Expression Proteomics Predicts Loss of RXR-γ during Progression of Epithelial Ovarian Cancer

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Abstract

The process of cellular transformation involves cascades of molecular changes that are modulated through altered epigenetic, transcription, post-translational and protein regulatory networks. Thus, identification of transformation-associated protein alterations can provide an insight into major regulatory pathways activated during disease progression. In the present protein expression profiling approach, we identified differential sets of proteins in a two-dimensional gel electrophoresis screen of a serous ovarian adenocarcinoma progression model. Function-based categorization of the proteins exclusively associated with pre-transformed cells identified four cellular processes of which RXR-γ is known to modulate cellular differentiation and apoptosis. We thus probed the functional relevance of RXR-γ expression and signaling in these two pathways during tumor progression. RXR-γ expression was observed to modulate cellular differentiation and apoptosis in steady-state pre-transformed cells. Interestingly, retinoid treatment was found to enhance RXR-γ expression in transformed cells and sensitize them towards apoptosis in vitro, and also reduce growth of xenografts derived from transformed cells. Our findings emphasize that loss of RXR-γ levels appears to provide mechanistic benefits to transformed cells towards the acquisition of resistance to apoptosis hallmark of cancer, while effective retinoid treatment may present a viable approach towards sensitization of tumor cells to apoptosis through induction of RXR-γ expression.

Introduction

A contemporary view of tumorigenesis is that transformation results as a multi-step process involving genetic, epigenetic, cellular and tissue-associated changes [1,2,3]. These effect alterations in several regulatory and functional networks within the cell that lead to a progressive acquisition of capabilities of self-sufficiency in growth signals, insensitivity to anti-growth signals, unlimited replicative potential, evasion of apoptotic signals, tissue invasion and metastasis, and sustained angiogenesis [4]. More lately, energy metabolism reprogramming and evading immune destruction have received recognition as additional hallmarks of transformation [5].

Epithelial Ovarian Cancer (EOC) is recognized as the fifth most common cancer and the highest cause of cancer-related deaths among women [6,7]. A limitation in EOC studies is the lack of identification of pre-neoplastic lesions that lead to rapid and aggressive metastasis, at which stage the disease is most frequently diagnosed. This is further made more complex from recent findings that suggest high-grade EOC to originate in the fallopian tube epithelia, in contrast to the classical opinion of the ovarian surface epithelium being the cell of origin [8,9]. Though contemporary proteome analyses provide a dynamic and efficient source of identification of tumor suppressors, oncogenes, cancer diagnostics and therapeutics [10,11,12,13], an extended understanding of the multi-step transformation events in EOC vis-a-vis altered molecular expressions among transformed and pre-transformed cells remains to be resolved.

The present study is based on proteomic profiling of an in vitro model of serous ovarian adenocarcinoma (SeOvCa) established earlier in our lab [14]. Briefly, we had established several single-cell clone derived cultures from the malignant ascites of a Grade IV serous ovarian adenocarcinoma patient. Nineteen of these underwent spontaneous immortalization and were established as continuous lines. The A4 clone was one of these clones. In its initial passages, it was seen to be slow-cycling and non-tumorigenic; however, around passage 20–25 it transformed into an aggressively tumorigenic clone with metastatic capabilities. This data suggests that early A4 cells, although lacking tumorigenicity had already acquired some of the features of transformation. Hence we referred to these as being pre-transformed (A4-P), while the transformed cells derived from A4-P cells were termed as A4-T. This provided us a suitable progression model of two functionally discrete cell groups derived from a single clone in the tumor. Proteome profiles of this A4 progression model resolved through 2-Dimensional Gel Electrophoresis (2DE) followed by MS (MALDI-TOF/TOF) led to the derivation of specific protein groups based on their exclusive and differential expression patterns.

Characterization of the functional networks defined by such proteins provided a clear insight into altered cellular functionality and major pathways involved in ovarian cell transformation. Of these, RXR-γ modulated cellular differentiation and apoptosis were exclusive to the pre-transformed cells. Modulation of retinol metabolism has been suggested in association with EOC
progression [15,16] in which decreased levels of CRBP1 (cellular retinol-binding protein) are considered a crucial step in progression of the transformation process [17]. However, the precise relevance of RXR-γ signaling remains largely uncharacterized. We resolved its functional role in the transformed cells of our progression model through induction of expression by treatment with selective retinoids including 9Cis-Retinoic acid (CRA), Adapalane (ADA) and 4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid (TTNPB). Such modulation of cellular differentiation and apoptosis by RXR-γ in SeOvCa further extends our current understanding of cellular transformation.

Results

Comparative protein expression analysis of A4-P and A4-T epithelial ovarian cancer progression model

The functionally different A4-P and A4-T epithelial ovarian cancer cells exhibit a distinct phenotype, with the former being spindle-shaped while the latter appear epithelial-like in morphology (Fig. 1A). 2-DE gels were prepared using proteome samples of A4-P and A4-T cells. Two technical sets of 2-DE analytical gels were prepared from each phenotype in each experiment, which was carried out in triplicate (total 6 replicates) and silver-stained. Scanned images were processed using PDQuest and proteins with differential expression were annotated. An average, 400-500 differential protein spots were thus demarcated. Annotation of spots led to the derivation of protein sets based on their expression patterns in each cell type. Towards identification of differential protein expression, selected protein spots were digested and mass spectra was generated in MS/MS analyses. GPS Explorer software (v.3.6) was used to submit the combined MS and MS/MS data from MALDI-TOF/TOF to Mascot against SwissProt database. For all proteins thus analysed, reasonable sequence coverage, low index of mass errors and high confidence interval (CI ≥ 95%) were obtained.

Derivation of the protein groups based on expression pattern

MS/MS based protein identification led to derivation of two groups of differentially expressed proteins (Fig. 1B). Group I comprised of proteins that were expressed qualitatively (exclusive-

![Figure 1. Expression profiling of the proteome of serous ovarian adenocarcinoma progression model.](image-url)
### Table 1. Details of proteins identified through 2DE followed by MALDI-TOF (MS/MS) analysis in Group I.

