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Title: SKOV3 cells containing a truncated ARID1a protein have a
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Keywords: Glucocorticoids, Glucocorticoid Receptor, SWI/SNF, ARID1a,
Transcription.

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Abstract: AT-rich interacting domain subunit 1a (ARID1a) is an essential SWI/SNF component frequently mutated in human cancers. ARID1a mutations have also been associated with glucocorticoid resistance, potentially related to the well-established role of the SWI/SNF complex in glucocorticoid target gene regulation. Glucocorticoids are steroid hormones important for regulating many physiological processes through the activation of the glucocorticoid receptor (GR). As GR interacts directly with ARID1a, we hypothesized that a truncating ARID mutation would interfere with GR-dependent gene regulation. Using high throughput RNA sequencing (RNA-SEQ) we show a restricted glucocorticoid response in SKOV3 cells, which contain an inactivating ARID1a mutation. We also show a lack of GR binding at the GR-dependent regulatory site in the Period 1 gene, which has previously been shown to require chromatin remodelling. Taken together, our data suggests that ARID1a may be required for regulation of a subset of glucocorticoid responsive genes. In the case of SKOV3 cells, in which ARID1a is mutated, glucocorticoid-dependent transcriptional regulation of these genes is significantly impaired.

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06 August 2017

Dear Editor

We thank you and your reviewers for your extremely insightful and kind comments about our manuscript. We have attempted to address all the recommendations in our revised manuscript. Unfortunately, all our attempts to transfect the SKOV cells (for either reintroduction of full length wild type ARID1a or knock-down of the existing ARID1a) proved impossible. Therefore, we have shown all the data obtained from our attempts to do as the reviewer asked, in our response to reviewers letter (Appendices). We are confident that with our revisions in text to clarify certain points, and include suggested discussion, that our manuscript would be of interest to your journal's audience. Therefore, we would be very pleased if you would consider our revised manuscript entitled 'SKOV3 cells containing a truncated ARID1a protein have a restricted genome-wide response to Glucocorticoids' for publication in Molecular and Cellular Endocrinology. We believe this work would still be of interest to those focused on ARID1a mutations in cancer as well as to glucocorticoid researchers.

Yours faithfully,

A handwritten signature in blue ink that reads "B. Conway-Campbell".

Becky Conway-Campbell

1. Formally, genetic manipulation of ARID1a (either rescue by wt protein overexpression or knockdown of the remaining wt protein) in SKOV3 may strengthen the role played by the mutated ARID1a allele in the impaired GR response with respect to other possible alterations (including the additional mutations identified by RNAseq) potentially present in this particular cancer cell line.

We agree with the reviewer that ideally we would over-express the full-length ARID1a in the SKOV3 cells and assess for changes in the RNAseq data. However several attempts have now been made to try to reintroduce the full length ARID1a construct back into the SKOV3 cells. Despite creating and obtaining a full length ARID1a construct complete with several tags and a Green fluorescent protein, transfecting the construct has proven incredibly difficult (See appendix A, B). Similarly trying to knock-down ARID1a in the SKOV3 cells has proved difficult (See appendix C). We now believe this to be impossible without further more invasive techniques such as electroporation, or methods outside our current expertise such as viral infection, both of which may also have many adverse effects on the cells and prevent us from properly deciphering the role of ARID1a. Therefore, we can only offer this comparison, which we have attempted to put into context appropriately in the manuscript.

2. Fig 1B, how do the Authors explain the partial retention of GR in the S100 fraction upon stimulation of SKOV3 cells ? Is ARID1a also expected to be involved in nuclear translocation of GR ? Should this be ARID1a-independent (see also point 1), this may suggest that glucocorticoid resistance of SKOV3 may have a heterogenous molecular basis.

The fractionation methodology used for these studies is known to yield a nuclear extract fraction containing the high salt extractable, chromatin bound proteins, and a S100 fraction composed of low salt extractable, non chromatin associated nuclear proteins and cytoplasmic proteins (Yang et al., 1996, 1997; Liu et al., 1999a/b; Deroo et al., 2002; Tago et al., 2004). In our hands, ARID1a is only present in the nuclear (chromatin associated) fraction of the SKOV3 and HeLa cells and therefore it is not

expected to be involved in the nuclear translocation of GR. However ARID1a may be involved in recruiting GR to the chromatin and this may explain why some GR is retained in the low salt S100 fraction. GR is not always depleted from the S100 fraction in every cell line in the presence of Dex, so this may also be a cell specific trait. In order to prove GR chromatin association is dependent upon ARID1a we would have to first be able to overexpress or knock-down the construct (see answer to question 1). We would like to thank the reviewer for this comment and have amended the text (line number 185-188) to further clarify this.

Figure 1. Data revealing the absence of ARID1a in our S100 fractions of both cell lines.

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Minor

3. Introduction, page 3: the last 15 lines or so, summarizing the results of the study, may be omitted or significantly shortened.

We would like to thank the reviewer for the feedback on this section that has now been significantly shortened.

4. Given the frequent occurrence of ARID1a mutations in cancer (perhaps also in patients not previously treated with glucocorticoids), do the Authors envisage that loss-of-function lesions, such as the one described in this study, may be only involved in secondary resistance to glucocorticoids or play additional roles in driving cancer formation (also regardless glucocorticoids exposure) ?

Again we would like to thank the reviewer for these questions. It is well known and largely published that ARID1a is a tumour suppressor. I have now included this into the introduction (line number 56-58).

Appendix A. Overexpression of a Full length ARID1a construct

A pcDNA6-ARID1a construct was obtained as bacteria in an agar stab (Addgene, UK). Bacteria were grown and plasmid DNA was extracted using a Qiagen maxi-prep kit (Qiagen, UK) following the manufacturer's instructions. SKOV3 cells were grown for 24 hours in normal growth medium in 10cm² plates to 70% confluence. The media was changed to a reduced serum Optimem (Gibco, Life Technologies, UK) an hour prior to transfection. A selection of quantity ratios of DNA to Lipofectamine2000 (Invitrogen, UK) were combined together and incubated for 5 minutes in Optimem at room temperature before being added to the cells (Table 1). Following an overnight incubation whole cell lysates were prepared for Western blotting. Anti-ARID1a antibodies (Bethyl Labs; A301-040A, A301-041A), a His-tag antibody (Cell signaling, 2365) and a loading control Beta Tubulin (Sigma, T4026) were used for protein detection.

