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Chromatin reader ZMYND8 is a key target of all trans retinoic acid-mediated inhibition of cancer cell proliferation

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ABSTRACT

All trans retinoic acid (ATRA), an active vitamin-A derivative, has been shown to regulate gene expression program and thus imparts anti-proliferative activity to cancer cells. Previously, we identified a dual histone reader ZMYND8 (zinc finger MYND (Myeloid, Nervy and DEAF-1)-type containing 8), to be a novel target of ATRA. In the present study, we attempted to decipher the detail mechanism of its transcription regulation. ATRA can reprogram the epigenetic landscape in the upstream regulatory region of *ZMYND8* thereby promoting its expression. Interestingly, there is a unique H3K27Me3 to H3K27Ac switch upon ATRA-treatment. We show here that ATRA causes dynamic changes in recruitment of transcription factor YY1 in concert with HDAC1 at *ZMYND8* promoter. Further, we show that ATRA treatment triggers an anti-proliferative activity in cancer cells through regulation of ZMYND8 expression. Subsequently, in 4T1-induced syngenic tumor mouse model, ATRA injection caused significant upregulation of ZMYND8. Overall our findings highlight a novel mechanism underlying ATRA-mediated changes in ZMYND8 expression which, in turn, activates the anti-proliferative program in a cancer cell. Thus, histone reader mediated modulation of epigenetic language could play a significant role in retinoid based therapeutic strategy which is well exploited to combat tumor growth.

1. Introduction

Chromatin is the physiological template for the diverse genomic processes ranging from transcription, replication repair and recombination [1,2]. Cells have evolved with a defined gene expression program of their own, which is regulated in a spatial and temporal manner [3,4]. Epigenetic alteration of gene expression is responsive to environmental cues which activate signal transduction cascades [5]. Further transcription factor dynamics also play crucial role in modulating cellular gene expression. Several small molecule modulators of chromatin structure can cause alteration in epigenetic language of chromatin as well as transcription factor dynamics [6]. All trans retinoic acid (ATRA), an active vitamin-A derivative, is one such ligand which causes significant changes in cellular transcription programs [7–9] and controls metabolism [10], organ development [11] as well as neoplastic transformation of the cells [12,13]. ATRA binds to the RAR/RXR nuclear hormone receptors which, in turn, activate transcription of its target genes by binding to Retinoic Acid Response Element (RARE) [14]. ATRA-induced distinct epigenetic modifications lead to transcriptional activation [15]. Further, RA-induction leads to release of corepressor complex encompassing HDAC and chromatin remodeling ATPases, in turn recruiting coactivator complex harboring HAT enzymes [16]. Incidentally, in several solid tumors as well as hematological malignancies, ATRA is used as one of the drug of choice [17,18]. Through the inhibition of cell proliferation, apoptosis and migration, ATRA shows potential anti-tumorigenic ability on colorectal [19], hepatic [20], glioma [21] and breast [22,23] cancer cells. The anti-proliferative effect of ATRA could be mediated by multi-functional transcription factors.

We have recently shown that Zinc finger MYND (Myeloid, Nervy and DEAF-1)-type containing 8 (ZMYND8) regulates several ATRA-inducible genes [24]. ZMYND8 was identified as a component of transcription coregulator complex. Generally, this complex is a large multi-protein assembly that can activate (*e.g.* Histone Acetyl Transferase complexes like CBP/p300 or PCAF) or repress transcription (N-CoR/SMRT or sin3-HDAC complex) [25]. Interestingly, ZMYND8 interacts with initiation competent RNA Polymerase II phosphorylated at Ser5 selectively [24]. It recognizes transcription activation signatures H3.1K36Me2/H4K16Ac and stimulates the gene expression in the presence of ATRA. ZMYND8 exerts its function by being associated with several lysine demethylase (KDM) machinery components, including KDM5A, KDM5C, or KDM1A [26,27]. In addition, ZMYND8 plays a significant role in embryonic neural differentiation, through its ability to interact with Xenopus REST Corepressor 2 (RCoR2) [28]. Thus ZMYND8 has a close association to the transcription network and can act as an activator or repressor in different context.

Abbreviations: ZMYND8, zinc finger MYND (Myeloid, Nervy and DEAF-1)-type containing 8; ATRA, all trans retinoic acid; IHC, immunohistochemistry; PBS, phosphate buffered saline.

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Recent evidences highlight the anti-tumorigenic ability of ATRA on breast cancer [22,23], while some subtypes are found highly sensitive [29]. For the use of ATRA as target based therapy, the detail knowledge of the mediators as well as molecular mechanisms underlying the sensitivity of the neoplastic cells to ATRA is of great importance. ATRA has been reported to impart its anti-tumorigenic ability through triggering several intracellular signaling pathways, including TGFβ, MAPK, Notch *etc.* [19,22,23]. However, ATRA-mediated alteration of epigenomic landscape has not been exploited yet. In the present report, we demonstrated that ATRA can modulate the histone modification status and transcription factor dynamics which is instrumental in *ZMYND8* up-regulation. Interestingly, *ZMYND8* was found to be a mediator of ATRA-induced anti-proliferative activity *in vivo*. Taken together, we show here for the first time that ATRA can indeed cause alteration in epigenetic modifications of chromatin, manifesting in differential expression of key cellular transcription factors which in turn inhibits cell proliferation.

2. Methods

2.1. Cell culture and treatment

SH-SY5Y and neuro2A cells were maintained in Dulbecco's modified Eagle's medium (DMEM): F12 and EMEM (Gibco) respectively. HeLa, MCF7 and 4T1 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). All cells were maintained at 37 °C humidified atmosphere with 5% CO_2. For treatment, cells were serum starved for 16 h and then 10 μM of all-trans retinoic acid (RA; Sigma, Mendota Heights, MN) was used for SH-SY5Y and MCF7 cells and at 20 μM for HeLa cells for indicated time.

