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Selected abstracts

The biology behind post-transcriptional regulation of microRNA expression

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ABSTRACT: Many mammalian miRNAs display tissue-specific expression patterns, a phenomenon that has so far been attributed to transcriptional regulation. Recently, differential processing of otherwise ubiquitous pre-miRNAs has emerged as an alternative, post-transcriptional mechanism to control miRNA function. In particular, we found miR-138 to be restricted to distinct cell types in the brain, while its precursor, pre-miR-138-2, is present in all tissues analyzed. We are currently investigating the molecular mechanism responsible for this regulation. In vitro and in vivo processing studies confirmed that HeLa cells do not process pre-miR-138-2 into miR-138. Interestingly, recombinant Dicer is able to cleave pre-miR-138-2, but the addition of HeLa cytoplasmic extract to the processing reaction inhibits cleavage. This result implies the presence of an inhibitory factor in HeLa cells and probably in all tissues not processing pre-miR-138-2 into miR-138. We are purifying this putative inhibitor employing classical chromatographic methods. After six purification steps, we have obtained a highly enriched fraction that strongly impairs processing of pre-miR-138-2 by recombinant Dicer. We envision that this inhibitor could be a founding member of a new class of proteins involved in the regulation of pre-miRNA processing.

MicroRNAs in biomedical research

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ABSTRACT: Microarrays to examine the global expression levels of microRNAs (miRNAs) in a systematic in-parallel manner have become important tools to help unravel the functions of miRNAs and to understand their roles in RNA-based regulation and their implications in human diseases. We have established a novel miRNA specific microarray platform that enables the simultaneous expression analysis of both known and predicted miRNAs obtained from human or mouse origin. Systematic confirmation of the array data with complementary methodologies suggests that the described microarray platform is a powerful tool to analyze miRNA expression patterns with rapid throughput and high fidelity. The approach presented here revealed novel expression signatures for known and novel computationally predicted miRNAs and is seen as a valuable tool in future studies to facilitate analysis of the biological function of miRNAs.

MicroRNAs in *C. elegans* development and human disease

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ABSTRACT: MicroRNAs (miRNAs) are a large class of short non-coding RNAs found in many plants and animals, where they often act as post-transcriptional repressors of gene expression. Here we report the generation and analysis of loss-of-function mutations in almost all known microRNA genes in *C. elegans*. Although we identify possible new roles for microRNAs in *C. elegans* embryogenesis and larval development, we find that the majority of microRNAs are not essential for development or viability of *C. elegans*. Moreover, whole microRNA families are not required for viability. This study represents the first comprehensive analysis of microRNA function in any organism and provides a unique resource for the study of microRNAs.

MicroRNA expression and function in zebrafish development

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ABSTRACT: MicroRNA expression can be easily studied in zebrafish embryos using LNA probes. Since many miRNAs are expressed in the vertebrate brain, we created a microRNA brain expression atlas for both the developing and adult zebrafish brain. To extend the brain microRNA repertoire, we cloned many new miRNAs from zebrafish and primate brain regions. As a next step, we have started to functionally characterize miRNAs in the zebrafish embryo. Since it is challenging to knockout a miRNA genetically in the zebrafish, we now successfully use morpholino oligonucleotides to inhibit miRNA expression and function during development. Using this strategy, we identified a miRNA involved in pancreatic islet development.

The nuclear aspect of RNAi in *C. elegans*

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ABSTRACT: RNA interference in *C. elegans* was discovered as a biological response to exogenously introduced double-stranded RNA. dsRNA commonly induces sequence-specific gene silencing at the post-transcriptional level. Recently, RNAi has been implicated in the initiation of chromatin-based silencing of repetitive elements in diverse organisms, ranging from fission yeast to mammals. I will discuss that (1) exogenous dsRNA can initiate transcriptional silencing of repetitive transgenes in the soma of *C. elegans*; (2) genes required for the RNAi response also play a role in the repression of endogenous targets, such as cyclin E; (3) a novel class of *C. elegans* short RNAs might have a role in large scale chromatin organization.