#### Sub-group I. Proteins qualitatively expressed in A4-P cells.

| Spot No. | Accession No. | Description of identified proteins | Function | SwissProt Accession | Gene name | Gene ID | Mass (Da)/PI | Peptide matched | Score (%) | Sequence coverage | RMS (ppm) | Validation method |
|----------|----------------|-----------------------------------|----------|---------------------|-----------|---------|--------------|---------------|-----------|-------------------|-----------|------------------|
| 1        | P07339         | Cathapsin D precursor             | Cell death/proteolysis          | CATD_HUMAN | CTSD      | 1509    | 44524/6.10   | 11            | 244       | 37                | 43        | 2D               |
| 2        | P29992         | Guanine nucleotide binding protein G(y) alpha subunit | Protein amino adic ADP-ribosylation | GNA11_HUMAN | GNA11      | 2767    | 42097/5.11   | 7             | 49         | 20                | 49        | 2D               |
| 3        | Q9P2J3         | Kelch like protein -9            | Ub1 conjugation pathway          | KLHL9_HUMAN | KLHL9     | 55958   | 69383/5.92   | 13            | 39         | 23                | 73        | 2D               |
| 4        | Q8NEV4         | Myosin 3A                        | Autophosphorylation and response to stimulus | MYO3A_HUMAN | MYO3A      | 53904   | 18596/9.0    | 21            | 41         | 14                | 64        | 2D               |
| 5        | Q8WXW3         | Progestrone induced blocking factor-1 | Progestrone mediator            | PIB1_HUMAN | PIBF1     | 10464   | 89719/5.77   | 13            | 43         | 20                | 30        | 2D               |
| 6        | Q8NEV4         | Guanine nucleotide binding protein G(y) alpha subunit | Protein amino adic ADP-ribosylation | GNA11_HUMAN | GNA11      | 2767    | 42097/5.11   | 7             | 49         | 20                | 49        | 2D               |
| 7        | Q15293         | Reticulocalbin 1 precursor      | endoplasmic reticulum lumen     | RCN1_HUMAN | RCN1      | 5954    | 38866/4.86   | 8             | 68         | 23                | 22        | 2D               |
| 8        | P08758         | Annexin A5                       | anti-apoptosis/blood coagulation | ANXA5_HUMAN | ANXA5     | 308     | 35783/4.94   | 18            | 243        | 66                | 23        | 2D               |
| 9        | Q13162         | Peroxisome proliferator 4        | I-kappaB phosphorylation/cell redox homeostasis | PRDX4_HUMAN | PRDX4     | 10549   | 30521/5.86   | 11            | 208        | 49                | 11        | 2D               |
| 10       | P09493         | Tropomyosin 1 α chain           | Actin binding and cellular dynamics | TPM1_HUMAN | TPM1      | 7168    | 32689/4.69   | 11            | 146        | 21                | 10        | 2D               |

#### Sub-group II. Proteins qualitatively expressed in A4-T cells.

| Spot No. | Accession No. | Description of identified proteins | Function | SwissProt Accession | Gene name | Gene ID | Mass (Da)/PI | Peptide matched | Score (%) | Sequence coverage | RMS (ppm) | Validation method |
|----------|----------------|-----------------------------------|----------|---------------------|-----------|---------|--------------|---------------|-----------|-------------------|-----------|------------------|
| 1        | P13010         | ATP-dependent DNA helicase II, DNA Repair and telomerase maintenance | KU86_HUMAN | XRCC5 | 7520    | 82521/5.55 | 17            | 194        | 24                | 20        | 2D: IB           |
| 2        | Q9Y696         | Chloride intracellular channel protein 4 | Cell differentiation and chloride transport | CLIC4_HUMAN | CLIC4     | 25932   | 28754/5.45   | 9             | 123        | 44                | 28        | 2D               |
| 3        | Q05823         | 2-5A-dependent ribonuclease (Ribonuclease L) | mRNA processing and protein phosphorylation | RNASEL | RNASEL     | 6041    | 83481/6.20   | 13            | 33         | 20                | 54        | 2D               |
| 4        | P49368         | T-complex protein 1, gamma subunit | Protein folding | TCPG_HUMAN | CCT3      | 7203    | 60364/6.10   | 12            | 56         | 22                | 24        | 2D               |
| 5        | P68104         | EF-1-alpha-1                      | Translation elongation | EEF1A1_HUMAN | EEF1A1     | 1915    | 50109/9.10   | 9             | 80         | 22                | 15        | 2D               |
| 6        | P09104         | Gamma enolase                    | Glycolysis | ENO2_HUMAN | ENO2      | 2026    | 47108/4.91   | 8             | 98         | 27                | 22        | 2D               |
| 7        | O00629         | Importin alpha-4 subunit         | Protein transport | IMA4_HUMAN | KPN4A     | 3804    | 57815/4.80   | 9             | 142        | 22                | 21        | 2D               |
| 8        | P06748         | Nucleophosmin (NPM)              | Anti-apoptotic function         | NPM1_HUMAN | NPM1      | 4869    | 32555/6.64   | 6             | 149        | 28                | 50        | 2D: IB           |
| 9        | P30101         | Protein disulfide-isomerase A3 precursor | Redox homeostasis and signal transduction | PDI3_HUMAN | PDI3      | 2923    | 56747/5.98   | 15            | 146        | 30                | 34        | 2D               |
| 10       | P14618         | Pyruvate kinase                  | Gycolysis & programmed cell death | KPYM_HUMAN | PKM2      | 5315    | 57769/7.95   | 15            | 121        | 35                | 51        | 2D               |
| 11       | Q12765         | Secernin 1                       | Exocytosis and proteolysis      | SCRN1_HUMAN | SCRN1     | 9805    | 46353/4.66   | 12            | 60         | 33                | 61        | 2D               |
| 12       | P02768         | Serum albumin precursor          | Starvation response and anti-apoptotic | ALBU_HUMAN | ALB1      | 213     | 69321/5.92   | 9             | 145        | 12                | 61        | 2D               |
Table 1. Cont.

