Progress Report

Research projects funded by the Childhood Eye Cancer Trust

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Project title: Use of Aptamers to increase the efficacy of *HMGA2* targeted therapy in Retinoblastoma

Date awarded & value:	**GBP 47,297.52 for 2 years**

Vision Research Foundation:	**GBP 23,511.53 for 2 years**
Missouri University:	**GBP 23,784.81 for 2 years**

CHECT grant update

As we faced delivery issues with HMA2 siRNA in our earlier work, we adapted a different strategy for delivering HMGA2 siRNA using nucleolin aptamer and bispecific linker (Lai *et al*, 2014). We conjugated nucleolin aptamer and HMGA2 siRNA using a bispecific linker sulfo SMPB. For this conjugation the 5' and 3' end of nucleolin aptamer was modified with amine group and fluorescein respectively. The 5' end of HMGA2 siRNA was modified with thiol group and all 'C' and 'U' of sense strand of siRNA was 2'fluoro modified to increase the stability.

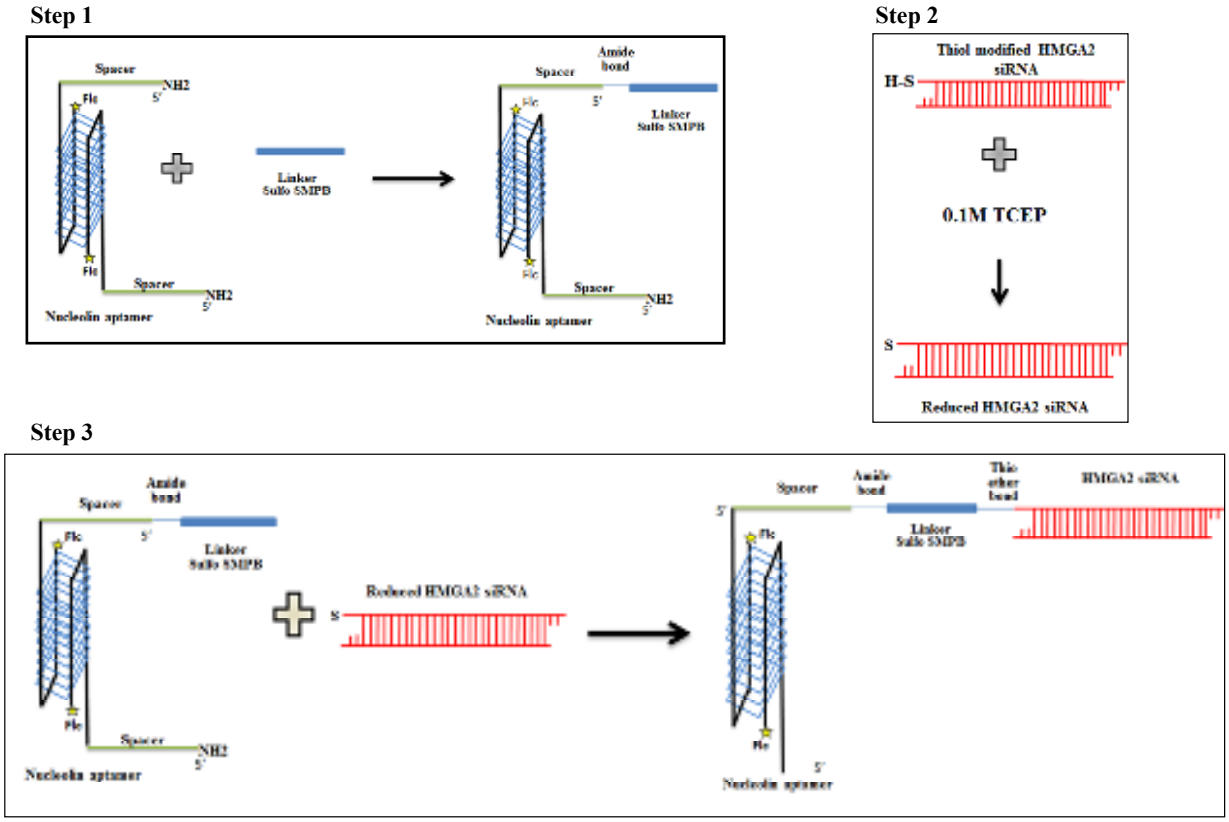

Figure 1: Schematic representation of conjugation of amine modified nucleolin aptamer and thiol modified HMGA2 siRNA using Sulfo SMPB linker.