MicroRNAs and inflammation

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ABSTRACT: Inflammation is thought to underlie many acute and chronic diseases including acute lung injury, asthma and chronic obstructive pulmonary disease. Since little is known about the role of miRNAs in inflammation we have examined their function during the release of cytokines and chemokines following activation of the innate immune response in both a cell and animal model. Studies in human A549 epithelial cells showed that exposure to the pro-inflammatory cytokine interleukin-1β (IL-1β) induced a time- and concentration- dependent release of interleukin-8 (IL-8) and RANTES which peaked at ~6hrs and gave EC50's of 0.01ng/ml and 0.3ng/ml, respectively. To identify miRNAs that might regulate inflammation, initial studies measured the differential miRNA expression profile and showed that IL-1β caused selective up-regulation of miRNA-146a and miRNA-146b. Investigation of the time- and concentration-dependency of IL-1β induced miRNA-146a/b expression demonstrated a rapid increase in miRNA-146a (70-fold) and miRNA-146b (20-fold) expression that peaked at 3-6 hrs and gave similar EC50's at ~0.3ng/ml. To investigate the functional role of miR-146a/b, we examined the effect of pharmacological modulation. Increased miR-146a/b expression following transfection with miRNA-146a/b mimics inhibited both IL-8 and RANTES release. In contrast, inhibition of miR-146a/b activity using antisense targeted to the miRNA guide strand or down-regulation of miRNA expression using siRNA, had no significant action. Identification of potential protein targets using predictive databases implicated IRAK1, TRAF6 and syntaxin-3, which are involved in IL-1β signalling and cytokine secretion. However, either exposure to IL-1β or transfection with miR-146a mimic had no significant effect upon protein expression. In parallel animal studies, we examined the differential expression of 104 miRNAs in mouse lung following exposure to aerosilised lipopolysaccharide (LPS). Following challenge, we observed rapid and transient increase in both the mean (4.3-fold) and individual (46 miRNAs) levels of miRNA expression which peaked at 3hrs. Crucially, this increase was correlated with a reduction in the expression of TNF-α, KC and MIP-2 suggesting a potential role for miRNAs in the resolution of inflammation. Examination of the individual miRNA expression profiles showed time dependent increases in miR-21, -25, -27b, -100, 140, -142-3p, -181c, 187, -194, -214, -223 and -224. These increases miR-223 were confirmed using in situ hydridisation and laser capture microscopy combined with RT-PCR and showed that miR-223 is localised to epithelium and inflammatory cells. Overall, these cell and animal studies suggest that the rapid induction of miRNAs is involved in the negative feedback regulation of the inflammatory response.

siRNA: Structure-based design and prokaryotic gene silencing

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ABSTRACT: In eukaryotes, small interfering RNA (siRNA)-mediated RNA interference (RNAi) represents a powerful reverse genetic tool and a promising strategy for drug development. We investigated the role of RNA

structure in RNAi and developed a structure-based program for selection of active siRNA. Major challenges in the design of siRNA and small hairpin RNA include reliable prediction of immunostimulation and off-targeting as well as the avoidance of interference with processing and action of cellular regulatory RNA. Considering the increasing number of parameters associated with siRNA activity can mean that the stringency of selection is too high to identify any candidate sequence. To overcome this limitation we developed a technique to expand the space of target-specific siRNAs by up to three orders of magnitude gaining access to many more active siRNA species.

Prokaryotes lack the RNAi pathway or major components thereof. Using potent computationally designed siRNA and complementing the prokaryotic repertoire with eukaryotic functions, specific silencing of chromosomal and episomal genes was achieved in Gram-positive and Gram-negative bacteria as well as in mycobacteria. Prokaryotic gene silencing is distinct from, and excels, antisense effects. Observed phenotypes ranged from transient gene knockdown to persistent gene knockout. This powerful technology can substitute for expensive conventional knockout strategies and opens promising perspectives regarding validation of prokaryotic gene functions and the development of novel anti-infectives.

Myc-regulated microRNAs: Friends and foes

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ABSTRACT: Recently the c-Myc oncoprotein has been shown to up-regulate expression of the miR-17-92 microRNA cluster in cultured cells. To investigate the interplay between Myc and miR-17-92 in genetically complex tumors and its role in tumor phenotypes, we developed a new xenograft mouse model for colon cancer. We found that K-Ras-transformed p53-null mouse colonocytes formed indolent, poorly vascularized tumors, whereas additional transduction with a Myc-encoding retrovirus elevated miR-17-92 levels and promoted vigorous vascularization and growth. Notably, miR-17-92-transduced cells formed larger, better-perfused tumors as well. Enhanced neovascularization correlated with downregulation of anti-angiogenic thrombospondin-1 (Tsp1). Provocatively, Tsp1 is a predicted target for the miR-17-92 cluster. Indeed, miR-17-92 knockdown with antisense oligoribonucleotides partly restored Tsp1 expression. These findings establish an oncogenic role for microRNAs in non-cell-autonomous Myc-induced tumor phenotypes. On the other hand, several other miRNAs were repressed in aggressive Myc-overexpressing tumors, suggesting that they might possess tumor-suppressive properties. Indeed, using a novel in vivo selection assay, we observed that restoration of their levels could sharply limit neoplastic growth in vivo, even when in vitro proliferation was unaffected. The involvement of the putative target genes in non-cell-autonomous tumor phenotypes is currently under investigation.

