MOLECULAR INSIGHTS AND OLIGONUCLEOTIDE BASED 
TARGETED GENE THERAPY AGAINST CANCER

by

NITHYA SUBRAMANIAN 
Master of Science (M. Sc Biotechnology)

Submitted in fulfilment of the requirements for the degree of 
Doctor of Philosophy
Registration No. 211640938

Deakin University, School of Medicine, Faculty of Health 

October, 2014
I am the author of the thesis entitled "Molecular Insights and Oligonucleotide Based Targeted Gene therapy against Cancers" submitted for the degree of Doctor of Philosophy.

This thesis may be made available for consultation, loan and limited copying in accordance with the Copyright Act 1968.

'I certify that I am the student named below and that the information provided in the form is correct'

Full Name: Ms. NITHYA SUBRAMANIAN
(Please Print)

Signed: [Signature Redacted by Library]

Date: 10/02/2015
DEAKIN UNIVERSITY
CANDIDATE DECLARATION

I certify the following about the thesis entitled “Molecular Insights and Oligonucleotide Based Targeted Gene therapy against Cancers” submitted for the degree of Doctor of Philosophy

a. I am the creator of all or part of the whole work(s) (including content and layout) and that where reference is made to the work of others, due acknowledgment is given.

b. The work(s) are not in any way a violation or infringement of any copyright, trademark, patent, or other rights whatsoever of any person.

c. That if the work(s) have been commissioned, sponsored or supported by any organisation, I have fulfilled all of the obligations required by such contract or agreement.

I also certify that any material in the thesis which has been accepted for a degree or diploma by any university or institution is identified in the text.

'I certify that I am the student named below and that the information provided in the form is correct'

Full Name: Ms. NITHYA SUBRAMANIAN
(Please Print)

Signed: [Signature Redacted by Library]

Date: 31/10/2014
Table of Contents

List of figures ...................................................................................................................................1
List of tables .......................................................................................................................................6
List of publications (during PhD; 2011-2014) ...............................................................................7
Research articles Published= 5 ........................................................................................................7
Research articles under review = 3 ..................................................................................................8
Research articles being submitted = 1 ........................................................................................ 8
Review article being prepared = 1 ...................................................................................................8
List of Publications (before PhD registration; 2007-2010) = 2 ................................................. 9
List of Conferences attended = 4 ................................................................................................... 9
List of abbreviations .........................................................................................................................10
Acknowledgements .........................................................................................................................13

Importance and novelty of the current study ..................................................................................1

Knowledge gaps which this study aims to fill .............................................................................1

Aim and objectives of the study .....................................................................................................2

1. To study the importance of Tiam1 and NCL in RB and aptamer based approach to target the cancer cells. ..................................................................................................................2
2. To fabricate and study the aptamerchimeric conjugates for therapeutic purpose ..................3
3. To target miRNA transcript and image cancer stem cell markers using aptamer and LNA modified aptamer constructs ..................................................................................................3
Hypotheses.......................................................................................................................... 4

ABSTRACT ........................................................................................................................... 6

PURPOSE .............................................................................................................................. 6

BACKGROUND ...................................................................................................................... 6

METHODOLOGY .................................................................................................................. 8

RESULTS .............................................................................................................................. 11

CONCLUSIONS .................................................................................................................... 15

CHAPTER 1 ......................................................................................................................... 17

REVIEW OF LITERATURE ................................................................................................. 17

1.1 CANCER .......................................................................................................................... 17

1.2 CURRENT TREATMENT MODALITIES AGAINST CANCER ..................................... 17

1.3 PAEDIATRIC CANCER .................................................................................................. 18

1.3.1 Retinoblastoma ......................................................................................................... 18

1.3.2 Treatment for RB ....................................................................................................... 21

1.4 TUMOR ANTIGENS IDENTIFIED FOR TARGETED THERAPY .................................. 21

1.4.1 TIAM1 .......................................................................................................................... 21

1.4.2 Nucleolin .................................................................................................................... 23

1.4.3 Survivin ....................................................................................................................... 26

1.5 APTAMER BASED TARGETING .................................................................................... 27
1.6 TARGETING CANCER STEM CELLS ..........................................................29
  1.6.1 EpCAM and EpCAM aptamer ...............................................................31
  1.6.2 CSC markers and aptamers .................................................................32
  1.6.3 miRNA-17~92 cluster and pri-miRNA aptamer ......................................34
1.7 ANTISENSE OLIGO BASED TARGETING ...............................................35
  1.7.1 Silencing RNA/short hairpin RNA .......................................................36
  1.7.2 DNAzyme .............................................................................................37
  1.7.3 Ribozyme .............................................................................................38
1.8 NANOCARRIER BASED TARGETING ....................................................39
  1.8.1 Targeting cancer using polymeric-PEI nanocarrier .................................40

CHAPTER 2 .....................................................................................................42

Materials and methods ..................................................................................42

*In vitro* materials and methods: .................................................................43
  2.1 Materials .................................................................................................43
  2.2 Fabrication of LNA modified aptamer chimera and truncation of CD44 aptamer ........................................................................44
  2.3 Cell culture .............................................................................................44
  2.4 Sample collection and Ethics statement ..................................................45
  2.5 RB tumor cells, cell lines –Total RNA isolation and Quantitative PCR .........45
a. Tiam1 .................................................................................................................................. 45
b. Oncogenes and cancer stem cell markers: ......................................................................... 46

2.6 Expression of NCL on tumors and cell lines: ................................................................. 46
2.7 Analysis of EpCAM with flow cytometry ........................................................................ 47
2.8 RNA aptamers ................................................................................................................... 47
2.9 Fabrication of EpApt-siEp chimeric construct ................................................................. 48
2.10 In vitro dicer Assay .......................................................................................................... 48
2.11 Synthesis and stabilization of PEI: sodium citrate nanocomplex .................................... 48
2.12 Fabrication of PEI-aptamer-siRNA nanocomplex ......................................................... 49
2.13 Characterization of the PEI-aptamer-siRNA nanocomplex ........................................ 49
2.14 Aptamer-doxorubicin conjugation and characterization ................................................ 49
2.15 Release and diffusion of doxorubicin from the aptamer-doxorubicin conjugates .......... 50
2.16 Synthesis of fluorescent copper-free click conjugates .................................................. 50
2.17 Aptamer DNAzyme conjugation ...................................................................................... 50
2.18 Stability of aptamer and aptamer conjugates ............................................................... 51
2.19 CD133^ve and ABCG2^ve cell isolation and aptamer uptake study .............................. 51
2.20 Aptamer, aptamer chimera and nanocomplex uptake study ........................................... 51
a. Cellular uptake of NCL aptamer on RB tumor cells and cell lines ........................................ 51
b. Uptake of EpCAM aptamer .................................................................................................. 52
c. Uptake of EpCAM aptamer chimera ................................................................................ 52
d. Uptake of EpCAM aptamer PEI-siEp nanocomplex .......................................................... 52
e. Visualization of aptamer-doxorubicin uptake with fluorescent microscopy ....................... 53
f. Uptake and Imaging of EpD-DIBO AF594 conjugates in cancer cells ............................... 53
g. Imaging of cancer cells using hyperspectral microscopy .................................................. 54
h. Uptake of survivin Dz and NCL Apt-Sur_Dz conjugates ..................................................... 54
i. Cellular uptake of the CSC marker aptamers .................................................................... 54

2.21 aptamer treatment, transfection and RNA interference ..................................................... 55
a. Transfection of Tiam1 constructs and RNAi of Tiam1 ......................................................... 55
b. Treatment of cells with NCL-aptamer and silencing of NCL in RB cells ............................. 55
c. Cell treatment to study silencing effect of the chimeric construct ..................................... 56
d. Silencing efficiency of the nanocomplex ......................................................................... 56
e. Transfection or treatment of cells with Sur_Dz or conjugate ............................................. 57
f. Treatment of cells with Pri-miRNA aptamer .................................................................... 57
g. Cellular activity of the CSC marker aptamers ................................................................... 57

2.22 Northern blotting .................................................................................................................. 57

2.23 RNA isolation and Quantitative PCR of mRNA .................................................................. 58

2.24 cDNA microarray analysis of Y79 cells treated with siTiam1 .......................................... 59

2.25 miRNA microarray analysis of WERI-Rb1 cells treated with NCL-aptamer ..................... 59

2.26 RNA isolation and quantitative real time PCR .................................................................. 60
2.27 Real-time Quantitative Reverse transcription PCR of miRNAs ............................................60
2.28 Immunohistochemistry of EpICD ..........................................................................................61
2.29 Functional activity of LNA chimera-qPCR and Immunofluorescence .................................61
2.30 Western Blotting ....................................................................................................................62
2.31 Immunofluorescence ..............................................................................................................62
2.32 Flow cytometry analysis of apoptosis ....................................................................................63
2.33 Flow cytometry analysis of cell cycle ....................................................................................63
2.34 Wound healing assay .............................................................................................................63
2.35 Matrigel invasion assay ..........................................................................................................63
2.36 Cytotoxicity assay – Lactate dehydrogenase (LDH) release assay .......................................64
2.37 Cell proliferation assay ..........................................................................................................64
2.38 Statistical analysis ..................................................................................................................64

In vivo Methods: ..........................................................................................................................64
2.39 Animal Care and Experimental Procedure ............................................................................64
2.39.1 Animal Welfare ............................................................................................................ 64
2.39.2 Housing and Feeding................................................................................................... 65
2.39.3 Drinking water ............................................................................................................. 65
2.39.4 Preparation of Animals ............................................................................................. 65
2.39.5 Animal Identification .................................................................................................. 65
2.39.6 Preparation of tumor cells .......................................................................................... 65
2.39.7 Subcutaneous injection of cells .................................................................................. 66
2.39.8 Formulation and Drug Dosage (Preparation of oligos and dosing solutions) ........ 68

2.40 OBSERVATIONS .................................................................................................................68

2.40 .1 Body weight ............................................................................................................. 68
2.40 .2 Tumor volume measurement ..................................................................................... 69
2.40 .3 Antitumor Activity .................................................................................................... 69
2.40 .4 Test/Control Value in % (% T/C) ............................................................................ 69
2.40 .5 Tumor growth inhibition (TGI) ................................................................................ 69
2.40 .6 Efficacy Criteria ........................................................................................................ 70
2.40 .7 Clinical Signs ........................................................................................................... 70
2.40 .8 Statistical Analysis ................................................................................................... 70
2.40 .9 Necropsy ................................................................................................................ 71
2.40 .10 Histology .............................................................................................................. 71

2.41 Serum miRNA isolation and Real time qPCR ............................................................... 71
2.42 Real time qPCR and Western blotting of the Y79 xenograft tumor tissues ................. 72
2.43 DESI MS of cell lines treated with aptamer and siRNA and Xenograft tumor tissues .... 72
2.44 Blood cell counts, biochemical and histology analysis .............................................. 72
2.45 Protein array for apoptotic markers and cytokines .................................................. 73
2.46 Structure prediction and dynamics ........................................................................ 73
2.47 Docking .................................................................................................................. 74

*In vitro* evaluation of the study oligonucleotides in RB and epithelial cancer models ................................................................. 75

CHAPTER 3 ........................................................................................................... 76

Expression of Tiam1 and NCL in RB: Potential Novel Targets for Therapy .......... 76

CHAPTER 3.1 ......................................................................................................... 77

Tiam1 gene knockdown inhibits invasion of RB ......................................................... 77

3.1.1 RESULTS ......................................................................................................... 77
3.1.1.1 Differential expression of Tiam1 in RB tumors .......................................................... 77
3.1.1.2 RNA interference of Tiam1 in RB cell lines ................................................................. 77
3.1.1.3 Tiam1 regulates apoptosis and viability in RB cells ..................................................... 79
3.1.1.4 Tiam1 silencing results in de-regulation of gene expression in Y79 cells ..................... 80
3.1.1.5 Significantly down-regulated genes in Tiam1 silenced Y79 cells ............................... 82
3.1.1.6 Significantly up-regulated genes in Tiam1 silenced Y79 cells ...................................... 83
3.1.1.7 Validation of de-regulated genes in Tiam1 silenced RB cell lines and primary RB tumors ..................................................................................................................................... 85
3.1.1.8 Tiam1 regulates actin polymerization, cell migration and invasion in RB cell lines. 87
3.1.1.9 Plasma membrane localization of Tiam1 is mediated by N-terminal PH domain .... 89
3.1.1.10 N-terminal PH domain regulates RB cell motility .................................................... 91

3.1.2 DISCUSSION ...................................................................................................................... 95

CHAPTER 3.2 ......................................................................................................................... 97

NCL regulates miRNA 17-92 cluster and lipid arrangement .............................................. 97

3.2.1 Results ................................................................................................................................... 97

3.2.1.1 NCL is overexpressed in RB ....................................................................................... 97
3.2.1.2 NCL aptamer perturbs RB cell growth and leads to cell death ................................. 100
3.2.1.3 NCL regulates onco-miRNAs expression in RB ......................................................... 107
3.2.1.4 NCL aptamer disrupts NCL - G-rich mRNA interaction thereby alters gene expression in RB .................................................................................................................................. 110
3.2.1.5 Lipid profile changes upon knocking down NCL and using NCL-aptamer .......... 113

3.2.2 DISCUSSION ....................................................................................................................... 116
CHAPTER 3.3.................................................................................................................................119
Chimeric survivin DNAzyme - NCLAPT targets cancer cells....................................................119

3.3.1 RESULTS..................................................................................................................................119

3.3.1.1 Fabrication of NclApt-Sur_Dz constructs ................................................................ 119
3.3.1.2 Stability of NclApt-Sur_Dz chimeric constructs ....................................................... 120
3.3.1.3 Cellular uptake of aptamer DNAzyme conjugates .................................................... 122
3.3.1.4 Functional activity of the conjugate ......................................................................... 125

3.3.2 DISCUSSION.............................................................................................................................127

CHAPTER 4 ...........................................................................................................................................129

EpCAM aptamer chimeric conjugates for therapeutic application .............................................129

CHAPTER 4.1 .......................................................................................................................................130

EpCAM aptamer-siRNA chimera regress epithelial cancer..........................................................130

4.1.1 RESULTS..................................................................................................................................130

4.1.1.1 Chimerization of EpApt with siEpCAM ................................................................. 130
4.1.1.2 EpApt-siEp: in vitro dicer mediated processing and cellular uptake ....................... 132
4.1.1.3 EpApt-siEp silences EpCAM specifically in cell lines and primary RB tumor cells 135
4.1.1.4 EpICD in primary RB tumors: regulation of cancer stem cell markers ................. 138
4.1.1.5 LNA modified aptamer siRNA fabrication and in vitro dicer processing .......... 140
4.1.1.6 Functional activity of LNA aptamer siRNA chimeras ............................................. 141

4.1.2 DISCUSSION.............................................................................................................................144
CHAPTER 4.2 ...............................................................................................................148

EpCAM aptamer mediated delivery of siRNA using polymeric nanocomplex ....148

4.2.1 RESULTS ...............................................................................................................148

4.2.1.1 Synthesis and characterization of PEI nanocomplex with EpCAM aptamer and siRNA 148

4.2.1.2 Cytotoxicity effect of PEI polymer on cells: .............................................................. 152

4.2.1.3 Cellular Uptake of PEI aptamer siRNA complex: ...................................................... 152

4.2.1.4 Silencing efficiency of the Nano complex: ................................................................. 155

4.2.2 DISCUSSION .............................................................................................................158

4.2.3 CONCLUSION ...........................................................................................................160

CHAPTER 4.3 ...............................................................................................................161

Targeted delivery of doxorubicin using EpCAM aptamer .................................161

4.3.1 RESULTS ...............................................................................................................161

4.3.1.1 EpCAM aptamer binds to RB tumor cells and cell lines: .................................. 161

4.3.1.2 Preparation of aptamer-Dox conjugates: .............................................................. 162

4.3.1.3 Release and diffusion of the drug from the aptamer-Dox conjugate ..................... 165

4.3.1.4 Targeted delivery and Uptake of Dox in cell line ....................................................... 166

4.3.1.5 Effect of aptamer–Dox conjugate on Cell cytotoxicity .............................................. 169

4.3.2 DISCUSSION .............................................................................................................170

CHAPTER 5 ...............................................................................................................173

EpCAM positive cancer cell imaging and onco-miRNA and cancer stem cell markers targeting using aptamer ..................................................173
CHAPTER 5.1 ........................................................................................................................................174

Imaging of EpCAM Positive Cancer Cells Using Novel aptamer- Fluorescent Conjugate ........................................................................................................................................174

5.1.1 RESULTS and DISCUSSION ........................................................................................................174

5.1.1.1 SPAAC based labeling of EpCAM aptamer and characterization ........................................ 174
5.1.1.2 Purification of DIBO-AF594 clicked aptamer ......................................................................... 176
5.1.1.3 Stability of conjugates ........................................................................................................ 177
5.1.1.4 Cellular uptake of conjugates ............................................................................................ 178
5.1.1.5 Cytotoxicity of conjugates .............................................................................................. 181

5.1.2 CONCLUSION ................................................................................................................................182

CHAPTER 5.2 ........................................................................................................................................183

Cancer stem cell marker and pri-miRNA targeting using aptamers ......................................................................................... 183

5.2.1 RESULTS AND DISCUSSION ......................................................................................................183

5.2.1.1 Pri-miRNA-17-92 aptamer abrogates maturation of miRNAs .............................................. 183
5.2.1.2 Pri-miRNA-17-92 aptamer affect cell cycle and leads to apoptosis .................................. 184
5.2.1.3 Pri-miRNA-17-92 aptamer inhibits cancer cell proliferation ............................................. 186

5.2.2 CONCLUSION ................................................................................................................................188

CHAPTER 5.3 ........................................................................................................................................190

Aptamers targeting cancer stem cell markers ......................................................................................... 190

5.3.1 Results ........................................................................................................................................190
List of figures

Figure 1. Targets used in the study. ........................................................................................................8
Figure 2. Schematic illustration of the study plan. ...................................................................................11
Figure 3. Flow of work: ..........................................................................................................................15
Figure 4. The cell cycle regulation mediated by the pRb. .................................................................19
Figure 5. Haematoxylin and eosin staining of RB tissue sections. ...................................................20
Figure 6. Diverse role of Tiam1 in tumorigenesis. ..............................................................................22
Figure 7. Regulation mediated by nucleolin in the cell. .....................................................................24
Figure 8. Functions of survivin that aids in tumorigenesis of cell........................................................27
Figure 9. Schematic representation of systemic evolution of ligands by exponential enrichment (SELEX). ..........................................................................................................................28
Figure 10. Schematic showing the cancer stem cell targeting. ..........................................................30
Figure 11. Cancer stem cell markers in RB. ..........................................................................................32
Figure 12. Antisense and oligonucleotide based targeting of cancer cells. .....................................36
Figure 13. Nanomaterials used for the drug delivery purposes. .......................................................40
Figure 14. Tiam1 constructs and expression of Tiam1 in RB tumors compared to normal retina. ........................................................................................................................................78
Figure 15. Knockdown of Tiam1 in Y79 and WERI-Rb1 cell lines using RNA interference. .............79
Figure 16. Pathways and gene ontologies deregulated upon TIAM1 silencing. ..............................81
Figure 17. Analysis of Apoptosis and viability of Tiam1 silenced Y79 and WERI-Rb1 cells. .................................................................82
Figure 18. cDNA microarray in Tiam1 silenced Y79 and validation of de-regulated genes in RB cell lines and tumors........................................................85
Figure 19. F-actin staining of Tiam1 deficient RB cell lines............................................................89
Figure 20. Invasion of Tiam1 deficient RB cell lines .................................................................90
Figure 21. Matrigel invasion assay of Tiam1 deficient RB cell lines ...............................................91
Figure 22. Localization of Full length Tiam1, C1199 Tiam1 and C580 Tiam1 in RB cell lines ................................................................................................................................................92
Figure 23. N-terminal PH domain maintains cell motility in RB cells. .................................................................................93
Figure 24. Tiam1 mediated signaling pathway. .........................................................................................................................94
Figure 25. The expression of NCL in RB tumor samples and cell lines. .................................................................................98
Figure 26. NCL expression on RB cell lines and stability of NCL aptamer. .................................................................101
Figure 27. NCL-aptamer binding and its effect on RB cell lines. .............................................................................................102
Figure 28. Cytostatic and cytotoxic effect of NCL aptamer on RB cells. ...............................................................................103
Figure 29. Effect of NCL-aptamer on RB cell lines. ..............................................................................................................104
Figure 30. Transfection in primary RB cells. .........................................................................................................................105
Figure 31. Cellular uptake of NCL-aptamer in the primary RB cells. ..................................................................................106
Figure 32. Changes in miRNA expression accompanying NCL-aptamer treatment. .........................................................108
Figure 33. Effect of NCL-aptamer on RB cell lines. ..............................................................................................................109
Figure 34. Effect of NCL-aptamer and siNCL treatments on gene expression changes in RB cell lines. ..........................................................111
Figure 35. Effect of aptamer and siNCL on surface lipid arrangement in RB cell lines and xenograft tissues. ...........................................................................115
Figure 36. Schematic representations of the assembly of aptamer-DNAzyme chimeric construct. .................................................................120
Figure 37. Stability of the DNAzyme and chimeric conjugates. .........................................................................................121
Figure 38. Cellular uptake of NclApt-Dz conjugates in MIO-M1 cells. ................................................................................122
Figure 39. Cellular uptake of Sur-Dz and chimeric conjugates in WERI-Rb1 cells. ..........................................................124
Figure 40. Cellular uptake of Sur-Dz and chimeric conjugates in Y79 cells. ........................................................................125
Figure 41. Knockdown of survivin using chimeric aptamer DNAzyme construct.................................................................126
Figure 42. Knockdown of survivin protein analyzed by Western blotting. ........................................................................126
Figure 43. Fabrication of EpCAM aptamer siRNA chimera and cartoon showing its processing in cell. .................................................................131
Figure 44. In vitro siRNA generation by Dicer. ..................................................................................................................133
Figure 45. Cellular uptake of the EpApt-siEp construct ........................................................................................................134
Figure 46. EpCAM knockdown using EpApt-siEp construct in MCF7 and WERI-Rb1. .................................................................136
Figure 47. Expression of GFP in primary RB cells.

Figure 48. Expression of EpCAM intracellular domain (EpICD) in RB.

Figure 49. Fabrication of the LNA modified aptamer siRNA chimera.

Figure 50. Knockdown of BIRC5 and STMN1 by L-EpApt-siRNA chimeras.

Figure 51. Illustration showing the cell specific silencing mediated by PEI nanocomplex fabricated with aptamer and siRNA.

Figure 52. Effect of citrate on the nanocomplex size and charge.

Figure 53. Characterization of the PEI-EpApt-siEp nanocomplex.

Figure 54. Effect of media and serum on PEI nanocomplexes.

Figure 55. Expression of EpCAM and binding of the nanocomplexes on cells.

Figure 56. Cellular uptake of the PEI nanocomplex by MCF7 cells.

Figure 57. Cellular Uptake of the PEI nanocomplex by WERI-Rb1 cells.

Figure 58. Expression of EpCAM post silencing using PEI Nanocomplexes.

Figure 59. Effect of PEI-Apt-siRNA complex on cell proliferation.

Figure 60. EpCAM expression on cell lines.

Figure 61. EpCAM aptamer binding on RB primary cells.

Figure 62. EpCAM aptamer binding on cell lines.

Figure 63. EpCAM aptamer structure and doxorubicin conjugation.

Figure 64. Diffusion of doxorubicin from the aptamer-Dox conjugate.

Figure 65. Uptake of aptamer-Dox conjugate on Müller glial cells addition in vitro.

Figure 66. Uptake of aptamer-Dox conjugate on the Y79 cell line post 2h addition in vitro.

Figure 67. Uptake of aptamer-Dox conjugate on the Y79 cell line post 12h addition in vitro.

Figure 68. Cell proliferation inhibition by aptamer-Dox conjugates in vitro.

Figure 69. Illustration showing the imaging of cancer cell by targeting EpCAM.

Figure 70. Reaction scheme and optimization of DIBO reaction concentration.

Figure 71. HPLC analysis of aptamer conjugates.

Figure 72. Characterization of aptamer conjugation and conjugate formation.

Figure 73. Stability of the aptamer-DIBO conjugate.
Figure 74. Cell surface binding of the aptamer conjugates ........................................................179
Figure 75. Dark field-fluorescent hyperspectral microscopic imaging of EpDNA-DIBO-AF594 in cancer cells ........................................................................................................................180
Figure 76. Fluorescent microscopy images showing the internalization of the aptamer conjugates ........................................................................................................................................181
Figure 77. Effect of conjugates on cell proliferation .................................................................182
Figure 78. Pri-miRNA aptamer inhibits the mature miRNA formation .....................................184
Figure 79. Effect of the Pri-apt on the cell cycle and apoptosis .................................................186
Figure 80. Pri-apt induced cell cytotoxicity and cell death .......................................................188
Figure 81. The expression of CSC markers and stathmin in RB tumour samples .................190
Figure 82. Expression of CD44 variants in RB tissues .............................................................192
Figure 83. Truncation of CD44 aptamer and CSC marker aptamer uptake on cancer cell lines .................................................................................................................................193
Figure 84. Aptamer binding to normal or non-malignant retina and RB tumor cells ...............196
Figure 85. Aptamer uptake on MCF7 spheroids- mammospheres ..........................................197
Figure 86. ABCG2+ve cell isolation from MCF7 cells, mammospheres and aptamer uptake study ..................................................................................................................................198
Figure 87. CD133+ve cell isolation from RB tumor and NR cells ............................................200
Figure 88. Functional activity of the CSC marker aptamers ...................................................201
Figure 89. RMSD trajectories of CD133 ExD2 and A15 aptamer .............................................202
Figure 90. Structure prediction of CD133 extracellular domain 2 and CD133 – A15 aptamer .................................................................................................................................203
Figure 91. Molecular dynamics of CD133 ExD2 and comparison of structures ......................204
Figure 92. Docking of CD133 ExD2 with aptamer and predicted interacting residues ..........205
Figure 93. Anti-tumor effect of NCL-aptamer and LNA-NCL-aptamer on Y79 xenograft model ........................................................................................................................................211
Figure 94. Anti-tumor, growth kinetics and biochemicals changes accompanying NCL-aptamer treatment ....................................................................................................................................213
Figure 95. H and E staining of sections ....................................................................................214
Figure 96. Effect of NCL-aptamer in vivo Y79 xenograft model .............................................216
Figure 97. Effect of NCL-aptamer and LNA-NCL-aptamer on surface lipid arrangement in Y79 xenograft tissues........................................................................................................................................218
Figure 98. Protein array for cytokine secretion and IHC analysis on LNA-NCL-aptamer treated xenograft samples. ........................................................................................................................................221
Figure 99. Illustration summarizing the role of NCL in cellular metabolism. .................................222
Figure 100. Tumor growth kinetics of MCF7 Xenograft treated with EpApt-siEp......................226
Figure 101. Changes in gene and protein expression upon EpApt-siEp treatment on MCF7 xenografts........................................................................................................................................227
Figure 102. Immunohistochemical analysis of EpCAM and PCNA in xenograft tissues. ...............229
Figure 103. Haematoxylin and Eosin staining of organ and tumor sections. .................................230
Figure 104. Protein array for apoptotic marker s, cytokine secretion and the IHC analysis on EpApt-siEp treated xenograft samples......................................................................................................................................232
Figure 105. Illustration summarizing the EpCAM aptamer siRNA chimera effect on the tumor growth inhibition. ........................................................................................................................................235
List of Tables

Table 1. Treatment schedule for Y79 xenograft .................................................................66
Table 2. Treatment schedule for MCF7 xenograft...............................................................67
Table 3. Formulation and dosage of oligos for injection in Y79 and MCF7 xenograft
models ..................................................................................................................................68
Table 4. The percent tumor growth and efficacy determination ........................................70
Table 5. Clinicopathological features of primary RB tumors and gene expression profile
by QRT-PCR .........................................................................................................................86
Table 6. Primer sequences for the selected panel of validated genes. ...............................88
Table 7. Clinicopathological details of the RB tumor and the NCL expression by IHC
studies ....................................................................................................................................99
Table 8. List of primers used in the NCL study .................................................................112
Table 9. Expression of EpICD in RB primary tumor by IHC. .............................................139
Table 10. Aptamer-siRNA chimeras used for targeted cancer therapy ..............................145
Table 11. Flow cytometry analysis of the binding of the EpDNA-DIBO-AF594 onto cell
lines .......................................................................................................................................179
Table 12. The percentage uptake of aptamers by primary RB tumors and normal retina....195
Table 13. Table showing the list of lipid masses and the corresponding lipid chemistry. ....219
List of publications (during PhD; 2011-2014)

Research articles Published= 5

   Link: http://pubs.rsc.org/en/Content/ArticleLanding/2014/CC/C4CC02996H#!divAbstract

   Link: http://www.jbiomedsci.com/content/22/1/4/abstract


   Link: http://www.molvis.org/molvis/v18/a285/
Research articles under review = 3


Research articles being submitted = 1


Review article being prepared = 1

List of Publications (before PhD registration; 2007-2010) = 2

   Link: http://vir.sgmjournals.org/content/90/8/1812.full

   Link:http://vir.sgmjournals.org/content/89/7/1579.short

List of Conferences attended = 4

1. Selected for poster presentation at “6th Bangalore Nano – 2013” held at Bangalore, INDIA

2. Travel grant and selected for Oral presentation at the “Asia-ARVO-2013” held at New Delhi, INDIA

3. Selected for Oral presentation at “4th International conference on Nanomedicine 2013” held at Sydney, AUSTRALIA

4. Best poster award for the presentation at “World congress on Biotechnology 2012” held at Hyderabad, INDIA
**List of abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>Ammonium Per Sulphate</td>
</tr>
<tr>
<td>APT</td>
<td>Aptamer</td>
</tr>
<tr>
<td>AWL</td>
<td>Aptamer With Linker</td>
</tr>
<tr>
<td>B2M</td>
<td>β-2-Microglobulin</td>
</tr>
<tr>
<td>BIR</td>
<td>Baculoviral IAP Repeat</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CO2</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer Stem Cells</td>
</tr>
<tr>
<td>CI</td>
<td>Choroid invasion</td>
</tr>
<tr>
<td>DAPI</td>
<td>4, 6 - Di Amidino -2- Phenyl Indole</td>
</tr>
<tr>
<td>DESI MS</td>
<td>Desorption Electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>DIBO</td>
<td>Dibenzooctonyl alkyene</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modification of eagle’s medium</td>
</tr>
<tr>
<td>Dox</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>DZ</td>
<td>DNAzyme</td>
</tr>
<tr>
<td>ECL</td>
<td>Electro ChemiLuminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetra Acetic Acid</td>
</tr>
<tr>
<td>EpApt</td>
<td>EpCAM aptamer</td>
</tr>
<tr>
<td>EpCAM</td>
<td>Epithelial cell adhesion molecule</td>
</tr>
<tr>
<td>EpD</td>
<td>EpCAM DNA aptamer</td>
</tr>
<tr>
<td>EpDT3</td>
<td>EpCAM RNA aptamer</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>F-DZ</td>
<td>Fluorescent DNAzyme</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Iso Thio Cyanate</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of Apoptosis</td>
</tr>
<tr>
<td>ICMR</td>
<td>Indian Council for Medical Research</td>
</tr>
<tr>
<td>IDT</td>
<td>Integrative DNA Technologies</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>LNA</td>
<td>Locked nucleic acid</td>
</tr>
<tr>
<td>MALDI TOF</td>
<td>Matrix assisted laser desorption</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi-drug resistance</td>
</tr>
<tr>
<td>mDZ</td>
<td>mutant DNAzyme</td>
</tr>
<tr>
<td>miR-17-92</td>
<td>miRNA 17-92 cluster</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MTT</td>
<td>(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)</td>
</tr>
<tr>
<td>NCL</td>
<td>Nucleolin protein</td>
</tr>
<tr>
<td>NCL-APT</td>
<td>NCL aptamer or as1411</td>
</tr>
<tr>
<td>NR</td>
<td>Normal retina or non-malignant retina</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimum cutting temperature</td>
</tr>
<tr>
<td>ON</td>
<td>Optic nerve</td>
</tr>
<tr>
<td>OTA</td>
<td>Ochratoxin A</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate Buffered Saline-0.1% ween 20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
</tr>
<tr>
<td>P-gp</td>
<td>P glycoprotein</td>
</tr>
<tr>
<td>PIC</td>
<td>Proteinase Inhibitor Cocktail</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly L Lysine</td>
</tr>
<tr>
<td>Pre miRNA</td>
<td>Precursor miRNA</td>
</tr>
<tr>
<td>Pri-miRNA</td>
<td>primary miRNA</td>
</tr>
<tr>
<td>pRB</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PSMA</td>
<td>Prostate Specific Membrane Antigen</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatise and tensin homolog</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative PCR</td>
</tr>
<tr>
<td>Ras</td>
<td>Rat sarcoma</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma gene</td>
</tr>
<tr>
<td>RB</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RBD</td>
<td>RNA Binding Domain</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>ROX</td>
<td>Carboxyrhodamine</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions Per Minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse Transcription</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Real Time quantitative PCR</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SELEX</td>
<td>Systematic evolution of ligands by exponential enrichment</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small inhibitory RNA</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline Sodium Citrate</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris Acetic acid EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffer saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris buffer containing 0.1% Tween 20</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TIAM1</td>
<td>T lymphoma invasion and metastasis protein</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>UV</td>
<td>UltraViolet</td>
</tr>
<tr>
<td>WB</td>
<td>Western blotting</td>
</tr>
</tbody>
</table>
Acknowledgements

I thank the almighty who was always with me during this study period. I thank him for giving me such a wonderful family and parents without whom the successful completion of the study was not achievable. I thank god for my well being and for the support he offered through my days by blessing me with great people around whom I acknowledge below.

I extend my heartiest thanks to Prof Jagat R. Kanwar, my principal supervisor; his guidance made this study possible. He is a person of constant encouragement and a role model. He is dynamic person with vision and knowledge for approaching complicated tasks. I learnt lots from him and his huge support helped to surpass difficult tasks. He was more than a guide, a well wisher to standby during my personal problems. His mentorship trained me as perfect researches with planning and developmental skills.

I am thankful to Dr S. Krishnakumar, Indian supervisor for giving me an opportunity to work under him and get his valuable guidance. His compassion for science and exploring complex fields without any hesitation and the expert’s contacts that he brought in during the study period helped to carry out my research topic without obstruction. He was just not a supervisor and helped to solve complex issues with his immense experience in the scientific field. His guidance improved my problem solving skills, planning and execution of work in meticulous way.

I wish to acknowledge Dr Rupinder K. Kanwar, my associate supervisor. She is very specific in drawing research plans with extreme practical knowledge. Her scientific zeal and designing of key experiments are major learning from her. She stands by as friend and sister and helped in molding me as good person and as scientific researcher. I am grateful to work under her guidance, as her help is inevitable to yield me success.

I am thankful to Deakin University for setting up “Deakin India Research Initiative” through which was able to register my higher degree and get research scholarship while working in the campus.

I thank School of Medicine, Head, staff and technical officers for the opportunity to work in safe environment that they have set up. Special thanks to Prof. Tes Toop, Mrs. Helen Barry, Mrs.
Helen Woodall and Mrs. Elizabeth Laidlaw and Ms. Monique for their help in setting up safe research. Mr. Nagsarath Pandurangi, Ms. Anuradha gupta and Ms. Gayathri Vedanarayan help to co-ordinate and solved my doubts during the process of my study. They were more helpful in finding out ways for approaching Deakin University for a given problem. Ms. Helen Woodall was always there to help with questions and to brief the academic processes and more helpful while I was in Deakin University.

I wish to thank Chairman emeritus Padmabushan Dr S. S. Badrinath, Sankara Nethralaya for providing a world-class research environment. I am thankful to almighty for getting to work under such an eminent person and get his blessings. His strenuous efforts to improve the research in India had provided a stage for my learning.

My sincere thanks to Dr H. N. Madhavan, Sankara Nethralaya for providing me an opportunity to work at Vision Research Foundation. He was always helpful and very inspiring person that I have ever come across. He is very influential person with unmatched knowledge and research planning skills.

I express sincere gratitude my department staffs Dr Nivedita Chatterjee, Dr Narayanan Janikaraman and Dr Sailaja Elchuri and all other departmental staffs at Vision research foundation and Sankara Nethralaya. Core lab facility is an extreme help by providing essential instrumentations, department of biochemistry, genetics and microbiology staffs were very kind in helping with instruments for carrying out all experiments without any hindrance.

Gurudevan for their help, motivation, constant support and for being with me for the past few years.

Finally I would like to thank once again my family members (Balasubramanian A, Akshayaa Shree), parents, in-laws and siblings for their constant inspiration, encouragement that supported and motivated to travel with success all through my aims in life.
Importance and novelty of the current study

There are various cancer targets and markers in the pipeline, but still there is a search for a lead that specifically targets cancer cells. This thesis studies specific addressing of cancer cells of retinoblastoma (RB) and cancers of epithelial origin, breast cancer and prostate cancer. The novelty and importance of this study are as follows:

- Unraveling the role of T-lymphoma invasion and metastasis (Tiam1) and nucleolin (NCL) in RB and analyzing its molecular networks for use as targets to attain better efficacy in cancer therapy.
- Approaches based on targeting of cancer stem cell (CSC) marker such as epithelial cell adhesion molecule (EpCAM) using aptamer-chimeras (drug/siRNA/nanocomplex) for cancer cell specific delivery.
- Nuclease resistant modifications of aptamer and aptamerchimera for better \textit{in vivo} stability and efficacy.
- Developing novel bio-orthogonal imaging agent, aptamers and truncated aptamers that can specifically bind to CSC markers that will serve for theranostic purpose.
- Extending the use of aptamer against CD44, CD133, ABCG2 and pri-miRNA 17-92 cluster, thus opening up translational capacity of these aptamers.

Knowledge gaps which this study aims to fill

Targeted therapy is the need of hour in cancer treatment. The well known cancer targets are Her2/neu, MUC1. These receptors are targeted using antibodies, affibodies or diabodies. Antibody derivatives are better therapeutic agents and carry few limitations such as larger size, immunogenicity and cytotoxicity caused by non-targeted delivery. In the case of RB, chemotherapy, brachytherapy and enucleation were the only choice of therapy and research is happening in targeted therapy. Current study aims to address the above mentioned drawbacks by untying new targets for therapy of RB and epithelial cancers, by

i. Studying novel oncogenes and their role in tumor cell survival. Using them for targeting cancer cells sparing the normal cells. The information on the targets such as TIAM1 and
Novelty and Hypotheses

nucleolin are minimal in RB, and hence this study will decipher the role of these targets and regulations they mediate in the tumorigenesis.

ii. Targeted delivery using aptamers which are smaller in size and target specificity with very little immune elicitation. We used an RNA aptamer EpCAM and its chimeric constructs for targeted delivery to CSCs of RB. The EpCAM siRNA chimeric construct is tested in EpCAM positive cancer model (xenograft) for its anti-tumor property.

iii. We explore the use of the EpCAM DNA aptamer for the fluorescent labeling by novel method that adopts bio-orthogonal reaction which is a strain promoted alkyne-azide conjugation. Additionally, a DNA aptamer against NCL is used for inhibiting the RB cells proliferation. Also the use of aptamer for therapeutic purpose will be addressed in the study using RB xenograft model. This will open new avenues of using aptameraptamer in eye cancer-RB which can be translated to other cancers too.

Aim and objectives of the study

Cancer cell specific delivery of drugs and siRNA and use of targeted imaging agents are still developed. In this milieu, unraveling new targets for therapeutic purpose and use of common markers that aids in cancer cell resistance is of importance. Use of targets such as Tiam1, an invasion protein, NCL, a major protein involved in transcriptional mechanism and use of cancer stem cell marker aptamer, open new avenues of therapeutic and diagnostic approach in the cancer biology and therapy.

The current study aims to answer the specificity, developing of anti-tumor and imaging agents. The broad aims of this study are achieved by fulfilling smaller milestones which are presented below:

1. To study the importance of Tiam1 and NCL in RB and aptamer based approach to target the cancer cells.

   The objectives set to accomplish the aim are, RB primary tumor samples were utilized to study the expression of the target antigen at protein and mRNA levels. The significance of the targets was studied using knockdown and/or aptamer based strategy. Followed by critical analysis of these mechanisms it adopts for exhibiting the anti-tumor property by
Novelty and Hypotheses

_in vitro_ assays and _in vivo_ using xenograft models. Quantitative PCR, Western blotting, immunofluorescence, immunohistochemistry, miRNA expression studies finally anti-tumor activity in nude mice-tumor xenograft model is studied.

2. To fabricate and study the aptamerchimeric conjugates for therapeutic purpose

The objectives set to accomplish the aim are, generate and characterize the EpCAM aptamer drug/siRNA chimeric conjugates – doxorubicin conjugates, aptamersiRNA chimeric construct and PEI siRNA aptamer fabricated nanocomplex using _in vitro_ assays. The best cell proliferation inhibitor without any toxicity and stable would be further analyzed under _in vivo_ condition using epithelial tumor xenograft model in nude mice for anti-tumor activity.

3. To target miRNA transcript and image cancer stem cell markers using aptamer and LNA modified aptamer constructs

The objectives set to accomplish the aim are, analyze the _in vitro_ efficacy of the novel EpCAM aptamer fluorescent conjugate generated by bio-orthogonal method for use in imaging of cancer cells and to explore the pri-miRNA17-92 cluster aptameraptamer (an onco-miRNA cluster targeting aptamer) and truncate and analyze the specificity of the cancer stem cell markers (CD44, CD133 and ABCG2) aptamers in primary tumor and CD133+ve primary tumor cells.

Overall, the objectives fulfill the broad aim by screening the best aptamer or aptamer chimeric conjugate with stable modification for the _in vivo_ analysis of anti-tumor activity.
Hypotheses

Broad hypothesis of the work was to develop nucleic acid based targeted gene delivery to understand the molecular mechanism(s) to kill the cancers cells and its future potentials for new generation real-time imaging of cancer.

Detailed chapter wise hypothesis:

Tiam1 is found to be de-regulated in most neoplasm and an earlier publication from our lab has shown that Tiam1 is over expressed in RB and it plays a critical role in invasion of the choroid and optic nerve by the RB tumor cells. Knockdown of Tiam1 expression leads cells to undergo changes in the actin filament polymerization and cellular cytotoxicity due to induction of apoptotic initiators. With this background we hypothesize that by silencing Tiam1 the RB cells will be inhibited from invasion and cells will undergo apoptotic death.

NCL, the other tumor target is usually present in nucleolus and shuttles to cytoplasm and it is expressed on the membrane surface under neoplastic condition and not under normal condition, but no data is available in RB tumor. We further hypothesize that the NCL present on the membrane of the RB tumor cell, has a role in signaling and tumor cell progression. By knocking down and using functional blocking aptamer the NCL role on cellular microprocessor and miRNA synthesis, regulations in lipid expression under in vivo condition in RB xenograft model are deciphered.

We would like to additionally target cancer stem cells though aptamer (anti-EpCAM, anti-CD133, anti-CD44 and anti-ABCG2) based strategy and hypothesize that the aptamers will have better effect against RB tumor cell proliferation. The EpCAM aptamer-doxorubicin chimera would have better cytotoxicity than the free drug due to specific delivery of drugs having minimal off target effects. We hypothesize that PEI nanocarrier fabricated with EpCAM aptamer-siRNA will exert cell specific silencing. Additionally by structuring aptamer-siRNA chimeras through prediction of secondary structure can be delivered without any nanocarrier. This aptamer-siRNA chimera will act as functional blocking aptamer and we believe that this aptamer will block the RB and epithelial cancer cell progression and exert tumor regression in xenograft model. Similar chimeras can be constructed with LNA modified for in vivo applications that would be more stable.
Cancer cell targeted imaging is an upcoming field, hence we wanted to extend the application of the DNA EpCAM aptamer for generating a novel bio-orthogonal fluorescent conjugate for better imaging. *We hypothesize that the generated construct is stable under physiological conditions without having any cytotoxicity that can be utilized for imaging purposes.* In addition, we truncated CD44 aptamer and utilized ABCG2 and aptamer binding to CD44 and ABCG2 positive cells for targeting RB cells and validate in ABCG2+ve and CD133+ve RB cells. *We hypothesize that these aptamers have potential application for RB or cancers with high CSC marker expression.* Targeting the miRNA cluster regulated by CSC marker, EpCAM is of interest as the effect will be enhanced. Thus *aptamer against the miRNA transcript can be hypothesized to have futuristic application.*
ABSTRACT

PURPOSE

Different combinations of aptamer constructs for the specific targeting are available; we attempted to test the new targets in RB and utilized aptamers targeting them. The aptamer in native form vs the chimeric forms were used for delivery of siRNA using fluro modified or LNA modified constructs or nanocomplex. Further pri-miRNA and cancer stem cell marker targeting aptamers were evaluated in vitro. The construct with best in vitro efficacy was tested in vivo in RB and epithelial xenograft model.

BACKGROUND

T lymphoma invasion and metastasis protein (Tiam1) is up-regulated in variety of cancers and its expression level is related to metastatic potential of the type of cancer. Earlier, Tiam1 was shown to be overexpressed in invasive RB cases and hence tested by silencing Tiam1 in RB cell lines (Y79 and WERI-Rb1) using siRN Astrategy.

Another target, nucleolin is differentially expressed in the cellular compartments and at the cell surface in malignant condition and translocate to nucleus. The surface NCL binds to various ligands and regulates the carcinogenesis, angiogenesis also interacts with microprocessor for regulating the onco-miRNA. The anti-tumor activity of NCL aptameris also shown in various cancers; however there is no data on RB regarding the expression of NCL protein, NCL aptamer activity and changes in lipid profile.

EpCAM has three different domains, Krishnakumar et al., 2006 reported the expression of EpCAM in RB. The current study revealed the intracellular domain (EpICD) expression in RB and its role in oncogenic signaling and also translocating/shuttling between cytoplasm and nucleus. The over-expression of EpICD was reported in other cancers and our study in RB primary tumor samples reveal the cancer stem cell (CSC) property of EpCAM, also regulated stem cell marker expression.
Chapter 1

Epithelial cancers over express epithelial cell adhesion molecule (EpCAM), also marker for cancer initiating cells and pluripotency marker and hence regards as potential target for cancer theranostics. EpCAM targeting larger molecules and conjugates involving antibody, affibody, ankyrin were approached earlier, yet chemical antibody, aptamer regards as convenient and efficient to target EpCAM positive cancers. In the current study, we chimerized the EpCAM aptamer, with siRNA targeting EpCAM mRNA (EpApt-siEp), thereby we achieve silencing of EpCAM in cells expressing surface EpCAM. This double selection for EpCAM may reduce off-target effects also able to inhibit its expression and functions as well as other cellular functions mediated by EpCAM.

Alternately siRNA delivery using PEI mediated delivery is one of the efficient method among the non-viral carriers. Targeted down-regulation of EpCAM in tumor cells using PEI, siRNA and aptamer nanocomplex based strategy is still unexplored and regards as current study interest. In addition to the nanocomplex targeting, delivery of doxorubicin (Dox) using an RNA aptamer against EpCAM was attempted to target only cancer cells, as drug alone lacks the specificity.

Fluorescent aptamer conjugates were generated using standard oligonucleotide labeling chemistries, but for the first time, we synthesized a novel EpCAM aptamer -DIBO-AF594 fluorescent conjugate using bioorthogonal chemistry, strain promoted alkyne- azide cycloaddition (copper free click) reaction (SPAAC). The ligation efficiency was optimized to yield. The obtained conjugate showed target specific binding and aided in imaging of various EpCAM positive cancer cell lines like MCF7, MDAMB453, WERI-Rb1 and PC3.

The miR-17~92 cluster encodes oncogenic miRNAs, also promotes RB formation, also reported to be regulated by the EpCAM. Antagomir and miRNA mimics based approaches are widely tried against oncogenic and tumor suppressor miRNAs. EpCAM silencing or antagomir based approaches can be tried against oncogenic miRNAs. Various targeting methods against miRNA cluster are still being developed. Current study focuses on the Pri-miR-17-92 aptamer (Pri-apt), which can potentially replace the mix of five antagomirs with one aptamer that function to abrogate the maturation of mir-17, mir-18a and mir-19b.
Cancer stem cell populations are reported from breast, pancreas, prostate, liver, ovarian, head and neck cancers, glioblastoma, melanoma and RB. Among these markers CD133, CD44, EpCAM and ABCG2 were found to be prominent markers in most of the cancers. Surface antigens such as ABCG2, MCM2, CD44, CD133, EpCAM, CD90, ALDH1, CD24 and alkaline phosphatase were found to be over-expressed on RB cancer stem cells. Hence targeting these molecules will effectively result in reducing the CSC population. Strategies using aptamer against CSC markers are being explored and in the current study we truncated to generated aptamer with better efficiency.

**Figure 1.** Targets used in the study. Categories of targets and aptamers used in the study and the chimera studied. The study majorly deals with 3 chapters. 1. Novel targets in RB, 2. Targeting epithelial cancers, RB using EpCAM as target ligand and finally, exploring other targets such as pri-miRNA 17-92 aptamer, cancer stem cell marker aptamers.

**METHODOLOGY**
Chapter 1

The status of the Tiam1, nucleolin, CSC marker expression in RB primary samples were studied using quantitative-real time PCR (qPCR) and Western blotting (WB) and Immunohistochemistry (IHC). The significance of the novel targets were studied by knockdown studies. The cDNA microarray of Tiam1 silenced cells to study the regulations altered by Tiam1 on the actin cytoskeleton interacting proteins, apoptotic initiators and tumorogenic potential targets. NCL expression was analyzed in primary human RB samples by WB and immunohistochemistry. miRNA microarray of the NCL aptamer treated cells was studied for revealing its importance in microRNA genesis. aptamer against NCL was tested for in vitro efficacy by studying the stability, cellular uptake, functional activity and induction of apoptosis in RB cell lines and in primary tumor cells.

Aptamer **chimeric conjugates were prepared** using different approaches.

1) NCL aptamer DNAzyme conjugate was prepared by complementary pairing using T-linker bridge in-between and characterized using agarose gel electrophoresis.

2) The chimeric siRNA construct was optimally designed, and the EpApt-siEp construct formed stem and loop structure.

3) The EpApt, siRNA decorated on PEI nanocore was constructed and characterized using dynamic light scattering and zeta potential measurements and electron microscopy. The gel retardation assay was performed to study the nanocomplex formation.

4) EpCAM aptamer doxorubicin conjugate was prepared by incubating both drug and aptamer and monitoring the changes in drug fluorescence emission by spectrofluorimetry and the drug release kinetics were studied by dialysis method.

