High Expression of Orphan Nuclear Receptor NR4A1 in a Subset of Ovarian Tumors with Worse Outcome

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ABSTRACT

Objective: Nuclear receptors (NRs) play a vital role in the development and progression of several cancers including breast and prostate. Using TCGA data, we sought to identify critical nuclear receptors in high grade serous ovarian cancers (HGSOC) and to confirm these findings using *in vitro* approaches.

Methods: *In silico* analysis of TCGA data was performed to identify relevant NRs in HGSOC. Ovarian cancer cell lines were screened for NR expression and functional studies were performed to determine the significance of these NRs in ovarian cancers. NR expression was analyzed in ovarian cancer tissue samples using immunohistochemistry to identify correlations with histology and stage of disease.

Results: The NR4A family of NRs was identified as a potential driver of ovarian cancer pathogenesis. Overexpression of NR4A1 in particular correlated with worse progression free survival. Endogenous expression of NR4A1 in normal ovarian samples was relatively high compared to that of other tissue types, suggesting a unique role for this orphan receptor in the ovary. Expression of NR4A1 in HGSOC cell lines as well as in patient samples was variable. NR4A1 primarily localized to the nucleus in normal ovarian tissue while co-localization within the cytoplasm and nucleus was noted in ovarian cancer cell lines and patient tissues.

Conclusions: NR4A1 is highly expressed in a subset of HGSOC samples from patients that have a worse progression free survival. Studies to target NR4A1 for therapeutic intervention should include HGSOC.

Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecologic malignancy and the fifth most common cause of cancer related deaths in women in the United States [1]. Despite advances in surgical and chemotherapeutic strategies, there has been only a modest improvement in 5-year overall survival over the past 20 years [2]. Although the majority of patients experience a response to primary therapy, approximately 75% will relapse within 2 years and will ultimately undergo repetitive treatments for disease recurrences until their disease becomes treatment refractory [3]. Thus, there is an unmet clinical need for the development of new therapeutic strategies that will provide meaningful improvements in outcomes for these patients.

EOC is a biologically heterogeneous disease and the identification of tumor-specific molecular alterations may provide opportunities for targeted therapy. Biologic agents such as bevacizumab have shown moderate benefit in the treatment of EOC; however, these agents can be associated with significant dose-limiting toxicities, thus prompting efforts to identify less toxic therapeutic strategies [4]. In this regard, nuclear receptor modulators are an attractive option due to their limited toxicity profile and ease of administration, especially in patients who are unable to tolerate cytotoxic chemotherapy or other biologic agents due to side effects or medical comorbidities [5, 6].

There are 48 known nuclear receptors (NR) that function as ligand-activated intracellular transcription factors, regulating important target genes and signaling pathways. Nuclear receptors play a role in many diseases including obesity, diabetes, dyslipidemia and cardiovascular disease [7, 8]. Steroid hormones, thyroid hormones and vitamin D3 are some of the most well-known targets of transcriptional regulation by NRs. Disruption of cell and tissue specific NR function can lead to the development and progression of several cancers including breast and prostate [9, 10]. Conversely, modulation of the estrogen and androgen receptors has resulted in the successful treatment of breast cancer and prostate cancer, respectively [10, 11]. Despite what is known about NR in other malignancies, their expression and function in ovarian cancer remains largely understudied.

Using The Cancer Genome Atlas (TCGA), we sought to identify critical NRs in high-grade serous ovarian cancers. We hypothesized that a subset of ovarian tumors would have similar NR expression profiles suggesting a common molecular pattern of tumorigenesis. Identification of patterns of NR expression may provide a potential avenue for further preclinical and clinical work which would harness the therapeutic potential of targeting these receptors.

