Results and Discussion
III. 1. Designing of Ribozyme against hTR
III.1.1. Designing Ribozyme and Visualizing Secondary Structure of Human Telomerase RNA (hTR)

To find out the possible target sites for ribozyme designing, the full length human telomerase RNA was analyzed for its possible secondary structures employing an M Fold program of Michael Zuker. RNA secondary structure is formed through hydrogen bonds between complementary RNA molecules {G-C, A-U, G-U}. These interactions can bring distant complementary sections of one RNA molecule in to close proximity (Zuker 2003).

The "M Fold" was developed in late 1980s and the 'M' simply refers to multiple; the software views possible secondary structure based on minimum free energy ($\Delta G$). The prediction of RNA secondary structures by energy minimization using nearest neighbor energy parameters began with Tinoco and colleagues (1971) and later on by others (Borer et al. 1974, Tinoco et al. 1971, Tinoco et al. 1973, Ulhenbeck et al. 1973 and Delisi and Crothers 1971). The efficient RNA secondary structure algorithms using dynamic programming methods were borrowed from sequence alignment programs developed by a number of different groups (Waterman and Smith 1978, Waterman 1978, Nussinov et al. 1978, Nussinov and Jacobson 1980, Zuker and Stiegler 1981, Sankoff et al. 1983).

The early programs computed single minimum free energy foldings of RNA sequence. The modified version of this was incorporated in University of Wisconsin GCG (UWGCG) package and later on as separate GCG package. The M FOLD server not only predicts secondary structure, it also predicts the hybridization, and melting temperatures which served as separate applications.

III.1.2. Folding parameters

RNA and DNA sequences may be linear or circular; the software by default treats a given sequence as linear. Still circular nucleic acids also can be used for the same. The folding temperature is fixed at 37°C for RNA folding, but any integral temperature may be selected from 0°C to 100 °C.

Ionic conditions may be altered only for DNA, and for RNA, the ionic conditions are fixed at $[\text{Na}^+] = 1\text{M}$ and $[\text{Mg}^{++}] = 0 \text{ M}$ for folding and these are equivalent to
physiological conditions. The choice of salt could be other than Na\(^+\). So Na\(^+\) may be considered equivalent to Li\(^+\), K\(^+\) and NH\(_4^+\) while Mg\(^{2+}\) is equivalent to Ca\(^{2+}\).

Bases in plotted structure may be annotated by pNum values which represents the number of ways that a base may pair in all foldings with in \(\delta \Delta G\) from the minimum energy. Low values indicate well defined bases, and value 0 or 1 indicates that a base is single stranded or always paired to a unique partner. The number of times that a base is single stranded in a computed folding is called its SS count number (Zuker 2003).

### III.1.3. Single Stranded Regions in Muliple Foldings of hTR

To assess the target accessibility by ribozyme, target RNA was analyzed for its optimal and suboptimal secondary structures using M FOLD software package. For targeting we selected GUC sites in hTR regions, and keeping in mind that GUC should fall in single stranded region. This M Fold program generated structures differed minimally in energies for suboptimal foldings. The full length sequence of hTR is represented below.

```
1 gggttgcgga gggtgggcct gggaggggtg gtggccattt tttgtcttaac cctaactgag
61 aagggccgtag gcgcgcgtct tttgctcccc gcgcgctgtt tttctcgctg actttcagcg
121 gcgcggaaaaag cctcgccctg ccgccttcca ccgttcattc tagagcaaac aaaaatgct
181 agctgcgcgg ccgtgcgtgct ccccggggga cctgcggcgg gttgcctgcc cagcccccga
241 accecgccctg gacgagcgggg tggcccgggg gcttctccgg aggcacccac tgccaccgcg
301 aagagttggg ctctgtctgc cgcgggtctc tgggggcaga gggcgaggtt caggccctttc
361 aggccgcagg aagaggaacg gagcgagctc cgcgcgcgcgg cgccattcccc tgagctgtgg
421 gacgtgccacc caggactcgg ctcacacagt c
```

The table 3.1 below shows the GUC sequences of hTR transcript that were used for folding. We have selected a few GUC of hTR and analyzed for conserved structures of single stranded regions. The foldings generates multiple secondary structure of target which displays varying degrees of differences in folded substrates. However,
some of the sequences still show conservation of their structural components at
different energy levels. High conservation of these structures among suboptimal
secondary structures increases the possibility of existence of true single strandedness
of target. First we analyzed the SS count which determines the frequency of certain
bases being single stranded at multiple foldings.

<table>
<thead>
<tr>
<th></th>
<th>Single Stranded Sequence</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37'-5' CCATTT TTT GTC TAAC CCTAA-3'</td>
<td>55</td>
</tr>
<tr>
<td>2</td>
<td>171-5' CAAAAAAT GTC AGCTGCT -3'</td>
<td>-187</td>
</tr>
<tr>
<td>3</td>
<td>215-5' CGGCGG GTC GCCTGCC-3'</td>
<td>-230</td>
</tr>
<tr>
<td>4</td>
<td>307-5' TTGGGCTCT GTC AGCCGC-3'</td>
<td>-323</td>
</tr>
<tr>
<td>5</td>
<td>318-5' GCCGCGG GTC TC TCGGG-3'</td>
<td>335</td>
</tr>
<tr>
<td>6</td>
<td>381-5' GAGCGA GTC CCCGCGC-3'</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1: The Presence of GUC in full length sequence of hTR shown in table for secondary structure analysis for its possible sub optimal structures.

The present analysis performed 21 folding at different energy levels and represented
the frequency of single stranded sequence at multiple foldings. The SS count results
of hTR shows the bases after 32 till 62nd base and 360-400 base to have wider window
of single strandedness. We chose the GUC sequence at 44th and 180th nucleotide with
in a single stranded region for designing ribozyme.

The structures were analyzed with truncated form of sequence in which 225 base
sequence were analyzed for single stranded regions (Fig 1A). The result shows the
GUC bases at 44 and 180th position falls 16 times as single stranded, and same is
repeated with full length hTR sequence of 451 bases (Fig 1B). In such a long substrate
RNA, the position of 44 and 180 shows same frequency of single strandedness. Other
structures found to be in single stranded regions were at the bases of 360-400th base
regions. One of GUC falls in such region may also fold in to unpaired regions, and
this window has sequence stretches with highest probability of assuming single
stranded forms. Further the structures created out of multiple foldings were viewed as
plots for conserved secondary structures.
Fig 3.01: Analysis of single strandedness of bases and its frequency in multiple folding of A) hTR 225 bases and B) 451 full length of hTR. The GUC at 180th position falls 16 times as single stranded structure. Bars below the base numbers are the stretch of window having single stranded sequence (40-60, 150-200, 350-400).
III.1.4. RNA Fold Finds Conserved Secondary Structures of hTR

Some of the selected GUC's seem to be conserved at structural level and the one finally chosen was 180th position of GUC. Out of 21 structures more than 7 structures were found to have (Fig 3.02-13) conserved structures at different energy levels.

The foldings from Fig 3.02-3.13 show alternative folding in which the selected GUC at 180th position was found to be in non base paired region except at 4 incidences out of 21 foldings and also found to have similar structures at different energy levels. The foldings of subset sequences show the sequence with conserved secondary structures (Fig 3.14: A, B, E, F) and dynamic nature of 180th sequence being single stranded (fig 3.14: G, I, J) for its full access of ribozyme bindings.

The selected GUC at 180th nucleotide was further folded after appending the designed ribozyme, and as expected the ribozyme finds its target by base pairing with flanking arms of GUCs of 180th position (Fig 3.16 and 3.17). Such designed ribozymes further has to be tested for invitro cleavage activity.

