

Anti-K-Ras siRNA to Treat Pancreatic Cancer.

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ABSTRACT

Four pancreatic cancer cell lines with the same point mutation at the 12th codon (GGT > GAT) of the *K-Ras* oncogene were used in this study: Panc1 and Panc 8.13 are both homozygous for mutant *K-Ras* (*mK-Ras*), Panc 10.05 is heterozygous for the mutation, and BXPC3 possesses both wild-type *K-Ras* (*wK-Ras*) alleles. Cells were transfected with Lipofectamine and either scrambled siRNA or *mK-Ras* siRNA for a period of six hours. Total *K-Ras* gene expression in these cells was then quantified at 24, 48, or 72 h after transfection by qPCR using *wK-Ras* and *mK-Ras*-specific primers. Results from these experiments show a significant down-regulation of *mK-Ras* compared to cells transfected with scrambled siRNA. After 24 h of treatment, Panc1, Panc 8.13, and Panc 10.05 cells showed significant down-regulation of *mK-Ras* mRNA, 52%, 50%, and 48% less, respectively, compared to scrambled siRNA control. On the other hand, results for BXPC3, the *wK-Ras* cell line, showed no significant change in *wK-Ras* mRNA throughout all time-points. Additionally, *mK-Ras* siRNA appears to significantly inhibit cell metabolic activity. These findings suggest that RNAi directed against point mutations in the *K-Ras* oncogene may be an effective method of to inhibit progression of pancreatic tumors. However, additional studies of nanoparticles-based siRNA delivery platforms are still required in order to determine if this approach can address the issue of intracellular delivery as well as to establish the potency of gene knockdown in such a system.

Keywords: Pancreatic cancer, cell metabolic activity, siRNA, *K-Ras*

1 BACKGROUND

Cancer of the pancreas is one the leading causes of cancer-related mortality [1]. Chemotherapeutic drugs used to treat pancreatic cancers are toxic and minimally effective, yielding a 1-4% 5-year survival rate after initial diagnosis [1]. Approximately 90% of pancreatic cancers possess point mutations in the *K-Ras* proto-oncogene [2]. The GTPase K-Ras is an early player in many signal transduction pathways involved in cell growth and cell division and mutations in the gene are involved in 30-

50% of total cases of cancer [1]. Point mutations in the 12th and 13th codons of *K-Ras* cause the K-Ras protein to permanently adopt the “on” GTP bound conformation, leading to constitutive activation of cellular mechanisms regulating growth and survival signals [1]. Because point mutations in *K-Ras* generally occur at the same codons, they are an attractive target for gene silencing technologies. Previous *in-vitro* and *in-vivo* studies have suggested that small interfering RNA (siRNA) designed against *mK-Ras* may be a potential therapeutic option for the treatment of pancreatic cancers [3]. However, the current methods of intracellular delivery of siRNA are limited to *in-vitro* applications due to lack of a medically credible means of selectively targeting and delivering siRNAs.

2 METHODS

Reagents

Cycloheximide (CHX), an inducer of apoptosis, was purchased from Sigma Aldrich and served as a positive control for in the cell metabolic experiments. Lipofectamine 2000, Power Sybr Green PCR master mix and RT-PCR primers were purchased from Invitrogen. The CellTiter Blue assays were purchased from Promega Corp.

Pancreatic Tumor Cell Lines and Cell Culture Conditions

The pancreatic carcinoma cell lines BXPC3 (K-Ras^{wt,Gly.wt,Gly}), Panc 1 (K-Ras^{Asp12/Asp12}), Panc 8.13 (K-Ras^{Asp12/Asp12}), and Panc 10.05 (K-Ras^{asp12/wt,Gly}) were purchased from American Type Culture Collection (Manassas, VA USA) (catalog # CRL-1687, CRL-1469, and CRL-2547, respectively) BXPC3 was cultured in Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-Glutamine, 100 U / ml penicillin, and 100 mg / ml streptomycin. Panc 10.05 cells were also cultured in RPMI 1640 and supplemented as described above with the addition of 10 U / ml of bovine insulin. Panc-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and supplemented as described above. All cells were grown in a humidified incubator at 37°C (95% air, 5% CO₂).

