RESEARCH PAPER

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A unique histone deacetylase inhibitor alters microRNA expression and signal transduction in chemoresistant ovarian cancer cells

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Abbreviations: BiNGO, biological networks gene ontology; DNMTI, DNA methyltransferase inhibitor; EMT, epithelial-to-mesenchymal transition; GO, gene ontology; HDACI, histone deacetylase inhibitor; NAC, *N*-acetylcysteine; ROS, reactive oxygen species; TF, transcription factor; TFBS, transcription factor-binding site

Previously, we demonstrated potent antineoplastic activity of a distinctive histone deacetylase inhibitor (HDACI), AR42, against chemoresistant CP70 ovarian cancer cells in vitro and in vivo. Here, in follow-up to that work, we explored AR42 global mechanisms-of-action by examining drug-associated, genome-wide microRNA and mRNA expression profiles, which differed from those of the well-studied HDACI vorinostat. Expression of microRNA genes in negative correlation with their "target" coding gene (mRNA) transcripts, and transcription factor genes with expression positively correlated with coding genes having their cognate binding sites were identified and subjected to gene ontology analyses. Those evaluations showed AR42 gene expression patterns to negatively correlate with Wnt signaling (> 18-fold induction of *SFRP1*), the epithelial-to-mesenchymal transition (40% decreased *ATF1*) and cell cycle progression (33-fold increased *14-3-3σ*). By contrast, AR42 transcriptome alterations correlated positively with extrinsic ("death receptor") apoptosis (> 2.3-fold upregulated *DAPK*) and favorable ovarian cancer histopathology and prognosis. Inhibition of Wnt signaling was experimentally validated by: (1) >2.6-fold reduced Wnt reporter activity, and (2) 36% reduction in nuclear, activated β-catenin. AR42 induction of multiple (type I or type II autophagic) cell death cascades was further supported by 57% decreased reliance upon reactive oxygen, increased mitochondrial membrane disruption and caspase independence, as compared with vorinostat. Taken together, we demonstrate distinct antineoplastic pathway alterations, in aggressive ovarian cancer cells, following treatment with a promising HDACI, AR42. These combined computational and experimental approaches may also represent a straightforward means for mechanistic studies of other promising antineoplastics, and/or the identification of agents that may complement epigenetic therapies.

Introduction

Precise regulation of post-translational protein acetylation/deacetylation is vital to normal physiological homeostasis, and includes essential biological processes such as transcription factor-to-DNA binding, chromatin structure (thus influencing gene expression), "chaperone"-mediated protein stabilization and intracellular transport.[1,2] Consequently, alterations in the balance of acetylation and deacetylation contributes to several pathologies, including cancer, metabolic disorders, neurodegenerative conditions and inflammation.[1,2]

In the late 1990s, mechanistic studies of a number of leukemia cell-differentiating hydroxamic acids revealed these agents to enhance histone acetylation, via the inhibition of histone deacetylase enzymes.[3] Subsequent preclinical and clinical studies led to FDA approval of two such "histone deacetylase inhibitors" (HDACIs), suberoylanilide hydroxamic acid (SAHA, vorinostat) and the cyclic peptide romidepsin (depsipeptide), for cutaneous

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T-cell lymphoma.[4] Likewise, recent clinical trials of hematologic cancers demonstrated efficacy of other hydroxamate HDACIs, including belinostat, panobinostat and SB939, while promising non-hydroxamate HDACIs include entinostat (formerly MS-275), apicidin, and an isoform-selective HDACI, mocetinostat (previously MGCD0103) (for more extensive HDACI review, see refs. 3 and 4). Preclinical studies by our group and others have similarly shown potential therapeutic efficacy of a "rationally designed" HDACI, AR42 (formerly OSU-HDAC42, Arno Therapeutics),[5] against multiple myeloma, adult T-cell lymphomas and solid tumors of the ovary, liver and prostate, both singly or combined with other agents.[6-12]

Various molecular mechanisms/processes have now been advanced to explain HDACI cancer cell-specific cytotoxicity (and non-toxicity toward normal cells), including upregulation of pro-apoptotic genes, downregulation of anti-apoptotic genes, abrogation of cell cycle checkpoints and increased production of reactive oxygen species (concurrent with diminished anti-oxidative response).[2,4] With some exceptions, however, many of those previously identified anticancer mechanisms were based on limited numbers of HDACI-altered genes, absent rigorous assessments of HDACI cumulative effects on signal pathways/networks.[2,4] In ovarian cancer specifically, clinical trials of single-agent HDACIs have shown marginal activity against high-grade serous (HGS) disease, but some efficacy against borderline cancer.[13,14] Several preclinical studies of HGS ovarian cancer, however, have demonstrated potent antitumor activity of HDACIs when combined with conventional therapies, likely via resensitization of drug-resistant subclones (reviewed in refs. 14–16). However, the precise mechanism(s) of HDACI-mediated chemosensitization, with regard to specific signal pathways/networks, remains somewhat speculative.[4,15]

In concert with aberrant protein acetylation, dysregulated expression of microRNAs (miRNAs) is also strongly linked to the progression of numerous solid tumors, including ovarian cancer.[14,17-21] Even so, relatively few reports have described HDACI effects on these non-coding, post-transcriptional gene product regulators. In chronic lymphocytic leukemia cells, the HDACI LAQ824 was shown to upregulate three silenced tumor suppressive miRNAs,[22] while HDACI treatment resulted in upregulation of 22, and downregulation of 10 miRNAs in breast cancer cells.[23] Similarly, numerous miRNAs were found dysregulated by vorinostat in colon[24] and lung[25] cancer cells (in concert with p53 gain-of-function), while LAQ824 altered the expression of over 60 miRNAs in breast cancer cells.[26] Another miRNA study, however, reported no HDACI effects on miRNA expression, using a panel of 91 cancer cell lines.[27] Consequently, in this follow-up to our previous study of AR42 chemosensitizing activity in vitro and in vivo,[6] we assessed the genome-wide effects of AR42 on both miRNA and protein-coding (mRNA) gene expression in drug-resistant ovarian cancer cells. Bioinformatic analyses of this data, combined with experimental validation, revealed AR42 to exhibit discrete (but likely cooperative) pleiotropic effects on multiple signal pathways that may jointly oppose progression of this lethal gynecological malignancy.

Results

HDAC inhibitor effects on microRNA and mRNA expression. Previously, we demonstrated AR42 to differentially resensitize drug-resistant ovarian cancer cells and xenograft tumors to cisplatin, in parallel with the well-known HDACI vorinostat.[6] Based on that work, and other studies showing HDACI effects on microRNA (miRNA) expression,[22-26] we further compared AR42 and vorinostat for differential effects on global miRNA and protein-coding gene (mRNA) expression levels. In accord with their similar structure/functional groups (hydroxamic acids, which act to chelate the essential HDAC cofactor Zn^{2+}),[5] the two HDACIs exerted comparable expression effects on a large number of genes (**Fig. 1A**). However, hierarchical clustering analysis also revealed distinct gene expression patterns ("clusters") between duplicate arrays of untreated, AR42- and vorinostat-treated CP70 cells (**Fig. 1A**). Likewise, while AR42 and vorinostat similarly altered the expression of several miRNA genes, we also observed the two HDACI miRNA profiles to distinctly cluster, as compared with the untreated (DMSO vehicle) control (**Fig. 1B and C**). These discrete miRNA and gene expression clusters validated sample reproducibility (thus qualifying the data for additional analyses), thus justifying further examination of the genome-wide effects of AR42 on signal transduction pathways.[28,29]