III.1.5. Folding of Ribozyme Appended with hTR Sequence

Once the target sequence has been folded and analyzed for conserved secondary structures, it is necessary to fold along with ribozyme sequence designed to check the ribozyme binding with its designated target sequence. The appended ribozyme has to find its target and bind the flanking arms of target GUC sites sequence. The result shown in the folding pattern reveals that the ribozyme at a few energy levels assumes its structure and binds the target site sequence specifically. The target sites in Fig 3.15 shows the sequences of hTR at 180 nt and Fig 3.16 and 3.17 show the ribozyme binding with target site sequence specifically.
Fig 3.02: Folding of full length hTR showing the different structures including bulge, loops, internal loops. The presence of GUC in loops are shown by arrow which may serve as target.
Fig 3.03: Folding of hTR showing bulges at reducing folding energy levels. Multiple folds are to compare the conserved structures of possible target sequences. Targets are shown by arrows.
Fig 3.04: folding of hTR at ΔG of -208.88 kcal/mole for comparing the conserved secondary structures.
Fig 3.05: Folding of hTR at $\Delta G = -209.91 \text{ k cal mol}^{-1}$ the predicted target site forms base pairing at this energy levels. But the flanking sequences are found to be single stranded.
Fig 3.06: Folding of hTR at $\Delta G = -208.44$ kcal mol$^{-1}$ shows the conserved structures of non base paired region of GUC at 180 the position shown with arrows.
Fig 3.07: Folding of hTR at $\Delta G = -210.94$ kcal mol$^{-1}$ which shows the accessibility of GUC at 180th position shown with arrows.
Fig 3.08: Folding of hTR at $\Delta G = -207.58$ kcal mol$^{-1}$, showing the presence of selected GUC at stem but in non base paired bulge region
Fig 3.09: Folding of hTR at $\Delta G = -210.80$ kcal mol$^{-1}$ shows the conserved single strandedness of GUC though present in stem of the loop structures
Fig 3.10: Folding of hTR at ΔG = -210.77 k cal mol⁻¹ showing conserved 180th position of GUC
Fig 3.11: Folding of hTR at $\Delta G = -205$ kcal mol$^{-1}$ showing selected GUC site at single stranded regions shown by arrow.
Fig 3.12: Folding of hTR at $\Delta G = -202$ kcal mol$^{-1}$ showing the conserved single strandedness of GUC at 180$^\text{th}$ position shown by arrow.
Fig 3.13: hTR folding at $\Delta G = -203$ kcal mol$^{-1}$ showing the single stranded ness of 180$^{th}$ nucleotide shown by arrow.
Fig 3.14: Conserved structure of hTR with respect to GUC at 180th position and its single stranded form in most of the foldings (A-J).
Fig 3.15: Folding of hTR and 180th position shown clearly as conserved single stranded internal bulge sequence.
Fig 3.16: Folding of hTR appended with designed ribozyme finding its target at 180th nucleotide sequence specifically, highlighted in box and shown with arrows.
Fig 3.17: Folding of hTR appended with designed ribozyme targeting 180th nucleotide showing its sequence specific target binding.
III. 1. 6. Discussion

Folding the hTR sequence at varied energy levels shows a few conserved single stranded regions. The sequence around 40-52\textsuperscript{nd} nt nucleotides showed highest possible single stranded region in the sequence. At 52\textsuperscript{nd} nt it shows the highest probable single stranded ness, but absence of GUC at these region prevents its use as target. The structure of bases at or around 180\textsuperscript{th} GUC nucleotides found to be conserved throughout multiple foldings.

In the present study we concentrated on targeting the catalytically relevant regions of hTR in order to inactivate telomerase. However, it will be interesting to target other regions of hTR (nt 44, 380) functioning as template, signal sequence respectively.

Though the target sequence at 44\textsuperscript{th} nt also seems to be good target, still it lacks conservation of structures at different energy levels, which prompted to take 180\textsuperscript{th} rather than 44\textsuperscript{th} nt as target sequence. The sequence at 3' end of the hTR is omitted since these sequences participate as signal sequence for localization of hTR rather than catalytically important. Studies have proved that the truncated version of hTR (32-224) can very well act as template and is essential and sufficient for catalytically active telomerase complex \textit{in vitro}. The pseudoknot sequence also has a role in dimer formation of hTR and the region 180 is found to be highly dynamic in nature and could serve as a better target for ribozyme targeting. The sequence of ribozyme appended hTR showed sequence specific targeting and could be manipulated for further analysis \textit{in vitro} and \textit{in vivo}.

Further folding of hTR appended with ribozyme shows the specificity at different energy levels though it also fails to identify its target sequences at few energy levels. The folding at Fig 3.16 and 3.17 confirms the sequence specificity of designed ribozyme against the 180\textsuperscript{th} nt of hTR target sequence. The sequence of hTR at 180\textsuperscript{th} 5'-GUC-3', the ribozyme pairing and cleavage sites are shown with arrow.
III. 2. Cloning of Ribozyme and hTR
III.2.1. Cloning of Ribozyme under T7 Promoter (pStuI vector)

To assess designed ribozyme, it has to be verified \textit{in vitro} for its cleavage efficiency. To make ribozyme transcripts \textit{in vitro}, the ribozyme- coding oligos were cloned under T7 or T3 promoter. To minimize the vector sequences co transcribed along with ribozyme, a modified bluescript vector with StuI restriction site overlapping with the transcription intiation nucleotide with reference to T 7 promoter was used (Yadava and Yadava 2000). The StuI digestion creates blunt ends for cloning of ribozyme coding sequences for creation of precise ribozyme transcripts \textit{in- vitro} which would be free from vector sequences. As an alternative, ribozyme coding oligos with T7 promoter were used for the same purpose.

III.2.2.Ribozyme Cloning and Sequencing

For cloning, the ribozyme coding oligos were end labeled, annealed and purified from non denaturing polyacrylamide gel following electrophoresis. The oligos contain degenerate base at conserved catalytic center of ribozyme (A/C) to clone wild type and mutant ribozyme (Fig 3.18, 19). The clones were further screened by digesting with Pvu II enzyme which creates 400 bp fragment in original constructs without insert and with insert it creates 440 bp (Fig 3.20A, B). In Fig 3.20A, lane 1 and 3 shows the presence of insert i.e., 440bp and lane 2 shows the absence of insert. In Fig 3.20B, lane 3 shows the presence of insert and other lanes represents clones without insert. The digested clones that show the differences in mobility when running in gel were considered for further confirmation with sequencing. The sequencing of positive clones shows the conserved sequences of ribozyme and mutant ribozyme (Fig 3.21).
Fig 3.18: Ribozyme cloning in pStuI vector shown schematically. The StuI site is used for cloning the duplexed oligos corresponding to sense and antisense sequence of the ribozyme with conserved catalytic sequence shown in red and blue colors respectively. M* is an ‘A’ in wild type and a ‘C’ in mutant ribozyme.
Fig 3.19: Electrophoresis of + and - strand oligonucleotide and their duplex through non-denaturing PAGE gel. Lanes 1 and 3 are sense and anti sense respectively and Lane 2 is annealed oligo showing shift compared to unannealed single strand oligos. The part of annealed oligo was eluted from gel for cloning purposes (15% non denaturing acrylamide gel).
Fig 3.20:  A) Representative picture of Screening patterns upon digestion with PvulI for presence of insert. Presence of insert has shown in lanes 1 and 3. B) Lane 3 shows larger Pvu II fragment than the vector and was verified by sequencing.
Fig 3.21: Sequencing complementary strands of ribozyme clones shown positive from restriction digestions. A) Sequencing of active ribozyme from T3 promoter site, showing the 5' GTTTC 3' (corresponding to 5'GAAAC 3' in the wild type ribozyme) and B) Sequencing of clone shows 5' GGTTC3' (corresponding to 5'- GAACC-3' in the mutant ribozyme).
III.2.3. Cloning of Human Telomerase RNA Component

The full length cDNA for human telomerase RNA component was prepared by using RNA from HeLa cells, amplified with telomerase RNA specific primers. The cDNA samples were further reamplified with modified temperature regime to avoid the co-migrants, which were found in cDNA samples (Fig 3.22A, B). The PCR products were purified and cloned in pGEM- T vector which contains T7 and SP6 promoter flanking the cloning site (Fig 3.23).

The hTR clones were screened by sequencing and confirmed with NCBI’s BLAST to confirm the sequences (Fig 3.24). The hTR was found to be cloned in reverse orientation with respect to T7 promoter, and sense strand with SP6 promoter. Sense strand transcripts of hTR for ribozyme cleavage assays, could be generated by SP6 RNA polymerase.

III. 2. 4. In vitro Cleavage by the Designed Ribozyme

To assess the designed ribozyme’s catalytic efficiency, in vitro cleavage assay were performed. The Constructs bearing ribozyme inserts were linearized and used for generating transcripts labeled with α32P UTPs. For monitoring cleavage products, generally the target sequences were labeled and ribozymes unlabeled. The transcripts were purified by G-50 column and quantitated in terms of specific radioactivity.

