

# Involvement of histone deacetylase 1/2 in adrenocorticotrophic hormone synthesis and proliferation of corticotroph tumor AtT-20 cells

Rie Hagiwara, Kazunori Kageyama\*, Kanako Niioka, Shinobu Takayasu, Mizuki Tasso, Makoto Daimon

Department of Endocrinology and Metabolism, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan

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## ABSTRACT

Cushing's disease is mainly caused by autonomous production of adrenocorticotrophic hormone (ACTH) from pituitary adenomas. In our previous study, a histone deacetylase (HDAC) inhibitor, trichostatin A, inhibited cell proliferation and ACTH production via decreased pituitary tumor-transforming gene 1 (PTTG1) in AtT-20 mouse corticotroph tumor cells. In the present study, we examined the effects of romidepsin, a potent and selective HDAC1/2 inhibitor, on cell proliferation and ACTH synthesis. To elucidate further potential mechanisms of romidepsin, we examined the effects of HDAC1/2 on proopiomelanocortin (*Pomc*) and *Pttg1* mRNA levels and cell proliferation. Small interfering RNA-mediated knockdown was used to decrease HDAC1 or 2. Romidepsin treatment decreased *Pomc* and *Pttg1* mRNA levels, and cell proliferation. The drug also increased *Hdac1* and decreased *Hdac2* mRNA levels. *Hdac1* knockdown decreased basal *Pttg1* mRNA levels and cell proliferation, but not *Pomc* mRNA levels. Romidepsin treatment decreases ACTH synthesis in corticotroph tumor cells. Romidepsin suppresses cell proliferation via PTTG1. HDAC1 is also involved in the proliferation of corticotroph cells via PTTG1.

## 1. Introduction

The hypothalamic-pituitary-adrenal (HPA) axis is activated under stress conditions. Corticotropin-releasing factor (CRF) plays a central role in controlling the HPA axis during stress [19]. CRF in the hypothalamic paraventricular nucleus, stimulates adrenocorticotrophic hormone (ACTH) production from the anterior pituitary [9]. ACTH then stimulates glucocorticoids such as cortisol production from adrenal glands. Cortisol in turn inhibits both CRF and ACTH productions. The presence of histone deacetylase (HDAC) can modulate ACTH and/or glucocorticoid signaling [7,13]. HDAC and/or pituitary tumor-transforming gene 1 (PTTG1) regulate cell proliferation in the pituitary corticotroph.

Cushing's disease is mainly caused by pituitary ACTH-producing adenomas [8,14]. This endocrine disorder is characterized by increased ACTH, leading to excess cortisol production from adrenal glands. This disturbs the normal cortisol feedback mechanism of corticotroph adenoma cells [15].

Tumor growth is generally determined by the balance between cellular proliferation, cell cycle progression, and apoptosis [10]. HDACs

contribute to regulating gene transcription. At least in part, modification of histone acetylation affects tumor growth [3]. Therefore, HDAC inhibitors are able to suppress tumor growth or cell proliferation [16,20].

Numerous HDACs have been identified and are grouped into four different classes [16]. Hydroxamic acid HDAC inhibitor compounds of class I (HDACs 1-3, 8, and 11) and IIb (HDACs 6 and 10) (e.g., trichostatin A and its derivative, suberoylanilide hydroxamic acid) show potent effects on inhibiting tumor growth [21]. Such HDAC inhibitors also suppress ACTH production and cell proliferation in corticotroph tumor cells [12,13]. In our previous study [13], trichostatin A decreased proopiomelanocortin (*Pomc*) mRNA levels in AtT-20 cells and reduced ACTH levels in the culture medium of these cells. Trichostatin A inhibited cell proliferation, and ACTH synthesis and secretion, via decreased PTTG1, a hallmark of pituitary tumor cell proliferation, in AtT-20 cells [13].

The current study used a selective HDAC inhibitor and small interfering (si) RNA-mediated knockdown to identify the role of HDAC1 or 2. Romidepsin is a potent and selective inhibitor of HDAC1/2 among class I compounds [16]. The drug also induces cell cycle arrest, cell differentiation, apoptosis, and altered gene expression in malignancies [11]. In

\* Corresponding author.

