Chapter 3

Materials and Methods

Materials

All the microbiological media such as Luria broth, Luria agar, antibiotics, gram’s staining kit, and other inorganic salts, Tris buffer, EDTA, SDS, etc were purchased from Himedia, Mumbai, India. All the solvents used were of analytical grade and were purchased from Qualigens, Mumbai, India. Cell culture mediums, RPMI 1640 and DMEM, FBS were purchased from Gibco, USA. Folate free RPMI 1640 and DMEM were obtained from Sigma, USA. Sodium bicarbonate, sodium pyruvate, trypan blue and trypsin were obtained from Gibco, USA. Geneticin was obtained from Merck, USA. All the chemicals for folic acid conjugation like, folic acid, EDC, Sulfo-NHS, DMAP were obtained from Sigma, USA. Restriction enzymes, λ Hind III ladder, 50 base pair ladder and other requirements for RT-PCR such as Taq DNA Polymerase, dNTPs, Verso cDNA synthesis kit, etc were purchased from Thermo Scientific, USA. TRIzol, Lipofectamine 2000, agarose, ethidium bromide were purchased from Life technologies Inc., USA. Primer for VEGF A, GAPDH and 18s were obtained from Sigma, USA. MTT and FITC were obtained from Himedia, Mumbai, India and MP Biomedicals, USA, respectively. Cyclosporine (Sandimmune) and Ketoconazole (Nizral) were purchased from Novartis, Switzerland and Johnson & Johnson, USA, respectively. Cyclophosphamide (Endoxan) was purchased from Baxter, Germany. Rodent diet, both normal and folate free were obtained from VRK nutrition, Pune, India.

Methods

Flow of work:

- Procurement and selection of minicells producing bacterial strain.
- Procurement of shRNAs specific for VEGF-A and BCL-2 gene and other expression vectors.
- Transformation of selected minicell producing strain with plasmid expression vector.
- Purification of minicells.
• Plasmid DNA isolation from minicells to confirm the presence of plasmid DNA in the minicells.
• Procurement and selection of cancer cell lines.
• Transfection of shRNA expression vectors in selected cancer cell lines for the validation of gene silencing.
• Packaging of shRNA in the minicells through transformation and minicells purification.
• Folic acid conjugation on shRNA packaged minicells.
• *In vitro* delivery of shRNA packaged, folic acid conjugated minicells in cancer cell lines and gene expression analysis by RT-PCR.
• Mechanistic study of minicell uptake.
• Development of immunosuppressive model for tumor xenograft model development in mice.
• Development of tumor xenograft model in immunosuppressive mice.
• *In vivo* delivery of folic acid conjugated minicells in mice bearing tumor xenograft.
• Analysis of tumor regression by measurement of tumor volume and gene expression analysis by RT-PCR.
• *In vivo* biodistribution study of folic acid conjugated minicells.

### 3.1 Procurement and selection of minicells producing strain

In order to develop minicells as a delivery system for shRNA, three minicells producing strains have been procured. *E.coli* K-12 X\(^{984}\) and *E.coli* P678-54 were kindly provided by Dr. John Wertz, Coli Genetic Stock Centre, Yale University, USA whereas *E.coli* PB-114 was kindly provided by Dr. Lawrence Rothfield, University of Connecticut, USA. Strain was selected in terms of highest minicells producing tendency. Gram’s staining was performed to observe number of minicells produce by each strain.

#### 3.1.1 Enrichment of *E.coli* K-12 X\(^{984}\) and *E.coli* P678-54

Enrichment of *E.coli* K-12 X\(^{984}\) and *E.coli* P678-54 minicell producing strains was done by employing following steps.

- The filter disc was opened carefully from the packet and kept it in the centre of LA agar plate containing 100 µg/ml streptomycin.
• Several drops of sterile LB broth have been put on filter disc.
• Broth was spreaded evenly in plate.
• Plate was incubated in incubator at 37° C temperature for overnight (16 hours)
• Growth was observed after incubation.

3.1.2 Enrichment of *E.coli* PB 114 minicell producing strain

• A single colony was inoculated from *E.coli* PB114 stab to 2 ml LB broth containing 50 µg/ml kanamycin.
• Tube was incubated at 37°C, 180 rpm overnight in shaking incubator.
• Growth was observed after overnight incubation.

3.1.3 Gram’s staining of *E.coli* K-12 X<sup>984</sup>, *E.coli* p678-54 and *E.coli* PB 114

Gram’s staining was performed in order to confirm the presence of gram negative organism i.e. *E.coli* as well as presence of minicells. It is a differential staining method for differentiating bacterial species into two large groups (Gram-positive and Gram-negative) based on the chemical and physical properties of their cell walls. Gram-positive bacteria have a thick cell wall made of peptidoglycan (50–90% of cell envelope) and as a result are stained purple by crystal violet, whereas gram-negative bacteria have a thinner layer (10% of cell envelope), when treated with decolorizer such as alcohol, it interacts with the lipids of the cell membrane and break it, so do not retain the purple stain and are counter-stained pink by the Safranin. The basic steps of grams staining are given below.

• Loop full of culture was taken and a smear was made in the centre of the slide.
• The smear was heat fixed by passing the slide on flame for 2 to 3 times.
• The smear was flooded with 2% crystal violet for 2 minutes.
• The smear was flooded with gram’s iodine for 1 minute.
• After that the slide was wash with decolouriser (95% ethanol) from one edge of the slide in a drop wise manner till the violet color fails to come out from the slide.
• The slide was flooded with 0.5% safranin for 2 minutes.
• After every step, slide was washed with tap water.
• The slide was air dried and observed under microscope in 100X oil emersion lens.
3.2 Transmission Electron Microscopy of *E.coli* PB114

Size and morphology of *E.coli* PB-114 was confirmed by Transmission Electron Microscopy (TEM). Following steps were performed.