Conjugation

The conjugation reaction was carried out at equimolar concentration of aptamer and siRNA with 8mM sulfo SMPB and 10mM TCEP. Briefly the aptamer was first incubated with sulfo SMPB at 37°C for 1 hour (Fig 1. step 1). Meanwhile the thiol modified siRNA was also activated with 10mM TCEP for 1 hour at 37°C (Fig 1. step 2). The aptamer and siRNA were purified using Princeton separations columns to remove the unreacted sulfo SMPB and TCEP. Then purified siRNA and aptamer were mixed together and incubated at 37°C for 2 hours (Fig 1. step 3). The conjugation was checked by loading in 10% non-denaturing PAGE. The aptamer and siRNA were conjugated with 50% efficiency which required purification to obtain the conjugate (Fig 2).

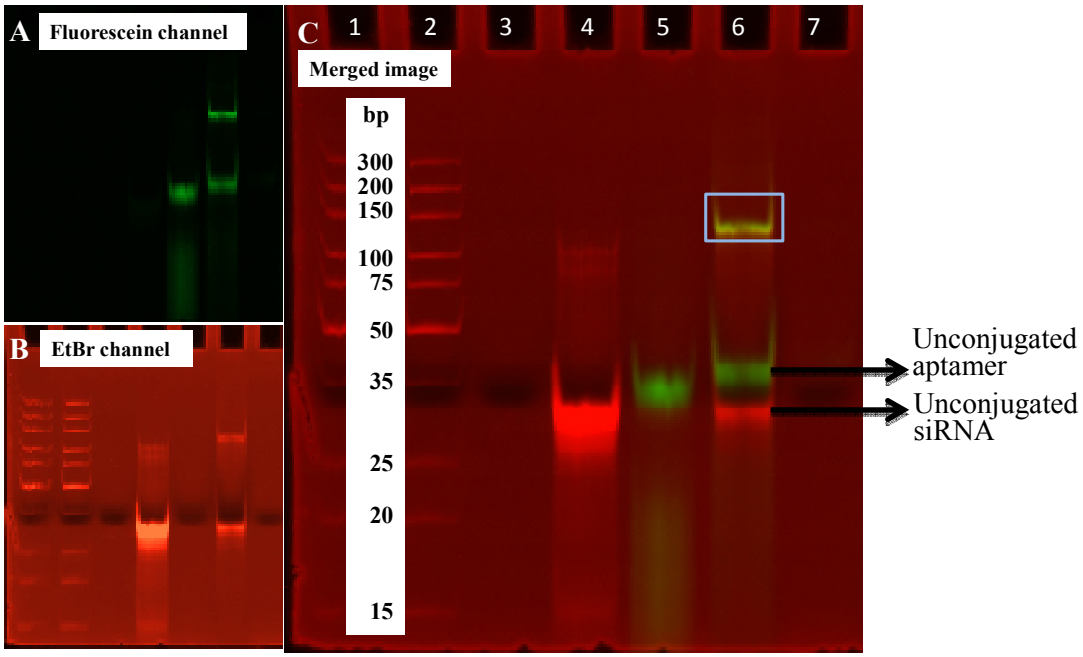

Figure 2: Conjugation of nucleolin aptamer and HMGA2 siRNA using bispecific linkersulfo SMPB. **A.** Fluorescein channel showing the presence of flc tagged aptamer in lane 5&6. **B.** Ethidium bromide channel showing EtBr staining of siRNA in lane 4&6. **C.**Merged image of fluorescein and EtBr channel. Conjugate band was observed in both fluorescein and EtBr channel showing the successful conjugation of aptamer and siRNA.