Functional analyses of AR42 alterations in protein-coding and microRNA gene expression. To examine the global bases for the antineoplastic effects of AR42, we first identified transcription factor (TF) binding sites (TFBSs) localized within 4,000 bp of the transcription start sites of AR42-differentially expressed genes (**Fig. 1A and B**), using the webtool ECR (evolutionary conserved region) Browser.[30] We then examined the expression of TFs matching those TFBSs, followed by assay of TF expression changes with those of their TFBS-possessing "target" genes.[30,31] The matched TF/target genes were then subjected to Gene Ontology (GO) analysis, using the web tool BiNGO,[32] revealing AR42-associated gene expression profiles to negatively correlate with the GO biological processes "epithelial-to-mesenchymal transition" (EMT, GO: 0001837) and "canonical Wnt receptor signaling" (GO: 0060070) (**Table 1**). Consistent with AR42 negative regulation of those tumor progression cascades, we also noted upregulation of genes repressive of the EMT (e.g., *ONECUT2*, *WT1*) and Wnt (e.g., *SFRP1 ICAT*, *TAK1*) pathways (**Tables 2 and 3**, respectively). By contrast, the expression of AR42-altered TF/target gene transcription positively correlated with the GO terms "negative regulation of cell cycle processes" (GO: 0010948) (**Table 4**) and "induction of apoptosis by extracellular signals" (i.e., extrinsic apoptosis, GO: 0008624) (**Table 5**). With regard to the latter two pathways, and in contrast with the Wnt and EMT cascades, AR42-upregulated agonists (*14-3-3*σ, *p21*, *DAPK* and *RIP*), and downregulated antagonists (*TopoII*, *BCL2*) of cell cycle arrest and death receptor signaling (**Tables 4 and 5**). Overall, these four AR42-associated GO pathways concur with our previously observed AR42 antineoplastic effects on ovarian cancer cells, including G_2/M cell cycle arrest (via repression of genes that stimulate that pathway), induction of epithelial morphology and extensive apoptosis (following

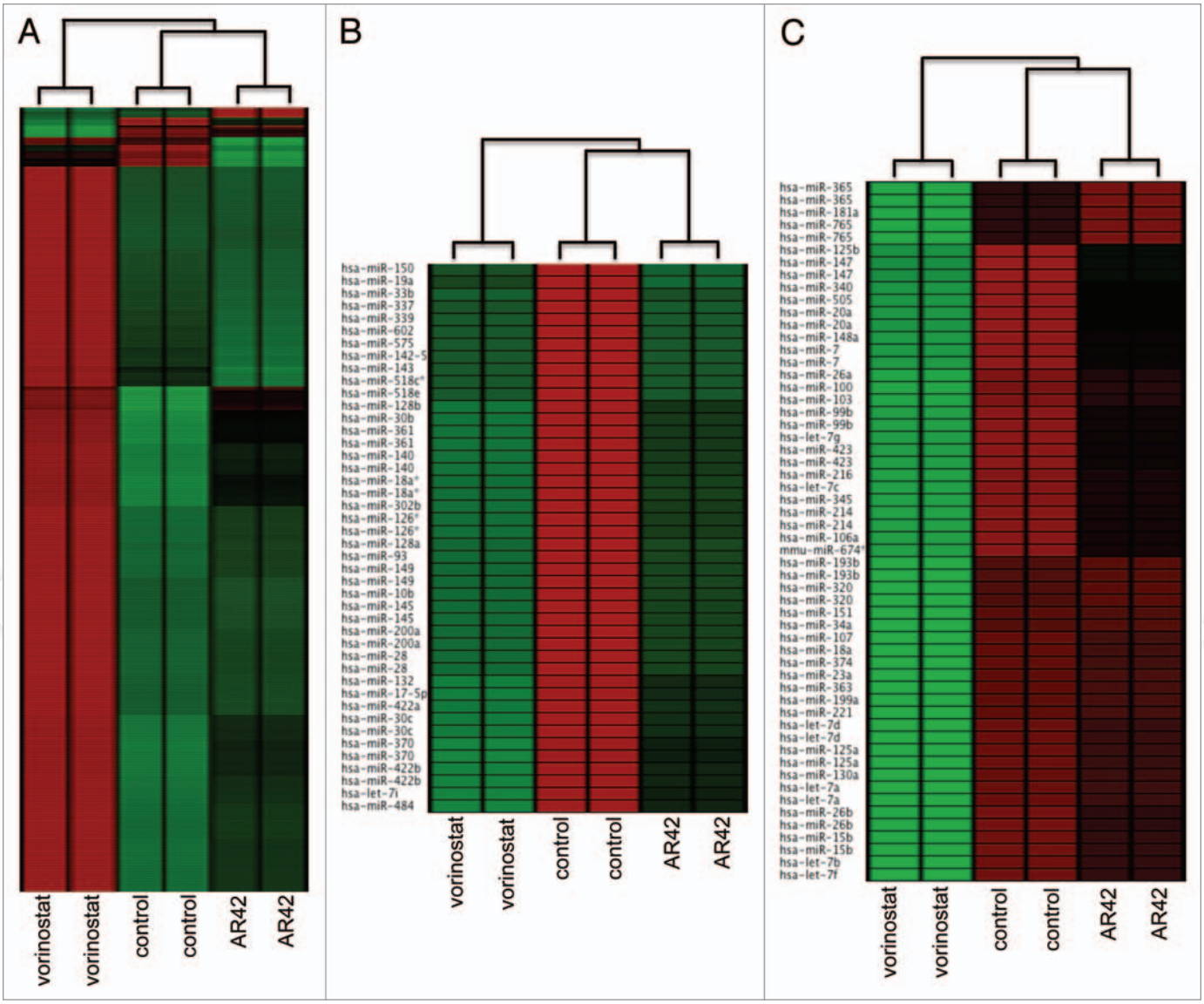

Figure 1. Unsupervised cluster analysis of (A) coding-gene expression (Nimblegen 385K Gene Expression); (B) downregulation mature microRNAs; and (C) upregulation of mature microRNAs. Microarray data (124-feature custom array)[71] was subjected to quantile normalization[72] and unsupervised clustering performed using EXPANDER.[29]

AR42 treatment in combination with the DNA-crosslinking agent cisplatin).[6]

Concordance of AR42 microRNA and mRNA expression profiles to pathway "gene expression" signatures. To further extend our functional analysis and provide greater mechanistic detail of AR42 antineoplastic activity, we examined drug-associated gene profiles for alignment to various published "gene expression signatures." To first affirm that AR42 effects were indeed due to deacetylase inhibition, we showed AR42 gene alterations to positively correlate with an "HDAC inhibitor common gene set" expression signature[33] (**Fig. 2A**), including upregulation of *CDNK1A* (*p21*^{CIP1}), a "benchmark" gene induced by all inhibitors of class I and class II HDACs.[2,4]

We also examined the AR42 transcriptome for expression of signature genes downregulated by EMT in breast cancer cells,[34] again supporting an AR42 inversely correlated expression profile (**Fig. 2B**). Likewise, for Wnt signaling, we compared AR42 changes in gene expression to a publically available Wnt-upregulated gene signature (KEGG pathway hsa04310).[35] Similar to the inverse EMT profile, AR42 downregulated the majority of those (Wnt-upregulated) signature genes (**Fig. 2C**), thus further supporting AR42 inhibition of Wnt signaling.

