Chapter 4

FUNCTIONAL RELEVANCE OF RXR-γ SIGNALING IN THE PROGRESSION OF EPITHELIAL OVARIAN CELL MALIGNANT TRANSFORMATION
**Summary**

Comparative expression profiling of our SeOvCa progression model led to the identification of differential expression of transformation-associated proteins. RXR-γ that belongs to the group of proteins exclusively associated with pre-transformed cells (EEx), is known to modulate two major regulatory pathways viz. cellular apoptosis and differentiation. These processes however, remain largely uncharacterized in EOC progression. Towards addressing the issue, we resolved the involvement of RXR-γ mediated retinol signaling in the two pathways during tumor progression. Interaction of RXR-γ with other nuclear receptors towards formation of heterodimer complex in pre-transformed cells initially affirmed its association with the respective cellular processes. Using specific retinoids, RXR-γ modulated cellular differentiation was further characterized through expression profiling of epithelial differentiation-specific markers. Retinoid-induced RXR-γ expression also appeared to modulate cell cycle phases and cellular apoptosis in steady state pre-transformed cells. Enhanced levels of RXR-γ induced through retinoid treatment significantly sensitized transformed cells *in vitro* towards apoptosis through the intrinsic pathways driven by Caspase-9. Further, treatment of xenografts derived from transformed cells with retinoids restricted their growth affirming the sensitization of tumor cells to apoptosis by RXR-γ. In conclusion, RXR-γ deficiency seems to provide mechanistic benefits to transformed cells towards acquisition of resistance to apoptosis which is a hallmark of cancer. Thereby, differential RXR-γ expression in A4 progression model system illustrated its role in the regulation of cellular differentiation and apoptosis during progression of serous ovarian carcinoma.
Introduction

Comparative protein profiling has been recognized as a comprehensive approach towards understanding the biology of cellular transformation and its underlying mechanisms (Aunoble et al. 2000; Havrilesky et al. 2001). Exploiting our SeOvCa progression model, we identified exclusive proteins associated with pre-transformed cells that reflect cellular functionality associated with the process of malignant transformation. This further led to the prediction of biological functions of key targets playing crucial roles in the process. Notably, the process of OSE cell transformation has been suggested to progress through the loss or disruption of differentiation mechanisms (Lippman et al. 1987; Lotan et al. 1990). Retinoids have been shown to regulate cell differentiation and proliferation, the two major pathways modulated through alternation in gene expression during cellular transformation (Lefebvre et al. 2005). Retinoids usually bind to RAR/RXR receptors and modulate transcriptional regulation of downstream targeted genes. Such involvement of retinoid receptors has been observed in epithelial cells of trachea (Zeisig et al. 2007). Involvement of polycomb group proteins in regulation of tumor suppressor genes during neoplastic transformation has also been observed and is induced by the PML-RARα fusion protein in acute promyelocytic leukemia (Martens et al. 2010; Villa et al. 2007). Earlier reports also have identified the involvement of retinol metabolism as an early event in EOC initiation and/or progression (Kuppumbatti et al. 2000; Roberts et al. 2002; Cvetkovic et al. 2003). Moreover, decreasing level of cellular retinol-binding protein-1 (CRBP1) has also been considered as being crucial to transformation. However, detailed involvement of retinoic acid receptors remains largely uncharacterized.

On this background, lowered RXR-γ levels exclusively in transformed cells could be an early significant event that necessitates further analysis. As outlined in the previous chapter, RXR-γ is associated with the process of cellular differentiation and apoptosis (Lotan et al. 1980; Fontana
et al. 1987; Lotan et al. 1990; Sporn and Roberts, 1991; De Luca et al. 1991; Koga and Sutherland, 1991). These pathways are known to be regulated by RXR-γ by dimerizing with retinoic acid and retinoic acid X receptors (RAR or RXR respectively) or with permissive heterodimer partners like PPAR-γ (Koga and Sutherland, 1991; Minucci et al. 1999; Benoi et al. 2001). We hence further resolved its functional role in our progression model and on treatment with selective retinoids including 9-Cis-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). Comparative expression analysis of cellular differentiation and apoptosis-specific molecular markers was also performed to examine consequence of activated RXR-γ signaling in both cell types in vitro as well as in vivo xenograft models.