Materials and methods

Analysis of Nuclear Receptor Expression in TCGA Samples Correlation with Outcomes

Publically available TCGA data were downloaded from TCGA data portal on 3/29/2011. We combined the downloaded expression profiles from three microarray platforms, including Affymetrix Human Genome U133a, Human Exon 1.0 and Agilent G4502A, using factor analysis. We also filtered out genes with mean expression level smaller than 50 based on the Human Exon platform. A total of 504 samples were included. Then we clustered the expression profiles using a “tight clustering” algorithm developed within our institution [12]. Only NR genes were included for the clustering. The tight clustering algorithm groups similar tumors based upon expression profiles and performs re-sampling evaluation to repeatedly cluster random subsamples of data, evaluate the stability of the identified clusters, and finally generates clusters of tumors with similar gene expression patterns. Tumor samples that do not exhibit gene expression patterns similar to any of the identified clusters are excluded. “Driver genes” of each cluster were identified using the gene-dominant and gene-dormant index method [13]. This method extends the signal to noise ratio to identify features as either dominant or dormant in a specific cluster compared to the other clusters.

Clinical data from the TCGA were analyzed to determine clinical outcomes based on cluster. Progression free survival was calculated from the date of diagnosis to the date of recurrence or censoring. Overall survival was calculated from the date of diagnosis to the date of death or date of last contact. Progression-free and overall survival were calculated for the patients within each cluster using Kaplan-

Meier methods. The Cox proportional hazard model was used to evaluate the expression of NR genes as continuous predictor variables. A p-value of <0.05 was considered statistically significant.

Normal human tissue mRNA, human ovarian tissue and ovarian cancer tumor specimens

The FirstChoice® Human Total RNA Survey Panel of pooled mRNA from 3 donors of 20 normal human tissues was purchased from Ambion (Austin, TX). Normal ovarian tissue and serous ovarian tumor specimens were obtained using a protocol approved by the Magee-Womens Hospital of UPMC Institutional Review Board.

Cell culture

The OVCAR3 and OVCAR8 cell lines were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS). OVCA432 and PEO1 cell lines were maintained in RPMI 1640 medium +10% FBS, supplemented with L-glutamine and 2mM sodium pyruvate, respectively. These lines have been continuously cultured in the Division of Gynecological Oncology at MWRI. The OVSAHO cell line was obtained through the Japanese Collection of Research Bioresources and was maintained in DMEM with 10% FBS. The CA-OV-3 cell line was obtained through American Tissue Culture Collection (ATCC) and was maintained in DMEM with 10% FBS. All cells were maintained in antibiotic free media at 37°C in a 5% CO₂ atmosphere and routinely screened for Mycoplasma contamination. All tissue culture medium and additives were purchased from Invitrogen, (Carlsbad, CA) unless otherwise indicated.

Patient-derived HGSOc xenografts (PDXs)

Patient-derived HGSOc xenografts were engrafted and propagated as previously described [14]. Briefly, tumor tissue from OVCA patients was finely minced, suspended in McCoy's 5A media (Invitrogen), engrafted into SCID/Beige mice (C.B.-17JlcrHsd-Prkdc^{scid}Lysf^{bg}; Harlan) via intraperitoneal injection. Upon sacrifice, the tumors were used to either passage for subsequent studies or were collected

for gene expression and immunohistochemical analysis. The PDX studies were approved by the local Mayo IACUC committee.

Immunoblotting

Cells from each cell line were lysed with RIPA buffer (1% IgePAL, 0.5% Sodium Deoxycholate (bwt), and 0.1% SDS) on ice for 30 minutes prior to harvest for Immunoblot analysis. Protein concentrations were determined via Bradford Assay (BioRad Hercules, CA) and measured using a SpectroMax M5e spectrophotometer (Molecular Devices Sunnyvale, CA). Samples were run on 4-15% gradient SDS acrylamide precast gels (BioRad Hercules, CA) and transferred to PVDF membrane (EMD Millipore Billerica, MA) overnight at 4°C. Rabbit anti-NR4A1 (IMG-528 Novus Biologicals Littleton, CO) and mouse anti- α -tubulin (T9026 Sigma-Aldrich St. Louis, MO) were diluted 1:200 and 1:1000 respectively in 5% non-fat dry milk/Blotto (150 mM NaCl, 20 mM pH 7.5 Tris-Base, and 0.1% Tween-20) and incubated on immunoblot membranes for at least 1 hour at room temperature. Membranes were subsequently washed of primary antibodies followed by probing with respective secondary antibodies at 1:10,000 or 1:25,000. Clarity reagents (BioRad Hercules, CA) were used to activate HRP signals for 2 minutes prior to imaging using Fujifilm LAS-3000 (Fujifilm Medical Systems Stanford, CT) for development.