| Spot No. | Accession No. | Description of identified proteins | Function | SwissProt Accession | Gene name | Gene ID | Mass (Da)/PI | Peptide matched Score (%) | Sequence coverage | RMS (ppm) | Validation method |
|----------|--------------|-----------------------------------|----------|---------------------|-----------|---------|--------------|---------------------------|------------------|-----------|---------------------|
| 13       | P30049       | ATP synthase delta chain          | ATP Catabolism | ATPD_HUMAN          | ATP5D     | 513     | 17479/5.38   | 3                         | 85               | 17        | 25 2D               |
| 14       | P43686       | 26S protease regulatory subunit 6B | Protein catabolic process | PRS6B_HUMAN         | PSMC4     | 5704    | 47337/5.09   | 11                        | 81               | 26        | 25 2D               |
| 15       | P35998       | 26S protease regulatory subunit 7 | Protein catabolic process | PRS7_HUMAN          | PSMC2     | 5701    | 48472/5.72   | 23                        | 367              | 52        | 34 2D               |
| 16       | Q92878       | DNA repair protein RAD50 (hRAD50) | DNA Unwinding | RAD50_HUMAN         | RAD50     | 10111   | 135797/6.48  | 22                        | 31               | 16        | 73 2D: IB           |
| 17       | P49411       | Elongation factor Tu, (EF-Tu) (P43) | Translational elongation | EFTU_HUMAN          | TUFM      | 7284    | 49510/7.26   | 15                        | 238              | 34        | 47 2D               |
| 18       | Q02790       | FK506-binding protein 4 (PPIase) | Protein folding | FKBP4_HUMAN         | FKBP4     | 2288    | 51641/5.35  | 9                         | 99               | 23        | 46 2D               |
| 19       | P10809       | Hsp60                              | Protein stabilization | CH60_HUMAN          | HSPD1     | 3329    | 61016/5.70   | 24                        | 708              | 45        | 28 2D               |
| 20       | P32119       | Peroxiredoxin 2                    | Anti-apoptosis and maintenance of redox homeostasis | PRDX2_HUMAN       | PRDX2     | 7001    | 21878/5.66   | 11                        | 390              | 40        | 23 2D               |
| 21       | P13489       | Placental ribonuclease inhibitor (IRA) | mRNA catabolism & angiogenesis regulation | RIN_HUMAN          | RNH1      | 6050    | 49810/5.71   | 17                        | 730              | 75        | 29 2D               |
| 22       | P31943       | Heterogeneous nuclear ribonucleoprotein H | mRNA processing and regulation | HNRH1_HUMAN        | HNRNPH1   | 3187    | 49067/5.89   | 14                        | 92               | 38        | 18 2D               |
| 23       | P06576       | ATP synthase beta chain, mitochondrial precursor | Angiogenesis & ATP synthesis | ATPB_HUMAN         | ATP5B     | 506     | 56525/5.26   | 15                        | 81               | 37        | 27 2D               |
| 24       | P45973       | Chromobox protein homolog 5        | Chromatin assembly and disassembly | CBX5_HUMAN         | CBX5      | 23468   | 22211/5.71   | 10                        | 144              | 60        | 24 2D               |
| 25       | P51571       | Translocon-associated protein, delta subunit precursor | Intercellular protein transport | SSRD_HUMAN         | SSR4      | 6748    | 18987/5.76   | 5                         | 236              | 36        | 16 2D               |
| 26       | P00441       | Superoxide dismutase [Cu-Zn]       | Apoptosis and cellular maintenance | SODC_HUMAN         | SOD1      | 6647    | 15795/5.70   | 3                         | 62               | 29        | 11 2D               |
| 27       | P61326       | Mago nashi protein homolog         | mRNA processing | MGN_HUMAN          | MAGOH     | 4116    | 17153/5.74   | 9                         | 280              | 54        | 17 2D               |
| 28       | O94832       | Myosin 1d                          | Cellular dynamics | MYO1D_HUMAN         | MYOD1     | 4642    | 116129/9.44  | 19                        | 46               | 15        | 52 2D               |
| 29       | P62306       | Small nuclear ribonucleoprotein F | Spliceosomal snRNP assembly | RUXF_HUMAN         | SRNPF     | 6636    | 9719/4.70    | 5                         | 226              | 33        | 31 2D               |
| 30       | P49720       | Proteasome subunit beta type 3     | Protein catabolism | PSB3_HUMAN         | PSMB3     | 5691    | 22933/6.14   | 8                         | 96               | 49        | 55 2D               |
| 31       | P61769       | Beta-2-microglobulin precursor     | Antigen processing and immune response | B2MG_HUMAN         | B2M       | 567     | 13706/6.06   | 2                         | 44               | 13        | 16 2D               |
| 32       | Q13011       | Delta3,5-delta2,4-dienoyl-CoA isomerase, mitochondrial precursor | Fatty acid beta oxidation | ECH1_HUMAN         | ECH1      | 1891    | 35971/6.61   | 6                         | 76               | 15        | 25 2D               |
| 33       | P25786       | Proteasome subunit alpha type 1   | Macropain subunit | PSA1_HUMAN         | PSMA1     | 5682    | 29337/6.15   | 7                         | 119              | 29        | 16 2D               |
| 34       | P50990       | T-complex protein 1, theta         | Protein folding | TCPQ_HUMAN         | CCT8      | 10694   | 59451.5      | 7                         | 46               | 15        | 52 2D               |
### Table 2. Details of proteins identified through MALDI-TOF (MS/MS) analysis in Group II.

#### Sub-group I. Proteins differentially up-regulated in A4-P cells.

| Spot No. | Accession No. | Description of identified proteins | Function | SwissProt Accession | Gene name | Gene ID | Mass (Da)/PI | Peptide matched | Score | Sequence coverage [%] | RMS (ppm) | Fold change (A4-P/T) | Validation method |
|----------|---------------|-----------------------------------|----------|---------------------|-----------|---------|-------------|----------------|-------|----------------------|-----------|----------------------|-------------------|
| 1        | P84103        | Splicing factor, arginine/serine-rich 3 | RNA splicing and processing | SFRS3_HUMAN | SFRS3 | 6428 | 19318/11.64 | 9 | 153 | 54 | 48 | 2.608228 | 2D: IB |
| 2        | O94925        | Glutaminase | glutamine catabolic process | GLS_HUMAN | GLS | 2744 | 73417/8.85 | 15 | 540 | 36 | 19 | 2.103207 | 2D |
| 3        | P31937        | 3-hydroxyisobutyrate dehydrogenase, mitochondrial | oxidation reduction | HIBADH_HUMAN | HIBADH | 11112 | 35306/8.38 | 8 | 199 | 27 | 33 | 2.88171 | 2D |
| 4        | P08754        | Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 | negative regulation of adenylate cyclase activity | GNAI3_HUMAN | GNAI3 | 2773 | 40375/5.51 | 14 | 166 | 36 | 30 | 4.086129 | 2D |
| 5        | P62873        | Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit alpha | Ras protein signal transduction/hormone signaling | GNB1L_HUMAN | GNB1L | 2782 | 37353/5.6 | 13 | 311 | 44 | 24 | 2.789543 | 2D |
| 6        | P31930        | Ubiquinol cytochrome C reductase complex core protein I | aerobic respiration/protein transport | UQRC1_HUMAN | UQRC1 | 7384 | 52585/5.94 | 20 | 592 | 53 | 16 | 3.247917 | 2D |
| 7        | P28331        | NADH ubiquinone oxidoreductase subunit | ATP metabolic process/apoptosis/protein transportation | NDUF51_HUMAN | NDUF51 | 4719 | 79465/5.89 | 21 | 249 | 42 | 35 | 13.41311 | 2D: IB |
| 8        | O43707        | Alpha actinin-4 | cellular component movement/protein transport/regulation of apoptosis | ACTN4_HUMAN | ACTN4 | 81 | 10478/5.27 | 31 | 462 | 37 | 38 | 2.19378 | 2D |
| 9        | Q07955        | Splicing factor, arginine/serine-rich 1 | mRNA splice site selection | SFRS1_HUMAN | SFRS1 | 6426 | 27597/10.37 | 6 | 172 | 43 | 29 | 6.715184 | 2D: IB |
| 10       | Q14974        | Importin beta subunit | NLS-bearing substrate import into nucleus/protein import & translocation | IMB1_HUMAN | IMB1 | 3837 | 97108/4.68 | 30 | 926 | 50 | 11 | 11.88048 | 2D: IB |
| 11       | Q86UE8        | Serine/threonine-protein kinase 1 (tousled-like 2) | intracellular signaling pathway/chromatin modification | TLK2_HUMAN | TLK2 | 11011 | 80606/8.6 | 17 | 45 | 15 | 73 | 6.09533 | 2D |
| 12       | P68363        | Tubulin alpha-ubiquitous chain | microtubule-based movement/protein polymerization | TUBA1B_HUMAN | TUBA1B | 10376 | 50120/4.94 | 11 | 88 | 33 | 28 | 4.44974 | 2D |
| 13       | P20700        | Lamin B1 | protein binding/structural molecule activity | LMNB1_HUMAN | LMNB1 | 4001 | 66237/5.11 | 20 | 220 | 37 | 26 | 2.990194 | 2D |
| 14       | P61978        | Heterogeneous nuclear ribonucleoprotein K (hnRNP K) | RNA splicing/mRNA processing/signal transduction | HNRNPK_HUMAN | HNRNPK | 3190 | 50944/5.39 | 18 | 425 | 40 | 37 | 2.46555 | 2D |
| 15       | P10121        | 78 kDa glucose-regulated protein | ER-associated protein catabolic process/anti-apoptosis | HSPAS_HUMAN | HSPAS | 3309 | 72888/5.07 | 14 | 753 | 52 | 18 | 26.707 | 2D |
| 16       | O75116        | Rho-associated protein kinase 2 | cytokinesis/protein amino acid phosphorylation | ROCK2_HUMAN | ROCK2 | 9475 | 160812/5.75 | 31 | 73 | 18 | 50 | 7.12232 | 2D |
| 17       | P22314        | Ubiquitin-activating enzyme 1 (A159 protein) | cell death/protein modification process | UBA1_HUMAN | UBA1 | 7317 | 117774/5.49 | 24 | 339 | 25 | 20 | 2.52764 | 2D |
| 18       | P05388        | 60S acidic ribosomal protein P0 | ribosome biogenesis/translational elongation | RPLP0_HUMAN | RPLP0 | 6175 | 34252/5.71 | 12 | 287 | 43 | 23 | 2.007637 | 2D |
| 19       | P62140        | Serine/threonine protein phosphatase PP1-beta catalytic subunit (PP-1B) | cell cycle/cell division/glycogen metabolic process | PPP1C_HUMAN | PPP1C | 5500 | 37163/5.84 | 7 | 91 | 19 | 45 | 16.02527 | 2D |
### Table 2. Cont.

