ORIGINAL

Retinoic acid receptor- α up-regulates proopiomelanocortin gene expression in AtT20 corticotroph cells

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Abstract. Cushing's disease is a disorder caused by excessive ACTH secretion from a corticotroph tumor of the pituitary gland. Although its standard therapy is a transsphenoidal surgery, innovation of novel medical treatments for the disease is urgently necessary. Retinoic acid (RA) has been reported to suppress adrenocorticotrophic hormone (ACTH) secretion in Cushing's disease. However, the role of RA receptor (RAR) in proopiomelanocortin (*Pomc*) gene expression remains uncertain. We here examined the involvement of RAR α in *Pomc* regulation using AtT20 corticotroph cells. Surprisingly, a synthetic RAR α agonist Am80 increased *Pomc* mRNA expression, CRH-induced ACTH secretion, and *Pomc* promoter activity. Small interfering RNA-mediated RAR α -knockdown suppressed both basal and Am80-induced *Pomc* promoter activity. RAR α -overexpression dose-dependently increased *Pomc* promoter activity. *Pomc* promoter mutation analysis revealed that both Tpit and NeuroD1 binding elements were responsible for the Am80-mediated effect. Am80 increased Tpit expression while RAR antagonist LE540 suppressed the increase. Tpit-overexpression increased *Pomc* promoter activity. Mammalian two-hybrid assay revealed that Am80 induced NeuroD1-RAR α interaction. NeuroD1-overexpression enhanced the Am80-induced *Pomc* promoter activity, which was suppressed by NeuroD1 truncated mutant-overexpression. RAR α thus positively regulates ACTH secretion/*Pomc* gene expression through interaction with NeuroD1 and Tpit expression increase. The present observation will be useful for the future development of the RA/retinoid-derived therapeutics of the disease.

Key words: RAR α , *Pomc*, ACTH

RETINOIC ACID (RA), a natural derivative of vitamin A, has numerous bioactivities [1-4]. We have previously reported its stimulating effects on nitric oxide production [2] and angiogenesis [3], suggesting the potential of RA as anti-atherosclerotic therapeutics. Additionally, due to its differentiation-inducing activity, RA exerts tumor-suppressing effects in acute leukemia cells [5] as well as in solid tumors [6-9].

Adrenocorticotrophic hormone (ACTH)-dependent Cushing's syndrome is a life-threatening disorder with many serious symptoms, mainly caused by ACTH-secreting pituitary adenoma (Cushing's disease). The standard therapy for Cushing's disease is a transsphenoidal surgery. However, since recurrence after the operation is not rare, its medical treatment is often required thereafter [10, 11]. Regarding Cushing's disease, although several drugs including bromocriptine, cyproheptadine, valproic acid, octreotide acetate, and rosiglitazone [12, 13] are reported to have therapeutic effect, its standard medical therapy is not yet determined [10, 11].

RA is reported to have anti-tumor effect and decrease ACTH secretion due to proopiomelanocortin (*Pomc*) gene suppression through AP-1, Nurr77, and Nurr1 in

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ACTH-secreting tumor cells [14] and in dogs with Cushing's disease [15]. Recently, beneficial effects of RA on patients with Cushing's disease have also been reported [16]. RA exerts its bioactivities through complicated mechanisms. All-*trans* RA (ATRA) binds to retinoic acid receptor (RAR), while its metabolite 9-*cis* RA (9cRA) binds to both RAR and retinoid X receptor (RXR). So far, it is unclear regarding the involvement of RAR on these RA effects.

In the present study, we therefore examined the role of RAR in *Pomc* gene regulation using pituitary corticotroph AtT20 cells. Surprisingly, we observed that a synthetic RAR α agonist Am80 rather increased *Pomc* mRNA expression, ACTH secretion, and *Pomc* promoter activity, indicating that the previously observed inhibitory effect of RA on ACTH secretion/*Pomc* transcription may be mediated via the mechanisms independent of RAR.

Materials and Methods

Reagents

Am80, HX630, and LE540 were previously described [3, 4]. CD2019 was kindly provided by Dr. Kojima (RIKEN, Saitama, Japan). Human corticotropin-releasing hormone (CRH) was purchased from Sigma (St. Louis, MO, USA).

