Chapter 2

GENESIS OF THE STUDY AND RESEARCH METHODOLOGY
2.1 Genesis of the study

As mentioned in the previous section, an \textit{in vitro} A4 progression model system of serous ovarian carcinoma (SeOvCa) was established earlier in our lab (Bapat \textit{et al.} 2005), became basis of the study that further led our interest to understand the molecular mechanism of SeOvCa progression. \textit{In vitro} culture, A4-P and A4-T cells exhibit phenotypes of early stage and advanced stage SeOvCa respectively. Aggressive, metastatic and tumorigenic potential of A4-T cells affirm its distinct growth properties and morphology from A4-P cells. We hypothesized that differential expression profiling of A4-P and A4-T proteome may provide an insight into the molecular alterations during transformation process. It may also suggest progressive acquisition of the hallmarks of cancer in the transformation process. We aimed to explore proteome of A4 SeOvCa progression model to identify transformation associated pathways and analyses of their function relevance in the disease progression. Functional characterization of some of the identified key targets further may help in understanding underlying mechanism and future implications for the prevention of disease.

2.2 Aims and Objectives of the study

The experimental objectives towards validation of the above hypothesis were as follows-

1) Analysis of differentially expressed proteins in A4 SeOvCa progression model by means of 2-Dimensional gel electrophoresis (2DE) followed by Mass Spectrometry.

2) Identification and functional characterization of differentially regulated proteins and their functional relevance to the molecular mechanism(s) of disease progression.
2.3 Study design

Signatures of pre-neoplastic lesions that lead to rapid and aggressive metastasis remain largely unknown in EOC and thus, become a major limitation in the diagnosis. Cellular heterogeneity and lack of suitable progression model system made situation more complex. Therefore, it has become increasingly important to identify altered protein profiles associated with early event of malignant transformation and advance stage of disease. A4 SeOvCa progression model system has provided an opportunity to investigate underlying mechanisms of the disease progression.

Present study comprise expression profiling of A4 SeOvCa progression model and functional analyses of key identified protein(s) found significantly enriched in individual cell types (A4-P and A4-T respectively). Aiming this, present work is divided into three parts as described above-

1. Protein profiling of A4 SeOvCa progression model to analyze differential expressed proteins in A4-P and A4-T cells. It includes 2DE mediated separation of total proteome and differential spot analyses & annotation. Analysis of selected protein spots based on their expression patterns and their identification through MALDI-TOF-TOF followed by database analyses. Prediction of protein-protein interaction and identification of functional pathways among the proteins identified within a group and expression validation of key identified proteins in order to affirm differential expression status (Figure 2.1).
A4 Progression model of SeOvCa

2DE (2-Dimension gel electrophoresis)

Profiling of A4-P and A4-T cells proteome

Analysis of differential expression patterns

Categorization of differential patterns in Qualitative and Quantitative groups

Sample processing

Peptide digestion

MALDI-TOF/TOF

Peptide mass-fingerprinting & MS/MS fragmentation

Data Mining

Database search

Data retrieval

Identification of proteins among two groups for A4-P and A4-T cells

Expression validation of identified proteins

PINA based within group protein-protein Interaction network analyses

Analyses of functional Groups (pathways)

Literature-based analyses

MetaCore analyses (Gene ontology)

Figure 2.1: Schematic diagram showing strategy towards profiling of expression of A4-P and A4-T cell proteome, identification, analyses of pathways, interactome and expression validation.

2. Functional analyses of key protein(s) identified in A4-P cells. Differential expression of such protein(s) to characterize important pathway(s) in pre-neoplastic cells. Approach for the functional analyses of such protein(s) for A4-P and A4-T cells was commonly demonstrated above (Figure 2.2).
Figure 2.2: Schematic approach towards functional characterization of key protein(s) found differentially expressed in A4-P and A4-T cells, which can be associated with the disease progression.