The equimolar amounts of transcript or 2 fold to 10 fold ribozymes (100 f mole target and 1000 fmol of Rz) were used to test the cleavage. The transcript of ribozyme and target sequences were mixed and denatured shortly and made to anneal slowly at 37°C. Addition of 10 mM MgCl2 provides catalytic efficiency to ribozyme and cleavage reaction is started at this point. The cleavage reaction was set for 3 hours. Fig 3.28 shows the truncated version of target RNA created with T7 appended primer and transcribed and cleavage reaction set with active Rz and mutant Rz. The mutant ribozyme serves as control. The cleavage of truncated hTR of 85 base produces 69 and 16 base RNA fragments confirming the cleavage activity of the ribozyme (Fig: 3.25). The present ribozyme 180 (Rz180) was taken for study because ribozyme against the template region of telomerase RNA has been found to be effective (Yokayama et al. 1998) in cell culture studies and already proven.
Fig 3.22: A) Typical pattern of RT-PCR amplicons of hTR showing co amplification of unprocessed transcript. B) Re amplification of cDNA with modified annealing temperatures. The reamplified products were used for cloning in pGEM-T vector.
Fig 3.23: Cloning of Full length Human Telomerase RNA component (hTR). The gel picture shows the RT PCR product used for cloning.
Fig 3.24: Sequencing of Cloned full length human telomerase RNA, using T7 primer. In this particular clone, hTR cDNA is cloned in reverse orientation with respect to T7 promoter.
Fig 3.25: Ribozyme mediated cleavage carried out with PCR generated truncated transcript of 85 base having target site, tested with active and mutant ribozyme. The undried denaturing PAGE gel showing the cleavage products of 69 and 16 bases as expected.
III.2.5. Cloning of Ribozyme in Mammalian Expression Vector

The pCI Neo contains mainly CMV promoter followed by intronic sequence to increase the stability of RNAs expressed from its cassettes. Down stream of introns have multiple cloning sites flanked by T7 and T3 promoters. The transcripts that are expressed form this construct will bear T7 sense and T3 sequence. The presence of T7 and T3 offers possibility of in vitro transcription and PCR amplification. The pCI-Neo has another transcriptional unit bearing SV-40 promoter containing neomycin phospho-transferase gene which confers the resistance against drugs for selection purposes. The continual exposure of cells to neomycin or G 418 selects the clones that got the vector stably integrated in to genomic DNA. The pCI Neo integrates in to genomic DNA of Cells during replication process and favors stable expression of cloned genes.

III.2.6. Cloning and Screening of Ribozyme

For cloning of Ribozyme pCI Neo was digested with Smal and annealed oligos were cloned by blunt end ligation at Smal site. The schematic diagram as shown in Fig 3.26 depicts the method used to clone ribozyme oligos in mammalian expression vector. The ribozyme bearing clones were screened by colony blotting. After cloning the vectors were transformed and cells were plated on agar. The membrane was cut to the size of plate and placed in plate to take the imprint of colonies and processed and probed with end labeled ribozyme-coding oligos (Fig 3.27).

The autoradiograph was superimposed on plates to identify positive clones for further screening. The clones, which were positive for inserts, were randomly taken for sequencing to confirm their orientation with respect to CMV promoter and screening of active and mutant ribozyme. The sequencing result confirms the integrity of ribozyme bearing clone as well as orientation with respect to CMV promoter system (Fig 3.28).
Fig 3.26: Cloning of Ribozyme Coding Oligos in pCI Neo mammalian expression vector. Immediately after CMV promoter there is intronic sequence which is followed by MCS as shown above (M in sequence denotes A or C nucleotide and K denotes T or G).
Fig 3.27: Colony blotting showing the positive colonies having Rz inserts, used for further confirmation by sequencing.
A) Active Rz

B) Mutant Rz

Fig 3.28: Sequencing of Rz positive clones identified from colony blotting A) Sequence of Active ribozyme B) Sequence of Mutant Ribozyme. Arrows shows the base 5'-GAAAC-3' for active Rz and as 5'-GAACC-3' for mutant Ribozyme, cloned in pCI -Neo mammalian expression vector.
III.2.7. Discussion

The cloning of ribozyme involves purification of annealed oligos to minimize or avoid single stranded, un-annealed oligo from interfering with cloning procedures. Once the annealed products were purified from gel with mild treatments it could be used directly for cloning purpose. The usage of pStu-I vector serves for transcribing only the catalytic RNA, as conventional vector system gives additional vector sequences in transcript, which may interfere with ribozyme’s kinetic efficiency. To avoid the vector derived sequences, pStu-I or T7 appended ribozyme sequences were used for *in vitro* transcription purposes. This vector also offers a good system for screening for inserts using restriction enzyme digestion patterns. The relatively low frequency of positive clones may be due to the secondary structure assumed by the ribozyme-coding oligonucleotides and the blunt end ligation used for cloning them.

The cloning of full-length hTR was carried out following RT-PCR with hTR specific primers. The designed primer falls upstream of template sequence of hTR and the amplicons consist of 440 bp out of 451 bp of full length hTR cDNA. We used stringent temperature regimens for PCR in order to avoid any non specific amplicons or any unprocessed transcripts. The hTR RNA can be transcribed in vitro using SP6 RNA polymerase from pGEM-T-hTR.

*In vitro* ribozyme cleavage assay was assessed by mixing the *in vitro* transcribed ribozyme and target hTR with appropriate cations (MgCl₂) and followed by (12% denaturing PAGE) gel electrophoresis. The cleavage of 85 base truncated version of target RNA (having the desired GUC sequence) theoretically should produce 69 and 16 base and same is confirmed from gel electrophoresis. After electrophoresis the denaturing gel was directly exposed for autoradiography as higher percentage of gels tend to be brittle upon drying. The ribozyme employed in our study though less efficient compared to the ribozyme against template region of hTR, still it is catalytically active *in vitro*.

Cloning of ribozyme under CMV promoter ensures high level of expression in the introduced cellular environment. The CMV promoter offers greater stability in terms of half life of the transcribed RNA under its promoter system. It may be mentioned that CMV based vector system is not specially designed for RNA expression, as it was mainly developed for protein expressions. Still CMV proved to be effective in
ribozyme studies. The neomycin transferase gene serves as a good selection marker in mammalian system.

Using colony-blotting approach allows numerous parallel clones to be screened. The pCI-Neo contains T7-T3 promoter containing multiple cloning site, which is 35 base pair sequence. Introduction of Rz oligo further increase to 75 base sequences and selecting the restriction site farthest of these sites doesn’t resolve the fragment unambiguously on agarose gels. As an alternative, colony blotting approach was carried out to screen the presence of inserts and to save the clones that are positive for inserts. Sequencing results further confirms the orientation of inserts with respect to promoter.
III. 3. Expression of Ribozyme and its Effect on Cells
III.3.1. Transfection, Selection and Ribozyme Expression in HeLa Cells

To assess the ribozyme's efficiency in vivo, the constructs representing vector alone, active ribozyme and mutant ribozyme were transfected in HeLa cells and selected with antibiotic G418 (400μg/ml), for 21 days. Cells surviving prolonged selection were propagated and analyzed for expression studies. The schematic of method carried out is shown in Fig 3.29.

Expression of transfected ribozyme was analyzed by RT-PCR coupled slot blot representing cells at varied time points post transfection. RNA from the transfected cells was reverse transcribed with T3 primer and amplified with T7-T3 primers, which is a component of expressed transcripts. The amplification was carried out at different PCR cycles to determine the level of expression as well as to avoid PCR cycle limited threshold parameters (Fig 3.30A). Slot blotting was carried out with control oligos and the PCR products confirmed that sequences are ribozyme specific (Fig 3.30B). The same PCR product was sequenced to confirm further the integrity of expressed ribozyme sequences (Fig 3.31). The sequencing reaction confirmed the integrity of expressed ribozyme sequences, importantly the conserved catalytic core of ribozyme shown with arrows and red bars above the sequence (Fig 3.31).

III.3.2. Analysis of cellular phenotype and cell cycle

Morphology and cell cycle related events of cells at different time points after transfection were recorded. The cells after transfection seemed to be under stress from 4th day onwards as the cells were exposed to high concentration of drug after 48 hrs of transfection. The cells had high granularity and abnormal shapes (Fig 3.32A, B). Most of the cells return to normal morphology after 15-17 days and still few cells were under stress. The selection continued for 21 days, by the time the surviving resistant cells proliferated as colonies. These polyclonal populations of selected cells were maintained for 120 days as stable transformants. Abnormal shapes such as rounded outline, granularity in the nuclei and vacuoles characterized the cells during selection. The selected cells become normal and properly flattened with normal cellular morphology.
Interestingly subset of the ribozyme transfected cells were showing elongated cellular phenotype from 60th day post selection, but very few such cells were observed in mutant ribozyme expressed cells (Fig 3.32.C). The elongated cells resemble the dendritic cells in morphology. Such transitions may reflect on induced terminal differentiation programmes in the cells.

In parallel the cells were examined with cell cycle event and presence of any apoptotic events by FACS. The cells were stained with propidium iodide and analyzed for DNA content and granularity. The forward and side scattering of general module was employed in FACS. The cells were initially gated from forward and side scattering to exclude the large cells or doublets of aggregated cells or cell debris. Fig 3.33A, B shows the gating of cells of same size and increasing DNA content. Those gated cells were further analyzed for DNA content and presence of apoptotic signatures. Generally alcohol extracts the apoptotic DNA, a small hypo diploid peak appears on as sub G1 peak in flow cytometry, indicating apoptosis.