Similarly, we compared AR42 expression changes to other gene signatures of cell cycle arrest and death receptor-mediated apoptosis. Those evaluations revealed that in contrast to the EMT and Wnt pathways, AR42 transcriptional changes positively correlated with cell cycle arrest (KEGG pathway map04110) and death receptor apoptosis (MSigDB identifier M16635) gene expression signatures[35] (**Fig. 3A and B**, respectively). Within the cell cycle arrest signature, we noted upregulation of the

Table 1. Gene ontology (GO) terms identified from gene expression changes following 48-h AR42 treatment

Gene ontology (GO) term	GO accession/ontology	Biological function
INDUCTION_OF_APOPTOSIS_ INDUCTION_BY_EXTRACELLULAR_SIGNALS	GO: 0008624 Biological Process	Programmed cell death initiated by extracellular ligand death receptor binding/signal transduction
NEGATIVE_REGULATION_OF_CELL_CYCLE_PROCESS	GO: 0010948 Biological Process	Inhibition of DNA synthesis, degradation of cyclin/CDK complexes, activation of checkpoints
EPITHELIAL_TO_MESENCHYMAL_TRANSITION	GO: 0001837 Biological Process	Loss of cell-cell contact, migration, invasion
CANONICAL_WNT_RECEPTOR_SIGNALING_PATHWAY	GO: 0060070 Biological Process	Embryonic development pathway, mitogenic signaling for cell proliferation

Table 2. Microarray AR42-induced expression changes in gene members of the GO term EPITHELIAL_TO_MESENCHYMAL_TRANSITION

Genes (gene symbol)	Fold change (AR42/Control)	Hypothesized role in EMT
PAX2	33.0	inhibitory (sequesters cdc2/cyclin B1 complex in cytoplasm)
GATA6	5.2	cell differentiation/specialization
WT1	1.3	negative regulator of EMT
SOX2	0.6	facilitator of EMT, induced pluripotency gene
ATF1	0.6	EMT-associated transcription factor
ONECUT2	2.8	tissue lineage commitment/differentiation

Table 3. AR42-induced changes in members of the GO term CANONICAL_WNT_RECEPTOR_SIGNALING_PATHWAY

Genes (gene symbol)	Fold-change (AR42/control)	Wnt signaling function
SFRP (SFRP1)	18.9	Wnt signal inhibitor
WIF (WIF1)	1.7	Wnt target gene repressor
PIN1	0.7	Wnt signal mediator
ICAT (CTNNBIP1)	2.2	Wnt signal inhibitor
APC	1.3	Wnt signal inhibitor
Naked (NKD1)	2.3	Wnt target gene repressor
CTBP (CTBP1)	0.9	Wnt target gene repressor
NLK	1.5	Wnt target gene repressor
DKK (DKK1)	1.2	Wnt signal inhibitor
Groucho (TLE1)	2.9	Wnt target gene repressor
Groucho (TLE2)	0.66	Wnt target gene repressor
Groucho (TLE3)	1.8	Wnt target gene repressor
TAK1 (MAP3K7)	2.8	Wnt signal inhibitor

cyclin-dependent kinase inhibitors *CDKN1A* (*p21*^{CIP1}), *CDKN1B* (*p15*^{INK4B}) and *CDKN2D* (*p19*^{INK4D}), in addition to the p53 target gene and coactivator *14-3-3σ* (**Table 4**). Interestingly, we also noted a slight (0.7-fold) downregulation of *p53* (**Fig. 3A**), which is overexpressed and dysfunctional in CP70 cells (likely contributing to chemotherapy resistance).[36,37] These gene signature results concur with our prior observation of AR42-associated cell cycle arrest and epithelial differentiation, and previous studies demonstrating AR42 repression of oncogenic cascades, concurrent with derepression of tumor suppressive regulators, in various cancer types.[5-11]

We also examined AR42 for disease-specific activity against ovarian cancer, by comparing AR42 expression patterns to two ovarian cancer clinically informative transcription signatures. Those analyses demonstrated AR42 expression changes to positively correlate with gene expression signatures for favorable prognosis[38] and low-grade serous (as compared with high-grade serous, HGS) disease[39] (**Fig. 4**). To further support specific anti-tumor activity against HGS malignancy, we also observed AR42 downregulation of various ovarian cancer oncogenic miRNAs, including miR-21 and miR-30 (**Fig. 1B**),[18,21] and upregulation of previously reported ovarian tumor-suppressive miRNAs, including those of the let-7 family and miRs 99, 100 and 125 [17,19,21] (**Fig. 1C**). Moreover, two other AR-42-upregulated miRNAs, miR-15 and miR-34 (a p53 target gene), were previously reported as epigenetically downregulated in ovarian cancer.[17,19] Taken together, these gene set enrichment and ontology analyses support AR42 anti-ovarian cancer activity by circumvention of two aggressive tumor progression cascades, EMT and Wnt, coincident with upregulation of two growth-limiting cascades, cell cycle arrest and death receptor signaling.

Experimental assessment of AR42 effects on Wnt pathway signal transduction. Based on our GO analyses (**Table 1**), we validated expression levels of selected genes from the various abovementioned pathways by quantitative, reverse transcription PCR (qRT-PCR), affirming upregulation of the Wnt antagonists *SFRP1* and *TAK1*, the cell cycle inhibitor *14-3-3σ*, and downregulation an EMT-activating TF gene, *ATF1* (**Fig. 5A**). To further assess the effects of AR42 on Wnt pathway signaling, we transfected CP70 cells with the Wnt luciferase reporter TOPFlash and its negative control, FOPFlash,[40] followed by 48-h treatment with vehicle or 1.0 μM AR42. As shown in **Figure 5B**, AR42 treatment resulted in > 2.6-fold decreased Wnt activity (luciferase activity). We also examined AR42 effects on the Wnt signal mediator β-catenin, which is dephosphorylated upon activation, followed by translocation to the nucleus.[40-42] Using an antibody specific for dephosphorylated (and thus activated) β-catenin, we determined that AR42 caused a 57% decrease in nucleus-localized protein and a 23% decrease in cytoplasmic

protein (**Fig. 5C and D**). Taken together, these computational and experimental results provide additional support for Wnt signal blockade as a mechanism of AR42 disruption of ovarian cancer malignant phenotypes.

Experimental evaluation of AR42 induction of extrinsic (death receptor) apoptosis. In accord with our AR42 functional analyses described above, several other HDACIs have also been demonstrated to induce cell death via "death receptor" (extrinsic) apoptosis,[2,15] a process that can occur in a mitochondria-independent manner (although "crosstalk" with mitochondrial apoptosis pathways may also take place).[43] Previously, we demonstrated that combined AR42/cisplatin treatment resulted in caspase activation in CP70 cells and xenografts.[6] While caspase induction associates with both the intrinsic (via intracellular signaling) and extrinsic apoptosis pathways,[43,44] caspase-independent cell death has also been associated with mitochondrial disruption.[44,45] Consequently, based on our gene ontology (**Table 5**) and gene expression signature analyses (**Fig. 3B and Table 5**), we assessed CP70 cell AR42 treatment in the presence or absence of the pan-caspase inhibitor z-VAD-fmk.[45] As shown in **Figure 5A**, caspase inhibition had little effect on AR42 dose-dependent growth inhibition. We then further investigated possible loss of mitochondrial membrane integrity and production of reactive oxygen species (ROS), two events that can also occur during extrinsic apoptosis and type II (autophagic) cell death[43-45] in 48-h AR42- and/or vorinostat-treated cells. With specific regard to ROS dependence, 48-h, HDACI-treated CP70 cells concurrently exposed to 15 mM of the antioxidant *N*-acetylcysteine (NAC) demonstrated a 37-fold increased 50% growth-inhibitory (GI_{50}) dose for vorinostat (thus showing vorinostat as 37-fold less apoptotic in the absence of ROS). In contrast, NAC treatment increased the AR42 GI_{50} by only 21-fold (**Fig. 6B**), suggesting AR42-induced cell death as less dependent on ROS production.

