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HDAC8 regulates neural differentiation through embryoid body formation in P19 cells☆

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ABSTRACT

Histone acetylation and deacetylation correlate with diverse biological phenomena through gene transcription. Histone deacetylases (HDACs) regulate deacetylation of histones and other proteins. However, as a member of the HDAC family, HDAC8 function during neurodevelopment is currently unknown. Therefore, we investigated HDAC8 function during neurodevelopment by examining embryoid body (EB) formation in P19 cells. HDAC8-selective inhibitor (NCC-149) (HDAC8i)-treated cells showed smaller EBs than non-treated cells, as well as reduced expression levels of the neuronal marker, NeuN. Additionally, HDAC8i treatment led to inhibition of cellular proliferation by G2/M phase accumulation and down-regulated cyclin A2 and cyclin B1 gene expression. Furthermore, two independent *HDAC8* knockout cell lines were established by CRISPR-Cas9, which resulted in smaller EBs, similar to HDAC8i-treated cells. These results suggest that HDAC8 regulates neural differentiation by exerting control of EB formation.

1. Introduction

Histone acetylation and deacetylation are epigenetic processes that regulate gene expression, and in turn, cellular function [1,2]. Histone acetyl transferases transfer acetyl groups to lysine residues on histones causing relaxation of the chromatin structure because of reduced interaction between DNA and histones. Histone deacetylases (HDACs) are a group of enzymes responsible for deacetylation of lysine residues on histones and non-histone proteins. Deacetylation of histones alters chromatin structure, thereby regulating downstream gene expression and subsequently many cellular processes. HDAC isoforms are classified into four groups: Class I (HDAC1, 2, 3, and 8), which are homologous to yeast RPD3; Class II (HDAC4–7, 9 and 10), which are homologous to yeast Hda1; Class III (Sirtuin 1–7); and Class IV (HDAC11) [3,4]. HDAC inhibition has extensively been shown to suppress proliferation and alter differentiation in tumor cells. Indeed, HDAC members are clinical target molecules for cancer management [5,6].

HDAC8 is a unique member of the Class I HDACs. HDAC8 shares only 43% sequence identity with other members of its class and has a shorter C terminal region [7]. Moreover, HDAC8 has a unique expression pattern and is abundantly expressed in the brain, kidney, and prostate [7,8]. Furthermore, HDAC8 is implicated in a number of diseases including neuroblastoma (with its high expression associated with poor prognosis) [9], and Cornelia de Lange syndrome (CdLS) [10,11].

However, little is known about the role of HDAC8 during development and differentiation of neurogenesis *in vitro*, which led us to hypothesize that HDAC8 inhibition might regulate these processes [9]. In this study, we investigated the involvement of HDAC8 inhibition on embryonic neurogenesis using retinoic acid (RA)-treated P19 cells as a model for neural progenitor cells, and a highly potent and selective HDAC8 inhibitor [10] that our group had

Abbreviations: CdLS, Cornelia de Lange syndrome; EB, embryoid body; ES cells, embryonic stem cells; FGF8, fibroblast growth factor 8; HDAC, histone deacetylase; HDAC8i, HDAC8-selective inhibitor; KO, knockout; RA, all-trans retinoic acid; SMC3, structural maintenance of chromosomes 3; WT, wild-type.

☆ We declare that this paper includes part of the doctoral dissertation of J. O. Makanga.

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previously discovery [12,13]. Moreover, using the CRISPR-Cas9 system, we constructed HDAC8 knockout (KO) cells and analyzed them. Here, we show that HDAC8 regulates neural differentiation through embryoid body (EB) formation.

2. Materials and methods

2.1. Materials

Anti-NeuN antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-β-tubulin antibody was obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Cell culture and differentiation of P19 cells

P19 embryonic carcinoma cells were grown on Minimum Essential Medium Eagle, Alpha Modification (α-MEM; Wako) containing 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA). Cells were passaged at least twice before differentiation. Cell differentiation was performed according to the procedure of Jones-Villeneuve et al. [14]. Briefly, P19 cells (1×10^6) were plated in medium with 0.5 μM all-trans RA (Wako) on bacterial ϕ10 cm dishes (Iwaki, Shizuoka, Japan) for suspension culture to EB formation. After two days, cells were harvested by centrifugation at 200 rpm for 5 min, with one-quarter of cells re-plated onto bacterial ϕ10 cm dishes containing 0.5 μM RA in medium. After two days, cells were collected as described above, and trypsinized. Next, 2×10^5 cells were plated onto poly-L-lysine coated 6 well plates (Nippon Genetics, Tokyo, Japan) in α-MEM without RA. EB cells were observed using a BZ-X710 microscope (Keyence, Osaka, Japan). EB area was measured using Image J [15].