Lane 1&2 ultra-low range DNA ladder, lane 4 siRNA alone, lane 5 aptamer alone, lane 6 conjugate, lane 3&7 empty.

To remove the unreacted siRNA and aptamer, PAGE gel purification of the conjugate was performed. The wells were combined for PAGE purification of the conjugate (Fig 3).

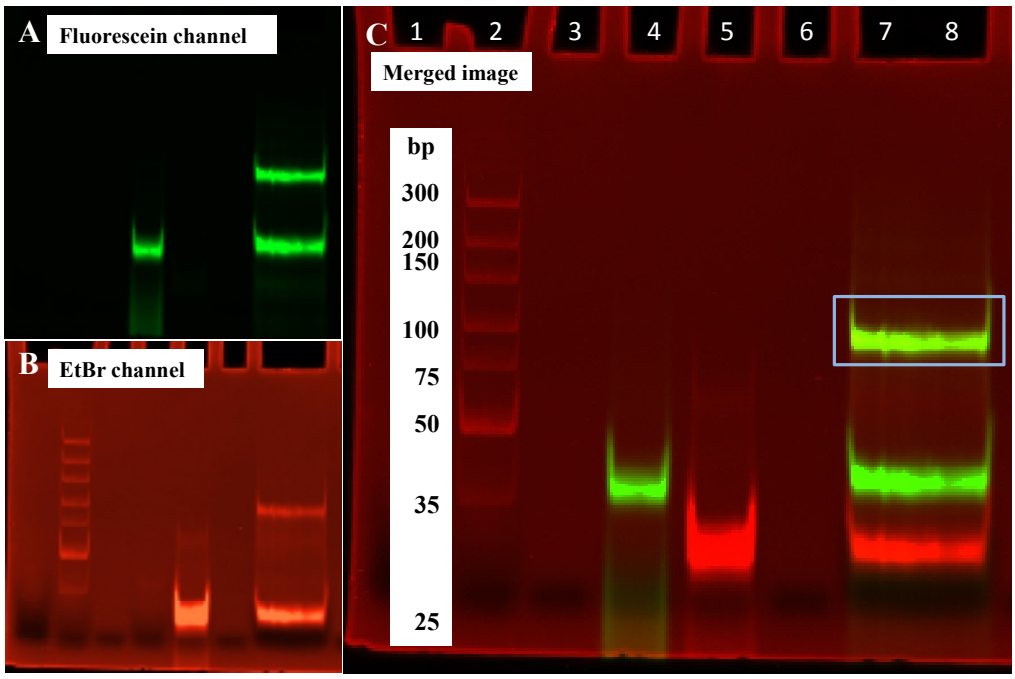

Figure 3: Purification of conjugate using 10% non-denaturing PAGE. **A.** Fluorescein channel showing the presence of flc tagged aptamer in lane 4 and 7&8 **B.** Ethidium bromide channel showing EtBr staining of siRNA in lane 5 and 7&8. **C.** Merged image of fluorescein and EtBr channel.

Lane 2 ultra-low range DNA ladder, lane 4aptamer alone, lane 5 siRNA alone, lane 7&8 conjugate, lane 1,3&6 empty.

The conjugate band was excised from the gel and was subjected to modified crush and soak method (Chen &Ruffner, 1996). The gel slice was crushed with 0.1M sodium acetate pH 6.0. The mixture was then incubated in boiling water bath for 5 mins followed by freezing at -80°C for 5 mins. Then it was spun down and the supernatant containing conjugate was collected. To remove sodium acetate, the conjugate was passed through Princeton columns and loaded onto gel to check the stability of the conjugate after purification. The conjugate remained intact after purification (Fig 4) and was used for further studies. The concentration of the conjugate was quantified using Nano drop spectrophotometer and the molar concentration was calculated based on the molecular weight of the siRNA.