2.2. Bioinformatics analysis of ZMYND8 promoter

Homology searches of human and mouse *ZMYND8* promoter were performed using BLAST program. The promoter sequences were analyzed by UCSC Genome Browser to identify the histone PTM enrichment (marked as A- 650 bp, B- 200 bp, C- 600 bp and D- 750 bp). Target Explorer and MatInspector programs were used to identify the RARE sequence in *ZMYND8* human and mouse promoter. RARE sequence was identified at −1239 to −1255 upstream region of human promoter. FLAG-RARα construct was purchased from Addgene (ID# 35555). This construct was used to reconfirm the identified RARE sequence by ChIP assays (detailed protocol mentioned below).

2.3. Reporter construct and site-directed mutagenesis

The 527 bp upstream promoter region of mouse *ZMYND8* gene was amplified by PCR using mouse genomic DNA as template and then cloned into pGL3 basic vector (Promega, Madison, WI) at *Kpn*I/*Hin*dIII site. Similarly, 873-bp upstream region of human *ZMYND8* gene was cloned into pGL3-basic vector at *Kpn*I/*Sac*I site. The primer sequences used to clone the promoter regions are mentioned in Table 1, where the restriction enzyme sites are underlined. All constructs were sequenced to confirm their identity.

The YY1-specific elements present in the upstream region of human and mouse *ZMYND8* promoter were mutated by PCR-based method using Quickchange Site directed mutagenesis kit (Stratagene) as per standard protocol [15].The wild-type clone of *ZMYND8* promoter in pGL3 vector was used as template. The wild type YY1-element (5′- ATGGC - 3′) was mutated to (5′- AGAGA - 3′; for human and 5′- AAAAA - 3′; for mouse genome). The mutations were confirmed by sequencing.

Full length ZMYND8 was cloned in pCMV-FLAG using GATEWAY CLONING SYSTEM (Invitrogen) as described previously [24]. To over-express ZMYND8, 1 μg of FLAG-ZMYND8 was transfected per 10^5cells/well in 6-well plate using Lipofectamine 2000 (Invitrogen). After 24 h of transfection, the cells were harvested for subsequent analysis.

2.4. Luciferase assay

For reporter assay, 5×10^4 cells were seeded on 12-well culture plates. After 24 h, pGL3-*ZMYND8* construct (wild type or mutated; 0.4 μg) and pRL-CMV vector (0.04 μg; Promega) was transiently co-transfected with Lipofectamine 2000 (Invitrogen). After 4 h of transfection, the medium was replaced with fresh complete one for next 16 h. pRL-CMV vector was used to normalize each transfection. In the following day, cells were harvested and firefly as well as renilla luciferase activity was determined in lysates using Glomax 96-microplate luminometer (Promega) and dual-luciferase reporter assay system following manufacturer's protocol (Promega). Each transfection was performed in triplicate and the experiments were repeated thrice.

2.5. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as per standard protocol. Briefly, crosslinking was done with 1% formaldehyde for 15 min and then the reaction was stopped by 0.125 M Glycine, followed by cell lysis in cell lysis buffer [5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% NP40 (with fresh protease inhibitor)] for 10 min in ice. This was followed by lysis with nuclei lysis buffer [50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS (with fresh protease inhibitor)] for 10 min in ice. After sonication of the chromatin, pre-clearing was done. Immunoprecipitation was set overnight with antibodies mentioned in Table 2. Next day, pre-blocked DYNA beads were added for binding to pulled chromatin complex. Beads were washed with RIPA buffer, high-salt buffer, LiCl buffer and TE consecutively for 10 min each. Following RNaseA and Proteinase K treatment, the beads were kept for de-crosslinking at 65 °C for 4 h. Phenol-chloroform extraction followed by ethanol precipitation was performed. The DNA pellet was dissolved in H_2O and used for Q-PCR analysis using gene specific primers (Table 3).

2.6. Quantitative Real-time RT-PCR (Q-PCR)

Total RNA was isolated from cells using TRI-reagent (Sigma) following the standard protocol. First-strand cDNA synthesis was done with RevertAid fast strand cDNA synthesis kit (Thermo Scientific) followed by Q-PCR assay was performed using Power SYBR GREEN mix (Applied Biosystems). The comparative C_T method ($\Delta\Delta C_T$) was used to measure relative gene expression where the fold enrichment was calculated as: $2^{-[\Delta CT\ (sample) - \Delta CT\ (calibrator)]}$. Here, ΔC_T is the C_T of target gene

Table 1
The sequence of the oligonucleotide primers used to clone specific region of *ZMYND8* promoter.

Gene name	Forward primer (5′- 3′)	Reverse primer (5′- 3′)	Tm (°C)
ZMYND8;Mouse	GGGGTACCGGAGCCCCAACAAATCAG	GGAAGCTTCTCTCTCTCTCTCTGCATCT	55
ZMYND8; Human	GGGGGTACCGGAAGTTTGTAGATCCAGGC	GGGGAGCTCGGCTCCTAGTTCTCACAGAA	55
ZMYND8;Mouse with YY1-mut	GGGCTCTGTCAGGAAAAAAAGGGAGATACCCTCTCC	GGAGAGGGTATCTCCCTTTTTTTCCTGACAGAGCCC	60
ZMYND8; Human with YY1-mut	CCTGTCTTTTTCTATGCACAGGAGAGATCTTGCTCACCCGAGC	GCTCGGGTGAGCAAGATCTCTCCTGTGCATAGAAAAAGACAGG	60