

RNAi2009: Non-coding RNAs: Bridging Biology and Therapy
18-19 March 2009, St Anne's College, Woodstock Road, Oxford, United Kingdom

J RNAi Gene Silenc (2009), 5(1), Suppl 1

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Selected abstracts (unedited)

(Published online 22 May 2009)

The role of microRNAs in tumor progression

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miRNAs are small 20-22 nucleotide long RNA molecules that regulate important cellular processes by suppressing expression of multiple mRNAs. Through the analysis of two tumor cell types, one of which exhibits an epithelial gene-expression profile (Type II) and one a mesenchymal gene-expression profile (Type I) (1), and which differ in their responses to the apoptosis-inducing activity of Fas ligand and Taxanes, we identified the miRNA let-7 as a marker for the more differentiated and less aggressive Type II cancer cells (2). Consistently let-7 has been shown to regulate the self renewal capacity and drug resistance of cancer cells. The early embryonic genes HMGA2 and IMP-1 were identified as prime targets of let-7 and are upregulated in advanced ovarian cancer (OvCa) patients (2, 3). A bioinformatics analysis identified 10 additional let-7 regulated oncofetal genes (LOGs) that might be upregulated in many human cancers giving cancer cells migratory and invasive properties of embryonic cells (3). A screen designed to identify miRNAs specifically expressed in E-cadherin positive cancer cells identified the miR-200 miRNA family as being highly expressed in epithelial cells. MiR-200 family members suppress ZEB1 and ZEB2, two transcription factors known to be powerful epithelial/mesenchymal transition (EMT) inducers through suppression of the expression of E-cadherin (4). We conclude that let-7 and miR-200 contribute to two key stages of cancer progression: 1) Let-7 suppresses the expression of a number of oncofetal genes preventing dedifferentiation of cancer and early cancer progression causing increased self renewal activity and increased resistance to chemotherapeutic drugs and 2) miR-200 maintains an epithelial phenotype, thereby preventing metastases by targeting factors that induce EMT. Loss of let-7 therefore allows early cancer progression whereas loss of miR-200 is expected to drive EMT resulting in more invasive cancers.

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Delivery systems and their use for single- and double-stranded RNA therapeutics

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Synthetic oligodeoxynucleotides (ODNs) or oligoribonucleotides (ORNs) can trigger immune defense mechanisms originally evolved to fight infectious pathogens. Toll-like receptors and RIG-I like receptors stimulate innate immune responses and their activation results in enhanced vaccination efficacy, defense against pathogen challenges, and results in anti-tumor activity. Similar to siRNA, specific delivery systems have to be employed for single-stranded RNA immune stimulators to result in efficient in vitro and in vivo immune activation. Several challenges have to be met such as the optimal route of administration and an efficient cellular uptake in specific intracellular compartments. In RNA interference, immune activation is becoming one of the issues with similarities to the beginning of the development of antisense ODN. However, specific RNA chemical modifications or delivery systems can be utilized to suppress such immune effects. Moreover, recent results suggest a promising perspective for the use of multifunctional silencing and stimulatory RNAs (sisRNAs).

Anti-tumor effects of polyethylenimine (PEI)-complexed siRNAs or shRNAs targeting tumor-relevant genes

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RNA interference (RNAi) is a powerful method for the knockdown of genes which are pathologically overexpressed in tumors. For the therapeutic exploration of RNAi, the identification of candidate tumor-relevant target genes, which may also include gene products considered 'undruggable' by other methods, as well as the development of strategies for the therapeutic application of siRNAs or shRNAs to induce RNAi *in vivo* are critically important. For safety reasons, the direct application of siRNAs may be preferable over DNA-based approaches; however, the poor stability, penetration and cellular uptake of naked siRNAs poses major challenges to any siRNA-based therapy. Consequently, any therapeutic application will rely on efficient siRNA delivery systems.

We have developed the non-covalent complexation of siRNAs with polyethylenimines (PEI) as a promising method for the therapeutic siRNA delivery. PEIs are water-soluble synthetic polymers with protonable amino groups which are able to form non-covalent complexes with siRNAs, mediating their full protection against nucleolytic degradation as well as their cellular uptake and intracellular release from the endosomal/lysosomal system. Critical for *in vivo* siRNA delivery are the PEIs or PEI derivatives being used, and we have introduced a linear PEI as well as the branched low molecular weight PEI F25-LMW as optimal with regard to efficacy of siRNA delivery and *in vivo* biocompatibility.