Table 1: Transfection ratios of DNA to Lipofectamine2000. Transfecting pcDNA6-ARID1a into SKOV3 cells.

Condition	PcDNA6-ARID1a (673.2 µg/ml) (µl)	Lipofectamine2000 (µl)	Optimem volume required for 1ml (µl)
Untransfected	0	30	970
Low DNA transfection	14	30	956
High DNA transfection	20	30	950
Very high DNA transfection	28	30	942

No change in the detection of ARID1a protein was observed (Figure 1). Beta tubulin is used as a loading control for SKOV3 whole cell lysates. Similarly, no His-tagged protein could be detected by Western Blot using an anti-His antibody in whole cell lysates or nuclear extracts in these cells (data not shown). As we had no positive control for the antibody we were therefore unable to determine whether the construct had been transfected into the cells.

Figure 1: Western blot showing no increase in the amount of ARID1a protein in whole cell lysates after attempted transfection of Addgene pcDNA6-ARID1a in SKOV3 cells. Beta Tubulin is used as an endogenous loading control.

B: Transfecting a full length ARID1a-GFPN1 construct

A fluorescent-tagged construct was considered to be beneficial in optimizing the transfection, in particular to aiding in the direct observation of the transfection efficiency during optimization. The following construct was created using a GFP-N1 backbone vector and incorporates a HIS-TAG (Figure 2). SKOV3 cells and HeLa cells (used as a comparison) were grown for 24 hours in normal growth medium in 10cm² plates to 70% confluence. The media was replaced with reduced serum Optimem (Gibco, Life Technologies, UK). A variety of concentrations of DNA to Lipofectamine2000 (Invitrogen, UK) were used to determine the best transfection efficiency. ARID1a-GFPN1 DNA and Lipofectamine2000 were incubated together for 5 minutes in Optimem before addition to the cells. Following an overnight incubation cells were observed using a fluorescence microscope (Leica Biosystems, UK).

Figure 2: GFPN1 vector and ARID1a insert from Addgene vector. A) Represents the two constructs, GFPN1 and pcDNA-ARID1a, which are digested and ligated to create ARID1a-GFPN1. B) Schematic of ARID1a-GFPN1 and the restriction enzymes used to create construct.

However no fluorescence was observed using Lipofectamine2000 so other transfection reagents were used. K2 transfection reagent (Biontex Laboratories, UK) was the most successful transfection reagent used. However even using the ‘In-Cell 1000’ to visualize transfected cells, there was no transfection of SKOV3 cells and very few cells transfected in HeLa cells (Figure 3). Consistent with this, Western blotting of SKOV3 cells again revealed a lack of increase in the ARID1a protein levels. As HeLa cells are regarded as relatively easy to transfect, the likelihood of overexpressing ARID1a in the SKOV3 cell line seemed highly unlikely without using a viral infection protocol, which we did not have access to.

HeLa cells transfected with ARID1A-GFP construct

Figure 3: ARID1a-GFPN1 transfection efficiency chart. Three full length ARID1a-GFPN1 clones (clone 1, 3 and 9) were transfected, using K2 transfection reagent, for 24 hours in HeLa cells. The majority of cells, indicated by the mean bar, do not show high cell fluorescence intensity compared to a GFPN1 transfected control and an untransfected (untrans) control (with only K2 transfection reagent). The mean fluorescence was very similar to that of untransfected cell group. Observation of cell images also revealed very few cells were fluorescent and suggested only a few individual cells were transfected.

C: Knock-down of ARID1a using shRNA with a pSUPER construct

Four sets of double stranded RNA oligos were designed to target ARID1a gene expression by short hairpin RNA (shRNA) knock-down when directionally cloned into a carrier vector, pSUPER (Table 2). Oligos contained a unique 19-nucleotide (nt) sequence derived from the ARID1a mRNA transcript, which was targeted for gene suppression. The forward and reverse oligos were designed to anneal and be cloned into the pSUPER vector, between the unique BglII and XhoI sites. This design enables the forward oligo to be positioned downstream of the pSUPER H1 promoter's TATA box. The ARID1a gene sequence and therefore the 19 nt mRNA sequence corresponds to the sense strand of the subsequent pSUPER produced siRNA. The antisense strand of the siRNA binds to this mRNA region to promote cleavage of the siRNA. The forward oligo also contains a 9 nt spacer sequence which separates the 19 nt target sequence within both the sense and antisense strands. The oligo 5' overhang corresponds to a BamHI site that is compatible with the plasmid's 3' BglII overhang therefore ligation destroys the BglII site producing a higher chance of obtaining positive clones. Cloning was successful and attempts were made to transfect DNA constructs into SKOV3 cells and HeLa cells.

Table 2: RNA oligos for shRNA ARID1a knock-down.

Target ARID1a Sequence	OLIGO Design
cgtgtgtggagaacttaga (7268~)*, UTR	5'-GATCCCCcgtgtgtggagaacttagaTTCAAGAGAtctaagttctccacacagTTTTTC-3' 3'-GGGgcacacacctcttgaatctAAGTTCTCTagattcaagaggtgtgtgcAAAAAGAGCT-5' (BamHI) (XhoI)
ccaacaacatggcggacaa (883~), ORF	5'-GATCCCCccaacaacatggcggacaaTTCAAGAGAttgtccgccatgttcttggTTTTTC-3' 3'-GGGggtgtgtgtaccgcctgttAAGTTCTCTaacaggcgggtacaacaaccAAAAAGAGCT-5' (BamHI) (XhoI)
tggcagaaggaggagactt (3834~)*, ORF	5'-GATCCCCtggcagaaggaggagacttTTCAAGAGAAagtctctcttctgccTTTTTC-3' 3'-GGGaccgtctctctctctgaaAAGTTCTCTttcagaggaggaagacggtAAAAAGAGCT-5' (BamHI) (XhoI)
gccaggagagcagagtaa (2379~)*, ORF	5'-GATCCCCgccaggagagcagagtaaTTCAAGAGAttactctgtctcttggcTTTTTC-3' 3'-GGGcggttctctctctctcattAAGTTCTCTaatgagacgagaggaaccgAAAAAGAGCT-5' (BamHI) (XhoI)
Oligos were purchased from Sigma, UK	

The construct DNA was incubated with Lipofectamine3000 (and Lipofectamine3000 additional reagent) (Invitrogen, UK) for exactly 5 minutes before addition to HeLa and SKOV3 cells. For a control, the PSUPER vector alone was transfected in the

same way into the cells. Unfortunately, the efficiency of the transfection was low, as observed under a fluorescent microscope, and failed to knock-down the 250kDa ARID1a protein band seen on Western blots (Figure 4).

