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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Targeting telomerase RNA (hTR) for degradation in breast cancer cells using antisense oligonuclotides has demonstrated a high level of cytotoxicity in vitro and in vivo. Cell death is rapid and specific to cells expressing telomerase. The antisense molecules used in this approach, however, have a number of drawbacks the most critical of which is the relative instability of the molecules which would be an important negative issue for systemic treatment of the disease. What these studies have successfully achieved is that they have provided the proof-of-principle that degradation of hTR triggers apoptosis in cancer cells. Recently we have investigated the use of small interfering RNA molecules targeting telomerase and demonstrated that they can cause complete degradation of the target hTR within a 2 day period and that this 'knockdown' can be sustained for up to 6 days. Scrambled siRNAs do not have any effect and the targeting siRNA does not affect normal cells which do not express telomerase. Preliminary studies suggest that siRNAs also cause extensive cell death over a 4 day period but have the advantage of doing this with only a single treatment. Because of the specificity of the siRNA as well as their stability we are transitioning the telomerase targeting project towards using siRNA molecules as a novel therapy for breast cancer.

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INTRODUCTION

Telomerase is the riboprotein enzyme complex which prevents the ends of chromosomes from shortening below a critical length in cancer cells. This enzyme is normally not expressed in the majority of human cells after an early point in embryonic development but is reactivated in the vast majority (95%) of highly malignant cancer cells. It is thought to be an essential requirement for the maintenance of cell viability in the cancer cells which express it. We have investigated whether inactivating telomerase in cancer cells using an antisense oligonucleotide approach targeting the RNA component of the enzyme will result in cell death. The oligonucleotides used carry a 2-5A moiety attached to the antisense molecule. 2-5A activates endogenous RNAseL which is normally found as an inactive monomer in the cytoplasm in most cells. In the presence of 2-5A the monomer dimerizes and become a potent RNAse. Thus, the antisense molecule targets a specific RNA and the recruitment of RNAseL then selectively degrades the target. The overall aim of the project, therefore, is to determine whether inactivating telomerase can be developed as a viable form of anti cancer therapy for breast tumors. The initial series of experiments were designed to establish the conditions of treatment which will produce effective cell killing.

BODY

The project to investigate targeting the RNA component of human telomerase in breast cancer as a novel therapy was initiated in September 1998 and the first two reports were submitted and accepted. In November 2000, I transferred my research group to Roswell Park Cancer Institute. As of September 2001 this grant was not yet transferred and so I was not able to resume the work on schedule as originally proposed. This grant was finally transferred on March 1st 2002 and so the report provided here covers the six months from 03/02-08/31.

To summarize the work so far, we have clearly demonstrated that the 2-5A-anti-hTR oligonucleotide H1 could induce apoptosis in all breast cancer cell lines tested. A scrambled oligonucleotide did not produce this response and neither did the oligo which carried a defective 2-5A moiety. To determine the specificity of the target sequence in the hTR RNA we designed 2-5A antisense from other regions throughout the molecule and found that the majority did not produce the apoptotic response. Since we designed the original oligo against the most open region of the hTR RNA (figure 1) we interpret these results to mean that accessibility of the antisense was determining the specificity.

Despite the very strong biological indication that targeting the RNA component of the human telomerase enzyme results in rapid and almost complete death of breast cancer cells in vitro and reduces the growth of tumors in the flanks of nude mice, we have repeatedly been unable to publish these results in high quality journals because of the criticism that we have not proven a mechanism behind the observed cell death. This has been a tricky technique and requires demonstrating that indeed the 2-5A anti-hTR specifically cleaves the hTR molecule. The main problem with this approach has been that the induction of apoptosis is not an all-or-nothing event and cells die over a 5-6 day period which is concomitant with the daily addition of the antisense molecule. Thus, although partial degradation of the target can be demonstrated, for some reason not all cells are equally affected in the heterogeneous culture, and so at any given time during the treatment there are always cells which are still expressing the target. This has been a frustrating aspect of the research since we clearly have an important biological effect but no clear mechanism. The same problem has also been repeatedly demonstrated using the apoptosis assays. Flow sorting of cells during treatment with the 2-5A anti-hTR oligo results in a clear demonstration of the increasing commitment of cells to apoptosis until they finally die out. However, since not all cells are responding to the treatment simultaneously we cannot easily quantify biochemical parameters associated with apoptosis such as annexin 5 release or cleavage of caspases. The same is true for the function of telomerase using the TRAP assay where, for the most part, even if there are 10% of cells expressing telomerase at any one time the assay is so sensitive that activity is demonstrable although reduced.