- 100 µl of overnight grown culture of *E. coli* PB-114 was applied to copper TEM grid for 5 minutes. During this time bacterial cells absorbed onto the supporting film.
- The rest of the solution was wicked from the edge of the grid by wedge of filter paper.
- Cells were observed in Transmission Electron Microscope (Tecnai 20, 200 kv, Philips, Holland) at an accelerating voltage of 90 kV.
- After that characterization was done in TEM and images were captured.

3.3 Procurement and maintenance of expression vectors

**Procurement and maintenance of shRNA expression vectors**

shRNA expression vectors specific for VEGF A (psNIPERDH1A1 and psNIPERDU6A2) and BCL-2 genes were kindly provided by Dr. Neeta Shrivastava (Pharmacognosy and Phytochemistry lab of PERD centre). shRNA expression vectors were maintained in *E. coli* DH5α. These vectors have been amplified and isolated from *E. coli* DH5α as and when required for the experiment. Moreover other plasmid based expression vectors such as pEZ 43G-D, pSUPERneo, pRNAT were also provided by Dr. Neeta Shrivastava. pEZ43G-D has ampicilin resistant gene as a selective marker and green fluorescence protein (GFP) as a reporter gene which express in bacteria. pSUPERneo is a backbone vector in which shRNA sequences specific for Scramble (control shRNA) and VEGF A have been cloned. Whereas pRNAT is a backbone vector used to clone shRNA specific for BCL-2 gene. pRNAT has GFP gene which express in mammalian cells.

**Note:** Expression vector for BCL-2 gene, pIPERDU6B has some inherent problem. We tried hard to identify and rectify the problem but we could not succeed. Hence, we move forward with shRNA specific for VEGF A, psNIPERDH1A1 and psNIPERDU6A2.
siRNA sequences for VEGF A

siRNA 1
Sense strand: 5’-CUUUCUGCUGCUCUUGGGUGtt-3’
Antisense strand: 3’-ttGAAAGACGACAGAACCCAC-5’

siRNA 2
Sense strand: 5’-UCAUCACGAAGUGGAAGtt-3’
Antisense strand: 3’-ttAGUAGUGCUUCACCACUUC-5’

3.3.1 Restriction enzyme digestion of shRNA vectors specific for VEGF A

Objective of the experiment was to check the integrity and confirm shRNA expression vectors. The Type II restriction enzymes recognize specific DNA sequences and cleave the DNA at fixed location at or near the recognition sites. Here, Hind III and Eco RI have been used to cut both the shRNA vectors. Hind III and Eco RI recognise at a site where shRNA sequence has been cloned. Hence, double digestion with Hind III and Eco RI yield cloned shRNA fragment. Following steps were performed to digest shRNA expression vectors.

- Purified shRNA vectors, psNIPERDH1A1 and psNIPERDU6A2 were sequentially digested by Eco RI and Hind III restriction enzymes according to the scheme given in the following Table 3.1.
- The reaction mixture was given a short spin for 30 seconds and incubated overnight at 37°C in dry bath.
- After overnight incubation enzymes were inactivated at 65°C and DNA samples were recovered by adding equal amount of absolute alcohol.
- DNA was pellet down by centrifugation at 12000 g for 20 min at 4°C
- Pellet was dissolved in 10 µl of TE buffer and digestion was confirmed by agarose gel electrophoresis.
Table 3.1: Restriction enzyme digestion conditions for both the vectors

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample DNA (µl)</th>
<th>HindIII (µl)</th>
<th>Eco R1(µl)</th>
<th>Buffer (µl)</th>
<th>Water (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single digestion</td>
<td>5</td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>Single digestion</td>
<td>5</td>
<td>1</td>
<td>-</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>Double digestion</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>13</td>
</tr>
</tbody>
</table>

3.4 Transformation of *E.coli* PB 114 with pEZ43G-D plasmid

Bacterial transformation is a process in which bacteria manage to uptake or bring in free/external DNA from surrounding medium. The purpose of this technique is to introduce a foreign plasmid DNA into bacteria and to use these bacteria to amplify the plasmid DNA. Cells can be artificially induced to take up DNA by treating them with calcium chloride. Culture of such cells that are capable of taking up DNA is said to be competent. When DNA is taken up by competent cells, cells are said to be transformed. Objective of the experiment was to transformed pEZ43G-D (plasmid having GFP (green fluorescence protein) as a reporter gene) plasmid in order to confirm segregation of plasmid DNA and protein into the minicells. Transformation of *E.coli* PB114 was performed as follows.

- 200 µl of culture was inoculated into 10 ml of LB broth containing kanamycin (50µg/ml) from overnight (16 hours) grown culture of *E.coli* PB114.
- Flask was incubated at 37°C with shaking till the cells reach to the log phase (O.D. = 0.4). Generally, a 3 hour shaking period is sufficient.
- 2 ml of culture was transferred aseptically in 2 ml microcentrifuge tube and cool down to 4°C by storing the tube on ice (all subsequent steps were carried out aseptically).
- Cells were centrifuged at 6000 g for 5 minutes at 4°C.
- Medium was removed and cell pellet was resuspended in 1 ml of 0.1 M CaCl$_2$ and incubated on ice for 15 minutes.
- Cells were centrifuged at 6000 g for 5 minute at 4°C. Supernatant was discarded and pellet was resuspended in 200 µl of 0.1 M CaCl$_2$. After that it was incubated on ice at 4°C for 30 minutes.
- Plasmid DNA was added in 3 different concentration, for instance, Test-1 (120 ng/1µl), Test- 2 (240 ng/2µl) and Test-3 (600 ng/1µl) to competent cells. It was mixed well and incubated on ice for 30 minutes.
- Heat shock was given to the cells at 42°C for 2 minutes in a water bath and rapidly transferred to ice to chill the cells for 2 minutes.
- Cells were transferred to 2 ml LB tube and incubated at 37°C for 30 minutes at 200 rpm in shaking incubator.
- Cells were transferred to 2 ml microcentrifuge tube and centrifuged it at 6000 g for 5 minutes at 4°C.
- Medium was removed and cells were resuspended in 100 µl of LB broth.
- These cultures were plated in different petridishes such as positive control (no ampicillin, no plasmid DNA), Negative control (ampicillin, 100 µg/ml, no plasmid DNA), Test-1(ampicillin, 100 µg/ml, kanamycin, 50 µg/ml), (120 ng/1µl plasmid DNA), Test-2 (ampicillin, 100 µg/ml, kanamycin, 50 µg/ml), (240 ng/2µl plasmid DNA), Test-3(ampicillin, 100 µg/ml, kanamycin, 50 µg/ml), (600 ng/1µl plasmid DNA).
- Plates were incubated at 37°C, overnight in incubator.
- After incubation plates were observed for the growth of transformed cells

### 3.4.1 Expression of GFP in transformed *E.coli* PB114

Objective of the experiment was to confirm the transformation of *E.coli* PB114 and to check segregation of plasmid DNA into the minicells and to observe expression of GFP in minicells. Expression of GFP was visualized by following steps.

