Chapter 3: Experimental procedures

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3.1 Cells, culture and in vitro model system

A4P and A4T cell cultures were maintained in a humidified tissue culture incubator at 37ºC, 5% CO2 atmosphere; fed with fresh medium [MEM(E) medium supplemented with non-essential amino acids (NEAA) and 5% fetal bovine serum (FBS; Invitrogen-Life Technologies)] every four days. Cells were routinely maintained in 25 cm\textsuperscript{2} tissue culture flasks (BD Biosciences) and were harvested with 0.25% trypsin/1 mM EDTA treatment when they were in logarithmic phase of growth.

3.2 Bisulfite genomic sequencing (BGS)

Principle

Bisulfite treatment of DNA converts cytosine residues to uracil, but leaves 5-methylcytosine residues unaffected. Thus, bisulfite treatment introduces specific
changes in the DNA sequence that depends on the methylation status of individual cytosine residues, yielding single-nucleotide information about the methylation status of a segment of DNA. Various analyses can be performed on the altered sequence to retrieve this information.

**Detailed methodology**

Bisulfite modification of genomic DNA (500ng-1µg) of A4P and A4T was performed according to manufacturer's protocols (EpiTect Bisulfite Kit; Qiagen). Primers for BGS were designed with Methprimer software, flanking the differentially methylated probes during A4 progression identified through MeDIP-chip (methylated DNA immune precipitation followed with promoter CpG island microarray; Li, 2007). Further, PCR amplifications was done with these BGS specific primers and 3µl of bisulfite modified gDNA in PCR mixture with final volume 25µl (Merck). The thermal cycler conditions were as follows: Initial denaturation at 95°C for 10min, 42 cycles of 95°C for 1 min, primer-specific annealing temperature (50°C-70°C) for 45 sec, 72°C for 1 min and final extension step at 72°C for 10 min. PCR products were gel purified using QiaQuick Gel Extraction Kit (Qiagen) and cloned into pGEMT easy vector (Promega). After transformation, at least 10 clones were selected following blue-white screening and sequenced by Big Dye Terminator method. Sequences were analyzed for methylation status of CpG sites using BiQ Analyzer software (Bock et al., 2005).

3.3 Semi-quantitative and quantitative reverse transcription PCR

3.3.1 RNA isolation

**Principle**

RNA extraction with Guanidinium thiocyanate-phenol-chloroform reaction primarily involves phase separation of an upper aqueous phase (containing mainly RNA) and a lower organic phase (containing mainly proteins).

**Detailed methodology**

Total RNA was extracted from cells using Trizol (commercial name of Guanidinium thiocyanate-phenol-chloroform) as per manufacturer's instructions (Invitrogen, Life Technologies). Briefly, cells were suspended in Trizol and lysed
by repeated pipetting and incubated at RT for 10 minutes to allow complete
dissociation of nucleosomal complexes. Chloroform (200μL per 1mL of Trizol)
was added to the tubes and mixed by vigorous shaking for 15secs followed by
incubation at RT for 3-5min. Samples were centrifuged at 12,000rpm for 15min at
4°C to separate the upper colorless aqueous phase from the lower red, organic
phenol-chloroform phase. The aqueous phase was transferred to fresh tube and
mixing with 500μL of iso-propyl alcohol precipitated the RNA. The samples were
incubated at RT for 10min and centrifuged at 12,000rpm for 15min at 4°C. RNA
was obtained as a white pellet, washed with 75% ethanol (1mL) at 15,000rpm for
10min at 4°C, air dried and dissolved in DEPC (di-ethyl pyro-carbonate) treated
water. RNA concentrations were determined with ND-1000 spectrophotometer
(Nano Drop Technologies, Wilmington, DE). The purity and the yield of RNA
were calculated by measuring the absorbance at 260nm and 280nm. Samples
showing absorbance ratio A260/A280 > 1.8 but < 1.99 were processed for further
studies.

3.3.2 cDNA synthesis

Principle
cDNA (complementary DNA) is synthesized from isolated RNA using the enzyme
reverse transcriptase (RNA-dependent DNA polymerase) like Moloney Murine
Leukemia Virus Reverse Transcriptase (M-MLV RT) that uses single-stranded
RNA or DNA in the presence of a primer (random primers/hexamers) and can
synthesize first-strand cDNA up to 7 kb.