Plasmids

The subcloned chimeric constructs containing the rat *Pomc* gene genomic DNA and luciferase cDNA (pGL3-Basic, Promega, Madison, WI, USA) were used for the transient transfection studies: rPomc-Luc (harboring the rat *Pomc* gene 5'-flanking region from -703 to +58 relative to the transcription start site upstream of the luciferase cDNA in pGL3-Basic), -359/+58-luc; -293/+58-luc; -169/+58-luc; -12/+58-luc. β -galactosidase control plasmid in pCMV (pCMV- β -gal) was purchased from Clontech (Mountain View, CA, USA). Murine RAR α , truncated NeuroD1 (residues 1-233) [17, 18], and Tpit cDNA were cloned by PCR from AtT20 cell RNA and cloned into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA) (RAR α -pcDNA3, NeuroD1-233-pcDNA3, and Tpit-pcDNA3). Murine full-length NeuroD1 cDNA was cloned into pCMV-HA expression vector (Clontech) (NeuroD1-pCMV-HA). rPomc-Luc was mutated using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA); NeuroD1 binding element in rPomc-Luc from 5'-AGGCAGATGGAC-3' to

5'-AGGttttcaaAC-3' (rPomc-Luc-Ebox_{Neuro}-Mut); Tpit binding element in rPomc-Luc from 5'-TCACACC-3' to 5'-gacCACC-3' (rPomc-Luc-Tpit-Mut). Murine RAR α cDNA was subcloned into pFN26A (Promega) (GAL4-RAR α). Murine NeuroD1 and Tpit cDNA were subcloned into pFN10 (Promega) (VP16-NeuroD1 and VP16-Tpit).

Cell culture

AtT20 cells were grown with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μ g/mL streptomycin. Cells were cultured in a humidified incubator at 37°C with 5% CO₂.

RNA preparation and quantitative RT-PCR

When AtT20 cells were grown to 50% confluence in 6-multiwell plates, they were exposed to Am80 for 2-8 days in DMEM supplemented with 1% resin and charcoal-treated (stripped) FBS. AtT20 cell total RNAs were extracted with TaKaRa FastPure RNA Kit (Takara Bio, Ohtsu, Japan) according to the manufacturer's instructions. Total RNAs were subjected to reverse transcription (RT) reaction using PrimeScript Reverse Transcriptase (Takara Bio) with random 6mer and oligo dT primers according to the manufacturer's instructions. Thereafter, obtained templates were used for quantitative real-time PCR (95 °C, 3 min for 1 cycle; 95 °C, 15 sec; 60 °C, 10 sec; 72 °C, 20 sec for 40 cycles) with iQ SYBR green Supermix (Bio-Rad, Hercules, CA) by DNA Engine thermal cycler attached to Chromo4 detector (Bio-Rad) as previously described [19]. The following primer sequences were used: *Pomc* (forward, 5'-CAGTGCCAGGACCTCACC-3', reverse, 5'-CAGCGAGAGGTTCGAGTTTG-3'), RAR α (forward, 5'-AGACACGCAGACGGGTTG-3', reverse, 5'-GAGGATGCCACTCCCAGA-3'), RAR β (forward, 5'-CACCGGCATACTGCTCAA-3', reverse, 5'-CAAA CGAAGCAGGGCTTG-3'), RAR γ (forward, 5'-CTGA TGAAAATCACCGACCTC-3', reverse, 5'-GGAATCT CCATCTTCAGGGTTA-3'), Tpit (forward, 5'-TCCTG TCTGTACCCACAGC-3', reverse, 5'-GGTCCACAG GCACGGATAGG-3'), NeuroD1 (forward, 5'-ACGCA GAAGGCAAGGTGTCC-3', reverse, 5'-TTGGTCATG TTCCACTTCC-3'), RXR α (forward, 5'-GGCTTCG GGACTGGTAGCC-3', reverse, 5'-GCGGCTTGATAT CCTCAGTG-3'), and 18S rRNA (forward, 5'-CTC AACACGGGAAACCTCAC-3', reverse, 5'-CGCTCCA CCAACTAAGAACG-3').

Measurement of ACTH concentration

When AtT20 cells were grown to 50% confluence in 6-multiwell plates, they were exposed to Am80 for 8 days in DMEM supplemented with 1% stripped FBS. Media and Am80 were freshly changed every 2 days. After stimulation with Am80, media were freshly changed and CRH was then added to the media at the concentration of 10 nmol/L, and the cells were incubated for 3 h. ACTH concentrations of the media were thereafter measured by ACTH (Rat, Mouse) EIA Kit (Phoenix Pharmaceuticals, Burlingame, CA, USA) according to the manufacturer's instructions.

Transient transfection and luciferase assay

When AtT20 cells were grown to 50% confluence in 24-multiwell plates, they were transiently transfected with 200 ng luciferase reporter plasmids and 50 ng pCMV- β -gal using Lipofectamine LTX and Plus reagent (Invitrogen) for 48 h according to the manufacturer's instructions. In some experiments, expression vectors were also transfected. The media were changed to DMEM supplemented with 1% stripped FBS, and the cells were incubated either with or without retinoids for the indicated times. After appropriate treatments, they were washed with PBS, and the cell extracts were prepared using Glo Lysis Buffer (Promega). Luciferase activity was measured using Bright-Glo reagents (Promega), and β -galactosidase activity was simultaneously measured. Data were normalized by β -galactosidase activity.