High-throughput RNAi screening: Initial steps toward creation of a functional map of the human genome

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ABSTRACT: Exploitation of RNA interference (RNAi) in the research laboratory has accelerated studies in functional genomics, gene mapping, pathway analysis, and drug target identification. Though initial applications were limited to single gene knockouts, advancements in nucleic acid synthesis, delivery, and small interfering RNA (siRNA) design have enabled expansion of the technology to genome-wide screens.

Successful merging of RNAi into high throughput applications requires the identification of critical attributes that distort the outcome of gene silencing studies. To that end, this presentation will review results of a recent multi-laboratory high throughput RNAi phenotypic screen for apoptotic induction and cell viability. The importance of assay design, reagent quality, positive and negative controls, and assay metrics will be discussed, highlighting the contribution that each parameter makes to data quality and false positive/negative rates. Consideration of these attributes prior to initiating screening will minimize the complexities of hit stratification and facilitate the development of an accurate functional map of the human genome.

Evolution of gene silencing technologies: Antisense oligonucleotides, siRNAs and shRNAs for target validation in pain research

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ABSTRACT: The vanilloid receptor TRPV1 is a central integrator of various noxious stimuli. We compared different approaches for gene silencing to inhibit TRPV1 expression in vitro and in vivo. In cell culture, siRNAs were found to be up to 1000fold more efficient than phosphorothioate oligonucleotides. Accordingly, repeated intrathecal injections of (unmodified) antisense oligonucleotides were required to reduce pain sensitivity of mononeuropathic rats. In contrast, a single bolus injection of a TRPV1-specific siRNA was sufficient to achieve a significant analgesic effect. A second independent siRNA

was used to confirm the biological effect. Mice treated with siRNAs against TRPV1 showed a drastically diminished response to capsaicin given intraperitoneally. Subsequently, transgenic mice expressing an shRNA against TRPV1 were generated. Continuous knockdown of the vanilloid receptor allowed detailed functional investigations. Expression of the shRNA was confirmed by RNase protection assays and knockdown of the target gene was demonstrated by the lack of binding of radioactively labelled resiniferatoxin (RTX), a TRPV1 agonist. Transgenic animals lacked capsaicin-induced hypothermia and showed drastically diminished nocifensive response to capsaicin injected into the hind paw. Furthermore, the sensitivity towards noxious heat stimuli in the 48°C and 58°C hot plate test was reduced. These results demonstrate the suitability of RNAi approaches for target validation in pain research. While intrathecal injection of siRNAs can be used for initial studies with transiently suppressed target gene expression, transgenic animal expressing shRNAs allow a more detailed investigation of the function of the gene of interest. Continuous knockdown of target gene expression by the introduction of an shRNA enconding sequence by recombinase-mediated cassette exchange (RMCE) into tetraploid blastocysts is much faster than the alternative approach to generate knockout animals by homologous recombination.

Antiviral RNAi approaches

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ABSTRACT: Human T cells can be made resistant to HIV-1 by stable expression of a shRNA that is directed against the viral RNA genome. Despite this potent inhibition, we demonstrated that HIV-1 can escape, e.g. by selecting a single point mutation in the targeted sequence. The escape routes nicely demonstrate the potency and sequence-specificity of RNAi-mediated inhibition, but also the weak point of this antiviral approach in a therapeutic setting. To block viral escape, we have selected multiple very potent shRNAs against several highly conserved target sequences within the HIV-1 RNA genome. Each individual shRNA is screened for its potency and its ability to block viral escape. This was performed in massive virus evolution studies, yielding a wealth of information on the impact of target site mutations on the efficiency of RNAi. These results will be discussed in the context of the siRNA-RISC-target RNA interaction.

The most potent shRNAs that also block or restrict virus escape will be used in a combination therapy. Experiments will be shown in which the presence of four shRNA inhibitors prevents the evolutionary escape of HIV-1. The construction of lentiviral vectors that express multiple shRNA cassettes poses several severe problems, leading to a significant reduction in vector titer. We will discuss these problems and present solutions that may be of interest for other applications. The future route towards an ex vivo gene therapy for HIV-AIDS will be discussed.