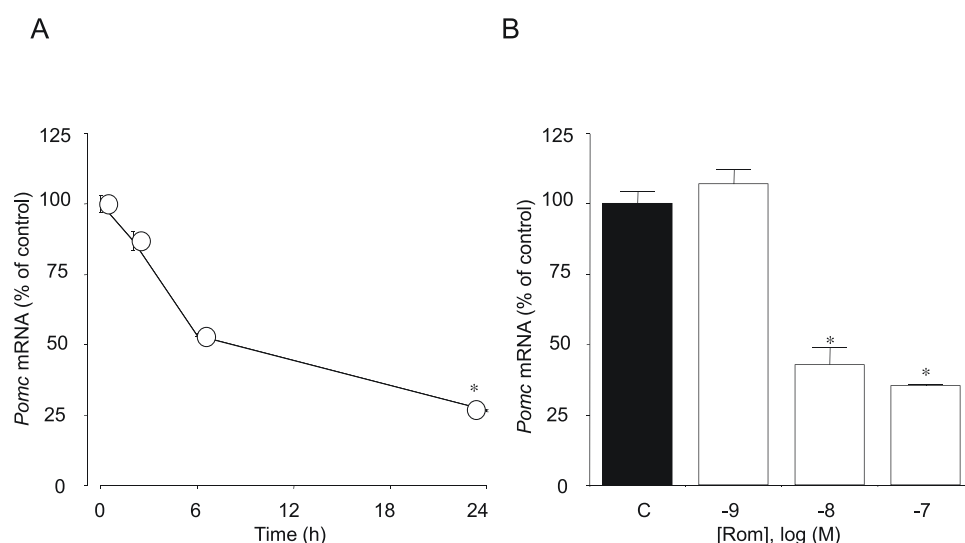
E-mail address: [kkageyama@hkg.odn.ne.jp](mailto:kkageyama@hkg.odn.ne.jp) (K. Kageyama).

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**Fig. 1.** Effects of romidepsin on *Pomc* mRNA in AtT-20 cells. **(A)** Time-dependent effect of romidepsin on *Pomc* mRNA levels. Cells were cultured in medium containing 100 nM romidepsin. **(B)** Concentration-dependent effects of romidepsin on *Pomc* mRNA levels. Cells were cultured for 24 h in medium containing 1 to 100 nM romidepsin. Data are expressed as means  $\pm$  SEM. \* $P < 0.05$  compared with time 0 or control (C). The cells were treated in triplicate, and the average of three independent experiments is shown ( $n = 3$ ).

the present study, we examined the effects of romidepsin on cell proliferation and ACTH synthesis in AtT-20 murine corticotroph tumor cells. To elucidate potential mechanisms of romidepsin, we then examined the roles of HDAC1/2 using siRNA, and its effect on *Pomc* and *Pttg1* mRNA levels, and cell proliferation.

## 2. Materials and methods

### 2.1. Materials

Romidepsin was purchased from ApexBio Technology (Houston, TX, USA).

### 2.2. Cell culture

AtT-20 murine pituitary corticotroph tumor cells were obtained from ATCC (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) containing 10 % fetal bovine serum (FBS), 100  $\mu$ g/mL streptomycin, and 100 U/mL penicillin at 37 °C in a humidified atmosphere (5% CO<sub>2</sub> and 95 % air), as reported previously [1]. Briefly, cells were cultured in 6-well plates at  $1.5 \times 10^5$  cells/cm<sup>2</sup> for 2 days before each experiment. On day 3, to remove the effect of factors contained in FBS, the cells were washed with DMEM supplemented with 0.2 % bovine serum albumin, then cultured overnight in DMEM without FBS prior to each experiment. Total cellular RNA was collected at the conclusion of each experiment and stored at -80 °C until the relevant assay.

### 2.3. RNA extraction

AtT-20 cells were incubated for the indicated times with medium alone (control) or medium containing 100 nM romidepsin. To examine the concentration-dependent effects of romidepsin, cells were incubated with medium alone (control) or medium containing 1–100 nM romidepsin. Total cellular RNA was extracted using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Total RNA (0.5  $\mu$ g) was used as a template for synthesizing cDNA using random hexamers as primers with the SuperScript first-strand synthesis system for the quantitative real-time polymerase chain reaction (qRT-PCR) (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