In conclusion, cellular differentiation and apoptosis were seen to be differentially modulated in our SeOvCa progression model as well as in response to retinoid-mediated activation of RXR-γ signaling.
**Results**

4.1 RXR-γ forms functional heterodimeric-complex with RXR-α, RAR-α, RAR-γ and PPAR-γ

To investigate involvement RXR-γ in the processes of cellular differentiation and apoptosis; we initially affirmed the qualitative (enhanced) levels of RXR-γ protein expression in pre-transformed cells (Fig.4.1A). Further, towards confirming physical association of RXR-γ with other nuclear receptors; co-immunoprecipitation assay was carried. This also affirmed the association of RXR-γ association with PPAR-γ, RAR-γ, RXR-α and RAR-α (Figure 4.1B-C).

Fig. 4.1: A. Validation of qualitative (differential) levels of RXR-γ between A4-P and A4-T cells. B. Co-immunoprecipitation with RXR-γ antibody with input, bead and isotype control (arrow indicates eluted immunocomplex). C. Immunoblotting in RXR-γ immunoprecipitated samples confirmed PPAR-γ, RAR-γ, RXR-α and RAR-α as interacting partners.
4.2 Retinoid treatment induce RXR-γ levels in A4-SeOvCa progression model

To evaluate RXR-γ mediated effect on cellular differentiation and apoptosis, specific retinoids viz. 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) were used to induce its expression. Thus, retinoid treatment enhanced protein levels of RXR-γ in A4-P cells; and interestingly, induced significant expression in A4-T cells as well (Fig. 4.2A-B). CRA and ADA individual treatment elevated RXR-γ levels in both cell types, though this induction was less effective in combination with TTNPB.

Figure 4.2: Retinoid treatment induces expression of RXR-γ between A4-P and A4-T cells. A. Immunoblotting showing relative expression of RXR-γ and β-actin in CRA, ADA or TTNPB retinoid treated A4-P (P) and A4-T (T) cells. B. Quantitation of relative RXR-γ 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).

4.3 Retinoid induced RXR-γ levels modulate cellular differentiation in A4-P cells

We further profiled the expression of some epithelial differentiation markers at the mRNA and protein levels in order to investigate the role of RXR-γ in cellular differentiation (Fig. 4.3A). At steady state, lower expression of E-cadherin (E-Cad) was observed in A4-P cells. Expression of
E-Cad further increased with synthetic RXR-γ ligand viz. CRA and also with ADA; CRA was given alone or in combination with ADA and TTNPB. Levels of E-Cad, Cytokeratin 18 (CK18) and Mucin-1 (Muc-1) were endogenously higher in A4-T cells and were upregulated in A4-P cells upon retinoid treatment. Synthetic retinoid ADA alone or in combination with CRA upregulated CK18 expression significantly in both cell types. Although, specific role of TTNPB in cellular differentiation is unknown, TTNPB treatment resulted in minor upregulation of differentiation markers. Statistical analysis affirmed significantly elevated levels of all three key molecules in cellular differentiation on retinoid treatment (Fig. 4.3B-C). In conclusion, the comparative profiling of CK18, E-Cad and Muc-1 on retinoid treatments showed significant increase in mRNA and protein levels in A4-P cells while expression of these markers was found endogenously high in A4-T cells (Fig. 4.3C).

Fig. 4.3 A. Expression profiling of epithelial differentiation markers at mRNA and protein levels. A. Expression of E-Cad, CK18 and Muc-1 (epithelial differentiation markers) at mRNA (top panel; Tr- transcriptional level) and protein levels (lower panel; Pr- protein level). B-C. Quantification of expression levels of E-Cad, CK18 and Muc-1 epithelial differentiation markers at mRNA and protein levels respectively.
4.4 Retinoid induced RXR-γ levels modulate cell-cycle phases

CRA in combination with TTNPB leads to apoptosis in A4-P cells, though cell cycle phases remain unchanged, whereas in A4-T cells enrichment of S phase population was observed. Treatment of TTNPB exerted no effect on cell cycle in both cell types. ADA treatment significantly enriched S and G2-M phase populations in both cell types, though unexpectedly G2-M population in ADA+TTNPB treated A4-T cells increased. In combinatorial CRA and ADA treated cells, S and G2-M phase populations were found higher in A4-T cells, while A4-P cells exhibited apoptosis. Addition of all three retinoids increased S phase population and apoptosis in both cell types (Fig. 4.4A-B). Statistical analysis of cell cycle data also showed a significant enrichment of G1-S phase population upon different retinoid treatment in A4-P and A4-T cells (Fig. 4.4B). In conclusion, retinoid induced RXR-γ levels modulate cell-cycle phases and increase population in S phase.