To establish whether there was a cell-cycle specific stage at which cells became susceptible to the 2-5A anti-hTR treatment we undertook cell cycle assays during the treatment period. There was apparently no change in the passage of the cells through the cell cycle during the treatment arguing against a susceptible phase which led to apoptosis. Synchronizing cells using blocking agents is difficult in cancer cell lines where the cell cycle is so disregulated. We have, however, achieved 60% sychronization using G1 arresting agents but, when these cells are released from the block in the presence of 2-5A anti-hTR, cell death occurred at the same rate seen in the parental culture. This observation argues that the response to 2-5A anti-hTR is not dependent on the stage of the cell cycle. We are still unable, therefore, to account for why different cells in a given culture are responding differently to the same treatment although we expect that it is a consequence of the short half-life of the oligo and the differential uptake using the lipofectamine approach that creates different intracellular concentrations in different cells.

We have, therefore, spent a lot of effort in trying to determine why the cells are dying which has been frustratingly unproductive over the past 6 months.

Formal demonstration of the mechnanism of 2-5A anti-hTR action has been difficult. It is also been a problem in predicting the pathways that lead to apoptosis. We anticipate that disrupting the telomerase function may expose DNA damage resulting from incomplete replication of the ends of chromosomes. However, since the cell lines we are using are p53 deficient it is clear that the apoptosis pathway is p53 independent. Although the existence of such pathways have been suggested from data from many different systems no specific pathways has been described. It is also of interest that the T47D cells are deficient in caspase 3 activity which is the major effector of apotosis and so the mechanism in these cells presumably involves other caspases. The other approach we have taken recently to use Affymetrix GeneChip experiments to survey gene expression change in cells treated with the 2-5A anti-hTR versus the same cells treated with the mismatch oligonucleotide, which does not produce a biological consequence of apoptosis. We treated cells in the standard way using 2-5A anti-hTR and then prepared RNA from cells after 8 hours and 24 hours and compared the gene expression profile using the Affymetrix HUGFL Chips which carry 6800 genes with that from cells treated with lipofectamine alone in the first instance.

In this experiment we clearly saw gene changes which were present in the 8 hour treatment as well as in the 24 hour treatment, we also saw gene changes that were present in the 8 hour treatment but which returned to normal after 24 hours, as well as changes in the 24 hour treatment but which were not seen after only 8 hours. From the list of gene expression changes, a sublist was compiled based on subjective interest level (i.e. possible functional significance) and examination of the data points on the actual genechip. From these genes, 10 were selected for verification of the results using real time quantitative PCR (RTQ). These genes included 3 genes associated with apoptosis: NIP, TRAIL and IPL; several growth factor related genes: BAP (Btk assoc'd tyr kinase); IGFBP5 and VEGF; a transcription factor: ID; an antiproliferative gene: BTG; and two genes for membrane bound proteins with unknown relevance: M6A and tissue factor (TF). Table 1 lists the fold change values obtained for each gene for a given cDNA sample. While the observed fold change did not always match the predicted GeneChip value exactly, the trends were typically correct. The exceptions were TRAIL at 8h and 24h and ID2, VEGF at 24h. See Figure 2. Because of the exquisite sensitivity and logarithmic nature of the assay, fold change values normally vary between +/-2 fold of the observed value which could explain some of the variability. For 2 genes: TF and IGFBP5, the RTQ fold change values were significantly greater compared to those predicted by Affymetrix GeneChip, e.g. 10-50X than expected. See Figure 3. These elevated values were found reproducibly over several experiments. It is not clear whether these represent variation between different cDNA samples and/or genechip limitations/effects.

