfering RNA
siRNP	small interfering ribonucleoprotein
ssRNA	single stranded RNA
TBE	Tris-borate-EDTA buffer
TGF-β	transforming growth factor-beta
TGS	transcriptional gene silencing
Th-cell	T-helper cell
TLR	Toll-like receptor
TMB	3, 3', 5, 5'- tetramethylbenzidine
TNF-α	tumor necrosis factor alpha
TRACP	tartrate-resistant acid phosphatase
TRAF6	TNF receptor-associated factor 6
TRITC	tetramethylrodamine isothiocyanate
TSAP	tartrate-sensitive acid phosphatase
USF	upstream stimulatory factor
UTR	untranslated region
WT	wild type

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by using Roman numerals (I-III) In addition, unpublished results are included.

I Muhonen P., Büki K., Parthasarathy R.N., Janckila A.J. and Väänänen H.K. (2007) Analysis by ***siRNA_profile*** program displays novel thermodynamic characters of highly functional siRNA molecules. Submitted

II Muhonen P., Tennilä T., Azhayeva E., Parthasarathy R.N., Janckila A.J., Väänänen H.K., Azhayev A. and Laitala-Leinonen T. RNA interference tolerates 2'-Fluoro-modifications at the Argonaute2 cleavage site. *Chem Biodivers* 4 (5), 858-873.

III Muhonen P., Avnet S., Parthasarathy R.N., Janckila A.J., Halleen J.M., Laitala-Leinonen T. and Väänänen H.K. (2007). Sequence and TLR9 independent increase of TRACP expression in monocyte-macrophage lineage by antisense DNA and siRNA molecules. *Biochem Biophys Res Commun.* 359:889-95

1 INTRODUCTION

Theoretically, specific DNA or RNA targeted knockdown molecules could be used to cure any disease that is caused by the too high expression of a deleterious gene. The approach to generate knock-out animals to gain information on the function of genes *in vivo* has been found to be labor intensive, time-consuming and expensive. Thus, knock-down models are an attractive alternative due to the possibility of also being able to study vital genes during embryogenesis.

RNA is a susceptible target for nucleic acid-based gene suppression as it exposes its unpaired bases for hybridization by other complementary single stranded nucleic acids. The common mechanism for oligonucleotide based gene suppression is a heteroduplex mediated induction of RNaseH. After induction, messenger RNA (mRNA) content is digested by RNaseH, which leaves the DNA molecule available to bind other complementary mRNA sequences. In an alternative mechanism, catalytic RNAs called ribozymes bind RNA sequence specifically and destroy it by cleavage which is RNaseH independent. Recently, short double stranded RNA molecules have been shown to induce a highly conserved RNA interference (RNAi) mechanism, which lead to gene suppression due to mRNA degradation, blockage of translation or heterochromatin modifications in the nucleus by short interfering RNA (siRNA) molecules. RNAi has challenged the bioinformatics to develop search algorithms for functional siRNA molecules among non-functional (I).

Soon after the use of nucleic acid-based inhibitors of gene expression became available, it was realized that certain modifications were necessary to improve their biological activity and delivery to target cells. The development of a variety of backbone modifications stabilized the antisense molecules without affecting their biological activity. Information attained from antisense modifications led to applications of modified ribozymes and further to DNAzyme molecules. Moreover, it was also shown that siRNA molecules, the active components of RNAi, can be chemically synthesized and modified (II), or expressed from vector backbones similar to ribozymes. The delivery challenges have been addressed with the introduction of anionic and cationic lipid formulations for packaging and delivering the nucleic acid inhibitors to target cells and tissues.

Nucleic acid-based methods provide powerful tools for targeted inhibition of gene expression; however, there are several concerns and limitations in the use of these technologies. Several reports have recently indicated unanticipated side-effects and unspecific knockdown of mRNAs. A CpG motif containing oligonucleotides has shown a pattern to induce strong innate and acquired immune responses and siRNAs to activate the interferon response pathways. These recently discovered and only partly examined side-effects require further experimentation to restructure combinations and modifications of nucleic acid knock down molecules without compromising biological activity but bypassing the side-effects.

The present study aimed to specifically knock down tartrate-resistant acid phosphatase (TRACP) by using gene knock down methods (III). TRACP is found in two isoforms originated from a single gene: TRACP 5a isoform is mainly expressed in antigen presenting cells such as activated dendritic cells and macrophages. TRACP 5b isoform is postranslationally modified from TRACP 5a and is secreted from osteoclasts, bone resorbing cells that are formed by the maturation and fusion of the monocyte-macrophage lineage cells. TRACP enzyme has two irons and a capacity to generate reactive oxygen species (ROS) via Fenton reaction. TRACP with its ROS generating activity has been previously suggested to be involved in bone matrix degradation in transcytotic route in osteoclasts, and in the pathogen presentation route in macrophages and dendritic cells. In addition, TRACP originated ROS are involved in bacterial killing of macrophages. To achieve the TRACP gene knock down, antisense DNA and RNAi methods were applied. Novel guidelines for siRNA molecule design were assembled to ensure highly efficient gene knock down *in vitro*. In addition, 2´-Fluoro-purine and 2´-Fluoro-pyrimidine substitutions were introduced into a ribose sugar ring of siRNA molecule to evaluate the effects of the modifications during RNAi (II). siRNA molecules efficiently knocked down TRACP in the CHO cell line stably expressing TRACP under a strong viral promoter. Surprisingly, in monocyte-macrophage lineage both DNA and RNA inhibitory molecules induced TRACP expression in a sequence and Toll-like receptor 9 (TLR9) independent manner (III). This induction of TRACP expression due to the exogenous DNA and RNA oligomers suggests a role for it in innate immunity against bacterial and viral invasion.

2 REVIEW OF THE LITERATURE

2.1 Gene silencing methods

2.1.1 Antisense DNA technology

The potential inhibition of gene expression by antisense DNA oligomers was discovered in 1978 (Zamecnik and Stephenson, 1978). Antisense molecules usually consist of 15- 20 nucleotides, which are complimentary to their target mRNA. There are several ways how antisense DNA oligomers interact with nucleic acids causing biological effects. The antisense DNA molecules may induce an RNAseH cleavage mechanism, which is the most commonly used function of antisense DNA oligomers to knock down genes. RNaseH is a ubiquitous enzyme that degrades RNA strands of RNA:DNA duplex. The precise recognition elements are not known; however, as short as four nucleotides long oligomers can induce RNaseH-mediated cleavage of a target transcript (Donis-Keller, 1979).

In addition, there have also been attempts to target DNA oligomers to sequences that affect mRNA stability, localization and mRNA accessibility for translation. Inhibition of translation may be obtained when oligonucleotides bind the 5'-cap region in mRNAs (Baker et al., 1992). Interruption of 3'-polyadenylation by oligonucleotides may theoretically destabilize pre-mRNA (Chiang et al., 1991). DNA oligomers in a variety of lengths targeting the translation initiation codon have been thought to inhibit translation by steric blockage of the ribosome. These represent additional mechanisms of action for antisense molecules. There are also several approaches to interfere with the excision of introns: DNA oligomers targeted at the sites of exon-intron boundary have been thought to inhibit the action of spliceosomes (Dominski and Kole, 1993; Kulka et al., 1989; Smith et al., 1986). Recently, a promising application of antisense oligonucleotide induced exon-skipping that re-directs dystrophin pre-messenger RNA processing was published (Harding et al., 2007; Wilton et al., 2007).

As unmodified DNA oligomers have low stability in biological fluids, the major challenge has been to stabilize oligonucleotide structures by a variety of modifications to improve their use as a drug. In general, three types of modifications have been tested; analogs with unnatural bases, modified ribose sugar or altered phosphate backbones. The stereochemistry at the 2'-position of the sugar has been shown to be a major determinant in the target RNA binding affinity and the activation of RNaseH (Zamaratski et al., 2001). Changes in the sugar that results a RNA-like oligomers, e.g. 2´-Fluoro or 2´-*O*-Methyl (2´-*O*-Me) substitutions, do not appear to serve as a substrate for RNaseH. In addition to alterations in the orientation of the sugar to the base, the backbone mutations e.g. methylphosphonates may also negatively affect RNaseH activation (Cazenave et al., 1989; Stein et al., 1988), however, phosphorothiate modified oligomers are excellent substrates for RNaseH (Stein et al., 1991).

The substitutions in phosphate analogs significantly influence their biological activity by affecting the electronic charge, polarity, size, hydrophobicity, basicity, and nucleophilicity properties of a nucleic acid analog. The characteristics of the oligonucleotide backbone determine how the nucleic acids interact with other nucleic acids or with proteins. A methyl substitution in the backbone lacks the charge, which reduces solubility and their cellular uptake (Miller et al., 1981), in contrast, boranophosphates mimic the natural phophodiesters in DNA: the element oxygen has six outer shell electrons and is isoelectric with borane (BH_3) also having six valence electrons (Shaw et al., 2000; Summers and Shaw, 2001). Phosphorothioate modified oligonucleotides have slightly reduced affinity to their complementary mRNA compared to unmodified DNA oligomers, but this is compensated by enhanced specificity. In addition, the first example of fully modified nucleid acid, 2'-deoxy-2'-Fluoro-D-arabinonucleic acid (FANA), has shown high RNA affinity and RNaseH activation capacity (Li et al., 2006).

Currently, there are about 30 clinical trials with antisense oligomers underway in different phases. Cancer is the major focus of these ongoing trials, although a number of other diseases are also involved. Vitravene™ (ISIS2922, fomivirsen), the first antisense drug to be approved by the FDA, has phosphorothioate oligomers designed for treatment of cytomegalovirus-induced retinitis in AIDS patients (Detrick et al., 2001; Geary et al., 2002). The targets for antisense treatment include genes that are involved in cell growth, apoptosis, angiogenesis, and metastasis. Unfortunately, compensatory pathways may be activated when the target is downregulated, therefore lowering the total antisense efficacy and specificity. Phosphorothioate linkages of DNA oligomers have been shown to interact with a wide variety of proteins, including laminin, bFGF, protein kinace C, DNA polymerase, telomerase, fibrinogen, phospholipase A_2, CD4, *Taq* polymerase and vacuolar ATPase (Bennett et al., 1994; Guvakova et al., 1995; Stein and Cheng, 1993; Yakubov et al., 1993). In addition, rapid induction of SP1 transcription factor has been reported (Too, 1998). The protein binding property may, on the other hand, protect oligomers from filtration and increase its half-life in serum. However, low doses of phosphorothioate oligomers in clinical trials are generally well tolerated.

Morpholino oligos were developed by Summerton and Weller (1997). Morpholinos were introduced first into developmental biology and later used in a wide range of model organisms. These DNA analogs have the riboside moiety of each subunit converted to a morpholine moiety (morpholine = C_4H_9NO) and use a non-ionic phosphorodiamidate intersubunit linkage instead of phosphodiester linkages. The morpholine ring contains one of four bases, adenine, cytocine, guanine or thymine. Unlike DNA oligomers, phosphorodiamidate morpholinos do not elicit RNaseH activity (Summerton, 1999). Morpholinos represent a neutral antisense molecules interfering gene expression either by binding and sterically blocking the translation machinery, or by altering splicing of pre-mRNA (Ghosh et al., 2000; Sazani and Kole, 2003). DNA modifications are shown in Figure 1.

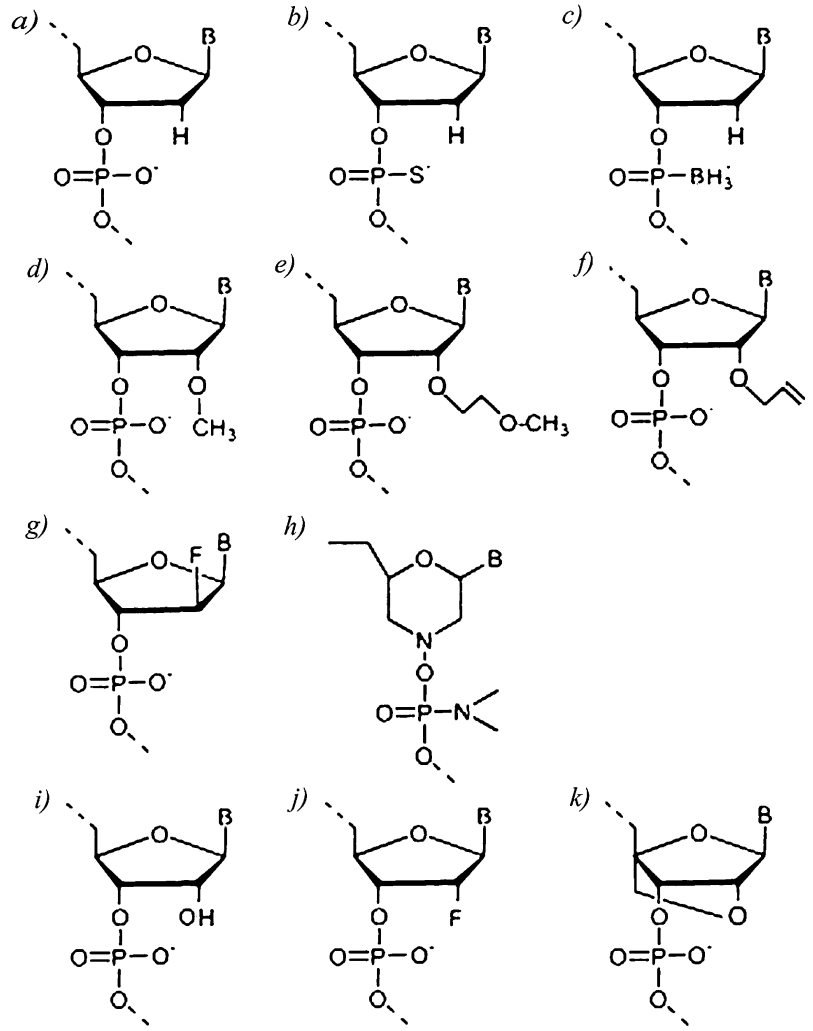

Figure 1. Schematic structures of modified DNA and RNA molecules. Commonly used backbone modifications of DNA (a) are phosphorothioate or (b) borane (c) modifications. The modifications of the 2´-position in the ribose sugar ring: 2´-O-Methyl (d); 2´-O-methoxyethyl (e); 2´-O-allyl.Fully modified nucleic acids are 2´-deoxy-2´-Fluoro-D-arabinonucleic acid (FANA, g) and morpholino (h). Commonly used RNA (i) ribose ring modifications are 2´-Fluoro- (j); 2´-O-Methyl (d); and locked nucleic acid (LNA, k) -substitutions to enhance endonuclease resistance of siRNA molecules.

2.1.1.1 CpG DNA oligomers

The mammalian immune system is equipped with pattern-recognizing receptors that detect foreign DNA. Bacterial DNA and synthetic oligomers containing a CpG unmethylated motif can potently activate immune responses e.g. via the TLR9 signaling system (Hemmi et al., 2000). These effects may interfere with therapeutic application of oligomers in gene silencing. CpG-motifs can be divided into three subsets due to their different responses in cells; i) natural killer (NK) cell activating

CpG-A motifs, ii) B-cell activating CpG-B motifs and iii) neutralizing CpG-N motifs (Kerkmann et al., 2003; Krieg, 2002).

The CpG-A motif is characterized by a central palindrome and poly (G) at the 5' and 3' end. The stimulatory effects are generally the induction of IFN type I by an IFNR-dependent pathway and strong activation of NK cells (Kerkmann et al., 2003). The stimulating mechanism has been unknown; however, it was recognized that CpG-A oligomers self-assemble to tertiary structures via G-tetrad formation of their poly(G) motifs. Spontaneous G-tetrad formation of CpG-A requires the palindrome sequence and physiological environment. CpG-A oligomers can be regarded as nucleic acid-based nanoparticles in the size range of viruses (Kerkmann et al., 2005).

The class of CpG-B oligomers have a fully phosphorothioate-modified backbone with one or more CpG motifs and no poly (G) motif (Krieg, 2001). CpG-B oligomers can induce B-cell stimulation and reduce NK cell stimulation, in contrast to CpG-A oligomers (Ballas et al., 1996; Liang et al., 1996). The negative charge of phosphorothioate CpG-B oligomers has been shown to influence to the receptor-oligomer contact on the B-cell surface leading to activation in a polyclonal manner (Liang et al., 1996; Liang et al., 2000). B-cells are induced to enter the G1 phase of the cell cycle and secrete interleukin (IL) -6 and IL-10 within a few hours of CpG-B oligomer administration (Redford et al., 1998; Yi et al., 1996).

There has been also a finding of immune neutralizing CpG motifs. Even high concentrations of calf thymus DNA (approx. 25 % CpGs unmethylated) does not cause immune activation. Further observations showed that demethylated CpG-motifs originated from the vertebrate genome do not trigger any immune response (Sun et al., 1997). The neutralizing CpG-N motif was identified as CpG dinucleotides in a continuous pattern, or preceded by a C, and/or followed by a G (Krieg et al., 1998). This motif has been shown not to induce Th1-like cytokine production; however, these CpG-N motifs were able to reduce the level of immune stimulation in response to bacterial DNA.

In addition to the effects of CpG motis mentioned above, the oligomer backbone has its own effect in stimulation: oligomers with different backbones and different sequence motifs can induce dramatically different profiles and kinetics of immune activation (Roberts et al., 2005; Sester et al., 2000; Tam et al., 1999; Zhao et al., 1996). The backbone modifications of DNA confering both nuclease resistance and improved cellular uptake are important factors mediating enhanced potency seen with phosphorothioated CpG-A and CpG-B oligomers. However, the immune stimulatory effects were abrogated if the cytosine was modified by methyl, bromo, or iodo modifications to the 5´position of the cytosine ring (Boggs et al., 1997).