Fig.4.4 A. Flow cytometry analysis showing cell cycle profiles of A4-P and A4-T cells upon retinoid treatment. B. Statistical analysis of cell cycle data represented percentage of cells in G0-G1, S, G2-M and apoptosis under different retinoid treatment.
4.5 Retinoid treatment led to apoptosis by sensitizing RXR-γ expressions in transformed cells

We also determined the extent of apoptosis in response to CRA, ADA and TTNPB treatments. At steady state, significant apoptosis was observed in A4-P cells in comparison with the transformed A4-T cells suggesting acquisition of resistance to apoptosis during transformation (Fig. 4.5A). CRA treatment also led to higher apoptosis in both cell types. RAR-specific agonist viz. Adapalene (ADA) alone and in-combination with other retinoids induced apoptosis in pre-transformed cells (Fig.4.5B), though it was found to be much higher in transformed cells. Retinoid mediated activation of RXR-γ expression was also found to correlate directly with higher levels of apoptosis. ADA in combination with either CRA or TTNPB showed significant apoptosis in transformed cells. Specific effect of TTNPB alone and with CRA and ADA retinoids remained undetermined in both cell types.

Fig. 4.5: A. RXR-γ is associated with cellular apoptosis in A4-P cells whereas its re-expression in A4-T cells upon retinoid treatment sensitizes these cells to apoptosis. A.
Annexin V-FITC flow cytometry data representing extent of apoptosis in A4-P and A4-T cells in response to retinoid treatments. B. Statistical analysis of data generated in A.

4.6 Stimulated RXR-γ/PPAR-γ expression led to significant apoptosis via intrinsic pathway in transformed cells

PPAR-γ forms heterodimeric complex with RXR-γ that leads to apoptosis mediated through intrinsic pathways that involve activation of caspase-9 and deactivation of Bcl-2 (Elrod and Sun, 2008). We thus investigated the levels of expression of Bcl-2, Snail (inhibits p53-mediated pro-survival signaling) and Caspase-9 (a key progressive modulator of intrinsic pathway of apoptosis) at transcriptional and protein levels (Fig. 4.6A-C) Upon activation of RXR-γ/PPAR-γ functional tetramer complex, Bcl-2 showed lower expression with stimulated RXR-γ in response to retinoid treatment in both cell types. Further, caspase-9 activation was also observed in both cells. 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), was also profiled. Snail levels were decreased on CRA treatment, though ADA treatment restores its levels in A4-T cells. At protein level, Bcl-2 was sharply down-regulated whereas levels of caspase-9 were upregulated in both cell types after retinoid treatment. Statistical analysis affirmed this along with RXR-γ and PPAR-γ while Snail expression was down-regulated in A4-P as well as in A4-T cells (Fig.4.6A).

Altogether, statistical analysis of mRNA and protein levels showed significant upregulation of RXR-γ, PPAR-γ and Cas-9 levels in A4-P and A4-T cells whereas levels of Bcl-2 and Snail were down-regulated in both cell types upon subsequent retinoid treatments (Fig. 4.6B-C).
Figure 4.6 A. Expression profiling of molecular markers associated with cellular apoptosis at mRNA (top panel; Tr- transcriptional level) and protein level (lower panel; Pr- protein level). B-C. Comparative expression of RXR-γ, PPAR-γ, Caspase-9 and Bcl-2 along with Snail at mRNA and protein levels in CRA, ADA and TTNPB treated sets of A4-P and A4-T cells.