In terms of DNA content and cell cycle events, there was no variation immediately following transfection. Cell cycle events were similar in cells transfected with wild type or mutant ribozyme transfected cells (Fig 3.34). The numerical representation also shows the uniformity of cellular phases and events which is indicative of undisturbed cell cycle events in ribozyme transfected cells.
Drug selection using G418

Transfect DNA into cells

24-48h at 37°C

Select stable transformants by adding
G418 over prolonged period of time (21 days)

All cells are having stably transfected
ribozyme construct

Fig 3.29: Schematic representation of methods carried out for assaying ribozyme expression in tissue culture systems.
Fig 3.30: A) Individual RT-PCR reactions using T7-T3 primers of RNAs expressed from cells transfected with Active ribozyme and mutant ribozyme constructs at different PCR Cycles. B) The PCR Products were further Slot blotted and probed with ribozyme coding oligos, showing the expression of ribozyme slot at 30th cycle of row C is only loaded with Rz Oligo and other slots at 35th, 40th cycle of C were left unloaded.
Fig 3.31: Sequencing of RTPCR products showing the conserved sequences of active ribozyme expressed from transfected cells. The sequence shown with red bar is ribozyme and arrow to show the conserved 5'-GAAAC-3' which is characteristic of active ribozyme sequences.
Fig 3.32 A: Phase Contrast Images showing the phenotype of cells transfected with constructs carrying vector alone, active ribozyme and mutant ribozyme. Cells were at stress for first 5 days. The images were obtained at 4th day after transfection. Fig 3.32. B) Images were taken at 8th day after transfection (Images at 400x).
Fig 3.32 C: Comparison of Cellular phenotype transfected with vector, active ribozyme and mutant ribozyme carrying constructs. Active Rz expressing cells appeared as elongated cells and very few cell also observed as elongated in mutant Rz expressing cells. Vector alone transfected cells appear as normal in phenotype. Images were taken at 69\textsuperscript{th} day after transfection.
Fig 3.33A: Gating of cells by forward and side scattering of active Rz transfected cells. The lower panel shows gating of 2N and 4N cells and excluding the doublets and debris for analysis.
Fig 3.33B: Forward and side scattering of cells transfected with mutant ribozyme. The lower panel shows gating of cells (green color) and excluding enlarged cells.
A) Active Rz

![Histogram showing population of cells transfected with A) Active ribozyme.](image1)

B) Mutant Rz

![Histogram showing population of cells transfected with B) Mutant ribozyme.](image2)

<table>
<thead>
<tr>
<th>Transfection</th>
<th>Sub G1(M1)</th>
<th>G1/G0 (M2)</th>
<th>S phase(M3)</th>
<th>G2/M (M4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active Rz</td>
<td>2.87</td>
<td>34.95</td>
<td>6.33</td>
<td>21.47</td>
</tr>
<tr>
<td>Mutant Rz</td>
<td>2.64</td>
<td>38.93</td>
<td>5.04</td>
<td>20.91</td>
</tr>
</tbody>
</table>

Fig 3.34: Histogram showing population of cells transfected with A) Active ribozyme B) Mutant ribozyme. Table in the bottom represents fraction of cells in different phases of cell cycle.
III.3.3. Reduction of Telomerase RNA level in Ribozyme Expressing Cells.

To assess the effect of ribozyme on hTR levels, we carried out northern blotting and semi-quantitative RT-PCR in transfected cells. In general, hTR is 451 bases long and associated with telomerase catalytic component. Free hTR is also found in cells as a varied length product which is expected as unprocessed transcripts. Collins et al (2006) reported that there must be telomerase RNA longer than previously defined length as unprocessed transcripts.

Northern blots of RNA from HeLa cells show reduction of hTR signal in ribozyme transfected cells (Fig 3.35). The observation of additional signals over the expected length may be attributed to unprocessed transcripts which are not confirmed in our study.

Semi quantitative RT-PCR was carried out to assess the level of hTR in ribozyme transfected cells. The RT-PCR carried out as two steps, once cDNA is made, an aliquot of cDNA was used for amplification purpose. The PCR products were internally labeled with α-32P label to monitor the signal intensity which is proportionate to amount of amplified transcripts. The results show that amplified products could be seen from 20th cycle onward in vector and mutant transfected cells (Fig 3.36A), and absent in ribozyme transfected cells. The products start appearing from 25th cycle onward in ribozyme transfected cells, and those levels are not comparable to the products in 20th cycle of vector and mutant ribozyme transfected cells, confirming the reduction of telomerase RNA in ribozyme transfected cells. The same results represented as semi logarithmic plot (Fig. 3.36B) and showing reduction of hTR by an order of magnitude.
Fig 3.35: Northern Blotting of hTR, probed with antisense truncated hTR sequence. Boxed area shows the actual expected hTR, but appearance of higher molecular weight bands may be co migrants or unprocessed hTR.
Fig 3.36: A) Semi quantitative radiolabeled RT-PCR at different cycle from RNAs derived from vector alone and active ribozyme transfected and mutant vector transfected cells. B) Semi logarithmic representation of hTR levels from the densitometric analysis (Fuji Phosphor imager) showing reduction of hTR levels over an order of magnitude in cells transfected with active ribozyme (---) that those transfected with vector alone (-----) and mutant ribozyme (-----).
III.3.4. Reduction of Telomerase Activity in Ribozyme Expressing Cells

The reduction in hTR should reflect in telomerase activity. To assess ribozyme efficiency in telomerase inhibition, extracts from transfected constructs were analyzed with TRAP assay. The samples from different time points post transfection were analyzed for telomerase activity. Telomerase extended products were found inspite of very feeble reduction of signal intensity in ladders compared to vector alone transfected cellular extracts (Fig. 3.37A). TRAP assay with 100ng extract showed visible reduction of telomerase activity in cells transfected with active ribozyme when compared to controls (Fig 3.37B). Next, serially diluted extract (100-300 ng) from all three cell lines were compared. The results show reduction or absence of products in cells transfected with active ribozyme (Fig 3.38, lane 4,7) as compared to those transfected with vector and mutant ribozyme at 100ng concentration (Fig 3.38, lane 3,6 and 5,8 respectively). But telomerase extended products started appearing in ribozyme transfected (lane 10) cells compared to vector and mutant transfected cells (Fig 3.38, lane 11) at higher concentration of protein extracts. This suggests partial lowering of telomerase activity.

III.3.5. Reduction of Telomere Length in Ribozyme Expressing Cells

Telomere length in transfected cells was visualized by southern blotting. The DNA from transfected cells was digested with Hinf I and Rsal and probed with end labeled telomere specific probes. There is reduction of telomere length in ribozyme transfected cells (Fig 3.39, lane 3) compared to vector (lane 2) and mutant ribozyme (lane 4). The reduction in telomere length is not different in cells transfected with vector or mutant ribozyme.
Fig 3.37: TRAP assay showing telomerase activity in cells transfected with vector, active ribozyme and mutant ribozyme employing 100ng or 300ng of protein equivalent in cell extract. A) TRAP assay for 60 days old culture, discernible reduction in telomerase activity in ribozyme transfected when compared with vector only at 300ng concentration. B) TRAP assay at 100ng extract showing reduction in ribozyme (Rz) transfected cells compared to proteins from vector and mutant ribozyme transfected cells.
Fig 3.38: Serial dilution of proteins from vector, ribozyme and mutant ribozyme transfected cells. At lower concentration of protein (100ng at lanes 3-5 and 200ng at lanes 6-8), reduction in telomerase activity observed in Rz transfected (lane 4 and 7) compared with vector (Lane 3, 6) and mutant ribozyme (lane 5, 8). TRAP assay with increasing concentration (300ng lanes 9,10, 11) of protein reveal, appearance of telomerase extended product in ribozyme expressing cells also (Lane 10) compared with vector and mutant Rz (Lane 9, 11).
Fig 3.39: Terminal restriction fragment analysis (TRF) in vector (lane 2), active ribozyme (lane 3), and mutant ribozyme (lane 4) transfected cells. Lane 1 is DNA from un-transfected cells as a control. Attrition of telomere length observed in ribozyme transfected cells (the maximum signal intensity shown with dents).
III. 3. 6. Discussion

For in vivo expression HeLa cells were used as it was earlier shown to have higher telomerase activity as well as it harbors human papilloma virus type 18 which is associated with inhibition of p53 and pRB (Horner et al 2004). This cell line also offers indirectly the p53-/- status and induced status of telomerase expression due to HPV E6, and E7 genes. These are considered as main oncogenes which deregulate most of the cell cycle control events directly or indirectly. It also facilitates the oncogenic status of cells and continued expression of telomerase. Introduction of p53 in HPV infected cells could inhibit the telomerase expression (Xu et al 2000, Kanaya et al 2000).

