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Selected abstracts

miRNA processing and its applications in RNAi

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ABSTRACT

Maturation of metazoan microRNA (miRNA) is initiated by RNase III Drosha and its cofactor DGCR8. Drosha accurately cleaves the local hairpin structure embedded in the long primary transcript (pri-miRNA) and thereby pre-determines the miRNA sequences. Although our knowledge on miRNA procssing has significantly advanced in recent years, it remains unclear how the Drosha-DGCR8 complex specifically recognize its substrates and determines the cleavage sites. Previous works suggested that the cleavage site may be decided by the distance (~22nt) from the terminal loop. Here, we propose a new model for pri-miRNA processing based on the evidence from computational analysis, systematic mutagenesis, and in vitro processing analysis. The substrate specificity of Drosha found in this study will facilitate the prediction of miRNA genes and the design of small hairpin RNAs for RNA interference applications.

miRNA expression in cancer cell lines and tumor samples

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ABSTRACT

MicroRNAs (miRNAs) are a recently recognized class of riboregulators. miRNAs are 21- 22 nucleotides in length and derived from longer precursors. The mature miRNAs function within large complexes to negatively regulate specific target mRNAs. In animals most miRNAs have an imprecise complementarity to their target mRNAs; basepairing with its target sequence - often in the 3' UTR - results in translational repression. The functional significance of most miRNAs is still unknown as is their involvement in disorders like cancer. The aim of our studies is to determine miRNA expression in cancer cell lines and in series of clinically well-annotated tumor samples and to examine the role of aberrantly expressed miRNAs for the pathogenesis of cancer.

Oligonucleotide arrays in which LNA capture probes are used for the detection of the full complement of human/mouse miRNAs were developed and tested in combination with various total RNA and miRNA labeling techniques. We will present data on the use of these arrays for miRNA expression profiling in the NCI panel of cancer cell lines, including chemotherapeutic sensitive and resistant cell lines, as well as samples of breast, lung and colon cancer. Investigated will be whether individual miRNAs or miRNA profiles correlate with important clinical and prognostic parameters like tumor size and type, tumor stage, response to chemotherapy, overall and disease free survival. Special attention will be given to the expression of the let-7 family of miRNAs – measured by Northern blotting and quantitative RT-PCR - in non-small cell lung cancer samples and corresponding normal lung tissues in relation to expression of the *RAS* oncogene.

miRNA expression signatures in cervical cancer

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ABSTRACT

Background: MicroRNAs (miRNA) are a recently discovered family of short non-protein-coding RNAs with diverse functions, including regulation of cellular differentiation, proliferation and apoptosis. It is

hypothesised that different populations of miRNAs are expressed in malignant versus normal tissues with general down regulation of miRNAs in tumoural samples. The interaction between miRNAs and specific oncogenes and cell cycle regulatory genes is not well understood.

This study interrogated the expression repertoire of 180 miRNAs in the context of cervical cancer using a recently developed technique, utilising stem loop primers for reverse transcription followed by real time TaqMan PCR.

Design: miRNA was purified from C33A (HPV negative) and CaSki (HPV 16 and 18 positive) cervical cancer cell lines using Ambion's mirVana™ miRNA isolation system. RNA extracted from histologically normal cervical tissue was used as a calibrator. miRNA expression profiles were examined using the Applied Biosystems TaqMan® MicroRNA Assays Human Panel - Early Access Kit (Part no. 4365409). Real time TaqMan PCR data was analysed by relative quantitation using the delta delta CT method. miR 16 and let 7 miRNAs were used as endogenous controls.

Results: Cervical cancer cell lines C33A and CaSki demonstrated distinct miRNA expression signatures in comparison with normal cervical tissue. Further, a differential profile of miRNA expression was observed between HPV positive and negative cell lines, with the predicted function of differentially expressed targets coinciding with a number of cell cycle regulatory molecules.

Conclusions: These findings highlight the potential importance of miRNA molecules in cervical cancer. It is interesting to speculate that the variations in miRNA expression in cervical cancer cell lines contribute to dysregulation of the cell cycle regulatory pathway in cervical cancer. Thus miRNA expression patterns may serve as potential biomarkers of pre-invasive cervical disease and potential therapeutic targets.

Studies of Argonaute proteins: Central mediators of RNA silencing

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ABSTRACT

RNA silencing regulates gene expression via mRNA degradation, translation repression and chromatin remodeling. Currently the most extensively characterised system is targeted mRNA degradation mediated by small interfering RNAs (siRNAs). siRNAs are 21-25 nucleotide double-stranded RNAs produced from double-stranded RNAs (dsRNAs) through the action of the RNase III enzyme Dicer. This process may have evolved as a cellular defence response to viral infection or the activity of transposable DNA elements. Related to the action of siRNAs are the mechanisms of silencing mediated by microRNAs (miRNAs). miRNAs closely resemble siRNAs in size and structure, but are encoded as stem-loop precursors in the genome, which are then processed through the actions of the RNase III enzyme Drosha together with Dicer. Many miRNAs regulate gene expression by binding and inhibiting the translation of mRNAs although in some instances, particularly in plants, miRNAs can also target mRNAs for degradation. A third mechanism of silencing operates at the transcriptional level, by targeting chromatin remodelling factors to heterochromatic regions. As with the siRNA and miRNA pathways, the specificity of nucleic acid targeting (perhaps this time DNA), is mediated by small RNAs.

These RNA silencing processes are mediated by large ribonucleoprotein complexes. In the RNA interference process, this complex is termed RISC (for RNA induced silencing complex) whereas chromatin remodeling effects are exerted by RITS (RNA induced initiation of transcriptional silencing). It is likely that these complexes share the same or a related Argonaute protein subunit. The mechanistic differences between siRNAs and miRNAs can be attributed partly to the degree of complementarity between the short RNA and its target.