### 2.4. QRT-PCR

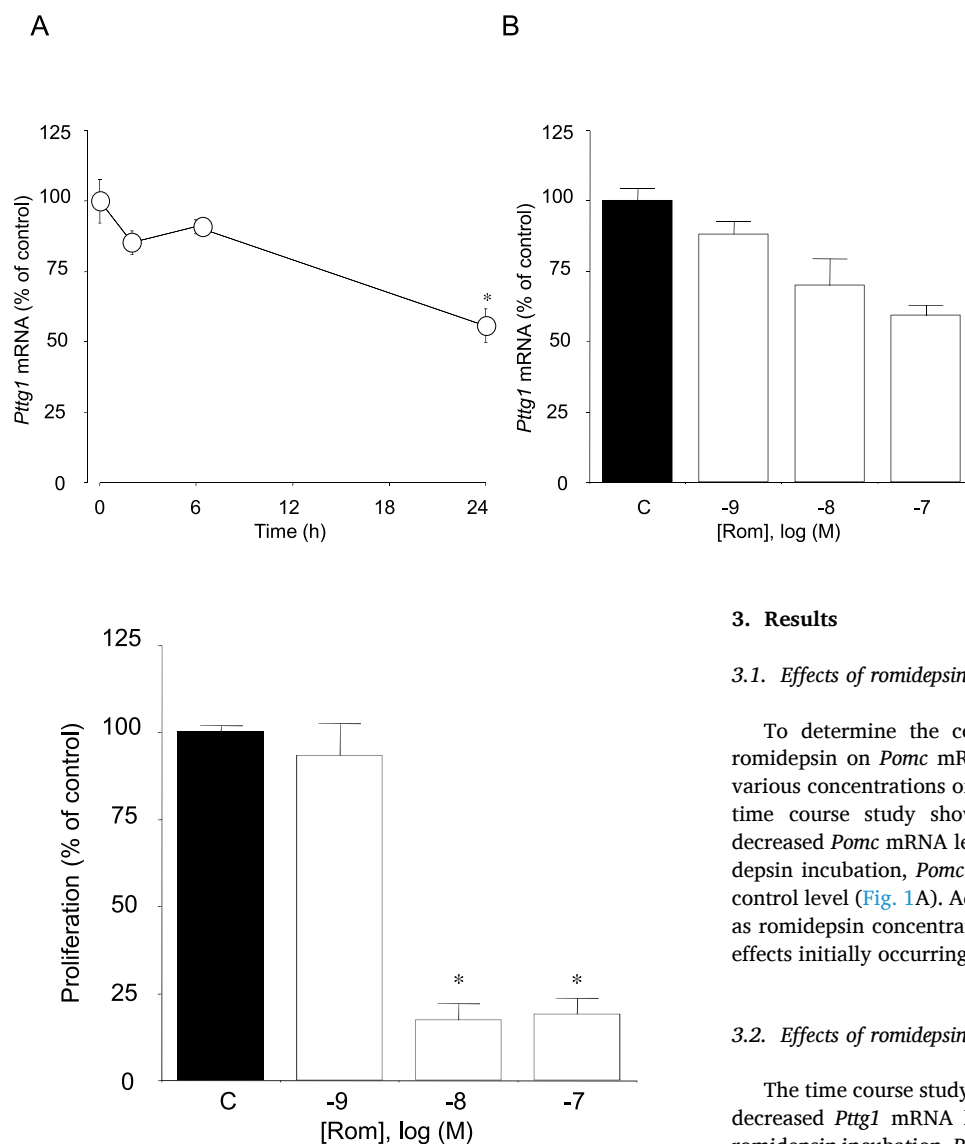
Total cellular RNA was extracted and cDNA was synthesized as described previously [11]. The resulting cDNA was subjected to qRT-PCR as follows. Transcript-specific primer and probe sets (Assays-on-Demand Gene Expression Products; Applied Biosystems, Foster City, CA) were used to perform the qRT-PCR to determine mRNA levels of the following mouse transcripts: *Pomc* (NM\_001278581.1), *Pttg1* (NM\_001131054.1), *Hdac1* (NM\_008228.2), and *Hdac2* (NM\_008229.2). To standardize gene expression levels,  $\beta$ 2-microglobulin (*b2mg*) was used as a reference gene. Across all treated samples, *b2mg* mRNA levels did not differ significantly from those of controls. The 25- $\mu$ L qRT-PCR reactions contained 1 $\times$  TaqMan Universal PCR Master Mix (Applied Biosystems) and 1 $\times$  Assays-on-Demand Gene Expression Products for each of the transcripts (Mm00437762\_m1 for *b2mg*, Mm00435874\_m1 for *Pomc*, Mm00479224\_m1 for *Pttg1*, Mm02391771\_g1 for *Hdac1*, and Mm00515108\_m1 for *Hdac2*), and 500 ng of cDNA. An ABI PRISM 7000 Sequence Detection System (Applied Biosystems) was used for amplification with the following thermal cycling conditions: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

To eliminate the risk of DNA contamination, transcript-specific primer sets as well as a TaqMan probe spanning exon–exon junctions were used in each of the qRT-PCR assays. All expression data are expressed as a function of the threshold cycle ( $C_T$ ) for quantitative analyses using ABI PRISM 7000 SDS (Applied Biosystems). Analyses that used diluted samples of genes of interest (*Pomc*, *Pttg1*, *Hdac1*, and *Hdac2*) and the reference gene (*b2mg*) revealed identical amplification efficiencies.