Caspase-independent cell death and ROS production may also associate with mitochondrial membrane permeation (and thus loss of mitochondrial membrane potential, $\Delta\Psi$).[43] To examine possible effects on mitochondrial integrity, we used a fluorescent $\Delta\Psi$ reporter, JC-1,[46] following 48-h CP70 cell treatment with 1.0 μM AR42 or 1.0 μM vorinostat. At normal $\Delta\Psi$, JC-1 self-aggregates in the inner mitochondrial space, with aggregated JC-1 fluorescing red. By contrast, disrupted $\Delta\Psi$ results in failed mitochondrial aggregation of the lipophilic dye, with the resulting (cytosolic) JC-1 monomers fluorescing green.[46] As shown in **Figure 6B**, following 48-h, 1.0 μM HDACI treatment, green fluorescence (i.e., monomeric and cytosolic JC-1) was predominant in AR42-treated cells (upper part), while red fluorescence (mitochondria-aggregated JC-1) was more pronounced in vorinostat-treated cells (lower part). These results suggest that at equal dose exposures, AR42 elicits greater loss of $\Delta\Psi$ than vorinostat, in possible partial independence from ROS production and caspase activity.[43,44]

Discussion

In an earlier study, we examined growth suppression and drug-sensitization of chemoresistant ovarian cancer cells and mouse xenografts by a distinctive histone deacetylase inhibitor (HDACI), AR42.[6] While several reports have associated HDACI anticancer activity with limited numbers of altered genes,[2,47,48] here we endeavored to assess global mechanisms (i.e., pathways/networks) of AR42 cancer cell-specific cytotoxicity, using a "systems biology" approach.[28,29,35] Moreover, with regard to the now well-established role of misexpressed (and frequently epigenetically altered) microRNAs (miRNAs) in ovarian tumorigenesis,[14,17-19,21] we also considered dysregulation of these short, non-protein-coding, translation-regulatory oligonucleotides.

Table 4. Selected AR42-altered genes in the GO term NEGATIVE_REGULATION_OF_CELL_CYCLE

Genes (gene symbol)	Fold-change (AR42/control)	Role in cell cycle progression/arrest
Chk2	1.3	G_1/S arrest (inhibits cdc25)
Wee1	1.5	G_2/M arrest (inhibits cdc2/cyclin B1 complex)
TopoII	0.8	Stimulation by DNA helicase activity involved in DNA replication
14–3-3σ	33.0	Inhibitory (sequesters cdc2/cyclin B1 complex in cytoplasm)
CDKN1A (*$p21^{WAF1A}$*)	22.4	Inhibitor of cyclin-dependent kinase-1
CDKN2B (*$p15^{INK4B}$*)	1.7	Inhibitor of cyclin-dependent kinase-4
CDKN2C (*$p18^{INK4C}$*)	1.8	Inhibitor of cyclin-dependent kinase-4
CDKN2D (*$p19^{ARF}$*)	2.2	G_2/M arrest (releases p53 from MDM2)

Table 5. Selected AR42-altered genes in the GO term APOPTOTIC_PROCESS

Genes (gene symbol)	Fold change (AR42/control)	Death receptor signaling function
ASK1	2.4	Signal kinase
APO2/TRAIL	1.7	Death ligand
BAX	1.6	Mitochondrial membrane permeabilization
BCL2	0.6	Inhibitor of mitochondrial disruption
BID	2.0	Permeabilizes mitochondrial membrane, facilitating the release of cytochrome *C*
DAPK	2.6	Signal kinase
DAXX	2.3	Adaptor/signal mediator
DR3	1.8	Death ligand receptor
MKK7	0.6	Mitogenic signal kinase
RIP	2.6	Apoptosis signal mediator
TRADD	1.4	Death domain adaptor (relays death signal from receptor)
TNFR1	1.7	Death ligand receptor

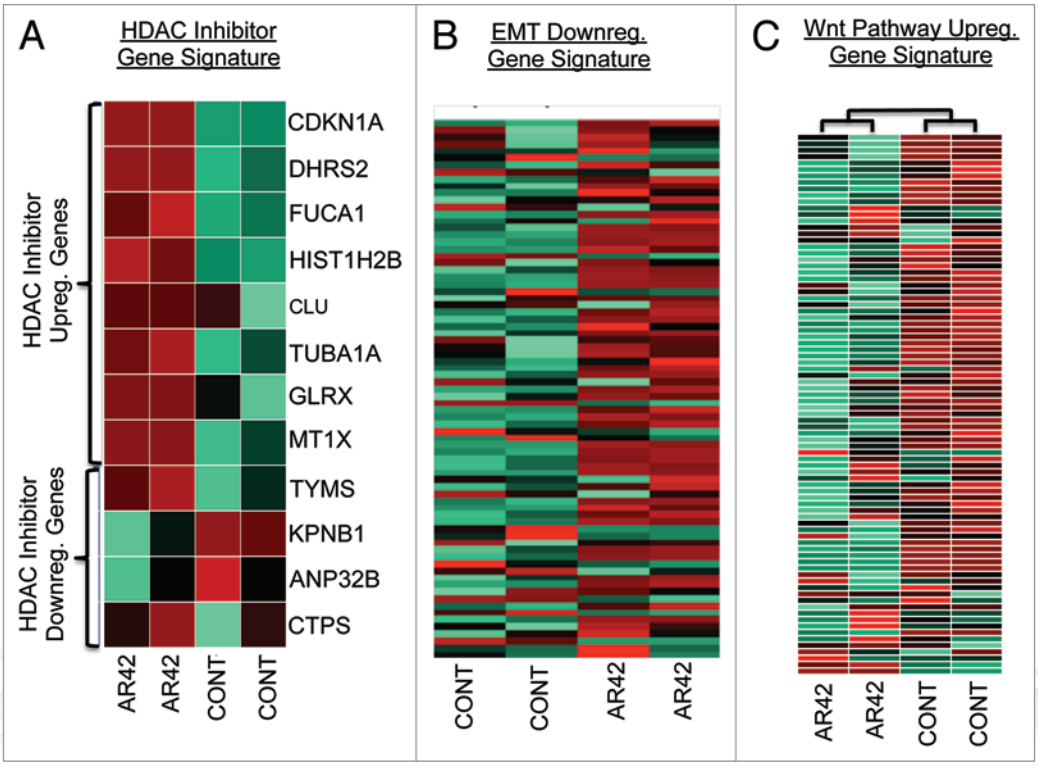

Figure 2. Comparison of AR42-associated gene expression changes for alignment with expression signatures for (A) genes commonly altered by 14 separate HDACIs;[33] (B) a set of 100 genes downregulated in cancer cells undergoing the epithelial-to-mesenchymal transition[34] and (C) an 84-gene signature of upregulated genes in cancer cells possessing active signaling of the oncogenic mitogenic pathway Wnt (KEGG pathway hsa04310).[35]

Although a few studies have examined HDACI antineoplastic miRNA alterations,[22-27] to our knowledge, the current work represents the first appraisal of monotherapeutic HDACI effects on miRNA expression, (in association with altered signaling pathways) in ovarian cancer.

In earlier studies as monotherapies, HDACIs were found to induce only small numbers of genes, with greater gene re-expression requiring cotreatment with another class of epigenetic derepressive agents, DNA methyltransferase inhibitors (DNMTIs).[47,48] Later reports, however, showed single-agent HDACIs to dysregulate (both induce and repress) considerably more genes.[2,4,8,9] Interestingly, one recent report demonstrated DNMTIs to only marginally enhance HDACI antineoplastic activity in ovarian cancer cells and mouse xenografts.[49] The basis for recent demonstrations of greater HDACI gene dysregulation is somewhat uncertain, but could be related to previously used suboptimal or toxic doses, non-ideal durations of treatment, or secondary "downstream" gene regulatory events.[2] Even more impressively (but also controversially), several HDACIs have now been reported to reverse other (histone deacetylation-unrelated) repressive epigenetic modifications, including methylation of deoxycytosine, gain of gene-activating and/or loss of gene-repressive histone modifications (e.g., lysine methylation).[50-52] Regardless of the extent of HDACI epigenomic reprogramming, however, it is widely held that these agents will be most beneficial when combined with conventional and/or targeted therapies, as demonstrated by our group[6] and others.[4,15,16,53,54]

In addition to chemosensitization, we previously demonstrated AR42 to differentially enhance protein acetylation and alter the expression of specific cell cycle-associated genes, in parallel with the HDACI vorinostat.[6] In the current work, comparisons of AR42- and vorinostat-altered microRNAomes and transcriptomes revealed distinct expression clusters (**Fig. 1**). This finding is consistent with earlier demonstrations of specific gene expression profiles for distinct HDACIs, possibly related to differences of inhibitor "fit" into the HDACI enzyme active site, HDAC-isoform preference(s), varying spatiotemporal HDAC enzyme location, and/or indirect (secondary, "downstream") transcriptional effects.[2] However, while AR42 effects on miRNA expression were not previously studied in-depth, our finding of its distinctive transcriptome profile, as compared with that of vorinostat (**Fig. 1A**), concurs with previously reported differences in gene expression by these two HDACIs.[6-9]