4.7 Sensitized RXR-γ levels reduce xenograft growth in-vivo upon retinoid treatment

As seen above, individual and combinatorial retinoid treatment re-sensitized RXR-γ in A4-T cells. In order to evaluate potential of retinoid regimes on transformed cells in vivo, xenograft study was carried out. Transformed A4 cells were xenografted in NOD/SCID mice to raise tumors and were observed for subsequent three weeks before initiating of retinoid treatment. Treatments were given as represented in the workflow (Fig. 4.7A).
Fig. 4.7 RXR-γ expression sensitizes cell death in A4-T tumors upon retinoid treatment. A. Experimental procedure illustrating retinoid treatment regime in NOD-SCID mice. B. Physiology of tumors in retinoid treated NOD-SCID mice prior to harvesting. C. Comparative tumor size of control (DMSO) and retinoid treatment.

Physiological status and relative tumor size of control and treated mice indicate reduced xenograft burden in mice treated with retinoids (Figs. 4.7B, 4.7C). Statistical analysis of mean tumor volume at each treatment point (towards day 21st) showed significant difference in
retinoid-vehicle versus control-treated (DMSO) tumor volumes (Fig. 4.8A). Further, at the 7th week, significantly decreased tumor volume in treated mice was noted (Fig. 4.8B). Maximum reduction in tumor burden was observed in combined retinoid treated tumors (Fig. 4.8C).

![Graphical representation indicating different tumor volumes in retinoid treated NOD-SCID mice at different time points.](image)

**Fig. 4.8** RXR-γ expression inhibits A4-T tumor growth on retinoid treatment. A. Graphical representation indicating different tumor volumes in retinoid treated NOD-SCID mice at different time points. B. Comparative tumor weight of harvested tumors. C. Comparative tumor volume of harvested tumors. The data shown are representative of three separate experiments (n=6 for in vivo experiment) and depicted as mean + SEM *p<0.05, **p<0.01, ***p<0.001.

Correspondingly, quantitative expression of RXR-γ in retinoid treated tumors indicates that these treatments restore levels RXR-γ in xenografts that could affect reduction in tumor growth. Combined effect of retinoids was found to be most lethal for tumor growth through resumed RXR-γ mediated apoptosis of tumor cells *in vivo.*
Upon treatment, RXR-\(\gamma\) 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 (Figs 4.9).

Fig. 4.9 Quantitative expression of RXR-\(\gamma\) in control and different retinoid treated tumors.
Discussion

Towards extending the current understanding of SeOvCa transformation, we aimed at characterizing the relevance of altered functionality of RXR-γ in the EEx sub-group during the process of transformation. A functional analyses and expression profiling approach was carried out to understand the relevance and molecular mechanism led by RXR-γ towards regulating cellular differentiation and apoptosis.

Upregulated levels of RXR-γ on retinoid treatment enhanced expressions of E-Cad, CK18 and MUC-1 that further modulated cellular differentiation in A4-P cells (Fig. 4.10A). Levels of these makers were endogenously high in A4-T cells that demonstrated unique character of SeOvCa where transformation is associated with expression of epithelial markers (Auersperg et al. 1997). Retinoid-induced RXR-γ expression also appeared to influence cell cycling and led to cellular apoptosis in A4-P cells at steady state; this emphasizes its involvement in maintenance and regulation of programmed cell death. Interestingly, CRA and ADA retinoid restored RXR-γ levels in A4-T cells, which in turn lead to cellular apoptosis. These effects were also identified in retinoid treated tumors, essentially confirming its role in restricting cell proliferation. Further, elevated levels of Cas-9 indicated that RXR-γ induces cellular apoptosis via intrinsic pathways. Lack of RXR-γ seems to facilitate mechanistic benefits to A4-T cells towards resistance to apoptosis (Fig. 4.10B). This character essentially reflects nature of transformed cells in order to prevent effects of oxidative stress and other targeted therapies as chemotherapy, radiotherapy etc.
In conclusion, we have characterized the pleotropic nature of RXR-γ signaling in our SeOvCa-progression model system. RXR-γ deficiency in transformed cells supports acquisition of resistance to apoptosis over pre-transformed cells; consequently, retinoid-sensitized tumor cells upregulate RXR-γ levels leading to significant cell death. Thus, RXR-γ signaling may be considered to be a potential gateway in the prevention of epithelial ovarian-transformation.