This presentation shows the therapeutic efficacy of PEI/shRNA- or PEI/siRNA-mediated RNAi in various mouse tumor models upon systemic treatment. The intraperitoneal administration of shRNA constructs targeting HMG2A reduced or abrogated the growth of subcutaneous or *i.p.* ovarian carcinoma xenografts, respectively. Anti-tumor efficacies of PEI/siRNA-mediated gene knockdown were evaluated in *s.c.* prostate or pancreatic xenografts for VEGF knockdown. The comparison of (i) PEI/siRNA-mediated targeting of the growth factor VEGF, (ii) treatment with the therapeutic anti-VEGF antibody Avastin and (iii) a combination of both revealed, dependent on the tumor model, comparable or additive anti-tumor effects. Other target genes employed in our studies included the HER-2 receptor in ovarian carcinoma and the growth factor pleiotrophin (PTN) in glioblastoma.

The observed anti-tumor effects are based on the efficient delivery of intact siRNA molecules into the tumors as determined in biodistribution studies, and the profound knockdown of the target gene. Furthermore, both PEIs show favourable properties with regard to biocompatibility/absence of unwanted side effects of the complexes or the free carrier at the concentrations used for therapeutic applications. Studies are now further extended towards chemically modified siRNA molecules and PEI derivatives to further increase knockdown efficacy and tissue specificity.

We conclude that the PEI-mediated systemic delivery of siRNA molecules targeting tumor-relevant receptors, growth factors or downstream signal transduction molecules represents an attractive approach in tumor therapy.

Keywords: RNAi-based therapy, siRNA, polyethylenimine, PEI, VEGF, anti-tumor therapy

Identification and characterisation of MCMV encoded miRNAs *in vitro* and *in vivo*

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MicroRNAs (miRNAs) are small, non-coding RNA molecules that specifically regulate gene expression at post-transcriptional level. Originally identified in a variety of organisms ranging from plants to mammals, miRNAs have now been found to be encoded by numerous viruses, especially herpesviruses. Viral miRNAs are thought to modulate both viral and host gene expression. We have identified 18 distinct miRNAs encoded by the murine cytomegalovirus (MCMV) by molecular cloning, sequencing and subsequent confirmation by northern blots. The MCMV miRNAs are expressed in small clusters with up to 3 distinct miRNAs created from a single transcript. Remarkably, we found that already after 24h post infection viral miRNAs constitute about 35% (706/1936 clones) and at 72h post infection they comprise more than 60% (785/1254 clones) of the total cellular miRNA pool. This indicates an important role of these miRNAs during productive MCMV infection. While the majority of viral miRNAs accumulated throughout infection there is evidence for abundance control for two of them. These miRNAs were polyuridylylated at their 3' end for subsequent degradation. We have created viruses with single miRNA deletions of the two most abundant miRNAs and report on the biological properties of the mutants.

Keywords: miRNAs, viral, cytomegalovirus, *in vivo*

Structurally modified anti-MDR1 siRNAs with selective 2'-O-methyl modifications: biological activity and nuclease resistance

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Small interfering RNAs (siRNAs) are potent tools for sequence specific gene silencing. However, the sensitivity of siRNA duplexes to nucleases and dependence of their interference activity on the nucleotide context of the

mRNA-target limit their biomedical application. Here, we showed that asymmetric “fork-siRNA duplexes” targeted to Multiple Drug Resistance gene (MDR1) suppressed the exogenous expressed chimeric EGFP/MDR1 in HEK 293 cells more effectively than conventional siRNA duplex. The data revealed that introduction of 2'-O-methyl modifications in nuclease sensitive sites of siRNAs did not reduce their silencing activity as compared to that their unmodified analogues. However, fork-siRNA duplexes containing 2'-O-methyl modifications were shown to induce the persistent silencing over a period of twelve days post-transfection, while the silencing effect of the unmodified analogues expired within six days. Thus, selective 2'-O-methyl modification improves the nuclease resistance of fork-siRNA duplexes and results in a prolonged gene silencing.