### 2.5. RNA interference experiments

*Hdac1/2* and control siRNA fragments were designed and purchased from QIAGEN. HiPerFect transfection reagent (QIAGEN) was used to transfect AtT-20 cells with the siRNA fragments according to the manufacturer's protocol.

Target mRNA levels in samples were determined from cells that were seeded in 6-well plates at a density of  $10 \times 10^4$  cells/well. Cultures were incubated for 24 h in 1 mL of culture medium control or experimental siRNAs, and then incubated in bovine serum albumin medium containing romidepsin or control for 24 h. *Hdac1*-specific siRNA (*siHdac1*; Mm\_Hdac1\_2) or *Hdac2*-specific siRNA (*siHdac2*; Mm\_Hdac2\_5) was added and the *Pomc*, *Pttg1*, *Hdac1*, *Hdac2*, and *b2mg* transcript levels were then assayed via qRT-PCR. Proliferation was measured from cells cultured in 200  $\mu$ L of medium containing siRNA fragments in 96-well



**Fig. 3.** Effects of romidepsin on the proliferation of AtT-20 cells. Cells were cultured for 48 h in medium containing 1 to 100 nM romidepsin. Data are expressed as means  $\pm$  SEM. \* $P < 0.05$  compared with time 0 or control (C). The cells were treated in triplicate, and the average of three independent experiments is shown ( $n = 3$ ).

plates ( $1.0 \times 10^4$  cells/well); the medium was changed after 24 h of incubation. Cell viability was measured 48 h post-transfection using Cell Counting Kit-8 (Dojin, Kumamoto, Japan).

## 2.6. Cell proliferation assay

AtT-20 cells were treated with 1–100 nM of 1 romidepsin for 48 h. Viable cells were determined by Cell Counting Kit-8.

## 2.7. Statistical analyses

Each *in vitro* experiment was performed three times. Samples were analyzed in triplicate for each group of experiments. Data are expressed as means  $\pm$  standard error of the mean. An analysis of variance was performed, followed by the shceffe's multiple comparison test.  $P < 0.05$  was considered significant.

**Fig. 2.** Effects of romidepsin on *Pttg1* mRNA in AtT-20 cells. (A) Time-dependent effect of romidepsin on *Pttg1* mRNA levels. Cells were cultured in medium containing 100 nM romidepsin. (B) Concentration-dependent effects of romidepsin on *Pttg1* mRNA levels. Cells were cultured for 24 h in medium containing 1 to 100 nM romidepsin. Data are expressed as means  $\pm$  SEM. \* $P < 0.05$  compared with time 0 or control (C). The cells were treated in triplicate, and the average of three independent experiments is shown ( $n = 3$ ).

## 3. Results

### 3.1. Effects of romidepsin on *Pomc* mRNA

To determine the concentration- and time-dependent effects of romidepsin on *Pomc* mRNA levels, AtT-20 cells were incubated with various concentrations of romidepsin for different periods of time. The time course study showed that 100 nM romidepsin significantly decreased *Pomc* mRNA levels ( $P < 0.05$ ). Within the first 24 h of romidepsin incubation, *Pomc* mRNA levels were decreased to 27 % of the control level (Fig. 1A). Additionally, *Pomc* mRNA levels were decreased as romidepsin concentrations increased ( $P < 0.0001$ ), with significant effects initially occurring at a 10 nM romidepsin (Fig. 1B).

