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Histone deacetylase inhibitors mediate pharmacological rescue and increase membrane expression of ABCG2 harboring the Q141K single nucleotide polymorphism

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ABCG2 is an ATP-binding cassette half-transporter that has garnered interest in pharmacogenomics as a modulator of oral drug absorption, drug excretion, and drug distribution through expression at the blood–brain barrier and the maternal–fetal barrier as well. A single nucleotide polymorphism (SNP) C421A in ABCG2, resulting in a glutamic acid to lysine mutation at amino acid 141 (Q141K), confers impaired protein expression and function. Pharmacogenomic studies have linked the SNP to increased exposure to substrate drugs including irinotecan, topotecan, sunitinib and gefitinib. We have studied the biology underlying altered expression and function. A large fraction of the Q141K variant is degraded before it reaches the cell surface, and variant at the cell surface has reduced transporter function. Q141K-ABCG2 is resistant to degradation by Endo H and levels are increased by the lysosome inhibitor, bafilomycin, suggesting that cell surface protein, although at reduced levels, is fully processed and folded. Proteosome inhibitors do not increase levels, arguing against retention and loss through ERAD (endoplasmic reticulum associated degradation). Pharmacological chaperones were used to evaluate impact on trafficking. Mitoxantrone (MX), a substrate previously noted to facilitate processing of ABCG2 mutants showed a small increase in Q141K-ABCG2 expression at the cell surface when imaged by confocal microscopy. Incubation with 50 μM of the dietary compounds dibenzoylmethane (DBM) and tert-butyl hydroquinone (TBHQ) increased expression of ABCG2 variant but did not improve localization to the cell surface. Romidepsin and other histone deacetylase inhibitors (HDIs), previously shown to increase levels of ABCG2 mRNA, were examined for impact on trafficking. A significant increase in total protein, surface expression, and function was seen in Q141K-ABCG2 variant when exposed to the various HDIs. Immunofluorescence analysis by confocal microscopy suggested Q141K-ABCG2 localization in the Golgi, with a dramatic shift to the plasma membrane following exposure to romidepsin and other HDIs. Results suggest that the Q141K-ABCG2 variant is trapped in the Golgi and that HDIs aid localization to the surface. Investigations into the mechanism underlying this HDI-mediated pharmacologic rescue are underway.

RNA and DNA based technologies

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Advancement of ALN-VSP, an experimental RNAi therapeutic for solid tumors

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Malignancies of the liver, including primary (hepatocellular carcinoma) and secondary (metastatic) tumors, represent a significant unmet medical need. We are developing the RNAi therapeutic, ALN-VSP, for solid tumors with significant liver involvement, and evaluation is currently ongoing in a Phase 1 clinical trial. ALN-VSP is comprised of lipid nanoparticle (LNP)-formulated small interfering RNAs (siRNAs) targeting VEGF and the mitotic kinesin, KSP (Eg5). Here, we report the efficacy of each siRNA component of ALN-VSP in orthotopic liver tumor models, as well as models of extra-hepatic tumors. To generate orthotopic liver tumors, human hepatoma or colorectal carcinoma cells were implanted directly into the livers of immunocompromised mice. These cell lines were also used to establish tumors at extra-hepatic sites including the lymph nodes and peritoneal cavity.
Histological analyses of tumors from mice treated with ALN-VSP demonstrated that each siRNA makes a distinct contribution to efficacy. ALN-VSP treatment leads to accumulation of aberrant mitotic figures (monoasters), a hallmark of KSP inhibition, in orthotopic liver tumors, as well as in extra-hepatic tumors of different origin. Evidence of therapeutic VEGF inhibition was shown by marked reductions in tumor microvessel density and intratumoral hemorrhage in orthotopic tumors. Similar results were obtained with a LNP formulation of the VEGF siRNA alone. Finally, we show that multi-dose administration of ALN-VSP significantly prolongs survival of mice harboring advanced orthotopic liver tumors. These studies show the contribution of both siRNAs in ALN-VSP to efficacy in tumor models, and demonstrate the therapeutic potential of ALN-VSP for the treatment of hepatic and extra-hepatic solid tumors.

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Therapeutic siRNA delivery against PKN3 improves the antineoplastic efficacy of gemcitabine in an orthotopic pancreatic cancer model