As vorinostat has now been extensively characterized and approved for human use for over five years,[2,3] we directed our focus on characterizing global anticancer mechanisms of the "third generation" HDACI, AR42.[5] To further evaluate AR42 mechanisms, at the biological pathway/network signal level,[28,29] we identified transcription factor (TF) genes whose expression correlated positively with that of their "target" genes (i.e., genes possessing specific binding sites for those TFs), followed by gene ontology (GO) assessment[32] (**Table 1**). Those analyses showed that expression of AR42-altered TF target genes (mRNAs) anti-correlated with the biological processes epithelial-to-mesenchymal

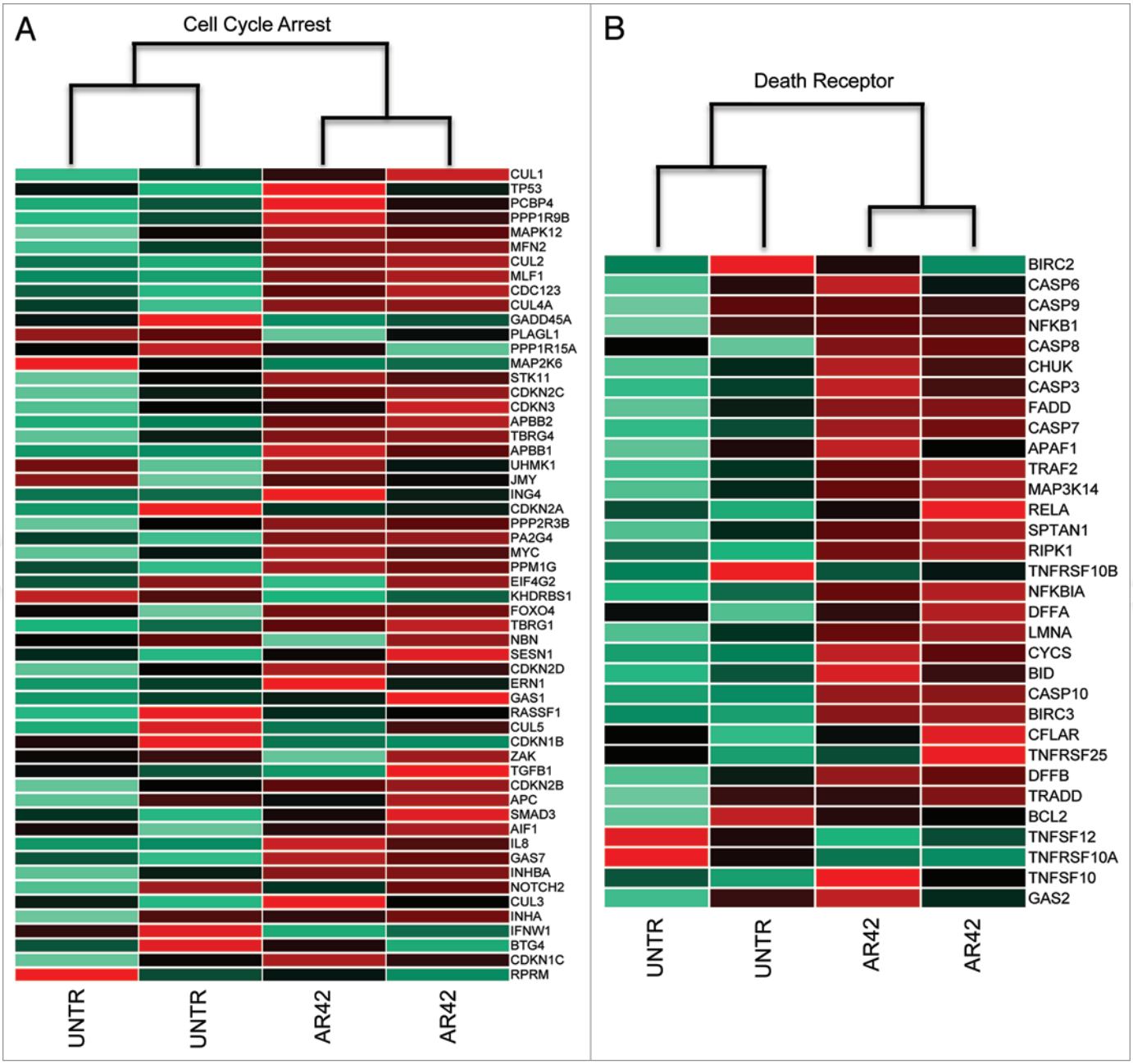

Figure 3. Assessment of AR42-altered expression patterns for signatures of (A) genes upregulated during cell cycle arrest (KEGG pathway map04110),[35] and (B) genes upregulated during "death receptor" (extrinsic) apoptosis (MSigDB identifier M16635).[35]

transition (EMT) (**Fig. 2B and Table 2**), with upregulation of EMT-opposing TFs (*GATA6* and *WT1*), and downregulation of EMT-promoting TF genes (*SOX2* and *ATF1*) (**Table 2 and Fig. 5A**). While primary high-grade serous ovarian tumors actually gain epithelial attributes (i.e., undergo "MET"), late-stage disease phenotypes, including metastatic dissemination ("peritoneal seeding"), chemotherapy resistance (as exhibited by CP70 cells) and angiogenesis likely associate with restoration of the EMT program.[14,55] Our current finding (possible EMT reversal) is consistent with our previous observation of AR42 induction of CP70 epithelial characteristics, based on cell morphology and the expression of multiple cytokeratins.[6] Additionally, while we did not examine Akt signaling in the current study, others have demonstrated that Akt signal inhibition (by AR42 and other agents) sensitized CP70 and other p53-dysfunctional, drug-resistant, ovarian cancer cells.[37,56] As AR42 has also been demonstrated to downregulate Akt activity,[7-9,12] blockade of that oncogenic signal pathway might represent another HDACI mechanism of resensitization of chemoresistant ovarian cancer cells (an antineoplastic effect we previously observed).[6]

Similar to EMT, gene set enrichment analysis[35] also demonstrated AR42 gene expression profiles to negatively associate