To understand the molecular mechanisms underlying RNA silencing we initiated structural and functional studies of Argonaute proteins. Argonaute proteins are highly conserved from humans to Archaea and are characterised by a C-terminal PIWI domain and in most cases, an N-terminal PAZ domain. I will describe our studies of the PIWI domain from *Archaeoglobus fulgidus* (AfPiwi) which has served as a model system for understanding eukaryotic Argonaute proteins. These studies have provided the following insights:

1. The identification of the component(s) of RISC responsible for the mRNA endonuclease activity, explaining how the site of cleavage within mRNA is specified.
2. Indicates how the guide (anti-sense) RNA strand is selected and incorporated into RISC/RITS, whereas the sense (passenger) strand is discarded.
3. Provides a rational for the observation that siRNAs (which are perfectly complementary to their cognate mRNA) cleave their target, whereas animal miRNA, which are only complementary within their 5' 2 to 8 nucleotides to their target mRNA, mediate translation repression.
4. Provides insights into optimal design of siRNAs for control of gene expression.

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MicroRNAs in Animal Development

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ABSTRACT

RNA silencing was discovered as an experimental tool; our laboratory is primarily interested in the biological functions. In one line of research we study RNAi as the machinery that protects the genome of *C. elegans* against transposition in the germ line. We now found that a single episode of RNAi can silence a gene in an inheritable fashion, over more than 40 animal generations. Off-target silencing can also inherit indefinitely.

A second research question is the role of microRNAs in vertebrate development. We previously performed computational whole-genome comparisons to predict hundreds of novel microRNAs (Berezikov et al, 2005). We now present experimental confirmation of many of those by a modified RAKE-microarray assay, and the discovery of many novel microRNAs by large scale cloning and sequencing from human, mouse, and zebrafish embryos, using different tissues and different stages. Following up on recent *in situ* microRNA detection experiments (Wienholds et al, 2005) we studied the expression of novel microRNAs. To address their role in development we knocked down miRNAs in zebrafish embryos.

Abrogation of gene silencing by DNA vector-based RNAi during apoptosis due to cleavage and inactivation of Dicer

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ABSTRACT

RNAi is increasingly used for studying apoptosis-related genes or cell death-related events with an assumption that RNAi process remains functional during apoptosis. However, gene silencing by RNAi requires active participation of host cell proteins including RISC and Dicer, and many cellular proteins are known to be inactivated during apoptosis. Therefore, we examined integrity of the RNAi process during apoptosis using a model system of DNA vector-based RNAi for silencing poly(ADP-ribose) polymerase-1 (PARP) gene. This model offers two advantages: (a) Gene silencing will be abrogated if either Dicer or RISC is inactivated during apoptosis since both are required for silencing; and (b) failure of RNAi in this model would be readily identifiable due to appearance of PARP or its apoptosis-signature 89 kDa fragment generated by action of caspase 3. We report here that DNA vector-based RNAi of PARP is abrogated in different cell types undergoing apoptotic death induced by a variety of agents. This was associated with a cleavage and inactivation of intact Dicer-1 protein from MW_r 230 kDa to a ~180 kDa fragment, whereas RISC member Argonaut 2 was not cleaved. In vitro cleavage assay with purified human Dicer-1 revealed caspases 3 and 7 to be principal caspases responsible for formation of this apoptosis-specific Dicer fragment. We also observed that caspase-cleavage of Dicer inactivates its catalytic function, as determined by its capacity to convert 27mer dsRNA substrate to the product 21mer dsRNA. In addition, silencing of GFP by 21mer synthetic dsRNA, that requires only RISC function and not the Dicer activity, was not abrogated during apoptosis. Thus during apoptosis, a specific targeting of Dicer, an essential upstream component of RNAi pathway, inactivates this process. Apoptosis-associated inactivation of Dicer can impact not only the experimental models of RNAi that depend on Dicer but also other normal gene expression control processes which depend on the Dicer function.

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KEYWORDS: *PARP-1, apoptosis, caspases, Dicer-1, Ago-2, DNA vector-based RNAi, dsRNA*

A new mechanism for siRNA inactivation

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ABSTRACT

The active agent of RNA interference (RNAi), short interfering RNA (siRNA), is known to vary in silencing capacity depending on the position in the mRNA target. Previous studies attributed this largely to biased strand loading into the mRNA cleavage complex. Here we present evidence for a new mechanism for siRNA inactivation based on the disappearance of mRNA cleavage products and improved silencing when introducing wobble-mutations close to the cleavage point in certain siRNAs. This supports the hypothesis that the RNAi complex can stall due to very high GC-content in the 5' end of the antisense strand, and opens a new avenue for research into siRNA activity.

siRNAs: From functional genomics to drugs

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ABSTRACT

Gene silencing by small interfering RNA (siRNA) has emerged as a useful technology for assessment of gene function. Because the RNAi machinery plays a fundamental role in controlling cellular processes, it has become apparent that treatment of cells with siRNA can lead to unintended "off-target" events that can complicate gene functional analysis and development of siRNA therapeutics. We have conducted detailed mechanistic studies of siRNA action in cells in order to more fully understand the basis for both potency and "off-target" activity. The results of these studies have lead to bioinformatic and chemical modification strategies that significantly enhance the properties of siRNA. Application of these strategies should broaden the application of siRNA across the drug discovery process and positively impact their direct application as therapeutic agents.