#### Sub-group I. Proteins differentially up-regulated in A4-P cells.

| Spot No. | Accession No. | Description | Function | SwissProt Accession | Gene ID | Mass (Da)/PI | Peptide matched Score | RMS (ppm) | Fold change (A4-P/T) | Validation method |
|----------|---------------|-------------|----------|---------------------|---------|-------------|---------------------|-----------|---------------------|-------------------|
| 20       | P03935        | DJ-1 protein (Oncogene DJ1) | cell death/regulation of androgen receptor signaling pathway | PARK7_HUMAN | 11315/19876/6.33 8 | 221 53 18 | 2.102286 2D | 2.102286 2D | 2.102286 2D | 2.102286 2D |
| 22       | P24934        | Elongation factor 1-beta (EF-1-beta) | | | 50631/6.11 18 | 324 44 31 | 2.078904 2D | 2.078904 2D | 2.078904 2D | 2.078904 2D |
| 23       | P24937        | Tropomyosin alpha 4 chain (Tropomyosin 4) | cellular component movement/muscle contraction | | 11315/19876/6.33 8 | 221 53 18 | 2.102286 2D | 2.102286 2D | 2.102286 2D | 2.102286 2D |
| 24       | P03936        | F-box/WD-repeat protein 1A | Wnt receptor signaling pathway/ubiquitin-dependent protein catabolic process | | 50631/6.11 18 | 324 44 31 | 2.078904 2D | 2.078904 2D | 2.078904 2D | 2.078904 2D |
| 25       | Q92972        | Transportin 1 (Importin beta-2) | interspecies interaction between organisms/protein import into nucleus | | 11315/19876/6.33 8 | 221 53 18 | 2.102286 2D | 2.102286 2D | 2.102286 2D | 2.102286 2D |
| 26       | P06733        | Alpha enolase 2-phospho-D-glycerate hydro-lyase | glycolysis/negative regulation of cell growth | | 11315/19876/6.33 8 | 221 53 18 | 2.102286 2D | 2.102286 2D | 2.102286 2D | 2.102286 2D |
| 27       | P13635        | Cytokeratin 10 | | | 50631/6.11 18 | 324 44 31 | 2.078904 2D | 2.078904 2D | 2.078904 2D | 2.078904 2D |
| 28       | Q03252        | Lamin B2 | Structural molecule activity | | 50631/6.11 18 | 324 44 31 | 2.078904 2D | 2.078904 2D | 2.078904 2D | 2.078904 2D |
| 29       | Q92973        | Tropomyosin alpha 4 chain (Tropomyosin 4) | cellular component movement/muscle contraction | | 11315/19876/6.33 8 | 221 53 18 | 2.102286 2D | 2.102286 2D | 2.102286 2D | 2.102286 2D |
| 30       | Q92974        | Vimentin | | | 50631/6.11 18 | 324 44 31 | 2.078904 2D | 2.078904 2D | 2.078904 2D | 2.078904 2D |

#### Sub-group II. Proteins differentially upregulated in A4-T cells.

| Spot No. | Accession No. | Description | Function | SwissProt Accession | Gene ID | Mass (Da)/PI | Peptide matched Score | RMS (ppm) | Fold change (A4-T/P) | Validation method |
|----------|---------------|-------------|----------|---------------------|---------|-------------|---------------------|-----------|---------------------|-------------------|
| 1        | P24937        | F-box/WD-repeat protein 1A | | | 50631/6.11 18 | 324 44 31 | 2.078904 2D | 2.078904 2D | 2.078904 2D | 2.078904 2D |
| 2        | P05787        | Cytokeratin 8 | | | 50631/6.11 18 | 324 44 31 | 2.078904 2D | 2.078904 2D | 2.078904 2D | 2.078904 2D |
| 3        | P05786        | Cytokeratin 8 | | | 50631/6.11 18 | 324 44 31 | 2.078904 2D | 2.078904 2D | 2.078904 2D | 2.078904 2D |
| 4        | P15954        | Protein disulfide isomerase A5 | | | 50631/6.11 18 | 324 44 31 | 2.078904 2D | 2.078904 2D | 2.078904 2D | 2.078904 2D |
| 5        | P32728        | Sporine synthase | | | 50631/6.11 18 | 324 44 31 | 2.078904 2D | 2.078904 2D | 2.078904 2D | 2.078904 2D |
| 6        | Q0410         | Importin subunit 3 | | | 50631/6.11 18 | 324 44 31 | 2.078904 2D | 2.078904 2D | 2.078904 2D | 2.078904 2D |
| 7        | P16252        | Ezhediospiasum precursor | | | 50631/6.11 18 | 324 44 31 | 2.078904 2D | 2.078904 2D | 2.078904 2D | 2.078904 2D |