The method adapted to analyze ribozyme expression was by using T7-T3 promoter primers. These promoter sequences are phage specific and absent in mammalian cells. The transcripts from CMV promoter have T7 and T3 phage promoter sequence flanking the ribozyme. The CMV promoter of mammalian expression vector (pCI-Neo) is followed by intronic sequence, which confers the stability of transcripts along with multiple cloning sites having termination sequence (for transcripts) that ends at the 3' into the T3 promoter sequences. The vector also contains neomycin phosphor transferase (neo) genes which can metabolize the G 418 drug used for selection purposes. The RNA used for RT-PCR were predigested with DNase and purified with phenol choloroform. The amplification was separately carried out as the expression level may be low or high and if it is very low it may need increased amplification cycle for detectable PCR products. From our studies we could visualize RTPCR products at 30 cycles onwards and carried out till 40th cycle. The PCR product is about 105 bases in length and same is confirmed by hybridizing the slot blots with ribozyme specific probe.

The sequencing of RT-PCR products further confirms the expressed sequence is active ribozyme and the sequences are conserved in vivo. The cells were maintained with periodic drug selection and expressions were analyzed at different time points after transfections.

The elongated cellular phenotype is characteristic of differentiation event in certain cells, and it is expected that the cells could be induced for terminal differentiation which may be telomerase dependent or independent. The cancer cells have uncontrollable proliferative capacity due to maintenance of telomeres. One way to
control its proliferation is to induce the differentiation programmes. The notion of treating tumor malignancies by forcing them to complete terminal differentiation was first suggested by Pierce (1961). The cancer cells are mostly differentiated cells but not terminally differentiated cells. They are metabolically active quiescent cells and many of therapeutic strategies opt for inducing terminal differentiation in different cancers including breast and colon cancer (Mueller et al. 1998, Nojiri et al. 1999). From our study the phenotype observed was similar to dendritic cells and studies with antisense and ribozyme against telomerase proved that the cells attain elongated phenotype and undergo differentiation programme (Kondo et al. 1999, Bagheri et al. 2007).

Northern blotting shows lowering of telomerase RNA in ribozyme transfected cells. Northern blot also reveals appearance of signal above the expected size limits, which may be due to unprocessed transcripts and to less extent due to non specificity, as hybridizations and washings were carried out under stringent conditions. The semi quantitative RT PCR product reveal the appearance of hTR from 16th cycle onward from vector and mutant ribozyme transfected cells while in ribozyme transfected cells those amplicons could be seen after a prolonged PCR.

Telomerase activity is assessed by standard enzymatic assay in which extracts were incubated with telomere-specific oligos. The efficiency of telomerase enzyme is monitored by the amount of product it generated in vitro. Telomerase extends the telomeric primer and further amplified with forward and reverse primer by PCR. The extended products are measured by its signal intensity with that of control 36 base ladder signal intensity. Arbitrarily, the reduction of telomerase activity can be assessed visually by absence or reduction of telomerase extended bands. Telomerase activity was diminished in ribozyme transfected cells at minimal concentration of protein extracts (3.37B). But when higher concentration of proteins was used, telomerase extended products start appearing (Fig 3.37A, 3.38 lanes 9, 10, 11). This indicates the partial inhibition of telomerase RNA and the residual hTR may be enough to carry out the telomerase function. At higher protein concentrations (300ng), the products seem to be reduced in vector transfected cell (Fig 3.38, lane 9). This is attributed to inhibitory activities of cellular debris or other inhibitors present in extracts.

Southern blotting reveals discernible reduction in telomere length in ribozyme transfected cells. The cells were maintained as stable transformants for 4 months and
observable reduction in telomere length needs prolonged maintenance of cultures. The present blot from our study was carried out at 45th day; it may need DNA from cells which are maintained for prolonged period of time in vitro. In TRF analysis, the signal intensity is maximal at a point which is used as reference for length analysis. Appearance of a streak of bands is a measure of telomere length. The southern blotting has a limitation that it cannot differentiate the length difference if not more than 1000 bases. The reduction of telomere length was not to the expected level which is also evident from partial inhibition of telomerase in our study. Yet some telomerase molecules are present which maintain telomere length, though there is reduction in telomerase and telomere length in ribozyme transfected cells. At this stage we do not rule out activation of alternative mechanisms of telomere maintenance in the ribozyme transfected cells.
III.4. Transcription Profiling of Transfectants
Ribozyme mediated effects on telomerase RNA, telomeric DNA and on telomerase activity were observed in transfected cells. Observation of changes in cellular morphology prompted us to compare the global gene expression profiling of transfected cells. Global gene expression analysis could be analyzed by subtractive cDNA hybridization, differential display PCR (Liang and Pardee 1992), serial analysis of gene expression (Velculescu et al. 1995) and by micro array. Though each technique has its own advantages and disadvantages, we opted for differential display technique considering the possibility of observance of novel genes and requirement of minimal amount of starting RNA material and ease of handling. Differential display technique is basically based on usage of "defined arbitrary primers" with the cDNA created with oligo dT producing arbitrary length products. We performed differential display PCR (DD-PCR) to analyze the differential expression pattern among the stable transformants and the molecular alterations upon ribozyme expressions. The differential display PCR was done in two batches corresponding to 30th day and 45th day post transfections. The schematic of the method carried out is presented in Fig 3.40.

III. 4. 1. Analysis for Differentially Expressed Genes

We examined the role of telomerase in modulating gene expression patterns which may in turn result in phenotypic changes observed. We explored the possibility of change in global gene expression pattern.

Aliquots of total RNA from cells transfected with vector, active ribozyme and mutant ribozyme were predigested with DNase, further purified and quantified. Equal amount (0.2μg) of RNA was taken from transfected cells and reverse transcribed with oligo dT primer terminating into G or C or A at the 3’ end. Each sample has 3 RT-PCR products and was amplified with 8 different defined forward primers. The products of all three sample amplified with primers were compared by electrophoresis and the products showing differential intensity were considered for further analysis.

The amplicons in Figs 3.41A (and 3.41 B), in lanes G-9, G-12 and 13 show differences in expression pattern (bands within boxes in lanes 9, 12, 13). Specifically transfectants with active ribozyme show signs of down regulation of some of the transcripts. Amplicons of oligo dT primers ending in A and C from 30 day old stable transfectants are not shown in the figure. The second batch DD-PCR was performed.
with 45 day old stable cell transfectants of vector, active and mutant ribozyme constructs (Figs 3.42, 43). The amplicons appearing in DDPCR of 45 days old transfectants differed from the amplicons of 30 day old transfectants. The boxed bands represent the differentially displayed products. A10, 12, 16 primer pairs show the differentially displayed products in Fig 3.42, and C-9, 11, 13, 15 of Fig 3.43. The differentially displayed bands were eluted and reamplified with same primers and either cloned or directly used for sequencing. Re-amplification of DNA from these bands yielded 150-220 bp amplicons (Fig 3.44).

III. 4.2. Reverse Northern Blotting, Sequencing and In Silico Analysis of Differentially Expressed Genes.

The cloned sequences were further confirmed to eliminate false positives arising out of arbitrary PCR primers. To eliminate false positive, reverse northern blotting was performed with PCR products blotted and probed with total cDNA as probe. Reverse northern blots of these amplicons probed with total cDNA probes confirmed the consistency of the amplicons as being part of expressed genes. The reverse northern blotting in Fig 3.45 shows the rows of 6 different differentially displayed products as a clone or PCR product. Only slots in the rows of c, d, and e showed positive transcripts and others proven to be false positive. The true positive DDPCR products were sequenced for further analysis (Fig 3.46-51).

The sequences of true positive clones were analyzed further for the gene it denotes. The in silico analysis of the sequences using BLAST (www.ncbi.nlm.nih.gov) reveals identity of some of the genes with those down-regulated specifically in ribozyme transfected cells. It will be of interest to define functions of respective gene products and their interplay with telomerase. The gene products so deduced were further analyzed with ‘STRINGS’ online software (a protein interaction network, www.strings.embl.de), for possible cross talks with cellular proteins (Fig 3.52). The genes corresponding to the differentially displayed PCR products include an enhancer invasion cluster (HEI-C) functioning in spindle fiber assembly, the ribosomal L23 and
Differential Display PCR

Fig 3.40: A schematic representation of differential display PCR technique and methods to identify the differential displayed products.
Fig 3.41 A: The differentially displayed product with transcripts amplified with oligo dT-G and with eight different primers (only six are shown). The boxed bands identified as differentially displayed sequences. First batch i.e., 30 days old post transfected cells. A-Vector alone, B -Active ribozyme and C- Mutant ribozyme transfected cellular transcripts.
Fig 3.41 B: Subsets of differential displayed products predicted to have positive or negative association with hTR and telomerase activity. A- Vector alone, B -Active ribozyme and C- Mutant ribozyme transfected cellular transcripts.
Fig 3.42: DD PCR of second batch 45 day post transfections, reveals the differential displayed products with oligo dT-A with eight different arbitrary primers. Boxed bands are differentially displayed products and varied with 30 day old post transfected gene expression profiles. A-Vector alone, B - Active Ribozyme, C- Mutant Ribozyme transfected cellular transcripts.
Fig 3.43: DD PCR products of cells 45 days after transfections. Products were of oligo dT-C with eight different arbitrary primers. Boxed bands are differentially displayed products. A- Vector alone, B- Active Ribozyme, C- Mutant Ribozyme transfected cellular transcripts.
Fig 3.44: Reamplified products from differentially displayed amplicon, reveals variation in size and the products were of 150-180 base pairs. M -100base pair markers, Numbers are different PCR product of differential displayed products. Many primary amplicons could not be amplified from eluate of the corresponding gel slices.