To determine whether any of the observed gene changes was linked to the induction of apoptosis in the treated cells, we next compared cDNAs from mismatch, antisense-treated cells with the same set of 10 gene primers. As shown in Figure 4, there were only small differences, if any, between the specific antisense and mismatch treated controls, typically within the 2-fold normal variation. Three genes, TF, IPL (implicated in fas pathways) and IGFBP5 had a reproducible induction of expression compared to mismatch controls. Tissue factor (TF) was not examined further since it was known to be IFN induced (a possible consequence of the 2-5A moiety). For IPL and IGFBP5, multiple cell lines were then tested to see if a similar induction of expression was observed correlating with the similar apoptotic responses. Figure 5 shows the results for these two genes on MDA468, U373 and HK cell lines treated for 24 hrs with either

the antisense or mismatch control oligonucleotide-2'5'A hybrid. The results showed that, there was no consistent induction of expression for either gene in the 3 different tumor cell lines examined even though they all responded to treatment by undergoing apoptosis.

To more quickly identify those genes which were distinctly induced/repressed in response to antisense hTR but not in the mismatch control, another GeneChip experiment was performed using only antisense hTR treated and mismatch hTR treated (at 8h post treated) as the RNA sources. Only 92 genes showed increased expression between the two of which only 15 had a sort score of $\geq$0.5 (the standard significance cut-off commonly use in these experiments). These 92 genes were then matched to the list of genes previously identified in the treated vs. untreated (lipofectamine) comparison described above. Only 10 genes were changed in BOTH experimental comparisons, e.g., treated vs. untreated AND treated vs. mismatch. After looking at the individual tiles on the chip, 4 of these 10 were eliminated due to artifact (dust, scratches) and 2 due to lack of signal leaving: BDP (Ca++ regulator), EPCR (centrosome assoc'd), GGF (heregulin/neu) and RAB8 (GTPase).

Real time quantitative PCR was performed for 6 genes on one or more of the cell lines (MDA468, MCF 7, HK, U373) comparing treated and mismatch treated at various time points. Table 2 contains the calculated fold change values. Unfortunately, no consistent change was detected in all 3 cell lines relative to the mismatch. The inability to identify differences in gene expression between the authentic antisense treated and mismatch treated may have been partly due to the early time point utilized. However, we feel that the gene expression changes are probably reflecting a stress response to the presence of high levels of oligionuclotides inside the cell rather than identifying pathways involved in the response to hTR poisoning. The other persistent problem is again that only small percentages of cells are induced to undergo apoptosis and so the gene expression changes that may be occurring in these cells is masked by the gene expression levels seen in the majority of the cells. At this point we feel that, although targeting telomerase offers great promise in the treatment of breast cancer, we need to explore other approaches of targeting hTR which are more robust and controllable.

Another limitation of these GeneChip experiments was that they only carry 7000 genes so it is possible that we simply missed the critical players in the response to telomerase damage. There are now chips available which carry a more comprehensive set of human genes and we could consider repeating these experiments but my feeling has been that the primary confounding issue is the heterogeneous response of the breast cancer cells to 2-5A anti-hTR treatment which is an issue we should address before committing to even more extensive analysis in a less than understandable system.

Clearly, we have induced a a profound biological response by targeting the RNA component of telomerase which should lead to a therapeutic option if a better understanding of the mechanism and a more controllable system can be established. With this in mind, and because of new developments in the biological sciences over the past 18 months, we have begun to investigate the possibility of using RNA-interference approaches to target telomerase as an alternative approach.

RNA inference (RNAi) is a phenomenon where specific double-stranded RNA molecules can selectively bind to the homologous target RNA and illicit degradation of the target. In this process an endogenous Rnase cleaves the double stranded RNA into small single stranded, small, interfering RNAs (siRNA) which mediate the degradation of the target, thus eliminating the function of that gene in the cells (Bernstein et al., 2001; Grishok et al, 2001; Knight and Bass, 2001).

We have designed an siRNA directed against the telomerase RNA template (hTR) from nucleotides 76-94 (the same ones used for the 2-5A targeting) in an effort to determine the effects of eliminating the RNA component required for telomerase activity (see figure 6). The siRNA duplex was chemically synthesized by Dharmacon Research Inc. Transient transfections were performed using either the complemetary siRNA (hTR) or an siRNA that contains several mismatches (mismatch) using the MCF7 cell line, which possesses an elevated endogenous level of telomerase activity.