RNA extraction and q-RT-PCR

RNA was isolated from samples using the Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Pittsburgh, PA). iScript RT Supermix (BioRad Hercules, CA) was used to convert mRNA to cDNA. The q-RT-PCR utilized SYBR green (BioRad Hercules, CA) according to manufacturer's instructions. Primer sequences were: NR4A1 forward (CACATTGTTGCCAAGACCTG), NR4A1 reverse (TGCTGGTGTCCCATATTGG), β actin forward (CCCTGGCACCCAGCAC), and β -actin reverse (GCCGATCCACACGGAGTAC). All primers were ordered from Integrated DNA Technologies

(Coralville, IA) with standard desalting. Each primer pair was verified using serially diluted template cDNA. The fold change for each gene was calculated using the $\Delta\Delta C_t$ method [15]. q-RT-PCR was performed in technical triplicates for each gene of interest. A reference gene (β -actin) was included to normalize for input cDNA.

Immunohistochemistry (IHC)

Formalin-fixed paraffin embedded (FFPE) tissues were cut into sections of 3-5 microns. Slides were then de-paraffinized in xylene and rehydrated. Fresh antigen retrieval buffer (0.01M Sodium citrate with 0.05% Tween-20, pH 6.0) was heated to boiling and the tissue slides were added to the pre-warmed buffer for 30 minutes. The tissue slides were then allowed to equilibrate to room temperature for 30 min. Endogenous peroxidase activity was blocked by immersing slides in 3% hydrogen peroxide solution for 5 min. After rinsing with ultrapure water, the primary antibody was added directly to the tissue sample (NR4A1, IMG-528 Novus Biologicals Littleton, CO) at 1:200 dilution in 1% bovine serum albumin (BSA) and incubated in a humidified chamber at 4°C overnight. The antibody was then linked using Envision Labelled Polymer-HRP Anti-Rabbit (Dako, Via Real Carpinteria, CA) for 30 min at RT in a humidified chamber. After rinsing, tissue was covered with Dako Cytomation DAB+ solution and incubated for 15 min before chromogen signal enhancement with DAB Sparkle Enhancer (Biocare, Concord, CA). Slides were counterstained in Harris Hematoxylin and Eosin (Sigma Aldrich) for 30 sec and dehydrated and cleared in the routine manner. The sections were mounted in Cytoseal (VWR, Radnor, PA) with appropriate coverslips. A Nikon 90-I microscope and NIS Elements Microscope Imaging software (Nikon, Melville, NY) was then used to image the slides.

Immunofluorescent Detection of Endogenous NR4A1

Cells were plated at the following concentrations using the Multidrop Combi into polystyrene 384 well plates (Greiner, Monroe, NC): 5×10^4 PEO1; 2×10^4 OVCAR8 and OVCA432, 4×10^4 OVSAHO and OVCAR3, and 2×10^4 CA-OV-3. Cells were allowed to settle and attach for 30 minutes prior to