Fig 3.45: Reverse northern blotting of differentially displayed products, using total cDNA as probe. The a, b, c, d, e are column containing many spots of differentially displayed products and only positive transcripts are confirmed with hybridizations and elimination of false positive PCR products. Arrows show the positive products. Absence of positive signals in a and b is false positive transcripts from DDPCR.
Fig 3.46: Sequencing of Differentially displayed products and \textit{in silico} analysis shows the sequence of ribosomal protein L23 gene.
757367 bp at 5' side: methionine adenosyltransferase II, beta isoform 1

Fig 3.47: Sequencing of Differentially displayed products and *in silico* analysis shows the sequence of methionine adenosyl transferase II gene.
>gi|113427929|ref|XM_001129608.1| PREDICTED: Homo sapiens coiled-coil domain containing 5 (spindle associated) (CCDC5), mRNA

Length=1002

Query 1  TCTCATGGAGAGTTTTTCCCCCTGCAATCTGCTAGCCTGGTCTAGGT 56

Sbjct181  TCTCATGGAGAGTTTTTCCCCCGCAATCTGCTAGCCTGGTCTAGGT236

Fig 3.48: Sequencing and BLAST search identifies the DDPCR product as Coiled coil domain domain containing 5 (CCDC5) or enhancer of invasion cluster (HEI-C).
Fig 3.49: Sequencing and BLAST search identifies DDPCR product as emopamil binding protein (sterol isomerase)
ref|NM_007209.3|Homo sapiens ribosomal protein L35 (RPL35), mRNA
Length=475  GENE ID: 11224  RPL35  | ribosomal protein L35 [Homo sapiens]

Query 76  GTACAAGCCCTGGATCTGCGGCCCAAGAAGACACGTGCCATGCGCCGCCGGCTNANCAA
Sbjct 280  GTACAAGCCCTGGACCTGCGGCCTAAGAAGACACGTGCCATGCGCCGCCGGCTCAACAA

Query 136  GCATGAGGAAAATCTGAAGACCAAGAAGCAACAGCGGAAGGAGCGGCTGTACCCTCTGCG
Sbjct 340  GCACGAGGAGAACCTGAAGACCAAGAAGCAGCAGCGGAAGGAGCGGCTGTACCCGCTGCG

Query 196  GAAGTATGCGGTCAAGGCCTGAG
Sbjct 400  GAAGTACGCGGTCAAGGCCTGAG

Fig 3.50: Sequencing and BLAST search identifies DDPCR product as ribosomal protein L35 coding sequence.
ref|NT_026437.11|Hs14_26604 Homo sapiens chromosome 14 genomic contig, reference assembly Length=88290585

Features in this part of subject sequence:

retinoblastoma-binding protein 11 isoform II

Query 1

```
GTCCATCCACTCTCTCCAGGTGAACCTTTGTTCTAAAAGATCCCTTAAATATGAATGCT 60
```

Sbjct3'2013

```
GTCCATCCCTATCTCTCCAGGTGAACCTTTATCTAAAAGGTACCTTAAATACAAATGCT
```

Query 61

```
GAATCCAATATTCAGTGTTTCTGTAAAAAAAAA 93
```

Sbjct 39772072

```
GAATCCAATATTCACCTATTTCTGTGAAAGAAAAA 39772104
```

Fig 3.51: Sequencing and BLAST search of positive clone identifies as retinoblastoma binding protein 2 isoform II
Fig 3.52: STRINGS search of protein networks (CCD5-Coiled Coil domain containing protein 5, JARDIA-Retinoblastoma binding protein 2, MAT2B-Methionine adenosyl transferase, of differentially expressed genes identified from DDPCR. The red color line shows the experimentally proven interaction with the reported proteins.
III. 4. 3. Discussion

Differential display PCR is a convenient technique to identify differentially expressed genes. DDPCR from cells 30 day and 45 day post transfection reveals alteration of transcripts in ribozyme transfected cells. Most of the transcripts were absent or down regulated in ribozyme transfected cells, and in two instances a new transcript appeared in ribozyme, the same was absent in both vector and mutant ribozyme transfected cells. Each group had 24 samples for comparison with 24 samples of other groups. Second batch DD-PCR results representing cells of 45 days post transfection came out with many differentially displayed products.

The main focus in cancer research and therapeutics is on identifying the underlying principles that govern the balance of tumor suppressor and activation of proto-oncogene and its involvement on cell proliferation and differentiation activities either in isolation or in concert. The behavior of molecules at different genetic status alters the patterns that are followed by strictly regulated molecular circuit. Carcinogenesis involves induction of proliferation and inhibition of cell differentiation and abrogation of cell cycle arrest programmes along one or more convergent pathways.

The main players of cell cycle p53 and pRb, in most types of cancer are either mutated or deleted. The tumor suppressor function of p53 involves activating its downstream effectors whose protein products may control cell cycle, apoptotic mechanism and senescence in response to various cellular stresses, preventing transformed and potentially tumorigenic cells from proliferation. The pRb products can control cell proliferation and promote differentiation (e.g., osteogenesis, adipogenesis and myogenesis) by enforcing several differentiation inducing transcription factors such as Myo D, CCAAT/enhancer binding protein β, Runx2, and glucocorticoid receptor. The p53 gets inactivated by either endogenous proteolysis or exogenous viral factors. Several viral oncoproteins, including simian virus 40 large T antigen and adenovirus E1A, have been found to bind strongly to hypophosphorylated Rb (DeCaprio et al. 1988; Whyte et al. 1988). By binding to strategic domains they functionally inactivate Rb, probably by displacing cellular proteins important for normal functions in negative cell cycle regulation (Jones et al. 1997)

In our studies, telomerase inhibition did not seem to induce any apoptotic signal although it is evidenced from other studies (Yatabe et al. 2002, Hahn et al. 1999). Alternatively, the non-induction of apoptosis in our studies, could be due to the
absence of functional p53 or pRB protein in the HeLa cells, as HeLa harbors human papilloma virus, (HPV18) and proteins coded by the viral early genes E6 and E7 are known to target p53 and pRb respectively causing their inactivation/degradation and these cellular proteins are main players in cell cycle regulation and apoptotic signaling events (Scheffner et al.1990, Boyer et al. 1996).

Appearance of elongated cellular phenotypes may also result from the interaction of HPV E6 and E7 gene products with their respective targets p53 and pRb. In such status cells might have lost the signals for G0/G1 arrest and instead, the ribozyme-transfected cells are promoted along the differentiation pathway. Some of the genes which are down regulated upon telomerase targeting may presumably in cross talk with hTR or be influenced by telomerase activity or under the regulatory net work of telomerase.

Most of the genes noticed for differential display of cDNAs corresponding to their transcripts, showed reduction of transcripts in cells expressing wt ribozyme; however we did find a few genes that were over expressed. Reverse northern blotting indicated the truly positive clones for further characterization. The list we present here of differentially displayed genes is by no means a comprehensive one, as the primer combination in this differential display PCR experiment could cover only 45% of the represented transcripts. Studies on genome-wide expression patterns associated with telomerase therefore gain in value (Liang et al. 1992, 1994).

Genes down regulated by hTR-targeting ribozyme and thus showing a positive association with hTR expression/availability are likely to be positively regulated by hTR or telomerase activity and function of those down regulated genes may open up novel therapeutic options in addition to telomerase itself.

One of the deduced genes codes fore L23a protein, a component of ribosome along with other proteins, which has also a role in rRNA maturation and in early stages of ribosome biogenesis. It also has a role in protein interaction with chaperone trigger factor in later stages of translation and in preventing the MDM2, an oncogene which triggers p53 degradation. The independent role of L23a have been studied in p53 -/- background as the reduction of L23a would disturb the ribosome biogenesis and rRNA maturation events, and the translation process will be at halt or disturbed, a molecular phenotype, which may be a prerequisite for apoptosis. It has been demonstrated that p53 and pRb deficient cells would not opt for a sudden apoptosis or
show any signals of apoptosis rather they proliferate and progressive reduction of ribosome contents and impaired biogenesis results in to cell death (Mantanoro et al. 2007). Another downregulated gene, the L35 protein is a basic protein from large subunit (50S) of ribosome in *E. coli* and 39S of mouse. L23a and L35 are close to the nascent polypeptide chain exit point in ribosome, having a role as a bridge with translocon in the translation process (Pool et al., 2002). The reduction of L35 again affects the translation process.