### 3.2. Effects of romidepsin on *Pttg1* mRNA

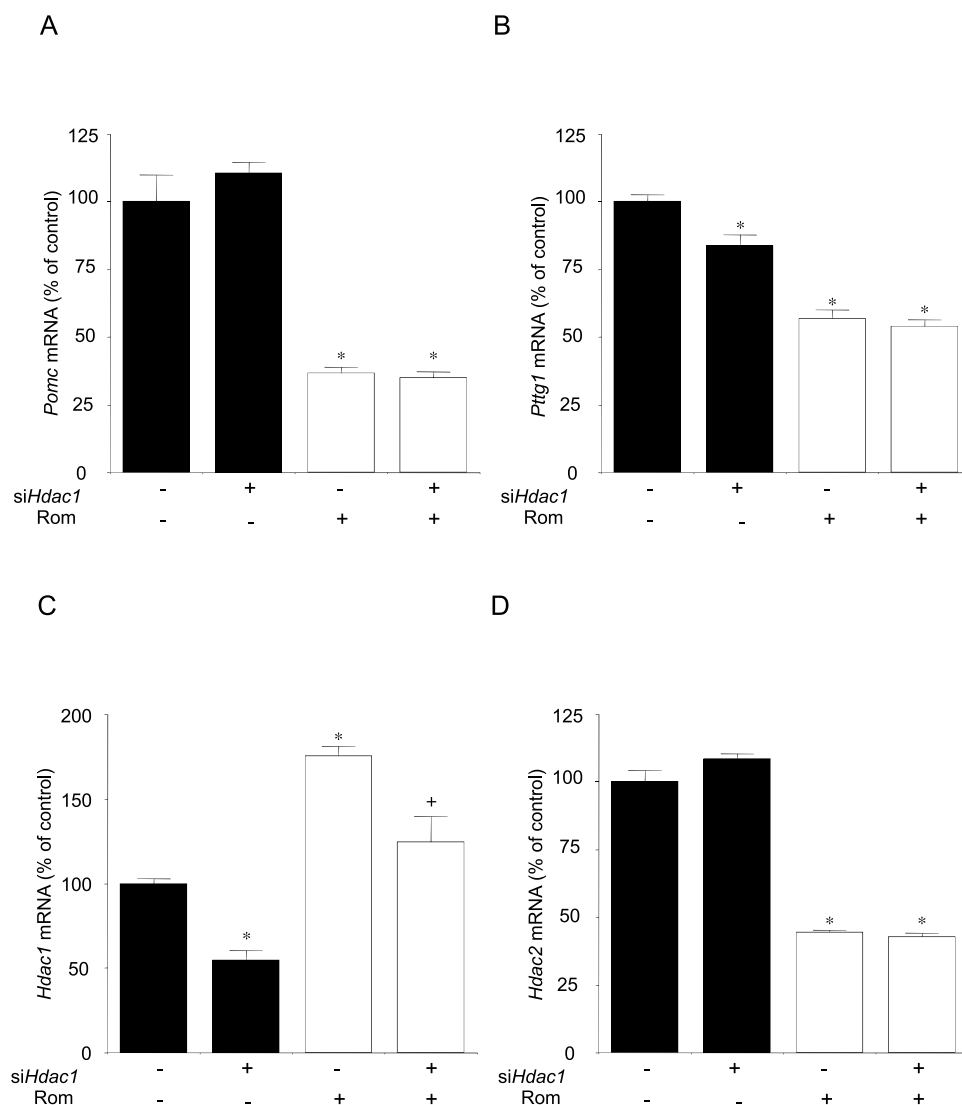
The time course study showed that 100 nM romidepsin significantly decreased *Pttg1* mRNA levels ( $P < 0.005$ ). Within the first 24 h of romidepsin incubation, *Pttg1* mRNA levels were decreased to 69 % of the control level (Fig. 2A). Additionally, *Pttg1* mRNA levels were decreased as romidepsin concentrations increased ( $P < 0.05$ ) (Fig. 2B).

### 3.3. Effects of romidepsin on cell proliferation

The proliferation of AtT-20 cells exhibited a concentration-dependent decrease under romidepsin treatments, with significant ( $P < 0.0001$ ) effects first observed at 10 nM (Fig. 3).

### 3.4. Effects of HDAC1 on *Pomc* and *Pttg1* mRNA levels

*Pomc* mRNA levels were reduced in cells treated with romidepsin, but not in cells transfected with siRNA against *Hdac1* (si*Hdac1*) (Fig. 4A). *Pttg1* mRNA levels were reduced both in cells transfected with si*Hdac1* and in cells treated with romidepsin (Fig. 4B). *Pttg1* mRNA levels were not further changed in cells transfected with si*Hdac1* and treated with romidepsin treatments (Fig. 4B). *Hdac1* mRNA levels were reduced by 55 % in cells transfected with si*Hdac1*, but they were increased (176 %) in cells treated with romidepsin (Fig. 4C). Romidepsin-induced increases in *Hdac1* mRNA levels were partially suppressed in cells transfected with si*Hdac1* (Fig. 4C). *Hdac2* mRNA levels were reduced by 45 % in cells treated with romidepsin, but not in cells transfected with si*Hdac1* (Fig. 4D).