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| Spot no | Accession no. | Description of identified proteins | Function | SwissProt Accession | Gene name | Gene ID | Mass (Da)/PI | Peptide matched Score | Sequence coverage [%] | RMS (ppm) | Fold change (A4-T/P) | Validation method |
|---------|---------------|-----------------------------------|----------|---------------------|-----------|---------|-------------|---------------------|---------------------|------------|------------------|-------------------|
| 9       | Q14764        | Major vault protein (MVP) mRNA and protein transport | MVP_HUMAN | MVP                 | 9961      | 99135/5.34 | 23           | 214                 | 28                  | 20         | 7.717112         | 2D                |
| 10      | P08238        | Heat shock protein HSP 90-beta protein folding | HSP90A1_HUMAN | HSP90A1          | 3326      | 83081/4.97 | 16           | 64                  | 17                  | 40         | 5.908433         | 2D                |
| 11      | Q99426        | Tubulin-specific chaperone B cell differentiation/nervous system development | TBCB_HUMAN | TBCB                | 1155      | 27308/5.06 | 9            | 79                  | 33                  | 45         | 3.162533         | 2D                |
| 12      | Q8BT78        | COP9 signalosome complex subunit 4 protein binding | COP54_HUMAN | COP54              | 51138     | 46240/5.57 | 15           | 207                 | 37                  | 17         | 2.618724         | 2D                |
| 13      | Q15372        | eIF-3 gamma (eIF3 p40 subunit) regulation of translational initiation | EIF3H_HUMAN | EIF3H              | 8667      | 39905/6.09 | 13           | 71                  | 28                  | 37         | 3.177448         | 2D                |
| 14      | P29692        | Elongation factor 1-delta (EF-1-delta) positive regulation of I-kappaB kinase/NF-kappaB cascade/ translational elongation | EEF1D_HUMAN | EEF1D              | 1936      | 30972/4.90 | 11           | 204                 | 43                  | 5          | 4.075992         | 2D                |
| 15      | Q15366        | Poly(rC)-binding protein 2 (Alpha-C2) RNA splicing/mRNA metabolic process | PCBP2_HUMAN | PCBP2              | 5094      | 38556/6.33 | 10           | 72                  | 35                  | 41         | 4.952897         | 2D                |
| 16      | P28070        | Proteasome subunit beta type 4 precursor anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process | PSMB4_HUMAN | PSMB4              | 5692      | 29173/5.72 | 7            | 96                  | 27                  | 28         | 3.783769         | 2D                |
| 17      | P27348        | 14-3-3 protein tau (14–3–3 protein theta) negative regulation of transcription, DNA-dependent | YWHAQ_HUMAN | YWHAQ              | 10971     | 27747/4.68 | 15           | 216                 | 55                  | 18         | 2.880806         | 2D                |
| 18      | Q13952        | Nuclear factor NF-Y protein chain C protein folding/regulation of transcription | NFYC_HUMAN | NFYC               | 4802      | 50271/5.78 | 3            | 66                  | 7                   | 15         | 4.573116         | 2D                |
| 19      | P60228        | Eukaryotic translation initiation factor 3 subunit eIF-3 p48 translational initiation/nuclear-transcribed mRNA catabolic process | EIF36_HUMAN | EIF36              | 3646      | 52187/5.71 | 11           | 49                  | 24                  | 36         | 8.501138         | 2D                |
| 20      | P13693        | Translationally controlled tumor p62(TCP1/p23) anti-apoptosis/cellular calcium ion homeostasis | TPT1_HUMAN | TPT1               | 7178      | 19583/4.84 | 7            | 70                  | 33                  | 20         | 3.649924         | 2D                |
| 21      | P08779        | Cytokeratin 16 cell proliferation/cytoskeleton organization | KRT16_HUMAN | KRT16              | 3868      | 51105/4.98 | 11           | 101                 | 24                  | 26         | 2.869215         | 2D                |
| 22      | P04792        | HspB1 Heat shock 27 kDa protein anti-apoptosis/cellular component movement/regulation of translational initiation | HSPB1_HUMAN | HSPB1              | 3315      | 22768/5.98 | 10           | 163                 | 46                  | 11         | 3.099848         | 2D                |
| 23      | P60842        | Eukaryotic initiation factor 4A (eIF4A-I) translation | EIF4A1_HUMAN | EIF4A1             | 1973      | 46125/5.32 | 21           | 574                 | 45                  | 40         | 6.608386         | 2D                |
| 24      | P17987        | T-complex protein 1, alpha (TCP1-alpha) protein folding/tubulin complex assembly | TCP1_HUMAN | TCP1               | 6950      | 60306/5.80 | 20           | 144                 | 38                  | 22         | 3.35298          | 2D                |
| 25      | P62258        | 14–3–3 protein epsilon apoptosis/induction of apoptosis by extracellular signals | YWHAE_HUMAN | YWHAE              | 7531      | 29155/4.63 | 16           | 257                 | 61                  | 30         | 2.060479         | 2D                |
| 26      | P12004        | Proliferating cell nuclear antigen (PCNA) (Cyclin) DNA replication/cell proliferation/mismatch repair | PCNA_HUMAN | PCNA               | 5111      | 28750/4.57 | 11           | 519                 | 52                  | 21         | 3.664895         | 2D                |
| 27      | Q01105        | SET protein DNA replication/nucleocytoplasmic transport | SET_HUMAN | SET                | 6418      | 33469/4.23 | 10           | 491                 | 38                  | 25         | 3.358717         | 2D                |
| Spot no. | Gene ID | SwissProt Accession | Mass (Da)/PI | Peptide matched Score | Sequence coverage [%] | RMS (ppm) | Fold change (A4-T/P) | Validation method |
|---------|---------|----------------------|-------------|-----------------------|------------------------|----------|----------------------|------------------|
| 28      | P27797  | CALR_HUMAN P04346    | 811 48112/4.29 | 14                    | 1180 49                | 35       | 11.82921 2D          | 2D: IB           |
| 29      | P28072  | PSMB6_HUMAN P34776   | 594 25341/4.80 | 7                     | 166 30                  | 15       | 27.70366 2D          | 2D              |
| 30      | P28072  | PRKAA1_HUMAN P04346  | 3500 40797/5.64 | 30                    | 861 69                  | 20       | 2.032384 2D: IB      | 2D: IB           |
| 31      | P09035  | ERLIN2_HUMAN P04346  | 11160 37151/5.54 | 9                     | 124 27                  | 20       | 4.06973 2D          | 2D: IB           |
| 32      | P14923  | JUP_HUMAN P04346     | 37288 81447/5.55 | 19                    | 210 30                  | 45       | 2.77087 2D          | 2D              |
| 33      | P03610  | KRT19_HUMAN P04346   | 3880 40729/5.44 | 30                    | 861 69                  | 20       | 2.032384 2D: IB      | 2D: IB           |
| 34      | P15311  | WARS_HUMAN P04346    | 7430 53125/5.49 | 10                    | 87 20                   | 47       | 2.423008 2D          | 2D              |
| 35      | P15310  | JUP_HUMAN P04346     | 7430 53125/5.49 | 10                    | 87 20                   | 47       | 2.423008 2D          | 2D              |
| 36      | P15309  | ERLIN2_HUMAN P04346  | 2534 42946/6.64 | 14                    | 129 34                  | 37       | 2.04263 2D          | 2D: IB           |
| 37      | P04181  | RUVBL2_HUMAN P04346  | 10856 51125/5.49 | 22                    | 292 47                  | 42       | 4.22873 2D          | 2D              |
| 38      | P04181  | RUVBL2_HUMAN P04346  | 7430 53125/5.49 | 10                    | 87 20                   | 47       | 2.423008 2D          | 2D              |
| 39      | P04181  | RUVBL2_HUMAN P04346  | 7430 53125/5.49 | 10                    | 87 20                   | 47       | 2.423008 2D          | 2D              |
| 40      | P04181  | RUVBL2_HUMAN P04346  | 7430 53125/5.49 | 10                    | 87 20                   | 47       | 2.423008 2D          | 2D              |
| 41      | P04181  | RUVBL2_HUMAN P04346  | 7430 53125/5.49 | 10                    | 87 20                   | 47       | 2.423008 2D          | 2D              |
| 42      | P04181  | RUVBL2_HUMAN P04346  | 7430 53125/5.49 | 10                    | 87 20                   | 47       | 2.423008 2D          | 2D              |
| 43      | P04181  | RUVBL2_HUMAN P04346  | 7430 53125/5.49 | 10                    | 87 20                   | 47       | 2.423008 2D          | 2D              |
| 44      | P04181  | RUVBL2_HUMAN P04346  | 7430 53125/5.49 | 10                    | 87 20                   | 47       | 2.423008 2D          | 2D              |
| 45      | P04181  | RUVBL2_HUMAN P04346  | 7430 53125/5.49 | 10                    | 87 20                   | 47       | 2.423008 2D          | 2D              |

**Table 2. Cont.**
ly) in either A4-P or A4-T (termed as EEx and LEx proteins respectively), while Group II includes proteins expressed at quantitatively different levels (minimum two-fold differential expression between the two cell types). Both groups were further divided into two sub-groups based on their expressions in respective cell types. Annotation of qualitative and quantitative expressions was performed within each replicative set of A4-P and A4-T cells. A total of 10 and 34 Group I proteins and, 31 and 48 Group II proteins were identified as being expressed in A4-P and A4-T cells respectively (Fig. 1C; Table S1). Tables 1 & 2 lists the identified Group I and Group II proteins with specific spot numbers, molecular and functional description along with the details of match peptides, protein score, sequence coverage (%) and relative expression fold-change.