2.3. Measuring cell proliferation activity by WST-8 assay

2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) assay (Dojindo,

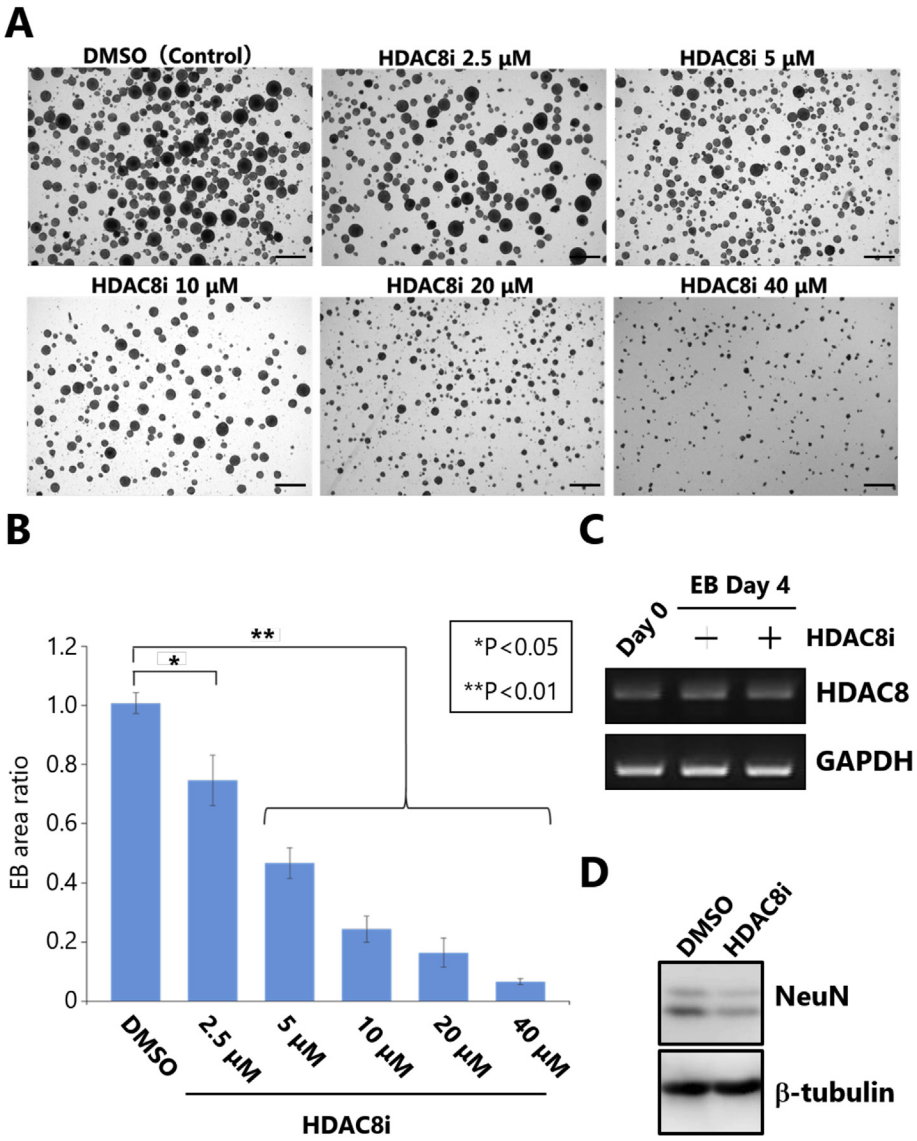

Fig. 1. Specific HDAC8 inhibition downregulates embryoid body size and neural differentiation activity. (A) P19 cells treated with HDAC8 selective inhibitor, NCC-149 (HDAC8i), in non-adherent conditions at 4 days. Phase contrast images of embryoid body (EB)-formed cells are shown (scale bar, 500 μm). (B) Graphical representation of EB area ratio. Data are mean ± SE of three sets of independent experiments. (C) *HDAC8* mRNA expression in P19 cells before and after aggregation in the presence or absence 25 μM NCC-149 (HDAC8i). RT-PCR was performed using 5 μg total RNA. *HDAC8* and *GAPDH* expression patterns are shown. (D) NeuN expression pattern in differentiated P19 cells with or without 5 μM NCC-149 (HDAC8i) during suspension culture. Differentiated cells were harvested at 3 days after re-adhesion, with 20 μg protein samples analyzed.