3. Analysis of functional relevancy of protein(s) identified and associated with significant pathways in A4-T cells. We put our emphasis to understand the regulation of such pathway(s) and their inevitability during neoplastic transformation (Figure 2.2).
2.4 Material and Method

2.4.1 Cell culture

Derivation of A4 ovarian cancer progression model of SeOvCa transformation was carried out from a patient diagnosed with Grade IV serous adenocarcinoma (Bapat, et al. 2005). A4-P and A4-T cells cultures were incubated in a cell culture incubator at 37°C in a 5% CO₂ atmosphere at 95% humidity. A4 cultures were maintained with medium-MEM (E) supplemented with 5% fetal bovine serum (FBS; Invitrogen, CA) and 1% nonessential amino acids. Cultures were harvested and reseeded on every 4 days.

2.4.2 In vitro treatments:

Natural retinoid ligand viz. 9 Cis Retinoic acid (CRA; 10μM) and synthetic retinoids Adapalene (ADA; 2μM; RAR agonist) or 4-{(E)-2-(5, 6, 7, 8 -Tetrahydro-5, 5, 8, 8-tetramethyl-2 naphthalenyl)-1-propenyl] benzoic acid (TTBPB; 10μM; RAR agonist) were used to treat A4 ovarian cancer progression model system for 48h. DMSO was taken as vehicle control. Concentration for xenograft study has been mentioned in in vivo section. Estradiol (E₂; 10nM) and cisplatin (4μM) treatments were given to A4-T cells for 48h.

2.4.3 Transient siRNA transfections

Small oligonucleotides (siRNA) of universal negative control, NPM1, RAD50 and XRCC5 were procured from MISSION siRNA (Sigma Aldrich) for transient knockdown in 24-well plate. During the procedure, 50μl of OptiMEM (Invitrogen, USA) and 3μl (10nMol) of siRNA duplex was taken in a sterile vial. Second vial constituted 50μl of OptiMEM and 2μl of Lipofectamine RNAiMax (Invitrogen). Contents of both vials were mixed and kept for 30min at room temperature (RT). 500μl DMEM containing 1% FCS was added to the mixture and added to the single well of 24-well plate. Cells were analyzed 48h after the transfection.
2.4.4 Protein extraction for 2-Dimensional gel electrophoresis (2DE)

Protein extraction from harvested cell pellets ($10^7$) of A4-P and A4-T cells was performed in 500μl ml of urea lysis buffer (8M Urea, 2M Thiourea, 100mM DTT, 2% CHAPS, 0.2% ampholytes and protease-inhibitor cocktail (PIC) (Amersham USB Guideline). Protein extract was allowed to be mixed for at least 15 minutes and incubated for 30 minutes at RT to facilitate proper protein solublisation. Extracted protein samples were further centrifuged (110,000g for 1h at 4°C) and suspension was collected. Protein concentration was estimated with 2DE Quant Kit (Amersham™, GE healthcare, USA) at 480nm (Beckman Coulter). Samples were aliquoted into working vial and stock stored at -70°C till further use.

2.4.5 2-Dimensional gel electrophoresis (2DE)

Extracted protein samples of A4-P and A4-T cells were run on first dimension based on their pl charges. In the process, 350μg of extracted lysate was taken to rehydrate 18cm immobilized pH gradient (IPG) strip (pH 4-7) and was incubated for overnight. Three step isoelectric focusing (IEF) voltage program was designed on Protean IEF cell (Bio-Rad) i.e. 50V for 20 min, 10,000V for 2h and 10,000V for 45,000V-hr. The IPG Strips were further reduced on incubation with equilibration/reduction buffer (6M Urea, 0.375M Tris pH 8.8, 2% SDS, 20% glycerol, 2% (w/v) DTT (Sigma) followed by alkylation step with the same buffer but containing 2.5% (w/v) Iodoacetamide (Sigma) instead of reducing DTT. Second dimension of 2DE was accomplished by running the IPG strips on 1mm thick 10% (w/v) SDS-PAGE on 150V. Six replicate gels were prepared from each cell type. Electrophoresed gels were stained with mass spectrometry compatible modified coomassie blue (Pierce, Thermo-Fisher) and silver stain. Image acquisition
of stained gels was done using Quantity One® software of VersaDoc™ system (Bio-Rad Laboratories, USA) with equal parametric values.