Categorization of functional pathways based on reported ontologies and expression validation

Gene ontology analyses further identified distinct molecular functionalities and pathways associated with the identified protein profiles in the two cell types (Fig. 1D.i). Thus, several cellular regulatory mechanisms including protein biosynthesis, cytoskeleton organization, signal transduction, regulation of apoptosis and protein degradation were largely enriched in and contributed to the functionality of A4-P cells. These were suggested to have a cross-talk with other pathways such as protein and energy metabolism, RNA metabolism, cellular differentiation and redox reactions.

Conversely, functional grouping of the proteins in A4-T cells comprised pathways associated with the classical hallmarks of cancer cells viz. resistance to apoptosis, energy metabolism, cell proliferation, angiogenesis and invasion and metastases (Fig. 1D.ii). Thus, molecules involved in associated with protein biosynthesis, folding and transport control the dynamic process of protein metabolism in transformed cells towards matching its proliferative activities.

Towards confirming levels of some of the identified proteins, their expressions were validated between A4-P and A4-T cells through immunoblotting (Fig. 1E; Fig. 2A,B,C,D). SeOvCa is unique in that, transformation is associated with expression of epithelial markers [21]. Enhanced vimentin expression in A4-P cells suggest mesenchymal while elevated levels of Cytokeratin 8 and 18 in A4-T cells correlate with epithelial features respectively (Fig. 1E), besides being in concordance with their cell morphology and the prevalent hypothesis.

Functional characterization of RXR-γ an exclusive Group I protein

Ten Group I proteins were expressed exclusively in the A4-P cells (EEx proteins). Literature-based functional annotation led to their categorization into four functional groups viz.

i. Cell differentiation and apoptosis – RXR-γ, PRDX4;

ii. Cell proliferation – GNA11, PIBF-1, ANXA5, Cathepsin D (CTSD);

iii. Mitosis and cytokinesis – KLH9;

iv. Epithelial-mesenchymal transition (EMT) – TPM1, ANXA5.

While all the above functions are relevant in the process of transformation, we focused on studying the functionality of cellular differentiation and apoptosis that is critical in maintaining tissue homeostasis and known to be regulated by RXR-γ at the transcriptional level by dimerizing with retinoic acid or retinoic acid X receptors (RAR or RXR respectively) or other permissive
heterodimer partners like PPAR-\(\gamma\) [22,23,24]. We thus decided to investigate the role of RXR-\(\gamma\) in our epithelial ovarian cancer progression model.

**RXR-\(\gamma\) interactions with nuclear receptors and modulation of cellular differentiation in A4-P cells upon retinoids treatment**

Retinoid treatment enhanced RXR-\(\gamma\) levels in A4-P cells; and interestingly, resumed significant expression in A4-T cells as well (Fig. 2E,F). CRA and ADA individual treatment elevated RXR-\(\gamma\) levels in both cell types, though this induction was less effective in combination with TTNPB. Towards validation of RXR-\(\gamma\) interactions with other nuclear receptors, co-immunoprecipitation and immunoblotting affirmed interactions with PPAR-\(\gamma\), RAR-\(\gamma\), RXR-\(\alpha\) and RAR-\(\alpha\) in pre-transformed cells (Fig. 3A, B). Evaluation of RXR-\(\gamma\) involvement in cellular differentiation was achieved through profiling epithelial markers E-cadherin (E-cad), Cytokeratin 18 (CK-18) and Mucin-1 (Muc-1) at gene expression and protein levels, in steady state and on exposure to natural viz. CRA and synthetic retinoids (ADA and TTNPB) (Fig. 3C). At steady state, lower expression of E-Cad was observed in A4-P cells. Expression of E-Cad further increased with CRA and also with ADA; CRA was given alone or in combination with ADA and TTNPB. Levels of E-Cad, CK18 and Muc-1 were endogenously higher in A4-T cells. Synthetic retinoid ADA alone or in combination with CRA upregulated CK18 expression in both cell types. Although, specific role of TTNPB in cellular differentiation is unknown, TTNPB treatment resulted in minor upregulation of differentiation markers. All three markers were enhanced in response to retinoid exposure in A4-P cells thereby affirming the involvement of RXR-\(\gamma\) in modulation of cellular differentiation. Retinoid treatment in the A4-T cells resulted in induction of RXR-\(\gamma\) without any significant alterations in the levels of these epithelial differentiation marker at gene expression and protein levels (Figs. 3D, 3E).

**Retinoid induced RXR-\(\gamma\) levels sensitize transformed cells towards apoptosis via intrinsic pathway**

Role of RXR-\(\gamma\) in mediating apoptosis in response to natural and synthetic retinoids was evaluated (Fig. 4A). At steady state, apoptosis was significantly lower in A4-T cells as compared to A4-P cells indicating acquisition of resistance to apoptosis during the transformation process. Apoptosis was enhanced in both cell types on exposure to CRA and ADA – either alone or in combination (Fig. 4B). While TTNPB by itself failed to induce cell death, in combination with ADA and CRA it sensitized A4-T cells to

Figure 2. Validation of differentially expressed proteins and induction of RXR-\(\gamma\) levels on retinoid treatments. Quantitative validation of the expression and fold change of some proteins, identified in both groups; Group I proteins, A, exclusively expressed in A4-P and B, in A4-T cell respectively; whereas D, group-II proteins quantitatively up-regulated in A4-P cells and E, in A4-T. E. Relative expression of RXR-\(\gamma\) and \(\beta\)-actin in CRA, ADA or TTNPB retinoids treated A4-P (P) and A4-T (T) cells validated through immunoblotting. F. Quantitation of relative RXR-\(\gamma\) expression in A4-P and A4-T cells. Statistical analysis showing test of significance (*=control A4-P and retinoids treated cells; $= control A4-P and retinoids treated cells). The data shown are representative of three separate experiments and depicted as mean ± SEM *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\).

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apoptosis. Retinoid mediated activation of RXR-γ expression was also found to correlate directly with higher levels of apoptosis (Fig. 2E,F). We profiled expression of the transcription factor Snail (that antagonizes p53-mediated pro-survival signaling through active repression of the pro-apoptotic molecules PUMA/BBC3, ATM and PTEN in ovarian cancer cells under stress; [19]) to evaluate the effect of RXR-γ led apoptosis on it. Caspase 9, a marker of intrinsic apoptosis pathway, upregulated during RXR-γ and PPAR-γ induction and Bcl-2 as markers of apoptosis [25]. Snail and Bcl-2 expression were reduced, while significantly elevated expression of RXR-γ, PPAR-γ and Caspase 9 were evident on retinoid treatment validated through RT-PCR. E. Quantitation of protein expression of E-Cad, CK18 and MUC1 makers in A4-P (P; line) and A4-T cells (T; dashed line) upon retinoids treatment validated through immunoblotting. Data shown are representative of three separate experiments depicted as mean ± SEM *p<0.05, **p<0.01, ***p<0.001.