The biological activity of selectively 2'-O-methyl modified anti-MDR1 siRNAs with nucleotide substitutions in the central part of either sense or antisense strand was investigated. We showed that three substitutions of C to U at 7 – 9 sites of the antisense strand resulting in the formation of GU wobble pairs greatly enhanced the silencing activity of the siRNA duplex.

The comparison of nuclease resistance and biological activity of selectively 2'-O-methyl modified double stranded (ds) and single stranded (ss) siRNAs revealed that selective modifications increased the resistance of ss-siRNA to nucleases, but did not enhance its silencing activity. This fact suggests that other properties rather than nuclease resistance are responsible for the much lower activity of ss-siRNAs as compared to ds-siRNAs.

This work was supported by RAS programs “Molecular and Cellular Biology” and “Basic sciences for medicine”, RFBR 08-04-01073, grant from SB RAS No. 5.10, INTAS/SBRAS grant No. 06-1000013-9117, FCP No. 02.512.11.2200.

Keywords: RNA interference, siRNA thermostability, asymmetric siRNA, fork-siRNA duplex, single strand siRNA, 2'-O-methyl modification, nuclease resistance

Fusion Genes: Lime leaf in the leukaemic armour?

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Chromosomal rearrangements frequently generate fusion genes, which may serve as tumour-specific targets for therapeutic approaches, if the tumour cell was “addicted” to this particular fusion oncogene. However, genes encoding transcription factors are frequently considered to be “undruggable” by conventional small molecule approaches. Furthermore, since the tumour-initiating or promoting effects of such fusion genes are often lineage-dependent, their function in tumour maintenance can only be examined

in the appropriate cancer cell context. We examined leukaemic fusion genes such as AML1/MTG8 or MLL/AF4 with siRNAs complementary to the fusion site of the corresponding transcript. Transient siRNA transfection demonstrated for AML1/MTG8 and MLL/AF4 an essential role in leukaemic proliferation and clonogenicity in cell culture. Moreover, transient siRNA application ex vivo prior to transplantation of leukaemic cells into immunodeficient mice significantly increases median survival. Notably, these siRNA-mediated effects are highly specific: leukaemic or haematopoietic stem/progenitor cells not expressing the target fusion gene are not affected by the corresponding siRNA. To further explore the therapeutic potential for these anti-leukaemic siRNAs, we have developed an immuno-polyplex approach for the targeted siRNA delivery to haematopoietic/leukaemic tissues. First results demonstrate efficient delivery and knock-down of targeted fusion genes in cell culture. Currently, we are examining the efficacy and toxicity of these delivery approaches in vitro, and we are initiating potency and toxicity in vivo studies.

Keywords: Fusion gene, oncogene addiction, leukaemia, chromosomal translocation, siRNA delivery, immuno-polyplex

U1 Adaptors: A novel gene silencing technology

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U1 Adaptors represent a novel gene silencing method where a bifunctional synthetic oligonucleotide recruits action of the U1 snRNP complex in the terminal exon of a targeted gene. Tethering of U1 snRNP prevents 3' end processing (polyA tail addition) by inhibiting poly-A polymerase (PAP), leading to degradation of that RNA species within the nucleus. Adaptors and siRNAs work via different mechanisms of action at different subcellular locations; the methods can be combined to increase the level of knockdown achieved using lower doses of both reagents.

Cellular uptake of naked functional oligonucleotides: Phosphorothioate-stimulated delivery of siRNA indicates new routes for cellular and intracellular transport

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The efficient cellular uptake of oligonucleotide-based drugs and tools in vivo is a fundamental and largely unresolved technical hurdle. This is also true for the therapeutic application of short silencing RNA (siRNA).

Further, even after entering cells there is insufficient transport of siRNA to the site of action where it encounters the RNA-induced silencing complex (RISC) and suppresses its target. In case of the majority of known delivery systems siRNA is captured in the endosomal compartment.

The phosphorothioate (PS)-stimulated uptake of naked extracellular siRNA into human cells represents a promising alternative because it delivers large amounts of siRNA into cells and makes use of a caveosomal rather than an endosomal pathway. So far, the biological activity of PS-delivered siRNA is moderate. In case siRNA accumulates in the perinuclear space which offers new strategies for its intracellular functional release.