moving to an incubator at 37°C/5%CO₂. Roughly 48 hours after seeding, cells were fixed with 50 µL/well fixative solution (PBS + 7.4% Formaldehyde for addition [made from 37% Formaldehyde stock with 10%-15% MeOH for stabilization) containing 2µg/mL Hoechst added with the Multidrop Combi and were incubated at room temperature for 20 minutes. Wells were then washed 3 times using the Biotek plate washer with 80µL/well 1x PBS, allowing 5 minutes between washes. Following the final PBS wash, 80µL/well permeabilization buffer (PBS + 0.5% Triton X-100) was added and the plates were incubated at room temperature for 10 minutes. Wells were then washed with 1x PBS and 80µL/well blocking buffer (PBS + 0.5% Tween-20) was added and the plate was incubated at 4°C overnight. After blocking, cells were incubated for 1 hour at room temperature with 25µL/well 1:100 rabbit polyclonal NR4A1 primary antibody (IMG-528 Novus Biologicals Littleton, CO) prepared in blocking buffer. Wells were washed 3 times with PBS using the Biotek plate washer. 25µL/well 1:750 goat anti-rabbit IgG secondary antibody (H+L) Dylight 550 (Thermo Fisher Scientific Pittsburgh, PA) in blocking buffer were added to each well and the plate was incubated at room temperature for 45 minutes. Wells were washed 4 times with PBS using the Biotek plate washer. 1x PBS was added to each well after the final wash and the plates were sealed. The plates were then imaged using the ImageXpress Ultra (IXU) confocal high-content imaging system (Molecular Devices Corp.).

Results

Nuclear receptor expression in TCGA serous ovarian tumor samples

Using publically available data from TCGA, we applied the previously described tight clustering algorithm to group HGSOC with similar NR expression profiles, which produced 5 distinct gene clusters (Figure 1A). Nuclear receptors within each cluster were identified as either “dominant” or “dormant” based upon gene expression levels (Table 1). We hypothesized that the overexpressed genes or “dominant drivers” within each of these clusters could potentially play critical roles in the respective subsets of HGSOC. Within the largest cluster of HGSOC (Cluster 1) the most significant driver genes contained 3 members of the NR4A subfamily of nuclear receptors: NR4A1 (Nur77, TR3), NR4A2

(Nurr1), and NR4A3 (Nor1). Because the NR4A subfamily encompasses the dominant drivers of the largest cluster of patient samples, we chose to further investigate the role and implication of these genes in additional studies.

Clinical outcome of tumors with high expression of NR4A subfamily

Clinical outcomes of patients included in each cluster were compared to determine whether NR expression differences are associated with patient outcomes. Patients with tumors in Cluster 1 who highly expressed NR4A family members had significantly ($p = 0.03$) shorter progression free survival (PFS) compared to the remaining 4 clusters (Fig 1B). Median overall survival for patients in Cluster 1 exhibited a trend toward worse outcomes as compared to Clusters 2-5 but did not reach statistical significance ($p=0.09$, Fig 1B). Subsequently, a Cox proportional hazard model was used to determine the individual contribution of each of the 3 NR4A to PFS. Of the three genes, only NR4A1 was found to be significantly associated with worse PFS ($p=0.0357$). Given these findings, we sought to further investigate the role of NR4A1 in pathogenesis of HGSOC.

NR4A1 expression of normal human tissue

We first queried NR4A1 expression in the normal ovary relative to other tissues. Therefore, human pooled mRNA was obtained from 21 non-diseased human tissues and qRT-PCR was performed. As compared to the majority of tissue types investigated, the expression of NR4A1 in the ovary is relatively high, with only skeletal muscle and tracheal tissue having higher levels of expression (Figure 2A). Figure 2B demonstrates IHC staining of normal ovarian tissue for NR4A1 and demonstrates nuclear localization of NR4A1. In addition, given that many ovarian cancers are thought to originate in the fallopian tube, we also investigated NR4A1 expression in the normal fallopian tube. As shown, similar to ovarian tissue, NR4A1 staining is primarily nuclear, and is limited to the tubal epithelium. In addition, our in silico analysis of Human Protein Atlas RNA seq data confirmed high expression of NR4A1 mRNA in the fallopian tube (data not shown).