The predominant effects of CpG DNA *in vitro* and *in vivo* are T helper cell 1 (Th1) -like including interferon (IFN) -γ and IL-12 responses (Klinman et al., 1996). The immuno-stimulatory effect of CpG-motifs has been useful when applied as an adjuvant with several types of vaccines (Davis et al., 1998; Lipford et al., 1997; Roman et al., 1997). Promising results have been obtained co-vaccinating immunosuppressive HIV-

patients with CpG7909 adjuvant and hepatitis B virus vaccine Engerix-B® (Cooper et al., 2005; Cooper et al., 2004). In contrast, it has been shown that immunostimulatory CpG oligomers may induce in early states hepatic injury and liver dysfunction in rats (Slotta et al., 2006). The ability of CpG oligos to preferentially activate Th1-type immune responses is therapeutically used in various diseases including cancer and infectious diseases. In addition, CpG oligomers may have potential in treating allergy and asthma, which result in T helper cell 2 (Th2)-type immune activation (Kline, 2000; Kline et al., 2002). CpG oligomers induced, Th1-derived cytokines IL-12 and IFN-γ are known to suppress allergen induced Th2 responses including IL-4, IL-5 and IL-13 secretion (Hessel et al., 2005; Kline, 2000; Kline et al., 2002). In addition to cytokine response, it has been shown that during antitumor treatment by monoclonal antibodies (mAbs) against tumor antigens, CpG oligomers have been shown to increase antibody dependent cellular cytotoxicity (Wooldridge et al., 1997).

2.1.2 Ribozymes

Ribozymes are catalytic RNAs that have the ability to break and form covalent bonds (Forster and Symons, 1987). Ribozymes have a biological role in the processing of RNA molecules and in the regulation of protein translation. Studies on the mechanism of RNA self-splicing have revealed details of the catalytic core and the secondary and tertiary structures of RNA folding leading to ribozyme-mediated cleavage; ribozymes cleave only at the specific location using base-pairing and tertiary interactions to help align the cleavage site. Cleavage reaction has been shown to happen in the presence of Mg^{2+} or other divalent cation (Dahm and Uhlenbeck, 1991; Perreault et al., 1991). Ribozymes can catalyze reaction with a single turnover when ribozymes are modified during the process. The capacity for multiple turnovers of ribozyme binding, cleavage and dissociation provides an advantage in gene function studies (Stage-Zimmermann and Uhlenbeck, 1998).

Ribozymes are divided into two classes on the basis of their size. Large ribozymes include group I and II introns, which are spliced out of their hnRNA, and the RNA subunit of RNAse P (Altman and Guerrier-Takada, 1986; Kirsebom, 2002). Small ribozymes includes hammerhead and hairpin structures (size 50 to 150 nt) and VS (Varkud Satellite) ribozymes (Lafontaine et al., 2001; Stage-Zimmermann and Uhlenbeck, 1998; Walter et al., 1998). These small ribozymes catalyse single turnover reactions; however, by modifications these small RNAs can be manipulated to induce intermolecular effects (*trans*-acting) and to cleave with multiple turnovers. The hammerhead ribozyme is the simplest in terms of size and structure and can readily be engineered to perform intermolecular cleavage on targeted RNA molecules. These properties make this ribozyme a useful tool for inactivating gene expression and a potential therapeutic agent. Structural modifications have been applied to enhance nuclease resistance. In contrast, some modifications that improve stability may dramatically interfere with the catalytic activity. It has been shown that the almost fully 2'-*O*-Me modified ribozyme had increased catalytic activity and nuclease resistance (Yang et al., 1992). However, partial 2'- *O*- allyl modification, 2'-Fluoro substitution in pyrimidines or two additional phosphorothioate substitutions in stem II increased the

serum stability but decreased catalytic activity compared to non-modified ribozyme. In one common modification, the 3' end is protected by inverted thymidine (iT), the fourth nucleotide is a 2'-C-allyl uridine surrounded by five unmodified ribonucleotides: the rest of the structure has a 2'-*O*-Me-ribose structure. This modification increases the ribozyme half-life from one minute to 10 days (Grunweller et al., 2003; Kurreck, 2003).

Several ribozyme structures have presently been reported to be in Phase I and Phase II clinical trials. The main obstacle may be the delivery of the ribozyme into the target tissue. The first clinical trials applying ribozyme activity were developed to inhibit replication of human immunodeficiency virus (HIV) (Brower et al., 1998; Rowe, 1996), and the infections of hepatitis C virus (HCV) and chronic hepatitis B virus (HBV) (Macejak et al., 2001; Michienzi et al., 2000).

DNAzymes are DNA analogues of hammerhead ribozymes that have the capacity to cleave RNA molecules in an enzymatic fashion (Feldman and Sen, 2001; Santoro and Joyce, 1997). Opposite to ribozymes, DNAzymes do not exist in nature. They have a structure of three domains: a central catalytic motif flanked by two arms (arm I and II) that in a sequence-specific manner bind to the target RNA. The catalytic activity is metal ion-dependent and the DNAzymes bearing a conserved 10-23 catalytic motif cut the phosphodiester bond between purine and pyrimidine. DNAzymes may catalyze multiple turnovers, thus having a potential in drug development. DNAzymes can be expressed endogenously; however, DNAzymes can also be phosphorothioate modified and this may also allow the systemic delivery *in vivo* without a transfection vehicle.

2.1.3 RNA interference (RNAi)

Post-transcriptional gene silencing (PTGS) mediated by a double stranded RNA (dsRNA) molecule represents an evolutionarily conserved defence mechanism against viruses and transposons (Hamilton and Baulcombe, 1999; Vastenhouw et al., 2003). Transcriptional gene silencing (TGS) has been associated with DNA and histone methylation (Chan et al., 2006; Matzke et al., 1989; Zilberman et al., 2003). In the 1990's dihydroflavonol 4-reductase or calchone synthase genes were introduced into petunias to evaluate the effect of increased expression. Surprisingly the method led to suppression of these genes at mRNA level (Napoli et al., 1990; van der Krol et al., 1990). Later, this gene silencing phenomenon was shown in *Caenorhabditis elegans* and named RNA interference (Fire et al., 1998). RNAi is highly a conserved gene silencing mechanism that utilizes double stranded RNA molecules to induce mRNA degradation or inhibits translation in sequence specific manner. The size of short interfering RNA (siRNA) molecules is restricted to 21 – 22 nucleotides by Ribonuclease III cleavage. mRNA degradation is mediated by activated RNA induced silencing complex (RISC). The nuclear-effector complex is named RNA induced transcriptional complex (RITS).

The first step of the RNAi pathway comprises the recognition of a dsRNA molecule and its processing into about 21 nt siRNA molecules with characteristic 2 nt 3´overhangs by the RNase II-like enzyme Dicer (Bernstein et al., 2001; Hammond et al., 2000; Zamore et al., 2000). The Dicer contains an N-terminal RNA helicase

domain, a Piwi, Argonaute, Zwille/Pinhead (PAZ) domain, two RNase III domains, and a C-terminal dsRNA binding motif. Second, siRNAs are handed over to the catalytic core of the RNAi machinery by a RISC loading complex (RLC)(Liu et al., 2003a). RLC has been suggested to sensor both the presence of the 5´-phosphate group and the 5´-end stability of the siRNA structure therefore determining which strand is associated with the core RISC protein Argonaute (AGO) (Hutvagner, 2005; Tomari et al., 2004). There have been two main models to illustrate the unwinding of the siRNA duplexes during RISC activation. The first model proposes that an ATP-depended helicase unwinds the siRNA molecule resulting one strand siRNA binding into AGO2 activating RISC (Nykänen et al., 2001; Zamore et al., 2000). However, recent results implicate an alternative model (Figure 2) in which the siRNA molecule is loaded into RISC as a duplex and the passenger-strand is cleaved in action by AGO2 as a first target (Leuschner et al., 2006; Matranga et al., 2005; Rand et al., 2005).

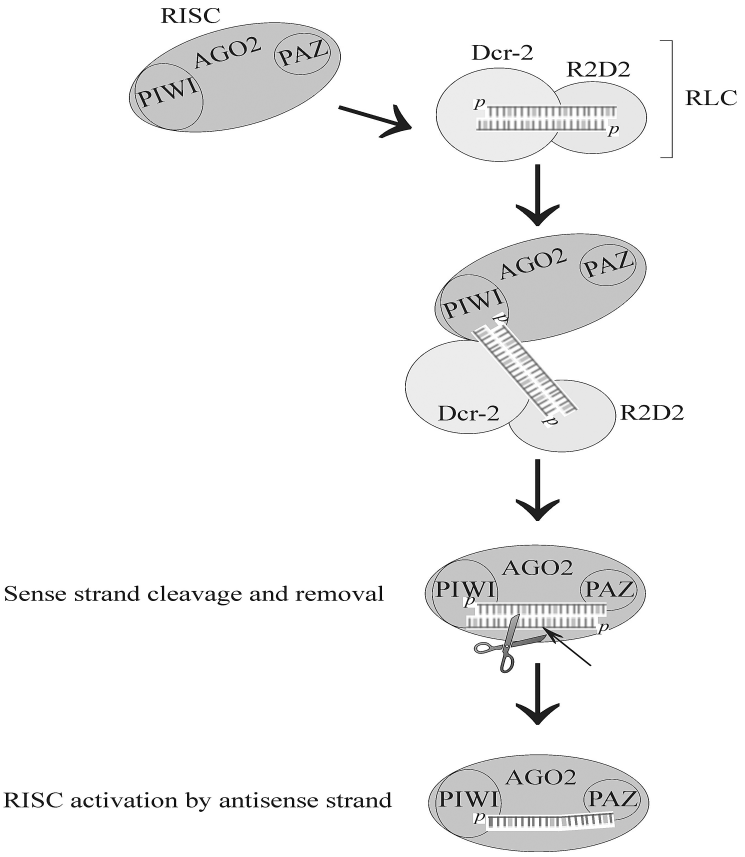

Figure 2. General summary of the current RISC assembly and activation model. Dicer (Dcr-2) associated with dsRNA-binding partner (R2D2) build the RISC loading complex (RLC). RLC senses the stability of the siRNA molecule 5´-end, and RLC directs the siRNA molecule into RISC in an asymmetry dependent manner. AGO2 slices the sense strand (also referred as a passenger strand) between the nucleotides 9 and 10, as counted from the 5´-end. PIWI domain binds to the phosphorylated (*p*) 5´-end and PAZ domain binds the 3´-end overhangs of the antisense strand (also referred as a guide strand) in the activated mature RISC. [Modified from Matranga et al. (2005)]

Argonaute proteins comprise an important role in RISC activation and function. The Argonaute protein family is defined by the presence of PIWI and PAZ domains. Detailed studies of PIWI and PAZ domains have revealed that the Ago-PIWI domain shows a RNaseH-like structure with a capacity to bind the 5´end of the siRNA antisense strand (Ma et al., 2005a; Parker et al., 2005; Song et al., 2004). The AGO-PAZ domain has been suggested to take action at the beginning of RISC activation by binding the two-base 3´overhangs of the siRNA antisense strand, which is released during siRNA-target binding (Lingel et al., 2004; Liu et al., 2004; Yan et al., 2003). Both siRNAs and microRNAs (miRNAs) can enter and activate RISC; however, far less is known about miRNA:RISC assembly than siRISC assembly.

2.1.3.1 Nuclear pathways

RNAi has been found to have a central role in heterochromatic gene silencing. Eucaryotic chromosomes are characterized by the presence of condensed tracts of heterochromatin. Heterochromatin surrounds the centromeric region and has a low density of expressed genes but a high density of repetitive DNA including transposable elements and satellite DNAs. Heterochromatin maintains compact and visible structure during mitosis therefore differing from the euchromatin that undergoes a typical cycle of condensation and unravelling (Heitz, 1928). Heterochromatin has diverse functions: maintenance of genome stability, proper chromosomal segregation, and prevention of telomere fusion. Heterochromatin is also involved in regulating gene expression mediating both repression and activation (Lippman and Martienssen, 2004; Piacentini et al., 2003; Verdel et al., 2004).

Methylation of the C^5 position of cytosine residues in DNA has been recognized as an important mechanism in epigenetic silencing. In addition to direct DNA methylation, histone modifications have also been defined as epigenetic modifiers. Histone methylation can be a marker for both active and inactive regions of chromatin. Methylation of lysine 9 on the N terminus of histone H3 (H3-K9) is a hallmark of silent DNA throughout heterochromatic regions whereas methylation of lysine 4 of histone H3 (H3-K4) denotes activity and is found predominantly at the promoters of active genes (Litt et al., 2001; Noma et al., 2001).

The link between RNAi and epigenetic silencing arose from the observation in plants that dsRNA could induce DNA methylation and heritable transcriptional silencing (Mette et al., 2000). Two possible models for the role of RNAi in heterochromatic silencing have been proposed. First, siRNA molecules may interact with DNA and histones via the RITS -complex (Verdel et al., 2004). Alternatively AGO may slice heterochromatic transcpits, products of RNA-dependent RNA polymerase (RdRP). These transcripts then recruit the histone modification apparatus (Martienssen, 2003; Motamedi et al., 2004). Recently, several connections between impared histone methylation and human diseases e.g. different types of cancer, congenital diseases and viral latency has been shown (Moss and Wallrath, 2007). In cancer cells, tumor-suppressor genes including *Rb*, *APC* and *BRCA1* have been silenced by extensive DNA methylation (Dobrovic and Simpfendorfer, 1997; Hiltunen et al., 1997; Stirzaker et al.,

1997). Substrate analogues of DNA methyl transferases (DNTMs), 5-azacytidine (Vizada™) and 5-aza-deoxycytidine (decitabine), have been used in clinical trials as DNTMs inhibitors in patients with myelodysplastic syndrome (Daskalakis et al., 2002; Silverman et al., 2002).

2.1.3.2 microRNA pathway

microRNAs (miRNAs) are endogenous, non-coding RNA transcripts ~22 nt in length that can form local hairpin structures (Figure 3). In the nucleus a microprocessor complex containing Pasha, a double strand RNA binding protein and Drosha, a class II RNase enzyme are located (Lee et al., 2003). This complex processes primary miRNAs (pri-miRNAs), ranging in size from several hundred nucleotides to several kilobases, into approximately 70 nt hairpin-shaped pre-miRNAs, the cleavage at approximately two helical turns from the loop structure (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004). Pre-miRNAs bear a two nt 3´-overhang which contribute to pre-miRNA export out of the nucleus to the cytoplasm by the RanGTP/exportin-5-depended mechanism (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003). In cytoplasm 3´-overhang of the pre-miRNA specifies the cleavage by Dicer near the stem loop into ~22 nt miRNA duplexes. Mature miRNA duplex enter RISC in an asymmetrical manner (Khvorova et al., 2003; Tomari et al., 2004) and activated miRISC leads to translational repression or mRNA degradation, depending on the miRNA complementary to the target mRNA.

lin-4 miRNA was first identified to have antisense binding sites in the *lin-14* 3´-untranslated region (3´-UTR) and repressed LIN-14 protein translation without change at the *lin-14* mRNA level (Lee et al., 1993; Wightman et al., 1993). miRNAs are named using an miR-prefix and a unique identifying number. Identical or very similar miRNAs have the same number, regardless of organism. Up to date, miRBase Target Database provides 640 published miRNA sequences for several vertebrata species (e.g. homo sapiens, mus musculus, rattus norvegicus), 77 for Drosofila melanogaster and 112 for Caenorhabditis elegans (Griffiths-Jones et al., 2006). miRNAs utilize the same protein complex machinery as siRNAs. miRNAs direct diverse regulatory pathways including developmental timing control, hematopoietic cell differentation, apoptosis, cell proliferation, and organ development (Brennecke et al., 2003; Chen et al., 2004; Johnston and Hobert, 2003; Xu et al., 2003).

There are now over 500 confirmed human miRNA genes (05/2007, miRBase, Sanger Institute). Each miRNA has been suggested to have the potential to regulate hundreds of mRNAs and their role in biological pathways is significant. miRNAs might be viable therapeutic targets for a wide range of diseases, including cancer (Krutzfeldt and Stoffel, 2006). miRNAs have been shown to have differential expression profiles in human cancer (Lu et al., 2005). These tumors also displayed reduced apoptosis (He et al., 2005). Gene expression analysis has also shown widespread reduction of miRNA gene expression e.g. *mir-143, mir-145* and *let-7* in tumors (Johnson et al., 2005; Michael et al., 2003; Takamizawa et al., 2004). Although most miRNAs display a reduced pattern in tumors, some miRNAs have oncogenic potential, e.g. miRNA

cluster, *miR-17~92*, has been shown to be overexpressed in some lymphomas and solid tumors (Eis et al., 2005; Metzler et al., 2004). This was the first evidence that non-coding RNA can function as a mammalian oncogene.

miRNAs do not encode proteins; therefore, they are not traditional targets of small molecule inhibitors. However, the widespread role of miRNAs makes them attractive as therapeutical targets. The base-pair interaction between miRNA and mRNA is the logical target for an inhibitor. miRNA-mRNA base-pair interaction has been interrupted by transfecting modified antisense RNA molecules complementary to miRNAs leading to blockage of the miRNA-RISC complex. These miRNA antisense molecules are termed antagomirs or anti-miRNA oligonucleotides (AMOs). Hutvagner et al. (2004) demonstrated inhibition of let-7 function in HeLa cells as well as in *C. elegans* larvae by using 31-mer 2´-*O*-Me-modified RNA oligonucleotides. Their results indicated that 2´-*O*-Me-modified RNA oligonucleotides blocked RISC function by binding sequence specifically to the miRNA guide strand. Esau et al. applied (2004) 2'-*O*-methoxyethyl (2'-*O*-MOE) phosphorothioate-modified antisense RNA oligonucleotides targeting miRNAs and demonstrated the potential inhibition of adipocyte differentiation by mir-143 antagomir. Locked nucleic acid (LNA) has been used as a probe for Northern hybridization to detect miRNAs (Valoczi et al., 2004). LNA substitution contains a methylene linkage between the 2´-oxygen and 4´-carbon position of the ribose ring. This bridge locks the ribose ring in the 3´-*endo* conformation. Combinations of modifications have also been used to inhibit miRNA function and used for e.g. investigation of mir-21 role in the regulation of apoptosis associated genes (Chan et al., 2005). Intravenous administration of antagomirs targeting miR-16 (abundant), miR-122 (liver-specific), miR-192 and miR-914 has resulted in the reduction of corresponding miRNA levels in liver, lung, kidney, heart, intestine, fat, skin, bone marrow, muscle, ovaries and adrenals (Krutzfeldt et al., 2005). Antagomir-122 was delivered into hepatocytes and treatment with antagomir-122 resulted in miR-122 degradation. miR-122 has been predicted to be involved in the regulation of cholesterol biosynthesis genes and loss of miR-122 has shown to led to reduced plasma cholesterol levels. These findings characterized the utility of antagomirs complementary to disease related miRNA sequences.