5) EpCAM DNA aptamer fluorescent conjugate using bio-orthogonal chemistry was synthesized utilizing the strain promoted alkyne azide reaction. The SPAAC reaction ligation efficiency was improved by freeze-thaw cycle and characterized by electrophoresing on gel, thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization – Time of flight (MALDI-Tof). Stability was also assessed under physiological condition.

6) CSC marker CD44 aptamer was truncated to yield shorter species that can be used for theranostic purpose.

Thus the aptamers, aptamer chimeric conjugates generated was studied for their in vitro efficacy.
1) Functional activity of the NCL-aptamer-DNAzyme was evaluated using qPCR, WB, LDH and MTT assay.

2) The EpApt-siEp construct was tested *in vitro* by dicer enzyme, to elucidate the mechanism of siRNA generation adopted.

3) The siRNA bearing constructs were analyzed for the specific delivery by the flow cytometry and fluorescent microscopy.

4) The silencing efficiency was studied using northern blotting or qPCR and WB.

5) The cell cytotoxicity or cell viability was monitored by the release of lactate dehydrogenase assay (LDH assay) and/or colorimetry based assay, using MTT assay.

6) The changes in cell cycle and apoptosis were measured using PI based staining and anti-annexin V-FTIC based assay by flow cytometry.

7) The truncated aptamers were tested for their specific binding onto cancer cell lines, spheroids of breast cancer cell line, primary tumor cells and on the ABCG2+ve and CD133+ve cells. The CD133+ve cells were isolated using the magnabeads based method from RB, while ABCG2+ve cells from mammospheres.

8) The aptamer uptake and internalization were performed by flow cytometry and microscopy.

*In vivo* efficacy was tested for the construct that was stable and better under *in vitro* evaluation.

1) RB and epithelial cancer xenograft models were generated in nude mice and tested the efficacy of the construct by injecting the drug either subcutaneously near the tumor site or intraperitoneal.

2) The animals were monitored until the end of study period by measuring the body weight and tumor volume. Biochemical assays for the liver and kidney function were performed.
RESULTS

The study involved analysis of the novel targets, aptamers targeting them and aptamer chimeric conjugates against oncogenes in RB and epithelial cancers. The results obtained are listed below:

- Tiam1 was shown to be overexpressed in retinoblastoma (RB). cDNA microarray of Tiam1 silenced cells showed gene regulations altered predominantly the actin cytoskeleton interacting proteins, apoptotic initiators and tumorogenic potential targets.
- The silenced phenotype resulted in decreased growth and increased apoptosis with non-invasive characteristics. N-terminal region is responsible for membrane localization of
Tiam1, also required for F-actin interaction with Tiam1 in the membrane edges that leads to ruffling, and also imparts varying invasive potential to the cell.

- NCL was overexpressed in RB patients’ tumor tissues at mRNA levels and found two fold greater in RB cytoplasmic protein levels as compared to normal retina.
- The expression of surface nucleolin confirmed by flow cytometry and microscopy ensured the NCL-aptamer binding and uptake by RB primary cells as well as standard RB cell lines, WERI-Rb1 and Y79 cells.
- Silencing of NCL or functional blocking of NCL using aptamer significantly affected the expression of G-rich, Bcl2 and MYCN mRNA levels in WERI-Rb1 and Y79 cells. Induction of changes in cell cycle and cellular toxicity resulted in the cell death of RB cell lines.
- There was significant (P<0.001) downregulation of miRNA-17-92 cluster reported to be highly upregulated in RB, upon knocking down of NCL or treating cells with NCL-aptamer.
- Y79 xenograft animal model showed tumor growth inhibition of about 28% and 65% in the LNA-NCL-aptamer treatment administered by subcutaneous (s.c.) near tumor site and by intraperitoneal (i.p.) modes with multiple injections respectively.
- The expression of cancer stem cell markers, PCNA, apoptotic and inhibitor of apoptosis proteins (IAPs) and the serum cytokines levels were altered.
- The serum miRNA-18a expression, potential biomarker in RB, was also downregulated in vivo upon the aptamer treatment.
- Significant changes in the lipid profile were observed by DESI MS of the aptamer treated cells and the xenograft tumor tissues treated by s.c. mode.
  - The over-expression of EpICD in RB primary tumor samples revealed the cancer stem cell (CSC) property of EpCAM and also regulated stem cell marker expression.
  - EpCAM silencing using siRNA or EpApt-siEp construct showed downregulation of pluripotency markers, SOX2, OCT4, NANOG, survivin and CD133 in RB cell line.
  - The EpApt-siEp was shown to be processed in vitro by dicer enzyme and the EpApt-siEp construct significantly silenced (P<0.005) EpCAM in epithelial solid cancers such as WERI-Rb1- RB cell line and MCF7 cells- breast cancer cell line, evaluated by qPCR, northern and Western blotting. The knockdown of EpCAM significantly inhibited (P<0.01) cell proliferation and induced cellular cytotoxicity.
Chapter 1

- The in vivo studies using epithelial cancer model, MCF7 xenograft, as a proof of concept showed tumor regression (P<0.001) with complete tumor growth inhibition without any toxicity in the animals confirmed histo-pathologically.
- The tumor tissues showed significant (P<0.05) downregulation of EpCAM, MRP1, ABCG2, BCL2, stathmin and survivin expression while upregulation of BAX and ATM upon EpApt-siEptreatment. Apoptosis and serum cytokine array results showed the cell death mediated though the intrinsic apoptotic pathway without immune elicitation.
  - The PEI nanocomplex synthesized with EpCAM aptamer (EpApt) and EpCAM siRNA (siEp) showed the particle size of 198 nm in diameter by dynamic light scattering and a surface charge of -30.0 mV using zeta potential measurements.
  - Gel retardation assay confirmed the formation of PEI nanocomplex fabricated with EpApt and siEp (PEI-EpApt-siEp). TEM analysis showed spherical shaped particles, with size of 151±11 nm. The difference in size between the hydrodynamic size and TEM measurements could be due to coating of the aptamer and siRNA on the PEI nanocore.
  - Flow cytometry analysis revealed that PEI-EpApt-siEp nanocomplex has superior binding to the cancer cells compared to the EpApt or scramble aptamer (ScrApt) or nanocomplex functionalized with scrambled aptamer(PEI-ScrApt-siEp).
  - The qPCR and Western blot result showed silenced EpCAM expression selectively in the PEI-EpApt-siEp treated cells than the PEI-ScrApt-SiEp. The PEI-EpApt-siEp loaded nanocomplex also showed inhibition of cell growth and proliferation of MCF7 and WERI-Rb1 cell lines.
  - The PEI nanocomplex fabricated with EpApt and siEp was able to target EpCAM tumor cells, deliver the siRNA and silence the target gene. This nanocomplex exhibited decreased cell proliferation than the scrambled aptamer loaded nanocomplex in the EpCAM expressing cancer cells.
  - Formation of physical conjugates of EpApt and Dox was monitored with spectrofluorimetry. Cellular uptake of aptamer-Dox conjugates was monitored though fluorescent microscopy. Drug efficacy was monitored with cell proliferation assay.
  - The EpApt but not the Scr-EpApt bound to RB tumor cells, the Y79 and WERI-Rb1 cells. However, both the aptamers did not bind to the non-cancerous müller glial cells. The
chimeric EpApt-Dox and the Scr-EpApt-Dox were synthesized and tested on the Y79, WERI-Rb1, and m"ller glial cells.

- The targeted uptake of the EpApt-Dox aptamer caused cytotoxicity in the Y79 and WERI-Rb1 cells but not in the m"ller glial cells. There was no significant binding or consequent cytotoxicity by the Scr-EpDT3-Dox in either cell line. The EpCAM aptamer alone did not cause cytotoxicity in either cell line.

- EpCAM aptamer (SYL3C)-DIBO-AF594 fluorescent conjugate was synthesised using bioorthogonal chemistry utilizing strain promoted alkyne-azide cycloaddition (copper free click) reaction (SPAAC).

- The ligation efficiency of SPAAC was improved by freeze-thaw cycle.

- The obtained conjugate showed target specific binding and aided in imaging of various EpCAM positive cancer cell lines like MCF7, MDAMB453, WERI-Rb1 and PC3.

- The Pri-miR-17-92 aptamer (Pri-apt), potentially can replace the mix of five antagomirs with one aptamer that function by abrogating the maturation of mir-17, mir-18a and mir-19b (P<0.05).

- Cellular changes upon transfecting the Pri-apt lead to the S phase arrest in WERI-Rb1 cells, apoptosis onset in both Y79 and WERI-Rb1 cell lines.

- There was increased cytotoxicity as measured by the lactate dehydrogenase activity in Pri-apt treated Y79 cells (P<0.05) and significant cell proliferation inhibition in both cell lines.

- CSC aptamers showed uptake on MCF7 spheres and in primary RB cells with minimal toxicity on the cell lines.

- The cancer stem cell marker aptamers preferentially bind to the CD133+ve and ABCG2+ve cells and showed minor binding on normal retina.
Chapter 1

**Figure 3. Flow of work:** *In vitro* efficacy evaluation of aptamer and aptamer chimeras to *in vivo* evaluation in xenograft models. The flow summarizes the constructs analyzed and taken up further for the *in vivo* study.

**CONCLUSIONS**

- The results obtained from our study show for the first time that Tiam1 modulates the cell invasion, mediated by actin cytoskeleton remodeling in RB.

- Our result shows the higher and differential expression of NCL in RB tumor tissues for the first time. Targeting the NCL in RB was able to downregulate the cancer stem cell marker, G-rich mRNA and onco-miRNAs expression. *In vivo* effect of NCL aptamers showed active tumor reduction and reduction in the serum onco-miRNA. We show the proof of anti-tumor effect of NCL aptamer (NCL-aptamer and LNA-NCL-aptamer) in RB by modulating the G-rich oncogenic and CSC marker mRNA, miRNA-17-92 cluster, apoptotic proteins and phosphatidylcholine (PC) expression.

- Our results collectively revealed that EpApt-siEp construct can potentially be used for eradicating EpCAM positive cancer cells as well as other CSCs while sparing normal EpCAM negative surrounding cells.
Chapter 1

- The PEI nanocomplex fabricated with EpApt and siEp was able to target EpCAM tumor cells, deliver the siRNA and silence the target gene. This nanocomplex exhibited decreased cell proliferation than the scrambled aptamer loaded nanocomplex in the EpCAM expressing cancer cells.

- The EpCAM aptamer-Dox conjugate selectively delivered the drug to the RB cells thereby inhibited cellular proliferation and not to the non-cancerous Müller glial cells. As EpCAM is a cancer stem cell marker, this aptamer-based targeted drug delivery can prevent the undesired effects of non-specific drug activity and will kill cancer stem cells precisely in RB.

- The obtained conjugate showed target specific binding and aided in imaging of various EpCAM positive cancer cell lines like MCF7, MDAMB453, WERI-RB1 and PC3.

- Results showed anti-cancer property of Pri-apt in retinoblastoma, which can readily be translated by developing appropriate vectors for the delivery of the aptamer specifically to cancer cells.

- The CD44, ABCG2 and CD133 aptamers exhibited specific binding to ABCG2+ve cells and CD133+ve RB primary cells and inhibition of cell proliferation in RB primary cells.
1.1 CANCER

Cancer is an abnormal or mutated cells growing in an uncontrollable manner. Cancer is a deadly disease that can be prevented from leading to mortality provided early diagnosis and proper treatment. From the report released by Indian council for medical research (ICMR), projections were made for predicting the raise of cancer cases from 979,786 in the year 2010 to 1,148,757 cases by 2020 (Takiar et al., 2010). Not all tumors are cancerous. Benign tumors grow is comparatively controlled and they don’t invade neighboring tissues or spread to other regions the body. Hence tumors of malignant types are more concerned as they are resistant to therapy and difficult to eradicate and to prevent from relapse. Though there are well known cancer causing agents or environmental factors or genetic factors or diseases that turn cancerous, still there are cancers that occur though undefined mechanism.

According to the classification of types of cancer by cancer research UK, based on the function that they are involved or the niche they belong into epithelial tissue cells, connective tissue cells and cells of blood and lymphatics. The epithelial tissues covers most of the parts of the body, rather it lines the internal organs and systems. They line lungs and abdominal cavity. Carcinomas are the cancer of epithelial cell origin and they account for about 85% of cancers. The other carcinomas includes lung, breast, prostate, renal, rectum, pancreas and liver based on the origin from the organ type.

1.2 CURRENT TREATMENT MODALITIES AGAINST CANCER

Current treatment modalities against cancer include chemotherapy, radiotherapy, tumor excision, targeted therapy using monoclonal antibodies, nanoparticles loaded with drugs etc. All these treatment modalities have their own drawback and the excision of tumor also cannot remove tumor cells completely from the body. Chemotherapy and radiotherapy based treatments are not cancer cell specific and they destroy healthy cells too. It also results in short term to long term
side effects such as hair loss, ulcers, loss of fertility etc. Monoclonal antibodies are one step ahead of all the fore mentioned therapies because of its specificity towards cancer cells. But they face a different drawback, such that high cost of synthesis and stability issues.

The treatment currently in action is broadly classified into chemotherapy, radiotherapy. Those cancers which cannot be cured of the any of the above said methods wherein the risk of metastases onsets before the cancer tissue or the organ is excised out of the body. One such procedure is mastectomy where in the affected breast is removed to prevent from invasion to other parts. There are various researches undertaken for targeted therapy some of them are in application successfully such as Herceptin®, Avastin®, Afinitor® and Gleevec®. So research involving targeted therapy is of greater interest.

1.3 PAEDIATRIC CANCER

There is considerable increase in the number of diagnoses with pediatric cancer closely accounting for 2-3% out of other cancers. The childhood cancers are: leukemia, neuroblastomas, cancers of the brain and nervous system, RB, Wilms tumor, rhabdomyosarcoma and osteosarcoma. The occurrence of the leukemia is found to be approximately 30% and RB accounts for 3% (Cancer Facts and Figures 2012, American Cancer Society). Based on an annual survey in India it’s found that there are 1200 cases for RB per year and India is the country accounting with highest rate of RB incidence. According to the statistical analysis the death due to childhood cancer in India accounts for 148000 per year. Amonth the cancers, RB incidences account for 1.9 to 12.3 and 1.3 to 6.7 millions in boys and girls respectively between 2009 to 2011 (Satyanarayana et al., 2014).

1.3.1 Retinoblastoma

RB is a malignancy of the retina, which occurs in the intraocular region of the children below the age of 5 years. The majority of RB cases are of 2-3yrs age and rarely at the age of 5 and in adulthood. The disease is fatal when diagnosed at an advanced stage. As earlier records, the incidence of RB in India is very high compared to the other developing countries. There are two types of RB based on whether it affects either the eyes (bilateral) or a single eye (unilateral). Unilateral RB is the most common RB. RB occurs due to the mutation in the Rb1 gene in the chromosome region 13q14, that encodes for the tumor suppressor protein RB protein (Rb
The disease occurs in two forms: hereditary and non-hereditary. Hereditary RB1 mutations are due to germ line mutations and the mutations are found in all cells hence have higher risk of occurrence of other cancers like malignant melanoma and osteosarcoma.

All human tumors in general have deregulated growth signaling pathways. The fate of the tumor cell during the proliferation is governed by the RB protein (pRb) and p107 and p130.

**Figure 4. The cell cycle regulation mediated by the pRb.** The pRb interacts with E2F and activates transcription and leads to cell cycle transition. The pRb-E2F complex is disrupted upon phosphorylation of pRb, thereby cell cycle progresses. The free E2F activates further cyclins and cyclin dependent kinases and PCNA that speedup DNA polymerization, results in replication of DNA and cell division. Image redrawn based on Nature review, Molecular cell biology.

pRb in its hypophosphorylated state blocks the proliferation by altering the function of E2F transcription factors. E2F controls the expression of genes involved in the cell cycle transition from G1 to S phase (Figure 4). When the pRb is phosphorylated its action on E2F is disrupted and which leads to cycle progression from G1 to S phase (Hanahan and Weinberg, 2000).

The systematic classification of RB was published earlier as Reese-Ellsworth classification. Recently Sastre *et al.*, has classified and it elaborates the guidelines of classification of RB and handling of the enucleated eye with RB, as per the instructions of International RB Staging Working Group (IRSWG) (Sastre *et al.*, 2009).

The RB cells are typically round in shape which has a basophilic nucleus and the nuclear cytoplasmic ratio is very high and the nucleus varies in its size. Viable cells appear as ribbon-like
areas and they alternate the necrotic area of cells. Viable cells appear around blood vessels and areas of calcification are common in highly necrotic tumors. Differentiated tumors shows clusters of RB cells which are called as Homer-Wright rosettes and Flexner-Winter-steiner rosettes. The RB eye ball and stained sections in the figure 5 shows tumors invading laminar regions, optic nerve and anterior regions of the eyes.

Figure 5. Haematoxylin and eosin staining of RB tissue sections. A. Photomicrograph showing the RB tumor cells invading till the post laminar region of the optic nerve (ON). B. RB cells poorly differentiated arranged around the blood vessels. C and D. Gross slides of surgical end of enucleated globe of a patient with RB showing the tumor cells invading the optic nerve and ON completely invaded by tumor cells.
Chapter 1

1.3.2 Treatment for RB

The treatment of RB lies overall in saving the life of the child and to prevent from metastasis. In past external beam radiation was the most used; due to increased mutational risk alternate therapeutic regimens have been chosen such as systemic chemotherapy and aggressive focal consolidation with cryotherapy, transpupillary thermotherapy, plaque radiotherapy, brachytherapy and intra-arterial chemotherapy. In spite of the above represented treatment methods, enucleation becomes necessary wherein the risk of retinal damage and invasion onsets. Post enucleation chemotherapy is instructed to prevent the residual tumor cells proliferation. Intra-arterial therapy is too in action.

1.4 TUMOR ANTIGENS IDENTIFIED FOR TARGETED THERAPY

Several research groups are working on the RB and epithelial tumors and trying to develop antitumor molecules. Potential tumor targets identified so far some of them are MUC1, HER2/neu, TGF-beta, VEGF, MDM4, p53, Hdm2, EpCAM, CD44, and NCL (Even-Desrumeaux et al., 2011). There are oligonucleotide based therapies available almost for all the targets which includes siRNA, miRNA, DNAzyme, ribozyme based targeting of the tumor antigen expression thereby causing lethality to cells (Lee and Jeong, 2014, Mei et al., 2014, Olie et al., 2000, Rawlings-Goss et al., 2014, Won and Lee, 2012). On the other sector peptides and proteins such as monoclonal antibodies, affibodies, darpins, ankyrins and peptide aptamers are of therapeutic interest (Heskamp et al., 2012, Li et al., 2014a, Malmberg et al., 2011, Mern et al., 2010, Sorensen et al., 2014).

1.4.1 TIAM1

T lymphoma invasion and metastasis protein (Tiam1) was first identified as an invasion and metastasis inducing gene using T lymphoma cells by proviral tagging and in vitro selection for invasiveness (Habets et al., 1994, Habets et al., 1995). Tiam1 is a guanine nucleotide exchange factor (Wheeler et al.) that mediates the specific activation of Rac1 (Mertens et al., 2003, Michiels et al., 1995, Hordijk et al., 1997). Small guanine triphosphate (GTP) binding proteins
belonging to Ras superfamily act as molecular switches for activation of cellular activities such as signal transduction, actin cytoskeleton remodeling, microtubule stabilization, centrosome reorganization and intracellular trafficking (Adams et al., 2010, Xu et al., 2010, Connolly et al., 2005, Sander et al., 1998). Aberrations or mutations of this protein lead to malignancy of the cell as summarized in the schematic below (Figure 6). In response to extracellular signals, GEFs play a major role by catalyzing the activation of GTP-binding proteins by dissociation of guanosine diphosphate bound to it. RhoA, Rac1 and Cdc42 are key proteins of Rho family that depends on GEFs for their activation (Rossman et al., 2005, Liu et al., 2006).

![Diverse roles of Tiam1 in tumorigenesis](image)

**Figure 6. Diverse role of Tiam1 in tumorigenesis.** Tiam1 is overexpressed in cancerous condition and its overexpression is linked to promote functions that activate the cell proliferation and migration. Tiam1 regulates cell cycle progression by activating small GTPase to further activate cyclin dependent kinases for the cell division. Tiam1 also regulates cell invasion by its presence and interaction on the plasma membrane. The membrane ruffling mediated by Tiam1 also helps in cell migration and metastasis. The cellular transformation to metastatic phenotype leads to secondary colonization and tumor spread to other areas.

Tiam1 has been linked with cancer progression and having growth promoting functions based on the tumor type. Overexpression of N-terminus truncated Tiam1 is found to impart oncogenic activity in NIH 3T3 cells (Michiels et al., 1997, van Leeuwen et al., 1995). Similarly, mutations in Tiam1 gene are able to transform NIH 3T3 cells (Engers et al., 2000). Oncogenic potential of Tiam1 was found to be present in various tumors with respect to the tumor grade and stage. The over expression of Tiam1 in breast carcinoma, nasopharyngeal carcinoma, hepatocellular
carcinoma, renal cell carcinoma, retinoblastoma, colorectal carcinoma, lung and prostate cancer has been previously reported (Qi et al., 2009, Yang et al., 2010, Adithi et al., 2006, Zhao et al., 2011a, Zhong et al., 2009, Liu et al., 2005, Engers et al., 2006). Tiam1 is negatively correlated in case of renal carcinoma, where it inhibits invasion by promoting E-cadherin mediated adhesion (Engers et al., 2000).

Tiam1 contains consensus myristoylation sequence at the amino terminus, two PEST sequences, a Ras binding domain (RBD), PSD-95/DlgA/ZO-1 domain (PDZ), two pleckstrin homology (PH) domains and DH domain (Collard et al., 1996, Crompton et al., 2000, Terawaki et al., 2010). The PH domain present in carboxy terminal next to DH domain is similar in all other GEFs. The DH domain is important for the protein-protein interaction (Stam et al., 1997). Presence of Tiam1 on the membrane surface is accomplished though the N-terminal PH domain, and not by the c-terminal PH domain or DH domain. Using truncated constructs of Tiam1, localization of Tiam1 in the membrane is shown to be necessary for the membrane ruffling (Crompton et al., 2000, Ceccarelli et al., 2007, Fleming et al., 2004).

1.4.2 Nucleolin

Nucleolin (NCL) also known as C23, represents as much as ~10% of total nucleolar protein in cell, is located on chromosome 2q12-qter and consists of 14 exons and 13 introns (Srivastava et al., 1990). NCL is a multifunctional protein that binds to DNA, RNA, and protein, and is expressed ubiquitously in rapidly dividing eukaryotic cells (Derenzini et al., 1995, Ginisty et al., 1999). NCL actively participates in the transcription, growth and proliferation of cell. It is involved in DNA and RNA metabolism (Cong et al., 2012, Daniely and Borowiec, 2000), rDNA transcriptions in nucleolus and translocate rRNA from the nucleus to cytoplasm and provides posttranscriptional regulations, also shuttles proteins from cytoplasm to nucleus (Storck et al., 2007, Fahling et al., 2005) and also acts as receptor for different types of ligands and growth factors and microbes on the cell membrane (Otake et al., 2007, Hovanessian, 2006, Fujiki et al., 2014). Ligand interactions with C-terminal domain of NCL on cell membrane having nine tripeptide arginine-glycine-glycine repeats, RGG or GAR domain were shown to induce signaling events (Hovanessian et al., 2010, Arumugam et al., 2010, Nisole et al., 1999, Joo et al.,
The surface NCL, which is present under diseased condition mediates signals from membrane to nucleus, ligand binding to NCL activates signal transduction events leading to intracellular Ca2+ membrane fluxes, K-ras induced MAPK signaling, actin cytoskeleton and cancer stem cell signaling and so on (Figure 7) (Inder et al., 2009, Hovanessian et al., 2000, Grinstein and Wernet, 2007, Hovanessian et al., 2010).

Figure 7. Regulation mediated by nucleolin in the cell. Schematic representation of functions that NCL (red oval) mediate for tumorigenesis. NCL influences on the post-transcriptional regulation by maintaining mRNA stability (mRNA turnover and translation). NCL aids in the rDNA replication, transcription and transport of the rRNA to cytoplasm and the ribosome biogenesis. The NCL also interacts with the microprocessor machinery for the miRNA biogenesis. NCL expression and presence over the different compartment of the cell decides the normal state of the cell. The NCL over expression on the cell surface and cytoplasm leads to tumorogenic state of cell. Image was partly adopted from Abdelmohsen and Gorospe, 2012.
Chapter 1

The presence of surface NCL molecule in cancerous cells in contrast to the non-cancerous condition regards as promising target for the cancer therapy (Abdelmohsen and Gorospe, 2012, Fujiki et al., 2014, Hovanessian et al., 2010).

To eradicate the cancer cell population without harming the normal cells is of potential interest. One such targeting agent, a 26-mer DNA aptamer, NCL aptamer (NCL-aptamer) also known as AS1411 (Antisoma, UK), binds to NCL on the cell surface, preferentially gets internalized and inhibits cancer cell growth sparing normal cells (Bates et al., 2009, Destouches et al., 2008, Reyes-Reyes et al., 2010). The NCL present on the membrane shuttles to nucleus and cytoplasm and is delivers the aptamer, which binds to NCL already present in the compartment (Soundararajan et al., 2009). The mechanism of action of NCL-aptamer studied on prostate cancer elucidated its role in interference with the NFk-B signaling, leading to alteration in the levels of NCL in cellular compartment, blocking DNA replication, perturbing cell cycle at S phase thereby inhibiting cell proliferation (Xu et al., 2001). Also the aptamertreatment altered the localization of protein arginine methyltransferase 5 (PRMT5) and NCL complex, tumor suppressor (ST7) protein expression, thus mediated cell death (Girvan et al., 2006, Teng et al., 2007). It has shown promising anti-cancerous activity in prostate cancer, breast cancer, leukemia, gastric cancer with no systemic toxicity in phase I trial (Mongelard and Bouvet, 2010, Watanabe et al., 2010). Phase II clinical trial is underway though multi-institutional efforts (Rosenberg et al., 2014).

The interaction of NCL with the G-rich RNA sequences and binding to G-quadruplex structures in the promoter regions of C-myc and in the 5’UTRs of VEGF, Bcl2 mRNAs mediates regulation of oncogenes and angiogenic stimulus critical for tumorigenesis (Abdelmohsen et al., 2011, Abdelmohsen and Gorospe, 2012, Gonzalez and Hurley, 2010). The role of NCL in rRNA transcription and maturation though DROSH-DGCR8 microprocessor machinery was know and recently reports on the miRNA biogenesis of 15a and 16, and on the anti-tumor activity against breast cancer though miRNA regulation were also available (Pickering et al., 2011, Pichiorri et al., 2013). The cell surface NCL has shown to cluster with lipid rafts before getting internalized into cells (Destouches et al., 2008).
1.4.3 Survivin

Survivin, an inhibitor of apoptosis family protein, known as BIRC5, coded by the baculoviral inverted repeats BIRC5. It’s a 16.5 KDa, existing as dimeric protein in the cell. It is expressed in the developmental stage of human tissues, but not much expressed in the normal differentiated tissues (Li, 2003). In cancer conditions this protein is found to be highly expressed, which in turn inhibits the apoptosis of cancer cells (Baratchi et al., 2010, Kanwar et al., 2010a, Kanwar et al., 2013). Also this protein is reported to be associated with chemo and radio therapy resistance, tumor recurrence and shorter patient survival (Du et al., 2014, Hinnis et al., 2007, Wang et al., 2012).
Survivin, a multifunctional protein is involved in mitosis, apoptosis and cell motility. Survivin also provides proliferative and metastatic advantages to tumor cells. Survivin is required for the chromosomal passenger complex (CPC) formation with aurora B kinase and inner centromere protein (INCEP) to align chromosomes for mitosis. Survivin interacts with hepatitis B X-linked interacting protein (HBIXP) and X-linked inhibitor of apoptosis protein (XIAP) thereby prevents the activation of caspase-9, an effector molecule of programmed cell death. The pro-apoptotic molecule Smac, released from mitochondria interacts with survivin for its anti-apoptotic function. Survivin can also promotes cell motility though activation of AKT and up-regulation of α5 integrin. Image was adopted from (McKenzie and Grossman, 2012).

Strategies so far explored for targeting survivin includes, LY2181308, EZN3042, antisence oligonucleotides (ASO), YM155, survivin mutant proteins and antagonist proteins (Du et al., 2014, Raetz et al., 2014, Sriramoju et al., 2014, Wiechno et al., 2014). The above mentioned set of oligonucleotide based therapies are in clinical trials and has few disadvantages and side effects. The above mentioned mutant survivin proteins are of better avenue, dominant negative survivin mutant (Surv-D53A, SurR9C84A) to compete with the wild type (Newton et al.) survivin in the cellular functions, thereby leads to initiation of apoptosis.

Under cancerous condition, survivin localizes more to the mitochondria and the mitochondrial survivin content determines the susceptibility to apoptosis (Li et al., 1998). Presence of survivin in mitochondria acts as inhibitor of apoptosis by inhibiting the Caspase-9 activation and additionally Caspase-3 (Figure 8) (Kanwar et al., 2013, Kanwar et al., 2010c, Tamm et al., 1998, Tirro et al., 2006, Xi et al., 2011). Thus the survivin over expressing cells inhibits apoptosis, thus having increased cell proliferation, drug resistance. Survivin was reported to be highly expressed and secreted in serum in RB patients (Shehata et al., 2010, Wang et al., 2006b). Survivin cross talks with many signaling pathways, heat shock proteins, hypoxic proteins and mediates oncogenic role in various cancers and in RB (Kanwar et al., 2001, Jiang et al., 2008, Sudhakar et al., 2013). This makes the protein one of the best targets, prognostic marker for cancer and is of current interest in the study.

1.5 APTAMER BASED TARGETING

Advances in molecular biology have revolutionized the treatment strategies for cancer. One such strategy is development of aptamers (Tuerk and Gold, 1990). Aptamers are the functional nucleic
acid ligands that are single stranded oligo-nucleotides having specific binding towards the target antigen. Aptamers could be DNA aptamers, RNA aptamers or peptide aptamers based on the library used for screening (Figure 9). They are generated by a molecular selection process called Systematic Evolution of Ligands by Exponential Enrichment (SELEX) (Ellington and Szostak, 1990).

![Diagram of SELEX process](http://apcam.co.uk/TechAptamers.html)

**Figure 9.** Schematic representation of systemic evolution of ligands by exponential enrichment (SELEX). SELEX procedure begins with the initial incubation of the ssDNA or ssRNA library with the target (protein or cell) followed by the removal of the unbound oligos. The eluted bound oligos were PCR amplified and used for next round of binding. This process involves iterative cycles of binding and finally cloning the eluted sequence and finding out the aptamer sequence bound to the target. Adapter from the website: http://apcam.co.uk/TechAptamers.html
Aptamers are smaller in size, easy to synthesize and have greater affinity coupled with excellent target specificity and better stability avoiding immunogenicity (Riechmann et al., 1988, Verhoeyen et al., 1988, Ellington and Szostak, 1990, Tuerk and Gold, 1990). Aptamers could be functional blocking aptamers such as NCL aptamer or osteopontin aptamer or target binding aptamer such as the Prostate-specific membrane antigen (PSMA) aptamer for prostate cancer and Mucin 1, cell surface associated (MUC1) aptamer for breast cancer (Ray and White, 2010). Aptamer chimerization has been carried out for diversifying their use for targeted therapy. Aptamer chimerization process aims to combine two aptamers or an aptamer with another non-aptamer moiety such as biomacromolecules, drugs or dyes (Kanwar et al., 2011b). One combining partner recognizes the target and the other effect the function of the target molecule (Goldstein et al., 1995, Song et al., 2005). Chimerization either through natural recombination or chemical engineering may result in diminishing the activity of one or both of the recombinating partners. Therefore, research investigations are required to study chimeric aptamers (Chu et al., 2006, Hicke et al., 2006, Huang et al., 2009, Hwang do et al., 2010, Iida et al., 2014, Kanwar et al., 2010b, Kanwar et al., 2011b, Kanwar et al., 2014, Vorhies and Nemunaitis, 2007).

1.6 TARGETING CANCER STEM CELLS

Cancer cells have different cell types among which exists a subset of cells, with features of stem cells, and are recognized as cancer stem/progenitor cells (CSCs/CPCs). According to the CSCs hypothesis this subset of cells, having characteristics such as extensive proliferation, self renewal and can differentiate to multiple lineages and thereby acts as tumor initiating cells (Visvader and Lindeman, 2008). Their existence has opened up a new avenue of drug targeting. Progenitor cells have the above said features and it could be hypothesized that the CSCs may arise from mutation of such progenitor cells which usually lack self-renewal character (Jordan et al., 2006).
According to cancer stem cell hypothesis (i) cancers originate from tissue stem cells or their progeny and (ii) tumors maintain a subpopulation that retains stem cell properties (Wicha et al., 2006). Several cancer stem cells express different cell surface markers with which they can be isolated. Putative CSC markers identified for cancers are as follows, in the case of breast cancer CD44, CD133, EpCAM, CD24, CD29, ESA, Sca1, ALDH1 and CD90 were reported as cancer stem cell markers. Similarly CD20, CD133, ABCG2, ABCB5, CD166, ALDH1 and CD271 were identified as melanoma stem cell markers (La Porta and Zapperi, 2013). Pancreatic cancer stem cell markers include CD24, CD133, CD44, ESA and EpCAM. CD133, CD15 and CD90 were reported to be putative glioblastoma stem cell markers. Prostate cancer stem cells can be identified using CD44, CD24, EpCAM, CD49f, CD133, P63, Sca1, ABCG2, ALDH1 and CD166 surface markers. CD44 and ALDH1 were found to be overexpressed on head and neck cancer stem cells (Klonisch et al., 2008, Medema, 2013). In the case of hepatocellular carcinoma CD133, CD44, EpCAM, CD13, CD24 and CD90 were reported to be cancer stem cell specific (Salnikov et al., 2009). Ovarian cancer stem cells possess surface markers such as CD133,
CD24, CD44 and CD117. Surface antigens such as ABCG2, MCM2, CD44, CD133, EpCAM, CD90, ALDH1, CD24 and alkaline phosphatase were found to be over-expressed on RB cancer stem cells (Khetan et al., 2011).

In case of breast tissue differentiation EpCAM+ cells acts as CSC/CPCs (Sarrio et al., 2012, Yamashita et al., 2009) and in hepatocellular carcinoma EpCAM+ alpha feto-protein+ cells show characters of CSCs/CPCs (Yamashita et al., 2008).

1.6.1 EpCAM and EpCAM aptamer

Cancer stem cells for a number of different malignancies are capable of unlimited self-renewal and differentiation leading to tumorigenicity, cancer recurrence, and metastasis (Al-Hajj et al., 2003, Bapat et al., 2005, Reya et al., 2001, Szotek et al., 2006). These cells are chemotherapy and radiation therapy resistant. Therefore targeting these cells with newer therapeutic agents will eradicate the relapse and metastasis. Epithelial cell adhesion molecule (EpCAM) is putative cancer stem cell marker and is dysregulated in a number of epithelial cancers (Baeuerle and Gires, 2007, Maetzel et al., 2009a, Munz et al., 2009). Earlier, we showed that EpCAM is overexpressed in RB tumors, with choroid or optic nerve invasion (Krishnakumar et al., 2004). Therefore it is an ideal target molecule for RB therapy. The EpCAM gene silencing using siRNA reduced RB cell proliferation (Mitra et al., 2010). Cancer immunotherapy by using a bispecific EpCAM X CD3 antibody to redirect the T lymphocytes for targeting the EpCAM positive CSCs reduced proliferation (Mitra et al., 2012). Various other immunotherapy based clinical trials on pancreatic, ovarian and gastric cancers using anti-EpCAM antibodies are in progress (Gires and Bauerle, 2010). Both RNA and DNA aptamers were developed against EpCAM and are available for the theranostic purpose (Shigdar et al., 2011, Song et al., 2013).
Figure 11. Cancer stem cell markers in RB. RB cells have heterogeneous population with subset of cells expressing stem cell markers. The CSC markers reported are EpCAM, CD133, CD44, CD90, CD24, ALDH1, p63, MCM2 and ABC transporters. EpCAM is reported for its pluripotency maintenance property. The AC133 epitope of the CD133 represents its undifferentiated state of cells. ABC transporters aids in pumping out of drugs thereby imparting resistance to the cancer cells. CD24, CD90, CD44 and its variants have varying role in cancer stem cell maintenance, mechanisms being unraveled.

1.6.2 CSC markers and aptamers

Retinoblastoma is a childhood eye cancer and cancer stem cell markers have also been identified for this tumor (Balla et al., 2009, Mitra et al., 2012, Mohan et al., 2006, Seigel et al., 2005, Seigel et al., 2007). Surprisingly majority of the markers are similar to those expressed in epithelial cancers. The CSC markers identified in RB and other cancers include CD24, EpCAM, ABCG2, MCM2, CD44, CD133, CD90, ALDH1, CD166, ScaI, p63 etc., (Figure 11). These markers have been found to be co-expressed in many cancers. Among these markers CD133, CD44, EpCAM and ABCG2 were found to be expressed in majority of the cancers as surface antigens. These surface antigens provide an opportunity for imaging and targeted therapy that will effectively result in reduction of CSC population. CSC targeting can be accomplished effectively using antibodies, affibodies and aptamers.

Aptamers are oligonucleotide sequence that binds to specific proteins. These aptamers possess several advantages such as reduced immuno-reactivity, free from contaminations, smaller size,
high specificity, ease of synthesis and eligible for modifications such as locked nucleic acid (Sahoo et al.) or unlocked nucleic acid (UNA) which will increase their stability (Shigdar et al., 2013a). Various fields utilize aptamers ranging from sensing of cancer cells, toxins, diagnosis to imaging of cancer cells. Cell based systemic evolution of ligands by exponential enrichment (Cell-SELEX) is used for developing aptamers against tumor spheres and brain tumor initiating cells which will be more specific to the cancer type being targeted (Sefah et al., 2013).

RNA and DNA aptamer targeting EpCAM are developed and used for their specificity to deliver drugs, doxorubicin and curcumin (Li et al., 2014c, Subramanian et al., 2012). Similarly, RNA aptamer against CD133 that binds to the AC133 epitope was shown to penetrate hepatospheres that represent 3D model of cell culture (Shigdar et al., 2013b). DNA aptamers were also isolated against CD44 and ABCG2 using recombinant proteins, stable cell lines and mammospheres. The first CD44 aptamer isolated was thioaptamer against the HA-binding domain of the CD44 which was tested against ovarian cell line (Somasunderam et al., 2010). Later RNA aptamer targeting CD44 was also developed; they were longer with more than 50 nucleotides (Ababneh et al., 2013). In continuation, CD44v10 DNA aptamer selected against exon v10 was able to inhibit the breast cancer migration (Iida et al., 2014). ABCG2 over expressing BHK21 stable cell line was used for developing A12 aptamer and the A35 aptamer was developed against the mammospheres from MCF7 cells and found internalization of these aptamers independent of clathrin and caveolin mediated endocytosis (Palaniyandi et al., 2012).

CD133 is an important CSC marker and is expressed in retinoblastoma and many other cancers. Therefore understanding its structure details is important for drug delivery purposes. The interacting residues within the CD133 extracellular domain 2 (ExD2) that harbours the AC133 epitope, modelling and structure prediction of the CD133 protein is necessary as crystal structure is not available. AC133 epitope maintains the undifferentiated state of the cells, while the refolding of protein results in disappearance of the AC133 epitope leading to the loss of cancer stem cell property (Kemper et al., 2010).

In the current study, we utilized ABCG2 and CD133 aptamers mentioned above (Palaniyandi et al., 2012, Somasunderam et al., 2010) and focused on evaluating the functional activity on RB cell lines and primary tumors. We also truncated the CD44 thio-aptamer and performed computational modeling of CD133 and its aptamer to decipher the interacting residues.
1.6.3 miRNA-17~92 cluster and pri-miRNA aptamer

MicroRNAs (miRNA) are short RNA sequences measuring between 21-25 nucleotides in size that are naturally synthesized by cells (Kim, 2005). The primary-miRNA (Pri-miRNA) is transcribed in the nucleus from the encoded gene by RNA polymerase II. The pri-miRNA is further processed in the nucleus by Drosha/DGCR8 microprocessor complex to form precursor-miRNA (pre-miRNA). The pre-miRNA is exported out of the nucleus and into the cytoplasm by exportin5, where it is processed by the enzyme DICER to form mature miRNAs (Wahid et al., 2010).

The role of various troublesome mature miRNAs in cancer had been widely studied (Wu et al., 2007). In some cases, several mature miRNAs that are involved in cancer are produced from the same primary-miRNA transcript. One such example is miR-17~92 oncogenic cluster (also referred to as OncomiR-1), where six mature miRNAs such as miR-17, miR-18a, miR-19a, miR-20a, miR-19b and miR-92 are derived from the same pri-miRNA transcript (Hong et al., 2010, Olive et al., 2010). The miR-17~92 cluster is overexpressed in many cancers such as breast, colon, lung, pancreas, prostate, stomach and medulloblastoma (Chen et al., 2011b, van Haaften and Agami, 2010, Volinia et al., 2006). Earlier we reported the overexpression of miR-17~92 in RB (RB)-child hood intraocular tumor. The miR-17~92 cluster expression was observed both in the primary tumor samples, RB cell lines and also in serum of RB patients (Beta et al., 2013, Kandalam et al., 2012).

Conkrite et al., elucidated the importance of this miRNA cluster behind promoting the oncogenesis and showed that expression of miRNA cluster with the background of RB gene (Rb) mutation and loss of p107 leading to brain metastasis(Conkrite et al., 2011). Later, this OncomiR-1 cluster was individually been targeted in several studies using specific antagomirs (Venturini et al., 2007, Jin et al., 2014, Tao et al., 2012). A RNA aptamer that targets the primary miR-17~92 transcript was obtained by Systemic Evolution of Ligands by EXponential enrichment (SELEX) to collectively inhibit the biogenesis of miRNAs in the cluster (Lunse et al., 2010). The miR-17~92 cluster and the paralog cluster miR-106-25b were shown to be efficiently blocked by locked nucleic acid modified seed sequence. The antagomir seed sequence
of miR-17 and miR-19 overlaps between both the miRNA clusters. The use of antagonir seeds in cell culture leads to decrease in cell proliferation, reduction in tumor growth, prevented metastasis and increased the survival rate upon testing \textit{in vivo} experiments (Murphy et al., 2013).

1.7 ANTISENSE OLGIO BASED TARGETING

The use of antisense molecules with hindering viral replication was elucidated by Stephenson and Zamecnik (Zamecnik and Stephenson, 1978). Catalytically active RNA molecules, Ribozymes (Rz) were discovered followed by the report of DNA having mRNA cleavage by high sequence specificity activity, termed as DNAzyme (Breaker and Joyce, 1994, Breaker and Joyce, 1995). It is also referred as Deoxyribozyme, also as DNAzyme (Dz), catalytic DNA or DNA enzyme, is single stranded synthetic DNA molecule which is catalytically active on nucleic acid targets (Bhindhi et al., 2007). DNAzyme is more stable, easy to synthesis and less expensive than ribozyme, siRNA and antisense oligonucleotide, and hence is preferred over other targeting approaches.
Figure 12. Antisense and oligonucleotide based targeting of cancer cells. Gene silencing was achieved efficiently with higher activity while lesser toxicity using the oligo based drugs such as siRNA/shRNA, DNAzymes, ribozymes.

On the other hand small interfering RNA (siRNA), antisense oligonucleotides (ODNs) and ribozymes have emerged as sequence-specific inhibitors of gene expression that may have therapeutic potential in the treatment of a wide range of diseases. Due to their rapid degradation in vivo, the efficacy of naked gene silencing nucleic acids is relatively short in its half life (Burnett and Rossi, 2012). The gene silencing attained by different types of oligonucleotides are summarized in figure 12.

1.7.1 Silencing RNA/ short hairpin RNA

Gene expression is regulated at numerous steps, ranging from initiation of transcription to post-translational modification until its degradation. The advent of small RNA-mediated gene-silencing mechanism (Zamore and Haley, 2005) was extensively investigated due to its important regulatory roles in diverse organisms. The gene silencing mechanism involves trans-acting small RNAs such as siRNAs and miRNAs as indispensable regulatory RNA elements. siRNAs are essential molecules in the RNA interference (RNAi) pathway. During RNAi, long double-stranded RNAs (dsRNAs) or short hairpin RNAs (shRNAs) are processed by the RNase III enzyme Dicer, and the resulting siRNAs of ~21 nt in length function as guides for specific mRNA degradation in the RNA-induced silencing complex (RISC)(Dorsett and Tuschl, 2004, Meister et al., 2004, Meister and Tuschl, 2004). In fact, RNAi is now widely used as a simple and effective method to knock down gene expression in diverse organisms. Furthermore, therapeutic applications of siRNAs are more explored and brought upto phase III clinical trials in human beings (Burnett and Rossi, 2012).

Compared to siRNAs, which transiently silences target gene expression shRNAs are constitutively expressed from promoters. They result in long-term gene silencing due to their extended transcription and biogenesis within cells. Although higher levels of shRNAs may be preferred to achieve maximum knockdown of the gene target. Such expression from RNA Pol III promoters tend to saturate the small RNA biogenesis, due to their strong transcriptional activity and results in severe toxicity (Castanotto et al., 2007). Thus shRNAs were expressed as a
multicistronic transcript from RNA Pol II promoter or with other non-RNAi therapies like ribozymes and small RNA decoys (Zhang and Rossi, 2010). Similar to other RNA-based therapeutics, the efficacy of siRNA/shRNA drugs depends on higher targeted delivery while lesser off-target toxicity and degradation. Currently clinical trials using different siRNA/shRNA delivery methods are ongoing (Burnett et al., 2011).

1.7.2 DNAzyme

Deoxyribozymes, referred as DNAzymes, catalytic DNA or DNA enzymes, are single stranded synthetic DNA molecules which are catalytically active on nucleic acid targets (Breaker and Joyce, 1994). DNAzymes being more stable, easy to synthesis and less expensive than ribozymes, siRNA and antisense oligonucleotides, are preferred for various targeting approaches. The 10–23 DNAzyme is composed of a catalytic domain of deoxyribonucleotides flanked by two substrate recognition arms. It can cleave any RNA substrate between an unpaired purine (A, G) and a paired pyrimidine (U, C) in the presence of Mg²⁺ (Breaker and Joyce, 1995). The 10–23 DNAzyme has exceptional catalytic efficiency. The general structure of the 10–23 DNAzyme is shown in Figure 12. The 10–23 DNAzyme has many advantages over other Dzs and antisense drugs, applied in antiviral and anticancer drug delivery (Xu et al., 2012).

DNAzyme against cancer cells was first developed against the variants of p210 bcr-abl gene and p190 variant (Wu et al., 1999). Cieslak et al designed DNAzymes against β1 and β3 integrins which are essential for cell-cell and cell-matrix interactions in tumors (Cieslak et al., 2003). DNAzyme that cleaves VEGFR2 mRNA was found to reduce 75% of tumor in mice (Dass and Choong, 2008). DNAzymes against β-catenin mRNA was found to inhibit colon cancer cells by reducing β-catenin transcripts (Choi et al., 2010). Dz13, Dz targeting c-Jun inhibited skin cancer and squamous cell carcinoma and is in the process of clinical trials in humans (Cai et al., 2012). DNAzyme targeting survivin mRNA was developed and found to cleave survivin mRNA efficiently in time dependent and dose dependent manner in pancreatic carcinoma cell lines (Liang et al., 2005). Several approaches have been used for DNAzyme delivery including PLL and PLGA microspheres, transferrin modified PEGylated polyplexes, dendrimers and nanoparticulate systems (Xu et al., 2012).
1.7.3 Ribozyme

Ribozymes are naturally occurring RNAs harboring catalytic activities having cis- or trans-cleavage of RNA at specific sequence sites. The mechanism behind this activity has been explored for targeted gene inactivation in cells. Among the antisense oligos, ribozymes act as important functional genomics tools, generally applied in drug target recognition and affirmation in drug discovery. The hammerhead RNA, like other naturally occurring ribozymes, is a metalloenzyme that requires a divalent metal ion, such as Mg$^{2+}$, to mediate catalytic cleavage (Scott and Klug, 1996). The types of Rz include, the hammerhead, hairpin, hepatitis delta virus (HDV), Varkud Satellite (VS), and \textit{glmS} ribozymes that catalyze sequence-specific intramolecular cleavage of RNA. They range between 50 and 150 nucleotides in length, and are known as the “small self-cleaving ribozymes.” The \textit{glmS} ribozyme are present in Gram-positive bacteria and functions as a riboswitch and were originally discovered as domains of satellite RNAs (Ferre-D'Amare and Scott, 2010).

Both hammerhead and hairpin ribozymes, are well known as small catalytic RNAs, that catalyze either cleavage or ligation of RNA with sequence specificity. Although they have been applied to gene therapy and gene discovery, by cutting the mRNAs of target genes, these ribozymes normally do not have the ability to regulate their catalytic activities. Therefore, allosteric control has been applied to the ribozymes (Scott and Klug, 1996).

Ribozymes that are coded by adenoviral system that can target and reprogram human cytoskeleton-associate protein 2 (hCKAP2) showed tumor retardation \textit{in vivo} in xenograft model (Lee and Jeong, 2014). A splicing variant of AIMP2 lacking exon 2 (AIMP2-DX2) is specifically generated by an alternative splicing process and is highly expressed in human lung cancer cells and the tissues of cancer patients. A specific ribozyme that can target and replace the splicing variant AIMP2-DX2 RNA with a new transcript selectively exerting therapeutic activity in AIMP2-DX2-expressing lung cancer cells by retarding the growth of cancer cells (Won and Lee, 2012). Unlike, siRNA or shRNA, ribozyme use in the cancer therapy is being studied and optimized.

Thus the oligonucleotide based targeting is much efficient and harbor lesser off-target effects. The non specific side reactions and the activities are controllable, thus preferred over other therapeutic approaches.
1.8 NANOCARRIER BASED TARGETING

Targeted drug delivery is a challenging task as it deals with biological system, wherein specific targeting of a receptor that differentially expressed under diseases is important. Nanoformulation is preferred while drug delivery systems are considered and they are formed using biological substances, such as albumin, gelatin and phospholipids for liposomes, to chemical substances, such as various polymers and solid metal-containing NPs (Figure 13). Polymer–drug conjugates due to high size variation are normally not considered as nanoparticles (NPs). As their size is controllable within 100 nm, they are too included in nanodelivery systems. These nanodelivery systems are designed to have drugs absorbed or conjugated onto their surface. Drugs are even encapsulated inside the polymer/lipid or dissolved within the particle material. Thus the drugs are protected from a hostile atmosphere or their adverse biopharmaceutical properties can be masked and replaced with the properties of nanomaterials. Additionally, nanomaterials accumulate at tumor site due to enhanced permeability and retention (EPR) effect. The EPR effect helps in site-specific accumulation of nanomaterial resulting in increased selective targeting.
Figure 13. Nanomaterials used for the drug delivery purposes. The NPs include nanocrystal, liposome, polymeric micelle, protein-based nanoparticle, dendrimer, carbon nanotube and polymer–drug conjugates. Image adopted from website: Nanomedicine 2012.