Interactions between the human double-stranded RNA binding proteins, TRBP, PACT, and Dicer to facilitate the production of siRNA

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ABSTRACT: Human Dicer requires double-stranded RNA (dsRNA) binding protein TRBP or PACT to mediate RNAi and microRNA processing. TRBP and PACT have a similar structure, but exert opposite regulatory effects on dsRNA-dependent protein kinase PKR. It is not understood whether TRBP and PACT are simultaneously associate with Dicer. In this study we show that TRBP directly interacts with PACT in vitro and in cultured human cells. TRBP and PACT form a triple complex with Dicer and facilitate Dicer-dependent production of small interfering RNA (siRNA). Silencing of both TRBP and PACT by short hairpin RNAs (shRNAs) in cultured human cells results in significant suppression of gene silencing mediated by shRNA, but not by siRNA, indicating that TRBP and PACT act primarily at the step of siRNA production. Collectively, our findings suggest a direct interaction between human TRBP and PACT. In addition, their simultaneous association with Dicer stimulates the cleavage of dsRNA or shRNA to siRNA. Our study significantly alters the existing model for the assembly and function of human Dicer complex that initiates RNAi.

Novel proteins involved in RNAi: Are scaffold attachment factor proteins involved in RNAi?

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ABSTRACT: Scaffold attachment factors (SAF) are nuclear matrix associated proteins that has been shown to be involved in many fundamental cellular processes, including RNA splicing, the regulation of transcription and the cells response to stress. Sequence analysis shows that SAF-B proteins contain several functional domains including; a N-terminal DNA binding region (SAP/SAF-box), nuclear localization signal, a central RNA recognition motif (RRM) and by Glu/Arg, Ser/Lys and Gly rich protein interaction regions. The presence of these highly conserved domains suggests SAFB proteins will have multiple cellular functions, a conclusion that is borne out by SAFB-1 being identified by

independent groups according to three differing biological properties/roles. It was isolated via its interaction with scaffold or matrix attachment regions (S/MAR) DNA, as HET a protein that down regulates the transcription of hsp27 by binding its promoter and as a protein that was recruited to stress bodies and associated with hnRNP. More recently SAF-B protein regulation and localisation has been found to be altered during apoptosis and SAF-B protein expression has been found to be associated with altered RNA splicing and maturation. In this presentation we show that SAFB expression and localisation in the presence of shRNA and microRNA species is altered; being strongly associated with translocation from the nucleus to the cytoplasm. We were unable to find SAF-B1 located in cytoplasmic P bodies or stress bodies though it was located within the cytoplasm close to P bodies. We are currently investigating whether SAFB-1 expression is linked to an alteration in the effectiveness of shRNA mediated gene knockdown.

Short RNA expression in T lymphocyte development

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ABSTRACT: Short RNA expression was profiled in several ordered stages of T lymphocyte development. The aggregate miRNA expression per cell was found to vary at different stages of development. These changes paralleled variations in total cellular RNA content, suggesting that global miRNA levels are co-regulated with the translational capacity of the cell. Expression of individual miRNAs was dynamically regulated during T cell development, with at least one miRNA or miRNA family overrepresented at each developmental stage. Changes in expression of miRNAs were characterized by temporal enrichments at distinct stages of development observed against a background of constant, basal expression of the miRNA. Enrichments in miRNA expression could be temporally correlated with depletions of the transcript levels of targets containing seed matches to the specific miRNAs, and may have specific functional consequences. MiR-181a, which is specifically enriched at the CD4+CD8+ (DP) stage of thymocyte development, can repress transcripts bearing the 3-R untranslated regions of Bcl-2, CD69, and the T cell receptor alpha chain, all of which are coordinately involved in positive selection.

Defining roles for RNA silencing in mammalian cells

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ABSTRACT: RNA silencing is a common term for homology-dependent silencing phenomena found in the majority of eukaryotic species. RNA silencing pathways share several conserved components. The common denominator of these pathways is the presence of specific, short RNA molecules (21-25 nt long), which serve as a template for sequence-specific effects including inhibition of translation, mRNA degradation, and transcriptional silencing. Cloning of endogenous small RNAs and data from mammalian cells lacking components of RNA silencing are very complex. Their analysis did not yield solid evidence yet for other physiological roles of RNA silencing than microRNAs (miRNA) pathway. In contrast to experimental interventions, degradation of mRNA induced by long dsRNA, generally termed RNA interference, seems to be very rare if present at all under physiological conditions. Similarly, physiological roles of RNA silencing in the nucleus and the mechanism of action of RNA silencing in the nucleus remain unknown. Therefore, repression of translation mediated by microRNAs is a dominant RNA silencing pathway in mammalian cells, where hundreds of genes can be affected by miRNAs in a single cell type.