Use of Dicer-substrate RNA duplexes *in vitro* and *in vivo*

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ABSTRACT

27mer RNA duplexes which are Dicer-substrates can be 100x more potent than corresponding 21mers. The dicing process itself affects potency and influences strand loading into RISC. We have defined methods to direct dicing to produce a single product where the AS strand is preferred for RISC loading, making design of high-potency 27mer siRNAs more predictable. Patterns for the incorporation of modified bases are described that improve nuclease resistance without affecting function as a Dicer-substrate. The utility of these compounds in several *in vivo* model systems was discussed.

Considerations for the development of siRNA-based drugs

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ABSTRACT

The successful application of siRNA drugs requires delivery systems that overcome their poor pharmacokinetics, limited biodistribution and inefficient intracellular uptake. An ideal vector will (i) be safe and well tolerated upon administration; (ii) have the appropriate pharmacokinetic attributes to ensure delivery to disseminated disease sites; (iii) deliver intact RNA to target tissue and release it inside the cell (iv) be non-immunogenic; and (v) be stable upon manufacture to facilitate production at commercial scale with uniform, reproducible performance specifications.

By application of the liposomal drug delivery paradigm, many of the limitations of the first generation non-viral delivery systems can be overcome. Here we will describe a modular delivery platform resulting in the encapsulation of siRNA in small, long-circulating particles called stabilized nucleic acid lipid particles (SNALP). SNALP are 75 nm lipid nanoparticles consisting of the RNA payload fully encapsulated in a lipid bilayer containing *diffusible* polyethylene glycol (PEG)-lipid conjugates. Following administration, the PEG conjugate dissociates from SNALP, revealing a positive charge and an increasingly fusogenic lipid bilayer, thereby transforming the particle into a transfection-competent entity. SNALP pharmacology can be modulated in a predictable manner by manipulating the composition of the SNALP lipid bilayer. For example, pharmacokinetics and biodistribution studies utilizing radiolabelled SNALP designed for hepatocyte uptake in mice demonstrated accumulation of up to 75 percent of the injected dose in the liver 24 hours after intravenous administration. SNALP mediated gene expression or RNA interference, mediated by siRNA, has been confirmed in several preclinical models of infectious and metabolic disease. Results establish the impact that SNALP formulated siRNAs could have in a number of diverse applications.

We show that while non-viral vector systems are generally considered to be non-immunogenic, the *in vivo* efficacy and safety of non-viral systems can be severely compromised due to the inherent immunostimulatory properties of their nucleic acid payloads, greatly potentiated by effective intracellular delivery. This has potential to manifest as activation of both the innate and acquired arms of the immune system. Here we will describe minimal modifications of siRNA that completely abrogate their immunostimulatory properties and concomitant toxicities while retaining potent RNAi. These results have important implications for the design and development of siRNA based drugs.

Phosphorothioate-stimulated uptake of siRNA by human cells: A progress report

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ABSTRACT

The cellular delivery of siRNA is a major hurdle of therapeutic drug development. Here, we describe that phosphorothioate (PTO)-derived oligonucleotides stimulate the physical cellular uptake of siRNA *in trans* in human cells. This is reflected by an apparent dose-dependent siRNA-mediated suppression of *lamin A/C* in primary human HUVEC cells. The PTO-stimulated cellular uptake *in trans* is

concentration-dependent, length-dependent, related to the phosphorothioate chemistry but not sequence-specific and related to a caveolin-mediated uptake mechanism. This work strongly suggests to explore PTOs as promoters for the delivery of biologically active siRNA to mammalian cells at doses sufficient for target-specific inhibition.

Current work indicates that the capturing of siRNA in the Golgi system prevents it from exerting biological activity though, conversely, this sub-cellular compartment seems to be promising for the delivery of siRNA because it is not related to TLR receptors and, thus, it might no be sensitive to a number of off-target effects.

REFERENCE

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The transition of RNAi from a research reagent to a validated tool

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ABSTRACT

The application of RNAi to target validation and functional genomics has revolutionized drug discovery and pathway analysis. Researchers are increasingly interested in performing large, genomewide screens using RNAi.

QIAGEN has developed an integrated system for gene knockdown and gene expression analysis using genomewide siRNAs and gene expression assays. This integrated approach transforms RNAi research reagents into standardized scientific tools.

This presentation included:

- Key steps in the functional validation of siRNA libraries including optimizing the workflow from siRNA transfection to monitoring of mRNA knockdown
- Lessons learned from analyzing knockdown efficiencies of thousands of siRNAs
- Use of validated siRNA libraries as a "proven-to-work" solution for screening

Synthetic oligonucleotides as therapeutic agents

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ABSTRACT

Synthetic oligonucleotides are currently being studied as therapeutic agents and are being evaluated in clinical trials based on various mechanisms, including immune modulation, antisense, siRNA, aptamers, etc. The presence of certain nucleotide motifs and structures in antisense, siRNA and aptamers has shown to stimulate immune responses. At least four molecular pattern recognition receptors, Toll-like receptors (TLR) 3, 7/8 and 9 have been known to recognize nucleic acids or their metabolites and induce immune responses. TLR9 recognizes synthetic oligonucleotides containing unmethylated CpG motifs, TLR3 recognizes double-stranded RNA, such as polyI.C and siRNA, and TLR7/8 recognize single-stranded RNA, ribonucleosides and other small molecules. We have carried out SAR studies to elucidate the structures and motifs required for immune stimulation through TLRs. Not only these studies provided novel synthetic agonists of TLRs but also provided the chemical modifications for oligonucleotides to suppress immunostimulatory activity in antisense oligonucleotides.