Figure 4: Western blot showing no knock-down in ARID1a protein levels following transfection with several pSUPER-ARID1a shRNA clones compared to the pSUPER vector alone. Lamin is used as an endogenous loading control for HeLa cell nuclear extracts.

SKOV3 cells containing a truncated ARID1a protein have a restricted genome-wide response to glucocorticoids

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Abstract

AT-rich interacting domain subunit 1a (ARID1a) is an essential SWI/SNF component frequently mutated in human cancers. ARID1a mutations have also been associated with glucocorticoid resistance, potentially related to the well-established role of the SWI/SNF complex in glucocorticoid target gene regulation. Glucocorticoids are steroid hormones important for regulating many physiological processes through the activation of the glucocorticoid receptor (GR). As GR interacts directly with ARID1a, we hypothesized that a truncating ARID mutation would interfere with GR-dependent gene regulation. Using high throughput RNA sequencing (RNA-SEQ) we show a restricted glucocorticoid response in SKOV3 cells, which contain an inactivating ARID1a mutation. We also show a lack of GR binding at the GR-dependent regulatory site in the Period 1 gene, which has

previously been shown to require chromatin remodelling. Taken together, our data suggests that ARID1a may be required for regulation of a subset of glucocorticoid responsive genes. In the case of SKOV3 cells, in which ARID1a is mutated, glucocorticoid-dependent transcriptional regulation of these genes is significantly impaired.

Keywords

Glucocorticoids, Glucocorticoid Receptor, SWI/SNF, ARID1a, Transcription.

Abbreviations

GR – Glucocorticoid Receptor

GRE – Glucocorticoid response element

SWI/SNF - SWItch/Sucrose Non-Fermentable

BAF – BRG1 associated factor

ARID – AT-rich interaction domain

FKBP5 - FK506 binding protein 5

Per1 – Period 1

DUSP1 – Dual specificity phosphatase 1

ChIP – Chromatin Immunoprecipitation

FPKM - Fragments per kilobase per million mapped reads

mRNA – Messenger RNA

1. Introduction

Glucocorticoids are among the most commonly used pharmacological agents due to their potent anti-inflammatory properties (1, 2). They are also extensively used as a cancer treatment due to their ability to induce apoptosis and promote cell cycle arrest (3, 4, 5). However despite their benefits, these drugs are often associated with several side effects (6, 7, 8, 9) and a significant proportion of patients develop glucocorticoid resistance (10, 11). ARID1a mutations have been linked to glucocorticoid resistance and are identified across a multitude of human cancers often associated with poor patient prognosis (12). ARID proteins have previously been found to be tumour suppressors and knockdown of their expression can promote cancer formation (13). ARID1a is an essential component of the ATPase driven SWItch/Sucrose NonFermentable (SWI/SNF) chromatin-remodelling complex. The protein's C-terminal has previously been reported to directly interact with

GR (14, 15) (Supplementary figure 1). ARID1a interacts with AT-rich sequences within the DNA (16) potentially recruiting GR to specific glucocorticoid response elements (GREs) to regulate gene expression through dynamic mobilization of the chromatin. Determining the functional role of ARID1a in GR signalling is therefore of great importance for understanding how mutations in the SWI/SNF subunit could contribute to glucocorticoid resistance.

Glucocorticoids act through the binding of GRs located in the cell cytoplasm bound to chaperone proteins (17, 18, 19). Upon ligand binding, GR translocates to the nucleus where it binds at GREs (20, 21, 22). Chromatin-remodelling by the SWI/SNF complex is a vital component of genomic GR signalling, with chromatin being dynamically opened and closed at GREs in target genes regulating the access of transcriptional machinery and RNA Polymerase II (23, 24). The SWI/SNF complex is comprised of a single ATPase, important for driving the remodelling activities of the complex, and several associated BRG-1 associated factors including ARID1a (BAF250a) (25). ARID1a could be an important subunit of the SWI/SNF complex potentially facilitating and fine-tuning this GR mediated transcriptional regulation for a subset of GR-dependent genes (26). Therefore absence of the functional ARID1a protein may disrupt GR-dependent gene regulation and provide one possible mechanism for development of glucocorticoid resistance.

SKOV3 cells are a human ovarian cancer cell line that has been extensively used as a model to study molecular mechanisms and outcomes of a mutation in ARID1a (27, 28, 29). SKOV3 cells contain a truncating mutation of ARID1a in exon 3; a Glutamine (codon CAG) is altered to a premature stop codon (TAG) at position 586 in the amino acid sequence (28). Such a truncating mutation results in loss of the reported GR binding domain so may impact on GR-dependent gene regulation. To test this, the glucocorticoid responsiveness of the SKOV3 cells has been assessed using RNA-SEQ expression profiling over a 6 hour Dexamethasone (Dex) time course. As a technical control for our analysis pipeline, we have conducted parallel assessment of the robustly glucocorticoid responsive HeLa cell line (30, 31) known to contain a full-length functional ARID1a.