The polymeric NPs are colloidal in nature with a size range of 10–1000 nm. They exist with spherical, branched or core–shell structures and are fabricated using biodegradable synthetic polymers, such as polylactide–polyglycolide copolymers, polyethyleneimine, polyacrylates and polycaprolactones, or natural polymers, such as albumin, gelatin, alginate, collagen and chitosan (Panyam and Labhasetwar, 2003).

1.8.1 Targeting cancer using polymeric-PEI nanocarrier

Polymer based nanocarriers show less immunogenicity, toxicity and has better nucleic acid delivery compared to the viral carriers. Polymeric nanocarriers such as polyethyleneimine –PEI
Chapter 1

are one of the most explored cationic carriers due to its high transfection efficiency (Luten et al., 2008). There are viral, non-viral nanocarriers available for the delivery of siRNAs (Subramanian et al., 2009, van den Boorn et al., 2011). Cell penetrating peptides and affibodies that can deliver siRNA are as well reported (Kim and Rossi, 2007). Among the polymeric nanocarriers, PEI has the best properties for the condensation of nucleic acids into a nanosized complex. The positively charged PEI polymer, complexes with negatively charged oligonucleotide though electrostatic interaction and forms a stable nanocomplex even in the presence of the serum (Grayson et al., 2006, MADY et al., 2011, Slita et al., 2007). Therefore, the PEI nanocomplex has been efficiently used for the delivery of siRNA using target specific antibody or affibody or aptamer. Also PEI nanocarriers were reported for their non-mutagenic property and not to induce inflammatory response (Beyerle et al., 2011a, Bonnet et al., 2008b).
CHAPTER 2

Materials and methods
**Chapter 2**

**In vitro materials and methods:**

### 2.1 Materials

Roswell Park Memorial Institute (RPMI) 1640 media, heat-inactivated fetal bovine serum (FBS), poly-L-lysine (PLL) were purchased from Sigma Aldrich (St. Louis, MO). Cell culture plastic wares (T25 flasks, 96, 24, 12 and 6 well plates, 10ml and 5ml serological pipettes, 0.22 μm low protein binding syringe filters were purchased from BD bioscience or Sigma (Bangalore, India). Microtips, aerosol resistant tips were purchased from tarsons, oxygen and thermostcientific (Chennai, India). Tri-Reagent, diethyl pyrocarbonate (DEPC), formamide, ammonium per sulfate, TEMED, sodium dodecyl sulphate, bio-process grade water free of DNase and RNase, were purchased from Sigma Aldrich (Bangalore, India). Other chemicals such as acrylamide, bis-acrylamide, tris, glycine, salts were purchased from SRL biotech. Chemicals were procured from Sigma Aldrich (Bangalore, India) unless otherwise stated.

Branched polyethyleneimine polymer (60 KDa, catalog #P3143) and sodium citrate (catalog #C8532) were purchased from Sigma Aldrich (Bangalore, India). EpCAM aptamer (EpApt) with 2’F pyrimidines and scramble aptamer (ScrApt) with 2’OMe pyrimidines carrying the sequence (5’GCGACUGGUUACCCGGUCG-3’) and SYBR green master mix (Finnzymes) was obtained from thermo fisher scientific (Lafayette, CO). Both EpCAM and scramble aptamer were fluorescein-labeled (FI), siRNA against EpCAM (SiEp) targeting the transcript region 5’-GGAACUAAUGCUAACUATT-3’ were purchased from Qiagen (Mainz, Germany). QPCR primers were obtained from Sigma-Aldrich (Bangalore, India).

The DNA aptamer against EpCAM was commercially synthesized from integrated DNA technologies (Coralville, IA, USA). The 42 nucleotide aptamer carrying 5’ azide modification had sequence 5’ /AzideN/CA CTA CAG AGG TTG CGT CTG TCC CAC GTT GTC ATG GGG GGT TGG CCT G -3’. The cyclooctynyl, alkyne with fluorescent dye, Alexa flour 594(DIBO-AF594) was purchased from invitrogen biosciences. Bioprocess grade water with no RNAse and DNase was used for the reaction. HPLC from Agilent, with ZORBAX bioseries oligo column, 80mm x 6.2mm ID, was used for analysis and purification of conjugates. Centrifugal spin-filters with 3KDa MWCO were used for desalting and concentrating of conjugates. ITLC strips with UV260 fluorescence was obtained from Sigma Aldrich (Bangalore,
India) and 4% agarose gel electrophoresis were used for monitoring the click reaction. Mass analysis was performed in MALDI-TOF using 3’hydroxypicolinic acid as matrix.

The NCL aptamer (NCL-aptamer) with and without FITC at the 5’end of 5’GGTGGTGGTGTTGTTGTTGTTGGTGG 3’ and LNA-NCL-aptamer with and without Cy5.5 carrying LNA modifications at 3 positions (2 modifications in the termini and one internally) in the NCL-aptamer (NCL-APT) sequence was purchased from Sigma Aldrich, India and Exiqon, Denmark respectively. Unlabeled aptamer was used for studying the functional effect of aptamer.

NCL APT with linker labeled with Cy5 (Apt-Linker) was synthesized from integrated DNA technologies (Coralville, IA, USA), survivin DNAzyme (Sur_Dz), mutant survivin DNAzyme (Sur_mDz) were synthesized from VBC biotech (Wien, Austria) and fluorescein labeled survivin DNAzyme (Sur_fDz) was obtained from Sigma Aldrich (Bangalore, India). DAPI (Sigma Aldrich, Bangalore, India), prolong gold anti-fade mountant (Invitrogen lifescience, Bangalore, India).

2.2 Fabrication of LNA modified aptamer chimera and truncation of CD44 aptamer

Cancer stem cell markers, EpCAM, CD133, CD44 and ABCG2 aptamers were studied. The LNA modified EpCAM RNA aptamer was fabricated by modifying the sequence with three LNA nucleotides at termini and one in the internal region. The LNA EpCAM aptamer-siRNA chimera (L-Ep-siRNA) were constructed as described in McNamara et., al. 2009. The secondary structure prediction of the chimeric construct was performed using RNA structure v5.3 software (Bellaousov et al., 2013) and Mfold online software (Zuker, 2003). The designed L-EpApt and L-Ep-siRNA chimeric constructs were commercially synthesized by Exiqon (Vedbaek, Denmark). The CD44 thioAPTs were truncated further to harbor the loop and the few nucleotides from the stem region, so as to maintain the predicted secondary structure. The TA1 and TA6 shown to bind to the ovarian cancer cells, were truncated and used in the study. The ABCG2 DNA aptamers, CD133 RNA aptamer A12, A35 and A15 were used as for this study.

2.3 Cell culture
Chapter 2

Human RB cell lines Y79, WERI-Rb1, breast Cancer cell line MCF7, MDAMB453 and prostate cancer cell line PC3 obtained from the cell bank, RIKEN bioResource center (Ibaraki, Japan) and non-cancerous Müller glial cell line gifted by Dr. Limb (MIO-M1) were included in the study. ARPE-19 cell line purchased from ATCC was obtained as gift from department of biochemistry and cell biology, VRF. Y79, WERI-Rb1 and PC3 cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS) and MCF7, MDAMB453 and MIO-M1 cell lines were cultured in DMEM media with 10% FBS (Gibco, Invitrogen, India) along with 1% pen-strep (Hi-Media, Mumbai, India). The cells were maintained in 5% CO2 incubator at 37°C.

2.4 Sample collection and Ethics statement

Primary RB tumor cells obtained from the enucleated eyes well resuspended in RPMI media and cultured in RPMI media containing 20% FBS with other similar conditions. RB tumor samples from enucleated eye balls were collected as part of therapy and utilized for research purpose anonymously. A general written consent was obtained from the parents/guardians of the patient undergoing enucleation. Retina (normal or non-malignant) samples were collected from normal cadaveric donor eyeballs from C U shah eye bank, medical research foundation, sankara nethalaya, India. The study was performed in accordance to the declaration of the Helsinki, at vision research foundation, after obtaining the approval from the ethics sub-committee (Institutional Review Board) of sankara nethalaya eye hospital [Ethical clearance. no. 136-2008-P, 240-2010-P, DBT-318-2012-P].

2.5 RB tumor cells, cell lines –Total RNA isolation and Quantitative PCR

a. Tiam1

The total RNA was isolated from transfected cells and fresh RB tumors by RNAeasy kit (Qiagen, Valencia, USA) and Trizol respectively. 1μg of total RNA was transcribed into cDNA using oligo-dT and random hexamers. The quantitative PCR was performed in applied biosystem 7500 by using SYBR-green (Thermoscientific, Mumbai, India). Comparative quantification (normal or non-malignant retinas primary RB tumors or untreated cells vs Tiam1 siRNA treated cells) was determined using the formula $2^{-\Delta\Delta Ct}$ and relative expression values normalized to the 18S or
β2M endogenous control were used for plotting. Experiments were performed in triplicate for the same samples.

**b. Oncogenes and cancer stem cell markers:**

Real-time PCR was performed to check the expression levels of oncogenes or potential cancer stem markers in RB tumors vs normal or non-malignant retina and between the cell lines used in the study. RNA extraction from the tumor cells, normal retina, Y79, WERI-Rb1, MIO-M1, MCF7, MDAMB453 and ARPE-19 cell lines was performed using Trizol based method followed by cDNA synthesis using Verso cDNA synthesis kit (ABgene, Epsom, Surrey, United Kingdom) with oligo dT primers and 1μg of the total RNA, QPCRs were performed using SYBR green based method using ~50ngs of cDNA for NCL and β2M, β2-micoglobulin. The thermal profiles are initial denaturation at 95°C for 10 mins, cycle denaturation for 95°C for 10 secs, annealing and extension at 60°C for 1 min. Data collections were set at extension step as well in the melt curve step. The quantitative PCR was performed in Applied Biosystem 7500 by using SYBR-green Dynamo HS master mix (Thermoscientific, Mumbai, India). The primer sequences for NCL and β2M were and other primers used in the study were listed in results chapter. Comparative quantification (normal or non-malignant retina vs primary RB tumors or cell lines) was determined using the formula 2^{-ΔΔCt} and relative expression values normalized to the β2M endogenous control were used for plotting. Experiments were performed in triplicate for each sample.

**2.6 Expression of NCL on tumors and cell lines:**

The expression level of NCL was studied by immunohistochemistry (IHC) of the RB tissue sections and normal retina, flow cytometry of the respective cells and by western blotting of the nuclear and cytoplasmic fractions extracted from the RB and normal or non-malignant retina (NR) cells for NCL. IHC was performed on de-paraffinized tissue section, post-antigen retrieval by pressure cooker method, using anti-NCL antibody (sc-56640, Santacruz biotech and anti-NCL, ab13541, Abcam) at 1:200 dilution with novolink minpolymer detection system (Leica biosystems). Briefly, post-primary block, followed by polymer incubation for 90mins followed by DAB chromogen based detection. Slides were mounted and observed under light microscope. To check the expression levels of NCL in nuclear and cytoplasmic compartments of RB and NR
Chapter 2

cells, nuclear and cytoplasmic proteins were extracted following the manufacturer’s instruction using NE-PER™ nuclear and cytoplasmic extraction kit (Thermoscientific, Rockford, USA). Western blotting was performed on nuclear and cytoplasmic lysate for NCL (anti-NCL, ab13541, Abcam) and β-actin (raised in mouse, Sigma Aldrich). To study the expression of surface NCL, flow cytometry was performed on unfixed RB cell lines as well on RB tumor cells obtained from enucleated eyes. Briefly, 2X10^5 cells were washed with 1X PBS followed by the addition of rabbit anti-NCL (abcam, 0.8μg/ml) for 2h at 4°C, FITC conjugated anti-rabbit (sc-2012, 1:300 dilution) for an hour followed by washing and flow cytometry analysis.

2.7 Analysis of EpCAM with flow cytometry

For studying EpCAM expression, cells were washed two times with 1X phosphate buffered saline (PBS; 1X PBS, prepared from 10X PBS, Himedia, Mumbai, India) and resuspended in blocking buffer (PBS with 0.02% sodium azide and 0.1% BSA [BSA]). The cells were incubated with the anti-EpCAM C-10 primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 1 h, washed twice with PBS, followed by incubation with the fluorescein isothiocyanate conjugated anti-mouse immunoglobulin-G secondary antibody (Sigma Aldrich, India) in blocking buffer for 45 min in the dark, and then followed by two washes with PBS. The fluorescence signal was read using flow cytometry (FACS Calibur flow cytometer; BD Biosciences, San Jose, CA), using the CellQuest software program (BD Biosciences).

2.8 RNA aptamers

EpCAM aptamer (EpDT3) and scrambled aptamer (Scr-EpDT3) with and without fluorescein (FI) fluorophore were custom synthesized by dharmacon Inc. (Lafayette, CO). The sequence of the aptamer is 5'-GCG ACU GGU UAC CCG GUC G-3'. Both aptamers have identical sequences except 2’ modifications. EpDT3 has 2'-Fluoro modified pyrimidines, and Scr-EpDT3 has 2’-OMe modified pyrimidines. Methyl modification at the 2’ position hampers binding of the aptamer to the receptor (Shigdar et al., 2011). Therefore, the aptamer served as negative control to study the functional role of the aptamers. A fluorescent (FI) labeled aptamer was used for the flow cytometry and microscopic studies. Aptamers were annealed at 70 °C before the conjugation or to the binding experiments.
2.9 Fabrication of EpApt-siEp chimeric construct

EpApt-siEp was constructed as described in Dassie et., al. 2009 (Dassie et al., 2009). In their study, stem and loop aptamer siRNA chimera with the strand swap (guide vs passenger strand) exhibited better silencing compared to other chimeric forms. Hence in the current study, we fabricated aptamer chimera by extending the aptamer sequence with siRNA sequence at its 5’end and 3’end. The fabricated EpApt-siEp carried siRNA targeting EpCAM. The EpCAM siRNA sequence used in the current study is taken from our previous publication (Mitra et al., 2010). The transcriptional changes accompanying knockdown of EpCAM using this siRNA is well validated before. As this siRNA efficiently silenced EpCAM, the same sequence was employed in the fabrication of chimeric construct. The secondary structure of the construct was studied by RNA structure v5.3 (Bellaousov et al., 2013) and Mfold software (Zuker, 2003). The designed construct was commercially synthesized by Dharmacon Inc (GE life sciences, Lafayette, CO).

2.10 In vitro dicer Assay

In vitro dicer Assay was performed to show that our EpApt-siEp chimera is being processed by dicer for the release of siRNA from the aptamer. To ensure proper secondary structure formation of the RNA chimeric construct, 1 μg of the sample suspended in a total volume of 10 μl nuclease free water was heated in boiling water (to denature the sample) for 10 min and allowed to gradually cool (to anneal the sample) to RT. A 10 μl reaction was set up by using 500 ng of the annealed RNA chimera, 1 μl of 10 mM ATP, 0.5 μl of 50 mM Mgcl2, 4 μl of 10X Dicer buffer and 2 μl (1 Unit) of Dicer enzyme. A control reaction was also set up without the enzyme. Both the reactions are incubated at 37°C for 18 h and 2 μl stop solution was added to stop the enzyme activity. The samples were run on a 15% polyacrylamide gel and visualized on a UV transilluminator (Recombinant Human Dicer Enzyme Kit Cat No: T510002).

2.11 Synthesis and stabilization of PEI: sodium citrate nanocomplex

PEI stock solution (100μg/ml) was prepared and the pH was adjusted to 6.0 using 1N HCl. The charge ratios of 1:1, 1:1.5, 1:3, 1:5 and 1:7 were prepared between PEI and sodium citrate based on the amine to the carboxylic groups present in the complexes (Zhao et al., 2011b). The reaction was incubated for 10 minutes in a reaction volume of 1ml. The size and the zeta-potential of the nanocomplexes were measured using Zetasizer Nano ZS (Malvern). The ratio 1:1.5 of PEI to
Chapter 2

sodium citrate was used for studying the cytotoxicity in the cell lines and also for synthesizing the PEI-EpApt-siEp and PEI-ScrApt-siEp nanocomplex.

2.12 Fabrication of PEI-aptamer-siRNA nanocomplex

The PEI concentration (0.3μg/ml) that was nontoxic to the cells was chosen and complexed with EpCAM aptamer (EpApt) and EpCAM siRNA (siEp). Different concentration of EpApt (100nM to 300nM) and SiEp (100 nM to 300 nM) were added to the PEI and incubated at room temperature for 10 minutes. The PEI alone and PEI-aptamer-siRNA (PEI-Apt-siRNA) nanocomplexes were electrophoresed on 2% agarose gel in TAE buffer to confirm the complex formation. The size and zeta potential of the nanocomplex saturated with aptamer and siRNA were measured using zetasizer. The size of PEI nanocomplex was tested in serum supplemented and serum free media using zetasizer.

2.13 Characterization of the PEI-aptamer-siRNA nanocomplex

The size and shape of the PEI-Apt-siRNA nanocomplexes were studied using Transmission electron microscopy (TEM). Briefly, TEM was performed for the PEI-Apt-siRNA nanocomplex (200 nM EpApt and 200 nM siRNA), mixing and dispersing with ethanol to form a dilute suspension and loaded onto the carbon coated copper grid. The PEI nanocomplex was imaged at 80V (TEM, Philips, CM12 STEM, Netherlands).

2.14 Aptamer-doxorubicin conjugation and characterization

Doxorubicin (fluorescent grade, Sigma Aldrich, Bangalore, India) was conjugated to EpDT3 and Scr-EpDT3 in conjugation buffer (0.1 M sodium acetate, 0.05 M NaCl, and 0.1 M MgCl₂). Aptamer-Dox conjugation was performed by increasing the molar ratios of the aptamer (0, 0.01, 0.1, 1, 3, 5, and 7 equivalents) to constant Dox (3 μM). Fluorescence quenching of the Dox due to the intercalation of Dox to the aptamer (aptamer conjugation) was monitored using spectrofluorimetry (SpectraMax M2 spectrofluorophotometer; Molecular Devices, Sunnyvale, CA) at a constant excitation at 470 nm (Bagalkot et al., 2006). The Dox-conjugated aptamers (1:1 molar ratio) were purified from the free Dox by passing though a NAP 10 column (G25-DNA grade column, GE Healthcare, Bangalore, India). Characterization of the intercalation reaction was monitored by decrease in the emission spectra of the conjugate reaction.
Chapter 2

2.15 Release and diffusion of doxorubicin from the aptamer-doxorubicin conjugates

Drug release and diffusion from the chimeric aptamer *in vitro* were studied by monitoring the passage of Dox under conditions that simulate the physiologic conditions (Bagalkot et al., 2006). About 100 μl of the aptamer-Dox conjugates were dialyzed (3.5 kDa cut-off, Pierce) in conjugation buffer at 37 °C. Samples were collected at various time intervals (2 h, 4 h, and 6 h) and were monitored with UV-VIS spectroscopy (Molecular Devices). Free Dox (50 μM) dialyzed in a similar way served as the control. The diffused Dox from the conjugate was measured at 470 nm excitation and 585 nm emission.

2.16 Synthesis of fluorescent copper-free click conjugates

The azide modified DNA was dissolved in DNase, RNase free water. The copper free click reaction was performed in 10mM HEPES buffer pH 7.0 with 10 nmol of aptamer as constant with varying concentration of alkyne, DIBO-AF594 (10 nmol, 20 nmol, 40 nmol and 60 nmol). The reactions were incubated at room temperature (RT) for one hour under constant shaking condition followed by freezing at -30°C for overnight and thawing at 4°C. Freeze thaw was repeated for analysis of higher yield. The preparatory reaction was performed with 50 nmol of aptamer and 50 nmol of DIBO-AF594 in 10 mM HEPES buffer pH 7.0. The purification of conjugate was performed using HPLC with following system. System: Mobile phase A- 20% acetonitrile, 80% 0.02 M sodium phosphate buffer pH 7.0, Phase B- A+ 1.0M Nacl, Gradient: 15% B to 75% B in 40 mins, with flow rate of 0.75 ml/min with UV 260 nm, 590 nm detectors. The DNA peak having absorbance at both 260 nm and 590 nm was eluted and analyzed for the conjugation by agarose gel electrophoresis.

2.17 Aptamer DNAzyme conjugation

In order to conjugate NCL aptamer (NclApt) with survivin DNAzyme (Sur_Dz), the aptamer was synthesized with poly T linker at the 5’ end followed by the complementary bases to Sur_Dz. This apt-linker was used for the conjugation with DZ. The reaction was similar to the conjugation described earlier by annealing method (Lai et al., 2014). Briefly, equimolar concentration of Apt-linker and Sur_Dz were mixed together and subjected for denaturation in boiling water bath for 10 minutes and annealed by gradual cooling. Similarly, NclApt-Sur_mDz and Ncl-Apt-Sur_fDz (fDz was fluorescently labeled with FITC) conjugates were also prepared.
The conjugate formation was analyzed and confirmed by subjecting them for agarose gel electrophoresis. 4% agarose gels with ethidium bromide (EtBr) were used for visualizing the conjugates. Non fluorescent DNAzyme conjugate was used for cell culture and further assays whereas fluorescent DNAzyme conjugate was used for microscopic and other internalization studies.

2.18 Stability of aptamer and aptamer conjugates

The stability of the conjugates generated by copper free click chemistry was tested under physiological condition using dialysis method. 100 µl of 5 µM conjugate was dialyzed against the phosphate buffered saline at 37°C for 2, 4, 6, 24 hour duration. The fluorescence emission at 620 nm was used for measurement and for calculating the amount released upon degradation.

2.19 CD133+ve and ABCG2+ve cell isolation and aptamer uptake study

CD133+ve cell isolation was performed from the RB primary tumor sample and normal retina using CELLection™ Pan Mouse IgG Kit (Invitrogen life science, India) following the manufacturer’s instructions. Similarly, ABCG2+ve cells were isolated from the mammospheres grown from MCF7 spheroid cultures. Cell suspensions were incubated with AC133 epitope binding anti-CD133 antibody or anti-ABCG2 antibody (Sigma Aldrich, India) for 4h at RT, followed by incubation with washed Dynabeads for 4h, separation using magnetic stand yields negative cells and incubation with DNAse I enzyme to release the positive cells for the antigens desired from the beads. All the cells were washed twice with 1X PBS and incubated with the CSC marker aptamers.

2.20 Aptamer, aptamer chimera and nanocomplex uptake study

a. Cellular uptake of NCL aptamer on RB tumor cells and cell lines

The NCL aptamer binding and uptake was performed on live, fresh, unfixed or non-permeabilized cells. Titration using various concentration of aptamer was performed by flow cytometry (0.5 µM, 1 µM, 5 µM) on Y79 cells. 500 nM and 1 µM were found to be the ideal concentration as it exhibited saturation. 500 nM of aptamer was added to RB tumor cells or cell
lines and incubated for 45mins and the cells were thoroughly washed with 1X PBS twice and analyzed by flow cytometry.

b. Uptake of EpCAM aptamer

The specific binding and uptake of the EpDT3 aptamer to the fresh tumors and RB cell lines was determined with fluorescein-labeled aptamers. The RB tumor cells (homogenized and suspended in PBS; n=4), the Y79 and WERI-RB1 cells, were washed twice with PBS (1X). The Müller glial cells were washed in PBS with 0.53 mM EDTA followed by two washes with 1X PBS. About 100 nM of FI-labeled RNA aptamers (EpDT3-FI and Scr-EpDT3-FI) were added to 2×10^4 cells resuspended in 100 μl binding buffer (PBS containing 5 mM MgCl₂, 0.1 mg/ml tRNA, and 0.1 mg/ml salmon sperm DNA). The cells were incubated on ice for 1 h followed by three washes in 1X PBS (Shigdar et al., 2011). The cells were stained with propidium iodide (Sigma Aldrich; 1: 20,000) for 5 min, and the signal was read with the flow cytometer (Mitra et al., 2011). The fluorescence excitation and emission were 488 nm and 530 nm, respectively.

c. Uptake of EpCAM aptamer chimera

Cellular uptake of FITC labeled aptamer chimeras by the target cells was studied by flow cytometry and fluorescent microscopy. 2X10^5 MCF7 and WERI-Rb1 cells were incubated with 50, 250, 500 nM EpApt-siEp chimera for 2 hours in RPMI complete media. After incubation, the cells were collected, washed twice with 1X PBS and acquired by flow cytometer. A 24 well plate containing coverslips that are coated with poly-l-lysine were seeded with 50,000 cells and allowed to grow for 24 hours. The cells were washed twice with media and were treated with 400nM EpApt-siEp chimera for 2 hours. The scrambled chimera was used as a control. The cells were washed twice with 1X PBS, fixed in 3.7% formaldehyde for 10 min, stained with DAPI for 15 min. The coverslips with cells were washed with 1X PBS and observed under Axio-Observer fluorescent microscope.

d. Uptake of EpCAM aptamer PEI-siEp nanocomplex

Cellular uptake of the PEI-Apt-siRNA nanocomplex was performed in MCF7 and WERI-Rb1 cells using flow cytometry (BD Science FACS Caliber). The 200nM of aptamer alone or PEI-Apt-siRNA nanocomplex (200nM EpApt and 200nM siRNA) were incubated with 2 X10^5 cells for 4 hours followed by washing with 1X PBS two times and the cells were analyzed using BD
Chapter 2

FACS Calibur. The unstained cells and the scrambled aptamer added cells served as controls. The uptake of the aptamer alone and PEI-Apt-siRNA nanocomplex were visualized using fluorescent Axio Observer microscopy (Zeiss, Germany). Briefly, MCF7 and WERI-Rb1 cells were seeded at a density of 20,000 per well in 24 well plate. The aptamer alone or PEI-Apt-siRNA nanocomplex was added at a reaction volume of 250μl after 24h. The cells were incubated with complexes for 4h, washed two times with 1XPBS followed by fixing with 4% paraformaldehyde and nuclear staining using DAPI and imaging.

e. Visualization of aptamer-doxorubicin uptake with fluorescent microscopy

The Y79 cells and the Müller glial cells (2×10^4 cells/ml) were grown on coverslips coated with poly-l-lysine. Cells were washed twice with media (without FBS) and treated with equal concentrations (1.5 μM) of aptamer-Dox and free Dox for 2 h and 12 h. The cells were washed twice with 1X PBS, fixed in 3.7% formaldehyde for 10 min, and stained with DAPI for 15 min. The coverslips with cells were washed with 1X PBS and were analyzed under a fluorescence microscope (Axio Observer, Carl Zeiss, Berlin, Germany).

f. Uptake and Imaging of EpD-DIBO AF594 conjugates in cancer cells

The specific uptake of the aptamer conjugates was confirmed using MCF7, MDAMB453, high expressers of EpCAM, WERI-Rb1. Moderate expresser of EpCAM and Muller-glial, low expresser of EpCAM. WERI-Rb1 cell line is non-adherent and hence collected directly, whereas the MCF, MDAMB453, PC3 and muller-glial cells were removed from the flask by trypsinization. Cells washing and collections were performed by centrifugation at 1000 rpm at RT for 5mins. The collected cells were washed and resuspended in PBS containing 2% or 4% FBS as blocking agent. DIBO-AF594 (500nM) or different concentrations (50, 125, 250, 500nM) of aptamer were added to the 2*10^5 cells in 100 μl volume and incubated for two hours at RT. The cells were washed two times with PBS and cell binding was acquired by flow cytometry. Cells were seeded in 24well plates with coverslip, coating with poly-l-lysine for the non-adherent cell line at a density of 2*10^4 per well. 24 hours after seeding, different inhibitors to study internalization was added incubated for 2 h, followed by washing of cells and addition of conjugates or aptamer alone to cells. The internalization of assessed by flow cytometry and fluorescent microscopy.
Chapter 2

g. Imaging of cancer cells using hyperspectral microscopy

The cell lines grown over coverslip (as mentioned in above section 4.1.3.5) were incubated with aptamer conjugate in at 37°C for 2h, followed by washing with 1X PBS and mounting on to special slides, for visualizing the internalization of the EpDNA-DIBO-AF594 using dark field imaging with CytoViva microscope, hyperspectral microscope (Auburn, AL) (Mathew et al., 2012). To visualize the presence of aptamer within the cells, Z-stacking was performed under dark field and fluorescence channels. Z-stacking from uppermost to lower most cell surface was done to study the internalization of aptamer EpDNA-DIBO-AF594.

h. Uptake of survivin Dz and NCLApt-Sur_Dz conjugates

Cellular binding was performed for checking the efficiency of the conjugate upon chimerization. Y79, WERI-Rb1 and MIO-M1 were harvested from 80% confluent flasks and washed twice with PBS at 1300 rpm for five minutes. Cells were resuspended in binding buffer containing 5 mM MgCl₂, 0.2% (w/v) sodium azide, 0.1 mg/ml salmon sperm DNA, 0.1 mg/ml yeast tRNA, 5% FBS in 1X PBS to a final count of 1x10⁶ cells/ml. 500nM concentration of Apt-linker alone and Apt-Sur_Dz conjugate were used for binding assay and were incubated at 25°C for 2 hours (Subramanian et al., 2012). The cells were washed twice with PBS to remove unbound conjugates. Then the cells were subjected for FACS acquisition. Fluorescent DNAzyme was used for imaging the conjugate binding and uptake in cells and delivery of DNAzyme into cells, internalization was studied following earlier published protocol (Subramanian et al., 2014). 500 nM of NclApt-Sur_fDZ conjugate, NclApt-linker alone and sur_fDZ alone were added to Y79, WERI-Rb1 and MIO-M1 cells grown on coverslips. After 2 hours of incubation the cells were washed twice with PBS, fixed with 4% paraformaldehyde for 10 minutes and counterstained with DAPI for ten mins. Then the fluorescent images were captured using Zeiss Axio vision fluorescent microscope.

i. Cellular uptake of the CSC marker aptamers

The uptake of fluorescently labeled aptamers by the target cells was studied by flow cytometry and fluorescent microscopy. 2X10⁵ MCF7, Y79 and WERI-Rb1 cells were incubated with 50, 250, 500nM aptamers for 2 hours in 1X binding buffer (Subramanian et al., 2012). After incubation, the cells were washed with 1X PBS and acquired by flow cytometer. Cells were seeded in 24 well plate containing coverslips that are coated with poly-l-lysine and aptamers wee
added for 2 h at 37°C, cells were washed twice with 1X PBS, fixed in 3.7% formaldehyde for 10 min, nuclear stained with DAPI for 5 min, coverslips were mounted and observed under Axio-Observer fluorescent microscope. Similarly uptake studies were performed in the primary RB tumor samples and normal or non-malignant retinathat were stored frozen. Tissues were cut, minced, washed with PBS and used for the study.

2.21 aptamer treatment, transfection and RNA interference

a. Transfection of Tiam1 constructs and RNAi of Tiam1

Full-length and N-terminal truncated C1199 and C580 Tiam1 containing a hemagglutinin tag at the 3′ end, cloned in the eukaryotic expression vector pUTSV1 (Eurogentec, Belgium) were procured from John G Collard, Netherlands Cancer Institute (Michiels et al., 1997). Tiam1 constructs were transiently transfected in Y79 and WERI-Rb1 cell lines with lipofectamine 2000 (Invitrogen) as prescribed in manufacture’s protocol. For Tiam1 silencing, the pre-designed short interfering RNA (siRNA) sequences targeting different regions of Tiam1 mRNA were purchased from Qiagen, Valencia, USA. The siRNA sequences are as follows, 5’-GGCGAGCUUUAAGAAGAAATT-3’ (sense) and 5’-UUUCUUCUUAAAGCUCGCGGT-3’ (antisense) for Tiam1_1 (T1), CAUGUAGAGCAGAGUUUUTT (sense) and 5’-AAACUCUGUCUCUACAUGTT-3’ (antisense) for Tiam1_5 (T5), 5’-GGUUCUGUCGCCCAAUAATT-3’ (sense) and UUAUUGGGGCAGACAGACCAG (antisense) for Tiam1_6 (T6). Briefly, the cells were transfected with 200nM of pooled siRNA sequences by using lipofectamine 2000 (Invitrogen, Bangalore, India). Scrambled siRNA (Qiagen, Germany) was used as a negative control in all experiments. The cells were collected after 48 h for further experiments.

b. Treatment of cells with NCL-aptamer and silencing of NCL in RB cells

For studying the NCL mediated regulation and cellular events in RB, NCL-aptamer treatment or knock down of NCL was performed. The effect of NCL-aptamer on Y79, WERI-Rb1 and MIO-M1 cell lines was studied by treating cells with various concentration of NCL-aptamer (0.5 μM to 20 μM). The effective dose or IC₅₀ concentration or a concentration with lesser cytotoxicity was chosen for further studies. The RB primary cells were also treated with 10 μM or 15 μM of NCL-aptamer in complete media for 48 h. Silencing of NCL in RB cell lines and primary RB
cells was performed by transfecting the siNCL in incomplete media followed by addition of complete media (serum containing media), incubated further to 48 h (cat no. SI03030965, Qiagen, Germany) at 200 nM concentration using lipofectamine 2000 (Invitrogen, Bangalore, India). In RB primary cells, pEGFP plasmid was transfected using lipofectamine 2000 to evaluate the metabolic activity. The uptake of FITC-NCL-aptamer on primary cells was also visualized using fluorescent microscopy. 48h post treatment or silencing cells were collected and subjected to cell cycle, cellular cytotoxicity and cell proliferation assay.

c. Cell treatment to study silencing effect of the chimeric construct

MCF7 and WERI-Rb1 (2X10^5 cells) were seeded in a 6 well plate. After 24 h of incubation, the cells were treated with 400 nM EpApt-siEp chimera. EpCAM siRNA (siEp) (200 nM) was used as control and was delivered to cells by lipofectamine 2000 (Invitrogen, Life technologies, Bangalore, India) mediated transfection. The aptamers were allowed to incubate in serum free condition for 4 h. After 4 h, media containing FBS was added and incubated further for 48 h.

d. Silencing efficiency of the nanocomplex

The effect of siRNA delivery to the cells was evaluated using quantitative PCR (qPCR) and Western blotting. MCF7 and WERI-Rb1 cells were seeded at a density of 2 X 10^5 cells per well of 6 well plate. After 24 h of seeding the PEI alone or PEI-Apt-siRNA nanocomplexes or lipofectamine-SiEp were added to cells in serum deprived media and incubated for 4 h, followed by the addition of serum containing media. The nanocomplex treated cells were incubated for 48 h to study the specific delivery of the EpCAM siRNA to the targeted cells. The total RNA from treated cells was extracted using Tri reagent (Sigma, Bangalore, Chennai) and the cDNA was synthesized using Verso cDNA synthesis kit. The levels of EpCAM expression were measured in MCF7 and WERI-Rb1 cell lines using TaqMan real time PCR reagents (Applied biosystems, Foster city, USA) using GAPDH expression as internal control. The immunoblot analysis was performed to check the EpCAM protein expression in both cell lines treated with the PEI-Apt-siRNA nanocomplexes or lipofectamine-SiEp.
Chapter 2

e. Transfection or treatment of cells with Sur_Dz or conjugate

The functional activity of conjugates on cells was studied by either transfecting Sur_Dz and Sur_mDz alone or by adding the conjugates to the cells. Briefly, cell seeding was performed as described in the cell culture section in incomplete media, 12.5 μM of NclApt-Sur_Dz and NclApt-Sur_mDz conjugates were added to the cells in incomplete media for 4h. The same concentration of Apt-Linker alone was added directly to the cells and Sur_Dz, Sur_mDz alone were also transfected as controls. Sur_Dz and Sur_mDz were transfected using Xtreme gene plus transfection reagent (Roche, Germany). After 4 h of transfection complete medium was added to the cells. Cells were collected 48 h post transfection and subjected for RNA isolation and protein extraction for quantitative real time PCR and western blotting respectively.

f. Treatment of cells with Pri-miRNA aptamer

The Pri-miRNA aptamer or aptamer 7 (Pri-apt) and control aptamer or aptamer F (con-apt) carrying the sequence 5’-CCUACCCGACACAAUUCUACUC-3’ and 5’-GCAACGAUGGUCCAACACCUCGCC-3’ respectively are purchased from Dharmacon (GE life sciences)(Lunse et al., 2010). Transfections were done using lipofectamine 2000 following the manufacturer’s protocol. Briefly, 400 nM of Pri-apt and con-apt were added to serum free media and lipofectamine 2000 alone was added to serum free media and incubated for 5 mins, followed by mixing both and incubating further for 15 mins. The mix was added to cells in serum free media and left for 4 h. Additional serum containing media was supplemented after 4h and incubated further for 48 h. Later to incubation, cells were collected and proceed for RNA extraction or for assays.

g. Cellular activity of the CSC marker aptamers

Cell proliferation was studied by MTT assay of silenced or aptamer treated cells. Transfected cells were incubated with 100μl of media containing 5 mg/ml 10μl of MTT and incubated for 3-4 h at 37°C. Then MTT was removed and 100 μl of DMSO was added and absorbance was measured at 570 nm. Experiments were performed in triplicate thice and the mean ±SD is plotted as graph.

2.22 Northern blotting
Chapter 2

Total RNA was isolated from MCF-7 untreated cells, siRNA transfected cells and aptamer-siRNA chimera treated cells by tri-reagent method. 2X RNA sample buffer (200 μl) was prepared by adding 100 μl of redistilled formamide, 33 μl of 37% formaldehyde solution, 4 μl of 0.5M EDTA (pH 8), 8 μl of 1M NaP (pH 6.8), 4 μl of EtBr (10 mg/ml) and 51 μl of DEPC treated water. The RNA samples (3 μg each) were added to equal volume of 2X RNA sample buffer and was heated at 65°C for 15 min to remove secondary structures. The samples were electrophoresed at 100 V in 1% denaturing NaP-formaldehyde agarose gel and Nap-formaldehyde running buffer made with DEPC treated water. The electrophoresed samples were transferred onto a nylon membrane by capillary transfer and cross-linked by baking at 80°C for 15 min. EpCAM siRNA was labeled with Biotin by Brightstar Psoralen-Biotin Kit method (Cat No: AM1480) and was used as a probe. The probe (100 ng) was hybridized onto the membrane by using ULTRAhyb-Oligo buffer (Cat No: AM8663) and EpCAM mRNA level was detected by using BrightStar BioDetect Kit (AM1930) by autoradiography.

2.23 RNA isolation and Quantitative PCR of mRNA

For studying the mRNA changes upon aptamer treatments and siRNA transfections was performed following RNA isolation, cDNA synthesis from the treated cells using previously published protocol (Subramanian et al., 2013). Briefly, the treatments were performed upto 48 h, Y79, WERI-Rb1, MCF7 and MIO-M1 cells were harvested and washed twice with PBS at 1300 rpm for five minutes. Total RNA were extracted by TRI reagent method. Briefly, to the cell pellet 500 μl of TRI reagent was added, vortexed, pipette to resuspend the cells and incubated for 5mins at RT. 100 μl of chloroform was added and vortexed and incubated for further 10 mins at RT followed by centrifugation at high speed for 15 mins. To the supernatant DNase I was added and incubated for 15 mins at 37°C followed by phenol: chloroform extraction, precipitation with isopropanol and ethanol wash. The RNA pellets obtained were dried well, resuspended in RNase free water and used for cDNA synthesis. cDNA was transcribed from 500 ng of RNA using thermo verso cDNA kit. Quantitative PCR based on SYBR green technology was carried out using Dynamo HS SYBR green master mix (Thermo scientific, GE life sciences). 50 ng of cDNA was used for real time qPCR for target gene analysis and the data was normalized to the untreated sample with the β-2-microglobulin as housekeeping gene. The list of genes and primers analyzed are tabulated in respective result chapters.
2.24 cDNA microarray analysis of Y79 cells treated with siTiam1

Whole genome microarrays were performed in Tiam1 siRNA treated Y79 cells along with untransfected Y79 cells. The experiment was performed in triplicates. In brief, 500 ng of total RNA was used for cDNA synthesis, followed by amplification/labeling using Total Prep RNA Amplification kit (Ambion Inc., Austin, TX) to synthesize biotin-labeled cRNA. The concentration of cRNA was measured by spectrophotometer (Nanodrop, ND-1000, Thermo scientific). Labeled, amplified cRNA (750 ng per array) was hybridized to a ver. 3 of the Illumina Human-Ht-12 BeadChip (48K) according to the Manufacturer's instructions (Illumina, Inc., San Diego, CA). The whole 48803 probes on the Human-Ht12 beadChip ver. 3 were used and the arrays were scanned with an Illumina Bead array Reader confocal scanner (BeadStation 500GXDW; Illumina, Inc.). Sample Gene Profile option of Illumina BeadStudio software was used to export the gene expression data.

2.25 miRNA microarray analysis of WERI-Rb1 cells treated with NCL-aptamer

miRNA microarrays were performed in duplicates for WERI-Rb1 untreated and NCL-aptamer treated cells. The RNA was extracted from biological duplicates of treated and untreated cells, followed by quality check using bioanalyzer. Hybridization was performed for the biological duplicates. The miRNA labeling was performed using miRNA complete labeling and hybridization kit (Agilent Technologies, Part Number: 5190-0456). The total RNA sample was diluted to 50 ng/µl in nuclease free water. About 100ng of total RNA was dephosphorylated along with appropriate diluted spike in control; (Agilent Technologies, MicroRNA Spike-In Kit, Part Number 5190-1934) using calf intestinal alkaline phosphatase (CIP) master mix (Agilent Technologies, Part Number: 5190-0456) by incubating at 37°C for 30 minutes. The dephosphorylated miRNA sample was denatured by adding dimethyl sulfoxide and heating at 100°C for 10 minutes and transferred to ice-water bath. The ligation master mix (Agilent Technologies, Part Number: 5190-0456) containing cyanine 3-pCp was added to the denatured miRNA sample and incubated at 16°C for 2 hours. The cyanine 3-pCp labeled miRNA sample was dried completely in the vacuum concentrator (Eppendorf, Concentrator Plus, Catalog Number: 5305000) at 45°C to 55°C for 2 hours. The dried sample was re suspended in nuclease free water and mixed with hybridization mix containing blocking solution (Agilent Technologies, Part Number: 5190-0456) and Hi-RPM hybridization buffer (Agilent
Chapter 2

Technologies, Part Number: 5190-0456) and incubated at 100°C for 5 minutes followed by snap chill on ice for 5 minutes. The samples were hybridized on the human miRNA version 3 array (Slide no: 252182715127). The hybridization was carried out at 55°C for 20 hours. After hybridization, the slides were washed using gene expression wash buffer1 (Agilent Technologies, Part Number 5188-5325) at room temperature for 5 minutes and gene expression wash buffer 2 (Agilent Technologies, Part Number 5188-5326) at 37°C for 5 minutes. The slides were then washed with acetonitrile for 30 seconds. The microarray slide was scanned using Agilent scanner (Agilent Technologies, Part Number G2565CA). The data was further analyzed using GeneSpring software.

2.26 RNA isolation and quantitative real time PCR

Total RNA was isolated by Tri-reagent method. The quality and quantity of the RNA was assessed by Biospec-Nano spectrophotometer and 1 μg of RNA was reverse transcribed to cDNA using a verso kit. qPCR was performed by SYBR green method using Dynamo HS mastermix for genes involved in the study. The reaction conditions were initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 45 sec, extension at 72°C for 45 sec. Final extension was for 10 min at 72°C. Meltcurve analysis was performed to verify the specificity of amplification. Comparative quantification was performed using the formula $2^{-\Delta \Delta Ct}$ and the log2 values are expressed as fold change regulation.

2.27 Real-time Quantitative Reverse transcription PCR of miRNAs

The changes in miRNA expression between the treated and untreated cells were detected by quantifying the mature miRNAs using reverse transcription– real-time PCR. All reagents, including the TaqMan® microRNA individual assays hsa-miR-17 (assay ID, 002308), hsa-miR-18a (assay ID, 002422), hsa-miR-19b-1 (assay ID, 002425), hsa-miR-20a (assay ID, 000580), hsa-miR-92 (assay ID, 002137), hsa-miR-330 (assay ID, 000544), hsa-miR-373 (assay ID, 000561), hsa-miR-196b (assay ID, 002215), hsa-miR-1 (assay ID, 000385), hsa-miR-152 (assay ID, 000474), hsa-miR-206 (assay ID, 000510), the TaqMan® microRNA Reverse Transcription Kit and the TaqMan® Universal PCR Master Mix without AmpErase® UNG, were purchased from Applied Biosystems (Joyvel, Chennai, India) and solaris qPCR Gene Expression Master Mix (cat. No. AB4352B, ABgene, Thermoscientific). Quantification was performed following...
the manufacturer’s protocol starting with 100-250 ng of the total RNA per cDNA synthesis. The RNU6B probe was employed to normalizing miRNA levels and untreated samples. The ABI PRISM 7500 Real-time machine (Applied Biosystems) was used. The innate expression of mature miRNAs of the miR-17-92 cluster was studied on MIO-M1, ARPE-19, Y79 and WERI-Rb1 and normal retina. Similarly the levels of expression of miR-330, miR-196b, mir-152 and miR-1 in RB tumors were studied were studied and compared with the normal or non-malignant retina and the normal cell lines.

2.28 Immunohistochemistry of EpICD

Paraffin embedded tissue sections were deparaffinized by serial treatments of Xylene and ethanol. Immunohistochemistry (IHC) was performed using novolink polymer detections system (Leica biosystems), following the instructions given by the manufacturer. The tissues were rehydrated and antigen retrieved under steaming in a pressure cooker containing (0.1 M citric acid, 0.1 M trisodium citrate, PH 6) buffer for 4 minutes (two whistles). The cooker was allowed to cool in running water and the tissues were rehydrated and added with 3% H₂O₂ for 5 min to block endogenous peroxidase activity. The tissues were washed twice with TBS, treated with protein block solution for 5 min to avoid non specific binding and added with primary antibody, EpICD (IMG6745A, Imgenex, Novus biologicals) (1:100 dilution) or NCL (anti-NCL, Abcam) (1:200 dilution) or PCNA (Sigma Aldrich) and kept at 4°C overnight. The detection was performed by using Novolink polymer IHC detection kit. The tissues were DAB stained for 5 min, counter stained by hematoxylin for 30 sec, washed twice with water. The slides were dehydrated, mounted with DPX, cover slipped and allowed to cure overnight before observing under Olympus microscope.

2.29 Functional activity of LNA chimera-qPCR and Immunofluorescence

The functional activity of the LNA chimeric aptamers were evaluated by adding to the WERI-Rb1 and MDA MB453 cells with siRNA transfection as positive control. The LNA-Ep-siSTMN1 and LNA-Ep-siBIRC5 were added at a concentration of 100 nM and siRNAs were transfected at 200 nM using lipofectamine 2000 (Invitrogen life science, India) in incomplete media for 4 h, followed by complete media addition. Cells were analyzed by qPCR (mentioned above) and immunofluorescence for the downregulation of STMN1 and BIRC5. Immunofluorescence was
performed following published protocol (Subramanian et al., 2013). Briefly, cells were seeded on Poly-l-lysine coated cover slips and transfected or aptamer treated, cells were fixed with 4% paraformaldehyde 48 h later, permeabilized, blocked and incubated with anti-STMN1 and anti-BIRC5 antibody (1:40) for overnight at 4°C followed by FITC conjugated anti-mouse secondary antibody (1:500) for 2 h at room temperature and DAPI nuclear stain was added. Intermittent washings were performed wherever necessary. The cover slips were mounted and viewed under Axio Observer fluorescence microscope (Zeiss, Germany).

2.30 Western Blotting

Transfected cells and RB tumors were collected and washed with 1X PBS followed by cell lysis in RIPA buffer (containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 1 %Triton X-100, 1 % Sodium deoxycholate, 0.1 % SDS and Protease inhibitor cocktail, Sigma). 50-100 μg of protein lysate was resolved on 8 % SDS Polyacrylamide gel and then electroblotted onto nitrocellulose membrane at 100V for 90min. The membrane was blocked in 5% skinned milk, further incubated with primary anti-Tiam1 polyclonal antibody (raised in rabbit, C-16, Santacruz) in 1:100 dilutions and anti-β actin monoclonal antibody (raised in mouse, Sigma) in 1: 2000 at 4°C for overnight. The membrane was washed thice with TBST (1M Tris-HCl pH-7.6 containing 0.1% Tween-20 and 0.8% NaCl) and incubated with HP-conjugated secondary antibody at 1:2500 dilution for 2h. The membrane was detected by superson signal west femto maximum sensitivity substrate (Thermoscientific, Rockford, USA). The blots are representative of three experiments.

2.31 Immunofluorescence

For immunofluorescence, cells were seeded on Poly-L-Lysine coated cover slips in 24-well plate. After 48h of transfection, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked with 5% BSA in PBS for 30mins. The cells were incubated with polyclonal anti-Tiam1 antibody (1:25) for overnight at 4°C. After PBS wash cells were incubated with FITC conjugated anti-rabbit secondary antibody (1:500) for 2h at room temperature then incubated with TRITC conjugated phalloidin at 1:300 dilution (Sigma Aldrich, St. Louis, MO) for 30 min whereas DAPI was used as a nuclear stain. The cover slips were mounted and viewed under Axio Observer fluorescence microscope (Zeiss, Germany).
2.32 Flow cytometry analysis of apoptosis

For the apoptosis assay, Annexin V kit from BD biosciences (San Diego, CA) was used. Briefly, cells were washed with ice cold 1X PBS twice and resuspended in 1X binding buffer and incubated with annexin V-FITC and Propidium iodide for 15 mins, followed by flow cytometry. Experiments were performed thice individually.

2.33 Flow cytometry analysis of cell cycle

Cellular events, metabolic activity and the mode of cell death were analyzed to understand the mechanism of aptamer action in RB cells. To study the effect of aptamer or aptamer conjugates activity on the cell cycle phases, cell cycle assay was performed using BD cycle-test kit. As per manufacturer’s protocol, 48 h treated cells were PI stained. Briefly, cells were washed twice with PBS followed by addition of solution A and incubated for 10 mins RT, solution B 200μl was added and mixed, incubated for 10 mins RT. Finally 150μl of PI solution was added and incubated for 10mins RT and flow cytometry was performed.