Multiple approaches to inhibit picornaviruses by RNA interference

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ABSTRACT

Coxsackievirus B3 (CVB-3) is a member of the picornavirus family containing an RNA genome in plus-strand orientation. Persistent infections with the virus may lead to the development of severe heart diseases. It is our aim to explore the potential of RNA interference to inhibit replication of CVB-3. To this end, we have recently developed efficient small interfering RNAs (siRNAs) targeting the viral RNA dependent RNA polymerase (RdRP). These siRNAs inhibit virus replication in plaque reduction assays by up to 90%. To investigate the mechanism of virus silencing in more detail, we generated strand-specific siRNAs either by sequence-design or by the introduction of modified nucleotides. These siRNAs enabled us to clearly demonstrate that only the viral plus-strand can be efficiently targeted in an RNAi approach, whereas the minus-strand, which occurs as an intermediate during replication, is resistant to siRNA-mediated silencing. CVB-3 is known to have a high error-rate during replication and can thus be expected to escape RNAi-silencing upon prolonged treatment. We have therefore developed an siRNA double expression vector (SiDEx), which generates two independent siRNAs simultaneously. This vector maintained its silencing capacity against the target RNA with an artificially introduced mutation in a reporter assay, whereas single siRNA-expression vectors lost their capacity to silence their respective target after

substitution of a base in the centre of the target site. As an alternative approach to cope with the problem of viral escape, we silenced the coxsackievirus-adenovirus receptor on the host cell and achieved a significant reduction of virus propagation as well. The current status of our efforts to employ siRNAs in *in vivo* models of CVB-3 infection will be discussed.

RNAi gene therapy to protect cells against HIV-1

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ABSTRACT

Double-stranded RNA can induce gene silencing via a process known as RNA interference (RNAi). The double-stranded RNA can be expressed as a short hairpin molecule (shRNA) with a base-paired stem of about 21-23 bp. Previously, we have shown that stable expression, via a retroviral vector, of a short hairpin targeting the Nef gene (shNef) of HIV-1 results in strong inhibition of HIV-1 replication. However, the application of this single shRNA was not enough to maintain inhibition. One of the hallmarks of RNAi, its sequence specificity, presented a way out for the virus, as single nucleotide substitutions in the target region abolished the suppression.

For the development of a durable gene therapy that prevents viral escape, it has been proposed to combine multiple shRNAs against conserved HIV-1 regions. Therefore, we have screened 86 different shRNAs targeting 19 highly conserved regions. We found several shRNAs that showed strong inhibition of virus production covering 8 target areas. Effectiveness of these shRNAs was confirmed in an independent reporter assay. For our gene therapy approach, we selected the lentiviral vector system. We show here that selected effective shRNAs expressed from a lentiviral vector are capable of inhibiting HIV-1 replication. We show, for the first time, that the expression of three different shRNAs from a single lentiviral vector resulted in similar levels of inhibition per shRNA as compared to single shRNA vectors. Moreover, the combined expression results in an additive effect of inhibition of virus production. Our data confirm that RNAi has great potential as an antiviral gene therapy approach and supports our effort in the development of this strategy for the treatment of HIV-1 infected individuals.

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Harnessing RNAi as a novel gene therapy: Targets and delivery

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ABSTRACT

Recently, RNA interference has emerged to be a promising therapeutic weapon. The advantages of RNA interference as a novel gene therapy against virus infection and malignant tumors line in the facts that RNA interfence silences target genes in an exquisitely specific manner, in principle, nearly all target cells and genes can be targeted, the price of siRNA processing is relatively cheap, and the silencing duration for target genes is suitable for therapeutics. However, the major obstacles hindering its immediate application in clinics include selection of targeting sequences and delivery of siRNA. Hence, the key issues of RNAi-based therapies are as follow: (1) How to select silencing targets for a particular disease; (2) How to efficiently deliver siRNAs into specific cell types in vivo? Our findings demonstrated that siRNA against HIV virus RNA and receptors on cell surface efficiently inhibited HIV infection in macrophages. Besides, our studies in mouse auto-immune hepatitis models demonstrated that injection of siRNA against Fas silenced the expression of the target gene on hepatocytes, and Fas-siRNA protects mice from fulminant hepatitis. For delivery of siRNA, we showed that hydrodynamic injection of siRNA into the tail veins of the mice can efficiently knock-down target gene expression in the liver and kidney. Injection of siRNA in renal vein is as efficient as hydrodynamic injection to deliver siRNA into the tubular epithelia of the kidney. Finally, our studies showed that using a fusion protein of antibody against gp120 and a positively charged peptide, protamine can specifically deliver siRNA into HIV infected cells *in vitro* and *in vivo*, and can specifically inhibit target gene expression.

Molecular mechanisms mediating immune evasion in African trypanosomes: Antigenic variation studied using RNAi in place of an immune system

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ABSTRACT

African trypanosomes are unicellular eukaryotes which cause African Sleeping Sickness in sub-Saharan Africa.

Trypanosomes provide a very manipulable experimental system for investigating antigenic variation and host-pathogen interactions. Trypanosomes are exposed to constant immune attack as they multiply in the blood of the infected mammal. Eventually, the host mounts an antibody response against trypanosomes expressing a given Variant Surface Glycoprotein (VSG) coat. However, as trypanosomes can switch to new VSG coat variants, these can (temporarily) evade antibody mediated lysis and form the next wave of infection. As trypanosomes have about 1000 *VSG* genes, a chronic infection can be mounted lasting for years.

We are investigating the molecular mechanisms behind trypanosome immune evasion. We have discovered that RNAi mediated ablation of *VSG* transcript triggers a rapid and specific cell-cycle arrest (*PNAS* 102: 8716-8721). Cells eventually die, leaving behind those that have switched to a new VSG variant not recognised by the RNAi. This has allowed us to develop a rapid and powerful experimental system allowing us to select for VSG switch variants completely *in vitro* using *VSG* RNAi in place of an immune system (*Mol Micro* 57: 1608-1622).