2.4.6 2DE image analyses and annotation of differential proteins

Demarcation and annotation of differentially expressed proteins among both cell types was performed with a dedicated image analysis software viz. PDQuest (advanced version 8.0 from Bio-Rad, USA). PDQuest software facilitated annotation of each and individual spot, unique identities were provided to these protein spots in all replicate gels. Analysis set were prepared based on identified proteins spots unique to A4-P and A4-T and common spots among two replicate groups with a 2.0 fold quantity variation threshold. Entire detected spot and annotation exercise led to identification of qualitative and quantitative expressed proteins across both gels. Total matched and unmatched spots were prepared to final images and differential proteins were identified in-between two cell types representative gels. In this exercise, match-sets (Master set) of six replicates of 2DE gels prepared and analyzed.

2.4.7 In-gel protein digestion and purification

Differentially identified protein spots were analyzed by PDQuest with reasonable statistical criteria followed by excision for in-gel digestion prior to identification. Selected spots were excised manually with the help of sterile sharp spot cutter. Coomassie/Silver stained slices were destained with 25mM ammonium bicarbonate (ABC). Spliced slices were dehydrated with a 2:1 mixture of 50mM ABC and 100% acetonitrile (ACN) for 3 times, 5 min each. Slices were reduced with 10mM DTT at 60°C for 1h. Further, slices were alkylated with 50mM iodoacetic acid for 15min at RT. Washing steps and dehydration of slices was repeated with 25mM ABC and ACN for 10 min and slices were finally vacuum dried. Protein digestion was performed with 50mM ABC containing 20ug/mL modified proteomic grade trypsin (Sigma-Aldrich) as per

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manufacturers guideline and placed on ice for 30min. 25 mM ABC was added onto the soaked gel to cover surface and digestion was continued overnight at 37°C. Digested peptides (in gel) were dried using vacuum concentrator and finally resuspended in 10μl of 20% ABC and 1% formic acid solution followed by extraction. Extracted peptides were processed through Zip-Tip pipette tips (Millipore, USA) for removal of existing salts in sample and extracted peptide mixtures were dissolved with matrix solution. The matrix used for MALDI analysis was α-cyano-4-hydroxycinnamic acid (Sigma) at (20) mg/ml in 50% ACN, 0.1% trifluoroacetic acid. Peptide and matrix solution were mixed, and 1 μl of the resulting solution was spotted on a stainless steel MALDI sample plate in an equal volume.

2.4.8 MALDI-TOF/TOF analyses and protein identification

Spectra from digested peptides were acquired on 4800 MALDI-TOF/ TOF mass spectrometer (AB Sciex, Framingham, MA) linked to 4000 series explorer software (version 3.5.3). Produced mass spectra were recorded in a reflector mode within a mass range from 800 to 4000Da, using a Nd:YAG 355nm laser while, acceleration voltage and extraction voltage were set on 20 kV and 18 kV respectively. Entire MS spectra were obtained from accumulation of 900 shots. Peptide standard kit (AB Sciex) was used for six point calibration of the instrument. MS/MS spectra were acquired with a total accumulation of 1500 laser shots and collision energy of 1Kv. On completion of MS survey scans, the data was processed to generate a list of precursor ions for interrogation by MS/MS. The lists of combined MS and MS/MS peak were explored using the GPSTM Explorer software version 3.6 (AB Sciex). Protein identification was done by MS/MS ion search using MASCOT (version 2.1) (http://www.martixscience.com) search engine with SwissProt database. Input search parameters were customized as follows: all entries and human taxonomy, trypsin digestion and one missed cleavage, fixed modifications: carbamidomethylation of cysteine residues, mass tolerance: 150ppm for MS and 0.4 Da for
MS/MS. Proteins having at least two unique matched peptides were selected with an identification confidence interval threshold of ≥ 95%.