**Fig. 4.** Effects of histone deacetylase (HDAC)1 on *Pomc* and *Pttg1* mRNA levels in AtT-20 cells. Cells were incubated with medium containing control small interfering (si)RNA or *Hdac1*-specific siRNA (*siHdac1*), and then with medium containing 100 nM romidepsin (Rom) or control medium. (A) Effect of HDAC1 on *Pomc* mRNA levels. (B) Effect of HDAC1 on *Pttg1* mRNA levels. (C) Effect of HDAC1 on *Hdac1* mRNA levels. (D) Effect of HDAC1 on *Hdac2* mRNA levels. Data are expressed as means  $\pm$  SEM. \* $P < 0.05$  compared with control siRNA and Rom (-). + $P < 0.05$  compared with either *siHdac1* and Rom (-) or control siRNA and Rom (+). The cells were treated in triplicate, and the average of three independent experiments is shown ( $n = 3$ ).

### 3.5. Effects of HDAC2 on *Pomc* and *Pttg1* mRNA levels

*Pomc* mRNA levels were reduced in cells treated with romidepsin, but not in cells transfected with siRNA against *Hdac2* (*siHdac2*) (Fig. 5A). *Pttg1* mRNA levels were reduced in cells treated with romidepsin, but not in cells transfected with *siHdac2* (Fig. 5B). *Hdac1* mRNA levels were not changed in cells transfected with *siHdac2*, but they were increased in cells treated with romidepsin (Fig. 5C). *Hdac2* mRNA levels were reduced by 26 and 54 % in cells transfected with *siHdac2* or treated with romidepsin, respectively (Fig. 5D). There was no additional effect on *Hdac2* mRNA levels in cells transfected with *siHdac2* and treated with romidepsin (Fig. 5D).

### 3.6. Effects of HDAC1/2 on cell proliferation

Cell proliferation was reduced by 80 % in cells transfected with *siHdac1*, but not *siHdac2* (Fig. 6). Romidepsin also decreased cell proliferation. However, cell proliferation was not further suppressed in cells transfected with *siHdac1* and treated with romidepsin (Fig. 6).