RNAi and the epigenetics of plant germline development

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ABSTRACT

All plants exist as two 'generations' and alternation between them is the principal event of their life cycles. These two generations are generally morphologically very different; in mosses the haploid gametophyte generation forms the major plant body, while the diploid sporophyte exists as a highly reduced plantlet - parasitic on the gametophyte. In flowering plants (angiosperms) these generations are reversed, with the diploid sporophyte generation constituting the plant body, and the gametophyte generation reduced to the pollen grain and the embryo sac – parasitic on the sporophyte.

There is little doubt that the plant life cycle is driven by epigenetic changes, but the role played by RNAi in these events remains to be determined. Exploration of epigenetic events associated with the formation of the germline and development of the gametophyte generation suggest that changes may take place to the RNAi system. For example formation of the sperm cells is accompanied by the activation of sequences normally silenced by RNAi, and recent data suggest that transcripts encoding some of the main elements of the RNAi system (eg *ARGONAUT*) are absent during at least part of the gametophyte phase. However, it is likely that the RNAi system functionally restored in the fertilisation products (of which there are two in plants; embryo and endosperm) since the asymmetric silencing of some alleles in the endosperm (parental imprinting) is accompanied by the appearance of non-translated short RNAs.

Drawing from work on arabidopsis, maize and tobacco we shall describe tests carried out on the RNAi system in the male and female germlines of plants, and discuss the functionality of the system in the gametophyte and fertilisation products. We shall also provide some preliminary data on the role of RNAi in imprinting, and on the plant homologues of a new family of transcription factors which, in flies and worms, plays an important role both in regulating RNAi and reproductive development.

Using genome scale RNA interference screens to investigate oncogenic signalling by Ras in human cells

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ABSTRACT

Transformation of cells by Ras and other oncogenes leads to profound changes in their gene expression programmes. Two decades of intense study of signaling pathways has led to a good understanding of many of the early signaling events induced on stimulation of oncoproteins such as Ras. In addition, recent advances in microarray technology have provided much information about the changes in transcription that accompany transformation. However, the key medium term events required to set up the transcriptional programme underlying malignant transformation are much less well defined, but may include a number of good therapeutic targets.

We have set out to study a number of aspects of cellular transformation using large-scale RNA interference libraries, including the 8000 gene NKI library. This library is in a retroviral vector that can be used either in selective screens in a pooled format, or in phenotypic screens in a high throughput format. To find novel signalling proteins regulated by Ras, we have undertaken a selective RNA interference screen for inhibitors of Ras induced senescence in human ovarian surface epithelial cells using pools of vectors targeting 96 genes each. A number of known critical Ras activated kinases have been identified in this way, including PI 3-kinase p110α and p70 S6K1. In addition, proteins not previously associated with Ras signalling have also been identified. One of these is MINK, a MAP4K which is activated by Ras through the Raf/ERK pathway. The role of MINK in Ras induced senescence appears to be through its ability to activate the p38 stress activated kinase, leading to induction of the cyclin-dependent kinase inhibitor p21. While p110α and p70 S6K1 are required both for Ras induction of senescence and transformation, MINK appears to play a significant role only in Ras-induced senescence.

Another screen has been carried out in a high throughput mode (one gene at a time) to study genes involved in the enhanced motility of invasive lung cancer cells carrying an activating mutation in K-Ras. Cells were transiently transfected with the RNAi vectors and a GFP vector and then their movement tracked by time-lapse video microscopy. In this way a number of novel proteins have been identified that affect cell motility, plus many proteins already known to be involved in this process. Subsequent characterisation has been carried out on a transcription factor that unexpectedly appears to play an important role in promoting invasiveness in human breast cancer. CUTL1 is important in providing the transcriptional programme needed for cells to move rapidly in two and in three dimensions, particularly being a regulator of integrin $\alpha 6$ expression. Low levels of CUTL1 expression prove to be a significant beneficial prognostic factor for long-term survival in breast cancer patients.

The use of this large-scale RNA interference library that targets a quarter of the human genome thus shows considerable promise in uncovering novel targets for cancer therapy.

The potential of RNAi for neurodegenerative diseases

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ABSTRACT

Endogenous pre-existing mechanisms of RNA degradation, known as RNA interference (RNAi), represent an attractive way to lower expression of targeted gene products. RNAi is an efficient method to manipulate gene function in mammalian cells either by transfection of short interfering RNAs (siRNAs) or by transcription of short hairpin RNAs (shRNAs) from viral vectors.

We have recently validated the proof-of-concept of RNAi-based knockdown of SOD1 mutant gene as a therapeutic approach for ALS (Raoul et al, 2005). We demonstrated that the delivery of a lentiviral vector carrying instructions that promote RNAi-based silencing of mutated SOD1 into lumbar spinal cord of an ALS mouse model significantly delays both onset and progression of the motoneuron disease. Nevertheless, the protective effect was restricted to the site of intervention and thus requires scale-up to reach a larger number of cellular targets for maximum clinical significance.

We are currently investigating the effect of virally RNAi-based knockdown of SOD1 mutant gene at onset of disease in transgenic mice. In parallel, we are also exploring the cell-type specific silencing of SOD1 mutant gene to better understand the role of different cells (such as muscle, oligodendrocyte, motoneuron) and their interaction in the pathogenic processes of familial ALS.

Mutations in the parkin gene are associated with autosomal recessive-juvenile Parkinsonism and lead to a defective E3 ligase activity of the parkin protein with the accumulation of parkin substrates. Similarly, mutations in the DJ-1 gene are associated with rare forms of autosomal recessive early-onset Parkinson's disease. Knock-out parkin or DJ-1 flies and mice do not exhibit nigral neuron death. The direct injection of lentiviral vectors encoding either parkin- or DJ-1-specific shRNA in the adult rat substantia nigra may avoid such compensatory mechanisms. We are currently investigating this approach to create new genetic models of Parkinson's disease and to better understand the pathophysiology of the disease.