To study the role of NR4A1 in ovarian cancer, we sought to compare the IHC staining pattern of normal ovarian tissue to that of patients with ovarian cancers (Figure 3). In addition to HGSOC, we also included FFPE samples with normal ovaries, and mucinous and endometrioid histologies. Of the 10 tissue samples from patients with normal ovaries, all stained positive for NR4A1. In each of those cases, NR4A1 was localized to the nucleus with no cytoplasmic staining noted. In contrast, the majority of the mucinous and HGS HGSOC showed significant cytoplasmic staining, in addition to the nuclear staining. Of the 4 endometrioid tumors included in this study, one exhibited nuclear positivity for NR4A1, and 2 of these tumors were noted to have cytoplasmic positivity.

Given that NR4A1 localization seems to differ between normal ovarian tissue and ovarian cancer specimens on IHC staining, we identified several serous ovarian cancer cells lines in which to further investigate these differences using preclinical models. Figure 4 shows an immunoblot for NR4A1 in 6 different HGSOC cancer cell line models. 4 The OVCA432 and OVCAR3 cell lines have significantly higher protein levels of NR4A1 than the CA-OV-3, OVCAR8, OVSAHO, and PEO1 cell lines. Of note are the two isoforms of NR4A1 on the immunoblot. There are multiple transcript variants of this protein that have been described and the role for each variant has not been clearly elucidated [16]. In order to further characterize heterogeneous NR4A1 nuclear or cytoplasmic localization, we used immunofluorescence analysis to evaluate the level and localization of NR4A1 in individual cells in these HGSOC cell lines (Figure 4C). We detected significant heterogeneity in the degree to which there is cytoplasmic positivity for NR4A1. Figure 4D demonstrates that OVCA432, OVCAR8, and CA-OV-3 cells have the highest levels of cytoplasmic NR4A1 staining with the median number of cells showing over 50% NR4A1 in the cytoplasm. Heterogeneous localization of NR4A1 is important when considering which cell population to focus on while studying the mechanisms related to the NR4A1 nuclear to cytoplasmic translocation and its potential implications on resulting phenotypes.

Expression of NR4A1 in PDX models

Data from TCGA indicates NR4A1 is upregulated in a specific subset of HGS ovarian cancer patients. In an attempt to externally validate this, we analyzed the mRNA expression of NR4A1 in a series of ovarian cancer subtype PDX models (Figure 5A). As with TCGA cases and ovarian cancer cell line models, PDXs displayed wide range of NR4A1 expression. NR4A1 expression did not correlate with histologic subtype of the PDX or with stage of the original tumor; however, sample size was limited.

Discussion

In this study we aimed to identify NRs of particular importance in ovarian cancer to guide further research of the role of NRs in ovarian tumorigenesis and ultimately allow for development of a targeted therapies. We identified the NR4A family of receptors, NR4A1 in particular, as portending a worse prognosis for patients within the TCGA dataset. The NR4A family members NR4A1, NR4A2, and NR4A3, are a group of closely related nuclear receptors with no known endogenous ligand (“orphan receptors”). This subgroup has been implicated in cell cycle regulation, neurological disease, inflammation and carcinogenesis [17]. The overexpression of genes within the NR4A subgroup has been documented in several other cancers including colon, pancreatic and lung [18-20]. Further, in bladder cancer, high NR4A2 expression is associated with high tumor grade, risk for distant metastasis, and overall patient survival [21].

Of the NR4A family members, NR4A1 has drawn considerable attention in the literature recently for its role in carcinogenesis and apoptosis. NR4A1 overexpression in breast cancer cell line xenografts results in altered inflammatory response and increased risk for metastatic disease [29]. However, cytoplasmic NR4A1 can contribute to apoptosis of breast cancer cells [22]. We were able to show that NR4A1 expression at baseline is relatively high within normal ovarian tissue as compared to a number of other human tissue types. Comparing IHC staining profiles between normal ovarian tissue and epithelial ovarian cancers, staining intensity appears stronger for the cancer specimens where NR4A1 localizes to both the nucleus and cytoplasm as compared to normal ovarian tissue where cellular localization is

nuclear only. Similarly, immunoblot analysis and immunofluorescence analysis of several HGSOC cell lines reveals significant heterogeneity both in NR4A1 protein levels as well as subcellular localization.