2.4.9 Immunoblotting

A4-P and A4-T cells were manually harvested (trypsin-EDTA treatment), washed with 1X Phosphate Buffered Saline (PBS) and whole cell protein was extracted in RIPA buffer (50mM Tris pH 7.5, 120mM NaCl, 5mM EDTA, 50mM NaF, 1mM DTT, 0.2mM Sodium orthovanadate, 0.5% NP40, 5mM PMSF and 0.5mg/ml protease inhibitor). A4-tumors were crushed with help of tissue homogenizer in RIPA buffer. Protein concentration was estimated using DC protein assay kit (Biorad) as per manufacturer’s instructions. From each sample, 40-50μg of extracted protein was taken and denatured at 95°C with reducing dye before to load on 12.5% Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE). Resolved proteins in SDS-PAGE were further electroblotted on methanol activated PVDF membrane (GE Healthcare, USA). The membrane with transferred proteins was blocked for 1h in 5% BSA in TBS buffer containing 0.1% Tween-20 (TBS-T). Membrane was washed thrice for 10 min each with TBS-T. Primary antibody against specific protein was incubated for 5h in TBS-T at RT or overnight at 4°C followed by three TBS-T washes. Membrane was further incubated in HRP-conjugated-secondary antibody (against the species wherein primary antibody is raised) solution for 3h with constant rocking. After 3 washes of 10min each with TBS-T, membrane was probed with chemiluminescence based ECL Plus western blotting detection system (GE Amersham™, Cat. No. RPN2132). Detection was performed on X-ray film followed by development and fixation. Detected protein expression was normalized with β-actin that was probed on the same blot after clearing with stripping buffer (100mM β-marcaptoethanol, 2% SDS and 62.5mM Tris (pH-6.8). Secondary antibodies, goat anti-mouse-HRP and goat anti-rabbit HRP were purchased from
2.4.10 Co-immunoprecipitation

Lysis/wash Buffer 1: 50mM Tris (pH 7.5), 150mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 5mM PMSF and 0.5mg/ml protease inhibitor cocktail (PIC).

Wash Buffer 2: 50mM Tris (pH 7.5), 500mM NaCl, 0.1% NP-40, 0.05% Sodium deoxycholate, 5mM PMSF and 0.5mg/ml PIC.

Wash Buffer 3: 50mM Tris (pH 7.5), 0.1% NP-40, 0.05% Sodium deoxycholate, 5mM PMSF and 0.5mg/ml PIC.

Protein extraction and concentration estimation was performed as described in immunoblotting section. Protein sample (1mg) was taken for pre-clearing with protein A/G beads (Amersham, GE Healthcare) on constant rotation for 3h on 4°C. Supernatant was collected and incubated with 5ug RXR-γ antibody on constant rotation for 1h on 4°C. Protein A/G beads (50μl) were incubated to bind antibody-protein complex for 3h on 4°C. Immunocomplex was washed alternatively with each of washing buffers for 20min on 4°C on constant rotation. 30μl of SDS-loading dye (0.063M Tris-HCl (pH 6.75, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptopethanol, and 0.002% (w/v) bromophenol blue) was added to washed Immunocomplex and denatured at 99° for 3min. Sample was centrifuged (12000k, 20sec) and collected into new vial. Eluted proteins were resolved on 2-4% denaturing SDS-PAGE at 80V followed by immunoblotting Details of RXR-γ antibody used in this section is provided in annexure I.