The transfection protocol was: Cells were seeded on a 6-well plates 24 hours prior to transfection in DMEM containing 10% FBS without antibiotics. Transfections were done at approximately 70% confluency. Two amounts of siRNA (for timecourse expt) were utilized, 80 pmoles or 240 pmoles as per recommended protocol (Dharmacon). SiRNA was incubated in Opti-mem (200ul) for approximately 10 minutes. Oligofectamine (6 ul) was incubated in Opti-mem (54 ul) for approximately 10 minutes. SiRNA and oligofectamine tubes were mixed gently and incubated (at RT) for 25 minutes. Following incubation, an additional 150 ul of Opti-mem was added. Cells were washed with PBS 1x after removal of media, and replaced with siRNA/oligofectamine mixture and placed in 37° incubator. Approximately 8 hours later, 1ml of DMEM (+10%FBS) was added to each well. Cells were counted and harvested at 48-96 hours post-transfection.

The results from these pilot experiments have been very encouraging. Semi-quantitative analysis of cell survival suggests that while cells treated with a scrambled siRNA molecule show no change in growth rate, whereas cells treated with the siRNA targeting molecule cause an apporiximately 60% reduction in cell numbers over a 4 day period. A control cell line, MRC5 which does not express hTR or telomerase, was completely unaffected by the siRNA strongly suggesting that we are seeing a specific response to the treatment. We are currently performing apoptosis assays to determine whether this is the cause of death. Analysis of the hTR RNA in MCF-7 cells using RT-PCR demonstrated complete absence after 2 days (figure 7) and furthermore, that this loss of the transcript was maintained for several days only reappearing after 7 days following the single initial treatment (figure 8). Control PCR reactions performed simultaneously using primers designed against GAPDH showed no changes. Thus these experiments demonstrate that it is possible to 'knock-down' hTR transcripts in breast cancer cells lines and that this effect is stable over a 6 day period with only a single treatment. Furthermore, the consequence appears to be cell death. This approach has obvious advantages over the need to treat every day with oligonucleotides, especially since the mechanism of siRNA action has been well studied. Over the next months we will extend these studies but I feel that this approach will provides a better opportunity to develop a novel strategy for breast cancer therapy targeting telomerase.

Task 1: Completed
Task 2: Completed
Task 3: In progress
Task 4: Completed
Task 5: Completed
Task 6: Completed
Task 7: Completed
Task 8: Suspended due to the nuclease sensitivity of the oligos
Task 9: Not initiated
Task 10: Not initiated

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration that targeting telomerase in breast cancer cells is not cell cycle dependent

- Gene expression screening identifies only minimal changes when the cells are treated with the targeting oligonucleotide versus the inactive mismatch oligonucleotide.

- Demonstration that small interfering RNAs can target hTR effectively to eliminate the RNA in the total populaiton of cells.

- A single treatment of siRAN can eliminate the target hTR RNA for up to six days represetning a considerable improvement over the 2-5A antisense approach.

- SiRNA knockdown of the hTR target is stable over a 4 day period with only a single treatment. Mismatch siRNA has no effect on cancer cells.

- SiRNA against hTR causes cell death in cells expressing telomerase only; normal fibroblasts that do not express hTR are unaffected by the treatment.

REPORTABLE OUTCOMES

Kushner D, Paranjape J, Bandyopadhyay B, Cramer H, Leaman D, Silverman RH, **Cowell JK**. 2-5A antisense directed against telomerase RNA produces apoptosis in ovarian cancer cells. Gynol. Oncol. 76; 183-192, 2000.

CONCLUSIONS

The major advance in our attempts to target the RNA component of human telomerase is in developing small interfering RNA molecules which can specifically inactivate it. The degradation of hTR results from only a single treatment with siRNA and sustains degradation over a 4-6 day period. During the 40 day period approximately 60% of the cells are killed following the single treatment. Cells which do not express telomerase are unaffected by the treatment as are cells treated with a scrambled siRNA. These results demonstrate specificity for the target. It is clear that siRNA approaches offer a much more stable way or killing cancer cells than any of the antisense approaches and operate through a well established mechanism. Future studies will concentrate on refining the treatment protocol and extending these studies into in vivo models.

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2. Grishok, A. Pasquinelli, A.E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D.L., Fire, A., Ruvkin, G., and Mello, C.C. (2001) Cell 106:23-34.