siRNA Design

Synthetic short double stranded RNA for siRNA targeting the GAT k-ras mutation at codon 12 of K-Ras (sense/antisense: 5'-GUUGGAGCUGAUGGCGUAG-3' TT/5'-CUACGCCAUCAGUCUCCAAC-3' TT) was designed based on studies by Rejiba et al [3], and its lack of potential for off target gene knockdown was verified using BLAST analysis. The K-Ras^{GAT} siRNA was purchased as ready-annealed, purified duplexes from Dharmacon. Scrambled control siRNA sequence was also purchased as ready-annealed, purified duplexes from Dharmacon.

siRNA Transfection and Gene Silencing Experiments

The day before transfection, cells were transferred to 6 well plates in appropriate cell culture media with the above mentioned supplements. The cells were then incubated at 37°C for 24 hours so that they would be ~ 70% confluent. Transfection of K-Ras^{GAT} was then conducted using lipofectmine 2000 according to the manufacturer's protocol. Briefly, the cell culture medium was replaced with serum free minimum essential medium (MEM) containing either, 0.5 ug/ml of K-Ras^{GAT}, 1 ug/ml of K-Ras^{GAT}, 2ug/ml of K-Ras^{GAT}, mixed with lipofectamine 2000, and incubated for 4 h at 37°C. A lipofectamine vehicle control and equivalent doses of scrambled siRNA were also run in the same manner. Thereafter, fresh medium containing 10% FBS was added and incubation was continued for an additional 1-2 days.

RT-PCR Analysis

Cells were plated in six well plates and treated as described above. After 24, 48, and 72 h of treatment cells were processed for RNA expression analysis. Cells were first rinsed thrice with ice cold 1 x PBS to remove media and residual siRNA. Cellular RNA was then harvested using TRIzol (Invitrogen) extraction as described by the manufacturer. Complementary DNA (cDNA) was synthesized using SuperScript® III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. The cDNA was then amplified using specific primers for K-Ras on a 7500 Fast Real-Time PCR System (Applied Biosystems). The results of the PCR were analyzed using the delta delta CT method.

Cellular Metabolic Activity Assay

Panc-1, Panc 8.13, Panc 10.05, and BXPC3 cells that were approximately 70% confluent were enumerated as described above and seeded into 96-well opaque plates (as recommended by the manufacturer, Promega Corp.) at 1.2×10^4 cells per well. Twenty hours later, cells were washed three times (1 x PBS) and the following treatments were added: untreated cells, 1ug/ml

of scrambled siRNA, 1ug/ml of K-Ras^{GAT}, or CHX (100 µg / ml) in optiMEM media. Upon 24, 36, and 48 h treatment, 20 µl of CellTiter-Blue Reagent (Promega Corp.) was added and allowed to incubate at 37° C for 4 h. The CellTiter-Blue reagent utilizes an indicator dye, resazurin to measure the metabolic capacity of cells. Viable cells retain the ability to reduce resazurin to the fluorescent resorufin. Nonviable cells are unable to reduce resazurin and thus do not generate the fluorescent dye. After incubation with the CellTiter-Blue reagent, the fluorescence signal (excitation wavelength, 555 nm and an emission wavelength, 590 nm) was quantified using a multilabel plate reader (Perkin Elmer, WALLAC 1420 VICTOR 2, Boston, MA, USA). We report results from a single experiment conducted in quadruplicate and representative of at least three independent experiments.

Statistical Analysis

All data analysis was performed using SPSS for windows, version 16.0.1. For all cellular metabolic activity assays, univariate ANOVA were performed at each time-point followed by a Dunnett *t*-Test post hoc multiple comparison to test each experimental group against its respective control. All Dunnett *t*-Tests performed were two-tailed.

