

# The histone deacetylase inhibitor trichostatin A alters microRNA expression profiles in apoptosis-resistant breast cancer cells

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**Abstract.** The development of drug resistance represents a major complication in the effective treatment of breast cancer. Epigenetic therapy, through the use of histone deacetylase inhibitors (HDACi) or demethylation agents, is an emerging area of therapeutic targeting in a number of ontological entities, particularly in the setting of aggressive therapy-resistant disease. Using the well-described HDAC inhibitor trichostatin A (TSA) we demonstrate the suppression of *in vitro* clonogenicity in the previously described apoptosis-resistant MCF-7TN-R breast carcinoma cell line. Additionally, recent work has demonstrated that these agents can alter the expression profile of microRNA signatures in malignant cells. Using an unbiased microRNA microarray analysis, changes in miRNA expression of MCF-7TN-R cells treated with TSA for 24 h were analyzed. We observed significant up-regulation of 22 miRNAs and down-regulation of 10 miRNAs in response to TSA treatment. Our results demonstrate that the HDACi, TSA, exerts anticancer activity in the apoptosis-resistant MCF-7TN-R breast carcinoma cell line. This activity is correlated with TSA alteration of microRNA expression profiles indicative of a less aggressive phenotype.

## Introduction

Despite significant advancement in the area of endocrine therapies and chemotherapeutics, nearly half of breast cancer patients exhibit *de novo* resistance, while the majority of

remaining patients ultimately progress to drug resistance (1). Drug resistant breast cancer is associated with poor prognoses (2,3), highlighting the critical need to develop novel therapeutics that are effective against these more aggressive forms of the disease. Epigenetic alterations, including aberrant DNA methylation and histone deacetylation, participate in cancer development and progression (4). Epigenetic aberrations lead to breast cancer chemotherapy resistance (5,6); hence, their reversal by inhibitors of DNA methylation and histone deacetylases (DNMTi and HDACi) may overcome it and are at present undergoing clinical testing, either alone or in combination with conventional chemotherapies (7).

Histone deacetylases (HDACs) and histone acetyltransferases (HATs) have important roles in the maintenance and function of chromatin by regulating the acetylation of histones. In addition, these enzymes have recently been shown to regulate the acetylation of many non-histone targets and therefore may represent a key means of post-translational regulation beyond their established roles in transcriptional regulation. The use of HDACi in the clinical setting is currently FDA-approved only for the treatment of progressive or recurrent cutaneous T-cell lymphoma following two other systemic therapies (8). Biologically, HDACi induce growth arrest, differentiation and cell death in breast cancer cells, but the underlying mechanism warrants further investigation.

In addition to direct regulation of mRNA gene expression, HDACis have been shown to alter microRNA (miRNA) expression in several human carcinomas including pancreatic (9), colon (10,11), gastric (12) and breast (13). microRNAs are small non-coding RNAs (18-22 nt) which function as an additional layer of regulation of mRNA stability and translation through 3'-UTR targeting (14). Through their ability to target the 3'-UTR of multiple genes, individual miRNAs can exert vast effects on mRNA-protein expression in cells. In cancer, miRNAs can function as tumor suppressors or oncogenes (15). We examined the effects of the HDACi trichostatin A (TSA) on the survival of the apoptotic-resistant MCF-7TN-R breast carcinoma cell line, as well as its effects on miRNA expression.

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Table I. microRNA microarray results for MCF-7TN-R cells treated with TSA (10  $\mu$ M) for 24 h.