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PCI facilitates endosomal escape and improves silencing efficiency, *in vitro* and *in vivo*

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For RNAi to occur, siRNA molecules need to be present in the cell cytoplasm. However, the physicochemical properties of siRNA (such as negative charge, hydrophilicity, and size) hamper the cell entrance of siRNA molecules. Delivery systems are generally used to overcome this difficulty by interacting with the siRNA and thereby protecting it, ultimately favouring cellular uptake of siRNA. Such systems, generally based on the use of cationic lipid- or polymer-based carrier molecules are referred to as lipoplexes or polyplexes, respectively, and are mainly taken up by cells through endocytosis. Once inside the endosomes, siRNA needs to cross the endosomal membrane in order to reach the cytoplasm, a process known as endosomal escape.

A technique named photochemical internalisation (PCI, Figure 3), which employs photochemical reactions to disrupt endocytic vesicles, has been applied to deliver molecules such as the proteins gelonin and saporin, and the chemotherapeutic agent bleomycin into the cell cytoplasm. PCI can also be successfully employed *in vivo*, for intratumoral delivery of siRNA.

Our treatment consisted of intratumoral (i.t.) injection of the photosensitizer disulfonated aluminum phthalocyanine AIPcS2a at day 0, followed two days later, by i.t. injection of the siRNA lipoplexes (either anti-EGFR siRNA or non-specific siRNA complexed with Lipofectamine, siEGFR/LF or siNS/LF, respectively). Six hours after the injection of lipoplexes, the tumour area was exposed to light. In this

study, two different light doses were employed, 75 J/cm² or 90 J/cm². Tumour volume was assessed. To determine the silencing efficiency, tumours were harvested two and three days after the treatment and immunohistochemistry techniques were employed to detect the EGFR expression in tumour tissue sections. In parallel, tissue lysates were prepared and western blot (WB) techniques employed for relative quantification of the EGFR protein expressed in the tumours.

EGFR expression was reduced 80% in A431 tumour xenografts when lipoplexes were administered with PCI, compared to 30% knockdown induced by the lipoplexes alone. EGFR silencing was prominent in the tumour center, while less apparent in the tumour rim. Single administration of PCI-facilitated siRNA-mediated EGFR-knockdown resulted in a tumor growth delay. Interestingly, upon recovery of tumour growth after approximately 5 days, tumours seemed to have larger blood vessels in the tumour periphery. A similar pattern has been previously reported by Vilorio-Petit et al. in A431 tumour xenografts, which appeared to be related with an altered VEGF-expression.

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Antiproliferative action of immunomodulating siRNAs: Dependence on their sequences

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The RNA interference (RNAi) approach is an efficient and widely used gene-silencing mechanism with great potential for gene-therapeutic applications. However, some siRNAs can also exert non-target-related biological effects, including immune-system stimulation. Nonspecific effects of siRNAs often depend on their sequences, search immunostimulated sequences allows to avoid unwanted side-effects of siRNAs and also to create high-effective immunomodulating agents, which can induce proliferation blockage, differentiation or apoptosis in cancer cells.

We find out that siRNA siInt (22 bp in length with 3 nt 3'-overhangs) partially corresponding to the MDR1 first intron region exert significant antiproliferative action on human carcinoma HeLa, KB-3-1 and neuroblastoma SK-N-MC cells, whereas siInt with the same sequence, but with 2 nt 3'-overhangs do not exert such effects. To investigate whether antiproliferative action of siInt is caused by activating IFN-related pathways, we examine expression levels of interferon-stimulated genes PKR, OAS1, STAT1, IFN α 2 and IL6 by real-time PCR, as a control interferon inducer we used synthetic analogue of long dsRNA poly(I:C). It was shown that siInt, transfected into human PBMC (peripheral blood mononuclear cells), HeLa or KB-3-1 cells, induce a potent interferon response.

To find most effective immunomodulating siRNA motifs we investigated antiproliferative action and immunostimulating activity of different version of siInt sequences. It was shown that introduction of mismatches in the middle of siInt sequence (10-16 positions) do not exert significant decrease of its antiproliferative activity, while mismatches at the ends of siInt sequence results in disappearance of antiproliferative action of investigated siRNAs.

The data suggest that siRNAs with defined sequences can serve as high-effective antiproliferative agents, stimulating innate immunity response.

This work was supported by RFBR (grant No. 03-04-48550-a), RAS programs "Gene-Targeted Biologically Active Compounds as Antiviral and Anticancer Drugs", "Physico-chemical biology" and "Fundamental science for medicine", SB RAS (Interdisciplinary grant No. 50).