Intracellular trafficking and localization of siRNA is a bottle neck for its biological activity

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ABSTRACT: This presentation summarizes data on the relationship between physical uptake of siRNA according to different modes of delivery, subcellular localization of siRNA, and siRNA-mediated target suppression. The model system is expression of laminA/C in human ECV304 cells. In sum, these data indicate that only a very minor fraction of intracellular siRNA is biologically active whereas major portions are captured in compartments in which they do not contribute to tagret suppression. Thus, intracellular release of captured siRNA is an option to increase its biological effectiveness.

Inhibition of microRNAs by synthetic oligonucleotide analogs

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ABSTRACT: MicroRNAs are an abundant class of non-coding RNAs that are involved in regulation of gene expression in many biological processes. MicroRNA 122 (miR122a) is a tissue-specific miRNA, being expressed only

in liver, where it constitutes 70 % of the total microRNA population. Its importance as a therapeutic target model has been highlighted by its involvement in lipid metabolism (1) and in the modulation of HCV infection. A neutral-backbone oligonucleotide based on peptide nucleic acids (PNA) can be used as a mir122 inhibitor in vitro. Both, human HuH7 cells and primary rat hepatocytes transfected with anti-miR122a(PNA) showed decreased levels of mature miR122, when analyzed by reverse transcription RT-PCR. Moreover, two mRNAs regulated by miR122 appeared to be affected by miR122 knockdown. The mRNAs corresponding to the cationic amino acid transporter-1 (CAT-1 or SLC7A1) and glycogen synthase from muscle (GYS1), both containing miR122a complementary sites on their 3'UTR regions, showed an increase in abundance when cells were treated with anti-miR122a(PNA). Oligo mixmers of the type LNA/OMe also showed particularly strong microRNA inhibition when transfected into HuH7 cells or primary hepatocytes. The results provide a platform for development of anti-microRNA PNA conjugates with enhanced cell delivery properties.

RNAi and disease in tropical monocot plants

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ABSTRACT: Post transcriptional gene silencing (PTGS) and RNAi pathways involved in plant defense were some of the first biological roles established for short interfering RNA molecules (siRNA). Small non protein coding RNA molecules are now known to be involved not only in plant protection against mobile genetic elements and viruses but also in plant development and plant stress response pathways. Whilst the recruitment of these pathways as a human strategy for plant protection predates the elucidation of the mechanism involved, this discovery has allowed for the development of more precise targeting of viral pathogens, in particular, and the possibility of gene knock down for functional genetic studies and biotechnological application. Our research aims to gather more information on RNAi and on disease elicited pathways in tropical monocotyledonous plants, through the study of RNAi pathway genes and investigations into the use of exogenously applied dsRNA for gene knockdown and plant protection.

Our current research on banana (*Musa acuminata*) focuses on gene expression studies to identify genes and possibly pathways related to disease resistance in wild cultivars. We have isolated homologues of Dicer Like 1 (DCL-1) from several varieties of banana and sequence information obtained to date suggests a high degree of conservation among the Musa. We are also studying the use of exogenously applied dsRNA including the use of coat protein sequences from the orchid virus Cymbidium mosaic virus (CP-CymMV) using a cucumber plant model. Preliminary data supports the ability of ds CP-CymMV RNA, but not ss CP-CymMV RNA, in reducing the viral load, suggesting that this may be a useful approach for developing disease resistant orchids.

Silencing of the expression of an antiapoptotic protein in cancer treatment - Preclinical data

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ABSTRACT: Insensitivity to apoptotic stimuli is one of most problems in cancer treatment. Inhibition of activity of proteins that belongs to IAP (Inhibitor of Apoptosis Protein) family is one of solutions of this problem. There are many papers about inhibiting or silencing survivin – the most studied member of IAP family. Expression of this protein is cell-cycle-dependend, but there is no evidence that expression of survivin causes carcinogenesis. In our studies we aimed to break blockade of apoptosis in cancer cell lines by decreasing protein level of the other IAP family member. This protein is involved in inhibition of caspases 3, 7, and 9.