Detailed Methodology
Reverse transcription reactions were set up using 2μg of total RNA, MMLV-RT
enzyme and random hexamers for 60min at 37°C. The reaction was terminated
by heating the samples at 70°C for 10min followed by snap chilling the samples
(Grillo and Margolis, 1990). The cDNA prepared were stored at -20°C till further
use.
3.3.3 Semi-quantitative PCR

**Principle**
Polymerase chain reaction (PCR) determines the gene expression levels by quantifying mRNA pre-converted into cDNA. The method relies on repeated thermal cycles of heating and cooling for melting, annealing and replication of DNA. During PCR progression newly generated DNA is being used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.

**Detailed Methodology**
For semi-quantitative PCR the cDNA was amplified using cDNA as template and primers flanking the specific gene sequences of interest, with thermo-stable *taq* polymerase and dNTP mixtures. PCR consisted of denaturation at 94°C for 45 seconds followed by 34 cycles of denaturation at 94°C for 30 seconds, annealing at optimized primer specific temperatures (50°C-70°C) for 45 seconds and extension at 72°C for 45 seconds, with a final extension at 72°C for 60 seconds was carried out. PCR products were electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining on a UV trans illuminator and images captured using gel documentation system and subjected to densitometry analysis (Syngene, Cambridge, England). mRNA expression of *Actin* and *GAPDH* was used as control.

3.3.4 Quantitative reverse transcription PCR

**Principle**
This allows for detection as well as quantification of nucleotide sequences. The strategy used involves quantification of the amplified product using DNA-binding dyes like SYBR green that produce fluorescence on binding to double-stranded (ds) DNA. An increase in DNA product during PCR therefore leads to an increased fluorescence intensity that is measured at each cycle, thus allowing DNA concentrations to be quantified.

**Detailed Methodology**
Real time-PCR was carried out using ABI Step One plus Real-Time PCR System in 96-well microtiterplate format using the ABI SYBR Green mix. PCR consisted
of denaturation at 94ºC for 45 seconds followed with 34 cycles of denaturation at
94ºC for 30 seconds, annealing at optimized primer specific temperatures (50ºC-
70ºC) for 45 seconds and extension at 72ºC for 45 seconds; a final extension at
72ºC for 60 seconds was carried out. Changes in the threshold cycle (CT) values
were calculated as:

\[ \Delta C_T = C_T \text{(test)} - C_T \text{(control)} \]

The fold difference was calculated as:

\[ \text{Fold difference} = 2^{\Delta (\Delta C_T)} \]

Housekeeping genes Actin and/or GAPDH were used to normalize gene
expression levels. Non-template controls were included to reduce the possibility
of amplification from contaminating DNA present in reaction mixture.

3.4 Chromatin immunoprecipitation (ChIP)-qPCR assay

Principle

Chromatin immunoprecipitation is the most common method for examining the
association of specific factors and modified histones with an endogenous DNA
region of interest, as well as for studying protein-DNA interactions at a genome-
wide scale. Since its initial development, the ChIP method has been expanded to
include ChIP-chip (CoC) and ChIP-Seq (CS) assays. Further, use of ChIP-qPCR
strengthens the ChIP studies by their validation.

Detailed methodology

Sub-confluent cells \((10^7)\) were cross-linked with 1% formaldehyde solution. Cells
lysate were prepared in immunoprecipitation (IP) buffer [16.7 mM Tris (pH 8),
167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, and 0.01% SDS]. Cells lysate
were sonicated to generate 400–1000 bp DNA using Sonicator (Sonics,Vibra
Cell). ChIP was performed after fragmenting (300–1000bp) genomic DNA from
A4P and A4T cells by sonication. For each immunoprecipitation, 4mg of
sonicated DNA was incubated for 12 h at 4ºC with H3K4Me3 / H3K9Me3 /
H3K27Me3 monoclonal antibodies (Upstate-Millipore). This was followed by
reverse cross linking to separate DNA from protein, and later DNA was
recovered by using Qiaquick DNA purification kit (Qiagen). qPCR analyses were
carried out using SYBR Green Mix (Life technologies) using specific gene
primers as described in above section. qPCR steps were consisted of
denaturation at 94°C for 45 seconds followed with 34 cycles of denaturation at
94°C for 30 seconds, annealing at optimized primer specific temperatures (50°C-
70°C) for 45 seconds and extension at 72°C for 45 seconds after cycling steps
final extension at 72°C for 60 seconds was carried out. Changes in the threshold
cycle (CT) values were calculated as;
\[ \Delta C_{T} = C_{T}(\text{test}) - C_{T}(\text{control}) \]
The fold difference was calculated as:
\[ \text{Fold difference} = 2^{\Delta (\Delta C_{T})} \]
For control housekeeping genes Actin or GAPDH were used to normalize the
gene expression levels.