3. Knight, S.W. and Bass, B.L. (2001) Science 2: 2269-2271.

APPENDICES

Tables 1, 2
Figures 1, 2, 3, 4, 5, 6, 7, 8

	Table 1: Affymetrix GeneChip Predictions vs. RTQ Values							
	Affy Predictions							
	8h (vs 0h)	24h (vs 0h)						
NIP	-2.7	-1.3						
BAP	-4.4	-1.9						
BTG	-1.9	3.4						
M6A	-3.6	-2.1						
TRAIL	-3.2	-3.1						
IPL	3.2	2						
ID2	-4.2	-2.2						
VEGF	-6.1	-1.7						
TF	5.7	2.7						
IGFBP5	-8.7	-1.5						
	RTQ Values							
	8h	24h	48h	72h	24hMM	48hMM		
NIP	-3.4,-2.0	-1.6,1.3	1.3	nd	2	1.3		
BAP	-4.5, -2.3	-2.8, -1.5	1.0, 1.2	1	1.6	1.3		
BTG	-2.0, -1.7	1.0, 1.0	3.7, 3.6	2.8	5.6	1.5		
M6A	-5.1, -2.4	-4.8, -2.0	-3.9, 1.6	-3.3	1.8	1.2		
TRAIL	1.7	3	1	nd	-4.0	-2.0		
IPL	1.6, 5.9, 15.8	-1.5, 5.3, 9.3	-1.8, 1.8, 3.8	6.3	1.8, 4.5	2.4		
ID2	-2.2, -2.5	1.0, 1.0	1.0, 1.0	1.7	3	1		
VEGF	-1.5, 1.3	3.4	5.7	nd	8	5.7		
TF	240, 117, 266	27, 30, 63	-2.7, -2.3, 1.0	-2.4	1.0, 1.6	-2.2, 1.0		
IGFBP5	-254, -223	-56, -68	-2.8	nd	-7.0	-2.3		
	U373/24H	U373/24MM		MDA/24H	MDA/24MM		HK/24H	HK/24MM
IPL	9.3	4.6		1.5	1.2		2.6	4.9
IGFBP5	-100.00	-12.80		-3.70	-12.50		-1.90	-1.80

Table 2: Fold Change in Gene Expression for Mismatch Identified Genes											
	(fractional number indicates decrease relative to untreated cells)										
	U373										
	8h	24h	48h	8hMM	24hMM	48hMM		1h	2h	4h	6h
BDP	0.75, 1.58	0.73	0.98	0.68, 0.99	1	1.03		0.69	0.86	0.86	0.98
EPCR	0.60, 1.13	0.66	1.24	0.81, 1.20	0.71	0.98		1.19	1.24	1.14	1.22
GGF	5.39, 1.07, 8.94	3.57, 1.92	1.34	4.31, 0.74, 5.86	0.95, 1.07	0.38		0.32	0.22	0.53	0.52
RAB8	0.47	0.29		1.46	0.54						
ZPF	0.37	0.3		5.02	1.25						
TRF	0.51	0.64		0.55	1.06						
	MDA468										
	8h	24h	8hMM	24hMM		8h ratio	24h ratio				
GGF	abs	abs	abs	abs							
RAB8	0.88	0.95	0.74	0.79		1.19	1.20				
ZPF	0.85	1.81	0.94	1.51		0.90	1.20				
TRF	0.89	0.39	0.78	0.56		1.14	0.70				
	MCF7										
	24h	24hMM		ratio trt/mm							
GGF	abs	abs									
RAB8	0.8	0.34		2.35							
ZPF	0.69	1.44		0.48							
TRF	1.11	0.44		2.52							
	HK										
	24h	24hMM		ratio trt/mm							
GGF	25.05	6.23		4.02							
RAB8	1.55	2.21		0.70							
ZPF	2.48	1.9		1.31							
TRF	3.23	4.2		0.77							