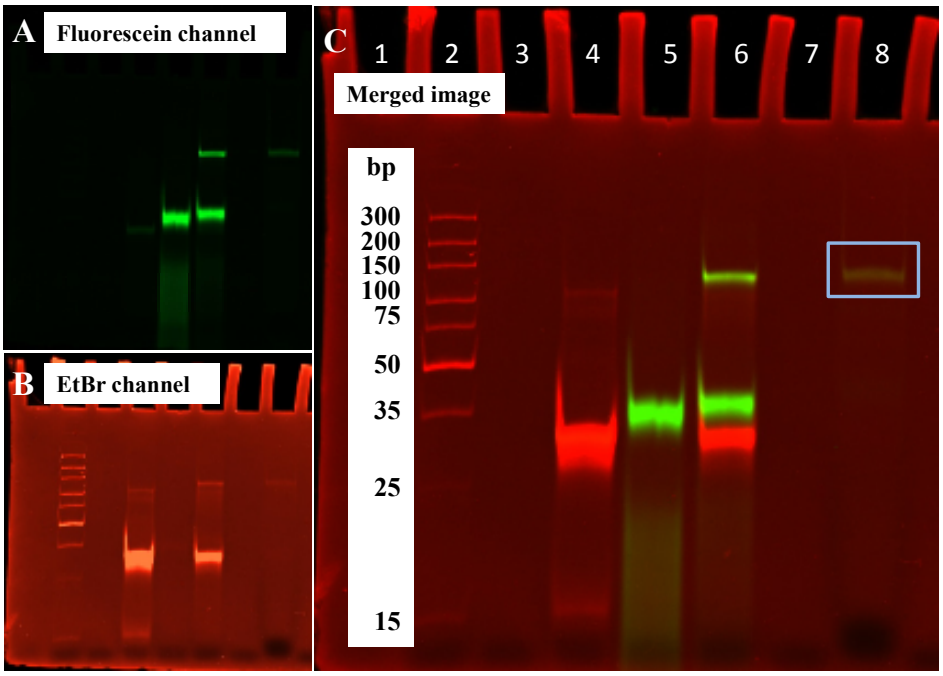

Figure 4: 10% non-denaturing PAGE loaded with conjugate. **A.** Fluorescein channel showing the presence of flc tagged aptamer in lane 5, 6&8**B.** Ethidium bromide channel showing EtBr staining of siRNA in lane 4, 6 &8. **C.** Merged image of fluorescein and EtBrchannel showing the purified conjugate similar to the unpurified one.

Lane 2 ultra-low range DNA ladder, lane 4siRNA alone, lane 5 aptamer alone, lane 6 unpurified conjugate, lane 8purified conjugate, lane 1,3&7 empty.

Cytotoxicity Assays

To study the cytotoxic effect and IC50 of the conjugate MTT assay was performed in WERI-Rb1(non-metastatic RB) and NCC-RbC51 (liver metastatic RB) cell lines. 8000 cells/well were plated a day before treatment in PLL coated 96 well plates. Conjugate of 50nM and 100nM concentration were used for the treatment. For better understanding of the effect of conjugate, aptamer alone and siRNA alone were also included. siRNA was transfected at 50 and 100nM concentration usingXtreme gene siRNA transfection reagent. Aptamer alone as well as conjugate was added directly to the cells in incomplete medium. After 4h of treatment complete media was added. After 48 hours media was removed and 100µl of incomplete media containing MTT was added and incubated at 37°C for 4h. After incubation the media was completely removed and

100µl of DMSO was added and the OD was read at 570nm. Experiments were carried out in triplicates in three sets.