ID	Serial no.	Average (TSA)	Average (DMSO)	p-value	logFC
hsa-miR-215	990	498.33	323.67	0.001	1.46
hsa-miR-657	725	206.33	573.67	0.002	-1.13
hsa-miR-139	524	886.33	783.67	0.003	0.88
hsa-miR-155	544	192.67	176.33	0.005	0.73
hsa-miR-146b	1501	182.67	178.00	0.005	0.66
hsa-miR-645	1681	181.33	519.67	0.006	-1.27
hsa-miR-544	127	374.67	305.67	0.006	1.01
hsa-miR-194	1541	362.67	319.67	0.007	0.95
hsa-miR-628	696	190.67	371.67	0.007	-0.60
hsa-miR-144	1498	148.00	115.33	0.008	0.94
hsa-miR-144	530	154.67	110.67	0.008	1.10
hsa-miR-559	1112	128.67	111.67	0.008	0.74
hsa-miR-128b	511	868.00	905.00	0.009	0.62
hsa-miR-143	529	204.67	143.67	0.009	1.13
hsa-miR-568	1121	145.67	130.33	0.009	0.74
hsa-miR-769-5p	744	230.33	502.33	0.010	-0.69
hsa-miR-1	1461	226.00	130.33	0.010	1.48
hsa-miR-497	645	296.67	639.67	0.011	-0.66
hsa-miR-632	1668	217.33	603.00	0.012	-1.12
hsa-miR-767-5p	1708	178.00	392.67	0.012	-0.73
hsa-miR-512-3p	660	155.33	362.67	0.013	-0.82
hsa-miR-191*	1537	206.67	138.33	0.014	1.25
hsa-miR-191*	569	285.33	155.33	0.015	1.65
hsa-miR-155	1512	163.67	150.67	0.015	0.70
hsa-miR-613	1649	246.00	624.33	0.016	-0.93
hsa-miR-202*	977	479.33	378.00	0.016	1.05
hsa-miR-636	704	226.67	705.33	0.018	-1.20
hsa-miR-486	1600	192.00	129.67	0.019	1.21
hsa-miR-622	1658	181.33	401.67	0.019	-0.79
hsa-miR-215	22	469.00	289.00	0.020	1.61
hsa-miR-22	27	1007.33	568.33	0.021	1.82
hsa-miR-875-5p	903	139.67	103.33	0.024	0.99
hsa-miR-620	1656	210.00	127.33	0.024	1.36
hsa-miR-370	581	1265.67	1152.67	0.026	0.88
hsa-miR-200c	7	272.67	278.00	0.026	0.60
hsa-miR-638	706	1096.67	1021.00	0.026	0.85
hsa-miR-630	1666	140.33	129.00	0.028	0.71
hsa-miR-208	14	191.33	168.67	0.028	0.74
hsa-miR-526c	121	321.33	215.33	0.028	1.27
hsa-miR-643	711	138.33	268.33	0.029	-0.61
hsa-miR-651	719	142.33	332.67	0.029	-0.84
hsa-miR-149	537	733.67	711.00	0.031	0.71
hsa-miR-589	1880	150.67	137.00	0.032	0.77
hsa-miR-768-3p	1709	994.33	1006.00	0.033	0.79
hsa-miR-22	995	935.67	635.00	0.035	1.65
hsa-miR-627	1663	133.67	102.00	0.035	0.96
hsa-miR-450	1588	270.67	571.33	0.036	-0.63
hsa-miR-620	688	198.33	136.67	0.036	1.17
hsa-miR-211	987	618.67	578.33	0.037	0.83
hsa-miR-346	84	625.33	604.67	0.041	0.77

Table I. Continued.

ID	Serial no.	Average (TSA)	Average (DMSO)	p-value	logFC
hsa-miR-1	493	256.00	184.67	0.041	1.11
hsa-miR-607	191	171.33	155.33	0.041	0.70
hsa-miR-153	1509	145.67	124.00	0.041	0.85
hsa-miR-621	1657	277.00	196.00	0.042	1.10
hsa-miR-668	1700	233.33	538.67	0.043	-0.74
hsa-miR-607	1159	105.33	254.00	0.043	-1.04
hsa-miR-129	1480	533.33	495.00	0.045	0.82
hsa-miR-335	76	175.67	134.00	0.045	0.96
hsa-miR-640	1676	188.67	447.00	0.047	-0.80
hsa-miR-524*	116	256.00	243.00	0.048	0.61
hsa-miR-663	731	681.33	757.67	0.049	0.60

## Materials and methods

**Cell generation and culture.** The apoptotic-resistant MCF-7TN-R cells were derived from MCF-7 cells grown in increasing concentrations of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) until resistance was established. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (10% DMEM). The detailed methods are described by Weldon *et al* (16).

**Clonogenicity assay.** Colony survival assays were performed as previously published (17). Briefly, cells were cultured in 10% FBS-DMEM media. Cells were seeded at a density of 2,000 cells/well in 2 ml of media in 6-well plates. Cells were allowed to adhere overnight at 37°C and treated on the following day with vehicle (DMSO) or TSA (0.1, 1, 10  $\mu$ M). After 10 days, media was removed and the cells were fixed with glutaraldehyde. Cells were stained with crystal violet (0.1% in 20% methanol) for visualization. Colonies >50 cells were manually counted and treatments normalized to vehicle control. Assays were run in quadruplicate with internal duplicates.