2.1.3.3 siRNA pathway

Foreign dsRNA is produced after viral infection, random transposon or transgene intergration into a host genom. An innate cellular defence system responds to these supplementary dsRNA elements by post-transcriptional silencing, which is commonly referred to as RNAi. The mediators of specific mRNA destruction are short siRNA molecules which are produced by enzymatic Dicer-mediated cleavage in the cell. siRNAs are ~21 nt in length and have characteristic 2 nt 3´- end overhangs (Figure 3 b). siRNA duplexes also contain 5´-phosphates and free 3´-hydroxyl groups (Elbashir et al., 2001a) . The 5´-phosphate has been shown to be essential for target mRNA degradation; however, rapid kinase activity in the cell has been shown to phosphorylate the 5´-ends of synthetic siRNA molecules (Nykänen et al., 2001).

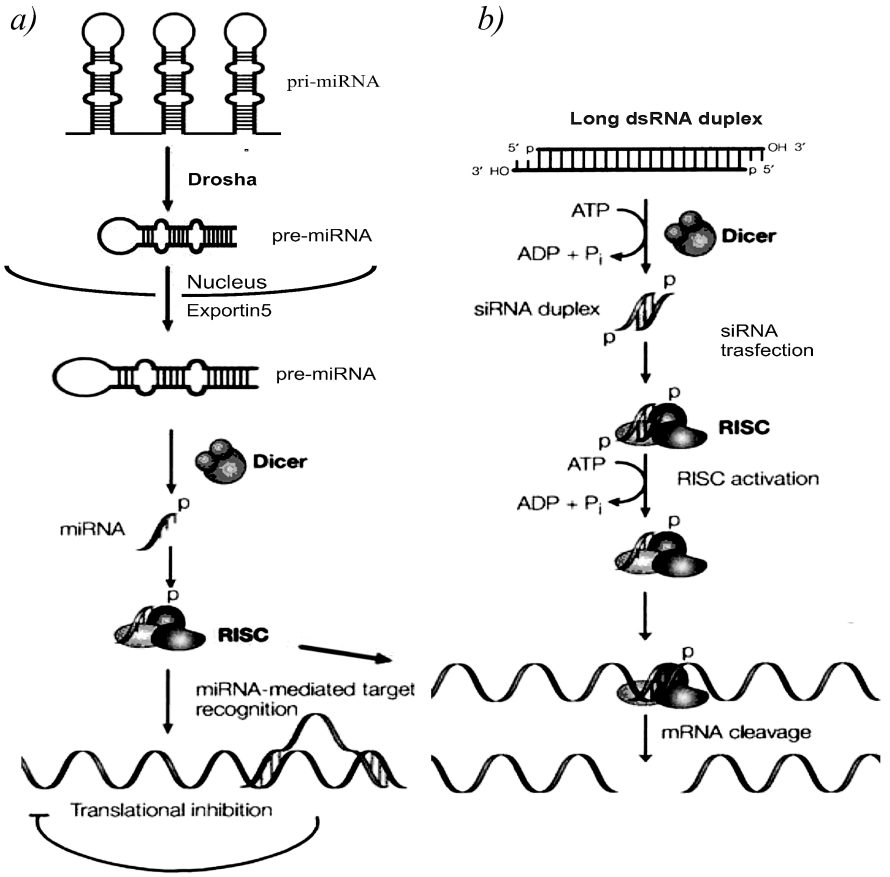

Figure 3. Schematic figure of miRNA and siRNA pathways. miRNA pathway (a) begins from endogenously produced primiRNAs that are processed to pre-miRNAs by Drosha in nucleus. Pre-miRNAs are transported by RanGTP/Exportin5 to cytoplasm. Dicer processes pre-miRNAs into mature miRNAs that activate RISC. Depending on the complementarity, miRNAs can either block target protein translation or lead to siRNA-like target degradation. siRNA pathway (b) starts at exogenously produced long dsRNAs that are cleaved by Dicer into ~21 nt long siRNA molecules. Alternatively fixed length siRNA molecules are transfected into cells. Antisense strand activates RISC and catalyse target mRNA degradation. [Modified from Dykxhoorn et al. (2003)]

Using siRNA for gene silencing is a rapidly evolving tool in molecular biology. There are several methods for preparing siRNA, such as chemical synthesis, *in vitro* transcription, siRNA expression vectors, and PCR expression cassettes. Irrespective of which method is used, the first step in designing a siRNA is to choose the siRNA target site. Further approaches have been addressed to siRNA structural characters: synthetic and modified siRNA molecules have been succesfully shown to induce RNAi in mammalian models both *in vitro* and *in vivo*. This evolves new biological challenges to further utilize siRNA molecules as a clinical tool.

2.1.3.3.1 siRNA design

The first siRNA desing rules were described by Tuschl et al. (2003) at their web page http://www.rockefeller.edu/labheads/tuschl/sirna.html. These rules were based on the results of systematical analysis of siRNA molecules in Drosophila cell lysates (Elbashir et al., 2001b; Tuschl et al., 1999). The Tuschl et al. (2003) rules recommend using siRNA duplexes composed of 21-nt sense and 21-nt antisense strands, paired in a manner to have a 2-nt 3'-end overhang (Elbashir et al., 2001a). The sequence of the 2-nt 3'-end overhang may have a small contribution to the specificity of target recognition restricted to the unpaired nucleotide adjacent to the first base pair. The targeted region should be selected 50 to 100 nt downstream of the start codon avoiding 5'- or 3'- UTRs and regions near the start codon. UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNP or RISC endonuclease complex. GC content was suggested to be between 30% and 70%. Tuschl et al. also recommended 3´-TT overhangs, not due to better activity over other nucleotides, but basically to help order siRNAs in the correct 5´- 3´- direction. In addition, BLAST-search (NCBI database) of the selected siRNA sequence against EST libraries is recommended to ensure that only one gene is targeted.

In addition to these basic rules, more precise thermodynamical factors were discovered distinguishing functional and non-functional siRNA sequences based on their internal stabilities and asymmetric properties (Khvorova et al., 2003; Schwarz et al., 2003). Internal stabilities of dsRNA molecules were calculated by using the nearest neighbour method (Freier et al., 1986). This revealed a strand bias of functional siRNAs: the 5´-end of the antisense strand showed lower stability than 3´-end. Non-functional siRNA exhibited a mirror stability signature. More detailed siRNA design rules were obtained by studying differences of nucleotide distribution between functional and non-functional siRNA sequences (Reynolds et al., 2004). They studied 180 siRNA sequences targeting every second position of the targets, firefly luciferase and human cyclophilin B. Their results showed the distinct effect of correct location siRNA molecule on its target sequence: the efficacy of two siRNAs targeting sequences only two nucleotides apart may vary significantly from highly efficient to non-efficient. In addition, Reynolds et al. (2004) first showed the effects of nucleotide distribution on siRNA efficacy.

Although the siRNA molecules used for RNAi have relative high stability and activity *in vitro*, there are motivations for chemical modifications for one or both of the strands. Primarily, chemical modifications are substituted to the dsRNA molecule to enhance the endonuclease stability and to increase the half-life of the siRNA molecules in blood circulation *in vivo*. It has also been suggested that chemical modifications may improve the biodistribution and pharmacokinetic properties that are important for therapeutic applications. The RNAi mechanism is sensitive for modifications disrupting the A-helix geometry of dsRNA, hindering the sense strand cut-off site or blocking the 5´-OH terminus of the antisense strand (Chiu and Rana, 2002; Rand et al., 2005; Schwarz et al., 2002; Schwarz et al., 2004), therefore, careful desing of modified siRNA molecules is required.

Backbone modifications (Figure 1) offer nuclease resistance but may lead to small loss in binding affinity (Braasch et al., 2003; Harborth et al., 2003). Cytotoxic effects were also observed when every second phosphate of the siRNA dyplex was replaced with phosphorothioate (Amarzguioui et al., 2003). RNA target destruction has been shown to be an Mg^{2+} dependent reaction. Further, it has been proposed that the non-bridging oxygen of the scissile phosphate in RNA backbone is an important factor in endonucleolytic cleavage. Therefore backbone modifications in the sense strand replacing the non-bridging oxygen may restrain the essential sense strand cleavage necessary for RISC activation (Schwarz et al., 2004). However, chirally pure boranophosphate modifications were more tolerated in the sense strand than phosphorothioate modifications (Wan and Shaw, 2005). Interestingly, single strand RNA molecules with a boranophosphate backbone have been reported to induce efficient and long lasting RNAi and have, therefore, been investigated as a novel class of therapeutic agents (Hall et al., 2006).

The observation that 2′-OH is not required for the RNAi pathway opened up new possibilities for chemical modifications (Chiu and Rana, 2003). Subsequently, substitutions at the 2′-position of the ribose have been extensively investigated, due in large part to the protection against endonucleases: 2′-OH of the ribonucleotide distinguishes RNA from DNA and it is a direct target for hydrolysis of the RNA backbone during RNase based degradation. Further, modifications with high preference for C3′-*endo* sugar pucker are well tolerated supporting the requirement for conformationally RNA-like A-type helical characteristics for effective gene silencing (Chiu and Rana, 2002; Chiu and Rana, 2003). Several results show that 2′-Fluoro substitutions in both sense and antisense strands are well tolerated (Braasch et al., 2003; Chiu and Rana, 2003; Harborth et al., 2003; Layzer et al., 2004). 2′-Fluoro substitutions increase the stability in human plasma. siRNA molecules with internal LNA modification retain full RNAi activity in mammalian cells (Braasch et al., 2003). It has also been suggested that incorporations of LNA in the siRNA molecule enhances bio-stability and may reduce undesired, sequence-related off-target effects (Elmen et al., 2005). 2′-*O*-Me-substitution has a protective effect against endonucleases; however its sterical properties are more bulky than the 2′-OH (Chiu and Rana, 2003; Cummins et al., 1995; Czauderna et al., 2003). There are several reports that siRNA molecules containing full 2′-*O*-Me substitutions into sense, antisense or both strands abolishes RNAi activity (Amarzguioui et al., 2003; Braasch et al., 2003; Chiu and Rana, 2003; Elbashir et al., 2001b). However, contradictory data have been presented demonstrating that fully 2′-*O*-Me modified siRNA mediate PTEN mRNA degradation (Kraynack and Baker, 2006). Interestingly, chemical modifications may change the physical properties of dsRNA molecules. 2'Fluoro-modification provide a significant increase in target binding affinity, ΔT_m of 2 to 4 °C per modification (Kawasaki et al., 1993). In addition, the LNA modified siRNA molecules exhibit T_m over 90 °C (Braasch et al., 2003). The extremely high thermodynamic dsRNA stabilities caused by chemical modifications does not seem to affect function of siRNA within RISC.

2.1.3.3.2 Off- target effects of siRNAs

In addition to target gene suppression by specific siRNAs, there have been several reports of non-specific gene target effects. Jackson et al. (2003) showed a large scale off-target gene regulation induced by siRNA molecules. Their data showed both sense and antisense strand derived off-target effects. Sequence alignment analyses revealed that even a short stretch (< 6-8 nt) of the siRNA changed the gene expression pattern not related to the target and was suggested to be due to cross-hybridization to transcripts of similar sequence. These findings were consistent with the data of miRNAs that seven nucleotides of 5'-end of the antisense strand play an important role in transcript silencing by stabilizing the miRNA-mRNA interaction. These results presented a challenge to siRNA design and to achieve accurate results by siRNAs, gene knock down phenotype should be confirmed by 3-4 siRNAs and with suitable nontargeting controls as described in Nature Cell Biology: Whither RNAi? (Editorial, 2003). A computational study of off-target effects showed that these effects are real but not restrictive for RNAi effects (Qiu et al., 2005). However, the length for siRNAs with high specificity and low probability for off-target reactivity were set for scales of 21 nucleotides; the length that was mostly observed efficient *in vivo*.

Non-specific effects of siRNA molecules may also be characteristic for extracellular dsRNA invasion including upregulation of type I IFNs α and β (Judge et al., 2005; Ma et al., 2005b). The 5´-UGUGU-3´ element incorporated in a siRNA sequence has especially been shown to have immunostimulatory potential (Judge et al., 2005). Upregulation of interferons mediate activation of the JAK/Stat pathway and global upregulation of IFN- stimulated genes (Sledz et al., 2003). Using synthetic siRNA molecules, Sledz et al. (2003) noticed at least a twofold induction of 52 out of 850 putative interferon-stimulated genes examined. Vector derived shRNAs have also shown induction of nonspecific genes, most of them downstream of the interferon pathway (Bridge et al., 2003). DNA vectors that are commonly used for stable shRNA expression under RNA polymerase III (pol III) promoters were shown to trigger an interferon response. This may not be noticed in some commonly used tumor cells having a defect in interferon response. Additionally, certain siRNA sequences and cell types induce activation of TNF-α and IL-6 (Sioud and Sorensen, 2003). Activation of nonspecific effects by siRNA molecules highlights several issues to consider in RNAi experiments: (i) siRNA sequence and method of siRNA production (ii) the target cell type or tissue (iii) the effects of the siRNA delivery mechanism and pathway (iv) the siRNA molecule and the delivery lipid ratio used for transfection (Heidel et al., 2004).

2.1.4 Comparison of gene knock-down methods

There has been a comparative *in vivo* study between siRNAs and antisense DNA oligomers in rats. siRNAs were found more effective than antisense oligos *in vitro* (Senn et al., 2005). Target knock-down molecules were administered by intracerebroventricul application: siRNA delivery was enhanced by use of cell detergents. Fluorescense labeled molecules showed that only antisense DNA oligomers had the capacity to be delivered into brain tissue and silence the target gene *in vivo*.

These results suggested the need for development of an optimized vector system for siRNAs to bypass the brain-blood barrier. However, in this study, the antisense DNA oligomers were 2'-*O*-MOE and phosphorothioate modified and thus endonuclease resistant: siRNAs were unmodified and more likely degraded in serum before reaching the target. In the other study, the specificity of siRNAs and antisense DNA oligomers were compared and examined by using microarray expression profiles in 48 and 72 h timepoints. The microarray analysis revealed that already at 48 h a target-dependent pattern of gene alterations was detectable; however, a large number of non-specific changes due to control nucleic acids were also present. These non-specific alterations become more apparent at the 72h timepoint suggesting the preferable short time points for result follow-up post-transfection to avoid false phenotypes (Bilanges and Stokoe, 2005). Most of the upregulated genes were involved in the cytokine pathway or in inflammatory response, and surprisingly Dicer-1, an essential part of the RNAi mechanism. The analysis revealed an antisense DNA oligomer- and siRNA-specific signature indicating that longer incubation time dramatically affects the global gene expression profile in a target gene independent manner.

Theoretically, ribozymes and DNAzymes should be better gene knock-down tools than antisense DNA oligomers due to their capacity to cleave multiple copies of target mRNA, whereas antisense DNA oligomers are expexted to cleave only one target. However, LNA modified DNAzymes showed a lowered target cleavage rate when compared to RNase H recruiting phosphorotioate modified antisense DNA oligonucleotides (Fluiter et al., 2005). A difficulty with ribozymes and DNAzymes *in vivo* is the balance with modifications that improve the stability and increases their half-life intracellularly and in serum. Modifications may also increase the affinity to the target mRNA and therefore, drastically reduce the catalytic activity, since release of ribozymes and DNAzymes after cleavage is a rate-limiting step in the catalytic cycle. Another factor important to ribozymes and DNAzymes is the requirement of the specific cleavage site that triggers the chemical step of the kinetic activity, not only the access to the site free for Watson-Crick base pairing.

2.2 Tartrate-resistant acid phosphatase

Acid phosphatase (AcP) activity is ubiquitously expressed in all human tissues. Various AcPs can be differentiated according to structural and immunological features, tissue distribution, and subcellular location. AcPs have been devided into four groups according to their cellular origin and to their sensitivity toward inhibition by (L+)-tartrate: 1) lysosomal; 2) prostatic; 3) erythrocytic; and 4) isoenzyme 5. Isoenzyme 5 was identified by its cathodal electrophoretic mobility at pH 4 and by its resistance to inhibition by (L+)-tartrate and was originally found in hairy cell leukemia cells (Li et al., 1970; Yam et al., 1971).

The most common term for isoenzyme 5 is tartrate-resistant acid phoshatase, TRACP. TRACP (AcP5, EC 3.1.3.2), is a metal containing enzyme that is common to bone and many other tissue macrophages. TRACP contain a catalytically active di-iron (Fe^{2+}/Fe^{3+}) cluster that is essential for its enzymatic activity (Vincent and Averill,

1990; Vincent et al., 1991). Because the bound metal ions confer an intense color on these enzymes, they are also known as purple acid phosphatases (PAPs). Reduction of the active site binuclear center to a mixed valency Fe^{3+}-Fe^{2+} form is required for activation and this corresponds to a shift in color from purple to pink (Halleen et al., 1998b). TRACP is synthesized as a low-active proenzyme that is activated by cleavage. The cysteine proteinases, papain and cathepsins B, K and L, have been shown to cleave the 34 kDa proenzyme into highly active two subunit isoforms: TRACP 5a and TRACP 5b (Ljusberg et al., 1999; Ljusberg et al., 2005). These isoforms have a different pH optimum; pH 4,9 for isoform 5a and ph 5.5-6 for isoform 5b (Lam et al., 1978). The carbohydrate content of the isoforms also differed with only 5a containing sialic acid (Lam et al., 1981). The protein structure of TRACP (Figure 4) can be described as a double β-sheet sandwich surrounded on both sides by α-helixes (Uppenberg et al., 1999). TRACP has N-linked glucolyzation sites and near the active site an uncovered protease sensitive loop has been determined (Guddat et al., 1999; Halleen et al., 1996; Lindqvist et al., 1999; Uppenberg et al., 1999).