Prediction of human targets for viral encoded microRNAs by thermodynamics and empirical constraints

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MicroRNAs (miRNAs) are small RNA molecules that modulate gene expression through degradation of specific mRNAs and/or repression of their translation [1]. miRNAs are involved in both physiological and pathological processes, such as apoptosis and cancer. Their presence has been demonstrated in several organisms as well as in viruses. Virus encoded miRNAs (vmiRNAs) can act as viral gene expression regulators, but they may also interfere with the expression of host genes. In particular, vmiRNAs may control host cell proliferation by targeting apoptosis regulators [2]. Therefore, they could be involved in cancer pathogenesis. Computational prediction of miRNA/target pairs is a fundamental step in these studies. Here we describe the use of miRiam, a novel program based on both thermodynamics features and empirical constraints, to predict vmiRNA/human targets interactions. More precisely, miRiam exploits target mRNA secondary structure accessibility and interaction rules, inferred from validated miRNA/mRNA pairs. A set of genes involved in apoptosis and cell-cycle regulation was identified as target for our studies. This choice was supported by the knowledge that DNA tumor viruses interfere with the above processes in humans [3]. miRNAs were selected from two cancer related viruses, Epstein-Barr Virus (EBV) and Kaposi-Sarcoma Associated Herpes Virus (KSHV). Results show that several of the mRNAs (such as BID, BAX, CASP3, CASP10, TP53) possess good binding scores to those miRNAs. Results suggest that during viral infection, besides the protein based host regulation

mechanism, a post transcriptional level interference may exist. This leads to the formulation of plausible hypotheses of vmiRNAs and human apoptosis gene involvement in cancer development. Future works will aim to provide experimental validations of estimated interactions and to extend predictions to other reasonable human transcripts, such as additional nodes of apoptosis pathway, cell-cycle regulators and genes involved in immune response. All the data will be freely available on the group website.

Keywords: microRNA, virus, apoptosis, cell cycle, cancer

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Development of Small Interfering RNA Selectively Activated in Target Cells

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Objectives: RNAi (RNA interference) technology became an important tool to modulate gene expression in vitro as well as in vivo. siRNA (small interfering RNA) molecules are easy to use with efficient gene silencing rates. Chemical modifications increase stability of siRNA. New algorithms and databases increase specificity of siRNA molecules, while new siRNA cocktails reduce side effects. In contrast, cell-specific silencing using specifically inactivated siRNA in co-cultures is not yet described. Here we present a new mechanism of target cell specific siRNA.

Mechanism: siRNA molecules are bound to short peptides that inhibit induction of RISC. These short peptides contain a specific sequence, which is the target sequence of peptidases, exclusively active in target cells. After transfection (here by electroporation), only in target cells, but not in others, peptidases cleave peptides and on this way activate siRNA. The peptide sequence used here (-L-E-V-D-) is the target sequence for caspase-4, expressed in Jeg-3 choriocarcinoma, but not in human embryonic kidney (HEK) cells. Successful binding was confirmed by mass spectrometry. To test this mechanism, we silenced GFP (Green Fluorescence Protein) expression by such modified specific siRNA in a GFP transfected Jeg-3 choriocarcinoma cell line in contrast to GFP transfected HEK cells. Flow cytometry and fluorescence microscopy were used to analyze GFP expression.

Results: We have been able to produce a covalent bond of peptides to siRNA molecules via two different linker molecules based on different chemical strategies. Results indicate, that in Jeg-3 cells modified siRNA became activated and was able to reduce GFP expression in

contrast to HEK cells lacking caspase-4 and unable to activate the modified siRNA.

Conclusion: This new mechanism may be useful for selective silencing of a single population in co-cultures. In the future the presented mechanism might be used for therapeutic siRNA applications.