Identifying a subpopulation of ovarian cancer patients with elevated levels of NR4A1 is critical to the future development of novel targeted therapies against NR4A1. A recent elegant study by Wilson and colleagues showed that NR4A1 contributes to platinum response in ovarian cancer and this is associated with platinum-induced nuclear export of NR4A1 which forms a proapoptotic NR4A1-bcl-2 complex [23]. They also indicate that later stage, metastatic serous tumors showed significantly lower NR4A1 expression, a finding that might seem at first counterintuitive to some of our studies. It is however possible that exact function of NR4A is very context-dependent and complex, and this should be the focus of further studies. It is of interest that a recent study by Zhou et al. showed that NR4A1 plays a role in TGF β -induced breast cancer cell migration (ie pro-oncogenic) and knockdown of NR4A1 decreases basal and TGF β -induced migration in MDA-MB-231 cells [24]. Our findings here show that NR4A1 is present in both the cytoplasm and nucleus of several ovarian cells lines under normal growth conditions. Nuclear or cytoplasmic localization appears to vary depending on the specific HGSOC cell line analyzed. Previous studies have indicated that the phosphorylation state of NR4A1, which is regulated by the MAP/JNK kinase and Akt pathways, play a role in nuclear to cytoplasmic translocation of NR4A1 [23, 25-31]. The MAP, JNK, and Akt signaling pathways are highly dysregulated in cancer and it will be interesting to see if there is a correlation between alterations in these pathways and localization of NR4A1 in ovarian cell lines, which would be an important aspect to consider while attempting to clarify the relevant biology as well as in drug discovery.

Finally, the bi-functional activity of NR4A1 appears to be dependent on its sub-cellular location with nuclear localization being responsible for the mitogenic effects and mitochondrial localization being important for the pro-apoptotic effects [32]. Many pro-apoptotic agents and stimuli have been reported to be associated with nuclear export and translocation of NR4A1 [17, 33]. Apoptosis induced in hepatocellular carcinoma cells by retinoids have been demonstrated to be NR4A1 dependent and to be

associated with localization of NR4A1 in the mitochondria [34]. In androgen sensitive LNCaP adenocarcinoma cells several apoptosis inducers consistently caused mitochondrial localization of NR4A1, and mutants that lacked mitochondrial localization failed to induce apoptosis [33]. Cytosporone B, a naturally occurring agonist of NR4A1, induces NR4A1 dependent apoptosis, modulates nuclear export [35], and retards xenograft tumor growth by inducing NR4A1 translocation to the mitochondria in gastric cancer cells [36]. It also demonstrates the bi-functional roles of NR4A1 in proliferation and survival – its nuclear role as transcription factor with effects on gene expression, and its role in apoptosis through its translocation to the mitochondria.

Developing a targeted molecule for the NR4A1 orphan nuclear receptor and applying the compound in the clinic is very conceivable. Safe and colleagues were able to develop a molecule, 1,1-bis(3-indolyl)-1-(p-substituted phenyl)methane (C-DIM) analog, which bind NR4A1 ligand binding domain [37]. These compounds bind nuclear NR4A1 and inhibit several pro-oncogenic NR4A1-regulated genes/pathways resulting in inhibition of mTOR signaling induction of oxidative and endoplasmic reticulum stress and inhibition of several growth promoting and survival genes [19, 38-40]. C-DIMs are synthetic analogs derived from indole-3-carbinol, which is a chemo-protective photochemical present in leafy green vegetables. Future studies should test whether C-DIM/NR4A1 antagonists and other NR4A1-targeting agents are effective for HGSOc cells, with the ultimate goal of targeting NR4A1 in a subset of ovarian cancer patients.