**microRNA microarray analysis.** MCF-7TN-R cells were plated at a density of  $2 \times 10^6$  cells in 25 cm<sup>2</sup> flasks in normal culture media (DMEM media supplemented with 5% FBS, 1% penicillin/streptomycin, 1% essential amino acids, 1% non-essential amino acids and 1% sodium pyruvate) and allowed to adhere overnight at 37°C, 5% CO<sub>2</sub> and air. The following day the media was changed to phenol red-free media (supplemented as above) and 5% charcoal-stripped serum was substituted for the 5% FBS. Cells were treated with TSA (10  $\mu$ M) or DMSO for 24 h. Cells were harvested in PBS, collected by centrifugation, and total RNA extracted using the miRNeasy kit (Qiagen) according to manufacturer's protocol. Enrichment for miRNA was not performed. Quantity and quality of RNA was determined by absorbance (260, 280 nm), and 5  $\mu$ g total RNA was used for microarray analysis. Microarray analysis was performed as we have previously described (18). Briefly, a custom microarray (19) was used to determine miRNA expression, using three biological replicates for each condition ( $\pm$  TSA). Low intensity probes (signal <100 in more than half samples)

were excluded from the analysis. Raw data was log-transformed and normalized by IQR. Clustering of miRNA expression data was performed using CLUSTER (20), with filtering to remove inconsistencies between replicates. For clustering, we first log-transformed the data and median-centered the array and genes, followed by average linkage clustering. Clustering results were visualized by TreeView (<http://rana.lbl.gov/EisenSoftware.htm>). Full array data is shown in Table I.

**Statistical analyses.** Colony assays were analyzed by one-way ANOVA with the Tukey's post-test (Graph Pad Prism V.4);  $p < 0.05$  was considered to indicate statistically significant differences. The Student's t-test was performed to evaluate the statistical significance of the cluster selection. For microRNA microarray data, the Welch's t-test was performed for each probe using their normalized signals, with p-values <0.05 considered significant as previously described (21).

## Results

**TSA inhibits drug-resistant breast cancer cell clonogenic survival.** We have previously described the generation of the MCF-7TN-R cells (16) which have acquired resistance to TNF $\alpha$ - and TRAIL-induced cell death. These cells have been characterized as highly aggressive and metastatic, and have been developed as a model system of chemoresistant breast carcinoma (22,23). To determine the effects of HDACi on apoptotic and clonogenic survival, MCF-7TN-R cells were treated with varying concentrations of TSA (0.1, 1 and 10  $\mu$ M) or vehicle control (DMSO) for 10 days. MCF-7TN-R cells treated with TSA at 10  $\mu$ M for 10 days demonstrated a decrease ( $p < 0.01$ ) in colony formation compared to vehicle-treated cells ( $62.37 \pm 6.45\%$ , Fig. 1).

**TSA induces microRNA expression in MCF-7TN-R cells indicative of tumor suppressive and anti-metastatic effects.** In human breast cancer, we and others have shown that specific miRNAs are significantly altered, as compared with normal breast tissue (21,24-26). Altered expression of specific miRNAs has been associated with poor prognosis (27), as well as breast cancer initiation, invasion and metastasis (28-31).

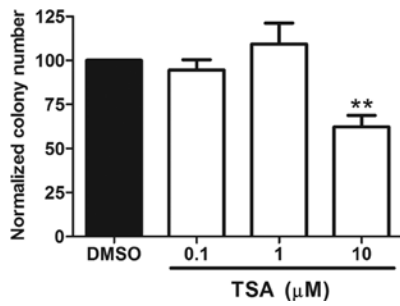


Figure 1. TSA suppression of MCF-7TN-R cell clonogenic survival. MCF-7TN-R cells were plated (2,000 cells/well) in 10% DMEM in 6-well plates and allowed to adhere overnight. Twenty-four hours later the cells were treated with vehicle (DMSO) or TSA (0.1, 1, 10  $\mu$ M) for 10 days. Colonies of  $\geq 50$  cells were counted as positive. Bars represent mean percentage clonogenic survival normalized to DMSO control cells  $\pm$  SEM. (\*\* $p < 0.01$ ).