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**Figure 3. RXR-γ interacts with a number of nuclear receptors and modulates cellular differentiation.** A. Co-Immunoprecipitation (Co-IP) with RXR-γ showing eluted Immunocomplex by silver staining. B. Validation of RXR-γ indicating interaction in Co-IP with RXR-γ with PPAR-γ, RAR-γ, RXR-α and RAR-α in A4-P cells validated through immunoblotting. C. Expression profiling of CK-18, Muc-1 and E-Cadherin at transcriptional (Tr) and protein (Pr) performed by semi-quantitative RT-PCR and immunoblotting in CRA, ADA or TTNPB retinoids treated A4-P (P) and A4-T (T) cells. D. Quantitation of mRNA expression of E-Cad, CK18 and MUC1 epithelial differentiation markers in A4-P (P; line) and A4-T cells (T; dashed line) upon retinoids treatment validated through RT-PCR. E. Quantitation of protein expression of E-Cad, CK18 and MUC1 makers in A4-P (P; line) and A4-T cells (T; dashed line) upon retinoids treatment validated through immunoblotting. Data shown are representative of three separate experiments depicted as mean ± SEM *p<0.05, **p<0.01, ***p<0.001.

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In vivo retinoid treatment significantly reduce xenograft growth in NOD-SCID mice through RXR-γ mediated sensitization of transformed cells towards apoptosis.

We further extended the above re-sensitization of RXR-γ levels in A4-T cells effects obtained in vitro to experimental tumors (Fig. 5A). Mean tumor volume (Figs. 5B,5C) at each treatment point along with mean tumor volume and weight at 7th week (Figs. S1C, S1D) showed significant reduction in retinoid treated mice tumors vs. those in DMSO treated controls. Overall, the combinatorial retinoid treatment was most effective. The distinctly upregulated RXR-γ expressions in the retinoid-treated tumors strongly suggest sensitization of transformed A4 cells to apoptosis. Combined effect of retinoids was found to be most lethal for tumor growth through resumed RXR-γ mediated apoptosis of tumor cells in vivo. RXR-γ levels were found significantly higher in all 5 sets including CRA, CRA & TTNPB, ADA, ADA & TTNPB and CRA, ADA & TTNPB; in comparison to DMSO vehicle control. This is a definitive correlation with RXR-γ stimulation and induction of apoptosis in these cells in vitro (Fig.5D).
Figure 4. RXR-γ levels sensitize cellular apoptosis in A4-T cells upon retinoid treatment. A. Annexin V-FITC assay data showing apoptosis in A4-P and A4-T cells on different retinoids treatment regimes; where i. having no retinoid treatment, ii. treated with CRA, iii. with ADA and iv. with both having alternative treatment of another synthetic retinoid i.e. TTNPB. B. Statistical analysis of apoptosis assay showing significant apoptosis.
Discussion

The existence of several histological sub-types that correlate with different cell(s)-of-origin in ovarian cancer [26] remains a hurdle in the establishment of representative progression models in this disease. This is in contrast to other malignancies such as prostate cancer in which such models have been applied over the last two decades in elucidating molecular mechanisms of disease [1,27,28,29]. In the present study, detailed exclusive and differential protein profiling of a progression model established earlier in our lab provided novel insights into altered molecular patterns during SeOvCa progression. A4-P cells with replicative immortality represent a pre-neoplastic stage while A4-T cells with aggressive and metastatic characteristics are representative of transformation and disease progression.

Our data affirms that the two functional states of the model are associated with distinct protein profiles. Within the group of proteins exclusive to the A4-P cells, characterization of the role of RXR-γ revealed a sensitivity of the pre-transformed cells to apoptosis and differentiation as described earlier [30]. Compromised RXR-γ levels are also reported in several malignancies including non-small cell lung cancer [31]; where it also has been reported that epigenetic silencing of RXR-γ correlated with decreased overall survival of patients [32]. In our pre-transformed cells, RXR-γ cooperates with PPAR-γ, RAR-γ, RAR-α and RXR-α to form functional heterodimeric complexes, where RXR-γ with PPAR-γ coordinates cellular apoptosis through the intrinsic pathway confirmed with elevated Caspase-9 levels. Further, we observed that RXR-γ activation in transformed cells re-sensitizes them to apoptosis as a synergistic effect of agonists that mediate cytotoxic effects in vitro as well as in experimental tumors (Fig. 6).

This is an important identification towards application of retinoid-based therapies. In this study, we characterized the pleotropic nature of RXR-γ signaling in our SeOvCa-progression model system. Loss of RXR-γ levels indicated to facilitate mechanistic benefits to transformed cells towards acquisition of resistance to apoptosis; consequently, retinoid-sensitized tumor cells upregulate RXR-γ levels leading to significant cell death.

The present proteomics approach is a first account of changes in SeOvCa that reflect on various transformation-associated functional pathways. Significantly, RXR-γ signaling could be a potential gateway in preventing disease progression. The elucidation of RXR-γ signaling extends contemporary approaches of cellular transformation in SeOvCa that can now be exploited further in development and evaluation of new therapeutic modalities.

Material and Methods

Ethics statement

All animal work was conducted with the National Centre for Cell Science (NCCS) Institutional Animal Ethics Committee (IAEC) approval of experiments in the NCCS Experimental...
Animal House (EAH) Facility, and was performed as per the norms, laws and policies laid down by the committee.

Cell culture, treatments and transfections
Derivation of the A4 progression model of pre-transformed and transformed SeOvCa cells (A4-P and A4-T cells) is described earlier [14,18]. Retinoid (RXR-\(\gamma\)-ligand) treatment was carried out using either natural retinoid viz. 9 Cis Retinoic acid (CRA;10 \(\mu\)M) or synthetic retinoids Adapalene (ADA; 2 \(\mu\)M; RAR agonist) or 4-[(E)-2-(5,6,7,8 –Tetrahydro – 5,5,8,8 –tetramethyl – 2 naphthale-nyl) – 1 -propenyl] benzoic acid Arotinoid acid (TTBPB; 10\(\mu\)M; RXR and RAR agonist) for 48h.

Sample preparation, 2-Dimensional gel electrophoresis (2DE) and image analyses
Cell pellets (10\(^7\)) of A4-P and A4-T were suspended in 500 \(\mu\)l of urea lysis buffer containing 8 M Urea, 2 M Thiourea,100 mM DTT, 2% CHAPS and 0.2% ampholytes with protease-inhibitor cocktail (Amersham USB Guideline). Cell extract was allowed to be mixed for at least 15 minutes and incubated for 30 minutes at room temperature to facilitate proper protein solublisation. Protein samples were further centrifuged (110,000g for 1 hour at 4\(^\circ\)C) and suspension was collected. Protein concentration was estimated with 2DE quant kit (GE healthcare) at 480 nm (Bekman Coulter). Prepared samples were run on first dimension (pI) followed by of second dimension in denaturing SDS-PAGE (Mw). A total of 350 \(\mu\)g whole cell protein lysate was taken on 18 cm immobilized pH gradient (IPG) strip (pH 4–7) and rehydrated overnight. A three step IEF voltage program was prepared to the strips on a Protean IEF cell (Bio-Rad): 50 V for 20 mins, 10,000 V for 2 hours minutes and 10,000 V for 45,000 V-hr. Strips were further reduced by incubation in the equilibration/reduction buffer (6 M Urea, 0.375 M Tris pH 8.8, 2% SDS, 20% glycerol, 2% (w/v) DTT (Sigma) and then alkylated the same buffer but containing 2.5% (w/v) Iodoacetamide (Sigma) instead of DTT.