2.4.11 RNA extraction and Semi-quantitative reverse transcription-polymer chain reaction (sQ-RT-PCR)
Trizol™ reagent (Invitrogen, USA) was used to extract total RNA from cells as per manufacturer’s guidelines (Ribaudo et al. 2001). Cells were suspended in Trizol and lysed by repeated pipetting at RT for 10min to allow complete dissociation of RNA complexes. 200μl of chloroform (for 1ml of Trizol) was added to the tubes and was mixed vigorous for 30sec and kept at RT for 5min. Sample was centrifuged at 12,000rpm for 15min at 4°C to separate the upper transparent aqueous phase from organic phenol-chloroform phase. The upper phase was collected and total RNA was precipitated by mixing with 500μl of isopropyl alcohol. Further, samples were incubated at RT for 10min and centrifuged at 12,000rpm for 15min at 4°C. A white pellet of RNA was washed with 75% ethanol at 12,000rpm for 10min at 4°C. RNA pellet was air dried and dissolved in DEPC (0.01% v/v) treated sterile water. Estimation of purity and yield of RNA was quantified by measuring the absorbance at 260nm and 280nm. Samples with absorbance ratio A260/A280>1.8 and <1.99 were considered pure. RT reactions were set up using 2μg (conc./20μl reaction) of total RNA, MMLVRT enzyme (4Unit/20μl reaction; Invitrogen, USA) and random hexamers (50ng/20μl reaction) for 60min at 37°C (additional- 2μl/ reaction 5X first strand buffer (Invitrogen), 10nM DTT, 0.5mM dNTPs and remaining Nuclease free water to make volume 20μl). The reaction was terminated by heating the samples at 70°C for 10min followed by quick chilling and cDNA samples were stored at -20°C. PCR was performed with specific primer sets. Reactions were set for different transcripts using 2μl of cDNA, 1 unit of Taq Polymerase (Invitrogen), dNTPs (200uM) and corresponding primers mix (100pmoles/20μl of reaction) in presence of MgCl2 (1.5mM). Amplification was performed under following steps-

a. Primary denaturation -94°C, 2min
b. Denaturation -94°C, 30sec
c. Annealing -60°C, 45sec
d. Extension -72°C, 45sec
e. Final Extension -72°C, 10min
f. Steps b to d were repeated for 30 cycles.
Amplified products were resolved on 1.8% Agarose gel at 80V per cm² of gel using 1X TAE buffer containing ethidium bromide. Gel was run for 30min and captured under gel documentation system (Syngene; Cambridge, UK).

2.4.12 Cell cycle analyses

Evaluation of different phases of cell cycle post retinoids treatment or silenced (NPM1, RAD50 & XRCC5) cells was performed through PI staining. Cells were harvested (Trypsin-EDTA), washed with PBS, and fixed in 70% chilled ethanol (dissolved in 1XPBS) for 15-30min at 4°C. Cells were centrifuging at 800X g for 8min and fixing solution was decanted. Cell pallet was suspended in PBS and incubated with 50µl Ribonuclease A (5mg/ml; Qiagen) for 30min at 37°C to degrade cellular DNA content. Cells were stained with 250µl propidium iodide (PI) (50µg/ml) for 1h in dark and analyzed on the FL- 2A channel of Flow cytometer (FACS Calibre, Becton Dickinson, San Diego, CA, http:// www.bdbiosciences.com) equipped with a 488nm argon laser at linear scale for cell cycle analysis. The data was analyzed by ModFit software (BD) and CellQuestPro (BD) to evaluate distribution of cells in different phases of cell cycle.

2.4.13 Annexin V-FITC apoptosis assay

Cells were harvested and washed twice with ice-cold 1X PBS. Cells (at least 1 x 10⁶ cells) were suspend in 1X binding buffer (0.1M HEPES (pH 7.4) 1.4M NaCl, 25mM CaCl₂). Further, cell suspension was transferred to a 5ml FACS tube and 5µl of Annexin V-FITC (BD, Bioscience) was added and kept for 5min and finally 5µl of PI (50µg/ml) was added. The cells suspension was mixed gently and incubated for 15min at 25°C in the dark and then 400µl of 1X binding buffer was added. Samples were acquired and analyzed at Flow Cytometer using FACS Canto II (Becton Dickinson) and DiVa software (Becton Dickinson).
2.4.14 In vivo studies

In vivo study was performed in Experimental Animal Facility, NCCS. Tumorigenesis experimentation was performed in non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mice models those were bred and maintained in the facility. In vivo study was conducted as per the norms, laws and policies of the institutional ethical committee. To generate in vivo tumors, A4-T cells (2.5x10^6) were injected subcutaneously in thighs of 4-6 week-old male mice. Mice were observed alternatively up-to 3 weeks for tumor formation. Injections of retinoids i.e. CRA, ADA and TTNPB as well in combination started while tumor size reaches 25-30mm^3 in volume, where DMSO given to vehicle control mice. Retinoids at 25μM CRA, 25μM CRA+10μM TTNPB, 5μM ADA, 5μM ADA +10μM TTNPB and 25μM CRA+5μM ADA +10μM TTNPB concentrations were injective twice per week directly into the tumor. Tumor size was measured in two perpendicular directions using Vernier's caliper. Mice were sacrificed and tumors were harvested at the end of 7th week.