We assumed that decreasing a level of this protein should sensitize cells to apoptotic stimuli and even could induce apoptosis without any additional agents. We had designed fifteen siRNA sequences against that gene and we have tested them using number of human cancer cell lines. Those sequences were delivered into cells by lipofection using Lipofectamine RNAiMAX (Invitrogen). The final concentrarion of siRNA was 50 nM. We chose A549 cell line as a lung cancer model, cell lines: JIMT 1, MCF7, MCF7bcl2 (MCF7 with bcl2 overexpression), MDA-MB-231 and MDA-MB-231bcl2 (MDA-MB-231 with bcl2 overexpression) as models of breast cancer and PC3 cell line as a model of prostate. In our screenings the first cut-off was defined by inhibition of proliferation, which was measured by MTT/MTS assay. Measurements were performed 24h, 48h and 72h after transfection. We also investigated if there was any difference in response after 48h between single transfection and double transfection with 24h interval.

In almost all cell lines tested the best silencing was observed 48h after transfection. The best silencing was achieved with sequence No. 7 – the number of viable cells was only 10% of the control. We observed induction of apoptosis only by siRNA molecule as well as we observed increase of sensitivity to apoptotic stimuli (by VP16). We

also observed decrease in mRNA and protein level after treatment with some of our sequences what was in correlation with inhibition of proliferation.

Among fifteen sequences designed we have chosen few which will be studied *in vivo*. Basing on our results we find this IAP member as potent target for anticancer therapy. Of course we need specific delivery system on which we are working.

Flexible ligation of double-stranded RNA

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ABSTRACT: Double-stranded RNA (dsRNA) is involved in a range of biological pathways including sense/antisense translation control, RNA processing, interferon responses and RNA interference. However, few molecular tools are available to analyse dsRNA as native hybrids. We describe a simple and flexible method for enzymatic ligation of duplex RNA adaptors to any dsRNA molecule without a need for prior sequence information; opening new possibilities to directly map, detect, clone and profile cellular dsRNA.

Characterisation of microRNAs in the human heart

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ABSTRACT: MicroRNAs are small, non-coding RNA species encoded within the human genome that regulate gene expression by inhibiting mRNA translation. Some microRNAs are important in developmental biology but the role(s) of microRNAs in the post-natal heart remain unclear. To begin to investigate the role(s) of microRNAs in the post-natal human heart, we collected cardiac MRI and positron emission tomography guided left ventricular biopsies from three groups: patients with normal ventricular function with or without diabetes (n=6 and n=7 respectively) and from patients with heart failure (n=6). Small RNA species were extracted from the biopsies and we determined the expression of 155 mature miRNAs using a TaqMan based real-time PCR method. The relative amounts of mature microRNA species ranged widely with the expression of most highly expressed microRNA being ~15,000 times higher than that of the lowest detectable microRNA. We observed that some microRNAs that were previously un-described in the heart (e.g. microRNA-30b) are expressed at levels comparable with that of microRNA-133, which has an important role in cardiac development. We compared relative microRNA expression levels between patient groups and identified differentially regulated species (fold change > ± 2 fold, nominal P value < 0.05) in patients with diabetes (14 species) or heart failure (44 species) as compared to the group with normal ventricular function in the absence of diabetes. The majority of differentially regulated microRNA species were downregulated in heart failure whereas they were evenly distributed in the diabetic heart biopsies (9 upregulated, 5 downregulated). Some microRNA species were specifically upregulated (e.g. microRNA-367, P<0.001, 4.4-fold and microRNA-342, P<0.001, 2.8-fold) in the diabetic heart as compared to the other two patient groups. A single microRNA that is up-regulated in both diabetes and heart failure, which are diseases of interrelated pathophysiology, is subject to ongoing functional studies for investigations of its role in the heart. These data describe the expression of microRNAs in the human heart in health and disease and indicate microRNAs may have important pathophysiological role(s) in the development of heart failure. As microRNA function can be inhibited by systemically delivered microRNA inhibitors this may have therapeutic implications for the treatment of heart disease.

RNA silencing in virus-infected plants

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ABSTRACT: Small RNAs of *Brassica juncea* infected by *Turnip mosaic virus* (TuMV) and/or *Turnip crinkle carmovirus* (TCV) were cloned. Short interfering RNA profiles of the viruses showed characteristic properties specific to viral species and are different between different viruses. Several sense and antisense siRNA hot spots were observed and they are also virus species specific. No relation between siRNA hot spots and good stem-loop structures was observed suggesting that hot spots are not simply products of heavily folded regions of virus genome, but alternative mechanisms might involve. Several brassica miRNAs were cloned and their corresponding miRNA genes were identified based on sequence homology to miRNA registry with miR168 having highest number. Further prediction of miRNAs based on available brassica ESTs suggested the presence of 8 additional miRNAs. Free energy calculation of siRNA and miRNA showed that their properties were different. Artificial siRNA design software using current rules did not suggest siRNA profiles similar to natural cases.