3 RESULTS

K-Ras Gene Knockdown

Results from siRNA mediated *mK-Ras* gene knockdown experiments are summarized in Fig. 1 A-C, and show a significant down-regulation of *mK-Ras* compared to cells transfected with scrambled siRNA. After 24 h of treatment, Panc1, Panc 8.13, and Panc 10.05 cells showed significant down-regulation of *mK-Ras* mRNA, 52%, 50%, and 48% less, respectively, compared to scrambled siRNA control. *mK-Ras* mRNA levels increased through the 48 and 72 h time-points. However, even after 72 h of transfection, *mK-Ras* mRNA remained significantly lower compared to scrambled siRNA treated cells. On the other hand, results for BXPC3, the *wK-Ras* cell line, which is shown in Fig. 1 D, exhibited no significant change in *wK-Ras* mRNA throughout all time-points.

Effect of Anti-K-Ras siRNA on Cell Metabolic Activity

The effect of *mK-Ras* siRNA on cellular metabolic activity in cell lines possessing homozygous and heterozygous point mutations in *K-Ras* is summarized in Fig. 2 A-C. By 48 h of treatment with *mK-Ras* siRNA, all three cell lines were found to have significantly lower metabolic activity compared to cells treated with an equivalent concentration of scrambled siRNA control. The effect that *mK-Ras* siRNA had on cell metabolic activity was greatest after 72 h, with Panc1, Panc 8.13, and Panc

10.05 cells exhibiting approximately 33%, 34%, and 47% less metabolic activity, respectively, compared to cells treated with scrambled siRNA control. On the other hand, the effect that *mK-Ras* siRNA had on BXPC3 cell metabolic activity, as shown in Fig. 2 D, was relatively small and only significant ($P = 0.046$) at the 24 h time-point.

4 DISCUSSION

Through the characterization of cells treated with *mK-Ras* siRNA our results have shown that these data provide initial proof-of-principle that siRNA may be a successful treatment for knockdown of *mK-Ras* and may serve as a valid approach to treating cancers with K-Ras point mutations. Also, our results indicate that the current siRNA design is specific to down-regulating only *mK-Ras* and not the *wK-Ras*. Furthermore, the results from the CellTiter Blue cell metabolic assays, suggest that siRNA has an inhibitory action in cells express the *mK-Ras* allele.

In conclusion, although these data are promising, additional studies of nanoparticles-based siRNA delivery platforms are still required in order to overcome the limitations and challenges of using naked siRNA *in-vitro*. Further studies must be conducted to address issues such as siRNA stability, cell-specific targeting, and intracellular delivery before siRNA approaches can move on to being utilized in *in-vivo* systems.