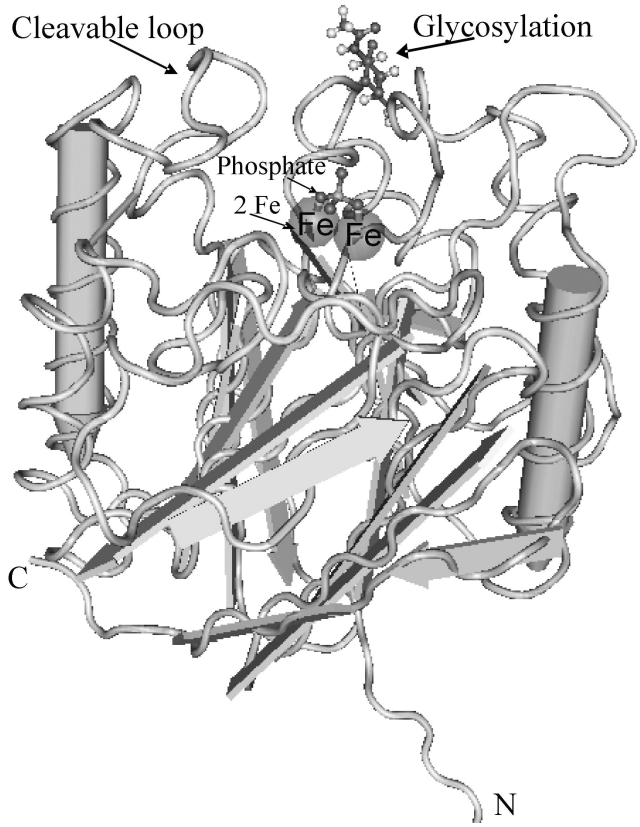

Figure 4. TRACP structure. The three dimensional structure of human purple acid phosphatase. The figure was produced with Cn3D version 4.1 and data was based on human TRACP protein structure analysis (Strater et al., 2005). The active site with two iron atoms and phosphate ligand is located at the interface between β-sandwich. The protease sensitive loop (residues 145-160) is located close to the active site.

2.2.1 TRACP activity in tissues

Studies in humans, mice and rats have shown that TRACP is widely distributed in normal tissues (Hayman et al., 2000b; Hayman et al., 1991; Hayman et al., 2001; Reddy et al., 1995a). mRNA levels of TRACP are expressed in the spleen, liver, linings of the gastrointestinal track, lung thymus, and skin. TRACP activity is highest in bone, spleen liver, thymus and colon. Lower activities were found from the lung, stomach, skin, brain and kidney. Traces of TRACP activity was measured in the heart, testis and muscle. Phosphatase activity does not correlate with the amount of the protein present (Hayman et al., 2000b). TRACP activity can also be detected in specialized macrophage progeny, namely in Kupffer cells of the liver, and in pulmonary alveolar macrophages (Yamamoto and Nagai, 1998; Yaziji et al., 1995). It has been shown that TRACP detection in diverse tissues is primarily due to the wide distribution of TRACP positive dendritic cells (Hayman et al., 2000a). In addition to osteoclasts in bone, TRACP expressing osteocytes have also been identified from areas at 200 μm distance from the bone-resorbing surface and TRACP positive osteoclasts (Nakano et al., 2004). Recently, endogenous TRACP expression has been detected in an other bone associated cell type; in osteoblast-like cells (Perez-Amodio et al., 2005). Further it has been shown that endocytosis of osteoclast derived TRACP by osteoblasts may lead to TRACP inactivation thus suggesting a regulatory role for TRACP in bone remodelling (Perez-Amodio et al., 2006).

An osteoclast is a type of bone cell that resorbs bone by removing the mineralized matrix. They are formed by the maturation and fusion of the monocyte/macrophage lineage cells. Osteoclasts are characterized by multiple nuclei (Figure 5). During active resorption, osteoclasts form specialized and polarized cell membrane structures: a ruffled border (RB), a functional secretory domain (FSD) and a sealing zone (Mulari et al., 2003; Väänänen et al., 2000). By using immunostaining techniques, high amounts of TRACP have been found in bone resorbing osteoclasts in which TRACP has been shown to localize in transcytotic vesicles and to co-localize with cathepsin K (Vääräniemi et al., 2004). By using the immunogold labelling technique, TRACP has been localized at the RB membrane compartments and in intracellular vesicular structures (Hollberg et al., 2005; Reinholt et al., 1990). It has been suggested that TRACP is activated by cathepsin K in acidic transcytotic vesicles locating near RB and resorption lacuna. Vesicles are neutralised closer to the FSD and the ROS generating activity of TRACP is activated. ROS generated by TRACP then finalizes the matrix degradation before secretion. In serum, TRACP 5b circulates as a complex that prevents formation of the ROS at the neutral pH (Ylipahkala et al., 2003). In plasma, TRACP concentration correlates with the bone resorbing rate (Janckila et al., 2003). Serum TRACP activity is increased in conditions in which bone resoption is enhanced: Gaucher´s disease, hyperparathyroidism, osteoporosis, and in some cancers (Chamberlain et al., 1995; Lam et al., 1984; Lau et al., 1987; Robinson and Glew, 1980). TRACP 5b is widely used as a cytochemical marker of osteoclasts and bone resorption (Chambers et al., 1987; Halleen, 2003; Minkin, 1982; Moonga et al., 1990; Zaidi et al., 1989).

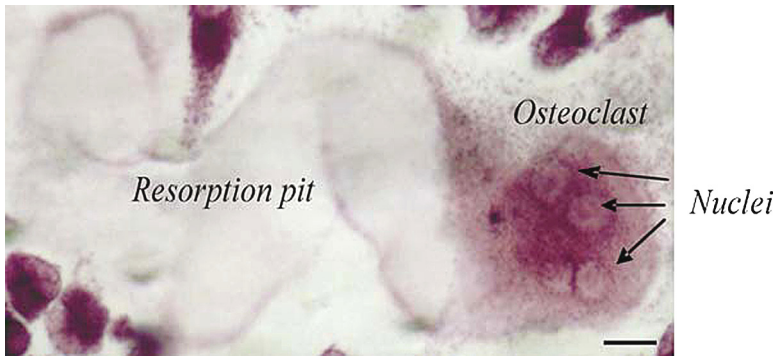

Figure 5. Resorbing human osteoclast visualized with TRACP staining. Bone resorbing osteoclasts are characterized as TRACP positive cells with multiple nuclei. During bone resorption osteoclasts migrate and form resorption pits. Bar=10 μm.

Dendritic cells (DCs) are critical in the activation of naive T cells and in the initiation of adaptive immune responses (Inaba et al., 1990; Munz et al., 2005). TRACP expression has been determined in DCs localized to the skin, spleen, thymus, lymph nodes, and epithelial surfaces of the gastrointestinal track (Hayman et al., 2001). Blood-derived CD80 antigen positive DCs has increased TRACP activity, suggesting a role for TRACP in antigen presentation. It has also been suggested TRACP may dephosphorylate osteopontin and therefore to be involved in the signaling of chronic T-cell demyelinating diseases (Chabas et al., 2001; Lang et al., 2001). By using TRACP knockout mice, it was shown that TRACP has a function in DCs and the regulation of the Th1 pathway (Esfandiari et al., 2006). DCs from TRACP-/- knockout mice stimulated with LPS showed a reduction in MHC class II and CD80 expression but enhanced IL-10 production when compared to wildtype. Increased secretion of IL-10 by DCs was suggested to be the mechanism involved in loss of Th1 response in TRACP-/- mice.

In the activated innate immune system, phagocytes produce ROS and reactive nitrogen species (RNS) to kill invading pathogens (Forman and Torres, 2002; Rossi et al., 1985). Antigenic peptides generated by fragmentation of pathogens within phagosomes are presented by major histocompatibility complex II (MHC II) molecules to the antigen receptors of T lymphocytes (Bona et al., 1973; Harding and Geuze, 1992). TRACP expression and its potential ROS production via Fenton reaction in antigen presenting cells suggest a possible role in antigen processing. In osteoclasts, there is evidence that ROS-mediated intracellular collagen fragmentation within transcytotic vesicles is facilitated by TRACP. A similar function of macromolecular fragmentation by TRACP has been suggested to exist in macrophages within their antigen-processing compartments. It has been shown that TRACP co-localizes with MHC II molecules and phagocytosed *Staphylococcus aureus* in alveolar macrophages (Bune et al., 2001; Räisänen et al., 2001). In addition, TRACP has been suggested as a biomarker for chronic inflammatory disease and, therefore, is an essential link between the skeletal and immune system (Janckila et al., 2003). Patients with rheumatoid arthritis have increased serum TRACP 5a isoform levels; a secreted product from both macrophages and dendritic cells (Janckila et al., 2002a).

2.2.2 Properties of TRACP

TRACP has been shown to act as a bi-functional enzyme with both protein tyrosine phosphatase activity and ROS generating activity. In addition, studies with uteroferrin related to TRACP suggest that TRACP may function as an iron transporter from mother to fetus (Buhi et al., 1982; Ducsay et al., 1984; Roberts et al., 1986). Iron has been shown to participate in the regulation of TRACP expression indicating a role of TRACP in iron metabolism (Alcantara et al., 1994; Reddy et al., 1995a).

2.2.2.1 TRACP as a phosphatase

An *in vivo* function of TRACP as a protein tyrosine phosphatase is to act as a counterpart of cellular protein tyrosine kinases thus playing an important role in various cellular events such as signal transduction, activation, proliferation and differentiation (Janckila et al., 1992; Nuthmann et al., 1993). TRACP can catalyze the hydrolysis of a wide range of phosphate monoesters and anhydrides, including the widely used substrates β-umbelliferylphosphate, p-nitrophenolphoshate and pyrophosphate (Marshall et al., 1997). Activation of TRACP is associated with the redox state of the di-iron center as well as with limited proteolytic cleavage in an exposed loop domain (Davis and Averill, 1982; Fagerlund et al., 2006). It has been shown that cathepsins K and L are capable of efficiently cleaving and activating recombinant TRACP *in vitro*; however, only cathepsin K was shown to co-localize with TRACP in rat long bones (Ljusberg et al., 2005; Vääräniemi et al., 2004).

No natural substrate has been identified, although phosphoserine containing osteopontin (OPN) and bone sialoprotein (BSP) has strongly been suggested (Andersson and Ek-Rylander, 1995; Andersson et al., 2003). OPN mediate different responses resulting in interactions with either CD44 or integrins (Denhardt et al., 2001; O'Regan and Berman, 2000). Removal of phosphate groups from OPN by TRACP have been suggested to inhibit osteoclast and choriocarcinoma cell migration possibly by weakening the interaction between the ligand and its integrin receptor (Al-Shami et al., 2005; Andersson et al., 2003). In TRACP and TRACP/LAP knockout mice, osteopontin has been shown to accumulate adjacent to actively resorbing osteoclasts (Hayman and Cox, 2003; Suter et al., 2001). There is also indication that TRACP may hydrolyze phosphate from the mannose-6-phosphate targeting sequence attached to lysosomal enzymes (Bresciani and Von Figura, 1996).

2.2.2.2 ROS generating activity

TRACP is capable of catalyzing the generation of reactive oxygen species (ROS). The native reduced form of TRACP has an Fe^{3+}-Fe^{2+} active site and ferrous ion is able to act as an electron donor (Halleen et al., 1999; Hayman and Cox, 1994; Sibille et al., 1987). The reactions involved in generation of ROS follows Haber-Weiss-Fenton chemistry as follows:

Hydroxyl radical formation:
$TRACP\text{-}Fe^{3+}\text{-}Fe^{2+} + H_2O_2 \rightarrow TRACP\text{-}Fe^{3+}\text{-}Fe^{3+} + OH^{\bullet} + OH^{-}$

The newly formed ferric ion is still redox active and able to react with H_2O_2 to form superoxide anion ($\bullet O_2^-$) and a ferrous ion.

TRACP regeneration and superoxide formation:
$TRACP\text{-}Fe^{3+}\text{-}Fe^{3+} + H_2O_2 \rightarrow TRACP\text{-}Fe^{3+}\text{-}Fe^{2+} + \bullet O_2^- + 2H^+$

The formed ferrous ion is again able to react by the Fenton reaction. Thus, a sequence of reactions generating both hydroxyl radical and superoxide anion occur, with continuous oxidation and reduction of the redox-active iron, making it possible for one enzyme to generate high amounts of highly destructive ROS as long as H_2O_2 is available. It has been shown that hydroxyl radicals in combination with superoxide anions cause protein fragmentation (Davies and Delsignore, 1987). So far, the mechanism producing large quantities of H_2O_2 in the transcytotic vesicles to promote ROS generation is unknown. It has been shown that ROS generating activity and phosphatase activity of TRACP are functionally independent and implemented by different mechanism (Fagerlund et al., 2006; Kaija et al., 2002). Replacement of His113 and His216 by site directed mutagenesis severely inhibited AcP activity but ROS generating activity remained intact.

Osteoclasts produce high amounts of TRACP during resorption (Alatalo et al., 2000; Minkin, 1982; Vääräniemi et al., 2004). In resorbing osteoclasts, TRACP is localized in transcytotic vesicles transporting matrix degradation products through the cell (Vääräniemi et al., 2004). It has been demonstrated *in vitro* that TRACP is able to destroy type I collegen, the major bone matrix component, by ROS (Halleen et al., 1999). This suggests that TRACP is facilitates the fragmentation of endocytosed material during bone resorption (Halleen et al., 1999; Halleen et al., 2003). In addition to this hypothesis, it has been shown that the receptor activator of the NF-κB ligand (RANKL) stimulation during osteoclastogenesis increases the intracellular level of ROS through a TRAF6 dependent manner indicating the importance of ROS during osteoclast differentiation (Lee et al., 2005). The ROS generating activity of TRACP has also been coupled to the immune defence system. Macrophages overexpressing TRACP showed increased levels of intracellular ROS. In alveolar macrophages, TRACP is colocalized with endocytosed *Staphylococcus aureus* and enhanced capacity for bacterial killing (Räisänen et al., 2005). TRACP has been also shown to colocalize with major histocompatibility complex II (MHCII) molecules (Räisänen et al., 2001). TRACP-deficient mice showed delayed clearance of *S. aureus*, after sublethal intraperitoneal inoculation of bacteria (Bune et al., 2001).

2.2.3 TRACP gene

Several mammalian TRACP genes have been cloned. The intron-exon structure of the TRACP gene is highly conserved in mouse, rat, pig, and human (Cassady et al., 1993; Ek-Rylander et al., 1991; Reddy et al., 1993; Vallet and Fahrenkrug, 2000). Southern blot analysis on human, pig and mouse shows that TRACP is expressed in a single gene, and no related sequence exists in many mammalian EST databases (Cassady et al., 1993; Ling and Roberts, 1993; Lord et al., 1990). The human TRACP gene locates on chromosome 19

(19p13.2-13.3) and on mouse chromosome 9 (Grimes et al., 1993; Leach et al., 1994; Lord et al., 1990). The overall intron-exon structure of mouse and human TRACP genes is conserved and consists of five exons with the translation initiation site ($A^{+1}TG$) located at the beginning of Exon 2 (Cassady et al., 1993; Fleckenstein and Drexler, 1997; Vallet and Fahrenkrug, 2000). The transcription product is 1.5 kb mRNA with an open reading frame of 969-975 bp encoding a 323-325 amino acid protein.

2.2.3.1 Promoter sequence and transcription factors

The promoter of TRACP gene, flanking the noncoding Exon 1, has been characterized in the human and mouse genes. There is relatively little sequence conservation upstream of the proximal promoter region between human and mouse (Reddy et al., 1995b). It has been suggested that TRACP expression may be regulated by tissue-specific promoters. It has also been proposed that the TRACP gene has three alternative 5´-untranslated regions that align with the first. These regions have been designated Exons 1A,1B and 1C (Walsh et al., 2003). TRACP Exon 1B transcript expression was highest in the kidney and liver. These tissues contain large populations of TRACP positive non-hematopoietic cells, particularly the kidney mesangial cells and the liver parenchymal cells. The Exon 1C promoter is suspected to regulate TRACP expression beyond the basal level in hematopoietic cells, such as osteoclasts and macrophages. The Exon 1A promoter function is unknown, however, and contradictory results about the expression of Exon 1A have been indicated (Angel et al., 2000; Walsh et al., 2003).

Interactions of transcription factors with the promoter region have been studied by several TRACP promoter-reporter experimental systems *in vitro*. Some transcription factors that are involved in osteoclast formation and activity have been proposed to interact with TRACP promoter. Cloning and characterization of the 5´-flanking region of mouse TRACP gene revealed numerous candidate transcription factor binding sites, including those for SP1, PU.1, GT-1, AP1 and H-APF-1 (Reddy et al., 1993). Interestingly, IL-6 has been shown to activate H-APF-1 enhancing gene expression (Majello et al., 1990). IL-6 can also stimulate osteoclast formation and activity (Girasole et al., 1992; Roodman et al., 1992). In addition, candidate binding sites for the microphthalmia transcription factor (MITF), MITF-related factors TFE-3 and TFE-C, PU.1 interacting protein Pip and upstream stimulatory factors (USFs) 1 and 2 have been found (Liu et al., 2003b; Luchin et al., 2000; Matsumoto et al., 2001; Partington et al., 2004). MITF and PU.1 have been suggested to co-operate to activate the process of osteoclast differentiation and transcriptional activation of TRACP gene (Cassady et al., 2003; Luchin et al., 2000; Luchin et al., 2001). However, there are also contradictory results indicating that PU.1 may not interact with the putative binding site in TRACP promoter region (Partington et al., 2004). MiTF has been shown to co-operate with TFE-3 and TFE-C to activate TRACP promoter (Mansky et al., 2002). TRACP expression is stimulated by RANKL, also in a NFκB and AP-1 independent manner, involving the USF 1 and 2, and a factor called YY1 (Liu et al., 2003b; Shi et al., 2004).