- Overnight grown culture of GFP transformed *E.coli* PB114 was taken.
- The smear was prepared by taking a small drop of overnight grown culture at the edge of slide
- The drop was spreaded thoroughly with the help of spreader and allowed to air dry.
• The smear was observed in fluorescent microscope under 100X oil immersion lens.

3.5 Minicell purification by penicillin lysis method

Objective of the experiment was to purify minicells from the parent cells and concentrate minicells for further experiments. This method is based on the principle of non dividing nature of minicells. Penicillin act on diving cells by inhibiting its cell wall synthesis. Hence, it kills parent cells but do not harm the minicells. The detailed method is given below.

- 100 ml of overnight grown culture of *E.coli* PB114 was taken
- It was centrifuged at 2,250 \(g\) at room temperature for 10 minutes
- The supernatant was separated and the pellet was discarded.
- The supernatant was centrifuged for 10 minutes at 10,000 \(g\) to pellet down the minicells
- Minicell pellet was resuspended in 50 ml of fresh broth.
- The suspension was incubated at 37°C with vigorous aeration for 30 to 40 min to permit reinitiation of cell growth.
- Penicillin was added at dose of 1,500 units/ml
- The flask was incubated for 30 to 45 minutes in shaking incubator at 37°C
- After incubation the broth was centrifuged at 2,250 \(g\) to remove any viable cells and cell debris
- The supernatant was separated and the pellet was discarded
- The supernatant was centrifuged for 10 minutes at 10,000 \(g\) to pellet the minicells
- Minicell pellet was washed in fresh broth, concentrated the cells and performed grams staining to check the purity of purified minicells.

3.5.1 Purification of minicells by ceftriaxone lysis and filtration

Penicillin lysis method could reduce majority of parent cell load. However, it could not totally eliminate parent cells as well as cell debris. Hence a better method which can eliminate all parent cells as well as cell debris and simultaneously give high yield of minicells is needed to be developed. Objective of the experiment was to purified minicells from parent cells by combining two methods i.e. ceftriaxone lysis and filtration to get ultrapure minicells. Detailed steps of developed method are as follow.
• 200 ml of overnight grown culture of *E.coli* PB-114 GFP was taken.
• Culture was centrifuged at 2,000 g at room temperature for 10 minutes,
• Supernatant was separated and the pellet was discarded.
• Supernatant was centrifuged for 10 min at 10,000 g to pellet the minicells.
• Minicell pellet was resuspended in 50 ml of fresh broth.
• Suspension was incubated at 37°C with vigorous aeration for 30 to 40 min to permit
  reinitiation of cell growth.
• After that ceftriaxone was added at a dose of 100 µg/ml.
• Flask was incubated for 30 to 45 minutes in shaking incubator at 37°C.
• After incubation broth was centrifuged at 2,000 g to remove any viable cells and cell
  debris.
• Supernatant was separated and pellet was discarded.
• Supernatant was centrifuged for 10 minutes at 10,000 g to pellet the minicells.
• Minicell pellet was washed in fresh broth and filtered through 0.45 µ filter.
• Filtrate was centrifuged for 10 minutes at 10,000 g to pellet minicells.
• Cells were filtered through 0.22 µ filter to remove any cell debris.
• Minicell pellet was washed in fresh broth, concentrated and plated on LB agar plate to
  check the presence of any viable parent cell.
• Cells have been observed under fluorescent microscope after each step of purification
  to assess the purity of purified minicells.
• Finally, purified minicells were inoculated in fluid thioglycolate medium and
  incubated for 14 days to check the presence of any slow growing contaminant.

### 3.5.2 Size measurement of purified minicells

Objective of the experiment was to study the average size of minicells at different stages of
purification by zeta sizer, Malvern and evaluate the purification by means of average size.
Average size and polydispersity index should decrease with the decrease in the parent cells.
Size was measured by employing following steps.

• Cuvette was filled with suspension of purified minicells (at various stages of
  purification) up to the mark given in the cuvette.
• After that cuvette was placed in the zeta sizer and size was measured.
3.5.3 Transmission Electron Microscopy of purified minicells

Morphology of purified minicells was observed by Transmission Electron Microscopy. Moreover, size of purified minicells was confirmed by the same. Following steps were performed to observed the morphology of minicells.

- 100 µl of purified minicells (from $10^{10}$ cells/ml) was applied to copper TEM grid for 5 minutes. During this time bacterial cells absorbed onto the supporting film.
- The rest of the solution was wicked from the edge of the grid by wedge of filter paper.
- Minicells were observed in Transmission Electron Microscope (Tecnai 20, 200 kv, Philips, Holland) at an accelerating voltage of 90 kV.
- After that characterization was done in TEM and images were captured.

3.5.4 Determination of minicell number by spectrophotometric method

Objective of the experiment was to find out total minicell number present in the final fraction of purified minicells. Minicells numbers were calculated by spectrophotometric method as reported by Giacalone et. al, 2006.