2. Methods

2.1 Cell culture

Human cervical cancer cell line, HeLa cells, and human ovarian adenocarcinoma, SKOV3 cells, were obtained from the European Collection of Cell Cultures (ECACC; Sigma-Aldrich), each as a frozen stock. Human HeLa cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine and glucose (Gibco, Life Technologies, UK), 10% foetal calf serum (FCS, Invitrogen, UK) and

1% penicillin/streptomycin (Gibco, Life Technologies, UK). Human SKOV3 cells were maintained in phenol red McCoy's 5a modified medium (PAA, UK), 10% FCS (Invitrogen, UK) and 1% penicillin/streptomycin (Gibco, Life Technologies, UK). Cells were cultured in 10cm² plates (for RNA extraction and RT-qPCR) OR 115cm² flasks (for ChIP) and maintained in a humidified incubator (LEEC Ltd., UK) at 37°C and 5% CO₂ until 70% confluent. Cultures were washed 3 times in PBS 24 hours prior to hormone addition and maintained in serum and phenol red free DMEM-F12 medium supplemented with 100 µg/ml BSA (fraction V) (GIBCO) and 10 µg/ml transferrin (Sigma). A final concentration of 100nM Dex was added to this culture medium.

2.2 Nuclear fractions

As previously described (32) the cells were resuspended in 300µl S1 buffer [10 mM pH 7.9 HEPES, 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM pH 8 EDTA] supplemented with 0.5 mM dithiothreitol (DTT), 50x complete protease inhibitor (Roche, UK), 2mM NaF and 0.2mM NaVan. Samples were briefly spun at 2000 x g at 4 °C and the cytoplasmic S100 fraction was removed. The nuclear pellet was resuspended in 1.2 volumes of cold S2 buffer [10 mM pH 7.9 HEPES, 400 mM NaCl, 1.5 mM MgCl₂, 0.1 mM pH 8 EDTA, 5% glycerol] supplemented with 2mM NaF, 0.2 mM NaVan, 0.5mM DTT and Complete protease inhibitor (Roche, UK). The pure nuclear fraction and cytoplasmic fraction were then stored at -80 °C. A bicinchoninic acid assay (Pierce, Rockford, IL) was used to determine protein concentrations.

2.3 Western blotting

As previously described in ref. Antibodies used were ARID1a A301-040A (Bethyl labs) and A301-041A (Bethyl labs), GR E20 (Santa Cruz, sc-1003), Anti Beta Tubulin (Sigma, cat: T4026) and Anti Lamin (Cell Signaling Technology, 2032). The membranes were washed and incubated with the appropriate secondary antibody, ECL anti-rabbit, cat: NA934V, or ECL anti-mouse, cat: NA931V (GE Healthcare, UK). Protein bands were detected using chemiluminescence; an enhanced chemiluminescence (ECL) reagent (EZ-ECL Biological Industries, USA).

2.4 RNA extractions and Real time qPCR

RNA was purified using membrane columns (RNAeasy minikit, Qiagen, UK) following the manufacturer's guidelines. The RNA sample was diluted using nuclease free water and 1mg of RNA was reversed transcribed into cDNA using a cloned Avian Myeloblastosis Virus (AMV) first strand synthesis kit (Invitrogen, Life Technologies, UK) following the manufacturers guidelines. Real-time

PCR assays were performed using SYBR (Applied biosystems) and primers (see Supplementary table 1).

2.5 Chromatin Immunoprecipitation

After treatment, cells were fixed for 10 minutes in 1% formaldehyde, quenched with 0.125M glycine and washed with PBS. Nuclear extracts were prepared as described in 2.2, then resuspended in 6 Vol MNase digestion buffer [50mM Tris-HCl, pH 7.5, 4mM MgCl₂, 1mM CaCl₂, 0.32mM sucrose, 2mM NaF, 0.2mM NaVan] with 2 units of MNase (cat: N5386, Sigma, UK) per 180µg chromatin for 15 minutes at 37 °C. MNase digestion was stopped with addition of 5mM EDTA pH8 on ice. The nuclear pellet was resuspended in 300µl sodium dodecyl sulfate (SDS) lysis buffer [1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8.1], supplemented with complete protease inhibitor (Roche Diagnostics, Burgess Hill, UK), 2mM NaF and 0.2mM NaVan. Lysates were cleared of debris by centrifugation at 16200 x g for 15 minutes at 4°C and the soluble chromatin was collected for chromatin immunoprecipitation (ChIP). For each ChIP reaction, 30µg samples were processed with H300 GR antibody (Santa Cruz sc-8992) or rabbit non-immune serum (Santa Cruz sc-2027). RT-qPCR was performed using SYBR reagents (Applied Biosystems) and the following Per1 primers.

Primer sequences (h, human; 5'-3')

Distal hPER1, F ACAGGACGGCTGTCGTTTTG

Distal hPER1, R CGCACTTGGGAACATCATGT

Distal hPER1, F TCATGTTCTCTTGGCTGGTG (33)

Distal hPER1, R GGCCCCCTCCTACTAATCC (33)

Proximal hPER1, F CTAGTCCGAAGTGGGCTGAC (33)

Proximal hPER1, R CCGGTCTTCTTGCTCGTTAC (33)

Exon 19 hPER1, F GCCTTGGTGCTCCCTAACTA

Exon 19 hPER1, R TCTGGAGTGCCCCATAAGGA

2.6 High throughput RNA Sequencing

High throughput RNA sequencing was performed (Bristol genomics facility) and the raw data was analysed using Galaxy (www.galaxyproject.org). Three lanes were uploaded for each RNA sample

and for each condition an n of at least 3 were assessed. The Cuff Diff parameters included were geometric library organization, pooled dispersion estimation, 0.05 False discovery rate, Min alignment count 10, multi-read correct, bias correction and cuff-links effective length correction. Differential gene expression was calculated as fragments per kilobase per million mapped reads (FPKM) values; summed fragments of each transcript with same gene ID. After Benjamini-Hochberg false discovery correction, genes with adjusted p values less than 0.05 were considered as differentially expressed genes.

3. Results

3.1 Decreased ARID1a protein expression in SKOV3 cells

We have confirmed a mutation (C1756T) in exon 3 of ARID1a (Supplementary Fig 2) resulting in a premature stop codon (Q586*) in SKOV3 cells as described previously (28). Interestingly, we can detect both transcripts in the sequencing data, consistent with a heterozygous mutation. Western blotting was used to assess whether this mutation resulted in loss of ARID1a protein (Figure 1). A strong 250kDa band was detected in the nuclear fraction of the positive control HeLa cells (Figure 1A) but not detected in HeLa cells after ARID1a siRNA knockdown (Figure 1B) confirming the band to be ARID1a. As shown in Figure 1A a 250 kDa band was also detected in the nuclear fraction of SKOV3 cells indicating the presence of full-length ARID1a. Using Image J Densitometry (<http://imagej.nih.gov/ij>) SKOV3 cells were found to express approximately 62 % of the full-length ARID1a protein expressed in HeLa cells. Taken together, the RNA-SEQ and Western blot results indicate that the ARID1a mutation in SKOV3 cells is heterozygous. ARID1a has been suggested to be a haplosufficient tumour suppressor with a large proportion of cancers containing a heterozygous ARID1a mutation (27, 34).