2.4.15 Immunofluorescence staining

Control A4-T and siNPM1, siRAD50, siXRCC5 transfected cells were grown on sterile glass cover slips in 24-well plate and treated with cisplatin and E2 for 24-48h wherever indicated. After 24h, media was decanted and wells washed with 1X PBS buffer. Cells were fixed with 4% paraformaldehyde and were kept for 10min on ice. Cells were rinse with ice-chilled 1X PBS and were permeabised with 0.1% Triton X-100 for 2min. Cells were washed with 1X PBS and rehydrated with it for 30min at RT. Blocking was performed with 10% Goat serum at RT for 30min. NPM1, RAD50, XRCC5, H2AX-γ and p53 antibodies in 2% goat serum (Dilution 1:50-200) incubated for 1h at RT. Cells were washed twice and incubated with secondary antibody for 30 min at RT. Cells were counter stained with Hoechst for nuclear and embedded in

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mounting medium. Images were acquired on confocal microscope (Leica, Germany). Detailed list of antibodies used in study is enclosed as annexure I.

2.4.16 In-situ fluorescein cell-death detection (TUNEL) assay

Requirements: 1X PBS, 4% paraformaldehyde (in 1X PBS) pH 7.4

Permeabilization Buffer: 0.1% Triton X-100 in 0.1% Sodium citrate (freshly prepared)

Control A4 and siRNA treated cells were grown, washed and fixed as describe in immunofluorescence section. Cells were permeabilised with corresponding buffer for 2min at ice and subsequently washed for 2 times with chilled 1X PBS. Labeling solution and enzyme solution (Roche) were mixed in equal volume and 50μl of TUNEL reaction mixture was taken onto single coverslip of 96 well plates. Cells with only labeling solutions were taken as negative control. 24 well plates were kept in a humidified chamber for 1h at 37°C. Cells were washed thrice and stained with Hoechst for nuclear staining. Stained samples were acquired and analyzed on confocal microscope (Leica, Germany).

2.4.17 Giemsa staining

Cells were grown in 24 well plate for 24h and treated with Cisplatin (4μM) and E2 (10nM) for next 48h. After the duration, cells were washed twice with chilled 1X PBS and fixed with ice-cold absolute methanol for 20 min at 4°C. Cells were rinsed once with 1X PBS and incubated with Giemsa stain for 30 min at RT. Cells were washed twice with 1X PBS and observed by microscopy (Olympus Co., Tokyo, Japan) at 40X magnifications.
2.4.18 Analysis of protein-protein interaction networks

Protein Interaction Network Analysis (PINA) platform was used to predict protein-protein interactions within a group. PINA as integrated platform used for protein network construction, analyses and visualization (Wu et al. 2009). PINA integrates protein-protein interaction data from public curated databases that were mined to generate the protein-protein interaction networks.

2.4.19 MetaCore™ based prediction and analysis of protein network

Network analysis of differentially expressed proteins within subgroups was performed using the MetaCore Analytical suite (GeneGo Inc., St. Joseph, MI, USA). MetaCore is a web-based computational platform designed for system biology that provides analysis of protein interactions in context of regulatory networks and signaling pathways. MetaCore was used to calculate the statistical significance (p-value) based on the probability of assembly from a random set of nodes (proteins) of the same size as the input list. To build the network of differentially expressed proteins, we applied the shortest paths algorithm to establish directed paths between the selected objects. Details of MetaCore algorithm and statistics are provided in annexure-II.

2.4.20 Statistical analysis

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 was used to determine the degree of significance in between the mean values, where p value <0.05 was considered significant. Analysis of variance (ANOVA) test was performed to compare values of gene, protein expression and tumor volume over time between treatment groups at a significance level of <0.05. Student-Bonferroni test was used to evaluate sub-comparisons to control the test-wise error rate.