- 1 ml of purified minicells from starting culture of 200 ml was poured in 1 ml cuvette.
- Optical density (OD) was measured at a 600 nm.
- Minicell number was determined according to following formula
  \[ \text{OD at 600} \times 5.0 \times 10^{10} / \text{ml} \]

3.6 Isolation of plasmid DNA from purified minicells

Objective of the experiment was to confirm the presence of plasmid DNA in the purified minicells. Plasmid DNA was isolated by alkaline lysis method. The method is simple, quick, easy to perform and most reliable to get pure plasmid DNA. Exposure of bacterial suspension to the strongly anionic detergent like SDS at high pH opens the cell wall, denatures chromosomal DNA and proteins, and release plasmid DNA into the supernatant. Although alkaline solution completely disrupts base pairing, the strands of closed circular plasmid DNA are unable to separate from each other because they are topologically intertwined. Native plasmid DNA can be recovered from the supernatant by addition of isopropanol. Detailed steps of employed methods are as follow.
Table 3.2: Composition of alkaline lysis solutions

<table>
<thead>
<tr>
<th>Alkaline lysis solution-1</th>
<th>Alkaline lysis solution-2</th>
<th>Alkaline lysis solution-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>50mM glucose</td>
<td>0.2 N NaOH</td>
<td>5 M Potassium acetate</td>
</tr>
<tr>
<td>25 mM Tris-Cl (pH 8.0)</td>
<td>1% (w/v) SDS</td>
<td>- 60 ml</td>
</tr>
<tr>
<td>10 mM EDTA (pH 8.0)</td>
<td>Prepare solution II freshly</td>
<td>Glacial acetic acid (96%) - 11.5 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Water                    - 28.5 ml</td>
</tr>
</tbody>
</table>

- $3.81 \times 10^{10}$ minicells/ml of purified minicells were taken (purified from 200 ml of culture).
- Minicells were centrifuged at 10000 g for 10 minutes at 4°C.
- Supernatant was decanted and the tube was dabbed on tissue paper.
- The pellet was resuspended in 100 µl of cold alkaline lysis solution-I and incubated on the ice bath for 5 min.
- 200 µl of freshly prepared alkaline lysis solution-II was added and the content of the tube was mixed by gently inverting the tube up-down 3 to 4 times. After that, the tube was incubated for 10 min on ice bath.
- 150 µl of cold alkaline lysis solution-III was added and mixed the content gently by inversion. Tube was incubated for 10 min on ice bath.
- The tube was centrifuged at 12,000 g for 10 min at 4°C.
- After that, the supernatant was removed carefully and transferred to a new tube.
- 0.7th volume of isopropanol was added, mixed and kept at -20°C temperature overnight.
- After the incubation the tube was centrifuged at 12,000 g for 20 min at 4°C.
- The pellet was washed with 70% ethanol.
- After that, tube was centrifuged at 10,000 g for 10 min at 24°C.
- The pellet was air dried and TE buffer was added to dissolve the DNA pellet.
- Sample was analyzed by agarose gel electrophoresis (0.9%) and spectrophotometric method to confirm the presence of plasmid DNA.
3.6.1 Determination of plasmid copy number

Objective of the experiment was to calculate the number of plasmid DNA segregated per minicell. Plasmid copy number was calculated by UV spectrophotometric method.

- 2 µl of plasmid DNA was diluted up to 200 µl with TE buffer
- Absorbance was measured at a 260 nm.
- Plasmid copy number present per minicell was determined by obtained absorbance

3.7 Selection, procurement and maintenance of cancer cell lines

- All the human cancer cell lines (A549, LNCaP, HeLa and KB) have been selected on the basis of overexpression of folate receptors. LNCaP, HeLa and KB have been selected as positive control whereas A549 was selected negative control, which has very least amount of folate receptor expression.
- All the human cancer cell lines (A549, LNCaP, HeLa and KB) have been procured from NCCS, Pune, India.
- A549 and LNCaP cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS and 1.0 mM Na pyruvate whereas HeLa and KB cell lines were maintained in DMEM medium supplemented with 10% FBS.
- Medium change has been done every alternate day.
- When flask becomes confluent, it has been split in the ratio of 1:3 by trypsinization.

3.8 Transfection of cancer cell lines

Transfection of all the cell lines was done by Lipofectamine™ 2000 transfection reagent. Lipofectamine™ 2000 Transfection Reagent is a lipid based proprietary formulation for the transfection of nucleic acids (DNA and RNA). Objective of the experiment was to transfect shRNA specific for VEGF-A gene in different human cancer cell lines in order to validate gene silencing by shRNA. Transfection scheme and detailed procedure are given as follow.

- ~1.0×10^5 cells/well have been seeded in a 24 well plate 24 hours prior to transfection. The recommended confluency for adherent cells on the day of transfection is 90-95%.
- 1 µg of DNA has been diluted in 50 µl of serum-free DMEM medium.
• 2 µl of lipofectamine reagent has been added into 50 µl of serum-free DMEM medium. Mixed immediately by pipetting.

• After 5 minute incubation, diluted DNA was combined with diluted Lipofectamine™ 2000 (total volume = 100 µl). It was mixed gently and incubated for 20 minutes at room temperature. 100 µl of complexes was added to each well containing cells and medium. To achieve even distribution of the complexes, it was mixed gently by rocking the plate back and forth.

• The plate was incubated at 37°C in a CO₂ incubator.

• Transgene expression was analyzed in the test well by growing them in selective medium 48 hours after transfection. Stable transfection was achieved by growing cells in selective medium for at least 10-15 days.

• Here, cells have been grown in medium containing geneticin for the selection of transfected cells.

• Transfection scheme has been given in the following Table 3.3.

• Transfected cells have been selected by gradual increase in the treatment of geneticin concentration.

**Table 3.3: Transfection scheme for all the cell lines**
3.9 Gene expression analysis from shRNA transfected cells

Objective of the experiment was to confirm the VEGF gene silencing in shRNA transfected cells. Total RNA was isolated from the cells using TRIzol reagent. TRIzol is a monophasic solution of phenol, guanidine isothyocyanate and other proprietary components which facilitate isolation of high quality total RNA. From the total RNA, cDNA was synthesized using verso cDNA synthesis kit. In the next step, cDNA was amplified by PCR using primer specific for VEGF A, 18s and GAPDH. Eventually, PCR products were analyze by agarose gel electrophoresis and relative gene expression was calculated in terms of GAPDH and 18s. Detailed procedure for this experiment is given below.