Finally and importantly for this study, both cell lines possess a full-length, functional GR indicated by a band detected at the expected size of 97kDa, which was detected in the nuclear fraction only after Dex treatment (Figure 1C, D). Similar GR protein levels were found for both cell lines in the absence of Dex. In contrast to the near complete depletion of GR from the S100 fraction after Dex treatment in HeLa cells, a visible GR band remains in the S100 fraction after Dex treatment in SKOV3 cells. This is highly suggestive of the presence of 'a pool of inactive GR' remaining after Dex treatment in the SKOV3 cells. This reflects an impairment of GR chromatin interactions or stability in SKOV3 cells potentially due to the loss of some full length ARID1a. ARID1a was not found in the S100 low salt cytoplasmic fraction for either cell line (data not shown). This is in contrary to previous suggestions that ARID1a is shuttled from the cytoplasm to the nucleus (35). As ARID1a appears to be associated

with the chromatin it thereby would not be essential for GR translocation from the cytoplasm. Despite this, there was still a robust activation response detected in the SKOV3 cells, with a significant translocation of GR to the 'active nuclear fraction'.

Figure 1: Western blots indicating the presence and subcellular localization of ARID1a and GR in HeLa cells and SKOV3 cells with or without Dex treatment. A) ARID1a protein is observed in the nuclear fraction of both cell lines. Lamin is used as an endogenous loading control specific to the nuclear extract fraction (NE). B) ARID1a is observed to be knocked down in HeLa cells using reverse transfections of a combination of four siRNAs for 48 hours. C) Western blot showing GR in HeLa cells in the nuclear extract (NE) and cytoplasm (S100) with or without Dex. The translocation of GR into the nuclear fraction upon Dex treatment indicates a functional GR protein. Beta Tubulin is used as a control specific to the cytoplasm. D) Western blot showing GR in SKOV3 cells with or without Dex in both the NE and cytoplasm (S100). An increase in GR into the nucleus upon Dex treatment indicates that GR is functional.

3.2 Basal expression profiling characterises cell-specific differences between SKOV3 and HeLa cells

It was first important to determine cell-specific basal expression differences between the SKOV3 and HeLa cells prior to assessing glucocorticoid responsiveness. Therefore, RNA-SEQ expression profiles of the two cell lines were compared in the absence of glucocorticoid (time 0hr). As expected there are several cell-specific expression differences at baseline (pre-treatment, time 0hr) as shown by the heat map, Figure 2A. By comparing these basal groups for both cell lines, 3171 genes were identified as being more highly expressed in HeLa cells compared to SKOV3 cells. Additionally, 3507 genes are more highly expressed in the SKOV3 cells compared to the HeLa cells (Supplementary table 1). This is the expression profile that defines each cell line, indicating expected cell-specific differences in their respective transcriptomes. Despite the difference in protein observed from

Western blotting, basal ARID1a mRNA levels are very similar in both cell lines. Basal GR mRNA levels in SKOV3 cells are approximately 3 times lower than the levels in HeLa cells. Importantly, there are large numbers of genes that are common to both cell lines (Figure 2B, C). Figure 2C shows a high correlation between basal expression values of genes with FPKM values greater than 1 for both HeLa and SKOV3 cells with a Pearson correlation coefficient of 0.86. Many commonly expressed glucocorticoid responsive genes such as GILZ (38), IRS2 and THBD (39) have similar basal expression levels. However, some largely ubiquitous genes such as Per1 and DUSP1 are expressed at lower levels in the SKOV3 cells; the basal mRNA levels are approximately 3 times lower for Per1 and 2 times lower for DUSP1 compared to HeLas. Some widely expressed genes such as DKK1 (40) have much higher basal expression in the SKOV3 than in the HeLas; basal expression is approximately 14 times higher in SKOV3 cells. However, it is predominantly the overall pattern of global changes after Dex treatment that is important for a comparison of the glucocorticoid responsiveness of these two cell lines.

Figure 2: Basal gene expression and genes regulated by Dex in HeLa versus SKOV3 cells. A) Heat map indicating the Log10 expression of genes after 0hr (control), 1hr, 3hr and 6hr Dex treatment in both HeLa and SKOV3 cells (includes all genes with a value of >1 FPKM at basal (0hr) gene expression for either cell line). Blue and Red represent lowest and highest expression levels respectively, with intermediate expression levels represented by the colour gradient indicated in the key. B) A Venn diagram to show the total number of significantly regulated Dex responsive genes, in HeLa cells compared to SKOV3 cells, that are regulated at any stage during the 1, 3 and 6 hour timecourse as determined by differential expression analysis of RNA-SEQ data as described by Trapnell and colleagues (36, 37). All genes shown are significantly differentially regulated at the $P < 0.05$ level and FPKM values > 1 . C) Pearson correlation coefficient ($r = 0.86$ and P value ($p = <0.001$ between genes with FPKM values > 1 at basal levels for HeLa and SKOV3 cell (in Log2). Data in red represents genes with $>1.5x$ fold expression in SKOV3 compared to HeLa cells. Data in blue represents genes with $>1.5x$ fold expression in HeLa compared to SKOV3 cells. A line of best fit is also shown. D, E) Volcano plot showing differentially expressed genes (of genes with basal levels FPKM values > 1) in HeLa cells (D) and SKOV3 cells (E) following 3 hours of Dex treatment. The negative Log10 transformed P values test the null hypothesis of no expression level change between basal level controls and cells with 3 hours of 100nM Dex treatment (y-axis) and are plotted against the average Log2 fold changes in expression (x-axis). Data for genes that were not differentially expressed are plotted in black. Data for genes that were differentially expressed (as determined by Benjamini-Hochberg correction analysis with $FDR = 0.05$ for RNA-SEQ) are plotted in blue ($P < 0.05$), green ($P < 0.01$) and red ($P < 0.001$). F, G, H, I) Venn diagram analysis shows overall numbers of regulated genes over the timecourse in each cell line. Significant gene induction (F, G) and repression (H, I) in HeLa (F, H) and SKOV3 cells (G, I) determined by differential expression analysis of RNA-SEQ data (36, 37; using Benjamini-Hochberg correction $FDR=0.05$), following Dex treatment of 1, 3 and 6 hours compared to 0 hour control. All genes shown are significantly differentially regulated at the $P < 0.05$ level and FPKM values > 1 .