3.5 Identification of IC\textsubscript{50} values of epigenetic drugs for A4 cells

MTT assays were performed to identify the IC\textsubscript{50} values of epigenetic drugs 5Aza-
dC, TSA, Curcumin and CBB1007 (5/3/3/7\textmu M respectively) following treatment of
A4T cells. Cells were seeded in a 96 well plate at density of 5 \times 10^3
cells/100\mu l/well and treated with epigenetic drugs (Range of concentration of
Drugs =1–10 \mu M) for 24, 48 and 72h. MTT (5mg/ml) was added to each well to a
final concentration of 0.5\mu g/\mu l and incubated at 37°C for 4hrs in an atmosphere
of 5% CO2 in 95% humidified air. The MTT crystals formed were dissolved in
solubilizing reagent [0.01N HCl in iso-propanol (v/v)] for 30min in dark at 37°C.
Absorbance was measured at 570nm with a background subtraction at 650nm
using a microplate reader (Molecular Devices, USA). The percent viable cell
number was calculated with respect to controls (untreated cells) considering later
as 100%.

3.6 Annexin V-FITC apoptosis assay

Cells were harvested and washed twice with ice-cold 1X PBS. 1 \times 10^5 cells were
suspended in 1X binding buffer [0.1M HEPES (pH 7.4); 1.4M NaCl; 25mM
CaCl2] and transferred to a 5ml FACS tube, 5\mu l of Annexin V-FITC (BD,
Bioscience) was added and incubated for 5min. Following this, 5\mu l of PI
(50μg/ml) was added to the cell suspension, mixed gently and incubated for 15min at 25°C in the dark. Finally 400μl of 1X binding buffer was added. Samples and flow cytometric data were acquired and analyzed using FACS Canto II (BD, Bioscience) and DiVa software (BD, Bioscience).

3.7 Immunofluorescence imaging
A4P and A4T cells were grown on sterile glass cover slips in 24-well plates and treated with epigenetic drugs 5Aza-dC, TSA, Curcumin and CBB1007 (5/3/3/7μM respectively for 24 to 48 hours). After treatment, media was decanted and wells were washed with 1X PBS buffer. Cells were fixed with 4% para-formaldehyde and kept on ice for 10min. Cover slips were rinsed twice with ice-chilled 1X PBS thereafter, permeabilized with 0.1% TritonX-100 for 10min, and washed with 1X PBS. Blocking was performed with 10% Goat serum at RT for 15min. PTGIS, MEST and RXRγ antibodies in 2% goat serum (Dilution 1:50-1:200) incubated for 1h at RT. Cover slips were washed twice with 1X PBS and incubated with secondary antibody for 30 min at RT. Cells were counter stained with Hoechst for nucleus and embedded in mounting medium. Images were acquired on confocal microscope (Leica, Germany).

3.8 PKH labeling of the cells
Principle
Horan et al. had developed PKH dyes by attaching fluorophore to a long aliphatic carbon backbone. PKH dye irreversibly binds to the lipid bilayer of the cell membrane without affecting cell growth and is equally partitioned among the daughter cells with subsequent cell division, resulting in the reduction of the fluorescence intensity that can be quantified by flow cytometry (Kusumbe and Bapat et. al. 2009).

Detailed methodology
Freshly harvested cells were suspended in MEM (E) with 5% FBS and centrifuged at 2000g for 5 mins, followed by subsequent washes in serum-free MEM (E)/1X PBS. 2 X 10^6 cells were suspended in 750 μL of Diluent C of the PKH labeling kit (Sigma), and 4 μM (19μL) PKH-26/67 dye in 750 μL was added
and incubated at room temperature (RT) for 7 mins. Labeling was terminated by adding 1.5ml of FBS for 3min and the cells were washed twice with serum free MEM (E) medium.

### 3.9 In vivo studies

#### 3.9.1 Animal Housing

Non-obese diabetic (NOD) /severe combined immunodeficiency (SCID) mice were used for the study, bred and maintained at the Experimental Animal Facility, National Centre for Cell Science (NCCS); all procedures involving animals were done in accordance with Institutional Ethical Animal Committee clearances, laws and policies. Animals were kept under sterile airflow conditions during the experiment.

#### 3.9.2 Generation of subcutaneous tumors in NOD/SCID mice

2 × 10^6 A4 cells (either unlabeled or PKH26/67 labeled) were subcutaneously injected into thighs of the six to eight week-old female NOD/SCID mice. After first week of injections, mice were daily evaluated for tumor growth (volume measurement). All mice were sacrificed after 28 days of injection and tumors were harvested. Harvested tumors were processed for single cell suspension or stored for mRNA and protein isolation at -80°C.