It has been reported that mouse TRACP promoter can be down-regulated by hemin (Reddy et al., 1995a). The responsive element GAGGC has been characterized both in

mouse and in human promoter region (Fleckenstein et al., 2000; Reddy et al., 1998). It has been suggested that TRACP from the porcine uterus may contribute to iron transport and metabolism (Buhi et al., 1982); however, it is not clear if iron or hemin have a role in regulating osteoclasts or bone resorption *in vivo*.

2.3 Toll-like receptors in innate immunity

Host defence against invading microbial pathogens is obtained by the immune system, which has two distinct patterns: innate immunity and acquired immunity. Both immunity systems recognize invading pathogens and trigger immune response to eliminate them prior to the occurrence of disease. The major players of innate immunity are phagocytic cells such as macrophages, neutrophils and dendritic cells (DCs). The primary roles of these cells are phagocytosis of pathogens in a non-specific manner, digestion of these, and presentation of pathogen-derived antigens to T cells. Toll-like receptors have been shown to recognize invasion of microbial pathogens and initiate a range of host defence mechanisms (Kopp and Medzhitov, 1999; Medzhitov et al., 1997). Cytoplasmic domains of *Drosophila* Toll and the mammalian interleukin-1 (IL-1) receptor are found structurally conserved proposing the role of TLRs in immune response (Belvin and Anderson, 1996).

The phylogenetic tree from all complete vertebrate TLRs in GenBank demonstrates that there are major TLR families that each has distinctive sequence characters (Roach et al., 2005). TLR families vary in the length of their leucine-rich extracellular domains. TLR7 family consists of nucleic acid patterns recognizing TLRs 7, 8 and 9. TLR1 family includes subfamilies TLR1, 2, 6, 10 and 14, and has specificity to lipopeptide molecule patterns. The TLR11 family includes TLR11-13 and TLR21-23 subfamilies. In humans, this family is present as pseudogenes: DNA sequences similar to normal genes are found but are non-funtional. Ligands are also mainly unknown in this family; however, TLR11 apparently recognizes uropathogenic bacteria and *Toxoplasma gondii* profilin has been shown to induce IL-12 production via TLR11 activation (Yarovinsky et al., 2005; Zhang et al., 2004). This suggests that the TLR11 family recognises protozoan pathogens. The TLR3 (for dsRNA), TLR4 (for LPS) and TLR5 (flagellin) families have not been divided into subfamilies.

2.3.1 The Toll Pathway

TLR-signaling pathways are initiated and finely regulated by the cytosolic domain of the Toll-like receptor, termed as the Toll/Interleukin 1 receptor (TIR)-domain. In TLR pathways there are four TIR-domain containing adapter proteins: MyD88 (myeloid differentiation factor 88); TIRAP (TIR-domain containing adaptor); TRIF (TIR-domain containing adaptor inducing IFN-β), and TRAM (TRIF-related adaptor molecule). Differential utilization of TIR-domain containing adaptors provides the specificity of each TLR-signalling pathway. Cellular activation proceeds through a signaling cascade involving IL-1 receptor-activated kinase (IRAK), TNF receptor-associated factor 6 (TRAF6), and NF-κβ translocation, culminating in the upregulation of genes involved in host defence (Aderem and Ulevitch, 2000). Host activation pathways are divided into MyD88-dependent and independent signaling pathways.

MyD88 has been shown to be essential in TLR pathways that lead to inflammatory cytokine production (Akira et al., 2001). MyD88 activate IL-1 receptor associated kinases (IRAKs) by phosphorylation that then associates with TRAF6, leading to phosphorylation of IκB. Nuclear translocation of transcription factor NF-κB after degradation of IκB leads to induced expression of inflammatory cytokine genes. In MyD88 deficient macrophages, response to ligands of TLR2, TLR7 and TLR9 has been impaired. However, stimulation with TLR4 ligand LPS resulted in activation of NF-κB, although in a delayed manner when compared to wild type macrophages. TLR4 ligand induced expression of several IFN-inducible genes (Kawai et al., 1999).

Bacterial DNA mimicking CpG motifs are recognized and bound by TLR9, leading to the rapid production of inflammatory cytokines and chemokines including IL-8/*CXCL8*, TNF-α, IL-6, IL-12, and type I IFNs. The recognition of CpG DNA oligonucleotides by TLR9 is dependent on accessibility to the 5'-end of the sequence, as the conjugation of large ligands at this end minimizes immunostimulatory activity. Immunostimulatory oligos containing two 5'-ends are significantly more potent than conventional CpG oligos containing a single 5'-end (Hemmi et al., 2000). The activation of TLR9 leads to polarized T-cell immune cells favoring Th1 cells (Klinman et al., 1996; Lipford et al., 1997). Recently, there are several research groups setting arguments against earlier results showing TLR9 and CpG motif independent immune response by DNA oligonucleotides. Vollmer et al. (2004) showed by TLR9 knock out and wild type mice that TLR9 can mediate either efficient Th1- or Th2-dominated effects depending on whether it is stimulated by CpG or certain non-CpG ODN. In addition, it has been shown that endosomally located host (self) DNA may activate TLR9, while cytoplasmic self DNA does not (Yasuda et al., 2006). TLR9-deficient neutrophils have been demonstrated to be activated in a CpG- and TLR9-independent manner (Alvarez et al., 2006; Trevani et al., 2003). These findings indicate the possibility of an additional DNA recognition mechanism against invading pathogens.

In osteoclast cell cultures it has been recognized that CpG motifs can regulate osteoclastogenesis. It has been demonstrated that the CpG-DNA motifs alter the osteoclastogenesis pattern in the RANKL mediated osteoclast differentiation process. CpG-ODNs have inhibitory effects onf RANKL activity in early osteoclast differentiation stages (Zou et al., 2002). Moreover, it has been shown that ligands for TLRs 2, 3, 4 and 9 (peptidoglycan, poly(I:C) dsRNA, LPS and CpG DNA, respectively) induce up-regulated TNF-α production in osteoclast precursor cells and inhibit their differentiation into mature osteoclasts (Takami et al., 2002). CpG DNA oligonucleotides also reduced expression of M-CSF receptor, a critical factor in the initiation of osteoclast differentiation. In addition, IL-12 levels have been shown to increase in CpG and CpG RANKL treated osteoclast precursor cells further supporting the findings that CpG oligonucleotides inhibit RANKL induced osteoclast differentiation (Amcheslavsky and Bar-Shavit, 2006). However, these osteoclast precursor cells maintained the phagocytic activity that may be critical to prevent pathogenic effects of microbial invasion on bone. In contrast, CpG DNA oligonucleotides have been shown to increase osteoclastogenesis in RANKL pretreated cells (Zou et al., 2003; Zou et al., 2002).

3 AIMS OF THE PRESENT STUDY

At the initiation of the present study, the role of TRACP had already been studied: However, the biological function of TRACP has remained unknown. TRACP has been proposed to be involved in immunity responses due to its ROS generating activity. The ROS generating activity of TRACP has also been suggested to be involved in bone matrix degradation during bone resorption. The original aim of the present study was to set up a TRACP specific knock-down model to study the loss-of-function phenotype in bone resorbing osteoclasts. This study focused on the RNA interference mechanism and its use as a gene silencing tool. The following specific aims were set for the study:

- Development of a proficient siRNA design program, and evaluation of the program functionality *in silico* and *in vitro*.

- To study the biological effects of 2′-Fluoro-purine and 2′-Fluoro-pyrimidine modified siRNA molecules.

- To downregulate TRACP by antisense DNA and RNAi methods in order to study the biological function of TRACP in human and mouse osteoclasts.

4 MATERIALS AND METHODS

4.1 Cell cultures

4.1.1 Human osteoclast culture (III)

Human *peripheral blood mononuclear cells* (PBMCs) were isolated by Ficoll-Paque (Pharmasia Biosystems, Sweden) sedimentation and adherence from peripheral blood as previously described (Husheem et al., 2005). One million cells were plated onto bovine cortical bone slices (150 – 200 μm thick) and cultured in 24-well plates containing fresh α-MEM supplemented with 10% heat-inactivated fetal calf serum (iFCS), M-CSF (10 ng/ml), RANKL (20 ng/ml), TNF-α (10ng/ml; Peprotech, UK) and dexamethazone (10^{-8}M; Sigma, USA).

Human *osteoclast precursor cells* from Cambrex (Poietics™, Cambrex Bio Science) were cultured in α-MEM supplemented with 10% iFCS, 20mM Hepes, 100 U/ml penicillin and 100mg/ml streptomycin (all purchased from Gibco, UK) and differentiated into mature osteoclasts in the presence of M-CSF (33 ng/ml; R&B Systems, UK), RANKL (66 ng/ml; Peprotech, UK) and TGF- β (added day three; 1ng/ml; R&B Systems, UK). Precursor cells were plated onto glass cover slips (∅ 14mm).

4.1.2 Mouse osteoclast culture (III)

Homozygote C57Black/B6 mice with NEO1500 cassette disrupting the TLR9 gene (Bioindustry Division Oreintal Yeast Co., Ltd, Japan) were used to study whether TRACP activation by CpG oligomers was TLR9 independent. Wild type C57Black/6 mice were used as a control. Bone marrow cells were isolated from 10 weeks old WT and TLR9 -/- male mice according to the method previously described (Hentunen et al., 1999). The cells were cultured for 11 days on bovine bone slices (10^6 cells / bone slice) in High-Glucose D-MEM supplemented with 10% iFCS, 20mM Hepes, 100 U/ml penicillin and 100mg/ml streptomycin (all purchased from Gibco, UK). Osteoclast differentiation was supported by addition of RANKL (20 ng/ml) and M-CSF (10 ng/ml) at day 0 and day 4.

4.1.3 CHO- TRACP cell line (I,II,III)

The efficiency of various TRACP siRNAs was tested in CHO cells overexpressing human TRACP (Janckila et al., 2002b). CHO-hTRACP cells were cultured in F-12K medium (Gibco Invitrogen, UK) supplemented with 10 % iFCS, 100 μg/ml Zeocin (InvivoGen, USA), 100 U/l penicillin and 100 mg/ml streptomycin (Gibco, Invitrogen Life Technologies, UK). Cells were regularly passaged at subconfluence and plated 24 h before transfection. A total of $5x10^4$ cells were added per well into 24-well plates and $5x10^3$ cells into 96-well plates.

4.2 RNAi setup

4.2.1 Specification of the siRNA_profile program (I)

The ***siRNA_profile*** program is based on findings of the asymmetric differences between functional and nonfunctional siRNAs (Freier et al., 1986; Khvorova et al., 2003; Schwarz et al., 2003) and on our studies of positional nucleotide differences and average dsRNA stability along the siRNA antisense strand. Some recommendations of Elbashir, S.M., et al. (2001a) were incorporated into the program and a novel, interactive and user-friendly siRNA design algorithm with multiple options for minimizing unspecific siRNA design was developed. The ***siRNA_profile*** program was written in the C-programming language. It was originally developed on Red Hat Linux 9.0 and compiled to an executable file. The CGI application was written in the Perl programming language. The application uses user-given input values from the siRNA_profile website to analyze and display the results. The ***siRNA_profile*** program, a printable introduction to the program and a help page are available at http://bonebiology.utu.fi/pimaki/main.html. The ***siRNA_profile*** program uses free energy values for the calculation of average internal stability profiles. The average internal stability profiles were calculated as the sum of stability in five nucleotide windows by using the nearest-neighbor method (Freier et al., 1986). Our program browses and calculates the target sequence from the antisense point of view, from the 5′ to the 3′direction. It does not utilize nucleotides on the mRNA beyond the 3′end of the siRNA, because this calculation method may easily lead to false bending of the profile.

mRNA or DNA sequence of the target gene, or a selected part thereof, can be pasted into the target sequence window. This program can also be used for profiling of already designed siRNA sequences by choosing a sense or antisense strand from the options-menu. It was suggested by Khvorova, A., et al. (2003) that the average internal stability of the 5′end is lower than of the 3′terminus in a functional siRNA. The ***siRNA_profile*** program specifies an energy difference that is the difference of calculated free energy values between the 5′terminus and the 3′terminus. The expected "energy difference" value should be 0.3 kcal/mol (Khvorova et al., 2003) or higher to ensure the activation of RISC primarily by the antisense strand and not the sense strand. Functional siRNAs also exhibit low overall internal stability region called the "energy valley" between 9 and 14 nucleotides in their profile, while non-functional siRNAs lack this feature (Khvorova et al., 2003). The user can define the exact location of the "energy valley"; the region between 9 and 14 nucleotides is suggested. In addition, the ***siRNA_profile*** program can be set to search the 5′AA patterns from the target mRNA, to eliminate sequences containing nucleotides in a 3-4 nt row, and to organize the siRNA sequences in a score-based order according to their nucleotide preferences. The heuristic scoring system of the program is based on findings of differences in nucleotide preferences of functional and non-functional siRNA sequences.

The ***siRNA_profile*** program recognises immunostimulatory elements incorporated in the siRNA sequences (Judge et al., 2005). According to the results of Judge et al.

(2005), by eliminating siRNA sequences containing 5´-UGUGU-3´ motifs the user can obtain more reliable knock-down phenotypes and avoid inflammatory responses. In addition, the program shows the positions of CpG motifs based on the knowledge obtained from antisense DNA oligomers containing CpG motifs leading to TLR9 dependent innate immunity responses. It has been shown that CpG motifs cause activation in human monocytes not only in DNA but also in single stranded RNAs (Hemmi et al., 2000; Sugiyama et al., 2005). CpG motifs are recommended to be rejected in case the ***siRNA_profile*** program is used for single stranded anti-miRNA oligomer analysis.

4.2.2 siRNA design (I)

Three siRNA sequences targeting human TRACP (NM_001611) were selected by ***siRNA_profile***. The following search parameters were used: siRNA candidate search limited to coding region 90-1067, siRNA length 19 nt, energy difference 1,2 kcal/mol, energy valley between nt 9 and 14. siRNA sequences with variable scores were selected and the absence of sequence homologies to off-target sequences was confirmed by BLAST search. One sequence illustrating a sense strand favoring energy profile was selected as a Bad Hit-control to estimate the selectivity of the ***siRNA_profile*** program. mRNA target sites of the TRACP siRNAs were the following: mRNA sequences; 143 siRNA 5'-TGGCCAATGCCAAGGAGAT-3'; 729 siRNA 5'-TCTGCAGTACCTGCAA GAT-3', 803 siRNA 5'-AGCGGCACCAGCGCAAGGT-3' and Bad Hit-siRNA 5'-GAAACAGCTGGCCGGCGGC-3'.

4.2.3 Enzymatic and synthetic siRNA synthesis, and transfection (I,II,III)

Enzymatic: siRNAs were synthesized by using T7 RNA polymerase for *in vitro* transcription. T7 RNA polymerase requires the first two nucleotides of the RNA transcript to be GG or GA to ensure efficient synthesis (Milligan et al., 1987). Therefore, an additional 5´-leader sequence was incorporated into both sense and antisense 21 nt long DNA templates. The sense and antisense templates were transcribed by T7 RNA polymerase separately. The resulting RNA transcripts were hybridized to create dsRNA to consist of 19 nt target specific dsRNA, 3´-UU overhang and a 5´-terminal single stranded leader sequence. The leader sequence was removed by digesting RNA molecules with a single strand specific ribonuclease, leaving the 3´-UU overhangs intact. The end product of enzymatic siRNA synthesis was a double stranded 21 nt siRNA molecule with 3´-UU overhangs.

Chemical synthesis: Oligomers were assembled on a Universal support USII-PS (Metkinen Chemistry) using a Biosset AM-800 synthesizer (0.8 μmolar scale, recommended RNA synthesis protocol). Corresponding 2'-F-RNA monomers were from Metkinen Chemistry and the RNA monomers were from Glen Research. The cleavage of oligomers off the solid support was achieved with 4N ammonia in methanol (0.5 ml, 30 min. at RT). Deprotection was carried out by adding 1ml of 7N ammonia in methanol and leaving the oligomer solutions at RT for 36 h. After oligomer cleavage and deprotection, oligomers were purified on a PolyWax LP (300Å,

5 µm, 4.6 x 100 mm) column (PolyLC) and finally desalted. Synthetic, single-stranded, 21 nt long RNA molecules were annealed to form 19 nt siRNAs with 3´-UU overhangs. 2´-F-pu or 2´-F-py modified sense (S) and antisense strands (A) were combined together to form nine different mixtures including unmodified siRNA molecule. For duplex formation, the single-stranded siRNAs were incubated in sterile, RNase-free hybridization buffer (100 mM potassium acetate, 2 mM magnesium acetate, 30 mM Hepes-KOH, pH 7,4), for 1 min at +90°C followed by 60 min at +37°C (Elbashir et al., 2002). All siRNA molecules were stored at -70°C.

siRNA transfection: To enhance the uptake of siRNA molecules, Mirus *Trans*-IT TKO® transfection reagent was used according to the supplied protocol (MirusBio, USA). 30 U of prime RNase inhibitor (Eppendorf, Germany) was added to the culture medium 30 minutes before siRNA additions to inhibit RNase activity in the medium. CHO-TRACP cells were cultured at 70% confluency and transfected by siRNA-Mirus complexes in a serum-free medium. siRNA molecules were used at 0,1 nM, 1 nM, 5 nM and 20 nM concentrations. Human osteoclast precursor cells (obtained from Cambrex) were cultured for 9 days before transfection in the presence of RANKL (66 ng/ml, Peprotech, UK) and M-CSF (33ng/ml, R&D Systems, UK), and supplemented with TGF-β (1 ng/ml, R&D Systems, UK) at day 3. The human osteoclast precursor cells were transfected by Mirus transfection reagent with control siRNA or TRACP siRNA (50 nM each) at day 9.