3.3 SKOV3 Cells Exhibit a Restricted Genome-Wide transcriptional response to Glucocorticoids

Visualisation of global changes in gene expression over the timecourse of Dex treatment (Figure 2A) revealed striking differences between the two cell lines, which is further shown quantitatively in the Venn Diagram analysis of Dex-regulated genes (Figure 2B). Figure 2D and 2E compares the glucocorticoid responsiveness of genes common to both cell lines and demonstrates greater fold change, seen as both higher inductions (Log_2 fold change > 0) and greater repressions (Log_2 fold change < 0) of a larger number of genes in HeLa cells (Figure 2D) compared to SKOV3 cells (Figure 2E). The total number of induced or repressed genes, over the full timecourse, for both cell lines is shown in by Venn diagram (Figure 2F, G, H, I). Over the full Dex timecourse, a total of 101 transcripts were upregulated in SKOV3 cells compared to 570 in HeLa cells (Figure 2 F, G). A total of 49 transcripts were downregulated by Dex in the SKOV3 cells compared to 350 in the HeLa cells (Figure 2 H, I).

Real-time quantitative polymerase chain reaction (qPCR) was used to validate differential regulation of a selection of targets identified in the RNA-SEQ analysis. Differential regulation of the well-known

Dex-regulated genes Per1, FKBP5, BIRC3, DUSP1, SLIT2, FR-alpha and SLIT3 is shown in Figures 3B, and 3C. Both cell lines had a significant induction of Per1 by 6 hours of Dex treatment, although induction was far more rapid in the HeLa cells reaching significant upregulation at 1hour (Figure 3A). DUSP1 was induced by 1 hour in HeLa cells but only showed a non-significant trend towards induction by 6 hours of Dex treatment in the SKOV3 cells. BIRC3 and FKBP5 were significantly induced in HeLa cells (Figure 3A) but not in SKOV3 cells, which is comparable to the RNA-SEQ data. FR-alpha, which is highly Dex inducible in some cancer cell lines, is not regulated by Dex in SKOV3 cells (Figure 3B) (41). SLIT2 and SLIT3 were not regulated by GR in SKOV3 cells, despite being repressed by glucocorticoids in the majority of ovarian cell lines (31) (Figure 3B). All these results were consistent with the RNA-SEQ differential expression data and provided supporting evidence for impaired GR-dependent gene regulation in SKOV3 cells. Overall RT-qPCR analysis confirmed the restricted transcriptional response shown by the sequencing data.

3.4 SKOV3 Cells Show a Delayed Transcriptional Response to Dexamethasone

RNA-SEQ analysis revealed fewer genes to be responsive to Dex following 1 hour of treatment in the SKOV3 cells compared to the HeLa cells. Only 11 genes were induced and 7 genes repressed after 1 hour of Dex treatment in SKOV3 cells. In comparison, 94 genes were induced and 17 repressed after 1 hour Dex treatment in HeLa cells (Figure 2 F, G). In HeLa cells, 72 genes (12.6% of all Dex inducible genes in HeLa cells) are induced throughout the Dex timecourse. A further 182 genes (31.9% of all Dex inducible genes in HeLa cells) were induced at both the 3 hour and 6 hour time points. Interestingly, in contrast to HeLa cells no genes are induced throughout the entire timecourse in SKOV3 cells. However, 21 genes were continually induced from the 3 hour timepoint (only 20.8% of all SKOV3 Dex inducible genes). Furthermore, some of the genes regulated in common between the two cells types exhibited a delayed induction in the SKOV3 compared to the HeLa cells. For Per1, gene induction was more gradual and progressed at each time interval in the SKOV3 cells in comparison to the control HeLa cells where induction reached a significant increase at the earlier 1 hour time point (Figure 3 A). A few other highly Dex inducible genes, DUSP1, GILZ, CEBPD, NFKBIA, DKK1 and ZFP36, similarly to Per1, are induced from 1 hour of Dex treatment in HeLa cells but induction in SKOV3 cells is only observed at 3 hours of Dex treatment. These genes are positioned in the central area of the Venn diagrams in Figure 3C and 3D showing genes induced by 3 hours and 6 hours of Dex treatment in SKOV3 cells respectively, compared to genes induced from 1 hour of Dex treatment in HeLa cells. These two diagrams indicate the genes that are regulated in both cell lines from 3 hours of Dex treatment. Despite a delay in the transcriptional response of Dex inducible genes in SKOV3 cells there is no observed delay in Dex repressed genes. Previous studies

have shown that the majority of downregulated genes are repressed less rapidly than the majority of upregulated genes are induced (42, 43). Therefore we cannot rule out that repression may occur at a later timepoint. Despite this, consistent with a delay in repression fewer genes are repressed in SKOV3 cells, 49 genes, in comparison to HeLa cells, 350 genes over the 6 hour timecourse. Conversely, in contrast to induced genes the vast majority of repressed genes have previously been shown to remain unaffected by the ablation of the SWI/SNF function (26). Overall this apparent loss of 'rapid' regulation of several genes in SKOV3 cells compared to HeLa cells and may suggest either a delay in GR binding, or a delay in the recruitment of required transcriptional components necessary for GR-dependent gene regulation. This could include binding of co-regulators such as the SWI/SNF complex or any factors of the transcriptional preinitiation complex including RNA Polymerase II.