Inhibition of ECHO virus 30 by RNA interference

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ABSTRACT: ECHO (enteric cytopathic human orphan) virus 30 is a member of the Picornavirus family containing a plus stranded RNA genome. ECHO virus 30 causes aseptic meningitis and a number of outbreaks have been documented. Current prospects of treatment are still rare. We therefore designed small interfering RNAs (siRNAs) targeting the 3D RNA dependent RNA-Polymerase (3DPol) of ECHO virus 30. In cell viability assays, we were able to show a significant protection of host cells two days post infection. We then developed a rapid and inexpensive method to transfer the siRNAs into according shRNA expression vectors. With this method, 6 different silencer plasmids were generated in a single shotgun cloning experiment. A key to inhibit ECHO virus 30 seems to be a knockdown of the host cell receptor, the decay accelerating factor (DAF). A combination of siRNAs directed against the receptor 3DPol prevents both the entry of ECHO virus 30 and its propagation. Based on these findings we developed siRNA double expression and siRNA triple expression vectors (SiDEx and SiTEx, respectively) which contain shRNA coding sequences under control of U6 promotors for simultaneous silencing of DAF and 3DPol. Experiments are currently ongoing to build up adeno-associated virus vectors for the efficient delivery of the multiple shRNA expression cassettes.

MicroRNA expression in haematological malignancies by expression profiling

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ABSTRACT: MicroRNAs are a recently discovered class of regulatory RNA molecules that play a key role in many cellular pathways. Whilst abnormal expression of microRNAs appears to be a common feature of many types of cancer, very little is known of their role in haematological malignancies.

Using a newly developed microRNA microarray platform (μRNA microarray) we investigated the microRNA expression profile of thirty-nine haematological cell lines including 11 T-cell and 20 B-cell lines as well as various B-cell and T-cell subpopulations. Unsupervised cluster analysis revealed that the microRNA expression profile of these samples in the main reflects their histological classifications. Over-expression of the miR-17-92 cluster was found in ~70% of cell lines and microRNAs associated with specific diagnoses were identified. Furthermore EBV-associated microRNAs were differentially expressed in EBV-infected cell lines.

In order to try and explain aberrant expression of microRNAs observed in haematological malignancies we examined expression levels of key components of the microRNA biosynthetic pathway by qRT-PCR. We found that compared with normal B and T cells that levels of components of the microprocessor complex (*i.e.* Drosha and DGCR8) are up-regulated in B cell neoplasms but down-regulated in T-cell malignancies. Up-regulation was also demonstrated in twenty-nine clinical cases of the B-cell malignancy diffuse large B cell lymphoma. The microRNA transporter protein Exportin-5 was up-regulated in both B and T-cell lines as were the Dicer accessory proteins TRBP and PACT. In contrast Dicer was found to be down-regulated in all cell lines tested compared to counterpart normal lymphocytes.

We conclude that aberrant expression of microRNA is a common feature of haematological malignancies that can in part be explained by changes in components of the microRNA biosynthetic pathway.

Proof-of-concept studies for the application of 'inducible' RNAi to silence a specific pathway and increase sensitivity of cellular toxicity screens

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ABSTRACT: *In vitro* cytotoxicity assays are often used to predict the toxicological liabilities of compounds, however these assays identify only a proportion of compounds that exhibit toxicity *in vivo*. It is therefore desirable to establish cell models that show increased sensitivity to a toxic challenge. The aim of this collaborative project between

Pfizer (Sandwich, UK) and the Karolinska Institute (Sweden) was to evaluate the application of inducible RNAi to conditionally silence key components of defence and survival pathways and hence 'sensitise' cellular toxicity screens. Studies evaluated the knockdown of NF-E2 related factor 2 (Nrf2) - a key transcription factor involved in the cellular oxidant defence mechanisms in HeLa cells.

Northern blot analysis demonstrated efficient knockdown (80%) of Nrf2 mRNA using two siRNA and shRNA clones. Subsequent western blot analysis demonstrated knockdown of heme oxygenase 1(HO-1) protein, transcriptionally regulated by Nrf2, using siRNA. Addition of the phenolic antioxidant *tert*-butylhydroquinone (tBHQ) is known to strongly induce HO-1. However, expression of the two different shRNAs (in the presence of tBHQ) each suppressed the HO-1 protein level approximately 7-fold, strongly suggesting that expression of the shRNAs down-regulates the Nrf2 pathway, and the oxidative stress response. Further research will evaluate the effect of several compounds (oxidants and non-oxidants) making use of the CellTiter-Glo Cell Viability Test (Promega) and GSH-400 Glutathione measurement (Oxis Research) to ascertain whether the knockdown of Nrf2 gene causes the cells to be more sensitive to compounds that cause oxidative stress.