4.2.4 siRNA stability in human plasma (II)

Unmodified and 2´-F modified synthetic siRNA duplexes were precipitated and diluted in RNase free water. Human plasma was added to the samples resulting in an 8 µM siRNA concentration in 90% plasma. The samples were incubated at +37 °C for 3 h, 24 h, 48 h or 72 h, with 5 µl aliquots collected at each time point and stored at -20°C. Samples were size-fractionated on a native 15% polyacrylamide gel (PAGE) in RNase free TBE buffer (0,09 M Tris base, 0,09 M boric acid and 9 mM Na_2EDTA). Following electrophoresis, ethidium bromide stained siRNA molecules were observed with an UV-transilluminator. Time-dependent siRNA degradation was evaluated from the gel using a Low Molecular Weight DNA ladder (New England BioLabs, UK) as a reference.

4.2.5 DNA oligonucleotides

A CpG- motif containing phosphorothioate oligodeoxynucleotides (PTO-ODNs) was obtained from TAG (TAG Copenhagen A/S, Denmark) and non-CpG PTO-ODNs were designed and manufactured by Biognostik® (Biognostik, Germany). CpG ODNs were added to the human osteoclast culture medium at days 4, 8 and 12, and the cultures were finished at day 15. With non-CpG ODNs, the ODNs were added to the human osteoclast culture medium at days 7, 9 and 11, and the cultures were finished at day 13. CpG and non-CpG ODNs were added to the mouse (WT and TLR9-/-) bone marrow derived cell culture medium at days 7 and 9, and the cultures were finished on day 11. A 5 µM concentration of the ODNs was used in each experiment. Sequences and details are in Table 1.

Table 1. Sequences of DNA oligonucleotides (III)

ODN	Sequence
CpG random (human)[1]	5′- GCA GTA GGC TA ***CG*** TT C -3′
CpG sense (human)[1]	5′- GG ***CG*** CT GCT CAT CCT G -3′
CpG antisense (human)[1]	5′- CAG GAT GAG CAG ***CG*** C C -3′
nCpG random (human)[2]	5′- GTA AAT TGA CCA GGA G -3′
nCpG AS1 (human)[2]	5′- CTG AGG AAG TCA TC -3′
nCpG AS2 (human)[2]	5′- AGT CTT CAG TCC CAT AGT -3′
CpG1 (mouse)[3]	5′- GCA GTA GGC TA ***CG*** TT C -3′
CpG2 (mouse)[3]	5′- CAT TTA CAT ***CG*** T CCT C -3′
nCpG1 (mouse)[2]	5′- GTA AAT TGA CCA GGA G -3′
nCpG2 (mouse)[3]	5′- GCA GTA GGC TAC CTT C -3′

[1] Custom ODNs manufactured by TAG (TAG, Copenhagen A/S, Denmark)
[2] ODNs designed and manufactured by Biognostik® (Biognostik, Germany)
[3] Custom ODNs manufactured by Biognostik® (Biognostik, Germany)

4.3 ELISA assays

4.3.1 TRACP specific immunoassays

4.3.1.1 Human TRACP 5b activity (III)

Human TRACP 5b activity was measured as previously described (Halleen et al., 2000). Briefly, the monoclonal antibody O1A (400ng/well) was incubated for 1 hour in anti-mouse IgG-coated microtiter wells (PerkinElmer Life Sciences, Wallac Oy). After washing cell culture samples and recombinant human TRACP as a calibrator (total volume 100 µl/well) were incubated in the wells for 1 hour. After washing, culture or lysate samples (total volume 100 µl/well) were incubated in the wells for 1 hour. Recombinant human TRACP was used as a calibrator. Detection of bound TRACP 5b activity was detected by using 8 mM 4-NPP as a substrate in a 0.1 M sodium acetate buffer, pH6.1 for 1 hour at 37 °C. The reactions were determined by addition of 25 µl of 0.32 M sodium hydroxide and the absorbance at 405 nm (A_{405}) was determined by using Victor II equipment (PerkinElmer Life Sciences, Wallac Oy).

4.3.1.2 Mouse TRACP 5b (III)

TRACP 5b activity was measured from mouse culture samples as previously described (Alatalo et al., 2000). Briefly, the polyclonal rabbit-anti-TRACP antiserum was diluted 1:1000 and incubated on anti-rabbit IgG-coated microtiter plates (PerkinElmer Life Sciences, Wallac Oy) for 1 hour. Detection of bound TRACP 5b was detected as above; however, incubation time at 37 °C was 2 hours.

4.3.1.3 Total TRACP (I,II,III)

Total TRACP protein was measured using a two-site assay with monoclonal antibodies O1A and J1B as described previously (Halleen et al., 1998a). Briefly, biotinylated O1A antibody (2 µg/ml) was incubated on streptavidin microtiter plates

(Innotrac Diagnostics Oy, Finland) for 1 hour. After washing ,culture or lysate samples (total volume 100 µl) were incubated in the wells for 1 hour. Recombinant human TRACP was used as standard. After washing europium (Eu^{3+}) labeled J1B antibody was incubated in the wells for 1 hour. After washing 200 µl of Enhancement solution (Wallac Oy) was added to the wells and shaken for 5 min. Fluorescence was measured by Victor II equipment (PerkinElmer Life Sciences, Wallac Oy).

4.3.2 Measurement of tartrate sensitive acid phosphatase (TSAP) activity (III)

Acid phosphatase activity was measured with and without (L+) tartrate using 4-nitrophenylphoshate as a substrate in sodium acetate buffer (pH 6.1 or 5.0). Samples were incubated for one hour at +37°C and the reaction was terminated with NaOH. Absorbance was read at 405 nm using model 2 Victor instrument (PerkinElmer Life Sciences-Wallac Oy, Finland) to evaluate the activities of tartrate-sensitive acid phosphatases (TSAPs) and TRACP.

4.3.3 Cytokine assays

4.3.3.1 Mouse TNF-α

Mouse TNF-α was measured by using commercial solid-phase enzyme-linked immunosorbent assay based on the sandwich principle according to the supplied protocol (HyCult Biotechnology, the Netherlands). Briefly, samples and a dilution series of mouse TNF-α standard (100 µl/ well) were incubated on anti-mouse TNF- α coated microtiter plates for 2 hours at +37°C. After washing, 100 µl of biotinylated mouse TNF-α antibody was used as a tracer and incubated for one hour at room temperature. After washing, 100 µl of diluted streptavidin-peroxidase conjugate was incubated in each well for one hour at room temperature. TMB (100 µl) was used as a substrate and the reaction was stopped after 20 min by equal volume of 2 M citric acid. The absorbance at 450 nm (A_{450}) was determined by using Victor II equipment (PerkinElmer Life Sciences, Wallac Oy).

4.3.3.2 Mouse IL-6

Mouse IL-6 was measured by using a commercial quantitative sandwich enzyme immunoassay according to the supplied protocol (R&D Systems, USA). Monoclonal antibody specific for mouse IL-6 was pre-coated onto microtiter plates. First, 50 µl assay diluent was pipeted to each well. 50 µl of samples and mouse IL-6 standard dilutions were incubated for 2 hours at room temperature. After washing, 100 µl of polyclonal antibody against mouse IL-6 conjugated to horseradish peroxidase was incubated for 2 hours at room temperature. After washing, mixed solution of hydrogen peroxidase and TMB was used as a substrate. The reaction was stopped after 30 mins by diluted hydrochlorid acid and absorbance was measured at 450 nm and absorbance 570 was used as a correction wavelength.

4.4 PCR methods

4.4.1 Reverse Transcription-PCR (I)

Total RNA was isolated from the bone marrow of tibia and femur by using RNeasy (Qiagen, USA) kit. Contaminating genomic DNA was removed with DNA-*free*™ (Ambion, USA). Levels of TLR7, TLR8, TLR9 and TRACP expression in WT and TLR9-/- mice were determined by RT-PCR (Robust II RT-PCR kit, Finnzymes, Finland). In the RT-PCR reaction 10 pmol of the following 5′-forward primers (mouse TRACP 5′-TCCTGGCTCAAAAAGCAGTT-3′; mouse β-actin 5′-CTTAGCCTTGGACCCATGAT-3′) and 10 pmol of following 3′-reverse primers (mouse TRACP 5′-ACATAGCCCACACCGTTCTC-3′; mouse β-actin 5′-GGACTCCTGTCTTACCCACAGT-3′) were used. In addition, 10 pmol of previously published 5′-forward and 3′-reverse primers of TLR7, 8 and 9 were used (Rodriguez-Martinez et al., 2005). Expression levels of β-actin were used as a reference. The cycling conditions were the following for TRACP, TLRs 7 and 8 and β-actin: reverse transcription 30 min at +40 °C followed by 30 cycles of 30sec at +94 °C, 30 sec at +55 °C and 45 sec at +72 °C. RT-PCR conditions for TLR9 were the same, but had 32 cycles.

4.4.2 Quantitative RT-PCR (II)

Total RNA was isolated from CHO-hTRACP cells transfected with 1nM siRNA molecules or vehicle by using the RNeasy kit (Qiagen, USA). Contaminating genomic DNA was removed with DNA-*free*™ (Ambion, USA). For quantitative RT-PCR analysis, 480 ng of total RNA was used for the reverse transcription (Superscript™ First-Strand Synthesis system, Invitrogen Life Technologies, UK). hTRACP mRNA levels were detected by using quantitative PCR and specific probes (ProbeLibrary, Roche Diagnostics, Switzerland). In the qPCR reaction, 300 nM of the following 5′-forward primers (CHO β-actin 5′-CTTAGCCTTGGACCCATGAT-3′, human TRACP 5′-GCAATGTCTCTGCCCAGATT-3′) and 300nM of the following 3′-reverse primers (CHO β-actin 5′-GGACTCCTGTCTTACCCACAGT-3′, human TRACP 5′-TGAAGTGCAGGCGGTAGAA-3′) were used. An online assay design software was used to identify matching probes (ProbeFinder; http://www.universalprobelibrary.com). Universal ProbeLibrary probe number 85 for CHO β-actin and number 60 for human TRACP were used at 200nM concentrations. Primers were purchased from Oligomer (Finland) and the Universal ProbeLibrary set was from Roche (USA). All cDNA samples were equally diluted 1:5 and 2 µl of the diluted sample was used in Probe Library qPCR in a 10 µl reaction volume (cycling conditions: 15 min at +95 °C followed by 40 cycles of 15 sec at +95 °C and 1 min at +60 °C.). qPCR was assayed with an ABI PRISM 7700 Sequence detector (Applied Biosystems, Foster City, USA) using ABsolute QPCR ROX mix (ABgene, Epsom, UK) at the Turku Centre for Biotechnology (Turku, Finland). β-actin levels were used for data normalization.

4.5 Immunohistochemistry (I,II,III)

Cells were fixed in 3% paraformaldehyde for 15 min. TRACP staining was performed using the Leukocyte Acid Phosphatase kit (Sigma, USA) in the presence of L(+) tartrate, and the nuclei were stained by Hoechst 33258 (Molecular Probes, USA). TRACP positive cells were counted under a light microscope (Leica Microsystems AG, Germany). Polyclonal rabbit anti-TRACP antibody (Alatalo et al., 2000) and TRITC-labelled donkey anti-rabbit secondary antibody were used for immunological visualization of TRACP (Jackson Immunochemicals, USA). The cytoskeleton was visualized using Alexa Fluor® 488 phalloidin (Molecular Probes Invitrogen, USA). To visualize TLR9 in human osteoclast, cells were first permeabilized with ice-cold 0.5% Triton X-100:PBS followed by incubation of primary mouse anti-TLR9 antibody (Imgenex, USA) in 0.5% BSA:PBS at +4°C overnight. FITC labeled secondary donkey anti-mouse (Jackson Immunochemicals, USA) was used for immunological detection of TLR9, and Alexa Fluor® 546 phalloidin (Molecular Probes Invitrogen, USA) was used to visualize the cytoskeleton. Samples were viewed with a Leica TCS-SP confocal laser scanning microscope equipped with an Argon-Krypton laser (Leica Microsystems AG, Germany).

5 RESULTS

5.1 *siRNA_profile*: analysis of large a database by the *siRNA_profile* program (I)

5.1.1 Scoring system functionality

The ***siRNA_profile*** scoring method was derived from sequences exhibiting only thermodynamically favourable siRNA profile targeting human CyclophilinB. The asymmetric energy profile of the siRNA sequence is not sufficient to eliminate the non-functional siRNA sequences; therefore, a scoring system was incorporated into the ***siRNA_profile*** program. It was adjusted based on our findings on nucleotide, purine and pyrimidine distribution along functional and non-functional siRNA sequences. The scoring method enhanced siRNA sequence candidate search when comparing results of hCyclophilinB siRNA hits with and without the scoring system (data is accessible in the ***siRNA_profile*** web page). The average gene silencing capacity of siRNAs with optimal thermodynamical parameters but without nucleotide preferences was 22.4 % of the baseline (minimum and maximum 5.4 % and 58.3 %, respectively, median 14.3 %). Including the scoring method (scores > 5) discarded nonfunctional siRNAs and average gene silencing capacity of selected siRNAs was 10.4 % of the baseline (minimum and maximum 5.4 % and 15.1 %, respectively, median 10.2 %). None of these selected siRNA sequences contained the immunostimulatory 5´-UGUGU-3´ element. To increase the number of analysed sequences, 2431 siRNA sequences with known efficacies (Huesken et al., 2005) were used to validate the functionally of the ***siRNA_profile*** program scoring method. All of the siRNA sequences (n=2431) were used for the correlation, also including the thermodynamically unfavourable siRNAs, and scores were calculated with the ***siRNA_profile*** program. The results suggested that our scoring method correlated with the expression level (r = -0.42, n = 2431). The scoring system is useful especially in the fine-tuning of siRNA candidate search.

The ***siRNA_profile*** program functionality was compared to five other available siRNA design programs (unpublished). Human cyclophilinB sequence region 193 – 390 (Reynolds et al., 2004) was fed into Deqor (Henschel et al., 2004), siRNA target finder (Ambion, USA), siDesign Center (Dharmacon, USA), EMBOSS explorer (gwilliam © rfcgr.mrc.ac.uk) and siDirect (Naito et al., 2004), and optimal siRNA candidate search options were used. The siRNA design results were compared to the results obtained by ***siRNA_profile*** by using the known efficacies of each siRNA sequences targeting the selected human Cyclophilin region. ***siRNA_profile*** selected six highly functional siRNA sequences: the average gene silencing capacity of selected siRNAs was 10.4 % of the baseline (minimum and maximum 5.4 % and 15.1 %, respectively, median 10.2 %). The other programs selected siRNA sequences as follows: Deqor average gene silencing capacity 14.8 % of the baseline (minimum and maximum 10.3 % and 22.8 %, respectively, median 13.1 %, n=4); siDirect average gene silencing capacity 14.8 % of the baseline (minimum and maximum 5.8 % and 32.2 %, respectively, median 14.9 %, n=9); siDesign Center (Dharmacon) average

gene silencing capacity 15 % of the baseline (minimum and maximum 11,2 % and 22.8 %, respectively, median 13 %, n=4); siRNA target finder (Ambion, USA) average gene silencing capacity 21.1 % of the baseline (minimum and maximum 8.2 % and 28.3 %, respectively, median 26.9 %, n=3) and EMBOSS explorer: average gene silencing capacity 23.1 % of the baseline (minimum and maximum 6.7 % and 54.1 %, respectively, median 16.1 %, n=8). The results showed that all the tested programs selected functional siRNA sequences with some variability, but the ***siRNA_profile*** program showed the highest selectivity. However, it was noticeable that all programs tend to select different siRNA sequences; even the mRNA region selected for siRNA candidate search was only 197 nucleotides long. The programs selected altogether 24 different sequences, and only 6 of them were selected by two or more programs. The other 18 sequences were individually selected by these six programs.

5.1.2 Novel energy profile findings

The average internal stabilities of functional and non-functional siRNAs were characterized. A large siRNA data set with 2431 individual siRNA sequences was fed into ***siRNA_profile*** (Huesken et al., 2005). siRNA profiles were divided into categories based on gene expression levels after siRNA silencing (gene expression level categories: ≤ 20 %, n=280; 20.1 %-30 %, n=455; 30.1 %-40 %, n=445; 40.1 %-50 %, n=405; 50.1 %-70 %, n=736 and >70 %, n=110). The results indicated mirror profiles for functional (gene expression ≤30%) and non-functional (gene expression >70%) siRNAs which is in good agreement with earlier results (Khvorova et al., 2003). However, in addition to asymmetric profiles, our results also showed that functional siRNAs exhibited siqnificantly lower general stability (I, see Figure 1).This novel feature was found to correlate siRNA energy profiles with their efficiencies. These results suggest that the low overall stability of dsRNA plays a significant role in the RNAi pathway and is recommended to be used as an additional guideline for highly efficient siRNA design.