RNA isolation

- Total RNA was isolated form 1×10⁶ cells using the TRIzol reagent (life technologies)
- The media was removed from 25 cm² flask and cells were trypsinised, centrifuged at 1300 rpm for 7 min at 4°C and resuspended in 2 ml of phosphate buffer saline (PBS) in microcentrifuge tube.
- Cells were again centrifuged at 1300 rpm for 7 min at 4°C
- Supernatant was removed and cells were resupended in 200µl of 1X PBS.
- Cells were again centrifuged at 1300 rpm for 7 min at 4°C to remove PBS.
- 700 µl of TRIZol reagent was added in microcentrifuge tube and pellet was resuspended by vigorous shaking.
- After that, 300 µl of chloroform was added and tube was shaken until milky emulsion is formed.
- Tubes were incubated at -20°C for 15 min and then centrifuged at 12000g for 15 min at 4°C
- Centrifugation separates the mixture into 3 phases, a red organic phase [containing DNA], an interphase [containing protein] and a colourless upper aqueous phase [containing RNA]
- The aqueous phase was transferred to a fresh tube and 0.4 ml of isopropanol was added. The samples were kept overnight at -20°C for precipitation
- After overnight incubation, tubes were centrifuged at 12000g for 10 min at 4°C. The RNA forms a pellet on the side and bottom of the tube. The supernatant was removed and the pellet was washed by adding 1 ml (minimum) of 75% ethanol
• The samples were centrifuged at 7500 g for 5 min at room temperature
• The supernatant was decanted and the pellet was air dried for 5-10 min
• 20 µl of TE buffer [100mM Tris Cl (pH 8.0), 10mM EDTA (pH 8.0) was added to the RNA pellet and the tubes were heated at 60°C for 10 min for complete dissolution of the pellet
• The RNA preparation was analysed spectrophotometrically at 260nm, 270nm and 280nm. The samples having ratio of absorbance at 260nm/280nm ≥ 1.9 and ration of absorbance at 260/270 nm ratio ≥ 1.2 were used for reverse transcriptase PCR analysis. The samples were stored at -80°C
• The concentration of RNA was calculated by the following formula. Concentration of RNA (µg/ml) = absorbance at 260nm (A260) × 40 µg/ml (single stranded RNA) × dilution factor.

cDNA synthesis

• cDNA was synthesized using Verso cDNA synthesis kit, following manufacturers’ instruction.
• Briefly, 1 µg (volume made upto 1 µl) of RNA of each sample was transferred to the 0.2 ml microcentrifuge tubes.
• Master mixture was prepared as shown in table below (Table 3.4).
• Master mixture was added to individual tubes containing RNA to make total volume of 20 µl and given a short spin (20 secs).
• The reaction tubes were incubated at 25°C for 5 min followed by the incubation at 42°C for the 1 h and then reaction was stopped by heating at 70°C for 15 min.
• cDNA was used to amplify gene-specific cDNA transcript.
Table 3.4: Components of cDNA synthesis reaction

<table>
<thead>
<tr>
<th>Components</th>
<th>Amount in µl (for 20µl reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA sample</td>
<td>1</td>
</tr>
<tr>
<td>5X cDNA synthesis buffer</td>
<td>4</td>
</tr>
<tr>
<td>Random hexamers</td>
<td>1</td>
</tr>
<tr>
<td>5 mM dNTPs (deoxynucleotide triphosphate)</td>
<td>2</td>
</tr>
<tr>
<td>RT enhancer</td>
<td>1</td>
</tr>
<tr>
<td>Enzyme mix</td>
<td>1</td>
</tr>
<tr>
<td>Water</td>
<td>10</td>
</tr>
</tbody>
</table>

**PCR reaction**

- Master mixture was prepared using the components as shown in table below (Table 3.6).
- PCR reaction was performed in gradient Veriti 96 well Thermal Cycler, Applied Biosystems.
- The PCR reaction profile include initial denaturation at 94°C for 90 seconds followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing for 60 seconds at 59°C and extension at 72°C for 60 seconds.
- Thereafter final extension was done at 72°C for 600 seconds.
- Amplified products were size fractionated on 1.5% agarose gel and visualized by ethidium bromide (EtBr) staining.

Table 3.5: Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF A</td>
<td>5’-TCTTCAAGCCCATCTGTGTG-3’</td>
<td>5’-TCTGCATGTTGATGTGGAC-3’</td>
<td>102</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-CATGAGAAGTGATGACACAGCT-3’</td>
<td>5’-AGTCCTTCCACGTACCCAGT-3’</td>
<td>113</td>
</tr>
<tr>
<td>18s</td>
<td>5’-GTAACCGTGGAAACCCATT-3’</td>
<td>5’-CCATCCGCGGTAGTAGCG-3’</td>
<td>130</td>
</tr>
</tbody>
</table>
Table 3.6: Components of master mixture used for PCR amplification.

<table>
<thead>
<tr>
<th>Components</th>
<th>Amount of components (µl) (for 50 µl reaction) per sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA sample</td>
<td>2 µl</td>
</tr>
<tr>
<td>Taq polymerase buffer (10X)</td>
<td>5 µl</td>
</tr>
<tr>
<td>MgCl₂ (25 mM)</td>
<td>3 µl</td>
</tr>
<tr>
<td>dNTPs (10 mM)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Forward primer (5 µM)</td>
<td>5 µl</td>
</tr>
<tr>
<td>Reverse primer (5 µM)</td>
<td>5 µl</td>
</tr>
<tr>
<td>Taq polymerase (1 U/ µl)</td>
<td>1.2 µl</td>
</tr>
<tr>
<td>Water</td>
<td>27.8 µl</td>
</tr>
</tbody>
</table>

**Gene expression analysis of VEGF A in shRNA transfected and normal cells**

VEGF A gene expression levels were determined by semi-quantitative reverse-transcription PCR (RT-PCR). Total RNA was isolated from the normal, pSUPERneoScramble (Scramble), psNIPERDH1A1, and psNIPERDU6A2 transfected A549, LNCaP, HeLa and KB cells using TRIzol reagent. RNA samples were quantified at OD 260/280 and 1 µg RNA was used to synthesized cDNA. cDNA was amplified using primer specific for VEGF A, 18s and GAPDH as described above. Water served as a non-template control (NTC). Amplified products were analysed on 1.5% agarose gel and visualized by ethidium bromide (EtBr) staining. Relative band intensities of various PCR products were calculated using Quantity One 4.6.9 (Basic) (Bio-RAD) software in the gel documentation system (BioRAD). Gene expression of VEGF A was normalized by two house keeping genes, 18s and GAPDH, respectively and represented as relative gene expression.