3.5 Loss of Prolonged Glucocorticoid gene regulation in SKOV3 cells

RNA-SEQ data revealed loss of induction of a large number of glucocorticoid responsive genes by 6 hours of Dex treatment in SKOV3 cells. This is in stark contrast to HeLa cells, where a large proportion of genes showed prolonged induction that remained upregulated at 6 hours (Figure 2 F, G). Only 20.8% of genes remained significantly upregulated from 3 hours of Dex treatment in SKOV3 cells compared to 44.6% of Dex inducible genes in HeLa cells. In SKOV3 cells, 90.9% of genes (10/11) induced following 1 hour of Dex treatment returned to baseline expression levels throughout the timecourse. Additionally 62.5% of genes (35/56) induced following 3 hours of Dex treatment showed loss of induction by 6 hours of treatment. In contrast, in HeLa cells, only 13.8% of genes (13/94) induced by 1 hour of Dex treatment showed loss of induction across the timecourse. Only 30% of genes (109/393) induced following 3 hours of Dex treatment returned to basal levels by 6 hours of treatment. In SKOV3 cells, 59% of Dex repressed genes do not remain repressed by 6 hours of Dex treatment. This is a large proportion of Dex repressed genes when in comparison 87.7% of repressed genes in HeLa cells remain downregulated at 6 hours of Dex treatment. The loss of prolonged Dex regulation of genes in SKOV3 cells throughout the timecourse may indicate a loss of GR-dependent gene regulation caused by the loss of ARID1a protein

A.

B.

C.

D.

Figure 3. Pattern of gene induction for common glucocorticoid responsive genes in SKOV3 cells compared to HeLa cells.

A - B) Fold change in mRNA expression normalised to β glucuronidase for SKOV3 cells (Purple) and HeLa cells (Blue) during a Dex treatment timecourse. Data is represented at mean $\pm$ Standard Error Mean (SEM). A) In both HeLa and SKOV3 cells the mRNA expression of Per1 is significantly increased following 6 hours of Dex treatment. In HeLa cells the mRNA expression of Per1 is also significantly increased at times 0, 1 and 3 hours. DUSP1 is significantly induced in HeLa cells at all timepoints of Dex treatment but only shows a trend towards induction over the timecourse in SKOV3 cells. Induction of BIRC3 and FKBP5 are also shown in HeLa cells but not in SKOV3 cells. For B) Two-way ANOVA with Dunnett's post-hoc test with all comparisons to the time 0 control were used and statistical significance indicated at $P < 0.05$ level (*), $P < 0.01$ (**), $P < 0.001$ (***), $P < 0.0001$ (****). C) Lack of regulation of commonly reported, ovarian specific, Dex regulated genes in SKOV3 cells FR-alpha, SLIT2 and SLIT3. Data is represented at mean $\pm$ SEM. One-way ANOVA with Dunnett's post-hoc test was used, with all comparisons to the time 0 hour control. No statistically significant differences were detected. C) Venn diagram analysis shows number of genes induced by 3 hour Dex treatment in SKOV3 cells compared to genes induced by Dex in HeLa cells at 1, 3 and 6 hours. D) Genes induced by 6 hour Dex treatment in SKOV3 cells compared to genes induced by Dex in HeLa cells at 1, 3 and 6 hours. For C) and D) all genes shown are significantly differentially regulated at the $P < 0.05$ level following Benjamini-Hochberg correction and FPKM values > 1 .

A.

B.

Distal PER1 GRE in HeLa cells

Proximal PER1 GRE in HeLa cells

C.

Distal PER1 GRE in SKOV3 cells

Proximal PER1 GRE in SKOV3 cells

Figure 4: GR binding at the Per1 proximal and distal GREs determined by ChIP studies. A) Schematic of the human Per1 gene and the position of the distal and proximal GRE sites. B) GR binding at the Distal and Proximal GREs in HeLa cells. C) GR binding at the Distal and Proximal GREs in SKOV3 cells. One way ANOVA with Dunnett's Post-hoc Test *** P<0.001.

3.6 SKOV3 cells have Decreased GR binding at a Chromatin Remodelling inducible GRE in Per1

Per1 was chosen as a candidate gene to assess GR binding. Per1 contains two GR binding sites, the first more distal site is a glucocorticoid and DNase1 hypersensitive GRE whereas the second proximal site is an inducible GRE site requiring chromatin remodelling (Figure 4 A) (44). Therefore, it provided a model for observing GR binding at a pre-accessible GR binding region, and at a site that is thought to require GR to mediate further chromatin remodelling potentially through the recruitment of the SWI/SNF complex. As both qPCR and RNA-SEQ suggested a delay in Per1 gene induction in response to Dex and an overall lower level of Per1 expression and induction in SKOV3 cells compared to HeLa cells it was important to determine whether these changes were due to the identified ARID1a mutation.

GR binding at the Per1 distal GRE region increased significantly following a 30 minute treatment with Dex in both cell lines compared to untreated controls (Figure 4 B, C). It should be noted that GR binding at the distal GRE was lower in the SKOV3 cells compared to that in the HeLa cells. At the proximal Per1 GRE region (within intron 1) GR binding increased following 30 minutes Dex treatment in HeLa cells but not in SKOV3 cells (Figure 4 B, C). This loss of GR binding at the proximal site may suggest decreased chromatin accessibility at this site. This could suggest that ARID1a is important for promoting the accessibility of this site for GR binding. This is potentially a mechanism for pre-setting the chromatin architecture prior to glucocorticoid treatment by primary chromatin remodelling. Alternatively this proximal GRE may not be accessible for GR binding in SKOV3 cells and may be pre-set by other mechanisms to be inaccessible to GR binding. This result becomes difficult to interpret with confidence in light of the lower GR binding result at the distal site in the SKOV3 cells relative to the HeLa cells.

3.7 Other gene mutations and loss of RNA expression detected in human SKOV3 cells

In addition to confirming the expected C to T mutation, which alters a Glutamine (codon CAG) to a premature stop codon (TAG) at position 586 in the amino acid sequence, identified in 32% of transcripts in SKOV3 cells (Supplementary Figure 2). Our RNA-SEQ data also confirmed a truncating mutation in exon 24 of BAF155 (SMARCC1) (45) (Supplementary Figure 3) and a mutation in PIK3CA (28) in which a Histidine (CAT) is changed to an Arginine (CGT) in 45% of transcripts (Supplementary Figure 4). Another important interaction of the ARID1a protein is the direct binding of the protein to

the cell cycle regulator P53 (46). However RNA-SEQ data revealed negligible P53 expression in SKOV3 cells, which is in contrast to HeLa cells.