2.34 Wound healing assay

The cells were grown on PLL coated culture plate until confluent in 1% serum medium. A wound was made by scratching a straight line using a 200 μl pipette tip. The cells were then washed twice, transfected and incubated further for 48 h with 10% FBS containing media. Images were taken under phase contrast microscope at 0 h and 48 h using AxioObserver fluorescent microscope.

2.35 Matrigel invasion assay

Y79 and WERI-Rb1 cells were counted 24 h post transfection of the siTiam1 and scramble siRNA and 5 X 10^4 cells in serum free media were seeded respectively in the rehydraded matrigel invasion chamber with 10% FBS containing media added as chemoattractant. Cells were allowed to incubate for 48 h. Chambers were removed, washed twice with 1X PBS, cleaned the matrigel using cotton plug, fixed cells in methanol, stained with crystal violet. The membranes were cut, removed and mounted with DPX mountant and viewed under 20X objective of Axio-Observer microscope.
2.36 Cytotoxicity assay – Lactate dehydrogenase (LDH) release assay

Cellular cytotoxicity was studied by measuring the activity of LDH using cytotoxicity detection kit plus (cat. No.04744926001, Roche) on RB cell lines treated with aptamer or aptamer chimera or aptamer conjugates or siRNA. For LDH assay, triton-X 100 lysed cell lysate was used as high control, media alone was used as low control, for treated and untreated cells, media supernatant was collected and subjected to the assay in 384 well formats. The supernatant and reagent mix were incubated for RT for 30 mins followed by addition of stop solution and reading at 470 nm. All experiments were performed in triplicate.

2.37 Cell proliferation assay

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate the percentage viability of silenced cells. Transfected cells were incubated with 100 μl of media containing 10 μl of MTT (5mg/ml) and incubated for 4 h at 37°C. Then media with MTT was removed and 100 μl of DMSO was added to each well the absorbance was measured at 570 nm. All experiments were performed in triplicate.

2.38 Statistical analysis

Statistical analysis was performed using unpaired student T-test for all experiments except for the cell viability assay, analysis of variance (one-way) is performed and data interpreted. The results are mean of the independent experiments. Each experiment consisted of 3 replicates. P values less than 0.001 were considered very significant and indicated with “***” and P ≤ 0.05 is considered as significant and indicated with “*”. Any P>0.05 was considered nonsignificance denoted as ‘NS’.

In vivo Methods:

2.39 Animal Care and Experimental Procedure

2.39.1 Animal Welfare

Animals were taken care as per the regulations of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and Association for
Chapter 2

Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines. The ‘Form B’ for carrying out animal experimentation was reviewed and approved by the Institutional Animal Ethics Committee (IAEC Protocol Approval No: SYNGENE/IAEC/430/10-2013).

2.39.2 Housing and Feeding

Animals were maintained in a controlled environment with 22 ± 3°C temperature, 50 ± 20 % humidity, a light/dark cycle of 12 hours each and 15-20 fresh air changes per hour. Animals were housed group wise and autoclaved corncob was used as a bedding material. The animals were fed, ad libitum, with certified irradiated laboratory rodent diet during the study period.

2.39.3 Drinking water

Fresh, potable drinking water, filtered though RO, was provided *ad libitum* after autoclaving to all animals via bottle fitted with nozzle.

2.39.4 Preparation of Animals

The animals were kept under acclimatization in the experimental room for a period of at least 5 days. A thorough physical examination was performed before selecting the animals and only animals that were apparently healthy were used for the study.

2.39.5 Animal Identification

Animals were individually numbered and the cage cards indicating the experiment, study number, date of tumor implantation, date of randomization, tumor type, mouse strain, gender, and individual mouse number were displayed on corresponding cages. After randomization group identity, test compound, dosage, schedule and route of administration were added.

2.39.6 Preparation of tumor cells

All procedures were performed in laminar flow hood following sterile techniques. Human RB cells (Y79) or epithelial cancer (MCF7) cells with a viability of >90 % was chosen for the study. Ideally 1 X 10^7 Y79 cells was resuspended in 200 μl of serum free media containing 50% of matrigel kept in ice.
Nude mice (Hsd: Athymic Nude-Foxn1\textsuperscript{nu}) housed in Individually Ventilated Cages (IVCs) was used for the present investigation. Y79 RB cell line was propagated in the animals by injecting the cancer cells subcutaneously in the flanks or back of the animals. The tumorigenicity of the MCF7 cells in mice is estrogen-dependent. Twenty hours prior to MCF-7 cell injection, animals were implanted with 17\(\beta\)-estradiol pellets (0.36 mg/pellet; 60-day release; Innovative Research of America, Sarasota, FL) into dorsal shoulder blade region of mice using trochar. Twenty four hours post implantation of pellets, MCF7 tumor cells (5 \(\times\) 10\(^6\) cells/animal) were injected subcutaneously in the flanks of the animals. The implanted area was monitored for growth of tumor. Once the tumor attained palpable, the animals were randomized based on tumor volume (TV\(\approx\)80mm\(^3\)) and dosing was initiated. The treatment schedule is given below:

Table 1. Treatment schedule for Y79 xenograft

<table>
<thead>
<tr>
<th>Y79 Group</th>
<th>No. of animals/group</th>
<th>Dose</th>
<th>Route</th>
<th>Dosing schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vehicle Control</strong></td>
<td>n=10</td>
<td>Sterile water for injection (100 (\mu)l)</td>
<td>s.c adjacent to tumor</td>
<td>For five animals: Once daily for 21 days \ For other five animals: Days: 0, 2, 4, 6, 8,10, 12, 14, 16 – 24</td>
</tr>
<tr>
<td>NCL Apt</td>
<td>n=8</td>
<td>25 nmol/animal</td>
<td>s.c adjacent to tumor</td>
<td>Cycle 1: On Days 0, 2,4, 6, 8,10, 12, 14, 16 Cycle 2: Once daily from Day 17 till Day 24</td>
</tr>
<tr>
<td>LNA NCL Apt</td>
<td>n=8</td>
<td>25 nmol/animal</td>
<td>s.c adjacent to tumor</td>
<td>Cycle 1: On days 0, 2 Cycle 2: Once daily from day 4 till day 10</td>
</tr>
<tr>
<td>LNA NCL Apt</td>
<td>n=5</td>
<td>25 nmol/animal</td>
<td>i.p injection</td>
<td></td>
</tr>
</tbody>
</table>
## Table 2. Treatment schedule for MCF7 xenograft

<table>
<thead>
<tr>
<th>MCF7 Group</th>
<th>No. of animals/group</th>
<th>Dose</th>
<th>Route</th>
<th>Dosing schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vehicle Control</strong></td>
<td>n=8</td>
<td>Sterile water for injection (100 μl)</td>
<td>s.c adjacent to tumor</td>
<td>Cycle 1: On Days 0, 2, 4, 6, 8, 10, 12, 14. Cycle 2: 20 - 24</td>
</tr>
<tr>
<td><strong>EpApt-siEp</strong></td>
<td>n=8</td>
<td>0.6 nmol/Animal</td>
<td>s.c adjacent to tumor</td>
<td>Cycle 1: On Days 0, 2, 4, 6, 8, 10, 12, 14. Cycle 2: On Day 20 all animals were dosed. 24 h post dosing 4 animals were sacrificed. The remaining 4 animals received dosing on Day 22 and Day 24.</td>
</tr>
</tbody>
</table>

s.c – Subcutaneous (two sites around the tumor; 50 μl per site)

i.p – intraperitoneal (100 μl injected at once)
2.39.8  Formulation and Drug Dosage (Preparation of oligos and dosing solutions)

In general, all the test compounds were dissolved in sterile water for injection which resulted in solutions at all prepared concentrations. The test item (NCL Apt and LNA NCL Apt) was freshly prepared by thawing of frozen aliquots from stocks on the days of administration. The preparation of test compounds is given below:

Table 3. Formulation and dosage of oligos for injection in Y79 and MCF7 xenograft models.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Y79 Treatment</th>
<th>Dose</th>
<th>Dose volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle</td>
<td>-</td>
<td>100μl/animal</td>
</tr>
<tr>
<td>2</td>
<td>NCL Apt</td>
<td>25nmol/animal</td>
<td>100μl/animal</td>
</tr>
<tr>
<td>3</td>
<td>LNA NCL Apt</td>
<td>25nmol/animal</td>
<td>100μl/animal</td>
</tr>
<tr>
<td>4</td>
<td>LNA NCL Apt</td>
<td>25nmol/animal</td>
<td>100μl/animal</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>S.No</th>
<th>MCF7 Treatment</th>
<th>Dose</th>
<th>Dose volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle</td>
<td>-</td>
<td>100μl/animal</td>
</tr>
<tr>
<td>2</td>
<td>EpApt-siEp</td>
<td>0.6nmol/animal</td>
<td>100μl/animal</td>
</tr>
</tbody>
</table>

2.40 OBSERVATIONS

2.40.1 Body weight

Body weights were measured once every three days during the study period. The percentage change in body weights was calculated.
2.40.2 Tumor volume measurement

The tumor volume was determined by two-dimensional measurement with a caliper on the day of randomization (Day 0) and then once every three days (i.e. on the same days when body weight was taken). Using a vernier caliper the length (L) and width (W) of the tumor was measured. Tumor volume (TV) was calculated using the following formula:

\[ \text{Tumor Volume (mm}^3\text{)} = L \times W^2 / 2, \]

Where, \( L = \) Length (mm); \( W = \) Width (mm).

Mean, Standard Deviation (SD) or Standard Error of Mean (SEM) were calculated for individual groups.

2.40.3 Antitumor Activity

Antitumor activity was evaluated as maximum tumor volume inhibition versus the vehicle control group. Data evaluation was performed using statistical software Graph Pad version 5.

2.40.4 Test/Control Value in % (% T/C)

Tumor inhibition on a particular day (T/C in %) was calculated by using the below formula:

\[
\frac{(\text{Mean TV of the test group on Day } x - \text{Mean TV of the test group on Day 0})}{(\text{Mean TV of the control group on Day } x - \text{Mean TV of the control group on Day 0})} \times 100%
\]

The minimum (or optimum) %T/C value recorded for a particular test group during an experiment represents the maximum antitumor activity for the respective treatment.

TV = Tumor volume (mm³)

2.40.5 Tumor growth inhibition (TGI)

TGI was calculated using the following formula:

\[ \text{TGI} = (1 - \text{T/C}) \times 100 \]
Chapter 2

Where, $T = (\text{Mean TV of the test group on Day } x - \text{Mean TV of the test group on Day } 0)$

$C = (\text{Mean TV of the control group on Day } x - \text{Mean TV of the control group on Day } 0)$

2.40.6 Efficacy Criteria

Group optimum $\%T/C$ values were used for activity rating as follow.

Table 4. The percent tumor growth and efficacy determination

<table>
<thead>
<tr>
<th>Score</th>
<th>Activity</th>
<th>$%T/C$</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>Inactive</td>
<td>$%T/C \geq 50%$</td>
</tr>
<tr>
<td>+</td>
<td>Moderate activity</td>
<td>$25% \leq %T/C &lt; 50%$</td>
</tr>
<tr>
<td>++</td>
<td>High activity</td>
<td>$10% \leq %T/C &lt; 25%$</td>
</tr>
<tr>
<td>+++</td>
<td>Very high activity</td>
<td>$5% \leq %T/C &lt; 10%$</td>
</tr>
<tr>
<td>++++</td>
<td>Partial remission</td>
<td>$%T/C &lt; 5%$</td>
</tr>
<tr>
<td>CR</td>
<td>Complete regression</td>
<td>No signs of tumor growth</td>
</tr>
</tbody>
</table>

2.40.7 Clinical Signs

Animals were observed for visible clinical signs once every three days during the study period.

2.40.8 Statistical Analysis

For the evaluation of the statistical significance of tumor inhibition, Two-way ANOVA followed by Bonferroni post test was performed using Graph Pad Prism v5. Any p-values $<0.05$ indicate statistically significant differences between groups.
Chapter 2

2.40.9 Necropsy

At the end of the experiment period, on the day of sacrifice blood was collected under light isoflurane anesthesia from all the groups for clinical assessment of liver function (SGOT, SGPT) and Kidney function (BUN, Urea). Additionally peripheral blood smears were prepared and were stained with May-Grunwald Giemsa stain and evaluated for Differential Leukocyte Count (DLC). Then the animals were sacrificed and representative animals from each group were photographed (Nude mice bearing tumor and harvested tumor alone). The subcutaneous tumor (Y79) tissue was harvested and divided into two parts (Snap frozen, formalin fixed) for further analysis. Necropsy was done to evaluate gross evidence of possible lesions in organs such as lungs, liver, spleen, kidney and heart. The collected organs were stored in 10% normal buffered formalin.

2.40.10 Histology

The tumor and collected organs were used for the assessment of therapeutic anti-tumor activity of the treatments by histological analysis.

2.41 Serum miRNA isolation and Real time qPCR

Serum miRNA acts as biomarker for the RB disease progression, especially the mir-17-92 cluster (Beta et al., 2013). For studying the changes in the serum miRNA levels, the isolation of serum miRNA was performed using Plasma/Serum circulating and exosomal RNA purification kit (Slurry Format) by following manufacturer’s protocol from n=2, of each group studied and additionally normal serum from nude mice was also included. Briefly, by mixing the serum (100 μl) with PS Solution A, which contains the separation matrix followed by PS Solution B and ethanol are then added, and the mixture is centrifuged. PS Solution C is then added to the pellet, and the slurry is loaded onto a provided mini filter spin columns. This is followed by washing of the bound RNA to remove the remaining proteins and other impurities. Finally, the purified circulating RNA is eluted into the 75 μl of elution buffer. As mentioned in the real-time PCR section, qPCR of the mature miRNA of miR-17-92 cluster was performed and Hs-18s rRNA was used for normalizing the fold expression.
2.42 Real time qPCR and Western blotting of the Y79 xenograft tumor tissues

For studying the changes in the G-rich mRNAs, cancer stem cell markers and oncogenes at mRNA level, qPCR was performed from the cDNA transcribed from the total RNA of tumor tissues (n=2 per group) extracted using RNeasy Qiagen kit method. qPCR was performed for the mature miRs of miR-17-92 cluster and for the miRNAs deregulated upon NCL-aptamer treatment (miR-330, miR-196b, miR-152 and miR-1). Western blotting was performed on the tumor tissues (n=2 per group) lysed with RIPA buffer and analyzed for the expression of FOXM1, Bcl2, Survivin and β-tubulin (antibodies from Santa Cruz biotech) following the protocol given above.

2.43 DESI MS of cell lines treated with aptamer and siRNA and Xenograft tumor tissues

The effect of siNCL and NCL-aptamer and the alternation in lipid profile was studied using Desorption ElectroSpray Ionization Mass Spectrometry (DESI MS). The treated cells (5x10^5 cells) were washed with PBS and resuspended uniformly to 15μl in 1X PBS, spotted on whatmann 42 filter paper and left for drying in laboratory condition for 5 mins. The sample was imaged in positive ion mode with methanol as solvent using conditions published earlier. Thermo Scientific LTQ XL mass spectrometer was used for experiments and it was coupled with DESI MS ion source from Prosolia. The snap frozen tumor tissues were sectioned using cryostat and thaw mounted on glass slide and analyzed by DESI MS using same conditions used for cell lines.

2.44 Blood cell counts, biochemical and histology analysis

At the end of the experiment period, on the day of sacrifice, blood was collected under isoflurane anesthesia from all the groups for clinical assessment of liver function (SGOT, SGPT) and kidney function (BUN, Urea). Additionally peripheral blood smears were prepared and were stained with May-Grunwald Giemsa stain and evaluated for differential leukocyte count (DLC). Then the animals were sacrificed and representative animals from each group were photographed (Nude mice bearing tumor and harvested tumor alone). The subcutaneous tumor (MCF7) tissue was harvested and divided into two parts (snap frozen, formalin fixed) for further analysis. Necropsy was done to evaluate gross evidence of possible lesions in organs such as lungs, liver, spleen, kidney and heart. The collected organs were stored in 10% neutral buffered formalin,
dehydrated and fixed paraffin blocks followed by sectioning for the histological analysis. Haematoxylin and eosin staining was performed on the organs and tumor section.

2.45 Protein array for apoptotic markers and cytokines

Protein array was performed to study the mechanism of EpApt-siEp and L-NCL-aptamer aptamer treatment (i.p. mode of injection) anti-tumor activity and to study the effect on apoptosis onset and inflammatory response. The protein arrays – Human apoptosis array, catalog# ARY009 and Mouse cytokine array panel A catalog# ARY006 (Proteome Profiler™; RandD Systems, Abingdon, UK) were performed following the manufacturer’s instruction. The xenograft mouse (vehicle control and EpApt-siEp treated) tissues and serum were prepared by normalizing their protein concentration, used for the array. The arrays were performed at an identical condition and developed the control and treated simultaneously using chemidoc XRS+ instrument (BioRad) using same exposure. Background signal normalization was performed and the integrated pixel density was measured using the imageJ software with the microarray profile plugin. The differences between the duplicate spots were used for calculating the standard deviation and expressed as error bar in the histogram plot.

2.46 Structure prediction and dynamics

With no significant structural homologue from the BLAST (Basic local alignment search tool), the extra cellular domain region of the CD133 protein was modeled by fold recognition techniques using I-TASSER (Zhang, 2008). Among the many structures generated, the one with high confident score(C-Score) was chosen as best model and was further refined using modeller9v7 software (Sali and Blundell, 1993).

The three dimensional structure of RNA was predicted using MC Fold-MC Sym (Parisien and Major, 2008) pipeline of tools. While the MC fold predicts the secondary structure of the target sequence, the MC Sym predicts the three dimensional coordinates for the secondary structure (predicted from MC Fold) with template from Nucleotide cyclic motifs (NCM).

All Molecular dynamics simulations were performed using GROMACS 4.5 (Pronk et al., 2013) in which the system was prepared by solvating the protein structure in SPCE model and RNA in TIP3P water models with OPLS as force field. The charge of the system was neutralized by
adding one CL ion for the protein system and 14 Na⁺ ions for the RNA. The neutralized systems were further energy minimized using steepest decent algorithm which was followed by equilibration by NVT and NPT ensembles. The simulation was carried out for 15 nanoseconds (ns) for the protein and 5 ns for the RNA using GROMACS package, and the coordinates were sampled at every two pico seconds for both the systems. Further, the Root mean square deviations (RMSD) trajectories of the molecular backbones of sampled coordinates were plotted against the time duration to observe the dynamics of the molecules. Similarly, the potential energy of all the evolved structural conformation was also calculated and plotted against time duration.

2.4.7 Docking

The interaction between the protein CD133 and A15 RNA aptamer was predicted by performing docking simulation using Hex Dock (http://hexserver.loria.fr) which calculates the docking poses on the shape complementarities using Fast Fourier Transformation. Based on HexDock scoring function, the best binding pose was chosen and was subjected to Nuc Plot (Luscombe et al., 1997) analysis to visualize the interactions between the docked complex.
In vitro evaluation of the study oligonucleotides in RB and epithelial cancer models
CHAPTER 3

Expression of Tiam1 and NCL in RB: Potential Novel Targets for Therapy

Summary of the chapter: This chapter emphasizes on unraveling newer therapeutic targets in RB; and using those targets for theranostic purposes through oligonucleotide technology. The role of Tiam1 protein in RB using knockdown strategy revealed its significance in the invasion of RB cells. Using truncated Tiam1 constructs, importance of different domains in cell migration was studied. NCL, another major target present on the cell surface of malignant cell is being reported for the first time in RB. NCL role in cell proliferation, oncogenic mRNA and miRNA stability, synthesis and membrane lipid arrangement were studied. The mechanism of action and pathways adopted by NCL aptamer under in vivo condition was too studied using protein arrays. As further extension, utilized the NCL aptamer for the delivery of DNAzyme targeting survivin, thus can potentially bring down tumorigenesis of RB.
CHAPTER 3.1

Tiam1 gene knockdown inhibits invasion of RB

3.1.1 RESULTS

3.1.1.1 Differential expression of Tiam1 in RB tumors

In our earlier study, we reported the expression of Tiam1 protein in RB tumors by immunohistochemistry (Adithi et al., 2006). In this study, we analyzed both the mRNA and protein levels of Tiam1 though qPCR and western blotting. The differential expression of Tiam1 in RB tumors (tumors with choroid invasion (CI) <3mm and CI>3mm), Y79 and WERI-Rb1 cell lines were compared to normal cadaveric human retina (n=2). On average of 7.56 ± 1.8, 3.3±0.3 and 5.3±0.5 fold were expressed higher in RB tumors, Y79 and WERI-Rb1 respectively (Figure 14B) using β-2 microglobulin for normalization in relative quantitative PCR. Tiam1 protein levels were higher in expression compared to the normal or non-malignant retina in Western blot. Tiam1 protein bands were observed at ~180 kDa, a loading control β-actin was detected at ~ 42 kDa in Western blot (Figure 14C). The protein bands intensity were represented as graph in Figure 14D.

3.1.1.2 RNA interference of Tiam1 in RB cell lines

To study the cellular events mediated by Tiam1 in RB, Tiam1 knockdown is performed transiently in Y79 cells with three different siRNA sequences (T1, T5 and T6) as mentioned in methods. After 48h of transfection, the silencing efficiency of each siRNA duplexes was determined by Real-time PCR (Figure 15A). Individual siRNA duplexes did not show significant down-regulation of Tiam1 in Y79. As per Parsons et al. (2009) the silencing efficiency was increased when all the three siRNA sequences were pooled and transfected (Parsons et al., 2009). 200nM of siRNA pools showed - 2.75 and -2.0 fold down regulation in Y79 and WERI-Rb1 respectively (Figure 15B). This was further confirmed at protein level by Western blotting (Figure 15C). The intensity of protein bands were measured using ImageJ software and percentage of Tiam1 expression upon siRNA treatment was calculated and represented as graph. In general, Tiam1 is localized in both plasma membrane and cytoplasm of untransfected cells but the expression level on plasma membrane was drastically reduced upon Tiam1 silencing in Y79 and WERI-Rb1 cells (Figure 15D).
Figure 14. Tiam1 constructs and expression of Tiam1 in RB tumors compared to normal retina. 

A. Full length Tiam1 consists of 1591 amino acids. Tiam1 has several specific domains such as, Myr: Myristoylation site; P: PEST sequences; PHn: N-terminal PH domain; CC: Coiled-coil region; Ex: Extended structure; RBD: Ras-binding domain; PDZ: PSD-95/DlgA/ZO-1 domain; DH: Dbl homology domain and PHc: C-terminal PH domain. Deletion of N-terminal myr and P sites leaves the active C1199 form of Tiam1 in truncated constructs. C580 contains only DH and C-terminal PH domain.

C. Western blot analysis of Tiam1 protein expression in RB tumors and normal or non-malignant retina shows Tiam1 at 180kDa and β-actin at 42kDa.

D. Densitometry analysis of the Tiam1 protein expression in RB tumors vs normal or non-malignant retina (n=2). RB1 to RB7 individual expression normalized with averaged NR is represented. Subramanian N et al., PLOS One 2013.
Chapter 3

Figure 15. Knockdown of Tiam1 in Y79 and WERI-Rb1 cell lines using RNA interference. A. Tiam1 silencing in Y79 cells using three siRNA sequences (T1, T5 and T6) targeting different regions of the mRNA at 50nM and 100nM concentrations. B. Down-regulation of Tiam1 in Y79 and WERI-Rb1 cells compared to scrambled siRNA mediated by transfection of 100nM and 200nM of pooled siRNA. The statistical analysis was calculated using student’s unpaired t-test and p<0.05 is indicated as asterisk. C. Western blot analysis of Tiam1 post silencing in Y79 and WERI-Rb1 cell lines. β-actin showing a band at 42KDa is used to normalize within sample and between siRNA transfected and untransfected cells, on the right is the densitometry analysis of western blot showing the relative band intensity of Tiam1 expression of siRNA transfected cells compared to scrambled siRNA transfected cells. The statistical analysis was calculated using students unpaired t-test and p<0.05 is indicated as asterisk. D. Immunofluorescence images of Tiam1 silenced Y79 and WERI-Rb1 cells showing the reduction of Tiam1 expression on plasma membrane compared to untransfected cells showing high level of Tiam1 expression on plasma membrane. The images were taken at ten fields under 100X oil immersion objective. Scale bar: 20μm. Tiam1 is shown as green and nucleus counterstained with DAPI fluoresces blue in color. Subramanian N et al., PLOS One 2013

3.1.1.3 Tiam1 regulates apoptosis and viability in RB cells

Further to understand the functional relevance behind the expression of Tiam1 in cellular activities such as apoptosis and viability, studies were carried out in RB cell lines, Y79 and
Chapter 3

WERI-Rb1 in the presence and absence of Tiam1. To elucidate apoptotic effects upon silencing of Tiam1 annexin-V assay was performed. Results showed 15% and 50% of late apoptotic cells in Y79 and WERI-Rb1 cells respectively upon siTiam1 (Figure 17A). Upon silencing with Tiam1, the cellular metabolic activity of Y79 and WERI Rb1 decreased by 25% in MTT assay (Figure 17B).

3.1.1.4 Tiam1 silencing results in de-regulation of gene expression in Y79 cells

cDNA microarray was performed to analyze the genes regulated upon Tiam1 knock down using Y79 RB cell line. Raw data files were normalized using GeneSpring GX v 12.0. A total of 790 transcripts were observed to be differentially expressed in Tiam1 silenced cells above 1.0 fold (p< 0.05) of which 302 genes were up-regulated and 488 genes were down-regulated. The ‘volcano plot’ arranges genes along dimensions of biological and statistical significance. The first (horizontal) dimension is the fold change between the two groups (on a log scale, so that up and down regulation appear symmetric), and the second (vertical) axis represents the p-value for a t-test of differences between samples (most conveniently on a negative log scale – so smaller p-values appear higher up). The first axis indicates biological impact of the change; the second indicates the statistical evidence, or reliability of the change (Figure 18A). Hierarchical clustering of differentially regulated genes was done using pearson uncentered distance matrix and average linkage rule to establish gene clusters that differentiate the two groups of samples (Figure 18B). Biological analysis of differentially expressed genes was done for gene ontology and pathways using DAVID tool (http://david.abcc.ncifcrf.gov/). Statistically significant ontologies and pathways were filtered based on p-Value <0.05 (Obtained using Fischer Exact Test) with Benjamin Hocheberg FDR correction (Figure 16). The data obtained from microarray analysis was deposited in NCBI’s Gene Expression Omnibus (GEO45130) as per MIAME guidelines.
Figure 16. Pathways and gene ontologies deregulated upon TIAM1 silencing. A. Pie chart represents significantly de-regulated pathways in response to Tiam1 silencing. B. Graphical representation of significantly de-regulated gene ontology. Subramanian N et al., PLOS One 2013
Figure 17. Analysis of Apoptosis and viability of Tiam1 silenced Y79 and WERI-Rb1 cells. A. *In vitro* cell apoptosis assay showing the difference in mean fluorescence intensity between Tiam1 siRNA treated and Scrambled siRNA treated Y79, WERI-Rb1 cells. On the right panel, overlay graph showing the Annexin V expression between the samples. B. MTT assay showing the significant reduction in cell viability in Tiam1 silenced RB cell lines. Error bar represents the standard deviation (SD) from mean of triplicates value and the statistical analysis was calculated using students unpaired t-test and p<0.05 is indicated as asterisk. Subramanian N et al., PLOS One 2013

3.1.1.5 *Significantly down-regulated genes in Tiam1 silenced Y79 cells*

Since Tiam1 acts as GEF, silencing of Tiam1 led to down-regulation of most of the RAS(rat sarcoma) oncogene family of small GTPases such as RAB8B, RAP2A, RHOT2, RAB3D, RAB14, RAB40B, and RABL3, actin cytoskeleton genes- *Homo sapiens* p21 (CDKN1A)- activated kinase 2 (PAK2), *Homo sapiens* CDC42 binding protein kinase gamma (CDC42BPG),
Chapter 3

Homo sapiens CD2-associated protein (CD2AP), Homo sapiens fibroblast growth factor 16 (FGF16), Homo sapiens actin-like protein (FKSG30), Homo sapiens microtubule-associated protein 1B (MAP1B), focal adhesion genes- Homo sapiens tubulin, delta 1 (TUBD1), Homo sapiens chondroadherin (CHAD), Homo sapiens myotilin (MYOT), Homo sapiens myosin light chain kinase 2 (MYLK2), Homo sapiens PDGFA associated protein 1 (PDAP1), Homo sapiens C-terminal binding protein 2 (CTBP2), Homo sapiens CREB regulated transcription coactivator 1 (CRTC1), Homo sapiens matrix metallopeptidase 27 (MMP27), Homo sapiens matrix metallopeptidase 28 (MMP28), Homo sapiens GRB2-associated binding protein 1 (GAB1), Homo sapiens calpain 7 (CAPN7).

3.1.1.6 Significantly up-regulated genes in Tiam1 silenced Y79 cells

Tiam1 knock down in Y79 cells resulted in up-regulation of apoptotic genes- Homo sapiens BCL2-associated X protein (BAX), Homo sapiens programmed cell death 7 (PDCD7), Homo sapiens DNA fragmentation factor (DFFB), Homo sapiens TNF receptor-associated factor 6 (TRAF6), Homo sapiens BCL2-like 12 (BCL2L12), GTP binding genes- Homo sapiens Rho family GTPase 2 (RND2), Homo sapiens RAS guanyl releasing protein 2 (RASGRP2), (multiple drug resistance genes) Homo sapiens ATP-binding cassette, sub-family G member 4 (ABCG4), Homo sapiens ATP-binding cassette, sub-family D member 1 (ABCD1), Homo sapiens cAMP responsive element binding protein 1 (CREB1), and Homo sapiens mitogen-activated protein kinase kinase kinase 4 (MAP3K4), Homo sapiens collagen, type VII, alpha 1 (COL7A1), Homo sapiens suppression of tumorigenicity 5 (ST5), Homo sapiens suppression of tumorigenicity 7 (ST7), Homo sapiens tumor protein p63 regulated 1-like (TPRG1L), Homo sapiens claudin 6 (CLDN6), Homo sapiens cadherin 15 (CDH15).
Chapter 3

A

B

C

D

VALIDATION IN TUMORS
Figure 18. cDNA microarray in Tiam1 silenced Y79 and validation of de-regulated genes in RB cell lines and tumors. Whole genome microarray of Tiam1 silenced Y79 cells was performed using Human-Ht12 beadChip ver. 3 platform. A. Volcano plot showing the genes arranged along biological and statistical significance. Fold change in the horizontal dimension and p-value based on t-test between treated and untreated samples. B. Hierarchical Cluster represents the expression profile of de-regulated genes upon Tiam1 silencing in Y79 cells, compared to untransfected cells. C. Real-time PCR results showing the mRNA expression of selected genes from microarray analysis in Tiam1 silenced Y79 and WERI-Rb1 cells. D. Relative fold change in the mRNA expression of selected panel of genes verified in primary RB showing the negative correlation to that of Tiam1 silenced Y79 and WERI-Rb1 RB cells. Error bar represents the standard deviation of triplicates value. Subramanian N et al., PLOS One 2013

3.1.1.7 Validation of de-regulated genes in Tiam1 silenced RB cell lines and primary RB tumors

From the de-regulated genes list, a panel of genes namely PAK2, MAP1B, RABL3, RAP2A, GAB1, MLCK2, MYOT, CALPAIN7, BAX, PDCD, TRAF6, DFFB were selected for the confirmation of microarray analysis by quantitative PCR in Tiam1 silenced Y79 and WERI-Rb1 cells (Figure 18B). The list of primer sequences used for the SYBR-green based qPCR is given in Table 6. Genes involved in actin cytoskeleton were down-regulated whereas apoptotic genes were up-regulated in post Tiam1 silenced Y79 and WERI-Rb1 cells. The mRNA expressions of these genes in both RB cell lines were consistent with microarray analysis. Similarly qRT-PCR was done to evaluate the correlation of the validated genes expression in primary RB tumors (Figure 18C). mRNA expression of these selected genes were negatively correlate with primary RB tumors. The average fold expression of 12 genes in 12 tumors normalized to two normal or non-malignant retina were, PAK2 (5.78), MAP1B (2.88), RABL3 (5.00), RAP2A (3.99), GAB1 (4.10), MLCK2 (3.47), MYOT (4.15), CALPAIN7 (3.17), BAX (-2.25), TRAF6 (-2.53), PDCD7 (-2.60), DFFB (-3.00). The list of the primary tumors used and its clinic-pathological descriptions, showing the expression levels of validated genes are given in Table 5. Tumors with CI<3mm showed lesser extent of changes in gene expression compared to tumors with CI>3mm (tumor 2, 3, 5, 6, 7). qPCR results were shown in Figure 18 and CI status were shown in Table 5. The expression level of Tiam1 is also directly correlating to the changes in gene expression of validated targets in RB tumor.
Table 5. Clinicopathological features of primary RB tumors and gene expression profile by QRT-PCR

<table>
<thead>
<tr>
<th>S.NO</th>
<th>AGE/SEX</th>
<th>Clinicopathological descriptions</th>
<th>PAK2</th>
<th>MAP1B</th>
<th>RABL3</th>
<th>RAP2A</th>
<th>GAB1</th>
<th>MLCK2</th>
<th>MYOT</th>
<th>CALPAIN7</th>
<th>BAX</th>
<th>TRAF6</th>
<th>PDCD7</th>
<th>DFFB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2/M</td>
<td>RB,PD,Focal RPE invasion,Focal CI&lt;3mm, Pre laminar&amp; Post laminar invasion of ON.</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>NS</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>2</td>
<td>2/M</td>
<td>RB,UD,CI&gt;3mm, Tumor cells invading in anterior border of sclera.</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>3</td>
<td>2/F</td>
<td>RB,PD, CI&gt;3mm, Pre laminar &amp; Post laminar invasion of tumor.</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>NS</td>
<td>↑</td>
<td>NS</td>
<td>NS</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>4</td>
<td>2/F</td>
<td>RB,WD,Focal Invasion of RPE, CI&lt;3mm, No invasion of ON.</td>
<td>↑</td>
<td>NS</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>NS</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>5</td>
<td>6/M</td>
<td>RB,CI&gt;3mm,Tumor cells touching the anterior border of the sclera.</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>6</td>
<td>3/F</td>
<td>RB,MD,Focal CI&gt; 3mm, Pre laminar&amp; Post laminar invasion of ON.</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>7</td>
<td>4/M</td>
<td>RB,PD,CI&gt;3mm, Pre laminar&amp; Post laminar invasion, Tumor invasion into anterior, middle&amp;posterior border of sclera.</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>NS</td>
<td>↓</td>
<td>↓</td>
<td>NS</td>
</tr>
<tr>
<td>8</td>
<td>1/F</td>
<td>RB,WD,Focal CI&lt;3mm</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>9</td>
<td>3/F</td>
<td>RB,PD,NoCI,Pre laminar &amp; Post laminar invasion of ON.</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>10</td>
<td>2/M</td>
<td>RB,PD,NoCl,Pre laminar invasion of ON.</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>11</td>
<td>13mon/M</td>
<td>RB,WD,Focal Invasion of RPE.</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>NS</td>
<td>↑</td>
<td>↑</td>
<td>NS</td>
</tr>
<tr>
<td>12</td>
<td>11/F</td>
<td>RB,MD,Focal CI&lt;3mm</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

F: Female; M: Male; RB: Retinoblastoma; PD: Poorly Differentiated; MD: Moderately Differentiated; WD: Well Differentiated; UD: Un-Differentiated; CI: Choroid Invasion; ON: Optic Nerve; RPE: Retinal pigmented epithelium ↑: Up-regulation above 1 fold change (Log 2 ratio); ↓: Down-regulation above 1 fold change (Log2 ratio); NS: Not Significant fold change. Subramanian N et al., PLOS One 2013
3.1.1.8 Tiam1 regulates actin polymerization, cell migration and invasion in RB cell lines

To investigate the involvement of Tiam1 in actin cytoskeleton regulation, F-actin staining was performed using phalloidin in Tiam1 silenced Y79 and WERI-Rb1 cells (Figure 19A). As mentioned above, Tiam1 was co-localized with actin at cell junctions. In case of Tiam1 knockdown, the cells exhibited lesser extent of actin polymerization at the cellular junctions and actin co-localization. Thus the results show that Tiam1 is essential for actin re-organization in RB cells. Additionally, the function of Tiam1 in cell migration was assessed by wound healing assay (Figure 19B). Tiam1 silenced RB cell lines showed impairment of cell migration, compared to untransfected and scrambled siRNA transfected RB cells. Silencing of Tiam1 in WERI-Rb1 had resulted in lesser number of cells invaded across the matrigel coated invasion chamber. This indicated the decrease in invasion potential depends on Tiam1 expression as it is not observed in the scramble siRNA transfected cells (Figure 20).
Table 6. Primer sequences for the selected panel of validated genes.

<table>
<thead>
<tr>
<th>GENE</th>
<th>PRIMER SEQUENCES (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAP2A</td>
<td>FP: AGA TCA TCC GCG TGA AGC</td>
</tr>
<tr>
<td></td>
<td>RP: CCC CAC TCT TCA GCA AGG</td>
</tr>
<tr>
<td>BAX</td>
<td>FP: TGG AGCTGCAGAGGATGATTG</td>
</tr>
<tr>
<td></td>
<td>RP: GAAGTTGCGTCAGAAAACATG</td>
</tr>
<tr>
<td>Rabl3</td>
<td>FP: TTGGGAGACTCAGGTGTGGGAAA</td>
</tr>
<tr>
<td></td>
<td>RP: CAGTTGGCACCAAATCCCTGTGTA</td>
</tr>
<tr>
<td>PAK2</td>
<td>FP: GAATGGGAAGGATCTGTTAGCAGTCA</td>
</tr>
<tr>
<td></td>
<td>RP: GCCATAAGCCTTCCGTAACC</td>
</tr>
<tr>
<td>MAP1B</td>
<td>FP: AAAGTGTCCAGGGGAGGCTTC</td>
</tr>
<tr>
<td></td>
<td>RP: CTCTCTGTACCCTTCCTCA</td>
</tr>
<tr>
<td>TRAF6</td>
<td>FP: TGGCATTACGAGAAAGCAGTG</td>
</tr>
<tr>
<td></td>
<td>RP: GTTCCATCTTGTGCAAACAAACC</td>
</tr>
<tr>
<td>DFFB</td>
<td>FP: GCCCTGCTTTTACCTCAGA</td>
</tr>
<tr>
<td></td>
<td>RP: CGTTCCGCACAGGCTGCTT</td>
</tr>
<tr>
<td>MLCK2</td>
<td>FP: GAGCTGAGGACCAGGAAT</td>
</tr>
<tr>
<td></td>
<td>RP: AGGTACAGACTGCCCCCAAAC</td>
</tr>
<tr>
<td>CALPA1N7</td>
<td>FP: ATCTGGAAAAGATCCAAGCT</td>
</tr>
<tr>
<td></td>
<td>RP: GCACGCTCTAAGACCAACAG</td>
</tr>
<tr>
<td>PDCD7</td>
<td>FP: TTGACCCAGGCTGCTAT</td>
</tr>
<tr>
<td></td>
<td>RP: AATCTCTGCTCCTGCTT</td>
</tr>
<tr>
<td>MYOT</td>
<td>FP: CGACTGCAAGTCTACATCA</td>
</tr>
<tr>
<td></td>
<td>RP: TGAATGAACCGTGGTGGTA</td>
</tr>
<tr>
<td>GAB1</td>
<td>FP: ACCTCAAGCCAGACAGAAAGT</td>
</tr>
<tr>
<td></td>
<td>RP: TCGAGCAAAACTCCTAGTGAT</td>
</tr>
</tbody>
</table>
3.1.1.9 Plasma membrane localization of Tiam1 is mediated by N-terminal PH domain

Since Tiam1 localizes along with F-actin and controls the actin cytoskeleton, we investigated which domain of the protein regulates the localization Tiam1 on plasma membrane in RB. RB cell lines (Y79 and WERI-Rb1) transfected with full length and C1199 Tiam1 showed the membrane localization and induced the membrane ruffling (Figure 22). In contrast, C580 Tiam1 was localized to the nucleus and failed to induce membrane ruffling.

Figure 19. F-actin staining of Tiam1 deficient RB cell lines. Tiam1 silenced Y79 cells and WERI-Rb1 cells were fixed, immunofluorescently labeled for Tiam1, nucleus stained with DAPI, stained with phalloidin and images were taken at 40X in ten fields. Bar represents 20μm. Subramanian N et al., PLOS One 2013
Figure 20. Invasion of Tiam1 deficient RB cell lines. Phase contrast microscope images of wound healing assay showing the cell migration pattern in Tiam1 deficient RB cell lines at 0h and 48h post silencing. Tiam1 knockdown cells were unable to migrate whereas the untransfected and control cells showed increased migration, migrated cells were indicated with arrows. The images were acquired using AxioObserver microscope at 5X objective with 1X optovar. Graph on the right of respective panel shows the number of cells migrated. Subramanian N et al., PLOS One 2013
3.1.1.10 N-terminal PH domain regulates RB cell motility

We observed that N-terminal PH domain, but not C-terminal PH domain modulates the localization of Tiam1 and induces membrane ruffling in RB cells. Further we determined the association of membrane localization of Tiam1 and cell migration in Y79, WERI-Rb1 cells. To elucidate this, wound healing assay was performed in Y79 and WERI-Rb1 cells. The cells transfected with full length Tiam1 and C1199 Tiam1 were showing more cell migration towards the wound, whereas C580 Tiam1 transfected cells showed delayed cell migration into the wound in both cell lines (Figure 23).
Figure 22. Localization of Full length Tiam1, C1199 Tiam1 and C580 Tiam1 in RB cell lines. A. Immunofluorescent images of Y79 cells. B. WERI-Rb1 cells transfected with Full length Tiam1, C1199 Tiam1 and C580 Tiam1 plasmids tagged with HA. Tiam1 and F-actin were stained as mentioned in the materials and methods. Full length Tiam1 and C1199 Tiam1 but not C580 Tiam1 were localized to plasma membrane. Unlike Full length Tiam1 and C1199 Tiam1, C580 Tiam1 could not induce membrane ruffling. Representative images were taken from 10 independent fields using AxioObserver fluorescent microscope at 100X and arrows indicate the membrane ruffling. Scale bar: 20μm. Subramanian N et al., PLOS One 2013
Figure 23. N-terminal PH domain maintains cell motility in RB cells. Phase contrast images showing the migration of Y79 and WERI-Rb1 cells transfected with Full length Tiam1, C1199 Tiam1 and C580 Tiam1 in wound-healing assay. White line indicates the original wound edge which made by a pipette tip. The images were taken from 10 different locations. Cells transfected with full length and C1199 Tiam1 showing significant increase in cell migration rate compared to the cells transfected with C580 Tiam1. The Images were captured at 5X objective in AxioObserver microscope. Subramanian N et al., PLOS One 2013
Chapter 3

Figure 24. Tiam1 mediated signaling pathway. Schematic representation showing the upstream and downstream effects of Tiam1 in intracellular pathways. External stimulants phosphorylate the receptor tyrosine kinase which in turn activates Ras-GTP, binds to RBD domain of Tiam1. Interaction of EphB, CD44 and Ankyrin Proteins with PHn domain of Tiam1 and CADM1 with PDZ domain leads to the activation of Rac-GTP at DH domain of Tiam1. The activated Rac-GTP involves in various signaling pathways, mainly on actin cytoskeleton. The binding of PHn domain to phosphoinositide facilitates the membrane localization of Tiam1. PDGF: Platelet-derived growth factor; EGF: Epidermal growth factor; RTK: Receptor tyrosine kinase; PIP3: Phosphatidyl inositol triphosphate; PIP2: Phosphatidyl inositol diphosphate; PHn: N-terminal PH domain; RBD: Ras binding domain; PDZ: PSD-95/DglA/ZO-1 domain; DH: Dgl homology domain; PHc: C-terminal PH domain; JNK: Jun N-terminal kinase; PAK: p21-activated kinase. Subramanian N et al., PLOS One 2013
3.1.2 DISCUSSION

Though Tiam1 has been shown to play a crucial role in actin cytoskeleton, cell migration and invasion, various intracellular pathways are involved in activation of upstream and downstream signaling of Tiam1 (Figure 24) (Connolly et al., 2005, Sander et al., 1998, Michiels et al., 1997, Ceccarelli et al., 2007). In particular, Tiam1 is required for activation of Rac1 and cdc42 to produce specific actin rich structures like membrane ruffling, lamellipodia, filopodia (Demarco et al., 2012, Yamauchi et al., 2005, Bourguignon et al., 2000a, Bourguignon et al., 2000b, Matsuo et al., 2003, Veluthakal et al., 2009) and for neurite outgrowth (Shirazi Fard et al., 2010). Addition to that, Tiam1 interacts with CADM1, Ephin and Arp2/3 to initiate Rac1 mediated actin cytoskeleton remodeling (Masuda et al., 2010, Tolias et al., 2007, Tanaka et al., 2004). Hence in the current study, we analyzed the importance of Tiam1 in RB cells using short interfering RNA (siRNA) mediated knockdown studies.

Silencing of Tiam1 in RB cell lines followed by cDNA microarray showed various pathways and genes altered, predominantly genes related to MAPK pathway, small GTPase, apoptosis and cell migration. The impairment of cell migration in Tiam1 silenced Y79 and WERI-Rb1 cells was proved by Wound healing and matrigel invasion assay. This correlates with the microarray analysis where the actin cytoskeleton genes were down-regulated in Tiam1 silenced Y79 cells resulting in lesser cellular migration potential (Figure 20). One of the down-regulated actin cytoskeleton gene in our microarray analysis is MAP1B (Microtubule-associated protein 1B) which is needed for axonal development, reported to be involved in neurite growth, neuron migration and metastasis (Tymanskyj et al., 2012, Gordon-Weeks and Fischer, 2000, Gonzalez-Billault et al., 2005). MAP1B is found to interact with Tiam1 thereby activating Rac1 and cdc42 and further inhibiting RhoA activity which leads to actin polymerization and axonal elongation (Montenegro-Venegas et al., 2010, Tortosa et al., 2011). MAP1B deficient cells exhibit a decreased cell migration and axonal development (Gonzalez-Billault et al., 2001, Takei et al., 2000). The other mechanism might be PAK mediated activation of MyosinII, a protein involved in stress fiber formation and contraction (Nakayama et al., 2005). The phosphorylation of myosinII light chain (MLC) by myosinII light chain kinase (MLCK) regulates actin-myosin II interaction (Shin et al., 2009). We observed that PAK2 and MLCK were down-regulated in Tiam1 silenced RB Y79 cells. Moreover, small GTPase subfamily Rab-like 3 (Rabl3) and actin-
binding protein Myotilin, promote motility, tumor cell survival (Li et al., 2010). The down-regulation of Rabl3 and myotilin might as well attribute to the suppression of cell motility in Tiam1 silenced cells. The expression level of these genes when validated in primary RB tumors showed differential expression correlating with their CI status. CI represents the invasion potential of the given tumor during the enucleation, which may or may not have undergone chemotherapeutic treatment. The tumors with CI >3mm compared to CI <3mm cases showed increased expression of pro-survival genes and decreased expression of apoptotic genes.

From our annexin-V assay results, a remarkable increase in apoptosis was observed. The mechanism of apoptotic induction might be due the up-regulation of pro-apoptotic gene BAX (Bcl2 associated X), PDCD7 (Programmed cell death protein), TRAF6 (Tumor necrosis factor receptor-associated factor 6) and DFFB (DNA fragmentation factor subunit beta) upon Tiam1 knockdown (Xu et al., 2002, Ouyang et al., 1998, Gavathiotis et al., 2012, Enari et al., 1998, He et al., 2006). Similar to our results, apoptotic cell death has been observed earlier upon Tiam1 silencing (Otsuki et al., 2003).