5.1.3 Design of a highly functional TRACP siRNA sequence by siRNA_profile

Three siRNA sequences targeting of human TRACP were selected by ***siRNA_profile*** (I, see Figure 2). siRNA sequences with variable scores were selected and the absence of sequence homologies to off-target sequences was confirmed by BLAST. One sequence illustrating a sense strand favoring energy profile was selected as a BadHit-control to estimate the selectivity of the ***siRNA_profile*** program. Selected siRNA candidates were tested in a CHO cell line stably expressing human TRACP (Janckila et al., 2002b). Mirus *Trans*-IT TKO® transfection reagent was used for siRNA delivery according to the supplied protocol (MirusBio, USA). The biological efficacy of siRNA molecules (n=3, siRNA concentration 10nM) was evaluated 48 hours post-transfection by using a target-specific ELISA assay (Halleen et al., 1998a). To ensure target knockdown, TRACP protein levels were measured from both culture medium and CHO-TRACP cell lysates (RIPA buffer: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 25 mM NaF, 0.2 mM Na_2VO_4, 1 mM PMSF, 1% NP-40 and Complete Proteinase Inhibitor Cocktail [Roche Diagnostics, Switzerland]). All statistical analyses were performed with one-way ANOVA and p-

values of < 0.05 were considered statistically significant. Three hTRACP siRNA candidates: 143 (score 9); 729 (score 2) and 803 (score 4), efficiently knocked down target protein expression (I, see Table 1.). Human TRACP expression remained insignificant during 48 hours despite the fact that TRACP was expressed under a strong viral promoter ($p<0.001$; compared to baseline). Even the efficacies of the low scoring siRNAs (no 729 and 803) were almost as efficient as the high scoring 143 siRNA. The functionality of siRNAs was most probably due to the high (1.2 kcal/mol) energy difference between the 5´- and the 3´- end that was used to allow functional siRNA sequence selection. When comparing the efficacy of these three optimally designed siRNA sequences to the BadHit- siRNA control having a reverse energy profile, the results showed that the siRNA favoring the sense strand instead of antisense strand failed to knock down the target: intracellular TRACP remained unchanged (I, see Table 1. and Figure 3). This indicated the crucial requirement of thermodynamical selection between the sense and antisense strand in advanced siRNA design program.

5.2 Evaluation of 2´-Fluoro-purine and -pyrimidine modified TRACP siRNAs (II)

The single-stranded 2´-Fluoro-purine and 2´-Fluoro-pyrimidine modified RNA molecules were produced and annealed so that all combinations of unmodified, 2´-F-pu and/or 2´-Fluoro-pyrimidine modified sense-antisense duplexes were obtained. When the biological efficacy of the combinations were evaluated, 7 out of 9 siRNA duplexes significantly downregulated TRACP protein secretion already at the lowest concentration of 0.1 nM (II, see Figure 3). However, all of the 2´-Fluoro-purine and 2´-Fluoro-pyrimidine combinations induced RNAi already at nanomolar concentrations. This is in a good agreement with previous results showing that 2´-OH is not required for the RNAi pathway (Chiu and Rana, 2003). The TRACP siRNA sequence includes both purine and pyrimidine at the AGO2 cleavage site in the sense strand. Therefore, it was possible to analyze the effects of 2´-Fluoro modifications in RISC activation and mRNA degradation in detail. Statistical analysis in Table 2. shows that 2´-Fluoro modification in either of the purine or pyrimidine in this critical site was well tolerated and rather improves than abolishes RISC mediated mRNA cleavage.

5.2.1 mRNA degradation

For mRNA quantitation, CHO-TRACP cells were transfected with 1nM of siRNA molecules. Quantitation of TRACP mRNA levels confirmed the gene silencing capacity as compared to the baseline (II, see Figure 6). Negative control siRNAs did not induce non-specific TRACP mRNA degradation and positive control siRNAs led to the target mRNA degradation as expected. All modified siRNAs knocked down the target mRNA at least 75% (as compared to baseline expression levels). The best mRNA knockdown effect was observed in samples treated with A1S3 (unmodified antisense strand, 2´-Fluoro-pyrimidine modified sense strand, gene expression 7.7 % of baseline) or unmodified A1S1 (gene expression 7.6 % of baseline).

Table 2. Detailed statistical comparison of the effectivity of 2´-Fluoro modified siRNA molecules (II)

	Dose (nM)	**A1S2**	**A1S3**	**A2S1**	**A2S2**	**A2S3**	**A3S1**	**A3S2**	**A3S3**
	0,1	ns	*	ns	ns	ns	***	ns	ns
A1S1	1	ns	ns	ns	ns	ns	ns	ns	ns
	5	ns	*	ns	**	*	*	ns	***
	20	ns	ns	*	*	ns	ns	**	*
	0,1		**	ns	ns	*	***	ns	*
A1S2	1		ns	ns	ns	ns	ns	ns	ns
	5		*	ns	*	ns	ns	ns	*
	20		ns	ns	*	ns	ns	*	ns
	0,1			*	ns	ns	ns	*	ns
A1S3	1			ns	ns	ns	ns	*	ns
	5			ns	ns	ns	ns	ns	ns
	20			*	*	ns	ns	**	*
	0,1				ns	ns	**	ns	ns
A2S1	1				ns	ns	ns	*	ns
	5				ns	ns	ns	ns	ns
	20				ns	ns	ns	ns	ns
	0,1					ns	*	ns	ns
A2S2	1					ns	ns	*	ns
	5					ns	ns	ns	ns
	20					*	*	ns	ns
	0,1						ns	ns	ns
A2S3	1						ns	*	ns
	5						ns	ns	ns
	20						ns	*	ns
	0,1							***	ns
A3S1	1							*	ns
	5							ns	ns
	20							*	*
	0,1								ns
A3S2	1								**
	5								ns
	20								ns

Nine synthetic 2´-Fluoro modified siRNAs were separately compared to each other at four concentrations (n=5-9). p-values varied between <0,001 and 0,033 and p< 0,05 was considered statistically significant (ns, non-significant; *, p< 0,05; **, p<0,01 and ***, p<0,001).
[II; additional data].

5.2.2 Plasma stability

Unmodified and 2´-Fluoro modified siRNA molecules were incubated in 90% human plasma at +37 °C. Samples were collected at various time points and siRNA stability was evaluated by measuring the migration of RNA samples in native PAGE. Results were normalized to baseline and the plasma stability of A1S1 (unmodified), A2S1 (2´-Fluoro-purine antisense strand) and A1S3 (2´-Fluoro-pyrimidine sense strand) were decreased time-dependently (II, see Figure 2). No obvious degradation of A1S2 (2´-Fluoro-purine

sense strand), A2S2 (both strands 2´-Fluoro-purine modified) or A3S3 (both strands 2´-Fluoro-pyrimidine modified) in human plasma was seen even after 72 h.

5.3 DNA oligomers activate TRACP in human monocyte-macrophage lineage (III)

The original aim of the study was to design endonuclease resistant antisense DNA oligomers (ODNs) with phosphorothioate backbone to knock down human TRACP (NM_001611). Random control ODN did not have any relevant homology to human sequences. We cultured human PBMC in conditions supporting osteoclast differentiation on bovine cortical bone slices. Antisense, sense and a random control CpG-ODNs (TAG) were diluted in sterile filtered (0,2 μm) water and added to the cultures in 5 μM doses at days 4, 8 and 12 during fresh medium change; water was added as a control. Cells were fixed and stained 15 min with the Leukocyte Acid Phosphatase kit for TRACP multinuclear cell detection. Medium TRACP activity was measured with in-house Elisa. The TRACP staining of the cells revealed that the mononuclear cells treated with PTO-ODNs were extremely TRACP positive compared to the cell control; no toxic effects were observed (III, see Figure 1). No differences in TRACP staining intensity or in the morphology between osteoclasts in different groups were detected. ODN addition affected TRACP expression significantly ($p<0.05$) already at day 8, four days after the first dose (III, see Figure 2). TRACP over-expression accumulated during the culture showing obvious induction of TRACP by CpG-ODNs compared to the baseline.

5.3.1 TRACP activation in monocyte-macrophage lineage is sequence independent

The CpG motif has been recognized as a ligand for TLR9 and, therefore, new ODNs with phosphorothioate backbone targeting human TRACP in two separate regions (AS1 and AS2) and a random control without CpG or GpC motifs were designed. non-CpG ODNs were diluted with sterile buffer, which was also added to culture medium and referred to as a baseline. Human PBMCs were cultured as described above. To test if the induction of TRACP expression was TLR9 and CpG motif dependent, 5 μM doses of non-CpG ODNs were added to PBMC culture consisting of mononuclear cell population at days 7, 9 and 11. On day 13 the cells were lysed; both intracellular and secreted TRACP were measured to monitor the TRACP activation due to nonCpG ODNs addition.The non-CpG ODNs induced TRACP expression already after the first dose and at day 11 the difference compared to the cell control was significant (III, see Figure 2). In addition, the levels of intracellular TRACP 5b were highly upregulated in non-CpG treated cells when compared to baseline ($p<0.001$).

5.3.2 Effects of siRNA molecules

Our next aim was to use the sequence specific siRNA molecules to knock-down human TRACP in human osteoclast culture. The efficacies of human TRACP siRNA molecules have previously been validated in CHO cell line stably expressing human TRACP under a strong viral promoter; the results confirmed that TRACP siRNA

molecules efficiently mediates RNAi in CHO-TRACP cells. Human osteoclast precursor cells (obtained from Cambrex) were cultured for 9 days before transfection in conditions supporting osteoclast differentiation. The culture medium was treated by RNase inhibitor 30 minutes before siRNA additions to inhibit RNase activity in the medium. The cells were transfected with or without Mirus *Trans*-IT TKO® transfection reagent with control siRNA or TRACP siRNA (50 nM each) at day 9. The effects of transfections were detected 24 h after transfection. Cells with three or more nuclei were counted and TRACP 5b was measured from the culture medium. Unexpectedly, TRACP specific siRNA molecules transfected by Mirus through endocytosis especially induced TRACP expression ($p<0.01$) in osteoclasts as compared to mock transfected cells (III, see Figure 3). In addition, TRACP siRNA molecules supplemented to the cells without transfection agent also significantly increased TRACP5b secretion ($p<0.01$, Figure 6, unpublished).

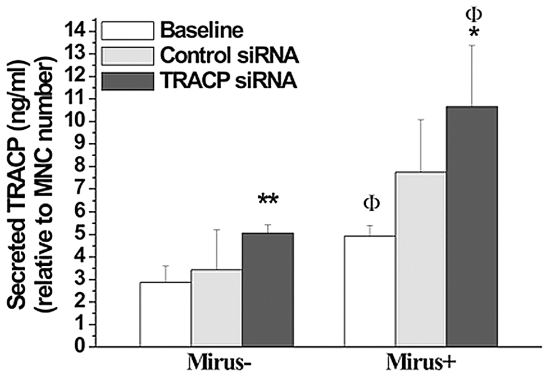

Figure 6. Induction of TRACP secretion 24 h after siRNA transfection (III, unpublished). The induction of TRACP expression in human osteoclast culture was studied after siRNA transfection without (Mirus-) or with (Mirus+) vehicle. Secreted TRACP5b was measured and the number of multinucleated cells (MNC) was calculated 24 h after transfection. TRACP specific siRNA significantly induce TRACP expression even delivered without a vehicle (unpublished). When siRNAs were delivered with Mirus, TRACP induction was more prominent (III). Values are means ±SD of triplicates; * $p<0.05$, ** $p<0.01$, compared to baseline.Φ $p<0.05$ compared to cells without transfection reagent (Mirus-).

5.3.3 TRACP activation in TLR9 knockout mice bone marrow derived osteoclasts

To evaluate whether or not the increased TRACP expression was TLR9 dependent, we cultured bone marrow -derived osteoclasts from WT and TLR9 knockout mice. The TLR9 knockout (TLR9-/-) mice were made in the C57Balck/B6 background and have been described previously (Hemmi et al., 2000). Both WT and TLR9-/- bone marrow derived osteoclasts were treated with CpG-ODNs or nonCpG-ODNs after 7 day osteoclast differentiation. 5 μM doses of ODNs were added to osteoclast cultures 7 and 9. Both CpG and non-CpG containing ODNs significantly increased intracellular TRACP 5b activity in both WT and TLR9 -/- osteoclast cultures in a similar manner (III, see Figure 4). However, CpG ODNs induced a somewhat stronger effect, surprisingly also in the TLR9-/- bone marrow cell culture (Figure 7, unpublished).

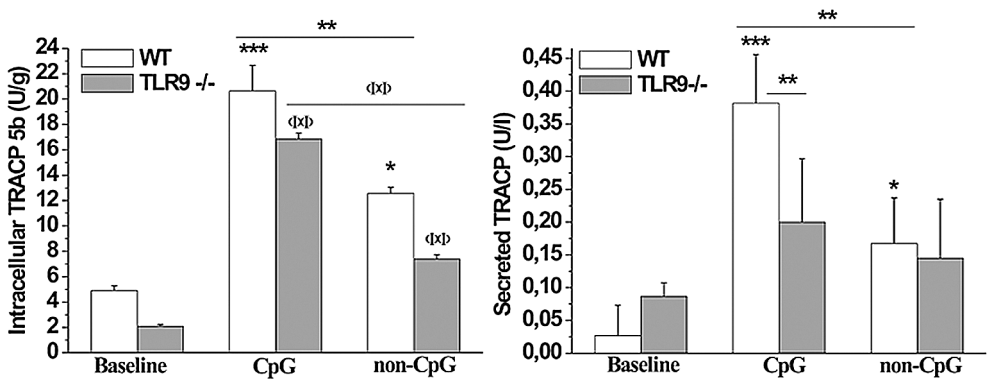

Figure 7. Increased intracellular TRACP5b after CpG and nCpG oligonucleotide treatment. Wild type and TLR9-/- bone marrow cells were treated two times with 5µM doses of CpG or nonCpG ODNs. Intracellular TRACP5b was siqnificantly increased in a TLR9 independent manner. However, the CpG motif showed slightly more capacity to induce TRACP expression (unpulished). Statistics: * $p<0.05$, ** $p<0.01$, ***$p<0.001$ compared to wild type baseline. Φ compared to TLR9-/- baseline. Horizontal lines represent the comparison between CpG and nonCpG treated cells.

5.3.3.1 Cytokine response after siRNA:Mirus transfection (unpublished)

Previous studies have shown that siRNA delivery into human cancer cells trigger significant induction of inflammatory responses (Yoo et al., 2006). Transfection of mouse bone marrow cells with the empty Mirus TransIT-TKO vehicle led to significant induction of TNF-α after 24 hours. Nuclei were stained with Hoechst 33258 (Molecular Probes, USA) to evaluate the nuclear morphology and no effects on apoptosis were present. The response of TNF-α induction was the same with bone marrow cells isolated from TLR9 -/- mice (Figure 8). Negative control siRNA:Mirus - complex induced cytokine secretion even further. Secretion of IL-6 was not affected by transfection reagent; however, negative control siRNA showed similar IL6 response compared to TNF-α induction.

5.3.3.2 TLR expression in bone marrow and in osteoclasts (unpublished)

Bone marrow total RNA was isolated from WT and TLR9-/- bone marrow and the levels of β- actin, TRACP and TLRs 7, 8 and 9 were analyzed by RT-PCR. Previously published TLR sequence specific PCR primers were used (Rodriguez-Martinez et al., 2005). TLR7 was not present in detectable amounts but TLR8, TRACP and β-actin showed equal expression levels in both WT and TLR9-/- bone marrow RNA samples (Figure 9a). As expected, TLR9 mRNA was found only in the WT bone marrow sample, not in RNA isolated from TLR9-/- bone marrow. TLR9 was microscopically visualized in human osteoclast by using TLR9 specific antibody. It has been previously suggested that TLR9 is not expressed in mature osteoclasts (Takami et al., 2002). Human osteoclasts were stained with TLR9 specific antibody and Alexa 546 was used

for detection of actin ring structures in resorbing osteoclasts. TLR9 was localized into the vesicular compartments of actively resorbing human osteoclasts (Figure 9 b). In mononuclear cells, the TLR9 staining was less intense and localized in sole vesicles, most likely in endosomes. TLR9 immunostaining was not present without cell membrane permeabilization, suggesting the lack of outer membrane localization of TLR9 in osteoclasts. The staining process with only FITC-labelled secondary antibody (no primary antibody present) showed no or a very weak signal (Figure 9c).

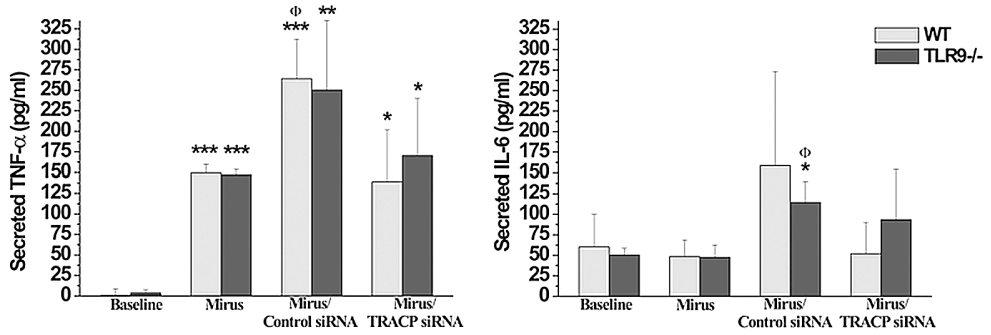

Figure 8. TNF-α and IL-6 responses by Mirus transfection reagent and siRNA molecules (unpublished). WT and TLR9 -/- bone marrow cells were tranfected with empty vehicle or with vehicle complexed with negative control siRNA or TRACP specific siRNA (25 nM doses). Left panel shows the increase of TNF-α secretion by Mirus vehicle and right panel shows the mild response of IL-6 by negative control siRNA. Values are means ±SD of triplicates; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to untreated baseline.Φ $p<0.05$ compared to Mirus transfected cells.