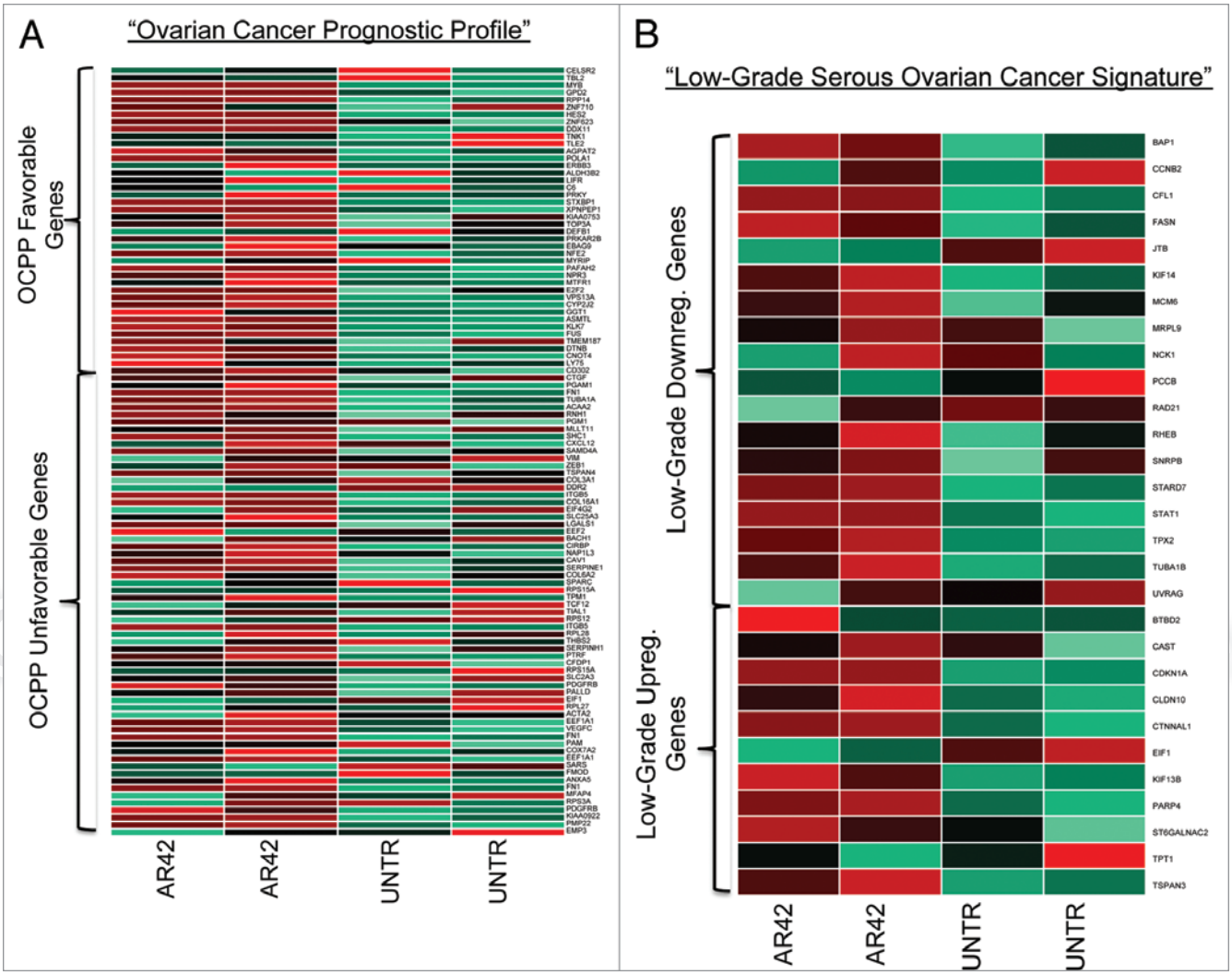

Figure 4. Ovarian cancer-specific gene signature alterations by AR42 (A) with positive vs. negative ovarian cancer patient prognosis. Upper group represents genes whose expression correlates with favorable prognosis; lower group represents genes expressed in unfavorable prognosis (reviewed in ref. 38). (B) AR42 alterations of genes correlated with low-grade ovarian adenocarcinoma histopathology upper gene group are upregulated with low-grade pathology, while the lower gene group represent those downregulated with low-grade pathology (reviewed in ref. 39).

with the EMT-dependent oncogenic signal cascade, Wnt (KEGG pathway hsa04310, **Fig. 2C**), while positively correlating with cell cycle antagonism (MSigDB_GO: 0007050, **Fig. 3A**) and extrinsic ("death receptor") apoptosis (MSigDB_M14971, **Fig. 3B**). We also note that in hepatocellular cancer cells, AR42 was found to potentiate type II, caspase-independent cell death via autophagy,[11] similar to vorinostat.[57] In the current work, we likewise saw AR42 downregulation of the anti-autophagy gene *BCL2*, and upregulation of two pro-autophagy genes, *CHAF1A* (*p150*) and *BECN1* (*Beclin1*) (**Fig. 1A**). However, we also observed downregulation of pro-autophagy (*UVRAG*, *BNIP3*), and upregulation of an anti-autophagy gene (*AKT1S1*) (**Fig. 1A**), thus confounding firm conclusions regarding that type II cell death process.

With specific regard to ovarian cancer, we also found AR42-induced gene transcription patterns to correlate with gene signatures of favorable ovarian cancer prognosis (**Fig. 4A**) and low-grade disease (**Fig. 4B**);[38,39] AR42 also upregulated various ovarian cancer-repressed microRNAs, including miR-99, miR-100, and the miR-125 and let-7 families[18-20,58] (**Fig. 1C**). Upregulation of miR-125 as a possible AR42 anti-ovarian cancer mechanism was also supported by a demonstration that miR-125 is repressed by epidermal growth factor signaling, and that miR-125 re-expression causes reversal of EMT.[20] Moreover, consistent with HDACI-mediated chromatin relaxation, we also observed AR42 to upregulate two miRNAs previously shown as epigenetically silenced in ovarian cancer, miRs 15a and 34 (**Fig. 1C**).[17,19]

Experimental examination of Wnt pathway signaling (by a luciferase reporter assay) demonstrated Wnt activity to be 2.6-fold downregulated by AR42, consistent with other reports of HDACI inhibition of the Wnt cascade.[42,59,60] While overactive Wnt signaling often correlates with activating β-catenin mutations (and/or inactivating mutations of Wnt antagonists), many cancers (including ovarian) exhibit elevated Wnt signaling in the absence of Wnt pathway genetic alterations.[41,42,61] These findings