Since endogenous Tiam1 is localized in both plasma membrane and cytoplasm, we were interested to find out which domain of the Tiam1 protein regulates the localization intracellularly in RB cells. We elucidated that N-terminal PH domain of Tiam1 mediates the membrane localization and invasion but not the C-terminal PH domain and targeting the N-terminal PH domain of Tiam1 affects the cell migration and invasion. Earlier studies showed that localization of Tiam1 to plasma membrane requires N-terminal PH domain. Also the membrane localization of Tiam1 is required for the activation of the c-Jun NH2-terminal kinase (JNK) thus leading to successful Rac mediated signaling (Michiels et al., 1997). N-terminal PH domain of Tiam1 has affinity towards phosphoinositides and its interaction with transmembrane domain accelerates the membrane localization. For the first time our data has additionally elucidated the importance of the N-terminal region with respect to the migration as it is directly correlating with actin cytoskeleton modulation in RB. From our findings, we suggest that Tiam1 can be utilized as a potential target for RB therapy. In future, docking studies to find potent inhibitors to RBD binding or alternatively aptamers targeting the N-terminal PH domain of Tiam1 need to be isolated, which have better application in vivo for drug delivery purposes.
CHAPTER 3.2
NCL regulates miRNA 17-92 cluster and lipid arrangement

3.2.1 Results

3.2.1.1 NCL is overexpressed in RB

The expression level of the NCL was analysed by qPCR on the tumor samples by normalizing the expression with the normal retina. The fold change in NCL mRNA expression level across the RB tumors varied between folds 1.5 to 7.6 fold of over-expression. The expression levels as analysed by comparing the cell lines to normal or non-malignant retinarevealed the highest expression of NCL in Y79 with 6.3 folds, followed by 4.4 folds in WERI-Rb1 and 2.0 and 1.4 fold in ARPE-19 and MIO-M1 respectively (Figure 25A).
NCL is present in nucleus and cytoplasm usually but during malignant conditions it is expressed on the membrane surface. Hence, immunofluorescence and flow cytometry was performed on live cells. Increased levels of surface NCL expression was observed in the case of RB tumor cells, ten RB tumors analysed by flow cytometry were found to be highly positive for the NCL expression and representative scatter plots showing the NCL expression and aptamer uptake are shown in figure 25B. NCL expression was studied by IHC in 15 cases and the expression levels were intensely observed in the membrane/cytoplasm of the RB. In NR section, the retinal cells showed faint nuclear staining and no cytoplasmic staining (Figure 25C, ia, ib).
Table 7. Clinicopathological details of the RB tumor and the NCL expression by IHC studies

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Age and sex</th>
<th>Histopathology report</th>
<th>NCL distribution</th>
<th>NCL expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3mon, F</td>
<td>RB, WD, No CI, No invasion of ON.</td>
<td>heterogeneous</td>
<td>60%</td>
</tr>
<tr>
<td>2</td>
<td>3y, F</td>
<td>RB, PD, No CI, No invasion of ON.</td>
<td>homogenous</td>
<td>40%</td>
</tr>
<tr>
<td>3</td>
<td>3mon, M</td>
<td>RB, PD, No CI, No invasion of ON.</td>
<td>membrane positivity</td>
<td>60%</td>
</tr>
<tr>
<td>4</td>
<td>2y, M</td>
<td>RB, MD, No CI, Prelaminar invasion of ON.</td>
<td>heterogeneous</td>
<td>50%</td>
</tr>
<tr>
<td>5</td>
<td>3y, F</td>
<td>RB, PD, No CI, Prelaminar invasion of ON. Tumor cells touching anterior fibres of sclera.</td>
<td>homogenous</td>
<td>80%</td>
</tr>
<tr>
<td>12</td>
<td>2y, M</td>
<td>RB, WD, focal RPE invasion, No CI.</td>
<td>membrane positivity</td>
<td>70%</td>
</tr>
<tr>
<td>9</td>
<td>2y, F</td>
<td>RB, FD, focal RPE invasion, No CI prelaminar, laminar and PL</td>
<td>heterogeneous</td>
<td>65%</td>
</tr>
<tr>
<td>8</td>
<td>2y, F</td>
<td>RB with focal retinoma, focal CI&lt;3mm, tumor touching anterior border of sclera, No invasion into prelaminar, laminar and PL</td>
<td>membrane positivity</td>
<td>50%</td>
</tr>
<tr>
<td>6</td>
<td>5mon, M</td>
<td>RB, WD, Focal CI&gt;3mm, No invasion of ON. Tumor cells touching anterior fibres of sclera.</td>
<td>membrane positivity</td>
<td>70%</td>
</tr>
<tr>
<td>7</td>
<td>9mon, M</td>
<td>RB, MD, invasion of iris stroma, tumor seen over anterior surface of iris. Multiple focal CI&gt;3mm, Prelaminar, laminar, PL invasion of ON</td>
<td>heterogeneous</td>
<td>70%</td>
</tr>
<tr>
<td>10</td>
<td>3y, M</td>
<td>RB, PD iris neo vascularization, tumor cells over iris, CI&gt;3mm, tumor cells invading anterior fibres of sclera, Prelaminar, laminar and PL invasion of ON</td>
<td>membrane positivity</td>
<td>80%</td>
</tr>
<tr>
<td>11</td>
<td>14y, M</td>
<td>RB, PD, iris neo vascularization, tumor invasion into anterior fibers of sclera, diffuse CI&gt;3mm, prelaminar, laminar and PL invasion of ON.</td>
<td>membrane positivity</td>
<td>75%</td>
</tr>
<tr>
<td>13</td>
<td>3y, F</td>
<td>RB, MD, CI&gt;3mm. Prelaminar, laminar and minimal PL invasion of ON.</td>
<td>membrane positivity</td>
<td>60%</td>
</tr>
<tr>
<td>14</td>
<td>3y, F</td>
<td>RB, MD, CI&gt;3mm. Prelaminar invasion.</td>
<td>membrane positivity</td>
<td>70%</td>
</tr>
<tr>
<td>15</td>
<td>2y, M</td>
<td>RB, PD, tumor cells on iris surface. No invasion of CI, prelaminar, laminar and PL invasion of ON. Section of ON show full thickness tumor invasion</td>
<td>membrane positivity</td>
<td>80%</td>
</tr>
</tbody>
</table>

F: Female; M: Male; RB: Retinoblastoma; PD: Poorly Differentiated; MD: Moderately Differentiated; WD: Well Differentiated; UD: Un-Differentiated; CI: Choroid Invasion; ON: Optic Nerve; RPE: Retinal pigmented epithelium
The staining pattern within the tumors varied, NCL was heterogenous in expression and on average 50% cells showing positive and few cases more than 70% positive (Figure 25C, iia, iib) as shown in Table 7. Upon correlating the invasion patterns by clinicopathological analysis with the NCL expression, we observed higher levels of NCL expression in the 8 out of 10 cases of CI and prelaminar invasion. The differential distribution of NCL under cancer condition in cellular compartments is studied. The expression levels between the NR and RB samples of nuclear and cytoplasmic extracts were studied by western blotting. The results showed over-expression of NCL in both nucleus and cytoplasm of the malignant retina compared to the non-malignant retina (Figure 25D). The densitometry analysis of Western blotting showed upregulation of NCL in cytoplasm of the malignant retina compared to the normal or non-malignant retina (Figure 25D below). Thus the NCL expression on membrane surface of RB cells aids in aptamer uptake by cells. NCL is expressed on the surface of the Y79, WERI-Rb1 cells as analysed by flow cytometry and microscopy of WERI-Rb1 also reveals membrane NCL (Figure 26A and B).

3.2.1. NCL aptamer perturbs RB cell growth and leads to cell death

The stability of the aptamer was assessed under physiological pH and varying serum concentrations. The aptamer was found to be stable in physiological pH in PBS and in 10% FBS (Figure 26C). The expression of surface NCL and uptake of NCL aptamer studied by IF showed surface and nuclear localization respectively (Figure 26B) by microscopy. The uptake of NCL-Apt in MIO-M1 vs RB cell lines showed preferential uptake by cancer cells sparing normal cells (Figure 27A). The morphological changes upon the NCL-aptamer treatment in RB, control cell lines are shown in the Figure 27B, the undivided cells remains as cluster of the cells due to the cells arrested in synthesis phase and unable to divide. The image taken under 5X objective clearly shows the clustering of cells. Such effects were not observed in the MIO-M1, non-malignant cell line (Figure 27B, right most panel).
Chapter 3

Figure 26. NCL expression on RB cell lines and stability of NCL aptamer. A. Microscopic images showing the expression of NCL in RB cell line, WERI-Rb1. WERI-Rb1 cells were stained using anti-NCL antibody (Abcam) followed by secondary anti-rabbit FITC. The NCL-aptamer was used at concentration of 500nM. B. Histogram overlay plots showing the expression of NCL in Y79 and WERI-Rb1 cell lines. C. Stability of the NCLAPT was tested in the 1X PBS and 10% FBS at pH7.0±0.2 upto 96h.
Figure 27. NCL-aptamer binding and its effect on RB cell lines. A. Histogram overlay plots showing the binding of NCL-aptamer to MIO-M1, WERI-Rb1 and Y79 cell lines. The NCL-aptamer was used at concentration of 500nM. B. Morphological changes accompanying treatment with NCL-APT in Y79 and WERI-Rb1 cell line. Phase contrast microscopic images of Y79 and WERI-Rb1 cells treated with NCL-aptamer (10μM) and MIO-M1.

The anti-proliferative property of the NCL-aptamer was tested in the RB and control cell lines by performing the MTT assay. The NCL-aptamer was tested from 0.5 μM to 20 μM in Y79, WERI-Rb1 and MIO-M1 cell lines and found to have cell viability around 40%, 42% and 90% in the respective cell lines at 20 μM (Figure 28A). The concentration of 10 μM was used in the other functional studies that has viability of around 60-65% in Y79 and WERI-Rb1 cell lines and uncompromised viability in MIO-M1 cell line (Figure 28A). The effect of aptamer on cell cycle was verified in the RB cell lines and MIO-M1 by PI staining and flow cytometry. We additionally included a LNA modified NCL-aptamer, LNA-NCL-aptamer. The MIO-M1 cells showed no significant change in the cell cycle phases. The histogram plot for the WERI-Rb1 treated with LNA-NCL-aptamer and NCL-aptamer clearly shows increase in the G0-G1 and S phase cells (5 to 10%) indicating arrest in S phase and leading to the cell death. Similarly, in Y79 increased arrest in S phase is observed around (20 to 30%) (Figure 29A).
Figure 28. Cytostatic and cytotoxic effect of NCL aptamer on RB cells. A. Graph showing the percentage cytotoxicity exhibited by the aptamers on Y79, WERI-Rb1 and MIO-M1 cells at the concentration of 0 to 20 µM at 48h. The error bar represents the standard deviation and the * indicates significance of P<0.05 and ** indicates significance of P<0.001.

The effect of NCL-aptamer, LNA-NCL-aptamer was studied in all cell lines, additionally the cytotoxic effect of co-drug treatment was evaluated in the Y79 and WERI-Rb1 cell line using LDH assay at 24h and 48h. The synergistic effect of the drug and NCL-aptamer, LNA-NCL-aptamer was observed in Y79 cell line by increasing the LDH activity by two fold at 24h and 5 fold at 48h. The WERI-Rb1 showed 0.5 fold with LNA-NCL-aptamer at 24h and 2.0 to 2.5 fold at 48h with both NCL-aptamer and LNA-NCL-aptamer (Figure 29B and C). The LDH activity in MIO-M1 cells were insignificant except that at 24h NCL-aptamer showed less than 0.4 fold increase in LDH activity (Figure 29B and C). The RB primary cells obtained from the enucleated eyes were cultured upto 8 to 10 days. The cells tested for the metabolic activity by transfecting the pGFP DNA showed expression of GFP at 24h of transfection. The expression was majorly on the periphery and the central region of RB tumor spheres showed lesser fluorescence due to physical nature of the larger cell clusters (Figure 30).
Figure 29. Effect of NCL-aptamer on RB cell lines. A. Cell cycle changes. The histogram plot showing the cell cycle changes accompanying the treatment of RB cell lines, Y79, WERI-Rb1 and normal retinal derived cell line, MIO-M1 with L-NA (LNA modified NCL-APT) and NCL-aptamer(NCL-APT). On the right respective graph shows the variation in percentage cell counts at different phases of cell cycle. B. Graph showing the percentage cytotoxicity was calculated by the LDH activity induced upon the NCLAPT (NCL-aptamer) (15 μM) and L-NA(LNA-NCL-aptamer) (15 μM) alone or with 1 μg/ml etoposide treated on Y79 and WERI-Rb1 cells at 24 h and 48h (C). The error bar represents the standard deviation (SD) from mean of replicate experiments and the * indicates significance of P<0.05 and ** indicates significance of P<0.001.
Figure 30. Transfection in primary RB cells. Expression of GFP plasmid in primary RB cells. Microscopic images of the expression of GFP in RB primary cells transfected with lipofectamine 2000. 24h after transfection cells were imaged with 20X objective. Cells were washed after 24h of transfection, with 1X PBS and imaged under 20X objective.
These cells exhibited uptake of the FITC-NCL-aptamer and the aptamer uptake was differential and majorly by the peripheral cells (Figure 31A). As the cells were actively transfected, silencing of NCL was performed to counter confirm the NCL mediated cellular activity, as exhibited by NCL-aptamer. NCL silencing (siNCL), at a concentration of 200 nM and aptamer, at a concentration of 15 μM was used to study its effect in cell cycle and proliferation. The cell cycle
changes studies in RB primary cells exhibited increase in G0-G1 phase cells by 15% and reduction in S phase around 14% (Figure 31B).

The cytotoxic effect of NCL-aptamer and siNCL studied by LDH studies showed the increased cytotoxicity in the NCL-aptamer treated cells than the siNCL cells. MTT assay revealed increase in cell death with siNCL compared to the NCL-aptamer. The decrease in LDH activity with siNCL treatment could be due to death of cells leading to lower levels of LDH secretion (Figure 31C and D).

**3.2.1.3 NCL regulates onco-miRNAs expression in RB**

NCL is reported for its involvement in the microprocessor machinery and regulation in the Dicer expression. We sought to study the effect of the NCL-aptamer in the mature miRNA expression. WERI-Rb1 cell line was chosen for the study as it showed higher regulation in the gene expression upon NCL-aptamer. The NCL-aptamer treated WERI-Rb1 cells were subjected to miRNA microarray and the global miRNA expression profiling was performed. The hierarchical clustering of the miRNA showed 46 miRNAs (Figure 32A) that are significantly downregulated (P<0.05) with 9 of them shortlisted shown in (Figure 33A). These miRNAs were chosen as they harbour the G-quadruplex or i-tetraplex structures in their promoter. These miRNAs majorly affects the pathways in cancer and metabolic pathways followed by cell adhesion and actin cytoskeleton changes (Figure 33B).
Figure 32. Changes in miRNA expression accompanying NCL-aptamer treatment. miRNA microarray. A. Hierarchical clustering of the miRNAs from the WERI-Rb1 control cell line post treatment with NCLAPT (NCL-aptamer). The color range basically indicates the status of expression (Yellow being neutral and towards green being downregulation) B. Graph showing the fold change in the miRNA levels - mir-196b, mir-330, mir-152 and mir-1 in RB cell lines normalized with MIO-M1. C. Graph showing the fold change in the mature miRNA levels of mir17-92 cluster, miR-330, miR-152, miR-1, miR-196b and miR-330 in Y79 cells treated with NCL-aptamer as represented above. D. Graph showing relative change in expression of mRNA levels upon treatment with NCL-aptamer on RB cell lines. The error bar represents the standard deviation and the * indicates significance of P<0.05 and all other treated samples compared to control were of significance of P<0.001 (not mentioned with any notation due to space constrain).

From the listed 9 miRNAs, the expression level of miR-196b, miR-152, miR-1 and miR-330 along with the miR-17-92 cluster in RB samples were analysed. The innate expressions of newly studied miRNAs were analyzed in RB cell lines (Figure 32B) normalized to the MIO-M1. The MIO-M1 showed the least expression of the miRNAs, followed by WERI-Rb1 and Y79 showed
upregulation of all the miRNAs. The innate expression of the mature miR-17, miR-18a, miR-19b, miR-20a were 1.0, 1.5, 5.0, 5.5 fold upregulated in Y79 and WERI-Rb1 than MIO-M1 (Figure 33C). The changes in expression of these miRNAs upon NCL-aptamer treatment in MIO-M1 showed no positive correlation (Figure 33D).

The shortlisted miRNAs from the microarrray and mature miR-17-92 were validated by qPCR using TaqMan based method in the WERI-Rb1 and Y79 cells treated with NCL-aptamer. The NCL-aptamer treatment showed downregulation of both listed miRNAs and the miR-17-92 cluster in both cell lines. The downregulation was efficient in WERI-Rb1 than Y79 cells (Figure 32C). The expression levels of these miRNAs under silenced condition in WERI-Rb1 were found to be downregulated (Figure 33E).

Figure 33. Effect of NCL-aptamer on RB cell lines. A. Hierarchical clustering of the shortlisted miRNA harboring the g-quadruplex structure in their promoters. The color range basically indicates the status of expression (Yellow being neutral and towards green being downregulation). B. Pathways affected by the miRNA regulated genes upon treating WERI-RB1 cells with NCL-aptamer (10μM), bottom panel shows the pie chart drawn from the output generated by Gene spring software. C. Graph showing the fold change in the miRNA levels(mir-17-92 cluster) between MIO-M1 cells and RB cell
Chapter 3

3.2.1.4 NCL aptamer disrupts NCL - G-rich mRNA interaction thereby alters gene expression in RB

NCL has been already shown for its binding to the AU-rich element of the Bcl2 mRNA and to other mRNA that has G-rich sequences in their 5’UTR (Abdelmohsen et al., 2011, Otake et al., 2007). Implication of NCL in cancer stem cell marker or pluripotent marker expression is not directly shown, hence we studied those alternations using the functional blocking aptamer, NCL-aptamer or by silencing NCL. Gene expression analysis of Bcl2, OCT4, SOX2, NANOG, MYCN and 18s were performed using the primers listed in table 8 by qPCR in WERI-Rb1 cells and Y79 showed downregulated upon NCL-aptamer treatment (Figure 32D). Western blotting also confirmed the presence of lower amount of Bcl2 in cells treated with NCL-aptamer, alternatively FOXM1 was found to be upregulated at protein levels in the NCL-aptamer treated cells (Figure 34A). mRNAs harbouring G-rich sequence in the 5’UTRs reported earlier were analysed in the current study and the expression levels of DUS1, MGAT, LRP, PDCD2, AKT and MG21 mRNA were downregulated upon treatment with NCL-aptamer in WERI-Rb1, could be due to the disruption of NCL-mRNA complex. There was downregulation MDM2 and upregulation of p53 indicating the repair mechanism initiated by it. Overall, the tumor antigen and stem cell markers expression were majorly shut down with the tumor suppressor p53 levels increase in the NCL-aptamer treated cells (Figure 34B).
Figure 3.4. Effect of NCL-aptamer and siNCL treatments on gene expression changes in RB cell lines. Changes in gene expression upon NCL-aptamer treatment. A. (i) Western blot showing the changes in FOXM1 and Bcl2 levels in the WERI-Rb1 cells, Y79 cells treated with NCL-aptamer (10 \( \mu M \)) for 48h. \( \beta \)-tubulin was used as loading control. (ii) Panel right to the western blots shows the relative expression levels calculated by densitometry. B. Graph showing the fold changes in expression levels of the NCL regulated mRNAs, Bcl2 and others upon treating WERI-Rb1 cell line with NCL-aptamer (10 \( \mu M \)) for 48h respectively. SYBR green based qPCR was performed to calculate the mRNA fold change. C. (i) Western blot showing the changes in NCL (nucleolin) levels in the Y79 and WERI-Rb1 cells transfected with siNCL (200 nM) for 48h. \( \beta \)-tubulin was used as loading control. (ii). Graph showing the relative expression levels of NCL (nucleolin) in Y79 and WERI-Rb1 cells transfected with siNCL (200 nM) for 48h.
expression levels of NCL as calculated by densitometry from the western blots. D. Graph showing the relative change in expression of the mRNA levels upon transfecting Y79 and WERI-Rb1 cells with siNCL (200nM) for 48h performed by SYBR green based qPCR. The error bar represents the standard deviation and the * indicates significance of $P<0.05$, others were of significance of $P<0.001$ and the non significant ($P>0.05$) were indicated with “NS”.

Table 8. List of primers used in the NCL study.

<table>
<thead>
<tr>
<th>Oligo</th>
<th>sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIR F</td>
<td>GACCACCGCATCTCTACATTC</td>
</tr>
<tr>
<td>BIR R</td>
<td>TGCTTTTTATGTTCCCTCTATGGG</td>
</tr>
<tr>
<td>STMN1 F</td>
<td>CTC GGA CTG AGC AGG ACT TTC</td>
</tr>
<tr>
<td>STMN1 R</td>
<td>ATT CTT TTG ACC GAG GGC TG</td>
</tr>
<tr>
<td>Oct-4 F</td>
<td>CTTGCTGCAAGAATGGGTGGAGGAA</td>
</tr>
<tr>
<td>Oct-4 R</td>
<td>CTGCAGGTGTTGGGGTTTCGAGGA</td>
</tr>
<tr>
<td>Nanog F</td>
<td>AGTCCTAAAAGGCAAACACACACACTTCT</td>
</tr>
<tr>
<td>Nanog R</td>
<td>TGCTGAGGGCTAGAGGTATTTTCGAGGC</td>
</tr>
<tr>
<td>Sox-2 F</td>
<td>ATGCACCGCTACGAGCTGTA</td>
</tr>
<tr>
<td>Sox-2 R</td>
<td>CTGTTCACCCCTCCCATTT</td>
</tr>
<tr>
<td>CD44s F</td>
<td>AAGGAGCAGCACTCAGAGA</td>
</tr>
<tr>
<td>CD44s R</td>
<td>TGGTGCTTGCTTGCTCCGTGAC</td>
</tr>
<tr>
<td>CD133 F</td>
<td>CTGGGCTTGCTGT TTATTATCTG</td>
</tr>
<tr>
<td>CD133 R</td>
<td>ACGCCTGTCTCTTTGGTAGTTG</td>
</tr>
<tr>
<td>MRP1 F</td>
<td>CGTCTACTCCAAACGCTGAC</td>
</tr>
<tr>
<td>MRP1 R</td>
<td>CTGGACCGCTGACCCCGTGAC</td>
</tr>
<tr>
<td>B-2-M F</td>
<td>TATCCAGGGTACTCCAAAGA</td>
</tr>
<tr>
<td>B-2-M R</td>
<td>GACAAGTCTGAATGCTCCAC</td>
</tr>
<tr>
<td>DUS1 F</td>
<td>GACGCCATCAGTCCAGTT</td>
</tr>
<tr>
<td>DUS1 R</td>
<td>GCACGGTAGAGCAAGAGAA</td>
</tr>
<tr>
<td>AKT1 F</td>
<td>TCTATGGCGCTGAGATTGTG</td>
</tr>
<tr>
<td>AKT1 R</td>
<td>CTGAATGTCAGGCTCCTTGT</td>
</tr>
<tr>
<td>PDCD2 F</td>
<td>CCCAGAGAAACAGGAGAATC</td>
</tr>
<tr>
<td>PDCD2 R</td>
<td>TATGCTTTTGTCACGTCTGGA</td>
</tr>
<tr>
<td>MGAT1 F</td>
<td>TGTGCAAGAGGTTGGCAAAC</td>
</tr>
<tr>
<td>MGAT1 R</td>
<td>GGGAGGGAGGAGGAGTATAG</td>
</tr>
<tr>
<td>LRP3 F</td>
<td>TGGCAAGACAGGAAGACTG</td>
</tr>
<tr>
<td>LRP3 R</td>
<td>TCTGAGATGACAGGTGTG</td>
</tr>
<tr>
<td>MG21 F</td>
<td>CTGGTCAAGCAGGAAAGCAC</td>
</tr>
<tr>
<td>MG21 R</td>
<td>GCTCACTGAGGACCTTCTC</td>
</tr>
</tbody>
</table>
The importance of NCL in maintenance of the mRNA stability was studied by performing knockdown study. NCL expression was downregulated upon silencing with 200nM of siRNA. Western blot analysis revealed 55% and 60% of NCL protein expression downregulation in Y79 and WERI-Rb1 respectively by densitometry analysis (Figure 34C). The expression level of the NCL mRNA was about 85% and 75% downregulated in the Y79 and WERI-Rb1 cells as analyzed by qPCR upon silencing. The changes in expression of G-rich mRNA such as AKT, MGAT, DUS2 upon siNCL in both the cell lines showed downregulation in addition to the MYCN and Bcl2 (Figure 34D).

3.2.1.5 Lipid profile changes upon knocking down NCL and using NCL-aptamer

As the lipid content of the cell is important in the cellular metabolism and to maintain the normal behaviour of cells, we studied the effect of NCL knockdown or blocking by silencing of NCL or with a functional aptamer. To begin with this, we did lipid profiling in positive mode using methanol as solvent by DESI MS. Positive ion mode is meant for the glycerophosphocholines or phosphatidylethanolamine (PC), galactoceramides (GalCer) and sphingomyelins detections (Gulcan et al., 1993). The lipid spectrum obtained for the Y79, WERI-Rb1 and MIO-M1 are presented in the Figure 35A. The difference in the intensities of the lipids $m/z$ 756.6, $m/z$ 768.6, $m/z$ 780.6, $m/z$ 804.6, $m/z$ 808.6 and $m/z$ 832.7 indicates the rearrangement and different levels of lipids in the membrane of normal to cancer cell lines. Also the imaging of cells treated and untreated showed decrease in the lipid expression (as observed by the decrease in the intensity) or presented on the membrane surface. The change in expression of lipids significantly differ from the siNCL, NCL-aptamer and LNA-NCL-aptamer treated cells of Y79 ($m/z$ 808.6, $m/z$ 782.6 and $m/z$ 754.6) and WERI-Rb1 ($m/z$ 810.6, $m/z$ 782.6 and $m/z$ 754.6) to untreated cells are presented as Figure 35B. In addition to the above represented lipids, spectral images of various treatments of Y79 cells ($m/z$ 810.6, $m/z$ 804.6 and $m/z$ 780.6), WERI-Rb1 ($m/z$ 832.6, $m/z$ 808.6 and $m/z$ 760.6) and MIO-M1 ($m/z$ 808.6, $m/z$ 782.6 and $m/z$ 754.6) were studied. There was drastic reduction in the intensity of lipid expression under the treated conditions in cancer cell lines with very less difference in the normal cell line, MIO-M1.
Figure 35. Effect of aptamer and siNCL on surface lipid arrangement in RB cell lines and xenograft tissues. DESI-MS based lipid profiling between RB cell lines and MIO-M1. A. DESI-MS spectra of Y79, WERI-Rb1 and MIO-M1 cells grown in cell culture. Cells were washed with 1XPBS twice and spotted on whatmann membrane and imaged using methanol as solvent using positive mode and data acquired from 50 to 2000 Daltons. Changes in the lipid expression accompanying the treatment with NCLAPT in RB cell line. B. DESI-MS spectral imaging of cells spotted on whatmann sheet and imaged using methanol as solvent, Y79 cells (i) and WERI-Rb1(ii) and MIOM1(iii) untreated or transfected with siNCL or treated with NCL-aptamer (10μM) or treated with LNA-NCL-aptamer (10μM) for 48h.
3.2.2 DISCUSSION

In the present study, we demonstrated first time the over expression of NCL protein in RB in comparison to normal retina. The cellular localization of NCL under malignant and non-malignant conditions varies and the surface localization is reported during malignant and diseased conditions and acts as receptors anti-HIV peptide, endostatins (El Khoury et al., 2010, Hovanessian, 2006, Hovanessian et al., 2000, Krust et al., 2001, Legrand et al., 2004, Nisole et al., 2002, Zhuo et al., 2010). Both the cytoplasmic expression and nuclear expression of NCL was high in RB compared to normal retina. However, the cytoplasmic localization of NCL was higher compared to the nuclear nucleolin, which could potentially aid in stabilizing the onco-mRNAs and for the shuttling of proteins to nucleus for the transcription activation under malignant condition. Similar observation of cytoplasmic localization in others cancers were also reported, in addition the half life of the surface nucleolin and the function of NCL ligand internalization were characterized (Hovanessian et al., 2010). The surface NCL was also further characterized for its interaction with other proteins and forms 500KDa complex in the plasma membrane (Krust et al., 2011). Our study clearly showed the presence of NCL on the surface of tumor cells and internalization of the NCL-APT exhibited the functional block of cell cycle at S phase and accumulation of cells in S phase followed by G0-G1 phase leading to cell death was evident in RB primary tumors and cell lines. These changes are in agreement with the observations reported regarding the G-rich oligonucleotides that lead to S phase arrest and inhibition of cell proliferation (Xu et al., 2001). The aptamer treatment in addition to the chemotherapeutic drug (chemodrug), etoposide treatment showed enhanced chemosensitization of RB. The silencing of NCL or NCL-APT or LNA-NCL-APT treatment of cells exhibited higher LDH activity leading to inhibition of RB cells proliferation. The interaction of NCL with G-rich mRNAs, AU-rich elements and its effect in the turnover of mRNA in disease conditions such as cancer are studied before (Abdelmohsen and Gorospe, 2012). The mRNA level of Bcl2 was downregulated upon treatment with NCL-APT or silencing NCL, additionally levels of 18s rRNA was also downregulated indicating the NCL in the rRNA transcription. The maintenance of stability of oncogenic mRNAs could be one of the important steps in tumorigenesis and role of NCL in mRNA stabilization and enhanced translation is shown in various diseases and in cancer. We observed similar changes in RB, oncogenes such as MYCN which is reported for its expression in aggressive tumor downregulated upon the perturbing NCL with aptamer or by
knocking down NCL. Although the knockdown of NCL was 60-65%, greater changes in the gene expression of G-rich mRNAs such as AKT1, MGAT, and DUS1 were observed upon the aptamer treatment. In addition, there was Bcl2, Bax downregulation and upregulation of p53, which implies the onset of tumor suppression activity (Saxena et al., 2006). The effect of NCL-APT on the WERI-Rb1 cell line was more pronounced in Y79, could be attributed to the aggressiveness and resistance of the cell type. The knockdown of NCL led to downregulation of the cancer stem cell markers, which could potentially be the mechanism behind our observation of synergistic and or sensitization of chemodrug etoposide in the RB cell lines.

The involvement of NCL in the miRNA maturation through microprocessor modulation is reported earlier. Specific set of miRNAs (miR-10a, miR-21 miR-221 and miR-222) expression was abrogated upon silencing of NCL in breast cancer, this study also showed the aggressiveness of breasts cancer was brought down by blocking NCL using aptamer or by silencing NCL (Pichiorri et al., 2013). Global miRNA profiling by us showed the downregulation of miRNAs with few upregulated miRNAs indicating the importance of the NCL in the miRNA biogenesis. We further studied the expression of oncogenic miRNA pertaining to RB in addition to the downregulated miRNAs.

The differentially expressed miRNA studied by us includes miR-196b, miR-373, miR-330, miR-152 and miR-1. The observation of miRNA downregulation upon NCL-APT treatment is related to the oncogenic miRNA and in addition to the regulation at transcription level leading to major halt of the miRNA expression. These miRNA promoter regions were predicted for the presence G-quadruplex and i-tetraplex structures that mediates the effect (Zhang et al., 2008). The NCL binding to the promoter G-quadruplex structure and in turn the transcription is reported in c-myc gene (Gonzalez et al., 2009b). The biophysical studies of the DNA sequences on the miRNA promoter predicted for the G-quadruplex structures are characterized (manuscript under review). Though there are reports on the tumor suppressor property of miR-152 and miR-1 in ovarian cancers (Xiang et al., 2014) and bladder cancer (Yoshino et al., 2011), these miRNAs were downregulated upon NCL-APT treatment, which could be attributed due to the promoter structure and NCL involvement which needs to be further investigated. The miR-330 reported as tumor suppressor miRNA in different cancer, but a recent report suggested inverse role in glioblastoma (Yao et al., 2014). In our study too, miR-330 was downregulated upon NCL-APT
treatment. In addition to the miRNA studied miR-196b was found to be significantly upregulated in the RB tumor (data not shown). The oncogenic role of mir-196b was already studied in glioblastoma and its expression was correlated to poor disease prognosis (Ma et al., 2012). The established onco-miRNA cluster and a serum biomarker of RB, miR-17-92 cluster (Beta et al., 2013) and the miR-196b were found to be downregulated upon aptamer treatment and silencing of NCL. The synthetic sequences derived from the miRNA promoters showed cytotoxic and cell proliferation inhibition in few targets, which will be further investigated.
CHAPTER 3.3

Chimeric survivin DNAzyme - NCLAPT targets cancer cells

3.3.1 RESULTS

3.3.1.1 Fabrication of NclApt-Sur_Dz constructs

Aptamer siRNA chimeric construct, aptamer ribozyme constructs were reported earlier, also aptamer Dz for the sensing of hemin, ochoatoxin A, adenosine and H2O2 form cancer cells (Chen et al., 2011a, Wang et al., 2014, Yang et al., 2012). In the present study, the NclApt-Sur_Dz construct was synthesized by the conjugation of the NclApt-linker with Dz. The schematic representation of the aptamer Dz chimera formation and the mechanism of the survivin mRNA cleavage are shown in figure 36A and B respectively. The reactions were performed by denaturation and annealing based method and the efficiency of conjugate formation was confirmed using agarose gel electrophoresis. The conjugate exhibited gel retardation due to its higher molecular weight compared to Apt-linker and Dz alone. Free Sur_Dz was found to be present after conjugation and hence further conjugation reactions were optimized with 50% of Sur_Dz in the reaction. As the NclApt-linker was not visible using at 254nm (UV-C), UV-A was used to visualize the conjugates and the acquired images are presented in figure 36C. The NclApt-linker is labeled with cy5 dye and hence were fluoresces, made to visualize effectively.

The conjugation of NclApt-Sur_Dz was checked in different buffers such as 1x SSC buffer and hybridization buffer. The conjugate in SSC buffer was observed to be the same as of the conjugate prepared in water as shown in figure 36D. The chimeric conjugate prepared with hybridization buffer showed noticeable difference in the size of conjugate compared to Sur_Dz and Apt-linker. This ensured the conjugation reaction was carried out perfectly and the optimized Dz concentration had hybridized with NclApt-linker and showed no unbound or free Dz or aptamer.
Figure 36. Schematic representations of the assembly of aptamer-DNAzyme chimeric construct. (A) The mechanism of action of DNAzyme in survivin mRNA cleavage. (B) NCLAPT carrying a linker followed by complementary region to the DNAzyme (Dz) was annealed with the Sur_Dz to synthesize the chimeric construct. Agarose gel electrophoresis of the conjugates. Conjugates were synthesized by annealing method. (C) Survivin DNAzyme, mutant DNAzyme, NCLAPT and conjugates (prepared in H2O) were loaded on 4% agarose gel. (D) Survivin mutant DNAzyme, NCLAPT with linker and conjugates prepared in hybridization buffer was electrophoresed on 4% agarose gel.

3.3.1.2 Stability of NclApt-Sur_Dz chimeric constructs

The chimeric conjugates NclApt-Sur_Dz, NclApt-Sur_mDz, Sur_Dz, Sur_mDz and apt-linker were checked for invitro stability under physiological temperature and pH using 1XPBS and in PBS containing 10%FBS. They were incubated up to 72 hours at 37°C and checked for their intactness onto 4% agarose gel. The gels were loaded with ladder separating PBS and FBS set of samples. The stability of Sur_Dz and Apt-Sur_Dz were uncompromised until 72h in PBS and FBS while the Sur_mDz and its conjugate showed minor changes in the intensities at 72h in 10%
FBS and not in PBS alone (Figure 37A-C). This shows that the conjugate is stable and can be used effectively for in vitro applications.

**Figure 37. Stability of the DNAzyme and chimeric conjugates.** The chimeric conjugates and Dzs were incubated in 1X PBS and 10% FBS up to 72 hours for checking the in vitro stability. (A) Survivin DNAzyme incubated in 1X PBS and 10% FBS up to 72h. (B) NclApt-Sur_Dz conjugate was also tested similarly (C) Sur_mDz incubated in 1X PBS and 10% FBS up to 72h. Cellular uptake of the NclApt-linker and NclApt-Sur_Dz conjugate. (A) Scatter plot of unstained cells, NclApt-linker and the chimeric NclApt-Sur_Dz conjugate uptake on WERI-Rb1 and MIO-M1 cell lines. (D) Cell lines and the primary RB cells were incubated with 500nM of the aptamer and the aptamer conjugates and acquired by flow cytometry. Scatter plot showing the unstained cells, NclApt-linker and the chimeric NclApt-Sur_Dz conjugate uptake on cells. The percentage positive cells in the lower right quadrant cells are labeled above the quadrant. (E) Graph showing the percentage uptake of aptamer with linker and the NclApt-Sur_Dz chimera by the WERI-Rb1, MIOM1 and RB primary cells. The error bar represents the standard deviation and the * indicates significance of P<0.05 and ** indicates significance of P<0.001.
3.3.1.3 Cellular uptake of aptamer DNAzyme conjugates

The conjugate was checked for binding efficiency in WERI-Rb1 and MIO-M1 cell lines. 500nM concentration of the NclApt-Sur_Dz and apt-linker was added, flow cytometry analysis showed binding of the conjugate on WERI-Rb1 to be 36.62% and the apt-linker showed 37.33% to WERI-Rb1 cells. The results showed no remarkable difference thereby confirming that the aptamer upon conjugation with the DNAzyme does not alter the binding affinity of the aptamer. In the case of MIO-M1 the NclApt-linker is binding nonspecifically up to 14.8%, which could be due to the insertion of the linker and Dz complementary sequence, but upon conjugation it got reduced to 0.66%. This depicts that the conjugate is efficient in targeting cancer cells and has very less affinity towards normal cells. This remarkable property of this conjugate helps in targeting cancer cells alone in mixed tumor population (Figure 37D). Similarly, RB tumors were also subjected for NclApt-linker and Apt-Sur_Dz binding. The affinity of these conjugates with tumor samples was extremely high and has uncompromised binding between Apt-linker and Apt-Sur_Dz, as shown in the histogram the percentage uptake (Figure 37D and E).

![Figure 38. Cellular uptake of NclApt-Dz conjugates in MIO-M1 cells](image)

As we wanted to study the delivery of the Dz to the cancer cells, we used fluorescent Dz and studied the internalization and delivery of the same. The NclApt-Sur_fdDz chimeric conjugate internalization was visualized and captured using Zeiss AXIO vision fluorescent microscope.
Chapter 3

The control cell line MIO-M1 showed no binding and internalization of the conjugate or NclApt-linker (Figure 38). For studying the internalization, from the surface to bottom of the cell, different Z-positions were viewed and found to observe the Dz delivery to the cells. WERI-Rb1 and Y79 cells showed binding and internalization of the conjugate, aptamers and DNAzyme. In the case of WERI-Rb1 cells, the NclApt-linker alone showed both cytoplasmic and nuclear localization, whereas the NclApt-Sur_fDz conjugate added cells showed NclApt more localized to the cytoplasmic region, while the Dz was delivered and present to the nucleus. The Dz alone exhibited more of membrane binding on the cells and feebly internalized. Thus the chimeric conjugate is efficiently getting internalized and also delivering DNAzyme to the cancer cells (Figure 39 and 40). In Y79 cells too similar results were observed for the NclApt-Sur_fDz conjugate, while the Sur_fDz alone was able to enter into the cells. Thus there were differential mechanisms adopted by the cell lines derived from same origin. Nevertheless, the aptamer was able to deliver the Dz to the cells.
Figure 39. Cellular uptake of Sur-Dz and chimeric conjugates in WERI-Rb1 cells. Microscopic images showing the cellular uptake of Sur_fDz, NclApt-linker and NclApt-Sur_fDz in WERI-Rb1 cells after 2h of addition. Cells were washed with 1X PBS and imaged under 100X objective. The green arrows indicates the Sur_fDz, red arrow indicates the NclApt-linker.
3.3.1.4 Functional activity of the conjugate

The functional activity of the chimeric conjugate was tested in RB cell lines, Y79 and WERI-Rb1 reported for its expression of survivin. In both the cell lines tested DNAzyme chimeric conjugate showed significant down regulation of survivin when compared to DNAzyme alone transfected or mutant DNAzyme or NclApt-linker and mDz chimeric conjugate (Figure 41). The
NcI Apt-linker which basically harbors the NCLAPT has property to downregulate survivin, hence the conjugate as expected to exert higher downregulation of the survivin. Down regulation of survivin with cancer cell specific targeting property of this conjugate serves to be a better platform for targeting cancer cells. Decrease in survivin levels leads to apoptosis of cancer cells thereby reduces the cancer population.

Figure 41. Knockdown of survivin using chimeric aptamer DNAzyme construct. The qPCR for survivin knockdown in NcI Apt-Sur_Dz conjugate treated Y79 and WERI-Rb1 cells. Graph showing the relative levels of survivin mRNA post treatment with NCLAPT-survivin DNAzyme conjugate and mutant conjugate in Y79 and WERI-Rb1 cells for 48h. The error bar represents the standard deviation and the ** indicates significance of P<0.001.

Figure 42. Knockdown of survivin protein analyzed by Western blotting. Y79 cells were treated with Dz alone or NCL-aptamer-Sur_Dz conjugates for 48h, followed by analysis of the survivin expression analysis by western blotting. β-actin was used as housekeeping control to normalize the survivin expression.
3.3.2 DISCUSSION

Survivin, a new member of the inhibitor of apoptosis protein (IAP) family, both inhibits apoptosis and regulates the cell cycle (Jiang et al., 2008, Oliveras-Ferraros et al., 2011, Wang et al., 2006b). It is overexpressed in all cancers including RB, but hardly expressed in most normal tissues (Moniri Javadhesari et al., 2013, Yakirevich et al., f sw). Therefore, it may be an attractive target for antitumor gene therapy (Kanwar et al., 2013, Kanwar et al., 2001, Kanwar et al., 2010c, Samarasinghe et al., 2012, Sriramoju et al., 2014). Oligonucleotide based targeting of cancer is the most explored and antisense oligonucleotide (ASO) against survivin has entered clinical trials and like such many in phase trials. Application of Dz and Rz were equally tried with siRNA and ASO as therapeutics. Chimerization of drugs, toxins, targeting peptides, aptamers, ASO, nanoparticles in combinations try to construct new conjugates for theranostic purpose in cancer (Chen et al., 2013, Dass et al., 2008, Li et al., 2007, Li et al., 2013b, Park et al., 2011, Ryoo et al., 2012).

Present study utilizes oncotarget, survivin, a protein overexpressed in cancer and a potent interest for therapeutic purpose (Kanwar et al., 2010a, Kanwar et al., 2011a). Survivin protein is approached using ASO, siRNA and mutant protein and gene therapy for example, LY2181308, EZN-3042, dNSurRC84A9 and survivin antagonists and so on (Raetz et al., 2014, Sapra et al., 2010, Talbot et al., 2010, Wiechno et al., 2014, Kanwar et al., 2001). Current study interests in chimerzing aptamer with a therapeutic Dz that targets survivin. The targeting aptamer chosen in the study is NCL, which is present in the cell surface of the cancer cells and preferentially uptakes the aptamer by different mechanism mediated by NCL. The aptamer shuttles from cell surface to nucleus and to cytoplasm and hence utilizing it for chimerization will aid in delivery of the Dz efficiently inside the cell, where it is expected to function to bring down the tumorigenicity. RB model was utilized in the current study as it overexpresses survivin, cell surface NCL as well found to internalize NCL APT. The results obtained in the present study revealed stable conjugate under \textit{in vitro} condition was able to target and silence survivin in cancer cells.

Our results elucidate the mechanism of action of chimeric aptamer-DNAsyme conjugate using NCLAPT and survivin Dz (NclApt-Sur_Dz) participates in the proliferation or growth of RB cancer cells. Chimeric NclApt-Sur_Dz was shown to be more effective than survivin Dz in down-regulating the expression of survivin, which is likely a direct result of the careful design of
Chapter 3

NclApt-Sur_Dz action against the survivin mRNA. Both the reduction in mRNA and protein expression was in the same order of magnitude and was in the order 50% in RB cells after 48h. Recently survivin DNAzyme were utilized in the MCF-7 breast cancer model and suggested possible mechanisms underlying their anticancer effects \textit{in vitro} (Zhang et al., 2012b). It was further reported that using Dz, against the expression of survivin significantly down-regulated the expression of pro-caspase-3 and 9, and concomitantly increase caspase-3 and caspase-9. Another study recently showed, DNAzyme against AKT1 to inhibit its expression were found that DRz1 significantly downregulated the expression of AKT1 in SW597 cells at mRNA and protein level (Yang et al., 2013). Flow cytometry assays confirmed qualitatively and quantitatively that DRz1 could suppress the growth, invasion and motility of SW597 cells. Meanwhile, anti AKT1 Dz further showed alterations in the expression of several molecules relating to cancer invasion and metastasis, such as VEGF and MMP-2.

Aptamer DNAzyme chimeric constructs were developed for the biosensors for the detection of ochatoxin A, \textit{Salmonella paratyphi} A in food industries and cytokines such as IFN-\(\gamma\) (Ning et al., 2014, Yang et al., 2012, Zhang et al., 2012a) and the usage of such conjugates for targeted delivery to cancer cells were not reported until now. In summary, our results demonstrated first time that chimeric aptamer-DNAzyme conjugate using NCLAPT with survivin DNAzymes (NclApt-Sur_Dz) could be used as a specific gene-targeting therapy to suppress progression of RB cancer as a proof of concept. This novel chimeric form may become powerful therapeutics of other cancer types in the future. Overall, down-regulating the expression and function of survivin may induce apoptosis and inhibit migration of cancer cells. Our results generally demonstrate that targeting survivin is a promising and potential new therapeutic option in combating cancer.
Summary of the chapter: Oncoproteins and their role in RB tumorigenesis and invasion were summarized in previous chapter. We further studied the therapeutic target, EpCAM which was utilized by other groups to target specifically the solid tumors. We report the expression and importance of the EpCAM intracellular domain for the first time in RB and its characteristic role in CSC maintenance. Similar to the previous chapter that deals with novel therapeutic targets, this chapter divulges on the use of aptamers and aptamer chimeric constructs targeting EpCAM for the therapeutic purposes. The aptamer siRNA chimeric constructs, aptamer fabricated polymeric nanoparticles with siRNA and aptamer doxorubicin conjugates were able to exert cancer cell specific delivery of the siRNA and drug. The proof concept was tested in RB cell line and MCF7 cell line, using a breast cancer cell line as a model.
CHAPTER 4.1

EpCAM aptamer-siRNA chimera regress epithelial cancer

4.1.1 RESULTS

4.1.1.1 Chimerization of EpApt with siEpCAM

EpCAM aptamer siRNA chimera was fabricated following the previously optimized structures of PSMA aptamer siRNA chimeric construct (Dassie et al., 2009). The stem and loop aptamer chimera with strand swap exhibited better silencing compared to other chimeric forms. Hence in the current study, we fabricated aptamer chimera by extending the aptamer sequence with the siRNA sequence at its 5’end and 3’end.

The fabricated EpApt-siRNA carried siRNA targeting EpCAM, a cancer stem cell marker that maintains pluripotency of stem cells and cancer initiating cells. The stem and loop structure designed manually by incorporating siRNA sequence targeting EpCAM, was additionally modified to harbor nuclease resistant modifications in the pyrimidines. The 5’end of the aptamer is end labeled with FITC, that aids in the monitoring the aptamer binding and uptake by cells. Additionally the 3’end of the aptamer harbor two U overhangs that aids in recognition and loading of dicer enzyme (Vermeulen et al., 2005). The EpApt shown in figure 43A, is 19ntd hairpin structured aptamer, chimerization involved in adding the siRNA sequence to the aptamer with an additional GC bridge that aids in stable bonding and strengthens backbone of the chimera. The constructed aptamer chimeric structure was predicted using RNA structure v5.3 and Mfold (Figure 43B). The EpCAM aptamer siRNA targeting EpCAM (EpApt-siEp) is synthesized with the above modifications for the study.
Figure 43. Fabrication of EpCAM aptamer siRNA chimera and cartoon showing its processing in cell. A. The EpCAM aptamer secondary structure prediction from Mfold online. B. EpCAM aptamer siRNA chimeric construct carrying the siRNA targeting EpCAM (EpApt-siEp) is folded using Mfold online and the aptamer is indicated in blue box and the siRNA inside red box. C. The EpCAM aptamer siRNA chimeric construct (EpApt-siEp) binds to the EpCAM receptor and gets internalized (1) and released in the cytoplasm where gets in to Ago complex with dicer enzyme (2) to generate siRNA. The siRNA loaded into RISC complex(3) binds to the EpCAM mRNA transported from nucleus to cytoplasm (4) and leads to mRNA degradation.

The functionality of the EpApt-siEp chimeric construct is tested by performing the in vitro dicer cleavage assay. The EpApt aids in binding to EpCAM and gets internalized and released in to the cell cytoplasm. The EpApt-siEp upon loading on to dicer, RNase III enzyme, gets processed to generate 21ntd antisense siRNA within the RISC complex. This complex binds to the target mRNA (EpCAM) translocated to cytoplasm from nucleus and creates translation block by cleaving the mRNA. Based on the functionality of the target gene, the cellular metabolism undergoes changes (Figure 43C).
4.1.1.2 EpApt-siEp: in vitro dicer mediated processing and cellular uptake

The process of generation of siRNA from the chimeric construct is vital for judging the functionality of the construct. Hence, we sought to test the dicer mediated processing of the constructs \textit{in vitro} for the generation of siRNA. Small RNAs of 21-23 ntd were reported to be directed show the gene target repression, independent of dicer involvement. The EpApt-siEp construct incubated with recombinant human dicer for 18h, was run on agarose gel and polyacrylamide gel. The siRNA of 21ntd size were digested and released from the construct, agarose gel electrophoresis in figure 44A, shows faint band corresponding to the synthetic siRNA and the aptamer alone (19ntd). This was further confirmed by running a non-denaturing PAGE, the dicer cleaved the construct and generated fragments of \~20-22bp length (Figure 4B).

We further sought to examine the stability of the construct under physiological mimicking condition. The aptamers incubated under conditions such as media without FBS, with 10% FBS and in 100% FBS for a duration of 96h showed to have minimal degradation at 72 and 96h (<10% degradation) in media and media with 10% FBS respectively. The construct in FBS was stable till 96h, except at 48h duration, there was slight degradation. Thus the constructs were found to be stable under physiological mimic conditions till 72h (Figure 44C).

The cellular uptake studies of the EpApt-siEp are necessary to substantiate the internalization of aptamer though receptor mediated endocytosis. The EpCAM aptamer (EpDT3/EpApt) has already been elucidated for the receptor mediated endocytosis. We used scramble aptamer, which carries 2’OMe modification in their backbone, which hinders the binding to the target, EpCAM as control. The scramble aptamer chimera (ScrApt-siEp) had resulted in non-specific binding to cells, upon chimerization the scramble aptamer no more retained its specificity (data not shown) and hence discontinued from the study.
Figure 44. *In vitro* siRNA generation by Dicer. The EpCAM aptamer siRNA chimeric construct was incubated with the recombinant dicer enzyme at 37°C for 18h. The reactions were performed without dicer as control reaction. A. Agarose gel electrophoresis. The reactions after terminating by adding stop solution were run on 2% agarose gel. Controls such as EpCAM aptamer and siRNA alone are run alongside. B. Polyacrylamide gel electrophoresis. Similar to the agarose gel, reactions with and without dicer enzyme were run on 15% PAGE and stained with EtBr. The processed 21bp siRNA and unprocessed construct were observed. Stability of EpApt-siEp. C. The EpCAM aptamer siRNA chimeric construct was incubated in media, media with 10% FBS and in FBS alone up to 96h. After the 96h time point reactions were electrophoresed on 2% agarose gel.
Chapter 4

**Figure 45. Cellular uptake of the EpApt-siEp construct.** A. The EpCAM aptamer siRNA chimeric construct was added to WERI-Rb1, MCF7 and MIO-M1 cells in binding buffer. B. Scatter plot showing the uptake of EpApt-siEp by the RB cell line, WERI-Rb1 and RB primary tumor cells. Cellular uptakes of the EpApt-siEp construct in primary RB (RB) cells. C. The EpCAM aptamer siRNA chimeric construct was added to primary RB cells in media without serum for 2h at 37°C followed by washing with 1X PBS. Microscopic images were taken at 20X objective under phase and FITC channels of control cells alone and cells with EpApt-siEp. Flow cytometry results are respective of experiments repeated more than thrice.

We found the cellular uptake of chimeric construct in WERI-Rb1, RB cell line model and MCF7 cells, breast cancer model. The Muller glial cell line, non-cancerous cell line, MIO-M1 express EpCAM at lower levels and they exhibited marginal insignificant binding of aptamer in comparison to the binding exhibited by the cancerous cell lines, WERI-Rb1 and MCF7. These cell lines exhibited uncompromised uptake by the targeted cell lines (Figure 45A). The construct uptake was additionally studied for uptake in primary RB tumors and found to exhibit higher binding efficiency to primary tumor cells than the cell lines, due to higher levels of EpCAM expression in tumors (Figure 45B). The cellular uptake of the EpApt-siEp-FITC in WERI-Rb1
cell line and primary RB cells showed to be 75% and 100% respectively at the concentration of 250 nM. The microscopy also revealed the binding of aptamer on RB primary cells.