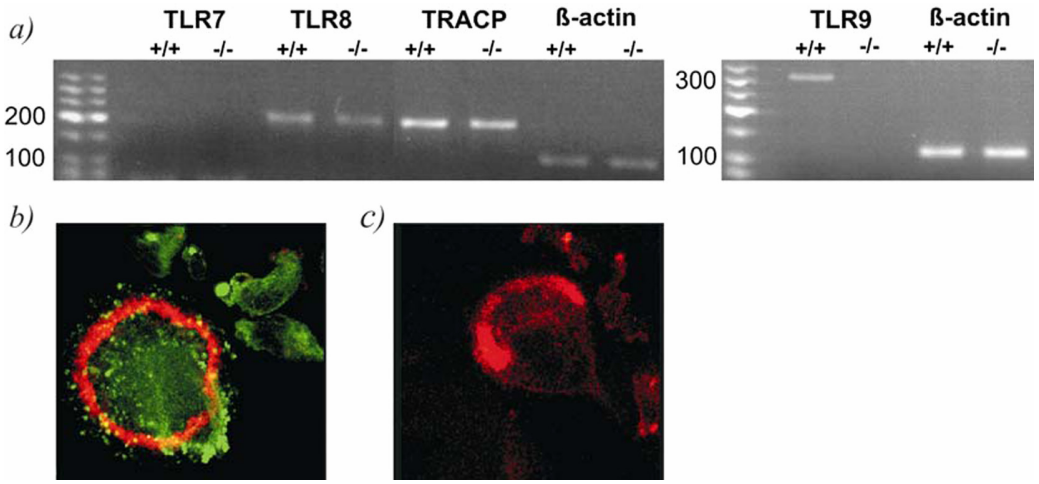

Figure 9. Toll-like receptors in bone marrow and in osteoclast (unpublished). mRNA levels of TLRs 7, 8 and 9, and TRACP were analyzed from both WT and TLR9-/- bone marrow to exclude the possibilities of altered phenotype of TLR9-/- strain (a). β-actin mRNA levels were used as a reference. Confocal image of TLR9 localization in human osteoclast (b), original magnification 100x, zoom 1.6. Green colour represents TLR9 staining, red colour represents actin. FITC-labeled secundary antibody shows no unspecific staining (c).

6 DISCUSSION

The first step in knocking down a target gene by RNAi is to design an efficient siRNA molecule. The sites of active siRNA molecules are not predictable, but there have been several systematic attempts by numerous siRNA molecules to correlate the siRNA features in function. First, Harborth et al. (2003) showed that siRNA efficacies varied with small positional shifts. Shortly after, both Khvorova et al. (2003) and Schwarz et al. (2003) showed that functional siRNA molecules have a weaker base pairing in the 5´-end than in the 3´-end of the antisense strand. Reynolds et al. (2004) used siRNA molecules that were targeted in every second position in the mRNA region of firefly luciferase and human CyclophilinB in their analysis. The results revealed base compositions and more detail base pairing strength data that are currently the principles of functional siRNA candidate sequence search.

The ***siRNA_profile*** program was first developed and adjusted by using the known siRNA data set of human CyclophilinB of Reynolds et al. (2004) in which siRNAs were designed to a target gene in every second position. Their data sets already showed the striking variation that closely lying siRNA sequences may have significant differences in their silencing efficacy, thus suggesting the need for cautious siRNA design. A heuristical scoring method was developed based on human CyclophilinB siRNA sequences to distinguish functional and non-functional siRNA sequences exhibiting favorable thermodynamical parameters but differential nucleotide preferences. Further, the functionality of the ***siRNA_profile*** program was evaluated by using a large data set of 34 target genes and over 2400 siRNA sequences with known efficacies (Huesken et al., 2005). The evaluation of siRNA stabilities of the siRNA sequences that were categorized based on the silencing efficacy revealed that highly efficient siRNAs have significantly lower overall stability than nonfunctional siRNA molecules (I). This low overall stability of functional siRNA is recommended as an additional guideline for designing highly functional siRNA molecules. By using this additional thermodynamical rule and novel heuristic scoring method, the ***siRNA_profile*** program was able to distinguish highly functional siRNA sequences from a pool of modestly functional siRNA sequences.

Recently, neural networks working on predicting siRNA efficacies *in silico* have been developed. There has been a number of various comparative analyses of siRNA design approaches (Saetrom and Snove, 2004; Yiu et al., 2005). The most recently published detailed comparison of four independent databases of over 3300 experimentally verified siRNAs revealed that BioPredsi, ThermoComposition and DSIR showed high siRNA predictional competence *in silico* (Matveeva et al., 2007). However, these three programs are mainly for large scale analysis purposes and are complicated to use for a basic researcher working on single gene knock downs. The ***siRNA_profile*** program was compared with five available web-based siRNA prediction programs and was found to be the most accurate. However, the comparison was performed with a short target region. The most striking finding was that the variability between the selectivity of different programs was due to their capacity to select different siRNA sequences

among the 80 available. This indicates the different weighting of available search parameters in each program. However, comparison of the different programs and growing number of validated siRNA data sets provides new information of functional siRNA sequences and therefore aids future siRNA design and miRNA sequence prediction.

The 2´-OH group of the ribonucleotide ribose ring distinguishes RNA from DNA and is required for nucleophilic attack during the hydrolysis of the RNA backbone and is therefore an attractive position for modifications. Interestingly, it has been shown that several substitutions in the 2´-position of the RNA ribose ring replacing 2´-OH groups improve the biological properties and *in vitro* utility of siRNA molecules, but also significantly increase the dsRNA melting temperature T_M. However, T_M can be measured precisely, but it is not a thermodynamic parameter and does not directly measure the hybridization affinity of RNA duplex. As a contrast, by the analysis of over 2400 siRNAs by the ***siRNA_profile*** program it was shown that low thermodynamical stability ($\Delta G°_{37}$) is crucial for the functionality of siRNA molecules (I). This suggests that the enhancement of the thermodynamical stability of the siRNA molecule by increasing the number of hydrogen bonds between Watson-Crick bases (increase of total C-G content) decreases siRNA efficacy, but the increased stability due to intermolecular changes in the 2´-position does not necessarily affect RISC functionality. It has been previously shown that the size of the 2´-substitution correlates with the stabilizing capacity; smaller substituents result in a greater duplex stability than larger ones (Freier and Altmann, 1997). It has been proposed that a network of water-mediated hydrogen bonds anchored by the 2´-OH group stabilize the A-helix form of the RNA duplex (Egli et al., 1996; Fohrer et al., 2006; Landt et al., 2005). The 2´-OH in RNA is the only H-bond donor in the backbone and brings water molecules into regions that are found hydrophobic in the DNA duplex, and exists as directional arms to build the water molecule framework in RNA duplex. It has also been suggested that the regularity of the water distribution in both minor and major grooves are important as structural water molecules in RNA-RNA or RNA-protein recognition. In contrast, organic fluorine has high electro-negativity properties, but when attached to carbon, it has low or no power to act as a proton acceptor in the formation of hydrogen bonds (Dunitz, 2004). This leads to the reorganization of structural water molecules due to the lack of supporting hydrogen bonds via the 2´-OH groups. However, fluorine-substitutions may shift the conformational equilibrium in the ribose moiety into more stable C3´-*endo* conformation and, therefore, may cause higher hybridization affinity in the dsRNA A-helix (De Mesmaeker et al., 1995; Guschlbauer and Jankowski, 1980). An A-helix having a narrow major groove and a shallow, wide minor groove has been shown to be strictly necessary for antisense:target mRNA recognition by activated RISC (Chiu and Rana, 2002). This may explain the enhanced efficacy of 2´-Fluoro-pyrimidine and- purine modified TRACP siRNA molecules having more favorable conformational ribose structure compared to unmodified (II).

Target mRNA is cleaved by activated RISC in a single site that is defined between nucleotides 9 and 10 where the 5´-end of the antisense strand is bound to the target

(Elbashir et al., 2001b; Hammond et al., 2000). Before mRNA cleavage, the sense strand of the siRNA molecule has been proposed to act as the first target of AGO2 and is cleaved and removed during RISC activation by the antisense strand (Matranga et al., 2005; Rand et al., 2005). The sense strand cleavage by AGO2 is crucial for proper RISC activation and function (Rand et al., 2005). Therefore, modifications such as rasemic mixture of phosphorotioate backbones or bulky 2´-*O*-Me substitutions in the cleavage site have been shown to inhibit sense strand cleavage and lead to impaired target mRNA cleavage (Leuschner et al., 2006; Rand et al., 2005; Schwarz et al., 2004). Phosphorothioate modification in the cleavage site replaces the non-bridging oxygen of the scissile phosphate by sulphur and therefore has been proposed to hinder Mg^{2+}-dependent sense strand cleavage (Schwarz et al., 2004). 2´-Fluoro-pyrimidine modified siRNA molecules with or without additional substitutions have already been used in *in vitro* and *in vivo* knock-down experiments (Layzer et al., 2004; Morrissey et al., 2005). Combinations of 2´-Fluoro-pyrimidines and -purines in the antisense and/or sense strand made it possible to study the biological effects of these modifications in more detail, also in the critical AGO2 cleavage site (II). The ***siRNA_profile*** program was used to design highly functional siRNA sequences specifically to knock down TRACP. The CHO cell line in which TRACP was stably expressed under a strong CMV promoter was used to monitor the siRNA efficacies and biological effects of 2´-Fluoro modified siRNAs (Janckila et al., 2002b). Antisense and sense strands of TRACP siRNA molecule were used for 2´-Fluoro-pyrimidine or -purine modifications. The results showed that all 2´-Fluoro-pyrimidine and –purine modified siRNA molecules efficiently knocked down the target protein and led to mRNA degradation. In addition, the replacement of 2´-Fluoro purine with 2´-Fluoro pyrimidine in the sense strand significantly increased siRNA efficacy; however, the 2´-Fluoro-purine substitutions allowed the use and protection of purine rich siRNA sequences. 2´-Fluoro-pyrimidine and -purine substitution increased T_M as expected; most of the modified siRNA molecules exhibited high plasma stability, and no toxic effects were observed. The most notable finding was that both 2´-Fluoro-pyrimidine and -purine modifications were well tolerated in the putative AGO2 sense strand cleavage site, and even showed increased efficacy compared to the unmodified TRACP siRNA molecule (II). These beneficial biological characteristics increase the *in vivo* and future therapeutical utility of 2´-Fluoro-pyrimidine and –purine modified siRNA molecules.

Recently, it has been shown that in addition to CpG-motif containing DNAs, siRNAs and shRNAs may trigger immune activation (Hemmi et al., 2000; Kariko et al., 2004). Efforts to understand the molecular mechanism by which dsRNA and bacterial DNA trigger the induction of inflammatory responses have led to identification of cellular sensors of viral and bacterial infections. There have recently been acknowledged two kinds of nucleid acid sensors signalling the production of interferons. One type of sensors are the family of cytosolic receptors known as the retinoic acid inducible gene I (RIG-I)-like receptors (RLRs). The other type of the sensor includes the members of the TLRs, especially TLRs 3, 7, 8 and 9. In addition, human monocytes that have been shown to express TLR8 instead of TLR3 and TLR9, respond to ssRNA and siRNA duplexes (Hornung et al., 2005; Judge et al., 2005; Sioud, 2005). TLR7 and 8 has also been suggested to be responsible for innate immunity responses originated by siRNA

molecules shorter than 30 nucleotides (Sioud and Furset, 2006). In addition, CpG motif DNA oligomers have been shown to directly modulate osteoclastogenesis. CpG ODNs induced TRACP positive cells in macrophage cultures, but not multinucleation or calcitonin receptor expression. Noticeably, CpG ODNs strongly increased RANKL-induced osteoclastogenesis in RANKL-pretreated bone marrow derived macrophage cultures , but inhibited RANKL- induced osteoclastogenesis when present from the beginning of the culture (Amcheslavsky et al., 2005; Takami et al., 2002; Zou et al., 2002). CpG-ODNs induce TNF-α expression, but reduce the expression of M-CSF receptor. This suggests that CpG ODNs support osteoclastogenesis of the osteoclast precursor cells that are already on the osteoclast differentiation path via TNF-α induction, but differentiation is inhibited in the early path due to the downregulated M-CSF receptor. Our original aim was to knock down TRACP in bone resorbing osteoclasts and study the loss-of-function phenotype. TRACP siRNA molecules that were found highly efficient in the CHO-TRACP cell line, were used to knock-down TRACP in osteoclasts differentiated from human osteoclast precursor cells. Unexpectedly, TRACP expression was significantly increased in the monocyte-macrophage lineage. Apparently, the increased TRACP expression was regulated by its own promoter, because no induction was seen when TRACP was expressed under a CMV promoter. DNA oligomers with and without CpG-motif designed to knock down TRACP gene also led to the same increase in TRACP expression. Random control siRNAs and DNA oligomers showed the same induction in the TRACP expression profile; thus the TRACP activation effect is not due to a regulatory feedback after gene knock down.

Interestingly, modifications in the 2′-position, including 2′-Fluoro, 2′-*O*-Me and 2′-H substitutions, have been shown to abrogate the immunostimulatory effects that are caused by naked siRNAs (Morrissey et al., 2005; Sioud, 2005; Sioud and Furset, 2006). Thus, the effects of fully 2′-Fluoro modified siRNA molecules to TRACP expression still needs to be determined. A sequence 5′-UGUGU-3 has been identified as a potential immunostimulatory motif within an siRNA sequence (Judge et al., 2005). Noticeably, TRACP siRNA sequences did not contain an immunostimulatory motif; however, the sequence of the negative control (Ambion, USA), which induced a strong TNF-α secretion, is unknown. In addition, TNF-α and IL-6 secretion were enhanced as a result of the sole transfection reagent. Moreover, TRACP siRNAs and the negative control siRNA were synthetised *in vitro* by using T7 that has been shown to generate 5′-triphosphate which further mediates interferon response via RIG-I (Plumet et al., 2007). In contrast, it has been shown that synthetic siRNAs having 2 nt 3′-end overhangs bypass RIG-1 (Marques et al., 2006). This demonstrates the sensitivity in siRNA design, synthesis, transfection and the whole concept of specific RNAi in mammalian cells (I,III).

The cellular aspects of the signalling pathway activating TRACP by exogenous DNA and RNA oligomers are unknown. It has been shown that dsRNA molecules are internalized by scavenger receptor-mediated endocytosis in *Drosophila* cells (Ulvila et al., 2006). In addition, it has been shown that different scavenger receptors have different ligand affinity to different CpG-motifs and these have been suggested to

interacts with TLR9: MARCO in macrophages has ligand affinity towards phosphorotioated CpG-B oligomers and scavenger receptor A binds to G-rich ODN aggregates (Jozefowski et al., 2006; Suzuki et al., 1999). Scavenger receptors have been accepted as an important part of innate immunity by recognizing and binding a wide variety of pathogens and initiating intracellular responses. Our results showed that the induction of TRACP expression by CpG, non-CpG DNA oligomers and RNA molecules was TLR9 independent suggesting that the signalling cascade may start from the receptor-ligand interaction during DNA and RNA molecule internalization bypassing the TLR9 pathway. Interestingly, there was some indication that CpG oligomers have more potent ability to induce TRACP expression, which may be an indication of stronger receptor affinity to CpG oligomers than to non-CpG oligomers.

In addition to host protection by Toll-like receptors, increase of TRACP expression by several DNA oligomers with and without CpG motif, and by siRNA molecules suggests TRACP being an important component of the host´s innate immune response during bacterial or viral infection.Though the biological function of TRACP has been intensively studied, it still remains unknown. The ROS generating activity of TRACP has been suggested to have several functions in TRACP expressing osteoclasts, macrophages and dendritic cells. In osteoclasts, ROS generated by TRACP are able to destroy type I collagen suggesting that TRACP may directly participate in bone resorption. Macrophages overexpressing TRACP show increased amounts of intracellular ROS and have increased bacterial killing properties (Räisänen et al., 2001). In contrast, mice lacking TRACP have disordered macrophage inflammatory responses and reduced clearance of pathogens. In addition, in alveolar macrophages TRACP has been shown to colocalize with major histocompatibility complex II (MHC II) and endocytoced *Staphylococcus aureus* in the antigen presentation route (Räisänen et al., 2001). The increased TRACP expression by DNA and RNA molecules in a TLR9 and sequence independent manner strongly suggests that TRACP is involved in both bone resorption and in the immune defence system in cells sharing analogous intracellular routes for transcytosis or antigen presentation.

7 CONCLUSION

Our user friendly siRNA design program will provide a new tool for researchers utilizing RNAi in their knock-down experiments. The ***siRNA_profile*** program was developed for selection of highly efficient siRNA design to save time and money in planning of loss-of function experiments and in the process of a new drug target validation. Detailed analysis of large siRNA data sets by the ***siRNA_profile*** program revealed additional guidelines to follow. The low overall stability of siRNA molecule is a particularly beneficial characteristic in addition to biased stabilities and favorable nucleotide distribution along siRNA to activate RNAi. To improve siRNA utility *in vivo*, 2´-Fluoro-purine and 2´-Fluoro-pyrimidine modifications were examined. Interestingly, 2´-Fluoro modifications in a putative sense strand Argonaute2 cutting site showed increased RNAi activation, indicating the beneficial steric structure of fluorine fitting into a RISC complex. Moreover, the plasma stabilities of 2´-Fluoro-modified siRNA molecules were prolonged showing the utility in therapeutical applications. In addition, our results presented a novel indication of the role of TRACP in innate immunity. RNAi mediated TRACP knock-down was highly successful when induced *in vitro* in a biological model in which viral CMV promoter replaces TRACP's own promoter. However, in the bone marrow derived monocyte-macrophage lineage, when the TRACP enzyme is expressed under its own promoter, both TRACP specific DNA oligomers and siRNA molecules highly upregulated its expression. This response was gene specific since tartrate-sensitive acid phosphatase levels were not changed. These results revealed an obstacle of using antisense DNA and RNAi gene knock down techniques in TRACP expressing monocyte-macrophage cells and tissues. The sequence and TLR9 independent specific upregulation of TRACP in cells of monocyte-macrophage lineage suggests that TRACP could act as a host protector against exogenous nucleotides.

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