REFERENCES

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5 FIGURES

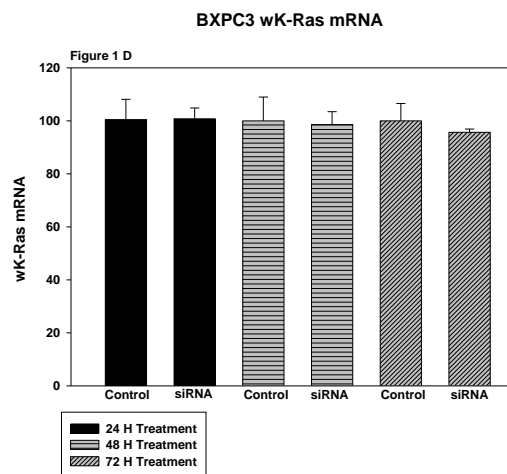
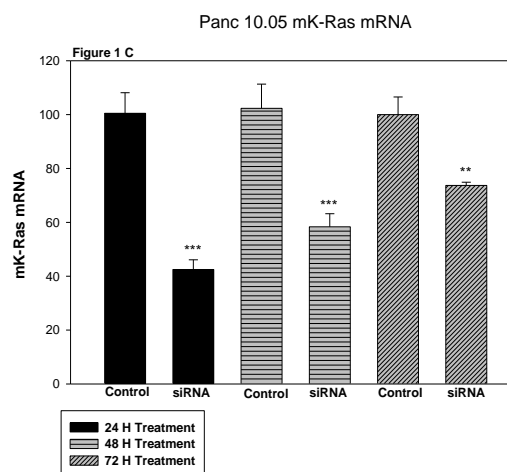
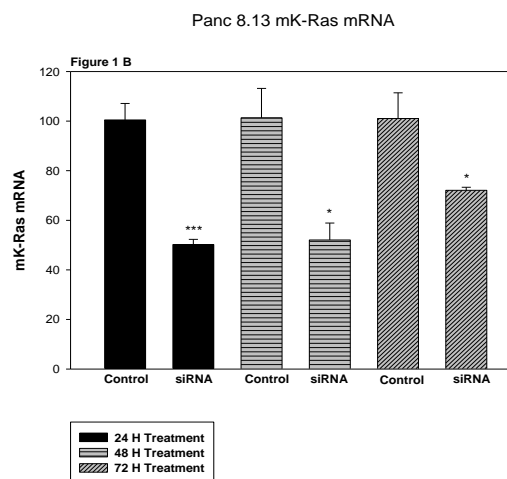
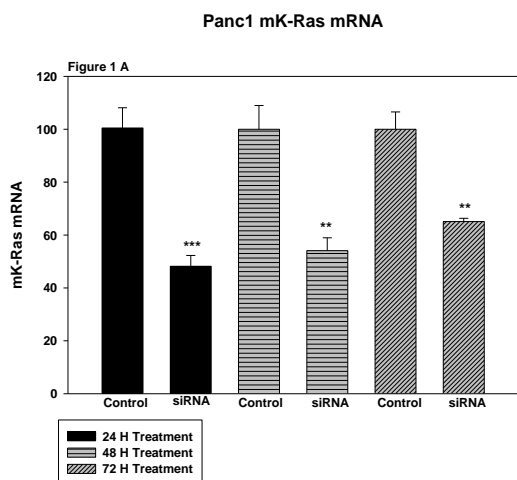


Fig. 1. *mK-Ras* mRNA in (A) Panc1, (B) Panc 8.13, (C) and Panc 10.05, cells and (D) *wK-Ras* in BXPC3 cells. Cells were seeded in 6 well plates at 5×10^5 cells per well. Twenty-four hours later, cells were washed (PBS) and treated as follows (in triplicate): $2\mu\text{g/well}$ scrambled siRNA or $2\mu\text{g/well}$ *mK-Ras* siRNA. Cells were then incubated for 6 h with treatments with $2\mu\text{l/well}$ Lipofectamine in 2 ml of

Optimem media. Cells were then washed again (PBS) and incubated in complete medium for 24, 48, or 72 h. After incubation, cells were harvested and stored in Trizol at -80°C . RNA isolation and purification and cDNA synthesis were then performed. Finally, qPCR was conducted on cDNA samples. Error bars represent standard error of the mean. * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$ compared to scrambled siRNA-treated controls at each time-point. Statistical analysis was conducted using t -Tests.