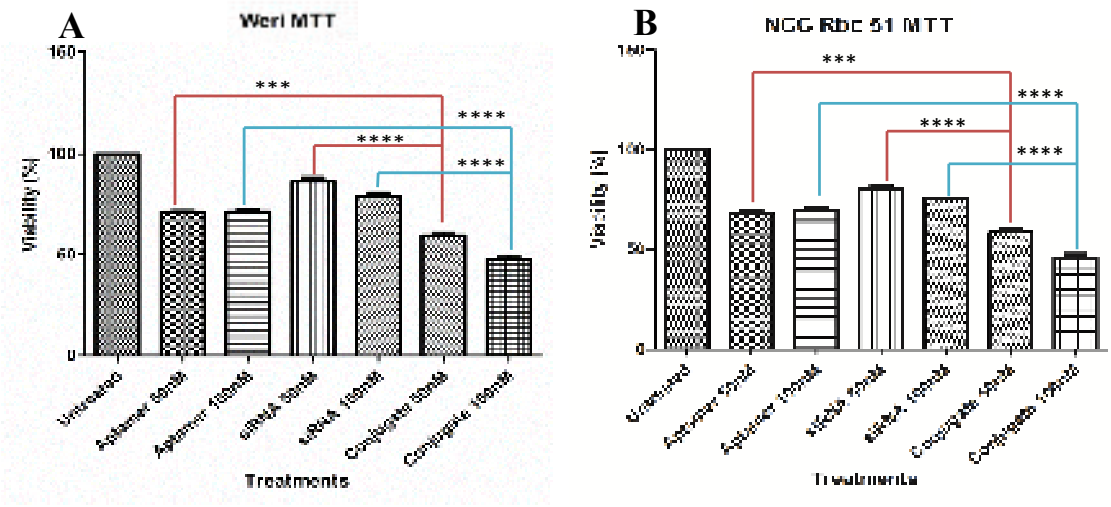

Figure 5: Cytotoxic effect of the conjugate on cell lines (MTT assay). A. Percentage viability of cells after 48h treatment in WERI-Rb1 cell line. B. Percentage viability of cells after 48h of treatment in NCCRbC51 cell line. P<0.0001

Number of families	1	WERI-Rb1			1	NCCRbC51		
Number of comparisons per family	21				21			
Alpha	0.05				0.05			
Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. siRNA 50nM	13.73	7.449 to 20.01	Yes	****	19.64	14.01 to 25.27	Yes	****
Control vs. siRNA 100nM	21.25	14.96 to 27.53	Yes	****	24.65	19.02 to 30.28	Yes	****
Control vs. Aptamer 50nM	29.02	22.74 to 35.30	Yes	****	32.22	26.59 to 37.85	Yes	****
Control vs. Aptamer 100nM	28.95	22.67 to 35.23	Yes	****	30.27	24.64 to 35.90	Yes	****
Control vs. Conjugate 50nM	40.69	34.41 to 46.97	Yes	****	41.58	35.95 to 47.21	Yes	****
Control vs. Conjugate 100nM	52.57	46.29 to 58.85	Yes	****	54.17	48.54 to 59.80	Yes	****
siRNA 50nM vs. siRNA 100nM	7.515	1.234 to 13.80	Yes	*	5.015	-0.6151 to 10.65	No	ns
siRNA 50nM vs. Aptamer 50nM	15.29	9.008 to 21.57	Yes	****	12.58	6.953 to 18.21	Yes	****
siRNA 50nM vs. Aptamer 100nM	15.22	8.937 to 21.50	Yes	****	10.63	5.005 to 16.26	Yes	***
siRNA 50nM vs. Conjugate 50nM	26.96	20.68 to 33.24	Yes	****	21.94	16.31 to 27.57	Yes	****
siRNA 50nM vs. Conjugate 100nM	38.84	32.56 to 45.12	Yes	****	34.53	28.90 to 40.16	Yes	****
siRNA 100nM vs. Aptamer 50nM	7.774	1.493 to 14.06	Yes	*	7.568	1.938 to 13.20	Yes	**
siRNA 100nM vs. Aptamer 100nM	7.703	1.422 to 13.98	Yes	*	5.620	-0.01050 to 11.25	No	ns
siRNA 100nM vs. Conjugate 50nM	19.45	13.16 to 25.73	Yes	****	16.92	11.29 to 22.56	Yes	****
siRNA 100nM vs. Conjugate 100nM	31.33	25.05 to 37.61	Yes	****	29.52	23.89 to 35.15	Yes	****
Aptamer 50nM vs. Aptamer 100nM	-0.07127	-6.352 to 6.210	No	ns	-1.949	-7.579 to 3.682	No	ns
Aptamer 50nM vs. Conjugate 50nM	11.67	5.391 to 17.95	Yes	***	9.357	3.727 to 14.99	Yes	***
Aptamer 50nM vs. Conjugate 100nM	23.55	17.27 to 29.83	Yes	****	21.95	16.32 to 27.58	Yes	****
Aptamer 100nM vs. Conjugate 50nM	11.74	5.462 to 18.02	Yes	***	11.31	5.675 to 16.94	Yes	***
Aptamer 100nM vs. Conjugate 100nM	23.62	17.34 to 29.91	Yes	****	23.90	18.27 to 29.53	Yes	****
Conjugate 50nM vs. Conjugate 100nM	11.88	5.600 to 18.16	Yes	***	12.59	6.964 to 18.22	Yes	****