The importance of miRNAs in these advanced breast cancer phenotypes raises the question of their further involvement in apoptotic resistance. Furthermore, based on the above result demonstrating MCF-7TN-R growth-inhibition by TSA, we performed microRNA microarray analysis of MCF-7TN-R cells after treatment with TSA (10  $\mu$ M for 24 h). As shown in Fig. 2, a number of microRNA expression changes were observed. In addition, the three biological replicates from vehicle-treated MCF-7TN-R clustered together and separately from TSA-treated cells, demonstrating high reproducibility between biological repeats as well as differential microRNA expression induced by TSA. Of the microRNAs significantly altered by HDACi treatment, 22 were up-regulated (Table II) and 10 were down-regulated (Table III). Their predicted (TargetScan and miRanda) or confirmed gene targets are provided.

## Discussion

Drug resistance, acquired or *de novo*, remains a major obstacle in the treatment of cancer (1). Progression to resistance represents one of the hallmarks of aggressive carcinomas with limited treatment options and poor prognoses (2,3). Epigenetic therapies, including HDACi, provide a novel class of treatment for therapeutically-resistant cancer patients (32), including breast cancer (33). Here we demonstrate the ability of the HDACi TSA to suppress *in vitro* clonogenic survival of the apoptotically resistant MCF-7TN-R cells. This cell culture data indicates that in progressive drug resistance or recurrent breast carcinoma, the use of HDACis may exhibit greater inhibitory effects on tumor cell survival. Numerous studies have analyzed gene expression changes in response to HDACi, with the goal of defining specific mechanisms of their anticancer activity (34). Recent studies have also demonstrated the regulation of microRNA expression changes in breast and other cancer cells treated with HDACi alone (9,10,13,35), or in combination with DNMTi (11,36-38). Overall, these studies revealed microRNA expression profiles suggestive of a less aggressive and more tumor-suppressive phenotype after treatment with epigenetic therapies. Consistent with those studies, our microarray results revealed that many of the microRNAs that were significantly up-regulated (Table II) following TSA treatment (compared to control) have been characterized as having tumor suppressive

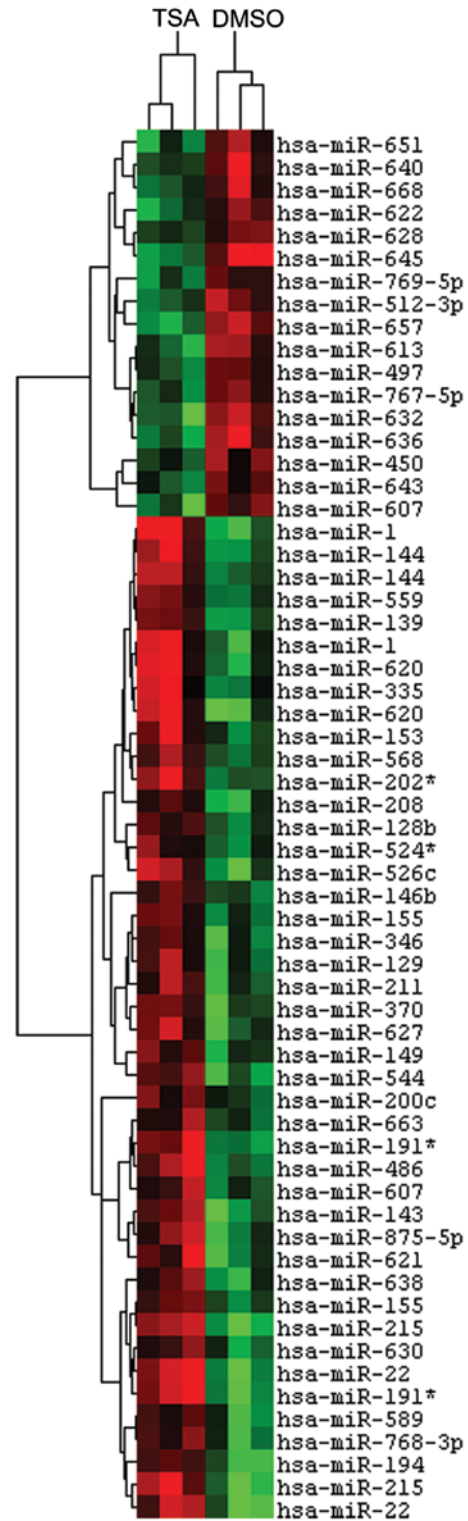


Figure 2. TSA regulation of microRNA expression in MCF-7TN-R. Heatmap of microRNA changes induced by treatment with TSA (10  $\mu$ M) after 24 h in MCF-7TN-R cells. microRNAs demonstrating statistically significant changes in expression are shown ( $p < 0.01$ ). Green indicates down-regulated expression and red indicates up-regulated expression of microRNAs. Individual samples are represented in columns while specific microRNAs are represented by rows as labeled.