4.1.1.3 EpApt-siEp silences EpCAM specifically in cell lines and primary RB tumor cells

The target specific delivery, siRNA generation and silencing capability were studied using WERI-Rb1 and MCF7 models. The expression level of EpCAM is higher in MCF7 cells than WERI-Rb1. The chimeric construct was first evaluated for the silencing of EpCAM in MCF7 cells, the northern blotting results normalized to the 28s rRNA, showed silencing of EpCAM about 40% in EpApt-siEp treated cells and around 35% in siEp transfected cells (Figure 46A and panel right to it). A quantitative analysis of the mRNA expression by qPCR showed better inhibition of EpCAM expression, -1.8 and -3.4 fold downregulation (73 and 90% inhibition of mRNA expression) in MCF7 cell line (P<0.01) and -1.4 and -1.0 fold downregulation (63 and 51% inhibition of mRNA levels) in WERI-Rb1cell line (P<0.05) (Figure 46B). The EpCAM downregulation was also observed at protein levels, WERI-Rb1 cells exhibited 47 and 43% and MCF7 exhibited 65 and 49% of downregulation of EpCAM at protein levels (P<0.05).
The effect of chimeric construct was tested in RB primary tumor cells for the silencing and functional activity. The primary RB cells were evaluated for the transfection efficiency using pGFP plasmid transfection. The transfected cells after 24h showed expression of GFP faintly by
50% cells, 25% cells showed moderate expression and 25% showed very high expression of GFP (Figure 47A). The confirmed the metabolic activity of the primary tumor cells. We then sought to analyze the effect of EpApt-siEp and siEp on these cells. The primary tumor cells showed inhibition of EpCAM expression by -0.5 fold and -2.4 fold in siRNA and chimeric construct cells respectively (Figure 47B). The cellular cytotoxicity as measured by LDH assay showed 37 and 35% increase in the LDH activity upon silencing of EpCAM using siRNA and EpApt-siEp. The EpApt-siEp construct significantly (P<0.001) downregulated EpCAM mRNA levels and caused cytotoxicity in the primary RB tumor cells (Figure 47C). The cell proliferation measured by MTT assay of the WERI-Rb1 and MCF7 cells showed significant cell proliferation inhibition with EpApt-siEp, while the EpApt or ScrApt alone did not show any effect in cell proliferation (Figure 47D).

**Figure 47. Expression of GFP in primary RB cells.** A. Microscopic images showing the expression of GFP transfected with lipofectamine 2000 in RB primary cells. 24h after transfection cells were imaged with 20X objective. Cellular changes accompanying knockdown of EpCAM knockdown using EpApt-siEp construct in primary RB cells, WERI-Rb1 and MCF7. B. The EpCAM mRNA levels were quantified by SYBR green based qPCR from the cDNA of control, siEp and EpApt-siEp treated RB primary tumor
cells. The graph shows the EpCAM mRNA levels normalized to β-2-microglobulin as housekeeping gene. C. The cellular cytotoxicity analysis of the RB cells with treatments was performed by calculating the LDH activity and normalization with untreated control cells. D. The percentage cell proliferation were quantified by performing MTT assay on the control, siEp transfected, EpApt-siEp, EpApt and ScrApt treated WERI-RB1 and MCF7 cells. The graph shows the % cell proliferation normalized to control cells. The error bar represents the standard deviation and the * indicates significance of \(P<0.05\) and ** indicates significance of \(P<0.001\). Experiments were repeated more than thrice.

**4.1.1.4 EpICD in primary RB tumors: regulation of cancer stem cell markers**

The cancer stem cell marker, EpCAM has been shown to be overexpressed in cancer initiating cells or cancer progenitor/stem cells (CPC/CSCs) (Gires et al., 2009, Gonzalez et al., 2009a, Mitra et al., 2012). The mechanism behind this property was regulated by the EpCAM activation mediated by intramembrane proteolysis, which involved the EpICD release from the cytosolic domain of EpCAM bound to EpEx in the extracellular region to nucleus (Maetzel et al., 2009b). We had reported the presence of EpCAM earlier in 2004 and in the current study we ought to study the expression of EpICD in the primary RB tumors (Krishnakumar et al., 2004). The immunohistochemistry results showed intense nuclear staining. The intensity of the nuclear staining varied among the tumors studied, with 40 – 60% cells positive and some cases showed 70-80% cells positive. The normal or non-malignant retinastudied did not reveal any evident nuclear staining (Figure 48A). The intensity and the percentage distribution of the EpICD positive cells in the tumor are presented in table 9.

The EpICD released from the nuclear protein complex by interacting with the FHL2, β-catenin and Lef-1 mediates gene transcriptions and aids in cell proliferation. The regulation of EpICD on the expression of pluripotency markers were reported earlier (Lu et al., 2010) and we were interested to study the modulation of EpCAM behind the expression of OCT4, SOX2, Nanog, CD133, CD44s. We additionally studied the expression of survivin levels upon silencing of EpCAM in RB cell line, WERI-Rb1. EpCAM silencing using the siRNA transfection or EpApt-siEp showed higher downregulation of SOX2, OCT4 and NANOG in siRNA transfected cells compared to the EpApt-siEp, whereas the CD133, CD44s and survivin levels were more downregulated in EpApt-siEp transfected cells (Figure 48B). We additionally studied the expression of SOX2, OCT4 and NANOG in MCF7 cells and found to be similarly downregulation of SOX2 and NANOG but not OCT4 upon silencing EpCAM using siRNA or
EpApt-siEp construct (Figure 48C). Regulation behind the OCT4 irresponsiveness is of future interest to study. Thus we were able to elucidate the maintenance of the undifferentiated state or stem cell state by the EpCAM though EpICD mediated regulation.

Table 9. Expression of EpICD in RB primary tumor by IHC.

<table>
<thead>
<tr>
<th>Tumor details</th>
<th>Intensity</th>
<th>Percent Positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>314/10</td>
<td>++++</td>
<td>90</td>
</tr>
<tr>
<td>435/03</td>
<td>+++</td>
<td>70</td>
</tr>
<tr>
<td>1002/03</td>
<td>+++</td>
<td>60</td>
</tr>
<tr>
<td>423/03</td>
<td>++</td>
<td>50</td>
</tr>
<tr>
<td>515/08</td>
<td>+++</td>
<td>65</td>
</tr>
<tr>
<td>935/10</td>
<td>+</td>
<td>20-30</td>
</tr>
<tr>
<td>403/08</td>
<td>++</td>
<td>40</td>
</tr>
<tr>
<td>679/10</td>
<td>++</td>
<td>60</td>
</tr>
<tr>
<td>544/10</td>
<td>+++</td>
<td>70-80</td>
</tr>
<tr>
<td>153/10</td>
<td>+++</td>
<td>70</td>
</tr>
<tr>
<td>520/11</td>
<td>+++</td>
<td>80</td>
</tr>
</tbody>
</table>

+= 20-30 number of cells have positive staining (%).
+++ = 31-60 number of cells have positive staining (%).
++++ = 61-80 number of cells have positive staining (%).
++++= 81 or more number of cells have positive staining (%).
4.1.1.5 LNA modified aptamer siRNA fabrication and in vitro dicer processing

The LNA modification (Figure 49A) was carried out at 3 positions in the EpCAM RNA aptamer (L-EpApt) and 5 LNA modifications on the chimeric 62mer construct was performed (Figure 49B and C). The LNA modification of the aptamer was carried out for the terminal nucleotides and the nucleotide G in the loop region. The stability of the aptamer chimera with the LNA modified aptamer chimera (siRNA- statmin and Survivin) is tested in plain media, FBS containing media (data not shown) and in FBS. The stability of the LNA chimeric construct even in 100% serum was uncompromised at 96h (Figure 49D). Thus in the in vivo conditions we
anticipate the LNA chimeric construct to perform better antitumor by sustained silencing activity. The LNA chimeras were checked for the dicer enzyme mediated cleavage and siRNA generation. The arrow indicates the processed siRNA generated due to dicer enzyme (Figure 49E).

Figure 49. Fabrication of the LNA modified aptamer siRNA chimera. A. The LNA modification – locking of the 2’ C with 4’ C of the ribose ring. B. The EpCAM aptamer secondary structure predicted from Mfold online with the LNA modification highlighted with blue circles. C. LNA modified EpCAM aptamer siRNA chimeric construct (L-Ep-siRNA) carrying the siRNA (targeting survivin and stathmin) is folded using Mfold online and the LNA modifications are indicated in blue circles and the siRNA inside red box. Stability of L-EpApt-siRNA. (D) The EpCAM aptamer siRNA chimeric construct was incubated in FBS alone upto 96h. After the 96h time point reactions were electrophoresed on 2% agarose gel. (E) The LNA chimeras (L-Ep-siBIRC5 and L-Ep-siSTMN1) were incubated with recombinant human Dicer enzyme at 37°C for 18h and electrophoresed on PAGE, stained with EtBr and visualized under UV transilluminator.

4.1.1.6 Functional activity of LNA aptamer siRNA chimeras

The LNA modified EpCAM aptamer (L-EpApt) binds to cells without compromising the binding affinity on to RB and breast cancer cell lines (Figure 50C). The L-Ep-siRNA chimeras were
efficiently cleaved by dicer *in vitro* and thus were proceed for testing for their cellular processing and functional activity for silencing. The L-Ep-siSTMN1 and L-Ep-siBIRC5 constructs were added to the cells and siRNA against stathmin and survivin (STMN1 and BIRC5) were transfected. The knockdown of target genes were analysed by performing qPCR for the STMN1 and BIRC5 post silencing or treatment upto 48h. Similar to the siRNA the chimeric constructs were able to silence STMN1 and BIRC5. The qPCR results in MDAMB345 and WERI-Rb1 are presented in figure 50A and B. The silencing mediated by L-Ep-siRNA chimera was significantly higher than the siRNA transfection. This is similar observation to that of the non-LNA chimera (manuscript under review). In addition to the mRNA level, protein levels were assessed by immunofluorescence assay for the STMN1 and BIRC5 in WERI-Rb1 and MDAMB453 cells (Figure 50C and D). The immunofluorescence assay of WERI-Rb1 cells showed downregulation of survivin in both the siRNA transfected cells and the chimeric aptamer treated cells, but the chimera treated cells showed fewer expression of survivin. In the case of stathmin silencing, LNA chimera had downregulated stathmin better than the siRNA transfection (Figure 50C upper and lower panel).
Figure 50. Knockdown of BIRC5 and STMN1 by L-EpApt-siRNA chimeras. The LNA modified EpCAM aptamer siRNA chimeric constructs were added at a concentration of 100nM directly to the cells in media without serum for 4h, followed by addition of media containing serum and incubated further for 48h. siBIRC5 and siSTMN1 transfections were performed using lipofectamine 2000, ScrApt was used as
negative control. qPCR was performed for BIRC5(A) and STMN1(B) by SYBR green based method and fold change in expression is plotted. Knockdown of BIRC5 and STMN1 by LNA aptamer siRNA chimeras in cell lines. Immunofluorescence of WERI-Rb1(C) and MDAMB345 cells (D) for Stathmin and survivin proteins post 48h of the treatment with LNA aptamer chimera or siRNAs. Cells were fixed, primary antibody for stathmin (Op-18) or BIRC5 (survivin) was added, secondary anti-mouse FITC, nuclear counter stained with DAPI, mounted and viewed under microscope.

4.1.2 DISCUSSION

aptamers are class of next generation therapeutics (Bruno, 2013), and RNA aptamers are more preferred as they are easy for chimerization and targeting the functional RNA molecules such as siRNAs, shRNAs, miRNAs and ribozymes (Bruno, 2013, Kanwar et al., 2010b, Kanwar et al., 2011b, Kanwar et al., 2014). Nevertheless, DNA aptamers were also equally exploited for cancer targeting (Marton et al., 2010). The backbone modification of aptamers to yield increased stability in vivo conditions and 2’ Fluoro, 2’ O methyl of bases and 3’ inverted thymidine (idT) are proved to be essential for nuclease resistance. aptamer siRNA chimerization only based on oligonucleotides were listed in table 10 and reports on liposome siRNA, using protein tag, polyethylene glycol (PEG) and polyethyleneimine (Kwiatt et al.) based chimeric nanoformulations for targeted delivery are also available (Li et al., 2014b, Liu and Gao, 2013, Bagalkot and Gao, 2011). Aptamer siRNA chimeras reported so far, are majorly targeting the prostate cancer (PSMA targeted), and followed by reports on integrin, Her2/neu and NCL APT (Table 10). Targeting cancer stem cells for the delivery of siRNA using aptamer based approach is still lacking and in the present study we bridged the gap using the EpCAM aptamer. In spite of the availability of CD133 (Shigdar et al., 2013b) and CD44 aptamers (Somasunderam et al., 2010), EpCAM, is of the interest due to the signaling mechanism it mediates to maintain the pluripotency and that maintains the undifferentiated stage of stem cells (Gonzalez et al., 2009a) which is also overexpressed in cancer cells that possess stem cells property (Munz et al., 2009, Simon et al., 2013).
### Table 10. Aptamer-siRNA chimeras used for targeted cancer therapy

<table>
<thead>
<tr>
<th>S.No.</th>
<th>aptamer (cancer type)</th>
<th>siRNA targeted and reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>αvβ3. RNA aptamer targeting alpha V and integrin beta 3.</td>
<td>EEF2. Eukaryotic Elongation Factor 2. (Hussain et al., 2013)</td>
</tr>
<tr>
<td>3</td>
<td>HER2. RNA aptamer targeting Human Epidermal growth factor Receptor 2. (Breast cancer)</td>
<td>BCL2. B Cell Lymphoma 2. (Thiel et al., 2012)</td>
</tr>
<tr>
<td>4</td>
<td>PSMA. RNA aptamer targeting Prostate Specific Membrane Antigen. (Prostate cancer)</td>
<td>DNAPK. DNA activated Protein Kinase. (Ni et al., 2011)</td>
</tr>
<tr>
<td>5</td>
<td>PSMA. RNA aptamer targeting Prostate Specific Membrane Antigen. (Prostate cancer)</td>
<td>PLK1. Polo Like Kinase 1. (Dassie et al., 2009)</td>
</tr>
<tr>
<td>6</td>
<td>PSMA. RNA aptamer targeting Prostate Specific Membrane Antigen. (Prostate cancer)</td>
<td>EEF2. Eukaryotic Elongation Factor 2. (Wullner et al., 2008)</td>
</tr>
<tr>
<td>7</td>
<td>PSMA. RNA aptamer targeting Prostate Specific Membrane Antigen. (Prostate cancer)</td>
<td>PLK1. Polo Like Kinase 1. BCL2. B Cell Lymphoma 2. (McNamara et al., 2006)</td>
</tr>
<tr>
<td>8</td>
<td>PSMA. Boranophosphate modified RNA aptamer. (Prostate cancer)</td>
<td>ERB2. ERBB2/Her2/neu. (Shaw et al.)</td>
</tr>
<tr>
<td>9</td>
<td>NCL. NCL expressed on cell surface. (lung cancer)</td>
<td>SLUG. Snail family zinc finger 2 NRP1. Neuropilin 1. (Lai et al., 2014)</td>
</tr>
</tbody>
</table>

As EpCAM is overexpressed in all cancer types including RB and breast cancer and has major role to play in the maintenance of cancer stem cell property and here first time we targeted its
expression and functions in the present study. Previously, we used the EpApt for the delivery of doxorubicin, an anti-cancer drug in RB cells and showed targeting to cancer cells while sparing the non-malignant cells (Subramanian et al., 2012). Later, we used EpCAM DNA aptamer (EpD) for imaging of the cancer cells that expresses EpCAM using bio-orthogonal chemistry based labeled aptamer (Subramanian et al., 2014). Chimeric EpCAM and NCL APT targeted superparamagnetic iron oxide nanoparticles (SPION) saturated lactoferrin, had shown promising anti-tumor property in vitro and in vivo (Kanwar et al., 2011b). In the present study, we were able to successfully fabricate and elucidate the target specific silencing of EpCAM using EpApt-siEp chimeric construct. The chimeric construct reported earlier targeting survivin siRNA requires one step annealing for the chimera generation. The current study was based on the earlier report showing the optimized structure for high efficient functioning of chimera in prostate specific membrane antigen (PSMA)-expressing tumors (Dassie et al., 2009) wherein not studied for EpCAM aptamer until now. The chimeric construct were processed though dicer, an RNase III enzyme family that generates, 19-21mer to be loaded onto RISC complex for the silencing of target gene. The EpApt-siEp chimeric construct carrying the nuclease stability modification were able to stabilize and prevent from degradation of the aptamer even in the 100% serum upto 72h. The chimeric construct was able to silence the EpCAM, analysis at protein level showed significant downregulation of protein. Although, the primary RB tumor cells showed 60% transfection efficiency, could be due to the spheroid like nature of the cell cluster and the cellular arrangement preventing the transfection complex to penetrate. While the siRNA mediated EpCAM downregulation was lesser the silencing mediated by the chimeric construct was higher. This shows the penetrating ability of the EpApt-siEp construct in the 3D cellular arrangement. This could be the potential mechanism adopted in the in vivo xenograft system for the tumor penetration and cellular internalization. Due to unavailability of large amount of human primary RB cells, mRNA levels and functional assays were only possible while protein based assays were not executable. The functional activity of the EpApt-siEp construct showed to be cytotoxic in primary tumor cells and was able to induce cell proliferation inhibition in WERI-Rb1 cells and MCF7 cell lines. Until, 2009, the role of EpCAM in cell proliferation was not elucidated and then it was found to be regulated though the intramembrane proteolysis and release of EpICD, that locates to nucleus for mediating transcription of gene aimed to promote cell proliferation (Maetzel et al., 2009b). Hence, we anticipated EpCAM
targeting using this construct to have higher anti-tumor activity, as the EpCAM expressing cells can only uptake the construct, and result in the inhibition of cell proliferation mediated by EpCAM silencing. Thus our construct undergoes double selection and specifically acts on EpCAM+ve cells leading to less off-target effect.

The role of intracellular domain of EpCAM, EpICD in the cancer stem cell signaling is elucidated previously (Gonzalez et al., 2009a, Lu et al., 2010) but there are no reports on the status of EpICD and its regulation in RB. Our study elucidates for the first time the presence of EpICD in RB. The correlation behind the EpICD localization to the nucleus may be attributed to the EpEx shedding and variation in the expression levels of EpICD within the RB patient samples observed. The regulation mediated by EpICD for the maintenance of undifferentiated form of human embryonic stem cells is studied (Lu et al., 2010), EpICD expression in cancer cells may help to gain resistant phenotype, that further lead to insensitive to chemotherapy. With this background, our results show that upon silencing of EpCAM the pluripotency markers, OCT4, SOX2 and NANOG gets downregulated and in addition the cell surface markers CD133, CD44. The level of survivin was significantly downregulated in chimeric construct treated cells. But in case of the breast cancer the gene regulation, varied with no significant change in OCT4 and NANOG expression upon silencing EpCAM, while SOX2 was downregulated. The effect of EGFR signaling alters SOX2 expression with fewer changes in OCT4 and NANOG. The downregulation of SOX2 alone was enough to bring down the self-renewable capacity of side population cancer with stem like characteristics (Singh et al., 2012).
CHAPTER 4

CHAPTER 4.2

EpCAM aptamer mediated delivery of siRNA using polymeric nanocomplex

4.2.1 RESULTS

4.2.1.1 Synthesis and characterization of PEI nanocomplex with EpCAM aptamer and siRNA

We have synthesized PEI nanocore and PEI-Apt-SiEp nanocomplex as shown in schematic representation (Figure 51). The illustration describes the process of PEI nanocomplex synthesis followed by siRNA and aptamer addition. The nanocomplex added to cells, specifically binds to the EpCAM receptor on the membrane and would release the aptamer and siRNA into the cytoplasm, leading to the silencing of EpCAM mRNA. PEI nanocore was prepared by stabilizing its charge using sodium citrate to form optimal core in aqueous solution. The size of the PEI nanocore depends on the ratios of citrate to PEI i.e., carboxyl group charge/amine group charge ratio (R). The PEI: citrate nanocore synthesized with different R ratios showed particles sizes ranging from 75 - 250 nm and zeta-potential ranging from 34 mV to 48 mV (positively charged). The ratio 1:1.5 resulted in optimum size 156 ±6.8 nm and zeta potential of 34.6 mV (Figure 52A and B). The nanocomplex formation was mediated by stabilization of the positively-charged PEI by negatively charged sodium citrate and further by the electrostatic interaction between the nanocore and the siRNA and aptamer. The PEI-Apt-siRNA nanocomplexes were synthesized in aqueous media with varying amounts of the aptamer and siRNA. To the PEI nanocore, siRNA was added first followed by aptamer. The aptamer was added finally to form the complex as it enables the complex to recognize the EpCAM on the cell surface. The addition of the aptamer and the siRNA together would lead to a lesser occupancy of aptamer on the particle surface thereby leading to lower binding efficiency of the particles; hence the stepwise complex formation was maintained. The synthesized complexes exhibited retardation on an agarose gel (Figure 52C). We observed that 200nM of aptamer and siRNA, were able to saturate the PEI: citrate nanocore beyond which free aptamer and siRNA were present, in addition to the complex retained in the well (Figure 52C, lane 4). Therefore, 200nM of EpApt and 200nM SiEp complexed with PEI-citrate (PEI-EpApt-SiEp) nanocomplex was used for further studies.
Figure 51. Illustration showing the cell specific silencing mediated by PEI nanocomplex fabricated with aptamer and siRNA. A. PEI nanoparticle is formed using sodium citrate as charge stabilizer, followed by the addition of siRNA and EpCAM aptamer to form the PEI-Apt-siRNA complex. This complex guided by the aptamer, binds to the EpCAM positive cells and delivers the siRNA in the cytoplasm resulting in target gene silencing and inhibition of cellular function pertained to it.

This nanocomplex exhibited a hydrodynamic diameter of 198±14.2 nm and zeta potential of -30.0 mV. The percent number distribution of the sizes of PEI nanocore alone and nanocomplex are shown in figure 52D. Figure 53A shows the zeta potential of the PEI nanocore alone and nanocomplex respectively. There is complete shift in the surface charge (34.6 mV) due to the aptamer and siRNA addition leading to negative surface charge (-30.0 mV). The TEM analysis exhibited a particle size of 151±11 nm. The TEM analysis of both PEI and PEI-Apt-siRNA nanocomplexes showed spherical particles (Figure 53B). The frequency of sizes of the particles as observed by TEM is plotted as histogram (Figure 53C).
Figure 52. Effect of citrate on the nanocomplex size and charge. Graph showing the hydrodynamic sizes (A) and surface charge (B) of PEI: citrate nanocomplexes formed using different ratio of PEI to citrate measured using zetasizer. (C) Agarose gel electrophoresis of nanocomplex. Titration of different concentration of aptamer and siRNA was carried out and loaded onto 2% agarose gel with ethidium bromide and checked for the retention of the PEI complex on the wells. Lane 3 shows 200nM of aptamer and 200nM of siRNA is required to saturate 0.3μg of PEI and the next highest concentration of 300nM of APT and siRNA respectively had some amount of free siRNA and aptamer (lane 4). Lane 5 and 6 indicates free aptamer and siRNA indicated with red and black arrow respectively. (D) Size distribution of particles. Histogram overlay plot showing the percent number distribution of the PEI nanocore alone and PEI-nanocomplex with aptamer and siRNA (hydrodynamic diameter in nm) measured using zetasizer.
Figure 53. Characterization of the PEI-EpApt-siEp nanocomplex. A. Graph showing the total counts of representative zeta-potential (mV) of the PEI nanocore and the PEI-Apt-siRNA nanocomplex. Transmission electron microscopic images of the PEI-nanocomplex. B. TEM images of the PEI nanocomplex left panel showing the uniformity of particle distribution and histogram showing distinct particles with a spherical shape C. Cytotoxicity of PEI. D. Graph showing the percentage cell proliferation upon treating with different concentration from 0.1 to 3mg/ml of PEI on MCF7 and WERI-Rb1 cell line till 48h. Inhibitory effect of PEI on the cell proliferation and mitochondrial activity was assessed by MTT assay.

We additionally studied the effect of serum on the size and the charge of the PEI nanoparticles prepared in aqueous media. For this, we added the prepared complexes to the RPMI media with and without the serum. The size of the nanocomplex in media with and without serum are plotted as overlay percent number distribution and exhibited very minimal difference (figure 54A) and the charge of the nanocomplex incubated in media with and without serum showed minor changes were found to be -18 mV and -18.7 mV respectively (figure 54B and C).
Figure 54. Effect of media and serum on PEI nanocomplexes. A. The hydrodynamic diameter of the PEI-Apt-siRNA complexes prepared in medium with and without serum were measured in zetasizer and plotted as histogram overlay plot against the percent number distribution. B. Graphs showing the zeta potential of the complexes prepared in medium with and without serum.

4.2.1.2 Cytotoxicity effect of PEI polymer on cells:
MCF7 and WERI-Rb1 were used to study the cytotoxic effect of the PEI on cells. The cytotoxicity of PEI was found to be lesser with decreasing concentration of the PEI i.e., 3μg/ml concentration showed higher toxicity, 0.3μg/ml and 0.1μg/ml showed lesser cytotoxicity, hence 0.3μg/ml was chosen to rule out any non-specific cellular effects that can be attributed by PEI (Figure 53D). Therefore, PEI nanocomplexes for the aptamer and the siRNA functionalization were carried out with the concentration of 0.3μg/ml of PEI.

4.2.1.3 Cellular Uptake of PEI aptamer siRNA complex:
The cell binding and uptake of the PEI-EpApt-SiEp complex were studied in MCF7 and WERI-Rb1 cell lines. Initially, we studied the expression of EpCAM in RB and the data generated by us
Chapter 4

in WERI-Rb1 cell lines is represented in figure 1A, chapter 4.1 (Subramanian et al., 2012). Similarly the expression of EpCAM in MCF7 is also studied earlier (Osta et al., 2004). The binding of EpCAM aptamer to breast cancer cells, MCF7 cell line is published earlier (Shigdar et al., 2011).

![Image](image-url)

**Figure 55. Expression of EpCAM and binding of the nanocomplexes on cells.** A. Histogram overlay plot showing the expression levels of EpCAM protein in MCF7 cells were evaluated using antibody based method and flow cytometry. B. Histogram overlay plot of MCF7 cells bound to EpApt/EpDT3 or ScrApt/ScrDT3. C. Histogram overlay plot of MCF7 cells bound to aptamer alone or PEI-Apt-siRNA complexes. D. Histogram overlay of WERI-Rb1 cells. aptamer alone or complexes were added to cells and incubated for 2h, washed and checked by flow cytometry. E. Flow cytometry analysis and histogram overlay plot showing the cells blocked with EpCAM antibody before incubating with EpDT3-FI with EpDT3 alone and unstained cells. F. Flow cytometry analysis and histogram overlay plot of cells blocked with antibody followed by incubation with PEI-EpApt-SiEp.

The expression levels of EpCAM in MCF7 cells are higher compared to the WERI-Rb1 cells (Figure 55A). Similar to the expression levels of the protein the aptamer binding was higher in MCF7 (Figure 55B). The uptake of aptamer and PEI-Apt-SiEp nanocomplexes was monitored using flow cytometry revealed that cells bound to PEI nanocomplex fabricated with EpApt-SiEp.
had increased fluorescent intensity compared to the cells bound with EpApt alone in both MCF7 and WERI-Rb1 cell lines (Figure 55C and D). The scrApt or PEI-ScrApt-SiEp did not show any binding onto the cell lines. The blocking of the cell surface EpCAM protein by the EpCAM antibody had decreased the binding of EpCAM aptamer alone or PEI-EpApt-SiEp (Figure 55E and F). The cellular uptake of the aptamer alone or the aptamer nanocomplex in MCF7 and WERI-Rb1 cells was visualized using fluorescent microscopy. The PEI nanocomplex on MCF7 and WERI-Rb1 cells showed intense membrane staining compared to the EpApt alone (Figure 56 and 57). The PEI-EpApt-siRNA nanocomplex exhibited greater binding than EpApt alone (Figure 6D and 7D). There was no binding when ScrApt or ScrApt-nanocomplex was used in both the cell lines (Figure 56C, E and 57C, E). Thus, the specificity of the EpCAM aptamer towards the target is in agreement with the above data.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase</td>
<td><img src="image" alt="Phase Image" /></td>
<td><img src="image" alt="Phase Image" /></td>
<td><img src="image" alt="Phase Image" /></td>
<td><img src="image" alt="Phase Image" /></td>
</tr>
<tr>
<td>FITC + DAPI</td>
<td><img src="image" alt="FITC + DAPI Image" /></td>
<td><img src="image" alt="FITC + DAPI Image" /></td>
<td><img src="image" alt="FITC + DAPI Image" /></td>
<td><img src="image" alt="FITC + DAPI Image" /></td>
</tr>
</tbody>
</table>

**Figure 56. Cellular uptake of the PEI nanocomplex by MCF7 cells.** The fabricated PEI complexes and free aptamer were added to cells and incubated for their uptake at 37°C for 4h followed DAPI counterstaining and microscopic evaluation. Images were taken at a 40X using AxioObserver fluorescent microscope. Legends on the top of phase image represent either the aptamer or nanocomplex added to the respective panels.
Chapter 4

Figure 57. Cellular Uptake of the PEI nanocomplex by WERI-Rb1 cells. The fabricated PEI complexes and free aptamer were added to cells and incubated for their uptake at 37°C for 4h followed DAPI counterstaining and microscopic evaluation. Images were taken at a 40X using AxioObserver fluorescent microscope. Legends on the top of phase image represents either the aptamer or nanocomplex added to the respective panels.

4.2.1.4 Silencing efficiency of the Nano complex:

The EpCAM silencing by PEI-EpApt-SiEp nanocomplex on both MCF7 and WERI-Rb1 cells were studied by monitoring the mRNA and protein level using qPCR and Western blotting, respectively. The PEI-Apt-siRNA nanocomplex was effective in silencing the EpCAM compared to the native siRNA transfected using lipofectamine 2000 (Figure 58A). The EpCAM gene was downregulated about 56 and 62% in SiEp treated MCF7 and WERI-Rb1 cells, while the treatment with PEI-EpApt-SiEp resulted in significant (P value >0.05) higher levels of downregulation of about 64 and 72% in MCF7 and WERI-Rb1, respectively. The downregulation was higher in WERI-Rb1 compared to MCF7 cells. EpCAM silencing was not observed in PEI alone or PEI-ScrApt-SiEp nanocomplex treatments. The EpCAM protein expression downregulated in PEI alone or PEI-ScrApt-SiEp (showed upregulation only in WERI-Rb1) treated cells which are in agreement with mRNA levels (Figure 58B, 3rd and 5th lane). A reduction in EpCAM protein expression was observed in PEI-EpApt-SiEp nanocomplex and siRNA treated cells (Figure 58B, Lane 2 and 4). The percent reduction in the proteins levels were quantified by densitometry of Western blots. A 35 and 33% reduction in EpCAM
expression was observed using lipofectamine mediated transfection of siEp in MCF7 and WERI-Rb1 cells, respectively.

**Figure 58. Expression of EpCAM post silencing using PEI Nanocomplexes.** A. Graph showing the fold change in the EpCAM expression levels, calculated by normalizing with untreated controls and using GAPDH as internal control gene. qPCR was performed post treatment with PEI alone or PEI-Apt-siRNA complexes or silencing using Lipo-siRNA. B. Immunoblots were performed for EpCAM and beta-actin to check the silencing efficiency at protein level using EpCAM C-10 primary antibody from Santa Cruz at 1:400 dilution, secondary ant mouse HP from Santacruz at 1:1000 dilution. C. Densitometry of the immunoblot normalized with tubulin-internal control and untreated control. The error bar represents the standard deviation derived from mean of three experiments and the * indicates significance of P<0.05 and ** indicates significance of P<0.001.
Furthermore, 38 and 52% reduction in EpCAM levels was obtained with PEI-EpApt-SiEp in MCF7 and WERI-Rb1 cells, respectively (Figure 58C). There was positive correlation between the mRNA and protein expression levels in the control, PEI-ScrApt-siEp and PEI-EpApt-SiEp group of MCF7 and WERI-Rb1 cells (R² value 0.99 and 0.86).

![Figure 59. Effect of PEI-Apt-siRNA complex on cell proliferation](image)

We performed MTT assay to evaluate the cell proliferation as the changes in EpCAM protein levels has effect on the cell proliferation. A significant decrease in the cell proliferation was observed in the cells treated with PEI-EpApt-SiEp nanocomplex compared to siRNA alone (P value <0.05) (Figure 59). In MCF7 and WERI-Rb1 cells, transfection with the SiEp showed 50 and 52% inhibition, respectively whereas the PEI-EpApt-SiEp showed higher inhibition (69% and 68% inhibition, respectively) in these cell lines (P<0.001). A negligible effect on the cell
proliferation was observed in the aptamer alone or PEI alone or PEI-ScrApt-SiEp nanocomplex treated cells. One-way ANOVA analysis showed significant difference (P < 0.0005) between the subgroups. Furthermore, to analyze the significant difference between the SiEp treated group and PEI-EpApt-SiEp group, unpaired student t-test was performed and found to have significant difference (P<0.005) in both the cell lines.

4.2.2 DISCUSSION

There are challenges behind the targeted delivery of siRNA specifically to cancer cells and nonviral carriers such as PEI, PLGA, liposomes are found to be better delivery systems (Gao et al., 2011, Lu et al., 2013, Miele et al., 2012). The PEI nanocomplex has good transfection efficiency for the siRNA delivery purposes even though various other polymers are available for carrying nucleic acids (Di Gioia and Conese, 2009, Godbey et al., 1999, Zhao et al., 2011b). The cytotoxicity of PEI being an issue is managed by using optimal levels that is non-toxic as well having uncompromised efficiency to deliver siRNA/pDNA. Charge stabilization of the PEI is found to be necessary to maintain uniform size (Urban-Klein et al., 2005). Earlier reports stating no mutagenicity and no immune response and use of PEI loaded with CD98 siRNA for inflammatory disease indicates higher possibility of use of PEI for therapeutic purpose (Beyerle et al., 2011b, Bonnet et al., 2008a, Laroufi et al., 2014). The size and charge of the PEI nanoparticles synthesized in the present study are in agreement with previous reports on PEI nanoparticle fabrications using sgc-8c and PSMA respectively (Bagalkot and Gao, 2011, Shahidi-Hamedani et al., 2013). In the PEI/pDNA polyplexes assembled with PTK7/sgc-8c aptamer showed particle size ranging from 160-275 nm. In the PSMA aptamer and siRNA chimera assembly on quantum dots surrounded by PEI (QD-PEI), surface charge drops down upon the siRNA and aptamer addition, which was observed in our study too. A negative charge was observed on the PEI nanocomplex in the presence of siRNA and aptamer compared to the PEI nanocore alone. The hydrodynamic size was higher compared to the size of the particles observed by TEM due to the fact that aptamer and siRNA present on the surface increased the electron dense nanocore size by 30nm (hydrodynamic size). The TEM gives the nanocore size and not the surface aptamer and siRNA molecules (Lu et al., 2009). The influence of the serum proteins on the nanocomplex binding to cells were managed by adding the complex in serum free media, followed by replenishment with serum containing media, to enhance the uptake of the
nanocomplex by the cell. The presence of serum in general helps to prevent cytotoxicity of the PEI by increasing membrane integrity of the cell but leads to lower cellular uptake of the particles (Clift et al., 2010, Patnaik and Gupta, 2013).

The cellular binding of the aptamer and process of uptake are under debate. The aptamers are very specific to the target protein and hence gets internalized by receptor mediated endocytosis whereas other mechanisms have been noticed for NCL-aptamer (Reyes-Reyes et al., 2010). The scramble nanocomplex had minimal or no binding on to the cell surface that shows the specificity of the complex. The increase in mean fluorescence intensity (MFI) of cells bound with nanocomplex fabricated with EpApt confirms that more than an aptamer is engaged per PEI nanocomplex. Hence binding of nanocomplex to a receptor compared to an aptamer alone results in the increase in MFI. Blocking the EpCAM receptor using a monoclonal antibody reduced the binding of aptamer alone and PEI-EpApt-SiEp nanocomplexes exhibiting lower MFI, confirming the receptor specific binding. The observation that less aptamer is required to saturate the cancer cells in the PEI nanocomplex compared to the EpApt alone i.e., 400nM of aptamer is required to show 90% cell binding in MCF7 cells compared to 200nM required in PEI nanocomplex, showed better performance/affinity of the nanocomplex. Thus we can bring down the required concentration of aptamer by preparing nanoformulation. The nanocomplex based formulation increases the bioavailability and reactivity of the aptamer to the receptor. Other group had similar observation while using quantum dot-PEI-siRNA-aptamer targeted to PSMA in prostate cancer (Bagalkot and Gao, 2011). The marginal binding unmediated by the receptor mediated endocytosis could be due to the free positively charged PEI groups, which might access the negatively charged cell membrane. Similar non specific binding was observed in the earlier reports (Hao et al., 2013).

The PEI alone or scramble aptamer targeted complex did not show any effect on the EpCAM gene expression where as the silencing efficiency of the EpCAM aptamer targeted nanocomplex had reduced EpCAM expression. The EpCAM silencing was considered as a read out for the delivery of the siRNA inside cells, as the aptamer was found to be internalized as observed by microscopy. The targeted gene silencing is better than the conventional lipofection. Other investigators had observed gene silencing effect using PEI nanocomplex targeted against PTK7 and PSMA (Bagalkot and Gao, 2011, Zhao et al., 2011b). EpCAM antibody-gold nanoparticle
based targeting using siEp and PEI also had similar observation (Mitra et al., 2013). The EpCAM aptamer doxorubicin drug conjugates promisingly targeted cancer cells (Subramanian et al., 2012). In the present study we fabricated a nanoformulation of PEI with aptamer and siRNA and showed the efficient targeting ability to the EpCAM positive cells. The targeted nanoformulation had better gene silencing activity than the conventional silencing mechanisms. This can be further translated to in vivo system for therapeutic purpose.

4.2.3 CONCLUSION

In conclusion, we have developed EpCAM aptamer and siRNA loaded PEI nanocomplex stabilized with citrate. The nanocomplex was characterized using zetasizer and TEM showed that the particles sizes were ~180nm with the mean surface charge of -18mV. The complex was able to bind to the EpCAM expressing cells specifically though EpCAM aptamer, which was shown by microscopy and flow cytometry. The delivery and silencing effect of EpCAM siRNA to the EpCAM positive cells (MCF7 and WERI-Rb1) is a novel approach for targeting the stem cell population, as EpCAM is regarded as a putative cancer stem cell marker. The scrambled aptamer directed siRNA did not have effect on the silencing of the EpCAM in the EpCAM positive cells, elucidating the aptamer specificity. This study shows that EpCAM aptamer fabricated PEI nanocomplex with siEp are able to target EpCAM positive cells and also able to deliver siRNA to the targeted cells with high specificity.
CHAPTER 4.3
Targeted delivery of doxorubicin using EpCAM aptamer

4.3.1 RESULTS

4.3.1.1 EpCAM aptamer binds to RB tumor cells and cell lines:

Equal EpCAM expression around 35-40% positive cells was observed in Y79 and WERI-RB1 cells lines, whereas non cancerous muller-glial cells showed no expression of the protein (Figure. 60A, B and C). The EpDT3-FI bound to 35% of the RB tumor cells (n=4) (Figure 61A and B), Y79 and WERI-RB1 whereas the Scr-EpD3-F1 did not bind to all the RB tumor cells or cell lines (Figure 62A and B). The muller-glial cells showed no binding for both the aptamers (Figure 62C).

Figure 60. EpCAM expression on cell lines. Y79, WERI-Rb1, and Müller glial cells were stained for EpCAM expression with indirect immunofluorescence followed by flow cytometry. A, B, C: Y79, WERI-Rb1, and Müller glial cells stained either with secondary antibody alone or primary anti-EpCAM (C-10) antibody, followed by secondary anti-mouse FITC antibody and flow cytometry. The overlay plot shows the positive population. (Subramanian et al., 2012)
4.3.1.2 Preparation of aptamer-Dox conjugates:

The site of Dox intercalation in EpDT3 aptamer, for cancer cell specific drug delivery is analyzed by RNA structure program version 5.3 (http://rna.urmc.rochester.edu/RNAstructure.html). The predicted EpDT3 secondary structure consists of hairpin structure and the site of Dox intercalation is between GC and CG sequence in the aptamer and has a single site for Dox intercalation (Figure 63A). Following the prediction, we optimized the aptamer-Dox conjugation assay and observed gradual quenching of fluorescence from Dox with increasing aptamer concentration (Figure 63B). The EpDT3-Dox and Scr-EpDT3-Dox conjugates generated were used for functional studies.

**Figure 61. EpCAM aptamer binding on RB primary cells.** Fluorescently labeled EpCAM aptamers were incubated with RB cells obtained from fresh tumor samples and analyzed with flow cytometry. Dead cells were gated out by gating the propidium iodide–positive cells. A, B, C: Scatter plots show control unstained RB tumor cells, tumor cells stained with EpDT3-FI shifting toward the right, and Scr-EpDT3-FI showing the least binding. D: Histogram overlay plot showing significant binding of EpDT3-FI to tumor cells compared to Scr-EpDT3-FI. (Subramanian et al., 2012)
Figure 62. EpCAM aptamer binding on cell lines. Fluorescently labeled EpCAM aptamers were incubated with RB cell lines and Müller glial cells and analyzed with flow cytometry. Histogram overlay plot showing distinct population of (A) WERI-Rb1 and (B) Y79 cells bound to EpDT3-FI compared to unstained and Scr-EpDT3-FI bound cells. C: Histogram overlay plot of Müller glial cells showing no distinct population upon incubation with EpDT3-FI or Scr-EpDT3-FI from unstained cells. (Subramanian et al., 2012)
Figure 63. **EpCAM aptamer structure and doxorubicin conjugation.** Structure prediction was performed using the RNA Structure program. A: Hairpin structure of the EpCAM aptamer with the predicted site for the doxorubicin intercalation represented as a red star. B: Fluorescence spectra of doxorubicin (3.0 μm) with increasing molar ratios of the aptamer (from top to bottom: 0, 0.01, 0.1, 1, 3, 5, and 7 equivalents) in conjugation buffer. (Subramanian et al., 2012)

Figure 64. **Diffusion of doxorubicin from the aptamer-Dox conjugate.** The time-dependent release of doxorubicin from aptamer-Dox conjugates was monitored by dialyzing free Dox and aptamer-Dox conjugates against conjugation buffer. Dox from the aptamer-Dox conjugate (closed square and triangle) and the free Dox (closed diamond) released were monitored from 2 to 6 h of the initiation of dialysis and represented as % cumulative release. Aptamer-Dox conjugates release Dox comparatively slower than free Dox. The error bar represents the standard deviation. Experiments were repeated more than thrice. (Subramanian et al., 2012)
4.3.1.3 Release and diffusion of the drug from the aptamer-Dox conjugate

The release and the diffusion of the drug from the Dox conjugated aptamer was studied under artificial conditions mimicking the role of the cell membrane (Figure 64).

The percent cumulative release of the Dox from the chimeric aptamers was one fold lesser than the free Dox. The dissociation of Dox from the Dox conjugated aptamer was about 20%, 37% and 45% by 2h, 4h and 6h, respectively. The free Dox had dissociated much faster than the aptamer-Dox (60%, 70% and 80% by 2h, 4h and 6h, respectively).
Figure 65. Uptake of aptamer-Dox conjugate on Müller glial cells addition in vitro. Free Dox, EpDT3-Dox, and Scr-EpDT3-Dox were incubated with Müller glial cells for 2 h and observed under an AxioObserver microscope. A, B, C, and D: 2 h incubated cells unstained, treated with free Dox, Scr-EpDT3-Dox, or EpDT3-Dox. E, F, G, and H: 12 h incubated post 2 h of treatment with free Dox, Scr-EpDT3-Dox, or EpDT3-Dox. (Subramanian et al., 2012)

4.3.1.4 Targeted delivery and Uptake of Dox in cell line

The EpDT3-Dox showed the target specific binding and delivery of Dox in vitro. Microscopic images with free Dox treated cells clearly show Dox localization in the nucleus at 2h for both the muller-glial cells and Y79 (Figure 65B and 66B), whereas with the EpDT3-Dox the localization was observed in the cytoplasm, faintly in the nucleus of Y79 at 2h (Figure 66C) and no such staining pattern is observed with muller-glial cells (Figure 65D and H). Scr-EpDT3-Dox conjugate showed marginal or no binding on muller-glial cells and Y79 (Figure 65C and 66D). Upon incubating the cells for 12h post treatment with the aptamer-Dox conjugates, localization for cells treated with EpDT3-Dox was majorly on nucleus in Y79 whereas, no staining in muller-
glial cells (Figure 65H and 67C). However, Scr-EpDT3-Dox did not show any detectable binding on both the cell lines (Figure 65G and 67D).

Figure 66. Uptake of aptamer-Dox conjugate on the Y79 cell line post 2h addition *in vitro*. Free Dox, EpDT3-Dox, and Scr-EpDT3-Dox were incubated with Y79 cells for 2 h and observed under an
AxioObserver microscope. A, B, C, and D: phase images of Y79 cells unstained, treated with free Dox, Scr-EpDT3-Dox or Scr-EpDT3-Dox and the respective right panel represents the merged fluorescence image. (Subramanian et al., 2012)
4.3.1.5 Effect of aptamer–Dox conjugate on Cell cytotoxicity

The cell cytotoxicity was evaluated by monitoring the metabolic rate of the cells by MTT assay. Free Dox showed toxicity in both cancerous and normal cell lines (Figure 68).

Figure 68. Cell proliferation inhibition by aptamer-Dox conjugates in vitro. The Y79, WERI-Rb1 and Müller glial cells were seeded 24 h before treatment with free Dox, and aptamers (EpDT3 and Scr-EpDT3) and aptamer-Dox conjugates (EpDT3-Dox and Scr-EpDT3-Dox; n=3) were added to cells in serum-free condition for 2 h followed by media change with 10% FBS. Cells were further incubated for 24 h and 48 h, and the MTT assay was performed. A: The graph represents the percent viable Y79 cells after 24 h and 48 h of treatment. B: Graph showing percentage viability of WERI-Rb1 cells treated with aptamer and aptamer-Dox. Cytotoxicity was observed with cells treated with free Dox and EpDT3-Dox. C: Graph showing percentage viability of Müller glial cells treated with aptamer and aptamer-Dox. Only free Dox treated cells exhibit significant cytotoxicity. The error bar represents the standard deviation and the * indicates significance of P<0.05. Experiments were repeated thrice. (Subramanian et al., 2012)
Free Dox showed 27%, 35% cytotoxicity at 24h, 70%, 60% cytotoxicity at 48h post treatment on Y79 and muller-glial cells respectively. Ep-DT3 Dox conjugate showed higher cytotoxicity in cancerous Y79 cell line compared to the non cancerous muller glial cells. The non chimeric aptamer alone exhibited reduced cellular toxicity compared to the aptamer alone. Ep-DT3-Dox conjugate showed 33% and 10% cytotoxicity at 24h, 66% and 25% cytotoxicity at 48h on Y79 and muller-glial cells, respectively. EpDT3 treated cells showed 19%, 5% cytotoxicity at 24h and 14% and 24% cytotoxicity at 48h post treatment on Y79 and muller-glial cells respectively. Scr-EpDT3-Dox conjugate and Scr-EpDT3 showed 18%, 16% and 27%, 28% cytotoxicity at 24h and 48h on Y79 cells, no cytotoxicity was observed at 24h while 22%, 18% cytotoxicity was observed at 48h on muller-glial cells (Figure 68A and B). Free doxorubicin showed 57 and 73% cytotoxicity towards WERI-Rb1 cells at 24h and 48h respectively. The EpDT3-dox and Scr-EpDT3-dox showed 59%, 68% and 96%, 97% cytotoxicity on WERI-Rb1 cells (Figure 68C).

4.3.2 DISCUSSION

EpCAM is a putative stem cell marker in breast, liver, colon, pancreas, and prostate tumors (Ray and White, 2010, Szotek et al., 2006, Terris et al., 2010). Recently, our group showed the correlation and presence of EpCAM and co-expression among the CSC markers (Mitra et al., 2012). EpCAM+ breast cancer and hepatocellular carcinoma showed the CSCs or CPCs phenotype (Sarrio et al., 2012, Yamashita et al., 2009). Hence, we used the EpCAM-targeted therapeutic approach for RB using an aptamer against EpCAM, and this is the first study using the EpCAM aptamer for targeted drug delivery in RB cells. EpCAM is ideal for drug targeting in RB because as this molecule is overexpressed in invasive tumors and is a putative cancer stem cell marker. The results clearly show a significant amount of EpCAM antigen was present in the Y79 and WERI-Rb1 cell lines compared to the Müller glial cells (Figure 60). In addition, the binding potential of EpDT3 and Scr-EpDT3 checked against RB fresh tumors, Y79 and WERI-Rb1, RB cells and Müller glial cells, showed 35% positive population in the RB tumor cells (n=4) and the RB cell lines (Figure 61 and Figure 62). This might be due to the heterogeneous population of cells in the tumor and cell lines expressing EpCAM. This is consistent with our
previous observation that EpCAM is expressed only in a subset of population of RB cell lines and only EpCAM+ Y79 cells have properties of CSCs (Mitra et al., 2012).

The EpCAM protein is overexpressed in RB cell lines. EpDT3-FI showed binding to RB cells but not to the Müller glial cells, which indicates the cancer cell–specific expression of EpCAM. In contrast, no binding was observed for the scrambled aptamer in the primary RB cells, Y79 and WERI-Rb1, and the Müller glial cells (Figure 61 and Figure 62). This is in agreement with previous observations that 2'-OMethyl modification of the pyrimidines in an aptamer hampers binding of the aptamer to the EpCAM receptor (Shigdar et al., 2011).