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Figure Legend:

Figure 1. TCGA *in silico* analysis. **Figure 1A** shows the heat map of nuclear receptor expression for tumors in the TCGA. Columns within the heat map represent TCGA tumor samples and rows represent individual nuclear receptors. The tight clustering algorithm utilized grouped the tumors into 5 clusters (see methods). **Figure 1B** shows progression-free and overall survival for the subset of patients with NR4A overexpression (cluster 1) as compared to those patients with lower NR4A expression. Median progression free survival of cluster 1 patients is significantly worse than that for those patients in clusters 2-5 ($p=0.03$). While there was a trend towards worse overall survival for those patients in cluster 1, the results did not reach statistical significance ($p=0.09$).

Figure 2. mRNA NR4A1 expression in normal ovarian tissue and fallopian tube tissue. **Figure 2A** shows q-RT-PCR data of NR4A1 expression in normal human tissue (from human pooled mRNA) graphed as relative expression to β actin. The expression of NR4A1 in the ovary is higher than most other tissues with the exception of skeletal muscle and tracheal tissue. **Figure 2B** demonstrates the immunohistochemical expression of NR4A1 in normal human ovarian tissue and fallopian tube tissue at 10x magnification. 1 demonstrates ovarian surface epithelium with underlying stroma while 2 demonstrates fallopian tube epithelium. The staining demonstrates strong nuclear NR4A1, but absence of cytoplasmic NR4A1 in normal human ovarian and fallopian tube samples.

Figure 3. NR4A1 immunohistochemical staining in benign and malignant ovarian tissue. **Figure 3A** is a table outlining histology of patient ovarian cancer samples analyzed and localization of NR4A1 staining. Positive NR4A1 staining was determined by observing at least 10% of cells in a given field with brown staining in the nucleus or cytoplasm respectively. **Figure 3B** shows representative images of immunohistochemical H&E and NR4A1 staining. Representative images are shown with 20x magnification and 40x insets.

Figure 4. NR4A1 in human serous ovarian cancer cell lines. **Figure 4A.** Immunoblot for NR4A1 in whole cell lysate of several cell lines representing HGS OVCA. **Figure 4B.** Densitometry analysis on immunoblot in A showing OVCAR-432 and OVCAR3 have significantly higher NR4A1 expression. **Figure 4C.** Representative immunofluorescent images of indicated cell lines stained for NR4A1 and Hoechst. Images are representative of 20x magnification with a digital zoom inset. **Figure 4D.** Analysis of total NR4A1 immunofluorescence signal in representative cell lines. Image analyses are representative of: PEO1(5 experiments), OVCA432(2 experiments), OVCAR8(3 experiments), OVSAHO(2 experiments), CA-OV-3(2 experiments), and OVCAR3(2 experiments). NR4A1 data from D was analyzed for nuclear/cytoplasmic localization. The fraction of NR4A1 in the cytoplasm was calculated by normalizing the integrated fluorescence intensity in the cytoplasm to the total integrated intensity in the cell (fluorescence in the cytoplasm and nucleus).

Figure 5. Quantitative Real-Time PCR data for total mRNA expression of NR4A1 in human patient derived xenografts of ovarian cancer. **Figure 5A.** Clinicopathological characteristics of the primary tumor sources used for generation of PDX models. **Figure 5B.** Gene expression corrected to respective beta-actin expression, followed by normalization to NR4A1 delta-Ct of MCF7 in order to observe fold change compared to MCF7.

Table 1. Dominant and dormant driver NR genes from TCGA dataset. Gene expression of those NRs included in the TCGA analysis on high-grade serous ovarian cancers was analyzed using a “tight clustering” algorithm (see methods section) and identified five patient clusters based on differential gene expression. Driver genes of each cluster were investigated with the gene-dominant and gene-dormant index method (21). This method extends the signal to noise ratio to identify features as either dominant or dormant in a specific cluster compared to the other clusters. The associated table identifies those genes included in the analysis and whether the gene of interest is considered dominant or dormant for each of the 5 clusters. Statistically significant drivers are highlighted.