**3.10 Chemosensitivity assay**

Objective of the experiment was to study change in chemosensitivity induced by transfection of cells with psNIPERDU6A2. To study this, both normal and transfected cells have been treated with different concentration of doxorubicin (10 nM to 10000 nM). After 72 hours of treatment MTT assay was performed, % cell viability and IC₅₀ were calculated. During
incubation at 37°C, a pool of cellular mitochondrial and cytosolic enzymes reduces the dye to its reduced purple colored insoluble formazan form. Hence, formazan production is indicative of cell viability. Formazan can be dissolved in DMSO and measured spectrophotometrically. The MTT assay is extremely well characterized and referenced to this day in the literature, and is often a gold standard to study cytotoxicity, chemosensitivity, etc. The assay was performed by employing following steps.

- $1 \times 10^6$ cells (A549, LNCaP, HeLa, KB, normal and transfected cells) have been plated in 96 well plate.
- After 24 hrs, cells have been replenished with respective medium have 2% FBS and treated with 10 nM, 100 nM, 1000 nM, 10000 nM of Doxorubicin and incubated for 72 hrs in CO2 incubator.
- After incubation, MTT was added (0.5 mg/ml, prepared in phenol red free RPMI 1640) and incubated for 3 hrs in dark.
- In the next step, 100µl of DMSO was added in each well and absorbance was taken in plate reader at 570 nm
- % viability was calculated by using following equation
  \[
  \text{Cell viability} \, (\%) = (A_{\text{test}}/A_{\text{control}}) \times 100
  \]
- Graph of cell viability against log concentration of doxorubicin was plotted and IC50 was calculated for each, normal and transfected cell line.

### 3.11 Transformation of *E.coli* PB114 with shRNA vectors

Objective of the experiment was to transform *E.coli* PB114 with pSUPERneoScramble and psNIPERDU6A2 in order to packaged pSUPERneoScramble vector and psNIPERDU6A2 in the minicells.

Procedure employed was same as section 3.4

### 3.12 Purification of minicells from shRNA transformed *E.coli* PB114

Objective of the experiment was to purify pSUPERneoScramble and psNIPERDU6A2 packaged minicells from the parent bacterial strain in order to develop shRNA packaged folic
acid conjugated minicells. Minicells were purified by ceftriaxone lysis method as discussed in section 3.5.1.

3.13 Isolation of shRNA vectors from purified minicells

Objective of the experiment was to confirm the presence of pSUPERrneoScramble and psNIPERDU6A2 in the purified minicells. Plasmid DNA was isolated by alkaline lysis method which is described in the section 3.6.

3.14 Quantitation of shRNA vector copy number

- Copy number of plasmid DNA present per minicell was determined by spectrophotometric method. Absorbance was measured at a 260 nm and quantity was determined based on absorbance.

3.15 Folic acid conjugation

Objective of the experiment was to conjugate folic acid on to the surface of bacterial minicells in order to active targeting of folate receptor present on cancer cells. Folic acid conjugation was performed using EDC and sulfo-NHS coupling agents. EDC and sulfo-NHS are very commonly used water soluble coupling agent which activates carboxyl groups to react with primary amine and form covalent amide bond. Here, free amines of bacterial surface protein were conjugated with $\gamma$ carboxyl group of folic acid. Following method was used to link folic acid on the minicells.

- (1× 10$^{10}$/ml) minicells were mixed with the folic acid solution dissolved in PBS (1.0 mL, 1mg/ml, total 5 mg, pH 7.6) in a 10-ml round-bottomed flask
- Then EDC (10.0 mg, 50 $\mu$mol) and sulfo-NHS (22.0 mg, 100 $\mu$mol) were added.
- Finally, DMAP (1mg) was added and final reaction mixture was make up to 5 ml.
- The reactions was proceed for about 16 h at room temperature, and the un-reactants were fully removed by quickly centrifuging at 1000 g for 30 s at room temperature for three times.
- The obtained FAs-modified minicells were redispersed in appropriate amount of PBS (pH 7.4)
• Obtained FAs-modified minicells have been loaded with FITC and observed in fluorescent microscope for its integrity.

• Moreover, optical density was also measured at 600 nm to analyzed integrity of minicells.

• Finally, Transmission electron microscopy was carried out to check minicells morphology after folic acid conjugation.

3.15.1 Quantification of folic acid conjugation

Objective of the experiment was to quantify amount of folic acid conjugated on the minicells. Amount of folic acid conjugation was quantified using spectrophotometric method by analysing reaction mixtures before and after folic acid conjugation. Folic acid was quantified by employing following steps.

- Standard curve of folic acid was prepared with a range of 4 to 28 µg/ml.
- Reaction mixtures before and after the reactions were centrifuged at 10000 g for 10 min at 24°C to remove minicells.
- After that reaction mixtures were analysed at 363 nm and the amount of folic acid were quantified.

3.15.2 Measurement of size and zeta potential of folic acid conjugated minicells

Objective of the experiment was to study the change in size after the folic acid conjugation and to determine zeta potential of the folic acid conjugated minicells. Procedure was same as section 3.5.2.