Table 1: Tukey's multiple comparisons for cytotoxic effect of treatments on WERI-Rb1 and NCCRbC51 cells

The conjugate showed significant decrease in viability in both WERIRb1 and NCCRbC51 cell lines compared to individual treatments of siRNA and aptamer (Fig 5). The effect of 50 and 100nM of aptamer was insignificant in both the cell lines, wherein the effect of 50 and 100nM of conjugate is greatly significant. Hence the effect of 100nM of conjugate was compared between the cell lines (Fig 6). This showed that there was no variation in the effect of conjugate on metastatic and non-metastatic cell line.

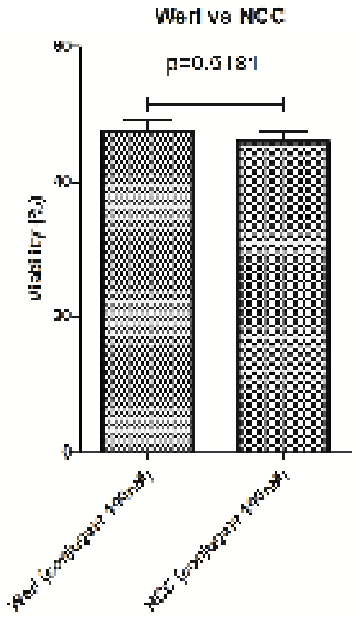

Figure 6: Comparison of effect of 100nM conjugate between metastatic and non-metastatic cell line. Percentage viability of cells from MTT assay for 100nM of the conjugate was compared for both the cell lines.

The supernatants of conjugate, aptamer and siRNA 48h treated WERI Rb1 cells were collected and LDH assay was performed using cytotoxicity detection kit (LDH) Roche. The assay was carried out as per manufacturer's protocol. Equal volume of supernatant and reagent was added and incubated at room temperature for 30mins. The OD was measured at 490nM. The LDH assay showed increased cytotoxicity in 100nM conjugate compared to 100nM siRNA (Fig 7).

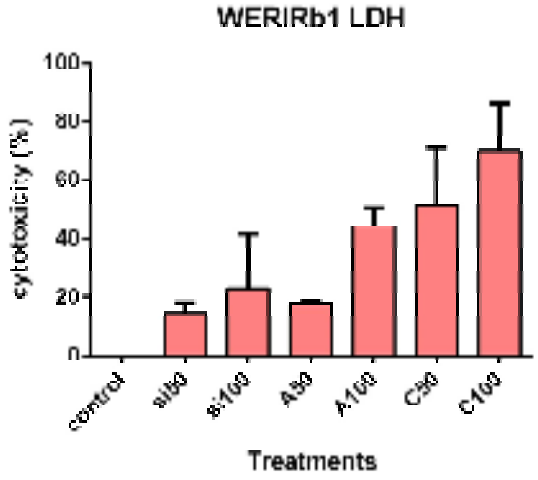

Figure 7: Percentage cytotoxicity measured by LDH assay in conjugate, siRNA and aptamer treated WERIRb1 cells.