4. Discussion

Previously luciferase reporter assays have been used to suggest ARID1a is important for GR activity (15). Here we provide evidence of a cell line with an ARID1a mutation with a restricted glucocorticoid response. Notably, SKOV3 cells possess a largely restricted GR-dependent transcriptional response following Dex treatment when compared to HeLa cells. There is also an apparent delay in transcriptional regulation as well as a marked loss of transcriptional regulation of GR-dependent genes over a 6 hour Dex timecourse in SKOV3 cells, which contrasts the dynamic and robust transcriptional response observed in HeLa cells. ChIP assays assessing GR binding at regulatory sites of the Per1 gene reveals a loss of GR binding at the proximal inducible GRE containing site in the SKOV3 cells despite significant binding at this site in HeLa cells. This could suggest that ARID1a is required to pre-set the accessibility of this GRE for GR binding. Although there are significant differences between the RNA transcriptional responses and GR binding to Dex treatment between these two cells, which could indicate differences due to the presence of a mutated ARID1a, these may also be due to inherent differences in cell type and related to differing cellular functions.

Multiple mutations identified in SKOV3 cells

Notably, the SKOV3 cell line has been widely used to study ARID1a (27-29), however the findings emerging from our genome-wide study now reveal these cells to be a less than ideal model for this purpose. In fact, several other mutations and loss of proteins have been observed in this cell line. It is also largely recognised that cell lines containing an ARID1a mutation do also co-occur with other gene mutations. Therefore loss of ARID1a alone may not result in such large-scale loss of glucocorticoid responsiveness and may be a result of several mutations. Recent studies have shown that the addition of a PIK3CA mutation as well as an ARID1a mutation can help promote tumour formation in ovarian cancer cells (47). PIK3CA encodes the catalytic subunit of PI-3K and mutations that co-occur with ARID1a often lead to increased PI-3K, and subsequently AKT, activity (47-49). PI-3K is also known to interact with GR (50). Therefore the PIK3CA mutation in SKOV3 cells may interfere with PI-3K-GR interactions and could be a reason for altered GR mediated gene regulation in this cell line. BAF 155, another subunit of the SWI/SNF complex, has also been shown to have a

truncating mutation (51). Although BAF57 and ARID1a (BAF 250) have been predicted to be the main mediators of an interaction of GR with the SWI/SNF complex using Protein interactions by structural matching (PRISM) techniques (52), other BAF subunits may also be important for this interaction. BAF155 was shown to be critical for subunit associations within the SWI/SNF complex (52), therefore a mutation in BAF155 may alter interactions between other subunits such as BAF57 and ARID1a within the complex. Interestingly deletion of the C-terminal of BAF155 has been shown to disrupt the ability of this subunit to regulate the stability of BAF57 (51, 52). Therefore the restrictive GR gene response to Dex treatment in SKOV3 cells may be due to loss of BAF57 from the SWI/SNF complex, which is important for an interaction of the complex with GR. P53 is another important factor known to directly interact with ARID1a (46) and it is now widely recognized that SKOV3 cells are P53-negative, which again is supported by our RNA-SEQ data. GR-dependent cell cycle arrest can often require P53 (53-55) and loss of P53 may impact upon regulation of a specific subset of GR-dependent genes. Therefore the differences in GR-dependent gene regulation observed may result from any combination of these mutations. Further studies assessing the impact of loss of other factors alongside loss of ARID1a may therefore be useful in assessing whether an impact on GR gene regulation occurs through a combination of mutations that frequently occur following ARID1a mutations observed in cancer.

Truncated ARID1a protein

Another factor which could contribute to the loss of glucocorticoid responsiveness is the potential presence of small truncated ARID1a protein of the first 585 amino acids of the N-terminal that may still be present in SKOV3 cells alongside the full-length ARID1a protein. There is a lack of evidence on the role of the ARID1a-NTD but previous studies have suggested it is required for GR activation and overexpression of the ARID1a-CTD using luciferase reporter assays results in loss of GR activity (15). An LXXLL motif has however been identified in the ARID1a-NTD (295-299 amino acids) (56), which may be important in binding coactivators of GR regulation. One previous study overexpressed LXXLL containing peptides that interact with ER (57). They showed that if these peptides mimic the interactions between the receptor and endogenous co-factors they can function in a dominant negative manner to disrupt interactions and prevent transcription (57). Therefore, the overexpression of such a small truncated ARID1a N-terminal protein containing an LXXLL may act as a dominant negative and interfere with the function of GR or other NRs that require interactions with specific cofactors. The presence of a small N-terminal LXXLL containing inactive ARID1a protein could potentially bind to and sequester certain co-factors thereby interfering with GR-dependent

gene regulation in SKOV3 cells. Future investigation may be aimed at further determining whether the ARID1a-NTD is translated and its role in GR-dependent gene regulation by studying the effect of overexpression of this domain in an ovarian cell line without an ARID1a mutation.

Summary

Despite the many caveats of the SKOV3 cell model, our genome-wide study has revealed a globally restricted transcriptional profile of GR regulated genes in these cells. This finding has potentially highlighted a functional role for ARID1a and the SWI/SNF complex in modifying the chromatin landscape for regulation of large numbers of GR regulated genes. Importantly, our study has lead to a potential mechanism to further explore in the clinical problem of glucocorticoid resistance, as ARID1a mutations have also been linked to glucocorticoid resistance in Leukaemia patients (12). Therefore further studies into ARID1a could subsequently identify subsets of cancer patients, which are likely to develop glucocorticoid resistance and could lead to identification of new therapeutic targets for these patients.

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Highlights

- SKOV3 cells have a truncated ARID1a
- SKOV3 cells exhibit a highly restrictive response to glucocorticoids
- We have identified impaired GR binding at a chromatin remodelling inducible site in the highly inducible glucocorticoid target gene, Per1.
- Consistent with reduced GR binding, Per1 shows an impaired and delayed transcriptional induction.

Supplementary Material

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