3.16 In vitro delivery of folic acid conjugated minicells

Objective of the experiment was to study the in vitro delivery of folic acid conjugated minicells in the selected human cancer cell lines. In this study all the cells have been treated with $10^{10}$ to $10^6$ folic acid conjugated minicells. Moreover, in order visualize minicells uptake, minicells have been loaded with FITC. Following steps were followed for FITC loading and subsequent delivery of minicells
**FITC loading**

Minicells have been incubated in FITC solution (1mg/ml) for 2 hours. After incubation minicells were washed three times with PBS in order to remove unbound dye. These minicells were used for the *in vitro* delivery experiment.

- A549 and LNCaP cells were grown in folate free RPMI 1640 whereas HeLa and KB cells were grown in folate free DMEM medium supplemented with 10% FBS.
- All the cells have been plated in 24 well plate at a density of $10^6$ cells per plate, a day before study.
- Next day all the cells have been treated with $10^4$ to $10^6$ FITC loaded folic acid conjugated minicells ($^{FA}$minicells$_{spNIPERDU6A2}$).
- All the treated cells have been incubated in CO$_2$ incubator for 1 hour.
- After incubation medium was removed and cells were washed with PBS three times.
- After washing, cells have been observed under fluorescence microscope for the minicells uptake.

### 3.16.1 Mechanism of minicell uptake

Objective of the experiment was to study the mechanism of delivery of folic acid conjugated minicells. To study uptake mechanism, primarily minicells have been delivered with and without folic acid conjugation at 37°C. In the third group folic acid conjugated minicells have been delivered at 4°C. Eventually in the last group folic acid conjugated minicells have been delivered in presence of free folic acid to observe the delivery in presence of free folic acid.

- A549 and LNCaP cells were grown in folate free RPMI 1640 whereas HeLa and KB cells were grown in folate free DMEM medium supplemented with 10% FBS.
- All the cells have been plated in 24 well plate at a density of $10^6$ cells per plate, a day before study.
- Next day all the cells have been treated with $10^9$ FITC loaded folic acid conjugated minicells ($^{FA}$minicells$_{spNIPERDU6A2}$) with and without excess folic acid and incubated at 37°C for 1 hour.
- Third group was treated with $10^9$ FITC loaded minicells$_{spNIPERDU6A2}$ and incubated at 37°C for 1 hour.
• Fourth group was treated with $10^9$ FITC loaded folic acid conjugated minicells ($^{\text{FA}}$minicells$_{\text{psNIPERDU6A2}}$) and incubated at 4°C for 1 hour.
• After incubation all cells have been washed three times with PBS and observed under fluorescence microscope.

### 3.16.2 Quantification minicells uptake by fluorimetry

Objective of the experiment was to quantify minicells uptake in KB and A549 cells after the in vitro delivery of FITC loaded $^{\text{FA}}$minicells$_{\text{psNIPERDU6A2}}$ in presence and absence of free folic acid. Cells were treated with 1% Triton X 100 to release bound FITC. Total protein concentration was determined. Uptake of minicells was expressed in terms of total fluorescence/mg of protein. Detailed method includes the following steps.

• A549 cells were grown in folate free RPMI 1640 whereas KB cells were grown in folate free DMEM medium supplemented with 10% FBS.
• All the cells have been plated in 24 well plate at a density of $10^6$ cells per plate, a day before study.
• Next day all the cells have been treated with $10^9$ FITC loaded folic acid conjugated minicells ($^{\text{FA}}$minicells$_{\text{psNIPERDU6A2}}$) with and without excess free folic acid and incubated at 37°C for 1 hour.
• After incubation all cells have been washed three times with PBS and observed under fluorescence microscope.
• In the next step cells were treated with 1% Triton X 100 for 20 min at 4°C.
• Total protein concentration of cells was determined by BCA method.
• After that cell lysate was analysed by fluorimetry. Excitation and emission was set at 490 and 515 respectively.
• Total fluorescence was represented per mg of protein.

### 3.17 In vitro delivery of $^{\text{FA}}$minicells$_{\text{pRNA}}$

Objective of the experiment was to study the expression of gene carried by expression vector followed by its delivery into the cells. To study this, pRNA expression vector (having GFP gene under U6 promoter) have been packaged in the minicells. Folic acid was conjugated on these minicells. In the next step these minicells have been delivered to the selected cell lines.
in vitro and expression of GFP was analyzed. Following procedure was employed to study gene expression.

- A549 and LNCaP cells were grown in folate free RPMI 1640 whereas HeLa and KB cells were grown in folate free DMEM medium supplemented with 10% FBS.
- All the cells have been plated in 24 well plate at a density of \(10^6\) cells per plate, a day before study.
- Next day all the cells have been treated with \(10^9\) \(\text{FA}_{\text{minicells}_{\text{pRNAT}}}\) and incubated at 37°C for 1 hour.
- After incubation all cells have been washed three times with PBS and replenished with respective medium.
- Cells were observed under fluorescence microscope for the expression of GFP after 72 hours.

3.18 In vitro delivery of \(\text{FA}_{\text{minicells}_{\text{psNIPERDU6A2}}}\) and gene expression

Objective of the experiment was to study the effective delivery of shRNA in the cells and whether it can silence the targeted gene, i.e. VEGF A. Detailed method is as follow.

- The study was done in all the four cell lines. There were three different groups, 1) \(\text{FA}_{\text{minicells}_{\text{Scramble}}}\) 2) \(\text{minicells}_{\text{psNIPERDU6A2}}\) 3) \(\text{FA}_{\text{minicells}_{\text{psNIPERDU6A2}}}\)
- \(10^9\) minicells from all the groups have been deliver in all the four cell lines and incubated for 1 hour in CO2 incubator.
- After incubation, cells were washed thrice with PBS and replenished with respective medium.
- RNA was isolated from all the cell line 72 hours after minicell delivery.
- Remaining procedure for gene expression analysis was same as section 3.8

3.19 Development of immunocompromised mice

Objective of the experiment was to develop immunocompromised mice for the tumor xenograft model development. Immunocompromised mice was developed by the treatment of cyclosporine, ketoconazole and cyclophosphamide. Cyclosporine interferes with the activity and growth of T cells which are key cells responsible for graft rejection. Ketoconazole is an antifungal agent which interferes with fungal synthesis of ergosterol, constitute of fungal cell
membrane. Moreover, it also inhibit cytochrome p450 enzyme CYP3A4 which metabolise cyclosporine. In this way ketoconazole help in prolong circulation of cyclosporine and simultaneously it protects from probable fungal infection. Cyclophosphamide is an alkylating agent it interferes with DNA replication. It also reduced neutrophil to a significant extent. Hence, treatment with cyclosporine, ketoconazole and cyclophosphamide suppresses majority of immune cells. Detailed procedure for the development of immunosupression is as follow.