#### 3.9.3 Epigenetic drug treatments in the NOD/SCID mice

Epigenetic drugs were administered at 15th day of xenograft tumor injection. Three doses of epigenetic drugs 5-Aza-dC, TSA, Curcumin and CBB1007 (5/3/3/7mg/kg respectively) were administered at tumor (subcutaneous) site on each 3rd day followed with seven days recovery period (Fig.1). During this period treated and control group of mice were monitored for tumor growth and drug cytotoxicity through tumor volume measurement and by measuring the weight of the animal routinely respectively.

Tumor volume was calculated as Volume = Length x (Width^2)/2 cm^3.
3.10 Preparation of single-cell suspensions from tumor tissue

Harvested xenograft tumors were minced with sterile scissors and scalpel blades, and subjected to collagenase digestion (Collagenase IA #C9891 and Collagenase XI #C7657; Sigma), that was performed for 40 minutes at 37º C. After digestion, collagenase was inactivated with fetal bovine serum (FBS), followed by a centrifugation step. The single-cell suspension was filtered through 40 μm filters, and the resultant suspension was washed twice with PBS to obtain single cell suspensions.

3.11 Cell cycle analysis and demarcation of euploid and aneuploid fractions

3.11.1 Propidium iodide staining

Principle

Propidium iodide (PI) binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4–5 base pairs of DNA. PI also binds to RNA therefore; to achieve exclusive DNA staining prior RNAase treatment is required. Once the dye is bound to nucleic acids, its fluorescence is enhanced 20 to 30 folds.
Detailed methodology

For the PI staining cells were fixed with chilled 70% ethanol for 30 min. Cell pellets were then suspended in 100 μL PBS and incubated with 1 mg/ml RNase (Type IIA; #19101; Qiagen) for 30min at 37°C. Cells were then stained with 50 μg/ml propidium iodide (PI- Sigma). Acquisitions were performed on the FACS Aria (Becton Dickinson), and data analysis carried out using DiVa software (Becton Dickinson) (Fig.2).

![Fig. 2 Representative analysis of PI cell cycle analyses through FACS DiVa software.](image)

3.11.2 Hoechst and pyronin Y staining

Principle

H33342 is a "vital" DNA stain that binds preferentially to A-T base-pairs allowing the estimation of DNA content. Pyronin Y interacts with double stranded RNA and double-stranded DNA by intercalation. Since interactions of Pyronin Y with DNA are suppressed in the presence of the DNA fluorochrome Hoechst 33342, Pyronin Y can be used as the RNA-specific fluorochrome. Determining the RNA content in addition to DNA allows discriminating cells in G0 from G1 cells.

Detailed methodology

Collagenase digested tumor cells were stained with Hoechst 33342 (B2261; Sigma) at 5 μg/mL for 45 minutes at 37°C in staining buffer (HBSS + 2% FBS). Pyronin Y (P9172; Sigma) was then added to 1 μg/mL, and cells were incubated for additional 45 minutes, prior to washing and analysis by flow cytometry.
Sorting of the cells was performed on the FACS Aria (BD, Biosciences) and data analysis carried out using DiVa software (BD, Biosciences) (Fig.3).

Fig. 3 Representative profiles of Hoechst/Pyronin Y cell cycle analyses using FACS DiVa software.

3.12 Flow cytometric immune-staining and combinatorial analysis of the PI and Hoechst/Pyronin Y staining

Single cell suspensions for flow cytometry analysis of tumor samples were obtained as described above. Cells harvested from culture were washed twice in ice-cold phosphate buffered saline (PBS). Permeabilization and fixation was done using 2% p-formaldehyde and 0.1% tritonX100 at 4°C for 10 mins each. Rest of the protocol was similar for immune-staining of antigens expect that all incubations were carried out at RT. The cells were stained with primary antibodies MEST, PTGIS and RXRγ at a concentration of 3µl per 10^6 cells for 1 hr followed with washing with PBS and addition of appropriate secondary antibody at 1:300 dilution for 20 mins followed with PBS washing. Staining with PI/Hoechst/Pyronin Y was done as mentioned above. The cells were then acquired on the BD FACS AriII and analysed using FACSDiva software.