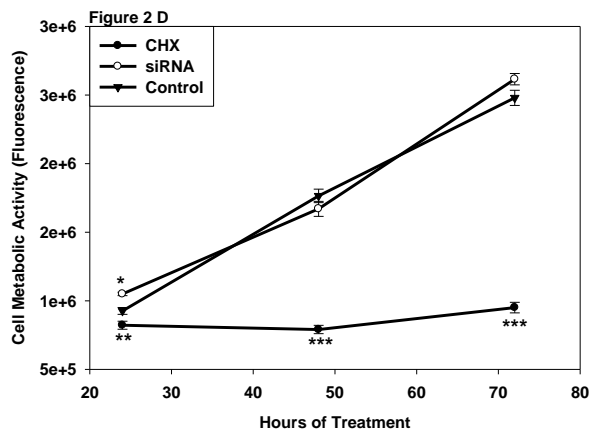
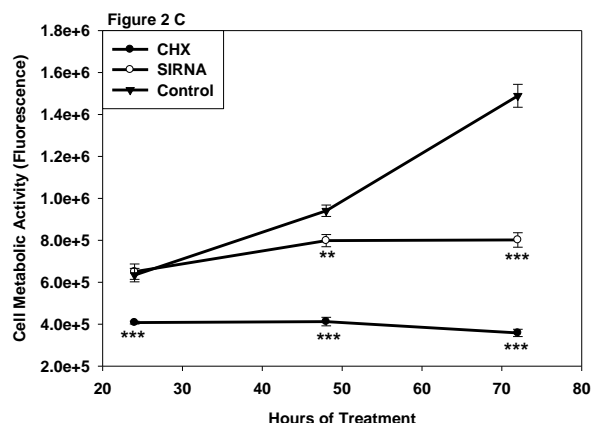
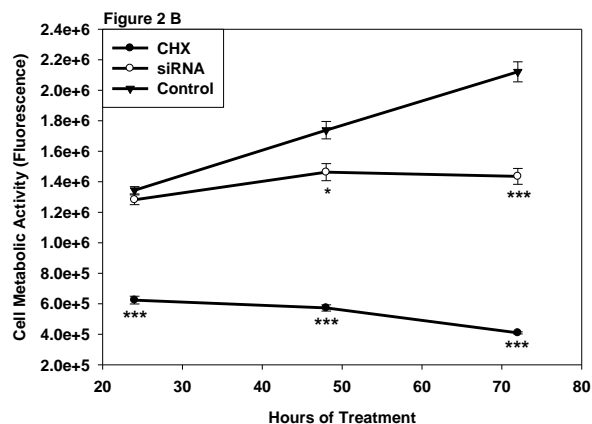
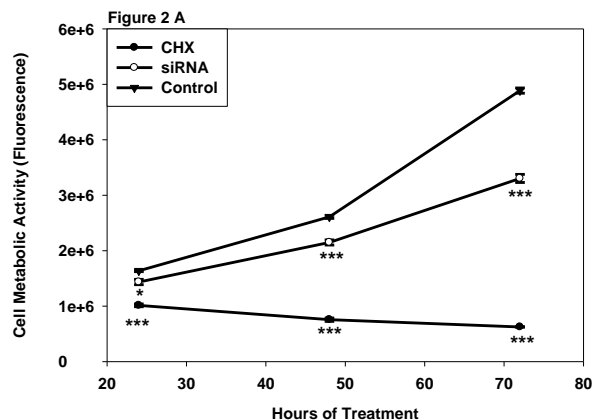


Figure 2. Cellular metabolic activity in (A) Panc 1, (B) Panc 8.13 (C) Panc 10.05 cells, and (D) BXPC3 cells. Cells were seeded into opaque 96 well plates at 1.2×10^4 cells per well. Twenty hours later, cells were washed (PBS) and treated as follows (in quadruplicate): control scrambled siRNA ($1 \mu\text{g/ml}$), *mK-Ras* siRNA ($1 \mu\text{g/ml}$), or CHX ($100 \mu\text{g/ml}$) in DMEM or RPMI 1640. Cells were then incubated for 4, 8, 12, 16, or 20 h with treatments. Subsequently, 20 μl of CellTiter-Blue reagent was added to each well and incubated at 37°C for 4 h. Fluorescence signals (excitation wavelength, 555 nm and an emission wavelength, 590 nm) were quantified using a multilabel plate reader (WALLAC 1420, VICTOR 2). Error bars represent standard error of the mean. * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$ compared to controls at each time-point. Statistical analysis was conducted using t -Tests.