KEYWORDS: *Amyotrophic Lateral sclerosis, SOD1, Lentivirus, RNA interference, small hairpin RNA, Parkinson disease, Parkin, DJ-1*

Death without stress

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ABSTRACT

The functioning of pro- and anti-apoptotic genes has largely been studied under conditions of applied stress. In order to investigate the balance of apoptotic forces under basal conditions we have employed RNA interference, induced by siRNAs. The p53 protein was used as a stress sensor and monitored by stabilization of p53 protein, phosphorylation at serine 15, and up-regulation of p53 target genes, such as p21 and HDM2. No activation of p53 was observed under the conditions of the experiments. Controls for selective RNAi knockdown included i) at least two distinct siRNAs for the target mRNA ii) demonstration that RNAi per se does not induce apoptosis in the cell models, iii) quantitative PCR for mRNA knowckdown plus immunoblotting for protein encoded by each targeted mRNA, and iv) phenotypic monitoring of siRNA-treated cells. The effects of a single does of siRNA were monitored up to 96 hours.

Clear-cut difference between epithelial cells of cancerous and non-cancerous origin were observed. For example, silencing SIRT1 (an NAD-dependent deacetylase) induced massive apoptosis in cancer cells but was without effect in non-cancer cells. Co-silencing experiments revaed that SIRT1 and Bcl-2 suppress separable apoptotic pathways in the same cancer cells. The 'Bcl-2 pathway' being dependent upon p53, Bax and caspase-2 whilst the 'SIRT1 pathway' was independent of these three pro-apoptotic mediators. However, both pathways converge and require Foxo4 (a member of Forkhead family of transcription actors) for apoptosis.

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Correcting dominant neurological disorders through allele-specific silencing of pathogenic mutants

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ABSTRACT

Dominant neurological disorders are often both progressive and incurable. A possible therapeutic strategy for these disorders is to target mRNA transcribed from the mutant allele. This strategy depends upon utilising two key features of RNA molecules: first, that they can form, or be part of, catalytic centres capable of cleaving multiple RNA substrate molecules; and secondly, that the catalytic core can be precisely targeted through RNA-RNA (or DNA-RNA) Watson and Crick pairing with the substrate.

The neuromuscular synapse is relatively large, has a well-defined function, is the site of dysfunction for several neurological disorders, and serves as an informative model for smaller and less accessible central synapses. The slow channel myasthenic syndrome is a dominant disorder of neuromuscular transmission caused by single missense amino acid substitutions of the AChR ion channel. We have demonstrated allele-specific down-regulation of pathogenic AChR mRNA by both RNAi (siRNA or in vitro transcribed shRNAi) in HEK 293 and muscle cell lines. Mutant AChR expression could be specifically suppressed to less than 20% of wild type. We have also demonstrated similar allele-specific gene silencing in cell lines using DNAzymes. The recent generation of animal models now allows us to test these molecules in vivo. Our results provide further encouragement for this approach in disorders where haploinsufficiency is not an issue.

Functional inhibition of poly(ADP-ribose) polymerase by siRNA technique

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ABSTRACT

Inhibitors of the nuclear repair enzyme poly(ADP-ribose) polymerase were found to protect cells and tissues against damages in oxidative stress. However, whether the pharmacological inhibitors exerted their cytoprotective effect by inhibiting nuclear PARP-1 activity or by an other mechanism has remained to be elucidated. To this end, PARP activity was inhibited pharmacologically or by suppressing PARP-1 expression by siRNA technique or by transdominantly expressing N-terminal DNA-binding domain of PARP-1 (PARP-DBD) in cultured cells. Cell survival, preservation of mitochondrial membrane potential and activation of cytoprotective kinase signalling systems were studied in hydrogen peroxide-treated WRL-68 cells. Our data showed that suppression of the single-strand DNA break-induced PARP-1 activation by pharmacological inhibitor, siRNA or by the transdominant expression of PARP-DBD identically protected cells from oxidative stress and induced the phosphorylation and activation of Akt. Furthermore, prevention of Akt activation by inhibiting the PI-3-kinase counteracted the cytoprotective effect of PARP inhibition. Microscopic data showed that PARP inhibition-induced Akt activation was responsible for protection of mitochondria in oxidative stress, because PI-3- and Src-kinase inhibitors diminished the protective effect of PARP inhibition. These data suggested that the pharmacological agents attenuated the oxidative stress-induced damages mainly by inhibiting nuclear PARP-1 rather then by an other mechanism, since the design of the siRNA and the PARP-DBD constructs were based on the nucleotide sequence of nuclear PARP-1.

KEYWORDS: *AKT, PARP, siRNA, transdominant expression of DNA-binding domain of PARP-1, mitochondrial membrane potential, oxidative stress, cytoprotection*

Dynamics of antiviral silencing in plants: A model study

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ABSTRACT

We study the antiviral properties of RNA silencing in plants. We extend our model of the RNA silencing pathway (1) with a replicating positive-strand RNA virus. In the model we assume that a cooperation of viral plus-strands leads to virion production. We study the system with and without amplification of the silencing response. The dsRNA that is synthesised via plant encoded RdRP does not function as an intermediate in viral replication.

Because the virus is selfreplicating, it is very sensitive for degradation of its dsRNA and plus-strand (sense) RNA. A small decrease in the number of available sense strands can

cause a strong decrease in the amount of virions produced. Amplification of the silencing response can reduce virus RNA levels dramatically: after an initial increase in virus RNA strands, RNA levels are so much decreased that virion production stops.