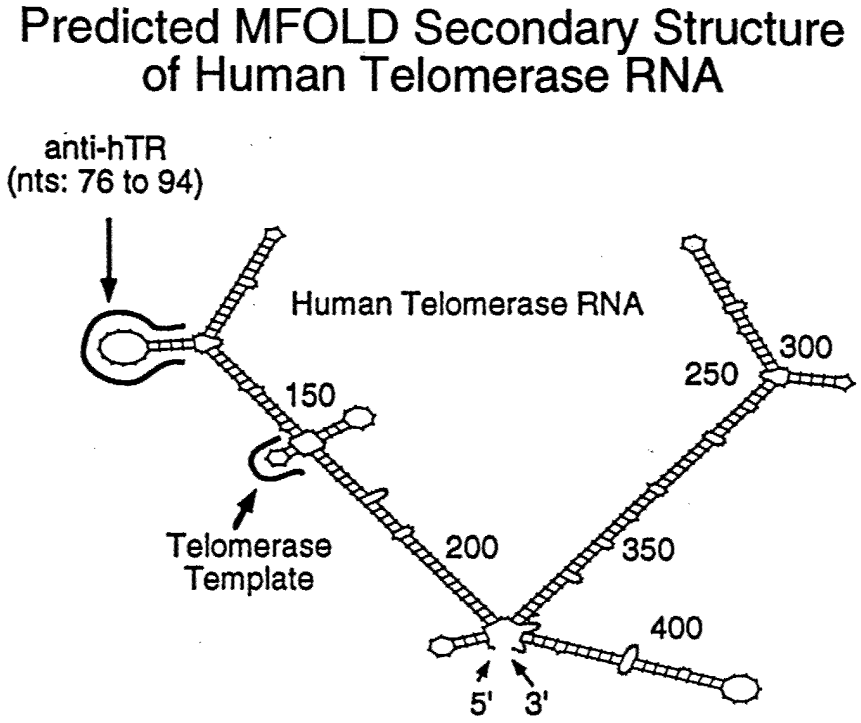

Predicted MFOLD Secondary Structure
of Human Telomerase RNA
anti-hTR
(nts: 76 to 94)
Human Telomerase RNA
150
250
300
Telomerase
Template
200
350
400
5'
3'