The methionine s-adenosyl transferase catalyzes the formation of s-adenosyl methionine (SAM), a donor of methyl group. In cancer SAM levels are decreased due to increased utilization of methyl groups as compared to abundant levels of SAM in normal cells. When the methionine adenosyl transferase level is reduced or inhibited, the methyl group abundance would be unavailable, preventing cancer cells from developing and maintaining their altered methylation patterns. In such instances, we expect methylation status changed in cancer cells and some of the genes may be relieved of methylation and may trigger other regulatory network which is necessary for cell cycle control or normal maintenance. SAM is also necessary for normal cell maintenance; the disturbance and unavailability of methyl group donor may result in many abnormal expressions which may select the cells for apoptosis (Chiang et al. 1996). Role of SAM as methyl donor is not limited to DNA methylation events but also extends to protein methylation and t-RNA maturation process etc (Amalric et al. 1977). From our studies we found reduction in methionine s-adenosyl transferase in cells with wt ribozyme targeting hTR, suggesting that it could be positively associated with telomerase activity or hTR.

Another gene which seems to be positively regulated by telomerase is the enhancer of invasion cluster since it is down regulated in ribozyme expressing cells. This is a conserved coiled coil domain containing protein 5 (HEI-C) which functions in maintaining spindle formation during mitosis and its reduction or knock down by siRNA causes perturbed metaphase to anaphase transition and disorganized mitotic spindles, and subsequent disintegration of cells (Einarson et al. 2004). Disintegration happened in such cells due to intact cell cycle check point machineries. We didn’t observe cellular disintegration perhaps due to their p53 and pRb negative status.

The role of pRB binding protein 2 (RBP2) is likely to be of significance since pRB is best known tumor suppressor; in addition, it also blocks proliferation and induces differentiation (Thomas et al. 2003). The free RBP2 blocks differentiation and pRb
inhibits the effect by binding with RBP2, and induces differentiation. In absence of pRB, down regulation of RBP2 can phenocopy the reintroduction of pRB in pRB-/­cells (Benevolenskaya et al 2005, 2007). RBP2 is also found to be modulating the chromatin structures. Recently it was shown to have a demethylase activity on trimethylated histones, and removes the methylated states of histone (H3K4 me3). In view of the association of trimethylated histones with euchromatic state of genome (Christensen 2007), the above observation would suggest that telomerase modulates chromatin through RBP2 protein in absence of functional pRBs (Ahmed et al.2004, Benevolenskaya et al. 2005). Hence reduction in RBP 2 level might cause heterochromatic region to switch in to active euchromatic region of chromosome and induce differentiation.

Emopamil binding protein (Sterol isomerase) belongs to sigma family of receptor, mutations that disrupts EBP's 3β -hydroxysteroid sterol Δ8 Δ7 isomerase activity impair cholesterol biosynthesis and cause X chromosomal dominant chondroplasia punctata, a rare disorder that is lethal in most males and causes patches of skin and bone in females due to random X inactivation process (Moebius et al 2003). Uptake of low density lipoprotein is through formation of coated pits and processed through endocytosis and hydrolyzed by lysosomal enzymes in to basic elements cholesterol, fatty acid and amino acids. The cholesterol liberated by lysosomal degradation is used for cell membrane synthesis. Cancer cells need large quantities of cholesterol for cell membrane synthesis and the control mechanism is some how deregulated. The reduction or absence of emopamil binding protein in ribozyme expressing cells may be defective in cholesterol biosynthesis. Impaired membrane synthesis may restrict cell propagation. Association of EBP in telomerase associated diseases like chondroplasia punctata also confirms direct or indirect association of EBP with telomerase.

In conclusion, the present study proves the association of some cellular genes with hTR, and also validated telomerase as a therapeutic target in p53-/­ and pRb-/­ status, and such treatment modalities will be effective in targeting telomerase. The study using ribozyme prompts to propose a hypothetical model of possible cross-talk or direct or indirect effect of hTR with necessary cellular events depicted as in Fig 3.59. The pathways mediating those gene and telomerase cross talk have to be established with experimental proofs.
III.5. Proteomic Alterations in Transfectants
III.5.1. Proteomic Profiling of Transfected Cells

The changes in whole genome transcript profiling prompted us to analyze the possible alterations in protein expression patterns. To analyze the proteome, 2D gel electrophoresis was carried out, which includes isoelectric focusing as 1st dimension and normal SDS PAGE as second dimension gel electrophoresis. Two-dimensional electrophoresis (2DE) of proteins is one of the highest-resolution analytical techniques available for the study of global protein expression patterns. The proteomic profiling by 2D gel electrophoresis methods allows one to identify a number of proteins that had been altered in their expression pattern.

III.5.2. Expression of Ribozyme Alters Proteomic Profile of Stable Transfectants

Cellular pellets of stable transfectants of vector, ribozyme and mutant ribozyme were lysed with urea buffer and isoelectric focusing was carried out in precast gels. After electrofocusing, conventional SDS PAGE gel was used for 2nd dimension PAGE gels as described under materials and methods. The spots were compared to locate the differentially expressed proteins. There were 9 proteins differentially expressed in ribozyme expressing cells and 4 from vector transfected cells (Fig. 3.53A, B). Most of the protein species were slightly basic in nature and were between 17 to 82 Kd molecular mass. At this stage we could extract significant mass characteristics for four spots from Ribozyme transfected and one spot from the vector transfected cells.

To identify the proteins, mass spectrometer was used i.e, MALDI-TOF (Matrix Assisted Laser Desorption and Ionization). The proteins of differentially expressed spots were lysed by trpsin digestion, resulting peptides were ionized by laser and fired and analyzed by MALDI-TOF (Bruker Daltonics). The represented peptide’s peaks m/z values were compared manually. A consistent presence of certain peaks was observed at 2370 and 2770 and 3250 (Fig 3.54-58). Those peaks considered as noise or contaminations were not taken for the analysis. The peptide peaks obtained for each sample were searched for their homology with the database of NCBI, EXPASY and SWISS PROT.

Spot 1 (Figs 3.53A, 54) is found to be exclusively expressed in vector transfected and absent from ribozyme transfected cells. The protein mass falls between 82-126 Kd
and MALDI created around 60 peaks and matched with interleukin 12 receptor beta 2 precursor, and 35 peaks created in MALDI TOF were matched with MASCOT’s database. The main peaks were 1024, 1306, 1474, 2241, 2382 m/z values. The molecular mass and gel pictures were compared and identity assigned.

The 6th spot (Figs 3.53B, 55) appeared between 82 -126 Kda marker. MALDI yielded 73 peaks spanning between 800-2800 of m/z ratios. The peaks were 904, 1045, 1064, 1233, 1261, 1319, 1492, 1583, 1604 and 1838 daltons and are identified to be ATR interacting protein. The highest peak observed had a m/z value of 1319 representing peptide ‘LQSLQSELQFK’.

The spot 7 of protein (Figs 3.53B, 56) from ribozyme transfected cells fell in the mass range of approximately to 82 Kda. The MALDI analysis created about 75 peaks and those are mainly 1035, 1178, 1191, 1233, 1299, 1319, 1739, 1790 and 1946. The peaks 1790 and 1946 converged, as single amino acid difference of cleavable amino peptide repeated (RR) at those sites (SIAVWDMASPTDITLRR). The matched peaks found homology to nearby significant value to the Beta transducin repeat containing isoform CRA-d.

The 8th spot (Fig 3.53B, 57) which has mass of between 38 to 82 kda from 2 D gel and the MALDI analysis created 100 peaks spanning from 800 to 3300 m/Z and those peaks with good intensities and matched with the available data base were identified as keratin 10. The matched peaks correspond to m/z of 806, 992, 994, 1002, 1089, 1117, 1164, 1200,1261,1299, 1356, 1364, 1389, 1433, 1492 and 1706, matched with Keratin 10 from the entire data base with significant score.