Positive-strand RNA viruses are known to produce more plus than minus-strands, i.e. minus-strands are used as a template more often. We show that when both strands would be used equally, the virus becomes oversensitive to RNA silencing. This mechanism could provide an extra driving force to bias viral replication towards production of plus-strands. Additionaly, the sensitivity of the virus for RNA silencing depends strongly on the timing and rate of virion production.

Plant genomes can contain integrated viral sequences of DNA viruses (2) as well as plus-strand RNA viruses (3). These sequences can be expressed, and it has been hypothesised that they function in the antiviral RNA silencing response. We test this hypothesis in our model, under the assumption that the integrated sequences do not contribute to viral replication. We find that integrated viral sequences can indeed decrease virus RNA and virion levels.

Currently we study co-evolution of plants and viruses, where plants can integrate viral sequences, and viruses can adjust their replication rate and virion production.

KEYWORDS: *Antiviral silencing, mathematical model, integrated viral sequence, positive strand RNA virus*

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Comparison of the activity of siRNAs and shRNAs against furin: a potential cellular target for HIV-1 inhibition

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ABSTRACT

RNA interference (RNAi) is a sequence-specific post-transcriptional gene regulation mechanism triggered by double-stranded RNA. HIV-1 replication can be inhibited by RNAi using synthetic siRNA or shRNA gene constructs directed against viral sequences. However, this approach has the disadvantage of viral escape through mutation of the target sequence. Targeting of cellular genes that encode viral co-factors could therefore present a solution against viral escape from RNAi. Furin is a membrane-associated cellular endoprotease that belongs to the subtilisin-like proprotein convertase family. Furin cleaves the HIV-1 precursor envelope glycoprotein gp160 into the biologically active gp120-gp41 trimer. Silencing of furin by RNAi may present a potential cellular target for inhibition of HIV-1. To study this, we designed and tested the activity of 7 siRNAs and 7 shRNA-expression plasmids targeting 7 furin sequences. We initially determined the si/shRNA inhibitory potential on luciferase-furine reporter constructs. Four si/shRNA pairs potently inhibited luciferase expression and 3 pairs showed marginal/no inhibition. Overall, we observed a correlation between the activity of a siRNA and the corresponding shRNA. si/shRNA that inhibited reporter-furin expression were also highly effective against furin. The impact on HIV-1 gp160 cleavage was tested by Western blot analysis. Inhibition of furin resulted in decreased gp160 processing. Experiments are in progress to directly determine the impact of furin silencing on HIV-1 infectivity.

KEYWORDS: *RNAi, HIV-1, furin, siRNA/shRNA comparison*

MicroRNA expression profiling of Diffuse Large B-cell lymphoma reveals differences between germinal centre-like and activated B cell-like subtypes

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ABSTRACT

MicroRNAs (miRNA) are a recently discovered class of short non-coding RNA molecules that negatively regulate gene expression. They have been shown to play a critical role in many biological functions. In humans about 320 miRNAs have been identified, some of which are expressed in a cell-specific and developmental stage-specific manner. Recently it has been shown that the expression profile of miRNAs can be used to subtype clinical cases (and cell lines) according to diagnosis with a greater degree of accuracy than traditional gene expression analysis. The identity of miRNAs associated with different lymphoma types however remains poorly defined.
Previous expression studies have revealed the presence of at least two subtypes of diffuse large B-cell lymphoma (DLBCL) representing the postulated cell of origin; those that are germinal center B cell derived (GCB-type) and those that are

activated B-cell derived (ABC-type). The latter subtype has been linked with poor prognostic outcome. It is not known whether these subtypes are also defined at the miRNA level. Therefore we examined the miRNA expression profile of DLBCL cell lines of defined subtypes as well as sub-populations of B-lymphocytes by microarray analysis. Consistent with recent publications, we found that *mir-18, 19b, 20* and *17-5p* (part of *mir-17-92* cluster) were up-regulated in cell lines but not in normal lymphocyte populations. Furthermore, cluster analysis showed that GCB-type cell lines (SUD-HL4, SUD-HL6 & SUD-HL10) have a distinct miRNA profile from ABC-type cell lines (OCI-Ly3 & OCI-Ly10). Most notably, high levels of expression of *mir-155, 221, 21* and *mir-222* were found in ABC-type cell lines whilst high levels of *mir-181a* were found in GCB-type cell lines. We looked at expression of these miRNAs in the cell lines as well as clinical cases of DLBCL by RNase-protection assay. Consistent with the microarray data, we found that *mir-155* was expressed in ABC-type cell lines but not GCB-type cell lines whilst the converse was true for *mir-181a*. Clinical cases showed similar patterns of expression but have still to be sub-typed according to immunohistochemical markers. Although still preliminary, our data suggests that miRNA profiling may be a useful tool in predicting the subtype of DLBCL cases and hence clinical outcome.

KEYWORDS: *DNA, RNA, As_2O_3, Binding constant, Binding mode, Conformation, FTIR, UV-Visible spectroscopy, Stability, Secondary structure*

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Improved *in vitro* transfection of mast cells and RNAi-mediated knockdown of the high-affinity IgE receptor, FcεRI