Figure 1

Figure 2: Affymetrix GeneChip Predicted Changes compared to RTQ Values

Figure 3: GeneChip Predictions vs RTQ Values for Gene Expression Changes

Figure 4: RTQ Treated vs Mismatch Controls

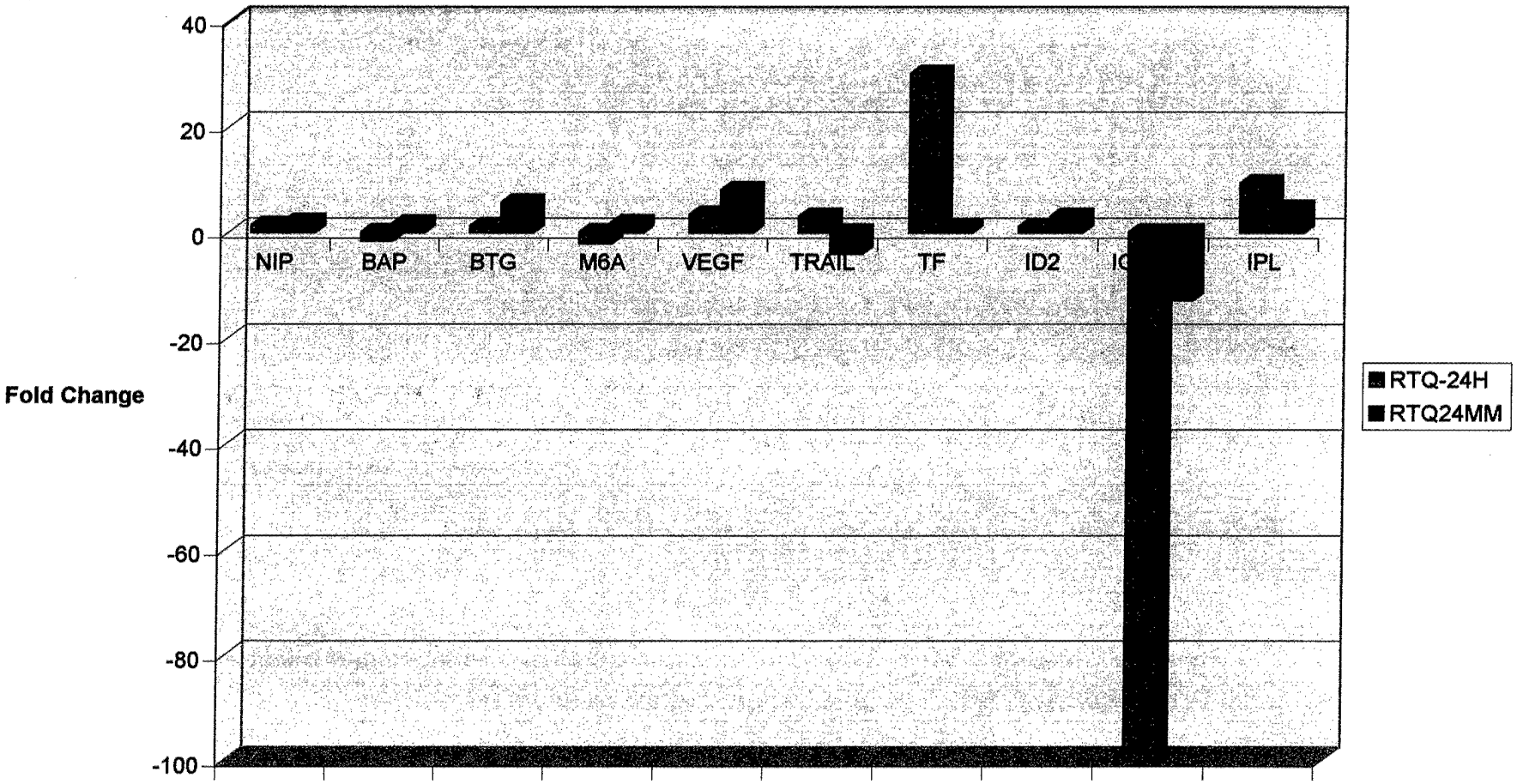
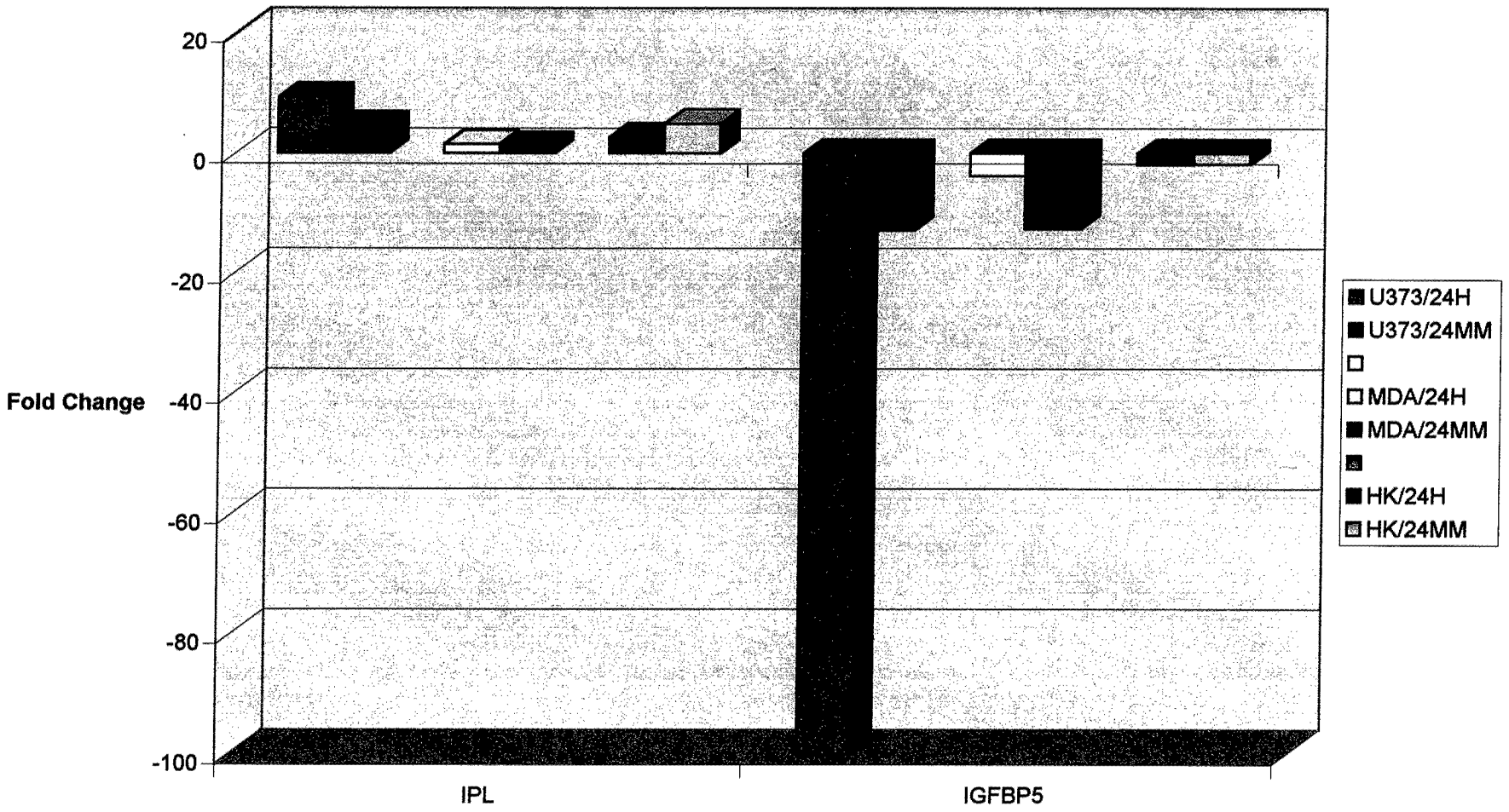