(miR-1, miR-143, miR-144, miR-191\*, miR-202\*, miR-486, miR-559), anti-migration/anti-metastasis (miR-22, miR-139, miR-194, miR-335), or anti-EMT (miR-215) roles in cancer.

Table II. Up-regulated miRNA following TSA treatment.

microRNA	Mean fold change	p-value	Description	Gene targets (ref.)
hsa-miR-1	2.44	<0.05	Tumor suppressor	cMET (39), TAGLN2 (40,41)
hsa-miR-22	2.69	<0.05	Anti-migration, cell cycle arrest	MYCBP, MAX
hsa-miR-139	1.61	<0.05	Anti-metastatic, tumor suppressor	ROCK2 (29), CDK6*, HOXB2*
hsa-miR-143	1.94	<0.05	Tumor suppressor	KRAS (42), BCL2 (43)
hsa-miR-144	1.97	<0.05	Tumor suppressor	Notch1 (44)
hsa-miR-153	1.70	<0.05	Tumor suppressor	BCL2 (45)
hsa-miR-155	1.53	<0.01	OncomiR	FOXO3A (46), SOCS1 (47), FADD and IKKE (48)
hsa-miR-191*	2.51	<0.05	Tumor suppressor	SPEN*
hsa-miR-194	1.70	<0.01	Tumor suppressor, anti-metastatic	CDH2, HBEGF, RAC1 and IGF1R (49)
hsa-miR-215	2.26	<0.01	Anti-EMT, cell cycle arrest	ZEB1/2 (50)
hsa-miR-202*	1.82	<0.05	Tumor suppressor	TGFBR2*, ROCK1*
hsa-miR-335	1.83	<0.05	Tumor suppressor, cell cycle arrest, metastasis suppressor	RB1/p105 (51), SOX4 and TNC (28)
hsa-miR-486	1.97	<0.05	Tumor suppressor	CD40 (52)
hsa-miR-526c	2.14	<0.05		
hsa-miR-544	1.74	<0.01		
hsa-miR-559	1.61	<0.05	Tumor suppressor	ERBB2 (53)
hsa-miR-568	1.59	<0.05		
hsa-miR-620	2.31	<0.05		
hsa-miR-627	1.93	<0.05		
hsa-miR-638	1.55	<0.01		
hsa-miR-641	2.02	<0.05		
hsa-miR-888	1.76	<0.05		

\*Putative targets as indicated by TargetScan and miRanda.

Table III. Down-regulated miRNA following TSA treatment.

microRNA	Mean fold change	p-value	Description (ref.)	Gene targets (ref.)
hsa-miR-500	-1.90	<0.05	OncomiR (54)	
hsa-miR-512-3p	-1.59	<0.05		cFLIP (55)
hsa-miR-607	-1.68	<0.05		
hsa-miR-613	-1.69	<0.05		LXR (56)
hsa-miR-622	-1.57	<0.05		
hsa-miR-632	-1.86	<0.01		
hsa-miR-636	-2.00	<0.05		
hsa-miR-645	-2.03	<0.01	OncomiR (57)	
hsa-miR-651	-1.60	<0.05		
hsa-miR-657	-1.91	<0.01		IGF2R (58)

Only one known oncomiR, miR-155, was found to be increased following TSA treatment, but with a mean fold change of 1.53 compared to control, this was the lowest change observed, although statistically significant. Of the microRNAs identified as significantly decreased (Table III) following TSA treatment (compared to control), two have been identified as oncomiRs (miR-500, miR-645) and three others have confirmed targets

involved in tumorigenesis (miR-512-3p, miR-613, miR-657). Taken together, these data indicate that HDACi treatment may promote an anti-tumor microRNA expression profile in the apoptotically resistant cell line MCF-7TN-R, providing novel therapeutic targets for the treatment of drug resistant breast cancer.



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