***In vivo* screening chemically modifications of RNA duplexes for their ability to reduce innate immune responses**

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Due to their sequence specific gene targeting activity siRNAs are regarded as promising new active compounds in gene medicine. But one serious problem with delivering siRNAs as treatment is the now well-established non-specific activities of some RNAs duplexes. Cellular reactions towards double stranded RNAs include the 2'-5' oligoadenylate synthetase system, the protein kinase R, RIG-I and Toll-like receptor activated pathways all resulting in antiviral defence mechanism. We have previously shown that antiviral innate immune reactions against double stranded RNAs could be detected in vivo as lowered mortality of siRNA-injected small fish after challenging them with a fish pathogenic virus as compared to control fish. This protection corresponded with an interferon response in the fish. Here we use this fish model to screen siRNAs containing various chemical modifications of the RNA backbone and find that it is possible to differentiate between the antiviral activities of these duplexes. We conclude that the fish in vivo model is a potent tool for gaining insight into the triggering of antiviral cellular reactions towards siRNAs in vertebrates. In further screens we will try to elucidate the role of the position of modifications and of strand bias of modifications. The overall perspective is to learn how to avoid triggering of non-specific antiviral responses and still allow uptake of siRNAs into RISC for specific gene silencing. The fish model is also used for screening various commercial and non-commercial delivery reagents with the same perspective.

Functional validation of genes related to local aggressiveness and metastatic potential in soft tissue sarcomas

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Soft tissue tumors are a heterogeneous group of mesenchymal tumors with variable clinical behavior and histological presentation. Clinically they can be separated in benign, locally aggressive malignant tumor and malignant tumor with metastatic potential. Sarcomas can also be divided according to the degree of aggressiveness in low grade, intermediate grade and high grade tumors. Tumor size, histological grade, depth, and status of surgical margins have been identified as prognostic factors. However, these prognostic variables do not explain biological differences in aggressiveness between sarcomas of similar clinical and/or histological aspects. In order to better understand the biology of sarcomas, as well as generate useful knowledge to the classification, diagnosis and prognosis, our group examined the gene expression of different types of tumors using cDNA microarray technology. In this study, 06 genes were found to be potentially involved in the process of local aggressiveness (SNRPD3, MEGF9, SPTAN1, AFAP1L2, ENDOD1 and SERPINA5), and 06 in metastasis (ZWINTAS, TOP2A, UBE2C, ABCF1, MCM2 and ARL6IP5). These potential new markers, once validated, may be useful to predict prognosis, and develop strategies to enable more selective treatment. In this sense, the project's goal is to validate the differential expression by real-time PCR, modulate the expression of validated genes by RNAi (lentiviral shRNA) in murine sarcoma cell line CRL-2799, and inject the silenced cell lines in C57BL6 murine experimental model. We currently have 8 genes validated by Real Time PCR (MEGF9, AFAP1L2, ENDOD1, SERPINA5, ZWINTAS, TOP2A, UBE2C, and MCM2). We have started the RNAi experiments with the MISSION® RNAi system (SIGMA) of the 8 genes, and preliminary data will be presented.

Financial support: CEPID/FAPESP

Keywords: Cancer, Sarcoma, local aggressiveness, metastasis, microarray, Real Time PCR, lentiviral RNAi, murine experimental model

Detection of microRNAs using a dual - fluorescence reporter/sensor plasmid in Zebrafish

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MicroRNAs (miRNAs) are 20–25 nucleotide long noncoding RNAs that have been found in a wide variety of organisms and shown to exert essential roles by regulating the stability and translation of target messenger RNAs (mRNAs) in animals and plants. Hundreds of miRNA genes have been found in diverse animals, and many of these are phylogenetically conserved. With miRNA roles

identified in developmental timing, cell death, cell proliferation in drosophila, haematopoiesis and patterning of the nervous system in mammal, and expression of *hela* cell and *Hox* gene in human, evidence is mounting that animal miRNAs are more numerous, and their regulatory impact more pervasive, than was previously suspected. In zebrafish, lots of miRNAs play important roles in developmental regulation. MiRNAs play important roles in the fine tuning of neural crest cell migration, regulation of neural *Hox* gene expression, and regeneration after tissue amputation in zebrafish. Here, we use a hsp dual fluorescent green fluorescent protein (eGFP) reporter/monomeric red fluorescent protein (mRFP) sensor plasmid, injected into zebrafish blastomeres embryos in utero or ex utero, to monitor the dynamics of specific miRNAs in individual live embryos. We make a class of transgenic fish for dual fluorescence reporter plasmid, and choose several microRNAs for a basic research. The dual fluorescence reporter/sensor plasmid allows the monitoring of miRNA appearance and disappearance in defined cell lineages during vertebrate development, which lays a foundation of miRNA research in zebrafish.