The optimal performance of the equimolar Dox and aptamer agrees with theoretical prediction of one Dox site in the aptamer (Figure 63A). The PSMA aptamer for Dox delivery had a single site predicted theoretically for the Dox conjugation (Bagalkot et al., 2006, Min et al., 2011). However, the Dox-to-aptamer ratios varied (0.5–2) in different practical applications. The slow diffusion of Dox from the aptamer-Dox conjugates compared to the free Dox is attributed to the physically bound state of Dox to the aptamer (Figure 65). Similar results were observed by Banglok et al. (Bagalkot et al., 2006). The free Dox localized to the nucleus in the RB and Müller glial cell lines. The nucleo-cytoplasmic presence of Dox in the Y79 cells and not in the Müller glial cells incubated with EpDT3-Dox. This indicates that the conjugation of the EpDT3 aptamer to the Dox did not impair the target finding ability of the Dox. The inability of Scr-EpDT3-Dox to localize to the nucleus indicates the targeted binding of the EpDT3 aptamer over the control aptamer. The target-specific binding of EpDT3 to EpCAM, a membrane antigen, resulted in the internalization of the aptamer-drug conjugate into the cytoplasm and finally into the nucleus resulting in sustained drug delivery to the nucleus of cells expressing EpCAM (Figure 65, Figure 66, and Figure 67). Other studies have obtained similar results in LNCaP and CCRF-CEM cancer cell lines (Bagalkot et al., 2006, Huang et al., 2009). EpDT3-Dox and Scr- EpDT3-Dox did not bind or get internalized in the Müller glial cells, proving the selective binding of the aptamer to the cancerous cells sparing the normal cells. The efficacy of the EpDT3-Dox drug delivery system in killing the Y79 cells and the WERI-Rb1 cells, and not the noncancerous Müller glial cells (Figure 68A, B and C) indicates the cancer cell–specific targeting of the drug. The aptamer binding to Dox spared the drug delivery to the normal cells and killed the cancer cells precisely. Therefore, EpDT3-Dox may reduce undesirable side effects associated with
chemotherapy. The Scr-EpDT3-Dox conjugate and the aptamer alone did not have a marked effect in inhibiting cell proliferation indicating the specificity of EpDT3 binding to the EpCAM-positive cells alone.

In conclusion, we have engineered a chimeric aptamer that binds to its target molecule and efficiently delivers the drug to the cancer cells. The aptamer-based targeted drug delivery prevents off-target effects of the drug Dox. This Dox conjugate can be applied as a therapeutic agent in all cancers overexpressing EpCAM. EpCAM aptamer–based drug delivery in the future can be potentially exploited with stable linking of the drugs for targeting EpCAM-positive cancer stem cells in RB as well as in other cancers. The aptamer-conjugated nanocarriers can be used for imaging tumors or as therapeutic systems for targeting EpCAM using chimeric aptamer-small interfering RNA for RB.
CHAPTER 5

EpCAM positive cancer cell imaging and onco-miRNA and cancer stem cell markers targeting using aptamer

Summary of the chapter: Therapeutic application of EpCAM aptamer and its conjugates were shown to be effective against the RB and breast cancer cell models. Current chapter utilizes bio-orthogonal chemistry, strain promoted alkyne azide cycloaddition for the fluorescent labeling of aptamer. The novel fluorescent aptamer was tested in EpCAM positive cancer cell lines, thus can be used for imaging of cancer cells. This chapter also deals with functional activity of the onco-miRNA-17-92 cluster aptamer using RB cell lines. This miRNA cluster is regulated by EpCAM thus CSC maintenance can also be modulated using this aptamer. Additionally the CSC marker aptamers were truncated and studied for their functional activity. Using molecular dynamics structure energy minimizations were performed to generated CD133 extracellular domain structure for docking with its aptamer to study the interaction. The CSC marker aptamers will be potent application in for theranostic purpose.
CHAPTER 5.1

Imaging of EpCAM Positive Cancer Cells Using Novel aptamer-Fluorescent Conjugate

5.1.1 RESULTS and DISCUSSION

5.1.1.1 SPAAC based labeling of EpCAM aptamer and characterization

The aptamer sequence (SYL3C) used in the current work has been reported for its EpCAM specificity (Song et al., 2013). This obviated the need to use a scrambled aptamer to prove the sequence specificity. Hence no random sequence was included in the present work. Instead, EpCAM low expressing cell line was used as control. Alexa-flour 594 (AF594) fluorescent dye conjugated DIBO was used for the cycloaddition with azide-end terminated aptamer to generate conjugate (EpDNA-DIBO-AF594) for fluorescent imaging (Figure 69).

The SPAAC reaction scheme for the conjugation of aptamer carrying terminal azide modification with an alkyne, DIBO is presented in Figure 70A. Initially, a set of experiments were performed by varying the equivalent ratio of aptamer to DIBO-AF594 (1:1, 1:2, 1:4, 1:6) at room temperature in 10 nM HEPES (pH 7.0) (Gibco, Invitrogen bioscience, Bangalore, India) buffer. However, it was observed that the change of ratios did not increase either the conversion or the yield in a significant way. At higher ratios of DIBO, we observed the purification of unreacted DIBO becoming cumbersome. Hence 1:1 ratio was used for further studies (Figure 70B).
Figure 69. Illustration showing the imaging of cancer cell by targeting EpCAM. Targeted imaging of cancer cells using EpCAM present on the cell surface using the fluorescent EpCAM aptamer conjugate generated by strain promoted alkyne-azide cycloaddition. (Subramanian et al., 2014) Chem comm.

Figure 70. Reaction scheme and optimization of DIBO reaction concentration. A. Scheme illustrating reaction of azide terminated EpCAM with DIBO-AF594 to generate fluorescent aptamer conjugate by strain promoted alkyne-azide cycloaddition. B. Gel electrophoresis of unpurified aptamer-DIBO reaction with increasing concentration of DIBO. (Subramanian et al., 2014) Chem comm.)
5.1.1.2 Purification of DIBO-AF594 clicked aptamer

Purification of the conjugate was performed using HPLC. Before proceeding to the purification, reaction optimization to get more yield of conjugate was attempted by multiple freeze-thaw. It was reported that freeze thaw can be effectively utilized to increase the ligation efficiency (Takemoto et al., 2012). It was observed that repeated freeze (-30°C) and thaw (4°C) more than twice increased the EpDNA-DIBO-AF594 formation (conversion from 35% to 75% HPLC analysis) (Figure 71).

![Figure 71. HPLC analysis of aptamer conjugates. A. Chromotogram of one cycle of freeze thaw reaction mix at Abs260nm and Abs590nm (B). C. Chromatogram of three cycles of freeze-thaw of aptamer conjugate at Abs260nm and Abs590nm (D). (Subramanian et al., 2014) Chem comm.)](image1)

The retention time corresponding to HPLC traces at 27.33 and 27.81 min were presumed to be regioisomers of the EpDNA-DIBO-AF594 (same mass by MALDI) and hence no attempts were made to separate them. The final purification of the EpDNA-DIBO-AF594 was carried out by collecting, pooling, concentrating and desalting the fractions corresponding to the retention time 27.33 and 27.81 min.
Chapter 5

Figure 72. Characterization of aptamer conjugation and conjugate formation. A. Thin layer chromatography of aptamer, DIBO-AF594 and the HPLC purified conjugate using Methanol and dichloromethane as solvent. The aptamer, unpurified mixture, HPLC purified mixture and the DIBO-AF594 were spotted and viewed under UV transilluminator. ((Subramanian et al., 2014) Chem comm.)

The reaction mix was purified by HPLC and the aptamer with Abs590 peak was collected and checked on agarose gel, TLC and confirmed by mass analysis (Figure 72B). Mass spectrum (MALDI-TOF) of the purified EpDNA-DIBO-AF594 is depicted in Figure 72A. The conjugates were desalted using amicon 3KDa MWCO columns by centrifugation and used for binding studies.

5.1.1.3 Stability of conjugates

The stability of the desalted conjugates was assessed in 1XPBS for duration of 24hours. The conjugates and free DIBO-AF594 when dialyzed against 1X PBS at physiological temperature, showed rapid release of 30% DIBO-AF594 at 2h and 100% release by 6h. The EpDNA-DIBO-AF594 was intact up to 72 hours (agarose gel electrophoresis) (Figure 73A). Also dialysis did not show any degradation or release of DIBO-AF594 or AF594 (Figure 73B).
5.1.1.4 Cellular uptake of conjugates

After successfully synthesizing and purifying the aptamer-fluorescent conjugate, focus was shifted to the binding and internalization studies on EpCAM positive cancer cell lines. Since the EpCAM is differently expressed in different cancer cell lines, particular importance was given to study the binding and internalization of the EpDNA-DIBO-AF594 in the following cell lines. Breast cancer cell line: MCF7, MDAMB453, RB cell line: WERI-Rb1, prostate cancer cell line: PC3 and low EpCAM expressing -Muller glial cell line: MIO-M1. We had studied percentage EpCAM expression in cell lines and found that MCF7 and MDAMB453 cell lines express EpCAM over 80 to 90% followed by PC3 and WERI-RB1 with 35 to 45%. The percentages are derived based on the percentage population (lower right quadrant) positive for EpCAM measured using flow cytometry and plotting the populations in scatter plot. The EpCAM expression in MIO-M1 cells was very less with 5% (data not shown). In order to differentiate between the membrane bound aptamer and the internalized ones, incubation was carried out at two different temperatures and were analyzed either by flow cytometry or fluorescent microscopy. Incubation at 4°C selectively inhibits active cellular uptake hence membrane bound aptamer could be determined (flow cytometry). Incubation at 37°C allows the aptamer to internalize hence it can
be visualized (fluorescent microscopy). The mean fluorescence intensity (MFI) of the total populations is represented in the Table 11.

Table 11. Flow cytometry analysis of the binding of the EpDNA-DIBO-AF594 onto cell lines. The averaged Mean fluorescence intensities from triplicate experiments were represented.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>MDAMB453</th>
<th>MCF7</th>
<th>Weri-Rb1</th>
<th>PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.7</td>
<td>6.8</td>
<td>3</td>
<td>5.02</td>
</tr>
<tr>
<td>DIBO alone</td>
<td>8.23</td>
<td>6.83</td>
<td>4.52</td>
<td>8.11</td>
</tr>
<tr>
<td>EpDNA-DIBO-AF594</td>
<td>33.4</td>
<td>40.25</td>
<td>28.54</td>
<td>13.05</td>
</tr>
</tbody>
</table>

It became evident from the table that the EpDNA-DIBO-AF594 bound cells showed higher MFI in all EpCAM expressing cells in line with their EpCAM expression levels. The EpDNA-DIBO-AF594 bound MDAMB453 and PC3 cells showed significantly higher MFI compared to DIBO-AF594 bound cells MFI. Although MIO-M1 control cell line was used for the study, the DIBO showed nonspecific binding (data not shown). The overlay plot representing the tabulated result is presented below (Figure 74).

Figure 74. Cell surface binding of the aptamer conjugates. A. Flow cytometric analysis of the conjugate and DIBO-AF594 binding to the cancer cell lines, breast cancer-MCF7 and MDAMB453, RB cell line and WERI-Rb1 cells. ((Subramanian et al., 2014)Chem comm.)
The internalization of the EpDNA-DIBO-AF594 was visualized using inverted fluorescent microscope (Carl Zeiss, Germany) as well by dark field imaging using CytoViva microscope (Auburn, AL). CytoViva imaging under dark field and fluorescence channels was performed to visualize the presence of aptamer within the cells by Z-stacking. Z-stacking from uppermost to lowermost cell surface clearly showed the presence of the fluorescence signal from the middle sections, indicating the internalization of aptamer EpDNA-DIBO-AF594. Cytoplasmic and nuclear staining was observed, due to the internalized EpDNA-DIBO-AF594 in MCF7, MDAMB453, WERI-Rb1, PC3 cells. Increased uptake of aptamer was observed in MCF7 and MDAMB453 followed by WERI-Rb1, while moderate intensity of fluorescence relating to lower aptamer accumulation was observed in the case of PC3 (Figure 75). The fluorescent signal from the internalized aptamer present in the cell cytoplasm and nuclear compartment is indicated by the white arrows. Fluorescent microscopy images showed similar internalization pattern for the cell lines mentioned above and no internalization was observed in MIO-M1 cells (Figure 76).
Chapter 5

The binding of aptamer EpDNA-DIBO-AF594 studied by flow cytometry and microscopy in MCF7, MDAMB453, WERI-Rb1, PC3 and MIO-M1 cells were in line with similar pattern of EpCAM protein expression in the corresponding cell lines. As DIBO alone was reported for its binding on to fixed cells, live cells without fixing were used in the study. Moreover, to minimize the non-specific binding of DIBO-AF594, a 2% FBS was used during the binding and internalization studies.

![Figure 76. Fluorescent microscopy images showing the internalization of the aptamer conjugates in PC3, MCF7 cells, WERI-Rb1 and MIO-M1. Upper panel has DAPI and AF594 merged and lower panel showing respective phase images. ((Subramanian et al., 2014) Chem comm.)](image)

5.1.1.5 Cytotoxicity of conjugates

The cytotoxicity of EpDNA-DIBO-AF594 was carried out in MCF7 (high EpCAM expression), WERI-RB1 (moderate EpCAM expression) and MIO-M1 (low EpCAM expression) cells. MTT assay was performed after 48 hours of treatment. The EpDNA-DIBO-AF594 was tested from
100 nM to 1 μM concentration. Significant toxicity was not observed until 1 μM of EpDNA-DIBO-AF594 (Figure 77).

![Figure 77. Effect of conjugates on cell proliferation.](image)

**Figure 77. Effect of conjugates on cell proliferation.** The cytotoxicity of the conjugates was evaluated by MTT assay after 48h of addition to cells. Control cells without any treatment are normalized to 1. Experiments are performed in triplicate thice. ((Subramanian et al., 2014) Chem comm.)

### 5.1.2 CONCLUSION

Thus EpDNA-DIBO-AF594 was successfully synthesized using bioorthogonal chemistry. It was also shown that the EpDNA-DIBO-AF594 can be successfully used to image EpCAM positive cancer cell lines. The EpDNA-DIBO-AF594 can be used up to 1μM concentration for imaging studies without compromising either cell proliferation or cellular metabolism. Although serum stability studies of the EpDNA-DIBO-AF594 up to 24h showed no significant degradation, there is 70% of degradation after 36 h. Hence for therapeutic application that requires longer stay of the EpDNA-DIBO-AF594 in physiological environment; suitable modifications may be required (2’OMe or LNA or 3’ idT). This novel concept can be potentially applied for similar *in vivo* studies and can be used for imaging of various cancers. Further work to incorporate radiometals to aid in PET imaging and for therapeutic purpose is currently underway.
CHAPTER 5

5.2 RESULTS AND DISCUSSION

5.2.1 Pri-miRNA-17-92 aptamer abrogates maturation of miRNAs

Oncogenicity of the mir-17-92 cluster is targeted using antagonirs and small molecule inhibitors in gastric cancer, lymphoma, multiple myeloma and myeloid leukemia. Earlier study (Lunse et al., 2010) has illustrated the aptamer binding to the high affinity binding site (HABS) within the pri-miRNA-17-92 in the miR-18a region. This has shown to interfere with further interaction by the Heterogeneous nuclear ribonucleoproteins-A (hnRNP-A), thereby leading to inhibition of mature miRNA synthesis. In the current study we wanted to explore further the application of APT in vitro using cell culture system. The previous study elucidated the inhibitory role using the cell lysate, and present study reveals the functional role of the aptamer targeting pri-miR-17-92 in RB model. This opens up higher possibility of translating the pri-aptamer (Pri-apt).

The Pri-apt and con-apt carry the sequence aptamer 7 and aptamer F respectively as listed in the materials and methods section were synthesised using 3’idT in its termini to protect from exonucleases and the pyrimidine are 2’Fluoro modified to provide nuclease stability. The aptamers were transfected to cells, as they were not studied for internalization. The functional role of the aptamer on the miRNA was studied by analyzing the mature levels of the miRNA-17, miR-18a and miR-19b. The pri-aptamer is hypothesized and shown experimentally to bind the pri-miR-18a region where it harbors the HABS present. When we transfected different concentration of the aptamer in Y79 and WERI-Rb1 cell line, we observed varying expression of mature miRNA-18a. We further used 400nM of the aptamer to transfect and analyze the functional inhibition mediated by the pri-aptamer. The pri-apt showed significant (P<0.05), 33%, 43% and 38% inhibition of mir-17, mir-18a and mir-19b synthesis in the transfected Y79 cells, while the con-apt did not show inhibition of the mature miRNAs and there was 52% and 28% upregulation of the miR-18a and mir-19b (Figure 78A). In the WERI-Rb1 cell line, pri-apt transfections lead to significant (P<0.05) downregulation of the mature miRNAs varying from 70%, 40% to 50% of mir-17, mir-18a and mir-19b respectively. The con-apt had not much interference with the miRNA expression except in the levels of mir-19b expression was analyzed.
Chapter 5

(Figure 78B). Thus we were able to observe the downregulation of the mature transcript of the miR-17, 18a and miR-19b under our experimental condition in vitro using RB cell lines.

**Figure 78. Pri-miRNA aptamer inhibits the mature miRNA formation.** A. Relative expression levels of the mature mir-17, mir-18a and mir-19b performed using TaqMan based qPCR and normalized to untreated control sample with RNU6B as endogenous control in Y79 cells transfected with Pri-apt and con-apt. the construct shown above illustrated the binding of the Pri-apt to the pri-miRNA-17-92 cluster and inhibiting the miRNA synthesis. B. Graph showing the relative changes in the expression levels of the mature miRNAs in WERI-Rb1 cell line accompanying pri-apt or con-apt transfection. Data represents mean ± SD. Experiments were repeated 3 times independently with similar results. **P<0.001; *P<0.05.

5.2.1.2 Pri-miRNA-17-92 aptamer affect cell cycle and leads to apoptosis

The miR-17~92 cluster regulates the cell cycle and the apoptosis by different process observed in different cancer models. In general, the expression of the cluster regulates the apoptosis by myelocytomatosis (MYC)-induced mechanism and also by the inhibiting theRas-induced senescence(Hong et al., 2010). The involvement of the PTEN, BIM, E2F for the onset of carcinogenesis in RB has been reported (Conkrite et al., 2011). However, other groups report strong relevance behind this cluster expression in the cells with stem cell property and our group has reported that the EpCAM regulates the expression of miR-17~92 cluster (Garg et al., 2013, Kandalam et al., 2012). This miRNA cluster and its paralog cluster were shown to promote cell proliferation and prevent from cell cycle arrest in somatic stem cells. Also the target E2F, a cell cycle mediator that controls other check points is regulated by E2F. Hence the effect on cell
Chapter 5

cycle was analyzed. The levels of pri-miR-17~92 as analyzed by qPCR (SYBR green) showed decrease in expression, which was inverse to the anticipated result (data not shown). As the mature miRNA formation is abrogated, we anticipated increase in the levels of pri-miRNA, instead found to be downregulated. Additionally we analyzed the levels of the MYC-N, ATM and Dicer mRNA levels. The level of MYC-N was downregulated, while ATM and Dicer were upregulated. Reports on the regulation of the cluster in the expression of K-ras, Dicer, HIF-1a, ATM has been shown before (Nittner et al., 2012, Taguchi et al., 2008, Tsang and Kwok, 2009, Wu et al., 2013, Yan et al., 2009). These background reports and events intrigued us to analyze the effect of the aptamer on the cell cycle and the apoptosis in the aptamer transfected cells. The Caspase 3/7 chemiluminescent assay also showed increase in activity upon transfecting Pri-apt (Figure 79). The Pri-apt transfected in Y79 showed 5% decrease in the S phase cells and in turn increase in the G0-G1 phase, while in WERI-Rb1 cells the Pri-apt induced S phase arrest about 9% and 10% decrease in G2-M population (Figure 78A and B). The apoptotic effect of aptamers studied by annexin-V-FITC assay showed 20% of annexin-V positive Y79 cells transfected with Pri-apt, while WERI-Rb1 cells showed 6% of annexin-V positive and 12% for PI positivity (P<0.05). Thus the Y79 cells exhibited early apoptosis while the WERI-Rb1 cells both apoptotic and necrotic population by 48h of transfection of aptamers under our experimental conditions (Figure 78C). Though the Y79 and WERI-Rb1 are derived from the same background of RB, the difference in the metabolic pathways they adopt and cellular signaling dictates the mechanism of aptamer functionality.
5.2.1.3 Pri-miRNA-17-92 aptamer inhibits cancer cell proliferation

The antiproliferative role of the antagonirs targeting the miR-17~92 cluster is studied in leukemia, lymphoma and solid cancers and found to be an effective target for the cancer (Jin et al., 2014). Studies on medulloblastoma showed antiproliferative and inhibition of invasion of the cells to brain upon treating with the antagonir seeds targeting miR-17 and miR-19b (Murphy et al., 2013). From our previous study on RB, transfections of antagonir mix targeting five miRNAs of this cluster were found to be effectively inducing apoptosis through Caspase-3

![Figure 79. Effect of the Pri-apt on the cell cycle and apoptosis. A. Graph showing the percentage cell count of the G0-G1, G2-M and S phase cells treated with pri-apt and con-apt in Y79 cells and (B) WERI-Rb1 cells. C. Scatter plot showing the anti-annexin-V FITC binding to the Y79 and WERI-Rb1 cells transfected with Pri-apt and con-apt. Percentage cells positive in the respective quadrant are given therein and the significantly varying populations were indicated with asterisk. Experiments were replicated twice and the result provided is representative of the experiments performed. D. Graph showing the normalized relative luminescence units obtained from the caspase 3/7 assay. Results represents from triplicate assay performed twice. Data represents mean ± SD. Experiments were repeated 3 times independently with similar results.**P<0.001; *P<0.05.](image-url)
Chapter 5

acivation and cell proliferation inhibition (Beta et al., 2013). In the present study, the Pri-apt was able to bring down the mature miRNA levels, and we were interested to study the caspase level. Also in the current study, we addressed the effect of the aptamer on cellular toxicity, caspase activation and proliferation. From the results obtained the proposed mechanism of action of Pri-apt was depicted in the Figure 80A. The cytotoxicity assessed by the lactate dehydrogenase activity showed decrease in the activity in WERI-Rb1 by 6%, while the Y79 cells showed significant (P<0.05), one fold increase in LDH activity (25%) upon transfection with Pri-apt. The con-apt did not show significant cytotoxicity in both the cell lines (Figure 3B). The extent of damage and the LDH expression varied with the cell type as the difference observed in the apoptotic mechanism mediated by the aptamer in the cell lines. Similarly, the cell proliferation inhibition was more pronounced in Y79 with significant (P<0.05), 35% decrease in proliferation rate and the WERI-Rb1 showed only 20%. The slow activity of the Pri-apt in WERI-Rb1 was utilized for studying the caspase mediated cell death upon inhibiting miR-17~92 cluster. The activation of caspase 3/7 by analyzed by chemiluminescent based assay, and found to have higher activity upon treating with Pri-apt after 48h (Figure 79D). The MIO-M1, control retinal cell line was transfected similar to the RB cell lines and assessed for the cytotoxicity and found to have uncompromised cell proliferation (Figure 80C). Thus the aptamer targeting pri-miR-17~92 cluster exerted anti-proliferative effect in the RB cell lines, and not the normal cell line, wherein the miR-17~92 cluster expression is low.
Figure 80. Pri-apt induced cell cytotoxicity and cell death. A. Graph showing the percentage cytotoxicity induced by the Pri-apt and con-aptamer on the transfected cells. The percentage cytotoxicity was calculated by normalizing to low control and high control, low control being no cytotoxic and the cytotoxicity in the untreated and transfected cell after 48h of transfection were expressed in the graph. B. Graph showing the percentage cell viable after transfection of the Pri-apt and con-apt for 48h duration and assessed by the MTT assay. Data represents mean ± SD. Experiments were repeated 3 times independently with similar results.**P<0.001; *P<0.05.

5.2.2 CONCLUSION

Treatment of RB by classical chemotherapy, brachytherapy and additionally by intra-arterial chemotherapy saves the vision and eye, still enucleation becomes choice at advanced stages(Dimaras et al., 2012, Theriault et al., 2014). The expression of cancer stem cell(CSC)
markers, invasion of tumor to the optic nerve or the choroid (Mitra et al., 2012, Mohan et al., 2006), vitreous seeds and the chemoresistance are the challenges faced for the treatment of RB (Munier et al., 2012, Parulekar, 2010). We had shown over-expression of miRNA 17~92 cluster and its regulation mediated by CSC marker, EpCAM. This miRNA cluster cooperates with the Rb pathway and is induced by the MYC-N oncogene (Rushlow et al., 2013). The mature miRNAs from the cluster were found secreted in the serum and acts as biomarker for RB (Beta et al., 2013). Thus our study evaluated the functional role of the Pri-aptamer on the RB cell lines and found that the aptamer was able to inhibit the mature miRNA formation with observation of pri-miRNA-17~92 getting downregulated and the mRNA targets of the cluster being upregulated, in turn inducing the apoptosis and cell proliferation inhibition. Developing chimeric delivery vectors or intramer based approach (Choi et al., 2006) and further modifying the aptamer for its stability using locked nucleic acid modification or unlocked nucleic acid modification will have better applicability in future (Campbell and Wengel, 2011, Mook et al., 2010). Thus current study confirms the proof of concept of cellular activity of the Pri-aptamer under metabolically active condition using two different cell lines derived from same disease model with elevated miR-17~92 expressions. Hence the Pri-aptamer has higher potential for application in other cancer types.
CHAPTER 5

CHAPTER 5.3
Aptamers targeting cancer stem cell markers

5.3.1 Results

5.3.1.1 Expression of CSC markers in RB

Expression levels of the CSC markers were analyzed by qPCR or Western blotting (WB). WB analysis of CD133, CD44, ABCG2 and EpCAM were performed with normalizations using β-actin, endogenous controls between the RB tumor samples and NR. The WB showed higher expression of CD133 in primary tumor samples with two bands at varying molecular weights. The first band at 90 kDa represents the native form of the protein while the 55kDa band may be a splice variant or arisen due to reducing or denaturing conditions on the protein. The differential observation was seen under cancerous condition and was absent in normal condition (Figure 81A). There was feebly upregulation of CD44 expression in tumor samples as shown in the lane (T1-T4) compared to normal retinal sample at the range of 200-230kDa (Figure 81B). The expression of ABCG2 protein was prominently higher in tumor samples compared to the normal retina (NR1 and NR2). The breast cancer, MCF7 cell line showed 67 and 72kDa band wherein the glycosylated protein, 72kDa is majorly observed (Figure 81C).

Figure 81. The expression of CSC markers and stathmin in RB tumour samples. The expression levels of the CD133, CD44 and ABCG2 by western blotting. The CD133 (A), CD44 (B) and ABCG2 (C),
protein Western blotting in Y79 cell line or MCF7, RB tumors and normal or non-malignant retina with the endogenous control β-actin.

5.3.1.2 Expression status of CD44 variants in RB

Due to the fact that the protein levels of CD44s were lesser expressed in the RB, we wanted to analyze the expression levels of CD44 variants. Under tumorous condition CD44 expresses into different splice variants. Hence before proceeding with the aptamer binding assay we wanted to study the variants expression. CD44 variants detection at the levels of mRNA was performed for the primary RB tumor samples by qPCR. The variant primers were designed between the exonic regions. The expression of CD44 was limited and varying between tumors and mostly found to be differential in the RB tumors (50%). The expression levels of the CD44v6, CD44v8, CD44v9 and CD44-T were around two fold upregulated in 50% of the cases, whereas the CD44v10 is mostly downregulated. Though 50% of cases showed CD44-S standard form, the expression levels were low in order to be considered for targeting. The presence of the variant proteins needs to be validated using variant specific antibodies especially for the CD44v8 and CD44v9 respectively.
Chapter 5

Figure 82. Expression of CD44 variants in RB tissues. Graph showing the relative fold change in the CD44 variant mRNAs in RB tumors and cell lines, WERI-Rb1 and Y79 normalized with adult normal or non-malignant retinaby SYBR green based qPCR method.

5.3.1.3 CD133, ABCG2 and CD44 aptamer: Truncation of CD44 aptamer and uptake by cell lines

The CD133 expression in RB between the RB and the NR (90kDa) were similar while the predicted variant or spliced form is overexpressed in RB (55kDa). CD133 targeting using AC133 epitope is well known and aptamer specific for this AC133 epitope and that loses its target binding upon differentiation is selected earlier and in the current study we utilized this CD133 aptamer, A15. Similarly, the aptamers that were targeted to the ABGC2 protein were isolated using cell-SELEX by utilizing the ABCG2 overexpressing BHK cells (A12), mammosphere cultures that overexpresses CD44 and ABCG2 were also used for aptamer selection (A35). These two aptamers isolated to target ABCG2 and ABCG2 and CD44 were studied in the RB. The aptamer uptake studies were performed on to the non-cancerous cell line, MIO-M1 followed by RB cell line, WERI-Rb1 positive for CD133 and breast cancer cell line, MCF7 positive for CD44 and ABCG2 positive was carried out.

The CD44 thioaptamer from earlier studies showed that the TTA6, TA1 aptamer binds the ovarian cancer cell lines. The results showed that higher affinity of TTA6 full length aptamer to the TTA1 aptamer and we first analyzed the binding of TTA6 full length onto WERI-Rb1, MDAMB453, MCF7 and RB primary tumor cells. Further truncations of the CD44 aptamer were performed for the TTA6 and TTA1 to obtained smaller aptamers with lower molecular weights and the truncated aptamer secondary structure predictions are given in figure 83Bi and Bii. The truncated TTA6 and TTA1 are referred as TA6 and TA1 (Figure 83).

The uptake of the CD133 – A15 aptamer in RB cell lines and control MIO-M1 cells showed specific binding and uptake by the cancer cells and not to the MIOM1 (Figure 83D). The A12, A35 and TA6 were also bound and taken up by cancer cell lines, sparing the MIOM1. MIOM1 did not show uptake of any of these aptamers. The truncated TA1 aptamer was not observed to bind to any of the cell lines (Figure 83E).
Figure 83. Truncation of CD44 aptamer and CSC marker aptamer uptake on cancer cell lines. (A) CD44 full length aptamer TTA1 and TTA6 clone obtained from combinatorial screening of DNA with thio-modification for stability. The aptamer is selected against the HA binding domain. (B) Mfold structure of the truncated aptamers. (i) shows the truncated aptamer (TA1) derived from the TTA1 and the TA6, (ii) derived from the TTA6. (C) Scatter plots showing the uptake of the LNA modified EpCAM (L-EpApt) aptamer on WERI-Rb1 and MDAMB453 cell lines. (D) FACS analysis and scatter plots of CD133-A15APT uptake at the concentration of 500nM to RB cell lines, Y79, WERI-Rb1 and MIOM1 cell line. (E) The scatter plots showing the uptake of ABCG2 (A12 and A35) aptamer and CD44 truncated aptamers (TA1 and TA6) binding to MIOM1, MCF7 and WERI-Rb1 cells with the percentage positive cells indicated on lower right quadrant.
Chapter 5

5.3.1.4 Cellular uptake of CSC marker aptamers by primary RB tumor cells

The CD133 aptamer, full length CD44, TTA6, truncated CD44, TA1 and TA6, ABCG2 aptamers, A12 and A35 were studied for uptake in the RB primary tumor cells as well in control, normal or non-malignant retina (NR) cells. The scatter plot showing the aptamer uptake with the percentage positivity or mean fluorescence intensity labeled in the same quadrant is shown in Figure 84A, B and C. The tumor cells showed very high uptake of the all the aptamers except the TA1 which showed lower binding even in the cell lines, could be that upon truncation, the aptamer affinity for the CD44 has lost. But the TA6 uptake was equally maintained with no compromise in its uptake. We tested two normal retinas for the aptamer uptake and both showed very less binding to the aptamers which signify the specificity of the aptamers against the cancer cells. Also the cancer cells can adopt mechanism in addition to the receptor mediated endocytosis for the uptake of the aptamers.

The FACS analysis of aptamer (A12, A35, TA1 TA6 and A15 aptamer) binding to the tumor samples (RB) were carried out and the percentage binding of each aptamers is tabulated in Table 84. The average binding of the aptamer A12 was found to be 56.21±2.59%. The average binding of the aptamer A35 was 58.072±5.11%. Both of these aptamers were found to be specific to ABCG2 protein in the tumor samples. The average binding of the aptamer TA1 was found to be 4.296±0.69% and the average binding of the aptamer TA6 was 68.496±0.57%. Both of these truncated aptamers were found to be specific to the CD44 protein in the tumor samples. The tumor samples showed very good binding of A15 aptamer having maximum binding of 95.81%. Interestingly all aptamers when subjected to binding with normal retinas, showed very less binding similar to that of MIO-M1 cells lines. This shows the specificity of the aptamer towards cancer cells.
Table 12. The percentage uptake of aptamers by primary RB tumors and normal retina. ABCG2 aptamer, truncated CD44 aptamer and CD133 aptamer uptake on cells.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Control cy3</th>
<th>A12 cy3</th>
<th>A35 cy3</th>
<th>TA1 cy3</th>
<th>TA6 cy3</th>
<th>Control FITC</th>
<th>A15 FITC</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB1</td>
<td>0.27</td>
<td>67.50</td>
<td>77.11</td>
<td>19.31</td>
<td>73.04</td>
<td>0.80</td>
<td>90.09</td>
</tr>
<tr>
<td>RB2</td>
<td>1.02</td>
<td>57.30</td>
<td>58.81</td>
<td>0.90</td>
<td>71.20</td>
<td>0.57</td>
<td>95.81</td>
</tr>
<tr>
<td>RB3</td>
<td>0.14</td>
<td>58.34</td>
<td>54.68</td>
<td>0.19</td>
<td>70.89</td>
<td>1.57</td>
<td>91.58</td>
</tr>
<tr>
<td>RB4</td>
<td>0.49</td>
<td>41.97</td>
<td>44.52</td>
<td>0.61</td>
<td>62.40</td>
<td>0.74</td>
<td>84.82</td>
</tr>
<tr>
<td>RB5</td>
<td>0.42</td>
<td>55.94</td>
<td>55.24</td>
<td>0.47</td>
<td>64.95</td>
<td>0.49</td>
<td>90.43</td>
</tr>
<tr>
<td>NR1</td>
<td>1.44</td>
<td>2.59</td>
<td>5.11</td>
<td>0.69</td>
<td>0.57</td>
<td>1.19</td>
<td>8.42</td>
</tr>
</tbody>
</table>

5.3.1.5 Breast cancer cell line and mammospheres preferentially uptakes CSC marker aptamers

The uptake of CSC marker aptamers was tested using 3D spheroid cultures and cancer stem cell maker, ABCG2 positive cells. The MCF7 spheroids were tested for the uptake of aptamer on intact spheroids. The ability of aptamer to penetrate the spheroids was evaluated by incubating the aptamer for 2h with spheroids. Except for the TA6 aptamer, other aptamers showed accumulation at specified sites, while TA6 showed more dispersed staining (Figure 85). The cells were isolated using Dynal magnabeads coated with anti-mouse IgG (Figure 86A). Mammospheres or MCF7 spheroids were grown by culturing the cells on uncoated plates for 15-18 days. The cells were given intermittent media change and used for evaluating the cellular uptake of the aptamers. All the aptamers showed increased uptake on the mammosphere cells except for the TA1 aptamer. Further ABCG2 +ve cells were isolated and aptamer uptake study was performed to confirm the uptake of aptamers by the cells expressing cancer stem cell markers. The A12 and A35 aptamers were more specific and showed very less uptake on to ABCG2 –ve cells. In spite of enrichment of cells, TA1 aptamer showed lesser binding and uptake (Figure 86B).
Figure 84. Aptamer binding to normal or non-malignant retina and RB tumor cells. (A) Scatter plots of ABCG2 (A12 and A35) aptamer and CD44 truncated aptamers (TA1 and TA6) binding to normal or non-malignant retina and RB tumor cells. (B) Scatter plots of CD44 full length aptamer TTA6 uptake on RB tumor cells. (C) Scatter plots showing the CD133-A15 aptamer binding to the RB primary tumor cells and normal retina. The percentage positive in the lower right quadrant or the total MFI is indicated therein. Binding experiments were performed in more than n=10, RB tumor samples and the results provided are representative of them which is repeated more than thrice. (D) Graph showing the percentage cellular uptake of aptamer by the RB and NR cells. Error bar represents the standard deviation between the samples.
Figure 85. Aptamer uptake on MCF7 spheroids- mammospheres. MCF7 cells were grown on agarose coated plates for more than 15 days. The spheroids were incubated with 500nM aptamers in 5% DMEM media for 2h, followed by washing with 1X PBS and imaging under Axiovision fluorescent microscope.
under 5X objective. Control mammosphere (A) A12 aptamer (B) A35 aptamer (C) TA1 aptamer (D) TA6 aptamer (E) and A15 aptamer bound and entered within the spheroids.

Figure 86. ABCG2+ve cell isolation from MCF7 cells, mammospheres and aptamer uptake study. A. Schematic representation of isolation of MCF7 monolayer, mammosphere culture and isolation of ABCG2 positive and negative. B. Graph showing the mean fluorescence of cell population up taken the ABCG2 (A12 and A35) aptamer and CD44 truncated aptamers (TA1 and TA6) cy3 labeled aptamers and CD133-A15 labeled FITC aptamer. Cell populations: MCF7, mammosphere–ABCG2 +ve and –ve.

5.3.1.6 CD133+ve cells tumor cells preferentially uptake CSC marker aptamers

To test the specific uptake of these CSC marker aptamers by the primary cancer cells, CSC marker based isolation of cells from the RB primary tumor and the normal retina was performed. RB primary cells were chosen as the innate expression of the stem cell antigens are higher as
well due to the higher uptake of aptamers. The expression of these proteins compared to the cell lines utilized in the study was higher in the RB cells. CD133 being a reputed marker for the stem and the cancer stem cells was used for the cancer stem cell isolation. The AC133 epitope binding monoclonal antibody was used for the isolation of the CD133+ve cells. Both the CD133+ve and negative cells were collected using the Dynal magnabeads coated with anti-mouse IgG (Figure 87A). Both the populations were subjected for the aptamer uptake study and revealed that the CD133+ve cells showed fold higher aptamer uptake compared to the aptamers uptake exhibited by the negative cells. The CD133+ve RB cells had differences of ~300 MFI than the negative RB cells for the A15 aptamer uptake, while the normal or non-malignant retinaisolated cells showed ~130 MFI difference. The enhancement in binding of the aptamer in the NRCD133+ve could be due to the innate CD133, also the negative cells too showed uptake of CD133, which could be by alternate cell internalization mechanism. The A12, A35, TA6 aptamers uptake on the CD133+ve and negative NR cells did not show any greater difference, the MFI were closer in both the uptake population. But the CD133+ve cells showed very high difference in uptake for A12 and TA6 while uptake of A35 also significantly higher. However, the TA1 aptamer binding was not enhanced inspite of the +ve cell isolation (Figure 87B).
Figure 87. CD133+ve cell isolation from RB tumor and NR cells. A. Schematic representation of isolation of CD133 positive and CD133 negative cells from the RB tumor and NR. B. Graph showing the mean fluorescence of cell population up taken the ABCG2 (A12 and A35) aptamer and CD44 truncated aptamers (TA1 and TA6) cy3 labeled aptamers and CD133-A15 labeled FITC aptamer. Cell populations: RB tumor and NR - CD133 +ve and CD133 –ve.
5.3.1.7 Functional activity of the CSC marker aptamers

The functional activity of the CSC marker aptamers using the RB cell lines and breast cancer cell lines and the primary RB cells by MTT assay showed inhibition of the cell proliferation or growth upto 15-20%. The RB primary tumor cells showed cell death upon CSC markerAPT treatment. The functional activity of the aptamers was more pronounced in the primary cells, as they express the CSC markers higher in amount than the cell lines. The A15 aptamer showed significant inhibition of cell proliferation of Y79 and RB1 cells. Similarly, TA6 aptamer showed inhibition on WERI-Rb1 and RB1 cells. In general results clearly indicate that CSC markers aptamers act better on primary RB cells that express CSC markers and uptakes aptamer at higher rate (Figure 88). Hence testing the aptamers in the markers overexpressing cells or cancer models will reveal their actual functional activity.

**Figure 88. Functional activity of the CSC marker aptamers.** Graph showing the percentage cell viability of the MIO-M1, MCF7, Y79, WERI-Rb1 and RB1 primary cells upon treatment with the cancer stem cell marker aptamers. The aptamers were treated at a concentration of 500nM for 48hrs and assessed for their functional activity by MTT assay. The control cells without treatment were normalized to 100. The error bars represents the standard deviation between the triplicate experiment, the * indicates P<0.05 and ** indicates P<0.001.
5.3.1.8 Structure prediction and dynamics

The stability of protein backbone can be related to deviations that the structure acquired during the simulation. Stable conformation of protein tends to maintain its rigidity and thus will be less prone to adapt more changes in dynamic nature. By calculating backbone RMSD trajectory of the protein, the conformational stability was assessed. Hence, in this study we performed Molecular Dynamics Simulation of the modeled CD133 extracellular domain and plotted the RMSD trajectory (Figure 89). The results inferred that during the course simulation, the protein tends to gain stable conformation after initial fluctuations. Within 5\textsuperscript{th} ns the backbone was observed to fluctuate up to 5 Å and gained a stable conformation after 7.5\textsuperscript{th} ns and maintained it till the end of 15\textsuperscript{th} ns.

![Figure 89. RMSD trajectories of CD133 ExD2 and A15 aptamer. A. C alpha RMSD trajectory of CD133 extracellular region during Molecular Dynamics Simulation. B. RMSD Trajectory of A15 RNA aptamer during Molecular Dynamics Simulation.](image)

Similarly, the A15 aptamer RMSD plot (Figure 89) also showed attainment of stability after 4 ns with a deviation within 1 Å. Molecular dynamics studies were performed to obtain optimal structure with lowest potential energy highlighted with red circles (Figure 90B and 90D). The optimal structure of both Protein (Figure 91A) and A15 aptamer (Figure 90C) obtained based on the lowest potential energy conformations sampled during the simulation process were further
utilized for docking study. The CD133 ExD2 structure with the highest c-score obtained from ITASSER, utilized for molecular dynamics yielded structure with the lowest potential energy which are overlayed or super imposed. The superimposed strucutes show differences in the conformation obtained upon stabilizing condition (Figure 91B).

Figure 90. Structure prediction of CD133 extracellular domain 2 and CD133 – A15 aptamer. Tertiary structure of CD133 extracellular domain 2 (ExD2) as predicted by ITASSER. Graph obtained from molecular dynamics simulation showing the lowest energy conformation as observed at 9580th frame for the modelled extracellular domain 2 of CD133 (B) and A15 CD133 aptamer (D). Atomic coordinates from the highlighted frame (red) was chosen for further docking studies. C. Structure showing the lowest potential energy conformation of A15 aptamer derived from Molecular Dynamics simulation.
5.3.1.9 Docking

Hex Dock returned 100 docked conformations for the protein and the aptamer, among which the best conformation was chosen based on HEX score (Figure 92A). NucPlot tool generates a 2D plot of the interactions in a docked complex. This tool was used analyzing the best docked conformation, which revealed the A15 aptamer to form two hydrogen bonds with Glu1 and Asp264 of CD133 ExD2 region. Moreover, the aptamer also showed non-bonded interactions with Thr229, Gly232, His236, Thr261, Thr257, Ala256, Asp260, Ser79, Thr75 and Leu267 residues of CD133 ExD2 region (Figure 92B). The predicted interacting sites were further utilized for future studies.
5.3.2 DISCUSSION

Cancer stem cells could act as potential seeds for metastases, chemoresistance and transformation. The existence of CSC is still a debatable topic. Their presence is recognized using markers that are not reliable; hence need to be dealt carefully. Thus the marker based validation of CSCs needs to be done, considering more than one marker for isolation is important. Also CSC markers studied using various cancer types express different set of markers, hence stringent techniques and counter confirmation becomes mandatory (Medema, 2013). CD133, ABC transporter proteins, CD44 and its variants were expressed widely in many tumors. CD44 variants are considered to be markers for CSCs than the standard form. Thus the present
Chapter 5

study molecules such as CD44 and CD44 variants, CD133, ABCG2 protein in RB opens up new information for the drug targeting purposes.

The CD44 aptamer earlier published was of longer in size and with modifications such as “thio” for stability (Somasunderam et al., 2010). But truncating aptamers will be bringing down its molecular weight desirable for its clearance from the systemic circulation if used for imaging purposes. Of the two truncation performed from aptamers that binds the CD44 hyaluronic acid (HA) domain, one aptamer retained its binding while compromising binding and uptake of the other aptamer. Later, another group developed CD44v10 aptamer that is too big for biological applications (Iida et al., 2014). The ABCG2 and mammosphere aptamers were also active in cellular uptake. Though cell lines were used as models, the RB primary tumor cells had acted as better systems as they expresses more stem cell markers. As an intermediate model, spheroid cultures were established to confirm the aptamer binding to cells. Moreover, spheroid culture cells express the markers at higher levels (Liao et al., 2014). Our results also showed better aptamer binding on to spheroid cultures than the cell lines. The aptamer uptake by RB primary tumor cells were superior to other cell types studied. Isolation of cancer stem cells using ABCG2 and CD133 antibodies and uptake studies using the positive and negative cells confirmed the stem cell marker specificity of aptamers studied (Palaniyandi et al., 2012). Studies by Shigdar et al., explored the use of EpCAM, CD133 RNA aptamers in various cell lines (Shigdar et al., 2011, Shigdar et al., 2013b). ABCG2 and CD44 aptamers were too studied in ovarian, breast cancer and other cell types. But reports are lacking on the studies regarding the CSC marker binding on to RB cell lines except for the study by our group on the EpCAM aptamer.

The existence of the stem cell markers in RB is reported by Balla et al., 2009 (Balla et al., 2009). Exploitation of the markers for the therapeutic purpose would be wise enough, as the specific delivery is still a problem in drug delivery. The intraocular delivery of drugs are still an issue and intra-arterial therapy has raised one step above to lower the systemic exposure of drug (Kanwar). The stem cell characteristics of the RB tumor cells and cancer stem cell marker status were studied. Our group had studied the EpCAM, MCM2, ABCG2 expression in RB tissues (Balla et al., 2009, Conklin and Sage, 2009, Mohan et al., 2006, Sage, 2012). Thus current study aptamers were of higher importance in applying to RB cells. The cancer cell exhibit stem cell like characteristics and reported for the very small embryonic like stem cell populations (Seigel et al.,
The use of lithium chloride for transformation from the stem cell state can be used for validating the aptamer binding to the untransformed cells (Silva et al., 2010). We too utilized lithium chloride and sodium butyrate as transforming agents to study the specific uptake of aptamer.

CD133 being an important marker for stem cells and cancer stems, understanding its structure details will help for drug delivery. But the crystal structure of CD133 protein is not yet performed and available. To study the interaction between the residues within the CD133 extracellular domain 2 (ExD2) that harbors the AC133 epitope, modeling and structure prediction of the CD133 protein is necessary. AC133 epitope maintains the undifferentiated state of the cells. The re-folding of protein results in disappearance of the AC133 epitope leading to the transformation of the cells thereby losing the cancer stem cell property. The A15 aptamer selected against the CD133 recombinant protein binds to the AC133 epitope bearing cells and that the binding abolishes with the transformation of cells. Thus the CD133 ExD2 harboring AC133 epitope was studied for its interaction with the A15 aptamer. Revealing the interacting residues of CD133 protein with A15 aptamer can aid in understanding the functional activity of the aptamer. Also the residues can be used for targeting using small molecules. Thus the aptamers studied by our group can be further modified with stability incorporating nucleotides and can be tested in other RB cell sub-types before proceeding for the clinical trials.
Chapter 6

In vivo xenograft study
In vivo analysis of the study oligos and oligo conjugates

The study oligos such as aptamers and aptamer chimeras were further studied for the in vivo efficacy. Aptamer and aptamer conjugate or chimeras that are stable and effective under in vitro conditions were further analyzed for the in vivo efficacy. The siRNAs utilized in the study to reveal the functional role of Tiam1 and NCL requires delivery vehicles which can specifically deliver to the cancer cells. In this background, aptamer and aptamer conjugates or chimeras are targeted molecules and hence from the current study aptamer derivates are further tested in vivo. Among the aptamers, NCL-aptamer was functional aptamer and both the LNA modified and unmodified aptamer showed downregulation of oncogenic miRNAs and apoptotic markers in RB. Hence study of the aptamer against RB xenograft, the most resistant cell type, Y79 cell line was used to generate xenograft. This will reveal the most probable effect that it can have in a resistant RB phenotype. Also the mode of drug delivery chosen were subcutaneously (s.c.) near the tumor site and intraperitoneal (i.p.). Subcutaneous mode was preferred as it can have maximum absorption by slow release of the drug in the systemic stream. The intraperitoneal mode chosen, will have systemic delivery, but will prevent from direct release into the blood stream. Also the NCL-aptamer DNAzyme conjugate is under study and will be a future target. The EpCAM aptamer is promising molecule, the aptamer siRNA chimeric construct showed downregulation of the pluripotency or stem cell markers, which are also presented on cancer stem cells. The EpCAM receptor being overexpressed in many epithelial cancers, can be tested for its in vivo efficacy for further clinical trials. EpApt-siEp construct studied in RB cell line and MCF7 epithelial cell line, was further subjected for in vivo efficacy study using MCF7 xenograft model itself. Due to the lower stability of the doxorubicin-aptamer and the PEI nanocomplex targeted siRNA delivery under the physiological condition. Hence the EpApt-siEp was tested in epithelial cancer model, MCF7 xenograft model. The in vitro activity tested for the other aptamer conjugate showed to be lesser than the NCL-aptamer and EpApt-siEp and hence they were promoted for in vivo study.
6.1 NCL aptamer inhibits the growth of RB in vivo

6.1.1 Y79 tumor xenograft growth kinetics and effect of NCL-aptamer and LNA-NCL-aptamer

Reports from several groups on the anti-tumor property of the NCL aptamer also our *in vitro* studies elucidated the anti-proliferative property in RB cell lines. The effect of NCL-aptamer in the growth of RB tumor *in vivo* was studied using Y79 xenograft model. Both subcutaneous and intraperitoneal mode of aptamer delivery was used. The animals dosed with NCL-aptamer and LNA-NCL-aptamer subcutaneously (*s.c.*) near the tumor sites showed reduction in the tumor growth and the difference in the mean tumor volume was 440mm$^3$ and 380mm$^3$ respectively (Figure 94A). There was tumor growth reduction of 22.2 and 26% in Y79 xenografts treated with NCL-aptamer and LNA-NCL-aptamer respectively (Figure 93A). Notably, the effect of LNA-NCL-aptamer by intraperitoneal (*i.p.*) injection showed better tumor reduction of about 65% (Figure 93B) with tumor volume of 780mm$^3$ in vehicle control group and 330mm$^3$ in *i.p.* mode treated group (Figure 94B, 93B insert in left corner). The NCL-aptamer, LNA-NCL-aptamers.*c* and *i.p.* groups showed no significant body weight loss or visible signs of abnormal behaviour and the dose levels were well tolerated (Figure 94C and D). The excised tumors aligned and photographed showed clear size differences between the tumor sizes (Figure 93C).
Figure 93. Anti-tumor effect of NCL-aptamer and LNA-NCL-aptamer on Y79 xenograft model. A. Graph showing the change in percentage tumor growth inhibition of the Vehicle control group injected with PBS subcutaneously near the tumor site, NCL-aptamer and LNA-NCL-aptamer at 25nmol per animal subcutaneously near the tumor site on alternate days till day 10, from day 12 daily dosed up to day 20. B. Percentage tumor growth inhibition upon treatment with LNA-NCL-aptamer by intraperitoneal route. C. Images of the Y79 tumor tissue from nude mice on day of sacrifice. D. Graph showing the fold change in expression levels of cancer stem cell marker TIAM1 and 18s rRNA in Y79 xenograft tissues post-treatment with NCL-APT. The vehicle control group was used for normalizing the fold expression and the β2M was used as internal control. VC-Vehicle control, NCL-APT-NCLAPT, LNA-NCL-aptamer LNA modified NCLAPT. The error bar represents the standard deviation and the * indicates significance of P<0.05 and ** indicates significance of P<0.001.