Figure 1. TCGA *in silico* analysis.

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Figure 2. mRNA NR4A1 expression in normal ovarian and fallopian tube tissue.

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Figure 3. NR4A1 immunohistochemical staining in benign and malignant ovarian tissue.

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Histology	Total	Nuclear Only	Cytoplasmic Only	Nuclear and Cytoplasmic	None
Benign	10	10	0	0	0
Mucinous	9	0	0	7	2
Endometrioid	4	0	1	1	2
High Grade Serous	6	0	0	5	1

Figure 4. NR4A1 in human serous ovarian cancer cell lines.

Figure 5. Quantitative Real-Time PCR data for total mRNA expression of NR4A1 in human patient derived xenografts of ovarian cancer.

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Table 1. Dominant and dormant driver NR genes from TCGA dataset.

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Nuclear Receptor	Cluster (Dominant)	p-value	Cluster (Dormant)	p-value
AR	5	0.1917	1	0.1431
ESR1	4	0.1938	1	0.7332
ESRRA	3	0.5610	2	0.0001
HNF4A	3	<0.0001	5	0.1358
NR1D2	5	<0.0001	3	0.0119
NR1H2	3	0.0220	1	0.1246
NR1H3	4	0.0488	2	0.7286
NR2C1	2	0.0004	3	0.0766
NR2C2	4	0.0801	1	0.5953
NR2E3	3	0.0189	5	0.0006
NR2F1	2	0.2902	4	0.0338
NR2F2	2	0.0021	4	0.4752
NR2F6	2	0.3093	4	0.6245
NR3C1	4	0.1591	2	<0.0001
NR3C2	4	<0.0001	2	0.0002
NR4A1	1	<0.0001	4	0.1686
NR4A2	1	<0.0001	2	0.2969
NR4A3	1	<0.0001	5	0.5827
NR5A1	3	0.0077	5	0.0416
NR6A1	3	0.0128	1	0.1253
PGR	2	<0.0001	5	0.0495
PPARA	4	0.0071	2	0.0003
PPARD	3	0.0011	5	0.1183
RARA	3	<0.0001	4	0.2198
RARB	1	<0.0001	2	0.7096
RARG	5	0.0002	2	0.0292
RORA	1	0.8749	5	0.1937
RORC	4	0.3249	1	0.2564
RXRA	3	0.1722	2	0.0316
RXRB	2	0.5655	1	<0.0001
THRA	2	0.7246	4	0.8499
THRB	4	0.0181	2	0.0245
VDR	1	0.0619	2	0.0076
GPER	3	0.0012	1	0.8310
PGRMC1	2	0.0027	4	0.0119
PGRMC2	4	0.8194	3	0.0078

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NR2C2	4	0.0801	1	0.5953
NR2E3	3	0.0189	5	0.0006
NR2F1	2	0.2902	4	0.0338
NR2F2	2	0.0021	4	0.4752
NR2F6	2	0.3093	4	0.6245
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NR3C2	4	<0.0001	2	0.0002
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NR4A2	1	<0.0001	2	0.2969
NR4A3	1	<0.0001	5	0.5827
NR5A1	3	0.0077	5	0.0416
NR6A1	3	0.0128	1	0.1253
PGR	2	<0.0001	5	0.0495
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PPARD	3	0.0011	5	0.1183
RARA	3	<0.0001	4	0.2198
RARB	1	<0.0001	2	0.7096
RARG	5	0.0002	2	0.0292
RORA	1	0.8749	5	0.1937
RORC	4	0.3249	1	0.2564
RXRA	3	0.1722	2	0.0316
RXRB	2	0.5655	1	<0.0001
THRA	2	0.7246	4	0.8499
THRB	4	0.0181	2	0.0245
VDR	1	0.0619	2	0.0076
GPGR	3	0.0012	1	0.8310
PGRMC1	2	0.0027	4	0.0119
PGRMC2	4	0.8194	3	0.0078