Expression level of HMGA2

The HMGA2 mRNA expression level after treatment was also quantified using realtime PCR. Briefly 3X10^5 WERIRb1 cells were seeded 24h before treatment in 6 well plates. 50 and 100nM of aptamer, siRNA and conjugate were used for treatments. The siRNA was transfected using Xtreme gene transfection reagent wherein the aptamer and conjugate was added directly to cells in incomplete media. After 4 hours complete media was added. The cells were collected for RNA isolation 48h post treatment. About 1µg of RNA was reverse transcribed using Thermo verso cDNA kit. Real time PCR was performed for HMGA2 and β2microglobulin using SYBR green master mix. Relative quantification 2^{-ddct} showed downregulation in siRNA and conjugate treated cells. Relative fold change in mRNA levels of HMGA2 is shown in fig 8.This shows that the aptamer mediated siRNA internalization has been achieved leading to reduced expression of HMGA2.

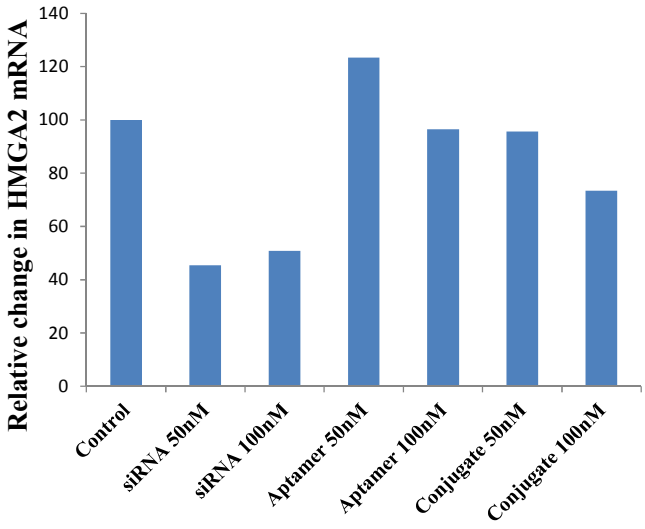

Figure 8: Relative fold change of HMGA2 mRNA in WERIRb1 cells upon treatment with HMGA2 siRNA, nucleolin aptamer and conjugate.

Conclusion

- The delivery of HMGA2 siRNA has been successfully achieved using nucleolin aptamer.
- Nucleolin aptamer being a functional aptamer was chosen for this delivery due to the advantage of being a shuttle protein. Results suggested that the Nucleolin aptamer and HMGA2 siRNA had synergistic effects in reducing the RB proliferation.
- Results from the real time PCR data on HMGA2 suggested that the combination of Nucleolin aptamer and HMGA2 siRNA may orchestrate HMGA2 dependent and HMGA2 independent pathways
- This nucleolin protein which is widely expressed on the surface of almost all cancers serves as a better platform for targeted delivery in cancerous conditions.

References:

Lai WY, Wang WY, Chang YC, Chang CJ, Yang PC, Peck K. Synergistic inhibitionof lung cancer cell invasion, tumor growth and angiogenesis using aptamer-siRNAchimeras. Biomaterials. 2014 Mar;35(9):2905-14. doi:10.1016/j.biomaterials.2013.12.054. Epub 2014 Jan 4. PubMed PMID: 24397988.

Chen Z, Ruffner DE. Modified crush-and-soak method for recoveringoligodeoxynucleotides from polyacrylamide gel.Biotechniques. 1996Nov;21(5):820-2. PubMed PMID: 8922620. Biotechniques 21: 820-822.

Publication from the grant

- Nalini V. Deepa P.R. Raguraman R. Khetan V. Reddy M.A. Krishnakumar S.Targeting HMGA2 in Retinoblastoma Cells in vitro Using the Aptamer Strategy. Ocul Oncol Pathol 2016;2:262-269 (DOI:10.1159/000447300)

Presentations:

- Abstract submitted to Asia Arvo Indian Eye Research Group Meeting to be held at Hyderabad in July 2016

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