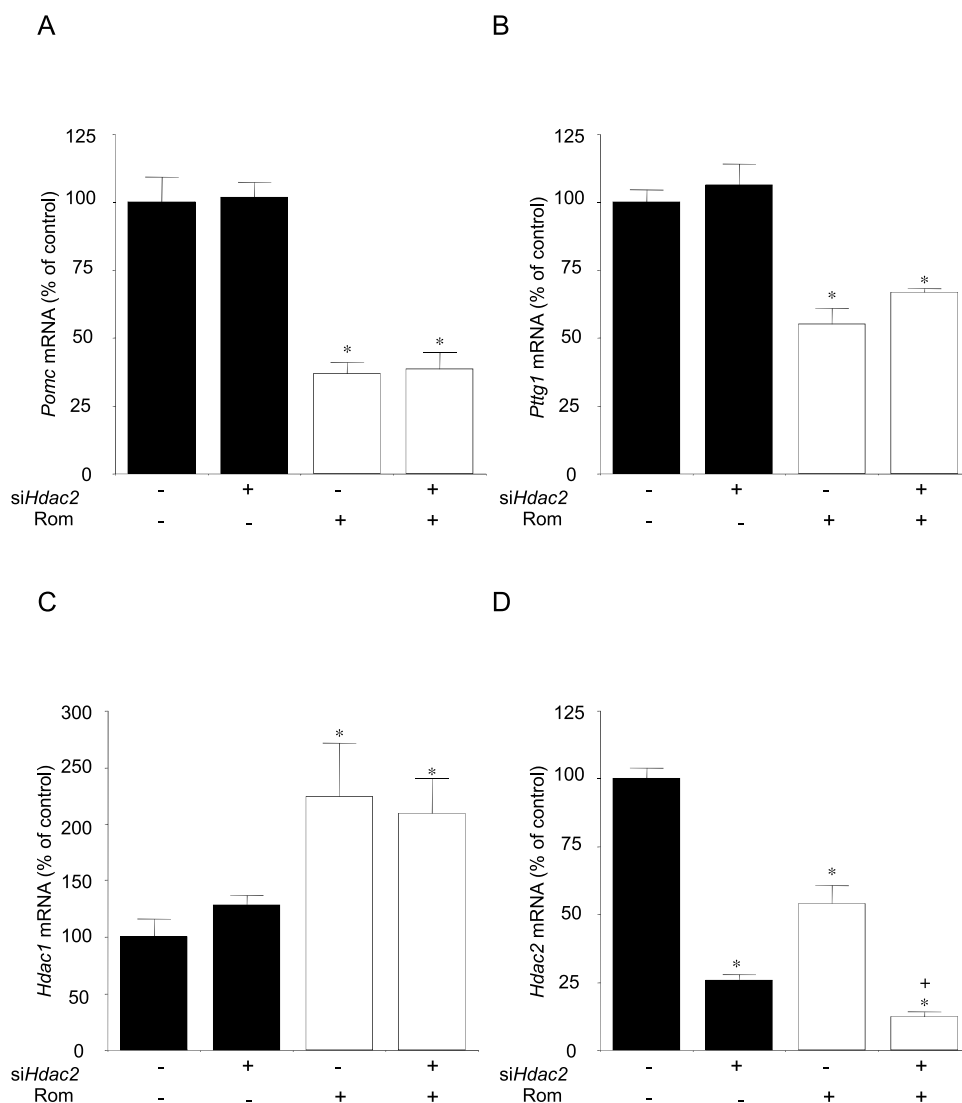
## 4. Discussion

In the present study, we found that treatment with the selective HDAC1/2 inhibitor, romidepsin, decreased *Pomc* mRNA levels in AtT-20

corticotroph tumor cells. Similarly, our previous study showed that a classic HDAC inhibitor, trichostatin A, decreased *Pomc* mRNA levels. These results suggest that the HDAC1/2 is involved in the autonomic synthesis of ACTH in corticotroph tumor cells. Although romidepsin decreased *Pomc* mRNA levels, *Hdac1* knockdown did not. HDACs remove acetyl groups leading to chromatin condensation, and HDAC inhibitors prevent it [16]. The protein levels of HDACs were not determined in this study. The effect of romidepsin, therefore, may be due to its inhibition of the HDAC1/2 activity, at least independent of *Hdac1/2* expression levels (Fig. 7). Additionally, romidepsin increased *Hdac1* mRNA levels. HDAC inhibitors diminish the level of histone acetylation, resulting in activating gene transcription [16,18]. As a molecular consequence of HDAC inhibition, the endogenous expression level of the enzyme could be increased.

Romidepsin is a potent and selective inhibitor of HDAC1/2 [16]. In fact, while 10 nM trichostatin A failed to suppress *Pomc* mRNA levels and cell proliferation in our previous study [13], the present study shows that 10 nM romidepsin significantly decreased them in a similar condition. It is also a systemic drug that has been already approved by the Food and Drug Administration for the treatment of patients with some types of T-cell lymphoma in USA. Therefore, the use of romidepsin is clinically advanced, compared to other HDAC inhibitors.

We also showed that romidepsin decreased *Pttg1* mRNA levels and AtT-20 cell proliferation. HDAC inhibitors repress *Pttg1* mRNA levels in



**Fig. 5.** Effects of histone deacetylase (HDAC)2 on *Pomc* and *Pttg1* mRNA levels in AtT-20 cells. Cells were incubated with medium containing control small interfering (si)RNA or *Hdac2*-specific siRNA (*siHdac2*), and then with medium containing 100 nM romidepsin (Rom) or control medium. (A) Effect of HDAC2 on *Pomc* mRNA levels. (B) Effect of HDAC2 on *Pttg1* mRNA levels. (C) Effect of HDAC2 on *Hdac1* mRNA levels. (D) Effect of HDAC2 on *Hdac2* mRNA levels. Data are expressed as means  $\pm$  SEM. \* $P < 0.05$  compared with control siRNA and Rom (-). + $P < 0.05$  compared with control siRNA and Rom (+). The cells were treated in triplicate, and the average of three independent experiments is shown ( $n = 3$ ).

a manner similar to heat shock protein 90 inhibitors [6]. PTTG1 contributes to prompt cell cycle progression and increased pituitary cell proliferation [4], and the overexpression of PTTG1 results in the proliferation of pituitary gonadotroph cells [5]. Our previous study [13] reported that trichostatin A induces cell death and attenuates cell cycle progression or causes cell cycle arrest in corticotroph tumor cells. In addition, *Pttg1* knockdown inhibits cell proliferation in corticotroph tumor cells [13]. These findings indicate that PTTG1 may be involved in the HDAC inhibitor-induced suppression of cell proliferation in corticotroph tumor cells. Thus, romidepsin would be expected to suppress cell proliferation via PTTG1 in corticotroph tumor cells (Fig. 7). Further, *Hdac1* knockdown decreased basal *Pttg1* mRNA levels and cell proliferation. HDAC1 expression may be responsible, at least in part, for the proliferation of corticotroph cells via PTTG1 (Fig. 7).