Figure 6. Schematic model showing modulation of cellular differentiation and apoptosis by RXR-\(\gamma\) during the progression of epithelial ovarian cancer. A. RXR-\(\gamma\) modulation at steady state in pre-transformed cells; retinoids treatment enhances RXR-\(\gamma\) levels and scale up apoptosis (upon RXR-\(\gamma\) interaction with PPAR-\(\gamma\)) and expression of epithelial differentiation specific markers (upon RXR-\(\gamma\) interactions with RAR-\(\gamma\), RXR-\(\alpha\) and RAR-\(\alpha\)). B. Deficiency of RXR-\(\gamma\) providing benefits of resistance to apoptosis to transformed cells; retinoid treatment induced RXR-\(\gamma\) levels sensitize these cells towards significant apoptosis.

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In-gel digestion and protein identification using MALDI-TOF/TOF
Protein spots in 2DE showing differential expression and satisfying the statistical criteria were selected and excised for in-gel digestion and further MS analyses. Spot excision was performed manually with the help of sterile sharp spot cutter. Briefly, the gel slices were destained in 25 mM ammonium bicarbonate, subsequently dehydrated with a 2:1 mixture of 50 mM ammonium bicarbonate:100% acetonitrile (ACN) for repeated 3 times each 5 minutes. Gel slices were reduced with 10 mM
DTT at 60°C for 1 hour. After cooling, gel slices were incubated for 15 min at room temperature with 50 mM iodoacetic acid. After washing and dehydrating the gel slices with 25 mM ammonium bicarbonate and ACN for 10 min, they were vacuum dried and tryptic digestion performed with 50 mM ammonium bicarbonate containing 20 μg/mL modified proteomic grade trypsin (Sigma-Aldrich) according to the manufacturer’s instructions and kept on ice for 30 min. Additional 25 mM ammonium bicarbonate was added and digestion was continued overnight at 37°C. Extracted peptides were completely dried using a speedvac and re-suspended in 10 μl of 20% Ammonium Bicarbonate and 1% formic acid solution.

After processing through the Zip-Tip pipette tips (Millipore, USA), 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% acetonitrile, 0.1% trifluoroacetic acid. Equal volumes of peptide and matrix solution were mixed, and 1 μl of the resulting solution was spotted on a stainless steel MALDI sample plate. Spectra of digested peptides were acquired on a 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 4000 Da, using a Nd:YAG 335 nm laser. The acceleration voltage and extraction voltage were set on 20 kV and 10 kV respectively. Six point calibration of the instrument was performed with peptide standard kit (AB Sciex).

All of the MS spectra were obtained from accumulation of 900 shots. MS/MS spectra were acquired with a total accumulation of 1500 laser shots and collision energy of 1 kV. At completion of MS survey scans, the data was processed to generate a list of precursor ions for interrogation by MS/MS. The combined MS and MS/MS peak lists were explored using the GPS™ Explorer software version 3.6 (AB Sciex). Protein identification was performed by MS/MS ion search using MASCOT® (version 2.1) [http://www.matrixscience.com] search engine against the SwissProt database. The search parameters were set as follows: all entries and human taxonomy, trypsin digestion and one missed cleavage, fixed modifications: carbamidomethylation of cysteine residues, mass tolerance: 150 ppm for MS and 0.4 Da for MS/MS. Identified proteins having at least two unique matched peptides were selected with an identification confidence interval threshold of ≥95%.

Co-immunoprecipitation and Immunoblotting
1 mg cellular protein extracted in RIPA buffer (1 M Tris pH 7.4, 4 M NaCl, 0.5 M EDTA, NP-40, 10% SDS) was incubated with 5 μg RXR-γ antibody for 2 h at 4°C. This was followed by overnight incubation with 20 μl protein-A agarose (Amersham, GE Healthcare). Complex-bound beads were collected through centrifugation at 12,000 g for 1 min, was washed with TBS (50 mM Tris-HCl, 150 mM NaCl, PMSF), resuspended in 2×SDS buffer and heated at 95°C for 5 min. Eluted proteins were resolved on 2–4% denaturing SDS-PAGE at 80 V followed by immunoblotting, that was performed as described earlier [19]. Details of antibodies used in the study will be made available on request.

Semi-quantitative reverse transcription-PCR
Semi-quantitative reverse transcription-PCR was performed under standard conditions as described earlier [19] and amplified products resolved on a 1.5% agarose gel; β-actin was used as internal control.

Cell cycle and apoptosis assay
Cell cycle analysis of transfected and retinoid-treated cells was done with PI (Propidium-Iodide) staining using standard protocols [20]. Data acquisition and analysis was performed on FACSCalibur (Becton Dickinson, San Diego, CA, http://www.bd.bdbiosciences.com) using ModFit analytical software. Annexin V–FITC apoptosis assay was performed as described earlier [20] using FACS Canto II (Becton Dickinson); DiVa software (Becton Dickinson) was used for data analysis.

In vivo studies
In vivo experimentation was performed in NOD/SCID mice bred and maintained at Experimental Animal Facility, NCCS; and carried out as per the norms, laws and policies of the institutional ethical committee. A4-T cells (2.5×10⁶) were injected subcutaneously (SC) in thighs of 4–6 week-old male mice and observed every 48 h till 3 weeks for tumor formation. Injections of retinoids i.e. 9Cis RA, ADA and TTNPB as well in combination started while tumor size reaches 25–30 mm³ in volume, where DMSO given to vehicle control mice. Treatments of DMSO, 25 μM 9Cis RA, 25 μM 9Cis RA+10 μM TTNPB, 5 μM ADA, 5 μM ADA +10 μM TTNPB and 25 μM 9Cis RA+5 μM ADA +10 μM TTNPB retinoids injections were given twice per week into the tumor of each mouse. Tumor size was monitored in two perpendicular directions using Vernier’s calliper; individual tumor weights and sizes were more precisely quantified in the seventh week after sacrificing mice to harvest tumors.

Statistical analysis
All experiments were carried out at least in triplicate; data are expressed as mean ± SEM of three independent experiments. The significance of difference in the mean values was determined using two-tailed Student’s t test; p < 0.05 was considered significant. ANOVA test was performed to compare gene and 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.

Supporting Information
Figure S1 A. PI based FACS analysis of cell cycle in A4-P and A4-T cells on different treatment regime of no retinoid treatment, treatment with CRA, ADA and with both having alternative treatment of another synthetic retinoid i.e. TTNPB, showing percentage of relative populations in different cell cycle phases. B. Quantitation of different cell cycle phases of A4-P and A4-T cells on different retinoid treatments. C. Graphical representation showing tumor volumes of retinoids treated NOD-SCID mice. D. Graphical representation showing tumor weight of retinoids treated NOD-SCID mice. The data shown are representative of three separate experiments and depicted as mean ± SEM *p<0.05, **p<0.01, ***p<0.001.

Table S1 Summary of total numbers of proteins identified between A4-P and A4-T cells through 2DE analyses followed by MALDI-TOF-TOF (MS/MS) identification.

[DOC]
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Author Contributions

Conceived and designed the experiments: SAB. Performed the experiments: RSK. Analyzed the data: RSK SAB. Contributed reagents/materials/analysis tools: SAB. Wrote the paper: RSK SAB.

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