Significant changes were not observed in the body weight, kidney function (BUN, creatinine) and liver function (SGOT, SGPT) parameters between the test groups (NCL-aptamer, LNA-NCL-aptamer) and vehicle control group. This indicates that there is no possible evidence of treatment...
related specific organ toxicity (liver/kidney) (Figure 94E). The differential leukocyte count (DLC) following treatment with NCL-aptamer, LNA-NCL-aptamer revealed no significant changes in the percentage of lymphocytes, neutrophils, monocytes and eosinophils when compared to vehicle control animals (Figure 94F). The current results from DLC indicate that there is no possible evidence of bone marrow suppression. The tumor tissue sections stained with haematoxylin and eosin stains from animals of untreated vehicle group and all treatment groups showed no significant differences and showed common characters in all the tissues. The organs collected, namely liver, lung, spleen, heart and kidney appeared normal across all groups with no deviations (Figure 95).
Figure 94. Anti-tumor, growth kinetics and biochemicals changes accompanying NCL-aptamer treatment. Graph showing the change in Tumor volume (A), percentage weight gain (C) of the Vehicle control group injected with PBS subcutaneously near the tumor site, NCL-aptamer and LNA-NCL-aptamer at 25nmol per animal subcutaneously near the tumor site on alternate days till day 10, from day 12 daily dosed upto day 20. (B) Graph showing tumor volume change and body weight gain (D) upon treatment with LNA-NCL-aptamer by intraperitoneal route. (E) Graph showing the levels of Blood Urea Nitrogen (BUN), Serum glutamic oxaloacetic transaminase (SGOT), Creatinin and serum glutamic pyruvic transaminase (SGPT) in the Y79 xenografts treated subcutaneously. (F) Differential white blood cell counts from the nude mice carrying Y79 xenograft after 24 days of treatment with PBS (VC group) or
Figure 95. H and E staining of sections. H and E staining of tumor sections (A) kidney (B) liver and (C) lung (D) spleen section (E) and heart sections (F) of VC or NCL-aptamer or LNA-NCL-aptamer group. Differential white blood cell counts from the nude mice carrying Y79 xenograft after 24 days of treatment with PBS (VC group) or NCL-aptamer or LNA-NCL-aptamer (groups) aptamer respectively.
6.1.2 NCL aptamer downregulated tumor and serum onco-miR expression in vivo

The NCL APT showed significant anti-tumor activity in highly aggressive Y79 xenograft model. The molecular mechanism behind the tumor growth reduction was addressed by analyzing the tumor mRNA and miRNA expression. Additionally, serum miRNA was studied for correlating the tumor growth changes. Interestingly, the expression of cancer stem cell markers was differentially expressed among the groups. The treated groups showed downregulation of CD133, CD44, MRP1, TIAM1 and 18s rRNA (Figure 93D). Western blotting for the FOXM1, Bcl2 and survivin showed downregulation of Bcl2 and survivin in the xenograft tissues (Figure 96A) with varying FOXM1 expression between the modes of treatments as shown by the densitometry (Figure 96B). The differential regulation in the gene expression upon treating with LNA-NCL-aptamer by altering the route of administration indicates the variation in the mechanism of action. Similar to the gene expression, tumor miRNA expression was studied for the mature miR-17-92 cluster and miR-330, miR-206, miR-196b, miR-152 and miR-18a and miR-19b-1. miR-196b was found to be downregulated in all the treated animals followed by the miR-330, miR-206, mir-196b and miR-18a was downregulated in all animals (Figure 96C).
Figure 96. Effect of NCL-aptamer in vivo Y79 xenograft model. A. Changes in protein expression accompanying treatment with NCLAPT on Y79 xenograft mice. Western blotting of proteins extraction from the n=2 tissues of the control group, mice treated with NCL-aptamer (25nmol), LNA-NCL-aptamer (25nmol) by s.c. route and LNA-NCL-aptamer (25nmol) by i.p. route. B. Graph on its right, shows the relative changes in the protein expression in treated tissues by densitometry analysis of the western blot. C. Graph showing the levels of Y79 xenograft tissue miRNA (miR-330, miR-206, miR-196b and miR-18a) post treatment with NCL-aptamer and LNA-NCL-aptamer. The vehicle control group was used for normalizing the fold expression and the Hs-RNU6B was used as internal control. D. Graph showing the levels of serum miRNA post treatment with NCLAPT. The normal serum (mice with tumor load) was used for normalizing the fold expression and the 18s rRNA was used as internal control. The error bar represents the standard deviation and the * indicates significance of P<0.05 and ** indicates significance of P<0.001.

Serum miRNA acts as bio-marker for the diagnosis and prognosis of the diseases. In RB, mir-17-92 cluster was found to be overexpressed in serum and hence changes in the serum miRNA levels is of greater interest during the treatment regimen. The relative changes in serum miRNA between the normal mice and Y79 xenograft showed over expression of miR-18a, whilst miR-17...
and miR-19b-1. The expression of miR-18a was downregulated in the treated groups, where the miR-17 expression was downregulated in all the NCL-aptamer treatment (Figure 96D).

6.1.3 Lipid profile changes in xenograft tissues post treatment with NCL-aptamer

The difference in the intensities of the masses of lipids between the Y79 cells grown in culture flasks and xenograft model were compared and found to have decreased intensity peaks (\( m/z \) 768.6 and \( m/z \) 810.6) and increased intensities (\( m/z \) 756.6, \( m/z \) 780.6, \( m/z \) 804.6, \( m/z \) 808.6 and \( m/z \) 832.6) which are closer to the adherent phenotype of cells except for the \( m/z \) 768.6 (Figure 97A). The Y79 xenograft treated with NCL-aptamer and LNA-NCL-aptamer by s.c. route and i.p. route were subjected to lipid profiling. The lipid profiling showed decrease in lipid intensities in the NCL-aptamers.c. treated tissues followed by difference in the LNA-NCL-aptamers.c. route (Figure 97B).
Figure 97. Effect of NCL-aptamer and LNA-NCL-aptamer on surface lipid arrangement in Y79 xenograft tissues. DESI-MS based lipid profiling between RB cell lines and MIO-M1. A. DESI-MS spectra of Y79 cells grown in suspension in cell culture and Y79 xenograft tissue in nude mice. B. DESI-MS spectral imaging of cryosection of the tissues and imaged using methanol as solvent of dispersion.
The spectral imaging of the masses that showed considerable differences between treatments is \( m/z \) 754.6, \( m/z \) 756.6, \( m/z \) 782.6 and \( m/z \) 810.6. Thus we were able to observe that blocking or silencing of NCL having impact in the lipid presentation or arrangement in the cells (Fig 97D). The mass observed by DESI MS corresponds to the lipids listed in the table 13 identified by MS/MS of the masses observed.

### 6.1.4 Effect of LNA-NCL-aptamer treatment in vivo on cytokine elicitation and apoptotic marker onset

The immunotherapy is greatly known for immune elicitation; similarly long DNA or RNA molecules too elicit the immunity. Hence, we wanted to study the effect of LNA-NCL-aptamer treatment in the cytokines expression in the mouse serum for 15 days. Compared to the vehicle control the aptamer treated groups showed significant (*, \( P<0.05 \); **, \( P<0.001 \)) increase in the expression of CXCL1, CXCL12, TIMP1, MCSF, i-309 cytokines, with decreased levels of IL2, ITAC, CCL2. Also there no significant difference in expression of TNF-\( \alpha \) and IFN-\( \gamma \). Thus there is mixed expression of the chemotactic cytokines and the mitogenic cytokines in the treated case (Figure 98A). The apoptosis array performed from the tumor tissues treated with PBS and LNA-NCL-aptamer showed significant difference in the apoptotic markers involved in extrinsic, intrinsic pathways. There was significant downregulation of XIAP, survivin, livin, cytochrome c, HSP27, clusterin and p21 with increase in the levels of Bad, Bel-xl, Fas, TNF\( \alpha \)R and p53 (Figure 98B). The LNA-NCL-aptamer treated tissues also exhibited decreased levels of the nuclear PCNA expression (Figure 98C).

**Table 13.** Table showing the list of lipid masses and the corresponding lipid chemistry.

<table>
<thead>
<tr>
<th>( m/z )</th>
<th>Lipid</th>
<th>( m/z )</th>
<th>Lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>754.6</td>
<td>([\text{PC}(34:4)+\text{H}]^+)</td>
<td>782.6</td>
<td>([\text{PC}(36:4)+\text{H}]^+)</td>
</tr>
<tr>
<td>756.6</td>
<td>([\text{PC}(34:3)+\text{H}]^+)</td>
<td>804.6</td>
<td>([\text{PC}(38:7)+\text{H}]^+, [\text{PC}(37:0)+\text{H}]^+)</td>
</tr>
<tr>
<td>760.6</td>
<td>([\text{PC}(34:1)+\text{H}]^+)</td>
<td>808.6</td>
<td>([\text{PC}(38:5)+\text{H}]^+)</td>
</tr>
<tr>
<td>768.6</td>
<td>([\text{PC}(35:4)+\text{H}]^+)</td>
<td>810.6</td>
<td>([\text{PC}(38:4)+\text{H}]^+)</td>
</tr>
<tr>
<td>780.6</td>
<td>([\text{PC}(36:5)+\text{H}]^+)</td>
<td>832.7</td>
<td>([\text{PC}(40:7)+\text{H}]^+, [\text{PC}(39:0)+\text{H}]^+)</td>
</tr>
</tbody>
</table>
Chapter 6

A

Y79
Vehicle control

Y79
L-NA i.p.

B

PCNA

Veh Control
L-NA i.p. inj

C

Y79
Vehicle Control

Y79
L-NA i.p.
Figure 98. Protein array for cytokine secretion and IHC analysis on LNA-NCL-aptamer treated xenograft samples. A. Mouse cytokine array performed on the vehicle control and LNA-NCL-aptamer (i.p.) treated nude mice serum collected on day 24 before sacrifice. The upper panel shows the Y79 xenograft vehicle control serum and lower to it is the serum from mice treated with LNA-NCL-aptamer (i.p.) upto 24 days. Graphs below shows the mean integrated pixel density of the each protein spot from the blot detected using chemiluminescence imaging and quantified using imageJ software using microarray profile plugin. B. Immunohistochemistry of the tumor tissues excised from the vehicle control mice and the mice treated with LNA-NCL-aptamer (i.p.) by intraperitoneal mode of injection. The levels of apoptotic markers between the vehicle control mice and the LNA-NCL-aptamer treated mice upto 24 days, was analyzed using 'human apoptotic array'. The labels next to the spot represent the protein and the integrated pixel density was quantified using imageJ software and plotted as graph (i, ii and iii). ‘i’ represents the targets that belongs to intrinsic apoptotic pathway, ‘ii’ represents extrinsic apoptotic pathway and ‘iii’ represents other key regulators of apoptosis. Data represents mean ± SD. Experiments were repeated 3 times independently with similar results.**P<0.001; *P<0.05.

6.1.5 Discussion

The in vivo studies for evaluating the anti-tumor activity of NCL-aptamer and LNA-NCL-aptamer in Y79 xenograft showed better activity in the intraperitoneal (i.p.) mode of delivery than the subcutaneous (s.c.) mode of delivery. The NCLAPT and the LNA-NCL-aptamer modified NCLAPT both showed similar results with LNA-NCL-APTAPT having 4% better efficacy in tumor inhibition under s.c. mode, while the i.p. mode of delivery of LNA-NCL-aptamer showed efficacy of 65% tumor growth inhibition. The treatment with NCL-aptamer and LNA-NCL-aptamer had downregulated the levels of Bcl2 and survivin. The increase in FOXM1 expression in the s.c NCL-aptamer and LNA-NCL-aptamer i.p. group and not in the s.c. LNA-NCL-aptamer group shows the different mode of action of the modified aptamers with respect to their mode of injection. FOXM1 is differentially expressed in cancers and upon arresting of cells during inhibition of cell proliferation and cell cycle events also reported for regulation though miRNA expression. These observation needs to be further investigated. The mRNA and miRNA expression in the tumor revealed partial response on the G-rich mRNA, with consistent downregulation of miR-18a and miR-196b in the treated tumors. The impairment of the miR-18a and miR-196b expression could be the mechanism behind aptamer activity in RB. The cellular balance between the expression of tumor suppressor and onco-miRNA, thus found to be modulated by NCL. The serum miRNA analysis showed downregulation of miR-17, miR-18a
and miR-19b were downregulated in the treated cases. Thus the multifunctional protein expressed under cancerous condition modulates various activities.

Figure 99. Illustration summarizing the role of NCL in cellular metabolism. NCL mediated major processes in the cell. It helps in 1 - gDNA replication and unwinding 2 – transport of mRNA and rRNA transcripts 3 - stabilizing the mRNA thereby modulating its half-life 4 – maturation of miRNA 5 – regulates secretion of onco-miRNA in serum 6 – regulates lipid arrangement. NCL aptamer perturbs NCL from participating in the vital processes thereby destabilizing the onco-mRNAs, synthesis and secretion of onco-miRNAs leading to cell proliferation, cell division inhibition and cell death by the onset of apoptotic pathway.

The retina majorly consists of glycerophospholipids and that in turn is classified into phosphatidylethanolamine (PE) and phosphatidylcholine (PC) and techniques used earlier were long process (Gulcan et al., 1993, Martinez et al., 1988). Recent report on the lipid imaging by MALDI mass spectrometry also revealed the PE, PC, other fatty acids distribution across the retina, RPE and optic nerve (Zemski Berry et al., 2014). Analysing the changes in the lipid content using DESI MS were reported in glioblastoma and in hepatocellular carcinoma. The considerable changes in the phosphatidylcholine studied in positive mode on the NCL
knockout or NCL-aptamer treated cells clearly showed the role of the NCL in the lipid metabolism or lipid arrangement on the cell surface. As the NCL is also presented in the cell surface, treatment of cells with aptamer could have lead to changes in the lipid, thus be putative mechanism to alter the lipids (PC) on cell surface, thereby preventing the oncogenic signals from the cell membrane. The xenograft tissues also showed lesser lipid distribution in the NCL-aptamer treated case, could explain the mechanism similarly implied in the in vivo conditions too. Similar observation upon using conditional knock on and off mice for the Myc-oncogene showed difference in the lipid profiling of hepatocellular carcinoma is reported (Perry et al., 2013). Also the importance of lipid profiling for the discrimination of the grades of astrocytoma report (Eberlin et al., 2010) shows evidently, that application of DESI MS has simplified the grading of cancers.

The protein array analysis supports the apoptotic onset by downregulation of the IAPs and increase in Bad and p53, cell proliferation inhibition. Also the cytokines levels were varying with some of the chemotactic attractants getting downregulated with minor or no changes in the levels of the TNFα and IFN-γ. Though our results and other reports showed tumor targeting and growth inhibition, still NCL-aptamer being a low molecular weight molecule gets excreted by renal clearance. Hence combining the aptamer with larger molecule can increase the half-life in serum, thereby enhanced permeation in tumor tissue; will result in better tumor reduction. The NCL-aptamer is used for the targeted delivery of nanoparticle to cancer, as well multimodal imaging of cancers and for delivering drugs to cancer cells. By preparing nanoparticle based preparations or pegylating the NCL-aptamer will help to retain aptamer in circulation and increased tumor uptake leading to increased tumor regression properties.

To conclude, our data reports for the first time, the NCL expression and its role in RB tumorigenesis. The knockdown of NCL lead to changes in mRNA and miRNA expression levels and in vivo inhibited the tumor growth effectively (Figure 99). Our data also substantiates the use of NCL-aptamer for targeting RB by intraperitoneal mode of delivery. Further studies are needed on the orthotropic RB model by improving the aptamer in preparing nanoformulation may target RB for future clinical trials.
6.2 EpApt-siEp regress epithelial cancer growth: *In vivo* xenograft study

6.2.1 Epithelial cancer MCF7 growth kinetics and effect of EpApt-siEp

The anti-tumor effect of the EpApt-siEp was studied in using the breast cancer *in vivo* model. MCF7 cells were injected in bilaterally ovariectomized nude mice supplemented with external estrogen. The dosing of EpApt-siEp was performed on alternate days from day 0 to day 14 and on day 20, animals were dosed, 24h later n=4 were sacrificed. The tumor growth kinetics showed significant reduction (P<0.01) in tumor volume, vehicle control showed 584 mm$^3$, while the treated animal showed 52 mm$^3$ mean tumor volume. The rest of n=4 in vehicle control group and EpApt-siEp group were dosed on day 22 and day 24. The mean tumor volumes on Day 33, for vehicle control group and EpApt-siEp were 922 mm$^3$ and 64 mm$^3$ respectively. The tumor growth profile during this period is shown in Figure 100A. The % tumor growth inhibition (TGI) for EpApt-siEp group at the tested dose level was found to be 102% (Day 33, # indicates p<0.001). On the day 33, terminal sacrifice, photograph of the VC group and EpApt-siEp group n=4 were taken (Figure 100D), the excised tumors were analyzed for the expression of EpCAM and cancer stem cell markers, apoptotic makers and drug resistant proteins.

![Figure 100A](image1.png)
![Figure 100B](image2.png)
Figure 100. Tumor growth kinetics of MCF7 Xenograft treated with EpApt-siEp. Female nude mice (Hsd: Athymic Nude-Foxn1tm, bilaterally ovariectomized) housed in individually ventilated cages (IVCs) were used for the present investigation. The tumorigenicity of the MCF7 cells in mice is estrogen-dependent. Twenty hours prior to MCF-7 cell injection, animals were implanted with 17β-estradiol pellets (0.36 mg/pellet; 60-day release; Innovative Research of America, Sarasota, FL) into dorsal shoulder blade region of mice using trochar. Twenty four hours post implantation of pellets, MCF-7 tumor cells (5 × 10⁶ cells/animal) were injected subcutaneously in the flanks or back of the animals. After 7-10 days post injection of cells, animals were randomized based on tumor volume (TV ≈ 80mm³) and dosing was initiated. Graph showing the (A) Tumor volume (B) % tumor growth inhibition of the Vehicle control group injected with PBS subcutaneously near the tumor site, EpApt-siEp was subcutaneously near the tumor site on alternate days. On Day 33, based on ethical reasons animals from all the groups were sacrificed. The subcutaneous tumor was harvested and photographed. *p < 0.001 significant difference when test group was compared with vehicle control group. Statistical analysis carried out by unpaired t-test using Graph PadPrism v5.0. C. Photographs of vehicle control and treated mice and the excised tumor (D). Body weight change, Haematology and biochemical parameters of xenograft animals. Graph represents the mean body weight (E) % differential leukocyte count (F) and biochemical parameters, BUN (Blood urea nitrogen), creatinine, SGPT (serum glutamic pyruvic transaminase) and SGOT (Serum glutamic oxaloacetic transaminase).

The results of DLC following treatment (EpApt-siEp) showed no significant changes in the percentage of lymphocytes, neutrophils, monocytes and eosinophils compared to vehicle control group. The results from DLC indicate that there is no possible evidence for the depression of bone marrow. Also there were no significant changes in the Liver function (SGOT, SGPT) and kidney function (BUN, Creatinine) parameters in EpApt-siEp treated group when compared with
vehicle control group (Figure 100B and C). This indicates that there is no possible evidence of treatment related specific organ toxicity (Liver/Kidney). Tissue sections were trimmed, processed, embedded in paraffin blocks, sectioned and stained with haematoxylin and eosin stains for microscopic evaluation. Histological observation of tumor sections showed no significant difference between the vehicle control group and EpApt-siEp group. Further histological analysis of lungs, liver, spleen, kidney and heart revealed no significant difference between the experimental groups (Figure 103).

Figure 101. Changes in gene and protein expression upon EpApt-siEp treatment on MCF7 xenografts. A. Graph showing the changes in MCF7 xenograft tumor tissue EpCAM, CD44s, CD24 and MRP1 mRNA levels post treatment with EpApt-siEp aptamer construct. The vehicle control 1 was used for normalizing the fold expression and the b-2 microglobulin was used as internal control. B. Graph showing the changes in STMN, BIRC5, Bcl2, Bax, ATM and CD44T mRNA levels post treatment with EpApt-siEp aptamer construct. C. Changes in protein expression by Western blotting of proteins extraction from the n=2 tissues of the control mice or mice treated with EpApt-siEp (0.6nmol) and terminated at 21 and 33days respectively and the densitometry is presented in (D).
The effect of EpApt-siEp in the expression of EpCAM and other cancer stem cell markers were studied on vehicle control group, day21 and day33 sacrificed treated groups (n=2). The gene expression analysis using qPCR showed downregulation of EpCAM and MRP1 significantly in the day21 and day33 treated groups. The levels of CD44s were high in day33 group, while day21 group showed lesser expression than vehicle control (Figure 101A). Additionally levels of Bax, Bcl2, stathmin (STMN1), survivin (BIRC5) and ATM were studied in vehicle control and both treated groups. The expression levels of Bcl2 seem to be downregulated, while the Bax was upregulated in the day21 group, around 2.5 fold upregulated, while in day33 group low level changes were observed. The level of ATM gene expression was upregulated in 50% of the day21 group and 100% of day33 group, which signifies the mechanism behind the tumor suppression. Similarly the levels of stathmin and survivin were downregulated in the day21 and day33 group, the stathmin levels were significantly downregulated on day33 group, while survivin was downregulated at higher levels in day21 than the day33 group. CD44T had minor changes in expression in the day33 tumor (Figure 101B). The expression of EpCAM and ABCG2 were analyzed by Western blot at protein levels, densitometry analysis showed downregulation of EpCAM (more than 60% downregulation) in day21 group and 50% of day33 group, while ABCG2 showed better downregulation (more than 70% downregulation) in day33 group when compared to day21 group (Figure 101C and D). Also the immunohistochemistry showed downregulation of the EpCAM and PCNA in the EpApt-siEp treated tissues (Figure 102A and B). Increased apoptotic bodies were observed in the treated tissues showing the possible mechanism by which the tumor reduction occurs. Thus we were able to elucidate the EpCAMdownregulation mediated anti-tumor effect of EpApt-siEp.
Figure 102. Immunohistochemical analysis of EpCAM and PCNA in xenograft tissues. Immunohistochemistry of the tumor tissues excised from the vehicle control mice and the mice treated with EpApt-siEp by intraperitoneal mode of injection. The levels of EpCAM and PCNA were studied and images are taken under 20x and 40x objective for EpCAM, 40x for PCNA.
Chapter 6

Figure 103. Haematoxylin and Eosin staining of organ and tumor sections. H and E staining of tumor sections of vehicle control and EpApt-siEp (RNA oligo labeled). Top and bottom panels are from two different animal. The Photographs are taken at 40X magnification. Mitotic figure (White arrow); Fibrovascular stroma (Yellow arrow); Apoptotic figure (Red arrow); Neutrophil (Green arrow). H and E staining of lung, spleen, heart and liver section of vehicle control (top) and EpApt-siEp (also labeled as RNA oligo). PT- portal triad; CV- central vein; Hp- hepatocytes; A-Alveoli; BV- Blood vessel; WP- White Pulp; RP- Red pulp; T- Tubules; G- Glomeruli.

6.2.2 Immunomodulatory and apoptotic effect of the EpApt-siEp chimera

The chimeric RNAs or long RNAs are known to elicit the immunity, hence we particularly wanted to study the effect of EpApt-siEp in the cytokines expression in the mouse serum of vehicle control group and day33 sacrificed EpApt-siEp treated groups. The cytokine array showed significant (*, P<0.05; **, P<0.001) increase in the expression of IL1RA, CCL2, G-CSF, CXCL1 and sICAM-1, while decrease in the levels of IL-16, M-CSF and TIMP-1 (Figure 104A).

The apoptosis array revealed difference in the expression of proteins of extrinsic, intrinsic and other regulators of apoptosis (Figure 5B). In the intrinsic pathway, Bcl-xl, Bcl2, cIPI-1 and survivin was significantly downregulated with significant increase in the BAX and pro-caspase-3 expression. There was upregulation of cleavage Caspase 3 and Bad and downregulation of cIAP-2 (Figure 104B i). The extrinsic pathway regulators were not significantly altered though FADD
and Trail2 showed decrease in expression. There were only minor changes in the extrinsic pathway regulators, but the levels of HPS60 were elevated, while HSP70, claspin and catalase were downregulated, hallmark proteins that inhibits the apoptosis (Figure 104B ii, iii). Thus the EpApt-siEp was found to mediate apoptotic cell death though intrinsic pathway.
Figure 104. Protein array for apoptotic markers, cytokine secretion and the IHC analysis on EpApt-siEp treated xenograft samples. A. Mouse cytokine array performed on the vehicle control and EpApt-siEp treated mice serum collected on day 33 before sacrifice. The upper panel shows the MCF7 xenograft vehicle control serum and lower to it is the serum from mice treated with EpApt-siEp upto 24days and studied upto 33days. Graphs below shows the mean integrated pixel density of the each protein spot from the blot detected using chemiluminescence imaging and quantified using imageJ software using microarray profile plugin. B. The levels of apoptotic markers between the vehicle control mice and the EpApt-siEp treated mice upto 24days and studied upto 33days, was analyzed using 'human apoptotic array'. The labels next to the spot represent the protein and the integrated pixel density was quantified using imageJ software and plotted as graph (i, iiand iii). ‘i’ represents the targets that belongs to intrinsic apoptotic pathway, ‘ii’ represents extrinsic apoptotic pathway and ‘iii’ represents other key regulators of apoptosis. The error bar represents the standard deviation and the * indicates significance of P<0.05.

6.2.3 Discussion

The in vivo assessment of the anti-proliferative and anti-tumor property of the chimeric construct dosed in alternate days upto 14 days followed by next cycle of dosing from day 20, 22 and 24
resulted in tumor regression. The construct elucidated high antitumor activity and was well tolerated without any toxicity in animals. The biochemical liver, kidney function and blood cell counts revealed the potentiality to use the drug safely in vivo. The treated groups were monitored till day 33 for any regrowth of tumors. Interestingly none of the mice treated with chimeric constructs developed any tumor. The molecular analysis of the excised tumors treated with EpApt-siEp chimeric construct showed downregulation of EpCAM at mRNA and protein level. In the breast cancer xenograft model, we analyzed series of markers, CD44 standard and total CD44 forms. The expression of the CD44 variants under malignant condition is reported (Li et al., 2013a, Sharma et al., 2014). Hence we wanted to study the CD44 levels, and we observed increase in the CD44s and CD44T in day 33 group. But the gene contributing majorly to the oncogenesis in breast cancer, survivin, stathmin, BCL2 were significantly downregulated upon treating with chimeric construct. The downregulation of stathmin, survivin would have synergistically functioned for the anti-proliferative effect of cells.

Remarkable increase in the expression of BAX was observed on day 21 group, but in day 33 group, the expression lowered down. The chimeric construct induced BAX expression and decreased BCL2 expression. The decrease in MRP1, motility related protein1, also reveals the mode of the anti-tumor property exhibited by the chimeric construct. Also 50% of the treated population showed significant increase in expression of ATM levels which is generally downregulated in breast cancer. The levels of ATM and the poor prognosis of the disease have been shown (Angele et al., 2000). Hence the increase in ATM expression upon chimeric construct treatment is an interesting finding for molecular mechanism that it has exerted in breast cancer. There is positive correlation behind the EpCAM and mir-17-92 cluster over expression in cancers (Kandalam et al., 2012) as the mir-18a targets ATM, thereby regulating its expression to aid tumorigenesis (Song et al., 2011).

Immune elicitation is commonly observed phenomenon in the case of antibody but not in aptamer based therapies. They revealed higher levels of TNFα and interferon-γ in comparison to before treatment or untreated candidates (Dranoff, 2004). Cytokine profiling and analysis in mice treated with EpApt-siEp showed increased levels of G-CSF, IL1ra, CXCL1, sICAM1, where in IL1ra can potentially block the inflammatory process mediated though IL1. Decrease in TIMP1, M-CSF could be the effect of treatment directly, as these are reported to be elevated in cancerous
conditions as well in poor survival of the candidates (Wang et al., 2006a). No significant changes in the TNFα or INF-γ levels were observed during the treatment, which are desirable. Also the apoptotic protein profiling revealed that the EpApt-siEp adopts the intrinsic pathway to mediate apoptosis. The inhibitors of apoptosis (IAPs) proteins were greatly downregulated with upregulation of Bcl-xl and Bad. Also the increase in cleaved Caspase-3, HSP60 and decrease in claspin, HSP70 and catalase supports the mechanism of apoptosis onset (Semple et al., 2007, Wong, 2011).

In conclusion, we were able to show the functional activity of the chimeric construct by targeted delivery to the EpCAM expressing cells and silencing of EpCAM expression. The observation of the EpICD in the RB opens up avenue for targeting EpCAM with Wnt signaling inhibitors which can synergistically enhance the therapeutic activity. The role of EpICD in regulating the cancer stem cell property in RB was elucidated for the first time and our group has already shown the relevance behind EpCAM and mir-17-92 cluster in RB. Upon treating the MCF7 xenograft model as a proof of concept for epithelial solid cancers, which expresses higher levels of EpCAM, we were able to elucidate first time the regression in the functional activity with the construct (Figure 105). As EpCAM is overexpressed in many cancers, our study also paves a way for the application of its potential anti-tumor agent as well as imaging (diagnosis) in future clinical studies.
Figure 105. Illustration summarizing the EpCAM aptamer siRNA chimera effect on the tumor growth inhibition. The EpCAM aptamer siRNA chimeric construct (EpApt-siEp) binds to the EpCAM receptor and gets internalized (1) and released in the cytoplasm where gets into Ago complex with dicer enzyme (2) to generate siRNA. The siRNA loaded into RISC complex (3) binds to the EpCAM mRNA (4) and leads to mRNA degradation (5). The EpCAM proteolysis leads to release of EpICD (EpCAM intracellular domain), shedding of EpEx (EpCAM extracellular domain) and EpTM (EpCAM transmembrane domain) (6) interacts with Wnt signaling mediators, b-catenin, FHL2 and TCF and complexes (7) to translocate to nucleus and regulate the gene transcription of pluripotency markers, SOX2, OCT4, NANOG, EpCAM, CD133 and CD44 (8) and downregulates the markers (9) and cell proliferation markers (10). The EpCAM silencing leads to apoptotic cell death by downregulation of survivin, IAPs, Bcl2, p27, HSP70, catalase and claspin, majorly mediated by intrinsic apoptotic pathway leading to cell death. Overall, the knockdown of EpCAM using EpApt-siEp chimera leads to the inhibition of the nuclear signaling mediated by the EpICD, thereby decreases the cancer stem cell marker expression and induces apoptosis and brings down the tumorigenicity.
Conclusions from the study

One of the leading problems worldwide is cancer and cancer metastasis. The major types of cancer in adults include lung cancer followed by gastric, liver, colorectal, breast and cervical cancer. In children, RB, leukemia, medulloblastoma are of major problem. RB cases are reported the second highest in India in the world. The early detection and targeted treatment is still a challenge for cancer. The current study answers and opens up new targets for targeting cancers. Targets studied earlier were used for theranostic purpose by constructing imaging conjugates and therapeutic siRNA, DNAzyme, miRNA and cancer stem cell aptamer and chimeric aptamers conjugates. RB tissue sample analysis showed Tiam1 protein elevated expression at mRNA and protein levels in the samples that showed choroid invasion more than 3mm and optic nerve invasion. Tiam1 involvement in invasion migration and metastasis of RB cell lines was studied using knockdown strategy. Functional studies using truncated constructs of Tiam1 revealed that the protein modulates actin cytoskeleton remodeling in RB. The study also revealed for the first time in RB the involvement of NCL. Targeting the NCL in RB was able to downregulate the cancer stem cell marker, G-rich mRNA and onco-miRNAs expression. In vivo effect of NCL aptamer showed active tumor reduction and reduction in the serum onco-miRNA load. We show the proof of anti-tumor effect of NCL aptamer (NCL-aptamer and LNA-NCL-aptamer) in RB by modulating the G-rich oncogenic and CSC marker mRNA, miRNA-17-92 cluster, apoptotic proteins and phosphatidylcholine (PC) expression.

Present study also utilized EpCAM protein expressed on apical surface of cancer cells for theranostic purpose using EpCAM aptamer (EpApt), wherein our results collectively revealed that chimeric aptamer siRNA, EpApt-siEp construct can potentially be used for eradicating EpCAM positive cancer cells as well as other CSCs while sparing normal EpCAM negative surrounding cells. Also the PEI nanocomplex fabricated with EpApt and siEp was able to target EpCAM tumor cells, deliver the siRNA and silence the target gene. This nanocomplex exhibited decreased cell proliferation than the scrambled aptamer loaded nanocomplex in the EpCAM expressing cancer cells. In addition to the chimeric siRNA construct, we also constructed the EpCAM aptamer-Dox conjugate that selectively deliver the drug to the RB cells there by inhibiting cellular proliferation and not to the noncancerous müller glial cells. As EpCAM is a
cancer stem cell marker, this aptamer-based targeted drug delivery will prevent the undesired
effects of non-specific drug activity and will kill cancer stem cells precisely in RB. The EpCAM
DNA aptamer fluorescent conjugate showed target specific binding and aided in imaging of
various EpCAM positive cancer cell lines like MCF7, MDAMB453, WERI-RB1 and PC3.

The study also explored the use of miRNA targeting aptamer, the anti-cancer property of Pri-apt
in RB, which can be readily tested under constitutive condition using appropriate vectors that can
further be translated for the delivery of the aptamer specifically to cancer cells. Furthermore, we
analyzed the CD44, ABCG2 and CD133 aptamers exhibited specific binding to CD133+ve RB
primary cells.
**Future perspectives**

From the current study *in vitro* characterization of many leads were done and the best two oligo conjugates were tested in *in vivo* systems using the RB and epithelial cancer xenograft model. As Tiam1 protein mediates RB cell migration, use of aptamers will have better blocking of function thereby leading to inhibition of cell invasion.

By modifying the other constructs and aptamers with nuclease stability modifications or incorporating PEG, better stability under *in vivo* condition can be achieved. The LNA modified NCL aptamer showed 65% efficacy against RB, can further be PEGylated for increased serum half life that will increase the EPR effect of the aptamer. Modifications that can covalently link the drugs such as chemotherapeutic agents will have synergistic effect. This will aid in increased activity and higher accumulation of aptamer and aptamer conjugates in the tumor tissue.

The use of miRNA aptamer for blocking the miRNA 17-92 cluster is novel and has potential application in RB therapy. The pri-miRNA aptamer can be chimerized or delivered to cancer cells specifically by conjugating aptamers or using vector systems that constitutively express the miRNA blocking aptamer to cells. This will have higher implication by incorporating such modifications. Also conjugating with transducible peptides or cell penetrating peptides will open up avenues for cell specific targeting of the miRNA aptamer.

From the current study the CSC marker aptamers were found to be effective in inhibiting the cellular acticity of primary RB tumor cells. They had higher penetrating capability and hence would be able to apply them for theranostic purposes. Also future use of the CSC marker aptamers and truncated aptamer constructs will assist in delivering therapeutic agents specifically to cancer stem cells. The understanding between the CD133 aptamer and CD133 ExD2 has revealed the possible interacting residues from the protein that can be exploited for understanding the importance of these residues in protein folding and interaction and inturn in stem cell maintenance.

Thus the study has generated informations that can be further utilized and developed as potential targeting and imaging agent against cancer and other diseases presenting with the study condition.
# List of Chemicals, Reagents and Antibodies

<table>
<thead>
<tr>
<th>S.No</th>
<th>Chemical / Reagent /Antibody/ Buffers</th>
<th>Purchased From</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4′,6-diamidino-2-phenylindole (DAPI)</td>
<td>Sigma Aldrich India</td>
</tr>
<tr>
<td>2</td>
<td>(3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) reagent</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>3</td>
<td>10X RT buffer</td>
<td>Thermoscientific, India</td>
</tr>
<tr>
<td>4</td>
<td>1M HEPES buffer pH7.0</td>
<td>HiMedia, India</td>
</tr>
<tr>
<td>5</td>
<td>3KDa cutoff amicon centrifugal filters</td>
<td>Millipore, India</td>
</tr>
<tr>
<td>6</td>
<td>3KDa cutoff dialysis membrane tubes</td>
<td>Thermoscientific, India</td>
</tr>
<tr>
<td>7</td>
<td>Acetone</td>
<td>SRL chemicals, India</td>
</tr>
<tr>
<td>8</td>
<td>Acrylamide and Bis-acrylamide</td>
<td>SRL chemicals, India</td>
</tr>
<tr>
<td>9</td>
<td>Agarose</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>10</td>
<td>Ammonium per-sulfate (APS)</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>11</td>
<td>anit-PCNA antibody</td>
<td>Santa cruz, Australia</td>
</tr>
<tr>
<td>12</td>
<td>Annexin V staining kit</td>
<td>BD bioscience, USA</td>
</tr>
<tr>
<td>13</td>
<td>Antibiotic antimycotic solution</td>
<td>Himedia, India</td>
</tr>
<tr>
<td>14</td>
<td>Anti-mouse goat horse radish peroxidise (HRP)</td>
<td>Santa cruz, USA</td>
</tr>
<tr>
<td>15</td>
<td>Anti-mouse-FITC</td>
<td>Santa cruz, USA</td>
</tr>
<tr>
<td>16</td>
<td>Anti-Rabbit- mouse horse radish peroxidise (HRP)</td>
<td>Santa cruz, USA</td>
</tr>
<tr>
<td>17</td>
<td>Anti-rabbit-FITC</td>
<td>Santa cruz, USA</td>
</tr>
<tr>
<td>18</td>
<td>Apoptotic array kit</td>
<td>R n D, Australia</td>
</tr>
<tr>
<td>19</td>
<td>Bovine serum albumin (BSA)</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td></td>
<td>Product Description</td>
<td>Supplier</td>
</tr>
<tr>
<td>---</td>
<td>---------------------------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>20</td>
<td>Bradford-Coomassie plus protein assay reagent</td>
<td>BIORAD, India</td>
</tr>
<tr>
<td>21</td>
<td>Brightstart detection kit</td>
<td>Ambion, USA</td>
</tr>
<tr>
<td>22</td>
<td>Calcium chloride</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>23</td>
<td>CD44, ABCG2 DNA aptamers</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>24</td>
<td>Chloroform</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>25</td>
<td>Chlormpromazine</td>
<td>Sigma Aldrich, Australia</td>
</tr>
<tr>
<td>26</td>
<td>Choloroform</td>
<td>SRL chemicals, India</td>
</tr>
<tr>
<td>27</td>
<td>Colchicine</td>
<td>Sigma Aldrich, Australia</td>
</tr>
<tr>
<td>28</td>
<td>Crystal violet</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>29</td>
<td>Cytokine array kit</td>
<td>R n D, Australia</td>
</tr>
<tr>
<td>30</td>
<td>DIBO AF594</td>
<td>Invitrogen, India</td>
</tr>
<tr>
<td>31</td>
<td>Diethyl pyrocarbonate</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>32</td>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>33</td>
<td>Dimethyl sulfoxide (hybridoma grade)</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>34</td>
<td>Dithiothreitol (DTT)</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>35</td>
<td>DNA ladder 1kb, 100bp, Ultralow range</td>
<td>MBI fermentas, India</td>
</tr>
<tr>
<td>36</td>
<td>DNA loading dye</td>
<td>MBI fermentas, India</td>
</tr>
<tr>
<td>37</td>
<td>DNase RNase free water</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>38</td>
<td>DNAzyme (mutant) survivin</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>39</td>
<td>Doxorubicin (Dox)</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>40</td>
<td>DTT</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>41</td>
<td>Dulbeco Modified Eagles’s Media (DMEM) high glucose</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>42</td>
<td>Dynal Magnetic Micro-beads separation kit</td>
<td>Invitrogen, India</td>
</tr>
<tr>
<td>43</td>
<td>Dynamo HS SYBR green universal mix</td>
<td>Thermoscientific, India</td>
</tr>
<tr>
<td>44</td>
<td>EpCAM DNA aptamer with azide termini</td>
<td>Integrated DNA Technologies, USA</td>
</tr>
<tr>
<td>45</td>
<td>EpCAM locked nucleic acid (LNA aptamer)</td>
<td>Exiqon, Denmark</td>
</tr>
<tr>
<td>46</td>
<td>EpCAM RNA aptamer and chimeras</td>
<td>Dharmaco, USA</td>
</tr>
<tr>
<td>47</td>
<td>EpCAM siRNA flexitubes (20nmol)</td>
<td>Qiagen, Germany</td>
</tr>
<tr>
<td>48</td>
<td>Ethanol</td>
<td>SRL chemicals, India</td>
</tr>
<tr>
<td>49</td>
<td>Ethylenediamine tetra acetic acid (EDTA)</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>50</td>
<td>Fetal bovine serum (FBS)</td>
<td>Gibco, Invitrogen, India</td>
</tr>
<tr>
<td>51</td>
<td>Formaldehyde</td>
<td>SRL chemicals, India</td>
</tr>
<tr>
<td>52</td>
<td>formamide</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>53</td>
<td>Glacial acetic acid</td>
<td>SRL chemicals, India</td>
</tr>
<tr>
<td>54</td>
<td>Glucose solution</td>
<td>sigma Aldrich, India</td>
</tr>
<tr>
<td>55</td>
<td>Glutamax solution</td>
<td>sigma Aldrich, India</td>
</tr>
<tr>
<td>56</td>
<td>Glycerol</td>
<td>SRL chemicals, India</td>
</tr>
<tr>
<td>57</td>
<td>Glycine</td>
<td>SRL chemicals, India</td>
</tr>
<tr>
<td>58</td>
<td>Haematoxylin</td>
<td>SRL chemicals, India</td>
</tr>
<tr>
<td>59</td>
<td>Immunohistochemistry kit</td>
<td>Leica systems, India</td>
</tr>
<tr>
<td>60</td>
<td>Indomethacine</td>
<td>Sigma Aldrich, Australia</td>
</tr>
<tr>
<td>61</td>
<td>Isopropyl alcohol</td>
<td>SRL chemicals, India</td>
</tr>
<tr>
<td>62</td>
<td>Isopropyl alcohol</td>
<td>SRL chemicals, India</td>
</tr>
<tr>
<td>63</td>
<td>Lactate dehydrogenase cytotoxicity kit</td>
<td>Roche, Australia</td>
</tr>
<tr>
<td>64</td>
<td>Lammeli buffer</td>
<td>Laboratory made</td>
</tr>
<tr>
<td>65</td>
<td>Lipofectamine 2000</td>
<td>Invitrogen, India</td>
</tr>
<tr>
<td></td>
<td>Product Name</td>
<td>Supplier</td>
</tr>
<tr>
<td>---</td>
<td>--------------------------------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>66</td>
<td>LNA-Ep-siRNA constructs</td>
<td>Exiqon, Denmark</td>
</tr>
<tr>
<td>67</td>
<td>Magnesium chloride</td>
<td>sigma Aldrich, India</td>
</tr>
<tr>
<td>68</td>
<td>Matrigel invasion chambers kit</td>
<td>BD bioscience, USA</td>
</tr>
<tr>
<td>69</td>
<td>Methylene blue</td>
<td>Invitrogen, India</td>
</tr>
<tr>
<td>70</td>
<td>mouse anti-CD133 monoclonal antibody</td>
<td>Miltenyi biotech, USA</td>
</tr>
<tr>
<td>71</td>
<td>mouse anti-ABCG2 polyclonal antibody</td>
<td>sigma Aldrich, India</td>
</tr>
<tr>
<td>72</td>
<td>Mouse anti-Bcl2 monoclonal antibody</td>
<td>Imgenex, USA</td>
</tr>
<tr>
<td>73</td>
<td>mouse anti-EpCAM antibody</td>
<td>Santa cruz biotech, USA</td>
</tr>
<tr>
<td>74</td>
<td>Mouse anti-EpCAM monoclonal antibody</td>
<td>Santa Cruz, USA</td>
</tr>
<tr>
<td>75</td>
<td>Mouse anti-survivin monoclonal antibody</td>
<td>Santa Cruz, USA</td>
</tr>
<tr>
<td>76</td>
<td>Mouse anti-tubulin monoclonal antibody</td>
<td>Santa Cruz, USA</td>
</tr>
<tr>
<td>77</td>
<td>Mouse-anti-beta actin monoclonal antibody</td>
<td>sigma Aldrich, India</td>
</tr>
<tr>
<td>78</td>
<td>MTT solvent</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>79</td>
<td>Nitrocelluloe (Ecl plus) membrane</td>
<td>GE Healthcare, India</td>
</tr>
<tr>
<td>80</td>
<td>Non Essential aminoacid solution (100X)</td>
<td>sigma Aldrich, India</td>
</tr>
<tr>
<td>81</td>
<td>NCLAPT</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>82</td>
<td>NCL LNA aptamer</td>
<td>Exiqon, Denmark</td>
</tr>
<tr>
<td>83</td>
<td>NCL siRNA flexitubes (20nmol)</td>
<td>Qiagen, Germany</td>
</tr>
<tr>
<td>84</td>
<td>NU-PER nuclear cytoplasmic protein extraction kit</td>
<td>Thermoscientific, India</td>
</tr>
<tr>
<td>85</td>
<td>Nylon membrane</td>
<td>Ambion, USA</td>
</tr>
<tr>
<td>86</td>
<td>Optimum cutting temperature (OCT) compound</td>
<td>Tissue-Tek, India</td>
</tr>
<tr>
<td>87</td>
<td>Paraformaldehyde</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td></td>
<td>Description</td>
<td>Supplier</td>
</tr>
<tr>
<td>---</td>
<td>-----------------------------------------------------------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>88</td>
<td>Phalloidin Fluorescein-isothyocyanate (TRITC)</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>89</td>
<td>Polyethyleneimine (60KDa)</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>90</td>
<td>Pri-apt and con-apt</td>
<td>Dharmacon, USA</td>
</tr>
<tr>
<td>91</td>
<td>Prolong gold anti-fade mountant</td>
<td>Invitrogen, India</td>
</tr>
<tr>
<td>92</td>
<td>Propidium Iodide (PI) stain</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>93</td>
<td>Protein ladder</td>
<td>Pierce, Thermo, India</td>
</tr>
<tr>
<td>94</td>
<td>Rabbit anti-CD44 monoclonal antibody</td>
<td>Abcam, USA</td>
</tr>
<tr>
<td>95</td>
<td>Rabbit anti-NCL monoclonal antibody</td>
<td>Abcam, USA</td>
</tr>
<tr>
<td>96</td>
<td>Rabbit anti-TIAM1 monoclonal antibody</td>
<td>Santa Cruz, USA</td>
</tr>
<tr>
<td>97</td>
<td>Radioimmunoprecipitation assay (RIPA) buffer</td>
<td>Laboratory made</td>
</tr>
<tr>
<td>98</td>
<td>RNase ZAP</td>
<td>Invitrogen, India</td>
</tr>
<tr>
<td>99</td>
<td>RNaseOUT</td>
<td>Invitrogen, India</td>
</tr>
<tr>
<td>100</td>
<td>Roswell park memorial institute (RPMI) media</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>101</td>
<td>Skim milk</td>
<td>HiMedia, India</td>
</tr>
<tr>
<td>102</td>
<td>Sodium Azide</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>103</td>
<td>Sodium Chloride</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>104</td>
<td>Sodium do-decyl sulphate (SDS)</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>105</td>
<td>Syringe filter low protein binding</td>
<td>Millipore, India</td>
</tr>
<tr>
<td>106</td>
<td>TaqMan mature miRNA assays</td>
<td>ABI biosystems, USA</td>
</tr>
<tr>
<td>107</td>
<td>Tiam1 siRNA (3-flexitubes 20nmol)</td>
<td>Qiagen, Germany</td>
</tr>
<tr>
<td>108</td>
<td>Tris base</td>
<td>SRL chemicals, India</td>
</tr>
<tr>
<td>109</td>
<td>Trisodium citrate</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>110</td>
<td>Triton X-100</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>No.</td>
<td>Item</td>
<td>Supplier</td>
</tr>
<tr>
<td>-----</td>
<td>-------------------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>111</td>
<td>TRIzol reagent</td>
<td>Invitrogen, India</td>
</tr>
<tr>
<td>112</td>
<td>Trypan Blue</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>113</td>
<td>Trypsin/EDTA (0.25%)</td>
<td>HiMedia, India</td>
</tr>
<tr>
<td>114</td>
<td>Tween-20</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>115</td>
<td>Ultraoligo hybridization buffer</td>
<td>Ambion, USA</td>
</tr>
<tr>
<td>116</td>
<td>Universal TaqMan master mix</td>
<td>Invitrogen, India</td>
</tr>
<tr>
<td>117</td>
<td>Versco cDNA synthesis kit</td>
<td>Thermoscientific, India</td>
</tr>
<tr>
<td>118</td>
<td>X-rays</td>
<td>Thermoscientific, India</td>
</tr>
<tr>
<td>119</td>
<td>Xtreme gene transfection 9 kit</td>
<td>Roche, India</td>
</tr>
<tr>
<td>120</td>
<td>Xtreme siRNA transfection kit</td>
<td>Roche, India</td>
</tr>
</tbody>
</table>
REFERENCES


LI, Y., BHINDI, R., DENG, Z. J., MORTON, S. W., HAMMOND, P. T. & KHACHIGIAN, L. M. 2013b. Inhibition of vein graft stenosis with a c-jun targeting DNAzyme in a cationic liposomal formulation containing 1,2-dioleoyl-3-trimethylammonium propane (DOTAP)/1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). *Int J Cardiol*, 168, 3659-64.


retinoblastomas without RB1 mutations: genomic, gene expression, and clinical studies. Lancet Oncol, 14, 327-34.