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Background: Since therapy of human pancreatic cancer remains a great challenge, the development of novel molecular targeted therapies is required that may improve the antineoplastic potential of chemotherapeutic agents. Particularly antiangiogenic approaches are of continued interest in this context. Recently, siRNA-lipoplex directed against protein kinase N3 (PKN3) has proven antiangiogenic properties in experimental tumor models. For this study we hypothesized that therapeutic application of PKN3-siRNA-lipoplexes may improve the antineoplastic efficacy of gemcitabine in an orthotopic pancreatic cancer model.
Materials/Methods: An orthotopic tumor model was employed using the human pancreatic cancer cell line L3.6pl. Animals (Balb/c nu/nu) received either PKN3-siRNA-lipoplexes (3x/week; 2.8 mg/kg siRNA) (ATU027; Silence Therapeutics, Berlin), or gemcitabine (50 mg/kg, 2x/week), or a combination of both per tail vein injections. Control animals received carrier (sucrose). Treatment started on day 11 post tumor cell implantation and the experiment was terminated on day 33.
Results: Single agent therapy with either gemcitabine, or PKN3-siRNA-lipoplexes both significantly reduced orthotopic tumor growth (tumor weight) ($p < 0.05$). Importantly, combination therapy further reduced pancreatic tumor growth ($p < 0.001$), and substantially diminished the occurrence of either lymph node or liver metastases. In vivo PKN3 knock-down was proven in lung tissues from mice receiving combinational therapy. Signaling analyses of tumor tissues and staining analyses are pending.
Conclusion: Therapeutic PKN3-siRNA delivery reduces growth and metastasis of human pancreatic cancer and improves the antineoplastic efficacy of gemcitabine. Thus, PKN3-Lipoplex-siRNA may be an effective approach for treating pancreatic cancer.

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Generation of microRNA-sensitive VSV vectors to reduce neurotoxic side effects

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Vesicular stomatitis virus (VSV) is an oncolytic virus which shows promise as a therapeutic agent for cancer, however, toxic collateral effects on the brain and the liver occur when it is administered at high doses. We hypothesized that by employing a novel strategy, termed microRNA-engineering, we could eliminate off-target replication and improve the therapeutic index. This approach involves the incorporation of target sequences of a specific miRNA (miRTs) into the viral genome, resulting in degradation of viral gene transcripts in cells in which this miRNA is highly expressed, without interfering with virus replication in cells not expressing the relevant miRNA.
We screened a panel of candidate miRNAs to select for those with high expression levels in the brain and/or liver and low levels in primary human HCC and CRC and cell lines. We then confirmed the miRNA-mediated regulation of the candidates in an *in vitro* reporter gene assay. Four tandem repeats of perfect complementary miRTs were cloned into the 3′-UTR of a luciferase reporter gene, and these plasmids were transfected into a panel of normal and tumor cells. We then selected only those miRTs which resulted in a reduction of luciferase expression in primary hepatocytes and neurons but not in tumor cells. As a final control, we tested the candidate miRNA expression levels in the presence vs. absence of VSV infection. Based on these preliminary experiments, we chose three miRTs which best fit our selection criteria, and created recombinant VSV vectors containing four tandem repeats of the miRTs in the 3′-UTRs of crucial endogenous viral genes. After characterization of these vectors in tumor and non-tumor cells *in vitro*, thorough toxicity and efficacy studies will be performed in mouse models of HCC and CRC. We ultimately aim to engineer next-generation viruses with one or more viral gene regulated by multiple miRTs. Because copy number of endogenous miRNAs is critical in restricting viral replication and could easily become saturated in the target tissues resulting

in "leaky" virus replication, we hypothesize that the incorporation of multiple miRTs could overcome this limitation and result in an even safer vector. Thus, this strategy has the potential to provide a basis for clinical application of VSV vectors for the treatment of primary and metastatic liver disease, as well as other cancers, in the future.

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Systematic functional analysis of microRNAs by transfection of 1129 miRNAs into prostate cancer cells

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The molecular mechanisms behind prostate cancer development, progression and therapy failure are incompletely understood, but the androgen receptor (AR) is almost invariably involved. Altered microRNA (miRNA) expression profiles have recently been demonstrated in a large number of cancer types, including prostate cancer. These short RNA molecules of ~21 bases regulate mRNA stability and translation by base-pairing mainly to the 3′-untranslated region (3′UTR) of their targets.
In this study, we analyzed the functional impact of miRNA gain-of-function on prostate cancer cell growth and survival as well as on the expression of AR protein. Seven prostate cell lines (LNCaP, LAPC-4, MDA-PCa-2b, 22Rv1, CWR-1R, RWPE-1, and EP156T) were screened with 1129 miRNA mimics (Ambion and Dharmacon), double-stranded oligonucleotides designed to mimic the function of endogenous mature miRNAs. Cell viability was measured with CellTiter-Glo® (Promega) whereas AR, cell proliferation (Ki67) and apoptosis (cPARP) were detected with specific antibodies using protein lysate microarrays as described in our recent publication on estrogen receptor targeting miRNAs (Leivonen *et al*., Oncogene 2009). When transfected to the prostate cancer cell lines, 68 of the miRNAs had anti-proliferative and 52 had pro-apoptotic functions. In addition, 52 miRNAs down-regulated the levels of the AR protein in all five AR-positive prostate cancer cell lines. Western blots were used to verify knock-down, and the direct functional role of the miRNAs was clarified by 3′UTR luciferase assays and by analyses of down-stream consequences of the knock-downs.
In summary, we have systematically defined those miRNAs that are critical for prostate cancer cell growth, survival and the expression of the androgen receptor. This integrated, multi-parametric profiling provides new systematic evidence on the role of miRNAs in prostate cancer as well as novel clues for therapeutic targeting by manipulating key pathways and cell survival pathways by miRNA gain-of-function.