Romidepsin increased *Hdac1* and decreased *Hdac2* mRNA levels in AtT-20 corticotroph. *Hdac2* mRNA levels were not further changed in cells transfected with *siHdac2* and treated with romidepsin, and both treatments had no effects on *Pomc* mRNA levels or cell proliferation. HDAC2 mRNA levels have been reported to be decreased in corticotroph tumors [17], but no such data exist for the expression of other HDAC subtypes. While decreases in or loss of *Hdac2* expression may not affect *Pomc* mRNA levels or cell proliferation, a loss of HDAC2 was reported to produce glucocorticoid resistance, developing the tumorigenic process in Cushing's disease [2].

The pan-HDAC inhibitor, suberoylanilide hydroxamic acid, is effective against human corticotroph tumor cells [12]. Although mouse corticotroph tumor cells were used in our study, further research should utilize human corticotroph tumor cells to determine whether these effects occur in humans.

In conclusion, the selective HDAC1/2 inhibitor, romidepsin, decreases ACTH synthesis in murine corticotroph tumor cells. Romidepsin appears to suppress cell proliferation via PTTG1. HDAC1 may be involved in the proliferation of corticotroph cells via PTTG1. Thus, romidepsin may be a potential new therapy for Cushing's disease.

## Funding

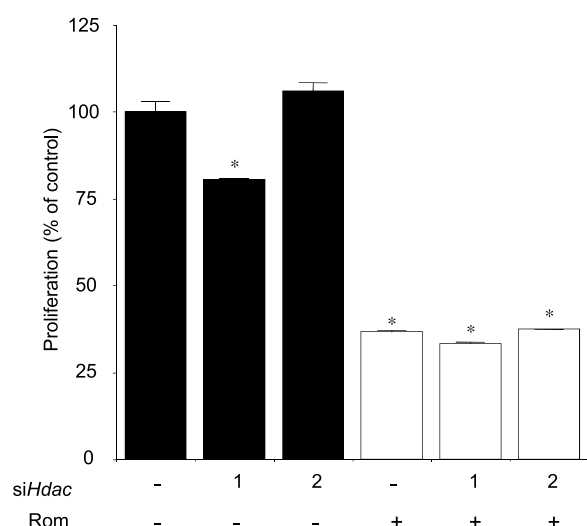
This research was not funded through specific grants from any funding agencies in the public, commercial, or not-for-profit sectors.

## Compliance with ethical standards

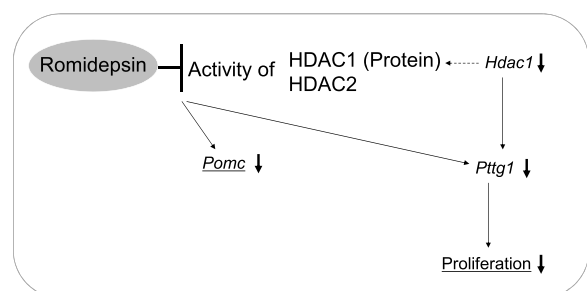
Human participants were not involved in this research.

## Authors' contributions

All authors participated in writing the manuscript and approved the final manuscript.



**Fig. 6.** Effects of histone deacetylase (HDAC)1/2 on the proliferation of AtT-20 cells. Cells were incubated with medium containing control small interfering (si)RNA or *Hdac1/2*-specific siRNA (si*Hdac1/2*), and then with medium containing 100 nM romidepsin (Rom) or control medium. Data are expressed as means  $\pm$  SEM. \* $P < 0.05$  compared with control siRNA and Rom (-). The cells were treated in triplicate, and the average of three independent experiments is shown ( $n = 3$ ).



**Fig. 7.** Proposed mechanisms of romidepsin and involvement of HDAC1/2. Romidepsin decreases *Pomc* mRNA levels in murine corticotroph tumor cells. Romidepsin also inhibits cell proliferation via PTTG1. HDAC1 may be involved in the proliferation of corticotroph cells via PTTG1.

## Declaration of Competing Interest

The authors report no declarations of interest.

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