This study has successfully demonstrated the feasibility of using 'inducible' RNAi to conditionally silence a specific pathway. However the project has encountered difficulties with the long term stability of the HeLa cultures. Therefore, further work will endeavour to produce 'stable' inducible cell systems and characterise the relative sensitivity and predictivity of these cellular models.

A foamy-virus system expressing small interfering RNA, for gene knockdown in mammalian cells

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ABSTRACT: RNA interference (RNAi) efficiently induces sequence-specific gene silencing in mammalian cells through transient transfection with short interfering RNAs (siRNAs) of 21–23 nucleotides or by transcription of short hairpin RNAs (shRNAs) from expression plasmid and viral vectors. However, for RNAi to be efficient, a suitable delivery method is needed. In contrast to virus-mediated RNAi, direct transfection of mammalian cells with chemically synthesized siRNA, hinders long-term, stable gene silencing. In the present study, we describe the delivery of a shRNA expressing cassette that targets GFP, using foamy virus-based retroviral vectors.

We are utilising a deleted (DeltaPhi), foamy-virus based vector (pΔΦmU6f), which can express shRNAs under the control of the mouse U6 promoter. We have further developed this vector (pΔΦmU6FMscvF) to co-express enhanced green fluorescent protein (eGFP) as a marker gene, under the control of Mscv promoter, for the identification of transduced cells by flow cytometry. Our reporter system is a clone of HT1080 human cell line that harbours a single copy of integrated FV provirus expressing GFP. In order to investigate the efficiency of FV-mediated RNAi, HT1080-GFP cells were transduced with virus, expressing either the hairpin RNA sequence against GFP or a scrambled sequence, the latter serving as our negative control. Preliminary experiments show that pΔΦmU6f _shGFP vector results in GFP silencing in 27% of the target population. The levels of GFP expression, as indicated by mean fluorescence intensity comparison, decreased by more than 20-fold (MFI: 57 _ 1390), relative to control uninfected or pΔΦmU6f_scrambled shRNA infected cells. This effect was sustained for more than 10 days.

Aim of this, preliminary study, is the evaluation of the silencing efficiency and further improvements of our own FV-based RNAi vectors, for their ultimate use in gene therapy approaches for haematological diseases. Overall, we have generated a foamy retroviral vector of high titer that expresses shRNA under the control of a mouse U6 promoter and demonstrated that foamy vector-mediated RNAi can substantially down-regulate GFP expression, in a GFP-expressing human cell line. Transduction of human cells with this vector results in sustained gene silencing over time. It is postulated that due to the nature of the promoter utilised, higher silencing efficiency should be achieved in murine, instead of human cells.

Decreased expression of microRNAs 143 and 145 in human colon cancers

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ABSTRACT: *Background/Aim:* MicroRNAs (miRNAs) are endogenously expressed RNAs 18-22 nucleotides in length that regulate gene expression through translational repression by binding to a target mRNA. It is thought that the expression level of miRNAs is frequently reduced in cancers because of chromosome aberrations and epigenetic changes. In this work, we examined onco-related miRNAs in colon cancer, resulting that miR-143 and -145 were shown to be low-expression in colon cancers tested. Furthermore, the expression levels in all the human cancer cell lines tested were also shown to be low, compared with those in their originating normal tissue cells. *Materials:* We

examined the expression in the samples from 6 advanced colorectal cancer patients and various kinds of human cancer cells. *Results:* In 5 of 6 colorectal cancer patients, the expression levels of miRNA-143 and -145 were significantly decreased compared with normal samples. The transfection of each precursor miRNA with the miR-143 or -145 to the low-expression cell lines such as human colon cancer DLD-1 and SW480 cells demonstrated growth inhibition. ERK5 was determined to be the target gene of miR-143. Genomic loci of both miRNAs were confirmed to be present more than one allele by genomic PCR in all the cell lines tested. The expression of miR-143 and -145 was inversely associated with cell growth and the expression level of miR-143 and -145 was significantly increased in the apoptotic cells, which were induced by the treatment with hydrogen peroxide or arsenic trioxide. *Conclusion:* These findings suggested that miRNAs-143 and -145 could be good tumor markers for colon cancer and provide an important clue in the study of the mechanism of oncogenesis involving miRNAs.