Figure 5: RTQ Results for Multiple Cell Types



siRNA Sequences and Target

siRNA duplex: GUG CUU UUG CUC CCC GCG CdTdT
dTdT CAC GAA AAC GAG GGG CGC G

mRNA target (5'-3'): GUG CUU UUG CUC CCC GCG C

siRNA scrambled duplex: GUG CUG UCG CUA CCA GCG CdTdT
dTdT CAC GAC AGC GAU GGU CGC G

Figure 6.

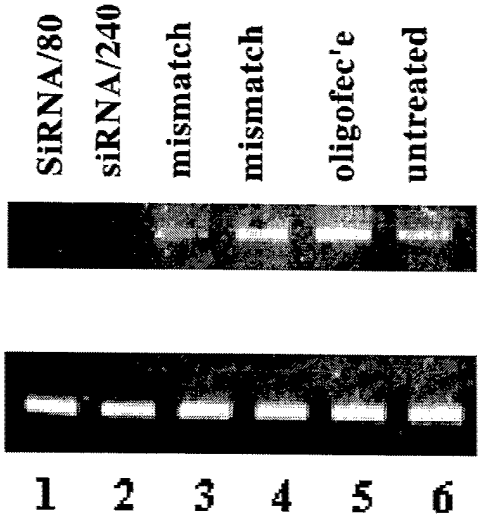


Figure 7: RT-PCR analysis of MCF7 cells treated with siRNAs at 2 different concentrations (80 and 240 picomoles). At both concentrations the siRNA completely eliminats hTR RNA after 2 days. The mismatch oligonuclotide has no effect at either concentration. treatment with oligofectamine alone also has no effect on hTR. These experiments strongly suggest the targeting siRNA is specific for hTR.

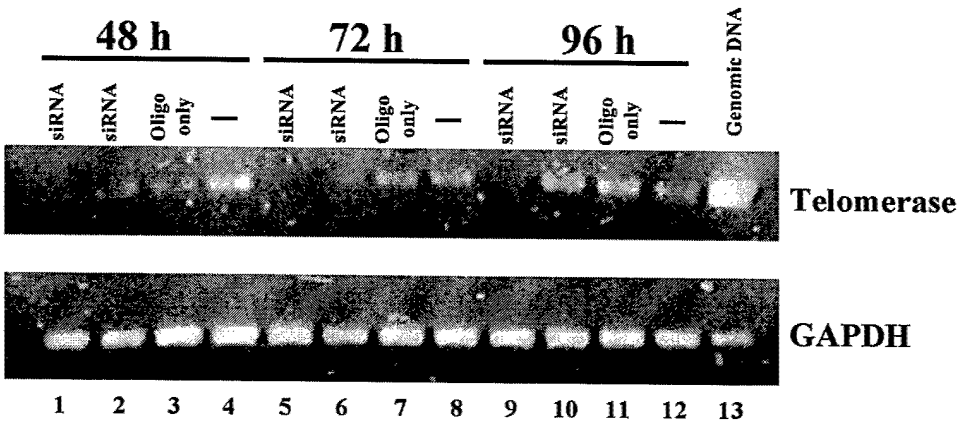


Figure 8: Analysis of the presence of hTR in MCF7 cells following a single treatment of siRNA against hTR. RT-PCR was performed on treated cells after 48, 73 and 96 hours. Two concentrations of siRNA was used in these experiments where the higher concentrations (right lane of the pair) were in fact less effective tahn the lower ones. this is a consistent observation in the field where too much siRNA is ineffective. Oligofectamine. the lipid carrier clearly has no effect on hTR.