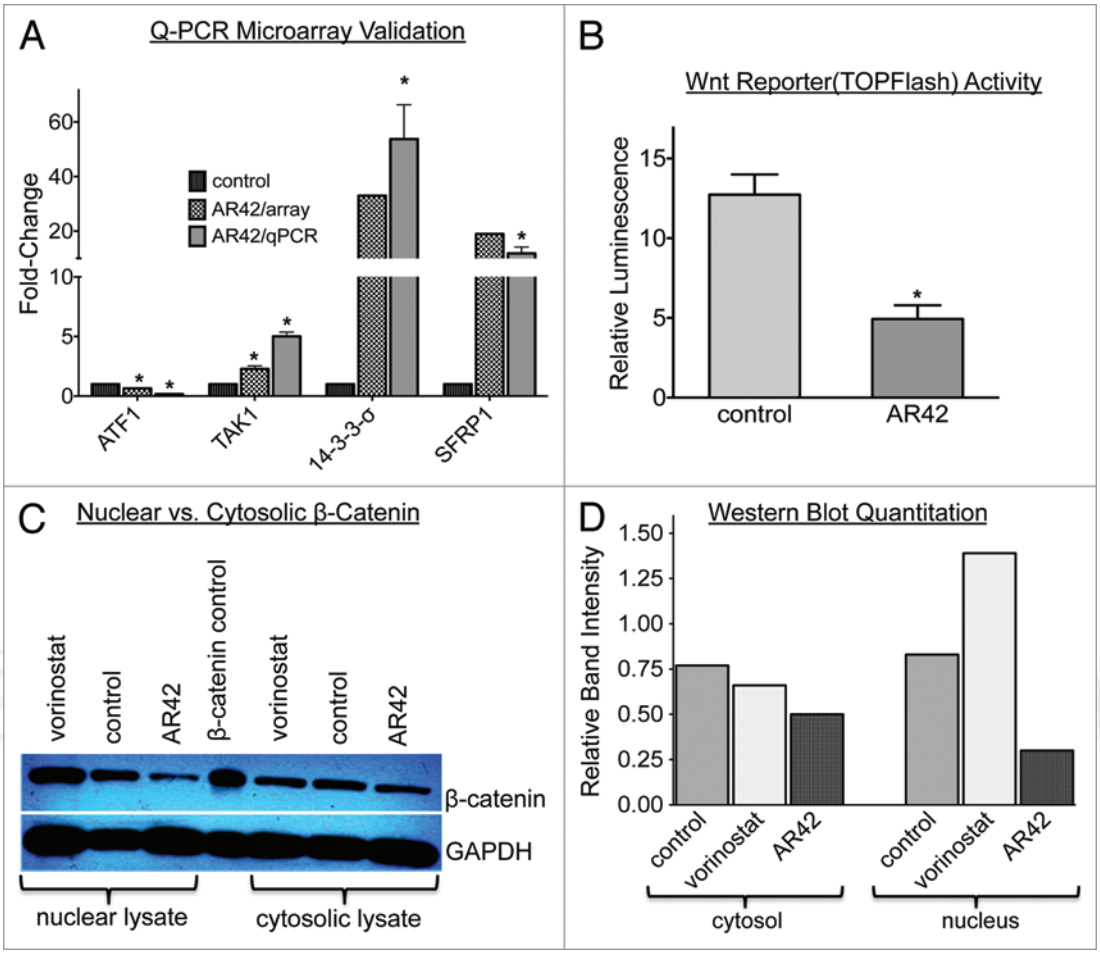

Figure 5. Experimental validation of AR42-decreased canonical Wnt signaling. (A) Quantitative PCR expression validation of selected microarray AR42-altered genes alter, including members of the Wnt signal pathway (*TAK1*, *SFRP1*), the epithelial-to-mesenchymal transition (*ATF1*), and cell cycle negative regulation (*14-3-3σ*). (*p < 0.05 vs. control). (B) Quantification of Wnt pathway signaling following 24 h, 1.0 μM AR42 treatment of CP70 cells transfected with a Wnt signal TOPFlash luciferase reporter.[40] Relative luminescence units (RLUs) were determined by normalizing the luminescence of TOPFlash-possessing cells to the total cellular protein from each lysate and to luminescence from mutant control vector ("FOPFlash")-transfected cells.[70] (C) Western blot analysis of cytoplasmic vs. nuclear extracts from CP70 ovarian cancer cells treated for 24 h with DMSO vehicle ("control," lanes 2 and 6), 1.0 μM AR42 (lanes 3 and 7), or 1.0 μM vorinostat (lanes 1 and 5). (D) Quantification of western blot using ImageJ software (*p < 0.05, vs. control).

suggest that epigenetic aberrations may play an analogous or even greater role in enhanced Wnt signaling. Indeed numerous studies have now demonstrated chromatin-related silencing of the Wnt antagonists *TAK1*, *SFRP1, ICAT*, *WIF1* and *DKK1*,[41,42] genes we also identified as upregulated by AR42 (**Figs. 2C and 5A and Table 3**).

EMT and Wnt also represent two interdependent pathways hypothesized to facilitate the genesis, proliferation and tumor propagation characteristics of "cancer stem cells" (CSCs), neoplastic progenitors believed to govern (or possibly exacerbate) most or all malignant phenotypes.[41,62] Additionally, the ovarian cancer cells used in this study, CP70, are highly aggressive, invasive and chemoresistant,[36,37,56] three traits associated with cancer "stemness."[14,55,62] CP70 cells also possess dysfunctional p53,[37] whose loss promotes self-renewal of normal and leukemia stem cells.[63,64] Interestingly, our gene expression microarray results indicated a slight downregulation of *p53* (0.7-fold, **Fig. 4A**), and upregulation of various p53 target genes including *TP53I5* (6.7-fold), *TP53I11* (4.3-fold), *TP53I3* (1.3-fold) and *TP53INP2* (1.3-fold) (**Fig. 1A**). These findings would concur with previous demonstrations that HDACIs can downregulate abnormal *p53* gene products, while also restoring "p53-like" pathways to cancer cells lacking functions (including apoptosis) of that tumor suppressor.[65-67] Notably, we also observed AR42 upregulation of a p53 target microRNA gene, miR-34, whose re-expression, in ovarian and other cancers, strongly associates with activation of numerous p53 response pathways.[17,68]

Epigenetic modifications also regulate differentiation states in normal stem cells and adult tissues,[1,16] and it has been shown that even highly aggressive cancer cells possess considerable phenotypic "plasticity," associated with chromatin-remodeling ability.[41,69] As HDACIs and DNA methyltransferase inhibitors (DNMTIs) are well-characterized differentiating agents, it is possible that these chromatin-relaxing agents could "reset" cancer progenitor cells

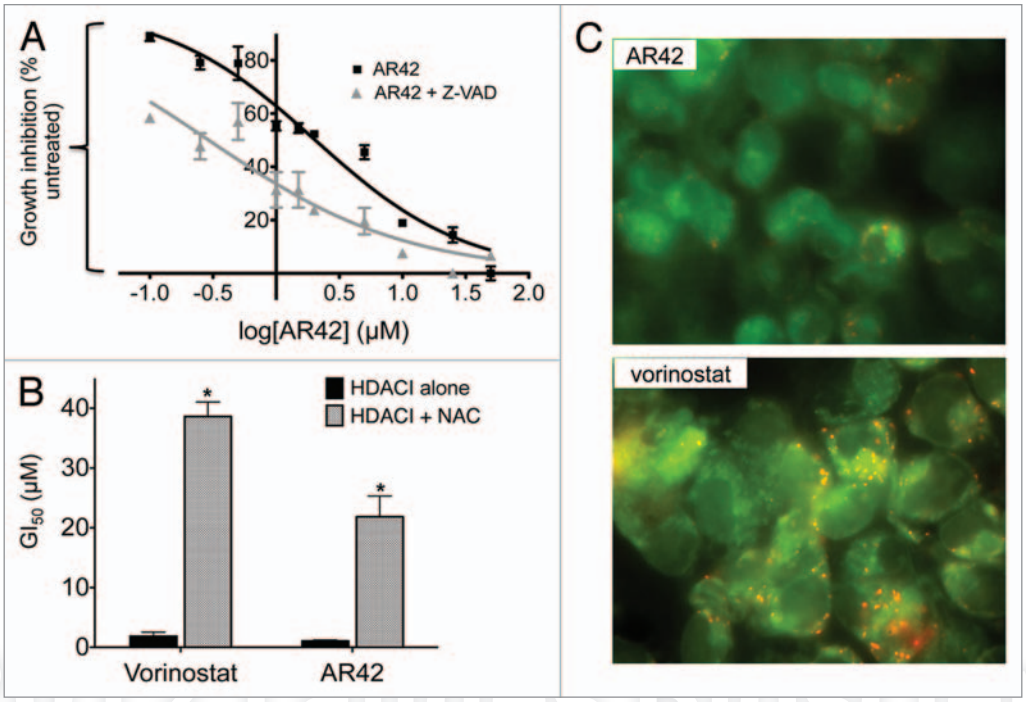

Figure 6. Experimental evaluation of the roles of caspase activity, reactive oxygen species (ROS) and mitochondrial disruption[43,44] in HDACI-mediated cell death. (A) Dose-dependent growth inhibition of CP70 cells treated for 48 h with increasing doses of AR42 in the continuous presence or absence of 20 μM pan-caspase inhibitor z-VAD-fmk. (B) HDACI dependence upon ROS, as determined by comparing 50% growth inhibitory doses (GI_{50} doses) of CP70 cells treated for 24 h with 1.0 μM vorinostat or 1.0 μM AR42 in the absence or presence of 15 mM antioxidant *N*-acetylcysteine ("NAC"). (C) Fluorescent micrographs of CP70 cells treated for 24 h with 1.0 μM AR42 (upper part) or 1.0 μM vorinostat (lower part) and exposed to the cell-permeable, mitochondrial inner membrane-accumulating fluorophore JC-1.[46] At normal membrane potentials, mitochondria-aggregated JC-1 fluoresces red, while loss of membrane potential results in JC-1 aggregation failure and monomer fluorescence emission of green.[46] (*$p < 0.05$, vs. control).

to a differentiation-competent, drug-sensitive phenotype,[14,16,54,62] thus decreasing the tumor subpopulation most responsible for perpetuating malignant phenotypes.[14,41,55,62]

Taken together, these combined computational and experimental approaches suggest that AR42 can upregulate tumor suppressor genes (including miRNAs) and antagonize specific tumor progression signal pathways, including two embryonic development cascades implicated in the genesis and maintenance of cancer stem cells. Overall, these findings suggest that AR42 (and perhaps other epigenetic modifiers), in possible combination with pathway-specific antagonists, might be clinically beneficial for the therapy of chemoresistant, high-grade serous ovarian cancer.