DNA content analysis performed by combining PI and Hoechst/Pyronin Y staining allows demarcation of cells in G0 and G1 phase of cell cycle which cannot be distinguished by classical propidium iodide (PI) staining. The number of cells within the euploid G0 and aneuploid G0 identified through Hoechst/Pyronin Y staining were subtracted from number of cells in G)/G1 of euploid and aneuploid fraction identified through PI staining in order to compute...
cells within G1 phase of cell cycle; the percentage of cells within the S phase and those within the G2/M phases were identified through PI staining alone.

### 3.13 Flow cytometric sorting

![Flow Cytometric Sorting Diagram](image)

**Fig. 4** An example of PKH analyses through FACS Diva software.

Single cell suspensions obtained through collagenase digestion of the PKH labeled tumors (untreated and treated) were subjected for flow cytometric sorting for three distinct fractions namely, PKH\textsuperscript{hi}, PKH\textsuperscript{lo} and PKH\textsuperscript{neg} depending on PKH intensities (Naik et al., 2015). Sorting of the cells was performed on the FACS Aria (BD, Biosciences) and data analysis carried out using Diva software (BD, Biosciences) (Fig.4). Sorting of the PKH\textsuperscript{hi}, PKH\textsuperscript{lo} and PKH\textsuperscript{neg} fractions was performed for functional assays including clonogenicity, wound healing, spheroid formation and soft agar colony formation.

### 3.14 Resolution of ITH

In order to understand the underlying mechanism of drug cytotoxicity, we delineated the specific cellular targets of each drug in relation to distinct cellular subsets resolved through flow cytometry based on the cancer stem cell (CSC) hierarchy, genetically unstable populations (aneuploidy) within xenografts and differential cell cycling (Naik et al., 2015). Briefly, label-chase of vital membrane dyes (PKH26/PKH67) resolves the CSC hierarchy as three cell subsets with a

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decreasing order of regenerative potential viz. PKH$^{hi}$ cells (quiescent CSCs), PKH$^{lo}$ (progenitors) and PKH$^{neg}$ (host and differentiated tumor cells).

Another parameter defined is genetic instability, established through DNA content profiling with propidium iodide (PI) staining that demarcates euploid and aneuploid fractions within the xenograft. PI staining also quantifies cells in basic cell cycle phases (G0/G1, S, and G2/M); supplementing this with combinatorial Hoechst-Pyronin Y staining (where Hoechst stains DNA and Pyronin Y stains RNA) further resolves individual G0 and G1 fractions. When all these three levels of resolution were superimposed 18 tumor subpopulations obtained defining the ITH (Fig.5).

### 3.15 Clonogenecity (Colony formation assay)

$5 \times 10^2$ sorted cells were added to each well of a 96-well culture plate in triplicate for each group. After incubation at 37 °C for 14 days, cells were washed twice
with PBS and stained with 0.05% crystal violet in 20% methanol. Colonies were photographed and counted with image J software (NIH, USA).

3.16 Soft Agar Assay

Cells from sorted tumor fractions were suspended in 2xMEM (Gibco) and mixed with equal volume of 0.5% low melting agarose (Sigma). This was layered above a 1% agarose pre-layer in 35 mm dish. Colonies developing after 3 weeks incubation at 37°C, 5% CO2 and humidified condition were stained with 0.005% crystal violet, photographed at 4x magnifications with Nikon DSLR and quantified using Image J software (NIH, USA).

3.17 Wound healing assay

1000 sorted cells of different tumor fraction were added into each well of a 96-well culture plate in triplicate for each group. Cells were cultured until 90% density. Thereafter, wound was created manually by scraping the cell monolayer with a 200μl pipet tip, and cultured further in medium without serum. To observe the wound healing process, area of the wounded region lacking cells was recorded for evaluation at 10x with phase contrast microscope (Olympus) at 6 hours interval till 72 hours. T scratch software was used to analyze photographs and quantify the open wound area.

3.18 Spheroid formation assay

Sorted cells of different tumor fractions were plated at a density of $5 \times 10^4$ cells/well in 24-well ultra-low attachment plates (Corning) in 1% FBS containing MEM (Gibco, United States). Fresh medium (1 mL) was added every other day to replenish medium. Developing spheroids were counted and photographed at 20x with inverted phase contrast microscope (Olympus) at day 6, 9 and 12.

3.19 Statistical analyses

Unless stated otherwise all experiments were carried out in triplicate; data values were expressed as mean ± SEM of three individual experiment sets. Two-tailed
Student’s t-test and Analysis of variance (ANOVA) test were used to determine the degree of significance in between the mean values, where $p$-value $<0.05$ was considered significant.