The 11th spot (Figs 3.53B, 58) eluted from 2D gel were between 17 to 32 Kd, and from MALDI analysis, we obtained 64 peaks including the noise and salt created peaks. The 64 peaks were searched for their homology and found hCG with significant score. The peptides1046, 1196, 1706, 3222 matched with hCG and the peptide peak of 3222 m/z confirms the hCG as represented protein in MALDI.
Fig 3.53: 2D gel electrophoresis of protein isolated from stable transfectants A) Proteins from vector transfected and B) from ribozyme transfected cells in 12% PAGE gel. M represents marker lane (3-10 represents pI values).
Protein spot 1: Interleukin 12 receptor, beta 2 precursor (Homo sapiens)

Fig 3.54: MALDI Analysis of peaks generated with digested peptides, and deduced as Interleukin 12 receptor, beta 2 precursor and its sequence. The sequences highlighted in red are matching peaks generated from peptides.
Protein spot 6: ATR interacting protein (Homo sapiens)

Fig 3.55: MALDI Analysis of peaks generated with digested peptides, and deduced as ATR interacting protein (ATRIP) and its sequence. The sequences highlighted in red are matching peaks generated from peptides.
Protein spot 7: Beta-transducin repeat containing, isoform CRA-d (Homo sapiens)

Fig 3.56: MALDI Analysis of peaks generated trypsin fragments, and deduced as beta-transducin repeat containing, isoform CRA-d and its sequence. The sequences highlighted in red are matching peaks.
Protein spot 8: Keratin 10 (Homo sapiens)

Fig 3.57: MALDI analysis of peaks generated with digested peptides, and deduced as protein keratin 10 and its sequence. The sequences highlighted in red are matching peaks generated from peptides.
Fig 3.58: MALDI Analysis of peaks generated with digested peptides, and identified it as hCG and its sequence. The sequences highlighted in red are matching peaks generated from peptides.

Protein spot 11: hCG 1993711
III. 5.3. Discussion

Two dimensional PAGE gel electrophoresis resolves a large number of proteins from whole cell proteome. The proteomic profiling by 2D gel electrophoresis and MALDI analysis can identify not only differentially expressed proteins, but also the phosphorylation and other post translational modifications. The advent of new techniques like MALDI –TOF simplifies the identification of peptide fingerprint in mass scale analysis as the whole genome sequencing, proteomic efforts have already defined the possible peptide reference of the proteins from particular cellular and tissue types (Walker et al 2002). Comparative proteomic profiling of normal cell and pathologic cell allows one to identify the proteins that may play role in pathology of diseases.

The proteomic profiling of vector and ribozyme transfected cells shows that there is marked difference in expression patterns as about 13-15 identifiable protein species were differentially expressed. The 2D gel electrophoresis pattern reveals that most of the proteins have mild basic pI (iso electric point) and fall in low molecular weight ranges (17-82 kda). Only very few were identified as high molecular weight proteins.

In view of some of the differentially expressed proteins like keratin 10, and IL12R, ATRIP, we try to propose the pathway that may be involved in induction of differentiation in the ribozyme expressing cells.

One of the proteins that is specifically down regulated in ribozyme transfected and over expressed in vector transfected cells is Interleukin 12 receptor (IL-12R), a transmembrane protein. Binding of IL12 with IL-12R leads to activation of JAK2, Tyk2, STAT3 and STAT4 transcription factors. TGF- β blocks the IL-12 induced activation of JAK/ STAT pathway. TGF-β also blocks the telomerase hTERT promoter. From our studies it can be suggested that down regulation of IL-12R may inhibit the JAK / STAT oncogenic pathway, and thereby inhibit cell proliferation.

Keratin 10 (K10) is major structural protein of the epidermis and recently its role was also proved in cell proliferation and differentiation. K10 expression is restricted to post mitotic cells of supra basal layer, and it is suppressed in wound and tumor cells. K10 inhibits proliferation by reducing cyclin D1 expression and pRb phosphorylation.

K10 binds with Akt, PKC ζ is a key signaling protein in phosphatidyl ionositol 3 kinase pathways (PI-3-K). Akt promotes cell survival by phosphorylating BAD
(Proapoptotic), and activate NFkB via regulating IkB Kinase (IKK), resulting in transcription of pro-survival genes. K10 binds with Akt and PKC ζ and prevents the translocation of these kinases to the membrane. Through this mechanism keratin 10 affects proliferation, differentiation and apoptosis (Paramio et al. 2001).

ATR (ATM and Rad 3 related) is homologous to Ataxia Telangietasia–mutated (ATM) and is related to PI-3-K. These kinases respond to DNA damage events and initiate signals for cell cycle arrest. Loss of ATR destabilizes genome and increases the risk of transformation in to cancer. ATR exists as stable complex with associated protein ATRIP (ATR interacting protein). Loss of ATRIP by siRNA causes the same phenotype as ATR loss. Depletion of ATRIP from cell causes a decrease in the intracellular levels of ATR and vice versa. ATR is proved to be negatively regulated by hTR (Kedde et al. 2006), when the level of hTR is increased the ATR level is decreased. ATR and ATRIP are positively associated with each other. Ribozyme mediated knocking down of hTR in the present study seems to cause ATRIP over expression and induction of DNA damage signaling events. Since HeLa cells have abrogated p53, cell cycle arrest does not follow.

β-transducin repeat containing protein isoform (β-TrCP), role of which is still not proved, is a components of β-catenin and Iκ Bα for proteasomal degradation. It negatively regulates Wnt/ β-catenin signaling and positively regulates NFκB signaling. Over expression of β-TrCP down regulates the catenin, and the isoform is predicted to have same function (Hart et al. 1999). In our study over expression of β-TrCP may inhibit β-catenin, and also raise the question about the plasticity of partly differentiated cells having stem cell like properties.

Specifically, the human telomerase RNA (hTR) is expressed in normal cells, though telomerase activity is nil. This raises the question about its function in absence of its catalytic counterpart. Recent evidences suggests that hTR increases wound healing potency of mouse skin (Gonzalez-Suarez et al. 001), and induce transformation capability together with RAS oncogene (Stewart et al. 2002 ). Its role in ATR regulation further proves that telomerase RNA have extra telomeric function.

In conclusion, from our studies including DDPCR and 2D –MALDI- TOF analyses, we could infer that in absence of the master regulators of cell cycle p53 and pRB proteins (as in the HPV-transformed cells like HeLa), cells fail to induce apoptotic signaling and adopting anti-apoptotic pathway generally mediated by PI-3-K, JAK-
STAT and Wnt pathways. This pathway may be converged together to maintain the proliferation status along with telomerase and hTERT expression to get the proliferative advantage. Upon ribozyme expression against hTR, cellular genes for retinoblastoma binding protein 2 (RBP2), human enhancer of invasion cluster (HEi-C or CCD5), ribosomal protein 23, 35 (RPL 23, RPL35), emopamil binding protein (EBP), methionine-s-adenosyl transferase (MAT 2A) and interleukin 12 receptor are predicted to have positive relation with hTR function whereas keratin 10, β-transducin repeat containing isoform (β-TrCP-CRA-d) and ATR interacting protein (ATRIP) have negative regulation or association with hTR. Haplo-insufficiency levels of hTR, and complete knockdown studies were not performed; we could create analogous situation with the help of the ribozyme. Increased level of hTR has an effect on cellular survival and to a certain degree regulates other cellular genes.

Our results for the first time, seem to suggest that knocking down hTR results in induction of differentiation and during the process, the cells inhibit PI-3-K pathway and may adopt TGF β pathway. It should be mentioned that HeLA cells are p53-/-.

From our own experimental proofs, STRING search of MALDI and DD-PCR results and other published sources we propose a hypothetical model on telomerase and its association with certain genes which provide proliferative, anti-apoptotic and differentiation functions to the cells.
Differentiation (TGF-β Pathway)

RBP2
HEI-C
RPL23
RPL35
MAT2A
EBP
IL12-R

Proliferation
PL-3-K Pathway

Apoptosis (TGF-β Pathway)

Keratin 10
ATRIP
βTrCP

Fig 3.59: Model depicting the pathway and genes that are positively and negative association with telomerase RNA possibly by inhibiting PI-3-K, Wnt, JAK/STAT pathway and integrate with TGF-β pathway. The positively associated genes RBP2 suppresses differentiation while HEI-C, RPL23, RPL35, MAT2A, EBP and IL12-R promote proliferation and inhibit apoptosis. Similarly, the negatively associated gene Keratin10 promotes differentiation while ATRIP and βTrCP promote apoptosis.

Since hTR is found to have role in regulation of certain cellular genes, it would be interesting to make a comprehensive study of the pathways involved. Complete knock out of hTR (instead of knockdown) in presence and in absence of hTERT and its changes in molecular signatures in cancer cells and in normal cells with functional p53 and pRb status need to be studied in greater details. In knock down studies, either ribozyme or siRNA can be expressed by strong promoter-expression system like lentiviral systems and co-localizing the therapeutic molecules to its target in nuclear or cytoplasmic compartment using certain signal sequences can add to the efficacy of the process. Usage of athymic or null mice for assessing the proliferative efficiency of hTR knocked out cells would give additional information on its role in proliferation.