Materials and Methods

Reagents, cell culture and HDAC inhibitor (HDACI) treatments. Cell culture reagents were obtained from Invitrogen Gibco/BRL. Except where specified, antibodies were purchased from KPL Laboratories. All other reagents were purchased from Sigma-Aldrich. For the specific study of advanced, chemoresistant ovarian cancer, we used the p53-dysfunctional CP70 ovarian cancer cell line previously shown resistant to platinum, adriamycin and ionizing radiation[36,37] (a kind gift from Dr Thomas Hamilton, Fox Chase Cancer Center). The HDACIs AR42[5] and vorinostat[3] were kind gifts from Drs Ching-Shih Chen and Samuel Kulp (College of Pharmacy, Ohio State University). For all HDAC inhibitor (HDACI) studies, CP70 cells (~70% confluency) were treated with vehicle (DMSO) control, 1.0 μM vorinostat or 1.0 μM AR42 for 24–72 h, harvested from culture plates, centrifuged and the pellets then further processed or stored at -80°C.

Mature miR and protein-coding (mRNA) gene expression. Total RNA was isolated from 24- or 48-h HDACI-treated or -untreated CP70 cells by suspension in TRIzol reagent (Invitrogen), as we have previously described in reference 70. Global analyses of mature miR expression were kindly performed by Dr Michael Thomson (Vanderbilt University), using a 124-feature custom microarray.[71] For gene expression analyses, total RNA was qualified (Agilent Bioanalyzer 2100, Agilent Technologies), reverse transcribed to cDNA, labeled with Cy3-conjugated nonamers (Roche NimbleGen) and purified and hybridized to Human Gene Expression 385K Arrays, according to the manufacturer's (Roche Nimblegen) protocol. Following washing, the hybridized arrays were then scanned (single color green) using an Axon GenePix Professional 4200A Scanner (Molecular Devices).

Data processing and functional analyses of miR and mRNA expression. Scanned microarray images (GenePix files) were aligned using center fiducial controls, and the gridded files imported into NimbleScan analysis software (v2.5), followed by quantile normalization,[72] and gene calls made using Robust Multichip Averaging.[72] MicroRNA (miRNA) expression data (.xlsx format), provided by Dr Thomson, was similarly processed

using quantile normalization.[72] Unsupervised hierarchical clustering (a control for reproducibility and distinctiveness of the treatment groups) was then performed, using the integrated software platform Expander (EXPression ANalyzer and DisplayER, acgt.cs.tau.ac.il/expander, Tel Aviv University, Israel).[29]

In this study, rather than restricting potential HDACI antineoplastic mechanisms to limited numbers of genes, we used a "systems biology" approach[28,29] to delineate specific signal pathway alterations and reveal possible transcriptional regulatory networks mediating the anti-ovarian cancer effects AR42. To that end, we compared genome-wide protein-coding gene (mRNA) and mature miRNA expression in AR42-treated vs. -untreated CP70 cells. Computationally, we first identified transcription factor (TF)-encoding genes transcribed in positive correlation with downstream "target" genes. Our rationale for the latter was that altered TF activity could result in pleitropic (and amplified) drug effects on specific pathways/processes.[28,29] By this approach, using a signal intensity fold change of 1.5, we identified TF genes whose expression positively correlated with AR42-altered genes having their cognate binding site motifs[31] using the visualization tool ECR (evolutionary conserved region) Browser (ecrbrowser.dcode.org).[30] Those TF/target gene sets were then subjected to functional analysis, using the Web tool BiNGO v2.31.[32] Following normalization and microarray processing, we identified all genes 1.5-fold up or downregulated by 48 h, 1.0 μM AR42 treatment. We then located the transcription start sites (TSSs) of those altered genes, as reported in research literature and publically available human genome databases.[30] Following identification of TSSs, we explored the presence of evolutionarily conserved transcription factor-binding sites (TFBSs) within 4 kb regions encompassing the TSSs, using the ECR browser (ecrbrowser.dcode.org).[30,31] Genes encoding TFs having cognate TFBSs within misexpressed (protein-coding) genes were then identified (also by a fold-change cutoff of 1.5) and subjected to BiNGO functional analysis[32] to determine gene ontology (GO) biological process terms (**Table 1**).

Assessments of mitochondrial (intrinsic) or death receptor (extrinsic) apoptosis. To examine AR42 caspase dependence, CP70 cells were treated for 48-h with 1.0 μM AR42, in the absence or presence of 20 μM pan-caspase inhibitor z-VAD-fmk (Promega). To assess the role of reactive oxygen species[43] in AR42-induced apoptosis CP70 cells were similarly treated in the presence or absence of 15 mM of the antioxidant *N*-acetylcysteine. Separately, CP70 cells were grown on microscope coverslips, treated for 48 h with 1.0 μM vorinostat or 1.0 μM AR42, incubated for 20 min with 500 ng of the cationic mitochondrion-accumulating dye, JC-1 (Cayman Chemical Company), and photographed using a fluorescence microscope (Carl Zeiss) with filters for excitation/emission wavelengths of 485/530 nm (green) or 535/590 nm (red). Under normal mitochondrial transmembrane potential ($\Delta\Psi m$), JC-1 self-aggregates within the mitochondrial inner membrane space, fluorescing red (multimeric JC-1). Loss of $\Delta\Psi m$, however (as occurs during mitochondrial disruption), results in JC-1 mitochondrial aggregation failure, with JC-1 monomers remaining in the cytosol, fluorescing green.[46]

Assessment of Wnt activity (β-catenin nuclear translocation). For western blot assays, CP70 cells were treated with vehicle (DMSO), 1.0 μM AR42 or 1.0 μM vorinostat for 48 h, followed by isolation of total cytoplasmic or total nuclear protein, using a nuclear extraction kit (Active Motif). Fifteen micrograms of nuclear or cytoplasmic protein (with β-catenin positive control cell lysate, Lab Vision) were then electrophoresed through 8% SDS-PAGE, transferred to nylon membranes, blocked with 5% milk and incubated overnight with diluted primary antibodies against activated (unphosphorylated) β-catenin (Lab Vision) and GAPDH (a gel-loading control, Millipore). Membranes were then washed and incubated with peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibodies for 60 min, washed again, placed for 1 min in SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific), and exposed to film (Kodak) for 2 min. Western blot band intensities were then determined by scanning and quantification using ImageJ Java-based imaging software (Research Services Branch, National Institute of Mental Health, rsb.info.nih.gov/ij).

Assessment of Wnt signal transactivity by luciferase reporter assay. For determination of Wnt signal activity at the pathway level, CP70 cells were grown in 6-well plates to 60–70% confluency. Using Lipofectamine 2000 (Invitrogen), cells were then transfected for 24 h with 1.0 μg of the luciferase reporter TOPFlash (a construct possessing four Wnt signal-transactivating TCF/LEF-binding sites ligated to the gene *luc*) or 1.0 μg of its negative control FOPflash (having four mutant *luc*-ligated TCF/LEF-binding sites).[40] Both TOPflash and FOPflash were purchased from Millpore. TOPFlash- or FOPFlash-transfected cells were then treated with DMSO vehicle or 1.0 μM AR42 or for 48 h, washed 2x with cold PBS, placed in 1x Reporter Lysis Buffer (Promega) and stored at -80°C for at least 1 h. Following one freeze-thaw cycle, Luciferase Assay Reagent (Promega) was added to the AR42-treated or -untreated cell lysates, with luminescence intensity detected by a TD-20/20 luminometer (Turner Designs). Raw TOPFlash and FOPFlash luminescence values were then normalized for protein concentration, and the normalized TOPFlash:FOPFlash ratios then used to quantify Wnt reporter activity in untreated vs. AR42-treated CP70 cell lysates.[70]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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