- Healthy mice (C57 BL/6), 4-6 week old, have been divided into 7 groups (n=6).
- Groups 1,3 and 5 have been administered 5mg/kg ketoconazol and groups 2, 4 and 6 have been administered 10 mg/kg ketoconazol by oral route every day for 7 days.
- Groups 1 & 2 have been administered 10mg/kg, groups 3 & 4 (20mg/kg), groups 5 & 6 (30mg/kg), cyclosporine by intra peritoneal route everyday for 7 days.
- No treatment was given to control group.
- All the animals have been kept on normal rodent food pellet.
- Animals have been given ampioxin (0.1µg/ml) by drinking water during the study.
- After completion of study, haematology has been carried out to confirm the immunosupression.

### 3.19.1 Development of tumor xenograft in mice

Objective of the experiment was to develop tumor xenograft model in immunocompromised mice C57 BL6 mice. To develop tumor xenograft, approximately 5 million cells were injected subcutaneously into the shoulder blade of immunocompromised mice. Detail procedure is given below.

- 4-6 weeks old immunocompromised male C57 BL/6 mice have been taken.
- Cylophosphamide was injected subcutaneously at a dose of 60 mg/kg -3 day and -1 day before tumor cells injection.
- Hair were removed by waxing from the shoulder blade of animal one day before tumor cell injection.
- 0.1ml of cells (approx 5 million of A549, LNCaP and KB cells) were injected subcutaneously in the right shoulder blade of mice.
- Tumor growth was observed at the site of injection
• After tumor development, tumors were excised to confirm the tumor by histopathological analysis.

3.20 *In vivo* delivery of $^{FA}$minicells$_{psNIPERDU6A2}$

Objective of the experiment was to study the *in vivo* delivery of psNIPERDU6A2 and its effect on tumor growth and angiogenesis.

• All the mice bearing tumor xenograft of A549, LNCaP and KB have been randomized and divided into four groups (n=6).

• All the mice have been kept in individually ventilated (IVC) cages, with a relative humidity of $60 \pm 5\%$ and a temperature of $25 \pm 2^\circ C$ was maintained. A 10:14 h light:dark cycle was also regulated for this animals.

• All the animals were kept on folate free rodent diet.

• Groups and their respective treatments are given in the following Table 3.7

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Saline</td>
</tr>
<tr>
<td>2.</td>
<td>$^{FA}$minicells$^{Scramble}$</td>
</tr>
<tr>
<td>3.</td>
<td>minicells$_{psNIPERDU6A2}$</td>
</tr>
<tr>
<td>4.</td>
<td>$^{FA}$minicells$_{psNIPERDU6A2}$</td>
</tr>
</tbody>
</table>

• $10^9$ of respective minicells have been administered intravenously 1st and 3rd week after tumor volume reaches 80-100 mm$^3$

• Tumor volume was measure every week externally by digital caliper using following formula.

  $$\text{Volume (mm}^3) = (A) \times (B^2)/2,$$

  where A is the largest diameter (mm) and B is the smallest diameter (mm).

• At the end of treatment tumor regression was recorded, tumors were excised and degree of angiogenesis was recorded in all the group of animals.

• Finally, RNA was isolated from the tumor to analyzed gene expression of VEGF A.
3.21 RNA isolation, RT-PCR and *In vivo* gene expression analysis

Objective of the experiment was to confirm *in vivo* delivery of psNIPERDU6A2 and the VEGF gene silencing in $^{FA}_{\text{FA minicells}_{\text{psNIPERDU6A2}}}$ treated mice. Detailed procedure is given below.

- Tumor was excised and 1mg of tumor tissue was weighed.
- 1 ml of TriZol reagent was added. Remaining procedure was same as section 3.8
- Relative gene expression of VEGF A was calculated.

3.22 *In vivo* biodistribution of $^{FA}_{\text{FA minicells}_{\text{psNIPERDU6A2}}}$

Objective of the experiment was to study distribution of $^{FA}_{\text{FA minicells}_{\text{psNIPERDU6A2}}}$ after intravenous administration. FITC loaded $^{FA}_{\text{FA minicells}_{\text{psNIPERDU6A2}}}$ mincells have been administered intravenously into the mice bearing tumor xenograft. After 3 hours, mice were sacrificed by CO$_2$ asphyxation. All the vital organs were extracted, cryosection were taken and observe under fluorescence microscope to study distribution of $^{FA}_{\text{FA minicells}_{\text{psNIPERDU6A2}}}$. Following method was utilized to study *in vivo* biodistribution.

- Mice bearing KB tumor xenograft have been taken (n=6).
- $10^9$ FITC loaded $^{FA}_{\text{FA minicells}_{\text{psNIPERDU6A2}}}$ have been injected intravenously through the tail vein.
- 3 hrs after injection, animals were sacrificed by CO$_2$ asphyxation.
- Vital organs such as liver, heart, lung, kidney, brain, spleen along with tumor have been removed.
- Organs were frozen at -80°C and cryosection were taken using Cryotome Cryostat, sections were stained with HE stain and observed under fluorescence and light microscope.

3.23 Statistical analysis

All the data were represented as mean±SD. One-way ANOVA followed by Bonferroni correction posthoc test was applied to determine the significance difference among groups. Probability values with $p \leq 0.05$ were considered to be significant.