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Novel approaches to enable molecular analysis of clinical samples with limited amount of tissue

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Molecular marker analysis of tumor biopsies is vital for understanding the molecular mechanisms underlying oncogenesis and for studying the molecular basis of response or resistance to targeted therapies. Molecular marker data gathered from such clinical trial samples offer great potential for predictive or prognostic diagnostics, and for identifying new targets or pathways to target for future drug development. Since archival tumor biopsies commonly exist as formalin-fixed and paraffin-embedded (FFPE), the ability to obtain high quality nucleic acids is highly compromised. In addition, the amount of tissue obtained from fine needle aspirates (FNA), core biopsies and certain tumor types such as lung can be extremely limited, further limiting the ability to evaluate biomarkers. In Oncology Biomarker Development Group at Genentech, we have focused our efforts over the last few years in optimizing approaches and methodologies for molecular analysis of such challenging samples, with the objective of (a) increasing the sensitivity of nucleic acid analysis from FFPE tissues, (b) minimizing the RNA/DNA input requirements for assays, and (c) maximizing the number of analytes assayed from a single tissue section, while ensuring robustness and quality of data obtained. We will discuss several technologies and methodologies, review strengths and weaknesses, and show validation data generated using clinical samples.

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In vivo pharmaceutical targets screening using lentiviral inducible-knockdown shRNA system

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Background: Small interfering RNAs (shRNAs) are able to suppress essentially any gene expression through the endogenous cellular process of RNA interference pathway and have been used in many studies to screen for therapeutic targets in various pathological conditions. In this project, we aimed to develop an efficient *in vivo* cancer target validation method using a lentiviral inducible-knockdown shRNA. To verify our approach, we used an essential cell-cycle protein polo-like kinase 1 (PLK1) as a proof of concept target.
Methods: An inducible shRNA construct targeting PLK1 or a control construct was transfected into colon carcinoma cell line SW620. PLK1 knock-down was quantified by real-time PCR. Inducible shRNA expression (marked by Turbo RFP) was monitored by fluorescence microscopy and flow cytometry. Xenografts were established by subcutaneous or orthotopic injection of PLK1 inducible-knockdown cells into nude mice. Therapeutic effect was assessed by calliper measurement of tumour size.
Results: Following doxycycline induction, PLK1 inducible-knockdown cells showed dose- and time-dependent PLK1 down-regulation, which was consistent with induction of shRNA expression. There was about 60% gene knock-down and 40% protein knock-down 72 hours post-induction. In the *in vivo* model, there was a decline in tumour growth rate in the PLK1 knockdown group compared with the control group. *Ex vivo* analysis showed significantly lower PLK1 gene and protein expression in the doxycycline-treated group compared with the control group.
Conclusion: The results support the anti-tumour effects of PLK1 down-regulation and confirm an efficient methodology for cancer target screening using a lentiviral inducible-knockdown shRNA system.

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miR-221 as potential therapeutic target for childhood hepatoblastoma

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Background: We aimed to elucidate the expression level of miR-221 in a large contemporary cohort of hepatoblastomas and to assess its potential as a therapeutic target. MiR-221 has been shown to be upregulated in other cancers but not in hepatoblastomas, which are rare hepatic malignancies of childhood with limited therapeutic options that may derive from hepatic progenitor cells. Overexpression of miR-221 in a mouse model of liver cancer stimulated growth of tumorigenic murine hepatic progenitor cells. MiR-221 targets the CDK inhibitor p27 and enhances cells growth in vitro.
Material and Methods: miR-221 expression was analyzed by real-time quantitative PCR in 12 human formalin-fixed paraffin-embedded hepatoblastoma resection or explantation specimens and matched non-tumorous liver samples of the same patients from the archives of the Department of Pathology at the University of North Carolina at Chapel Hill. Patient characteristics were as follows: Gender: 5 females and 7 males; mean age: 33 months (range 3–144 months). In two patients, subsequent lung metastases were also analyzed and compared with the primary tumor. Assays were performed in triplicate using Taqman MiRNA methodology (Applied Biosystems). RNU66 was used for normalization and the fold-expression changes of miR-221 were calculated by the $2^{-\Delta\Delta Ct}$ method.
Results: MiR-221 is markedly upregulated in hepatoblastomas compared to matched non-tumorous liver parenchyma. Hepatoblastomas that metastasize to the lungs have even higher miR-221 levels than those that do not metastasize. Suppression of miR-221 by antagomirs may be a novel therapeutic strategy in primary and metastatic childhood hepatoblastomas.
Conclusions: MiR-221 is an oncomir that stimulates the onset of tumors when compared with vector-infected cells, possibly through p27 and/or DDIT4 downregulation. In this study of hepatoblastomas, miR-221 is upregulated in tumor samples and promotes tumor progression and metastasis. The use of synthetic inhibitors such as antagomirs of miR-221 may prove to be a promising novel approach to treatment of hepatoblastomas.