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ABSTRACT

Many allergic disorders, including asthma, atopic dermatitis (eczema) and food allergies are mediated by the antibody class known as immunoglobulin epsilon (IgE). Severely atopic or allergic individuals typically have elevated levels of serum IgE, produced by B cells that have become committed to IgE production. Secreted IgE antibodies are rapidly captured by the high affinity IgE receptor (FcεRI), a tetrameric protein found on the surface of resident mast cells and circulating basophils. The presence of antigen on receptor-bound IgE causes receptor cross-linking, triggering the release of factors including histamine, leukotrienes, prostaglandins and cytokines which mediate the allergic response. Reduction of high affinity IgE receptor expression on mast cells using RNA interference has therapeutic potential for ameliorating IgE-mediated allergies. The ability to test this hypothesis *in vitro* has been challenging, however, due to the inadequacy of existing chemical transfection protocols for siRNA delivery, with which it is difficult to deliver sufficient siRNA to haematopoietic suspension cells whilst at the same time preserving cell viability. We have overcome this problem by developing a transfection co-culture system where both increased transfection efficiency and preservation of cell viability in suspension cells is achieved by co-culture with adherent feeder cells. The use of fluorescent dye labelled feeder cells and fluorescent protein expression plasmids enables flow cytometric analysis to accurately discriminate between target and feeder cell populations and to assess transfection rates. Following co-culture with feeder cells, transfection of a variety of mast and B-cell lines with eGFP or DsRed reporter plasmids led to an increase in transfection rate by one to two orders of magnitude compared with cells transfected in suspension alone. An equivalent increase in transfection rate was observed with primary bone marrow-derived mast cells (BMMCs). Moreover, a similar increase in transfection rate was observed in all cell lines tested when fluorescently-labelled siRNAs were used. Transfection of cells with siRNAs targeting either the alpha or beta chains of FcεRI resulted in loss of the tetrameric receptor from the surface of BMMCs at 48 and 72h post-transfection, as assessed by concomitant fluorescent antibody-mediated detection of the expression of the alpha subunit of FcεRI. These results suggests that siRNA-mediated FcεRI knockdown is a viable strategy for inactivating mast cells, and that the transfection rates in difficult-to-transfect suspension cell lines can be improved using a co-culture system.

In vitro and *in vivo* delivery studies of siRNA-peptide conjugates

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ABSTRACT

siRNA is a novel reagent for targeting RNA in cells and reducing gene expression. However, its major limitation is the need for a suitable carrier for specific delivery into cell cultures

or animal tissues without associated toxicity. In the work presented here, we use different cell penetrating peptides (CPPs) for *in vitro* and *in vivo* delivery of various siRNAs in the absence of other transfection reagents. CPPs were conjugated to the siRNA sense strand (OMe/DNA or RNA) by a disulfide linkage and subsequently hybridized to the complementary antisense RNA strand. Amongst the CPPs studied were Penetratin, Transportan and Tat (residues 48-58). CPP-siRNA conjugates were evaluated initially using either an *in vitro* model targeting plasmid-encoded *firefly* luciferase or by targeting a disease-relevant endogenous gene coding for p38α MAP kinase. The ability to modulate levels of endogenous MAPK14 (p38α) expression is important in validating the role of the p38 pathway in disease models and in establishing new drug targets. The results showed that free CPP-5'-siRNA (Luc) conjugates were unable to knockdown *firefly* luciferase expression at low concentrations (up to 500 nM), when analyzed by luminescence 24 hr post-transfection in HeLa or HepG2 cells. In contrast, it was found that certain CPP-siRNA(p38) conjugates were able to knockdown p38α MAP kinase expression. HeLa cells were incubated with CPP-siRNA(p38) conjugates in serum-free media for 24 hr and p38 expression normalised to 18S rRNA was determined by quantitative real-time PCR using Taqman probes. Following 24 hr transfection, p38 knockdown was achieved with CPP 5'- and 3'-conjugated siRNA. The highest activity was observed for the Penetratin 3'-conjugate, which gave ~ 50% knockdown ($P < 0.01$) of p38 expression (n=4) at 10 μM. Certain CPP-siRNA conjugates were evaluated in an *in vivo* mouse model, by targeting p38α MAP kinase in lungs following intratracheal instillation. p38 mRNA knockdown efficiency, duration and siRNA recovery from the mouse lung appeared to be dose-dependent over a 24-hour period in the absence of delivery reagents/CPPs. Conjugation of Penetratin or Tat did not appear to improve significantly upon knockdown efficiency. Whilst recovery of siRNA from lung homogenates was impaired due to CPP conjugation, our findings suggest that the knockdown efficiency paradox compared to our *in vitro* data may relate to sequence-dependent siRNA instability.

Prevention of a myocardial pathogen by RNA interference

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ABSTRACT

One of the crucial pathogens of severe heart diseases like acute myocarditis and dilated cardiomyopathy is coxsackievirus B3 (CVB-3) a member of the picornavirus family. They are small, non-enveloped RNA viruses containing a (+)-stranded genome which is qualified for the application of RNA interference.

We developed highly efficient small interfering RNAs (siRNAs) against the viral 3D RNA dependent RNA polymerase (RdRP). With some of these siRNAs we were able to inhibit CVB-3 proliferation up to 90% in a plaque reduction assay. In GFP reporter assays, an effective RdRP-downregulation was found by means of short hairpin RNA (shRNA) expression. In addition we achieved a degradation of our target under utilisation of micro RNA-type siRNA expression vectors.

As an alternative approach, we inhibited the expression of CVB-3 specific receptors on a human cell line. The receptor of utmost importance for mediation of CVB-3 entry is the coxsackievirus-adenovirus-receptor (CAR). We found different siRNAs that almost completely silence CAR expression on HeLa cells 48 hours after transfection as long as 6 days. Plaque reduction assays showed that siRNA induced knock-down of CAR reduces virus reproduction. There are various well known splice variants of the receptor. One of them (called CAR 4/7) forms homodimers with the mature CAR on host cells, interacts with CVB-3 and prevents a viral infection. Based on this fact we cloned the sequence of CAR 4/7 upstream of an siRNA in a micro RNA environment (co-cistronic expression) that selectively silences the full-length transmembrane CAR. This construct is supposed to simultaneously decrease full-length CAR and block virus binding CAR domains.

KEYWORDS: *Picornavirus, coxsackievirus-adenovirus-receptor, short hairpin RNA*