Small interfering RNA

HiPerformance Validated small interfering RNAs (siRNAs) for RAR α (SI01397067) and negative control siRNA (sense 5'-UUCUCCGAACGUGU CACGUdTdT-3' and antisense 5'-ACGUGACACGU UCGGAGAAdTdT-3') were obtained from Qiagen. When the cells were grown to 50% confluence in 24-multiwell plates, transiently transfected with 20 pmol siRNAs with reporter plasmids using Lipofectamine 2000 reagent (Invitrogen) for 48 h according to the manufacturer's instructions.

Cell counting assay

When AtT20 cells were grown to 50% confluence in 96-multiwell plates, media were changed to 100 μ L/well of DMEM supplemented with 1% stripped FBS and the cells were incubated either with or without Am80 for 3 days. Then 10 μ L/well of Cell Counting Kit-8 solution

(Dojindo, Kumamoto, Japan) were added to the media, and the cells were further incubated for additional 2 days. The optical density at 450 nm (reference 600 nm) was measured using a multiplate reader.

Statistical analysis

All data are presented as mean $\pm$ SEM. Statistical analyses were performed with ANOVA followed by Fisher's LSD post hoc test.

Results

Effects of Am80 on *Pomc* gene expression and CRH-induced ACTH secretion

We first examined the effect of a synthetic RAR α agonist Am80 on *Pomc* gene expression levels in AtT20 cells. Treatment of AtT20 cells with Am80 significantly increased *Pomc* mRNA expression (Fig. 1A). Next, we determined the effect of Am80 on ACTH secretion from AtT20 cells. As shown in Fig. 1B, Am80 significantly augmented the CRH-induced ACTH secretion from AtT20 cells to the media, although it did not affect ACTH secretion in the absence of CRH (data not shown).

Effects of Am80 on *Pomc* promoter activity

We next examined the effect of Am80 on *Pomc* promoter activity. Am80 gradually increased *Pomc* transcriptional activity from 24 to 48 h (Fig. 2A), also in a dose-dependent manner from 10^{-8} mol/L in AtT20 cells (Fig. 2B). Stimulation with CRH strongly increased *Pomc* transcriptional activity as well as Am80, and co-administration with both Am80 and CRH synergistically enhanced it (Fig. 2C). Next, we tested the effect of Am80 on AtT20 cell viability using WST (water soluble tetrazolium salt)-8 to clarify the role of Am80 in cell viability or proliferation. Am80 slightly increased AtT20 cell numbers from 10^{-9} mol/L (Fig. 2D).

Effects of RAR α on *Pomc* gene expression

We next examined the effect of RARs on *Pomc* transcriptional activity. As shown in Fig. 3A, AtT20 cells expressed RAR α and γ isoforms, but rarely β isoform in both unstimulated and Am80-stimulated conditions. Since RAR β and γ were not increased by Am80 in AtT20 cells irrespective of RA responsive element (RARE) in their promoter regions [20, 21], we examined the effects of an RXR agonist HX630 [22] on RAR isoform expression. HX630 increased both RAR β and γ in AtT20 cells (Fig. 3A). We next tested selective RAR agonists on

Fig. 1 Effects of Am80 on *Pomc* gene expression and ACTH secretion from AtT20 cells. (A) Effects of Am80 on *Pomc* mRNA expression in AtT20 cells. AtT20 cells were treated with Am80 (1 μ mol/L, 8 days) or 0.1% ethanol as vehicle control. Data represent mean $\pm$ SEM (n = 3), percent of vehicle group. (B) Effects of Am80 on ACTH secretion into the media from AtT20 cells. AtT20 cells were treated with Am80 (1 μ mol/L, 8 days) or 0.1% ethanol as vehicle control, the media were then freshly changed and CRH was added to the media at 10 nmol/L. The cells were thereafter incubated for 3 h. The secreted ACTH to the media for the last 3 h was determined by ELISA. Data represent mean $\pm$ SEM (n = 6), percent of vehicle group. *** $P < 0.001$, * $P < 0.05$, vs. vehicle group.

Fig. 2 Effects of Am80 on *Pomc* gene transcriptional activity and cell viability of AtT20 cells. (A-C) Time course analysis (A), dose response analysis (B), and CRH co-administration (C) on *Pomc* transcriptional activity. AtT20 cells transiently transfected with rPomc-Luc and pCMV- β -gal were treated with Am80 (A, at 1 μ mol/L for indicated times; B, at indicated concentrations for 48 h; C, at 1 μ mol/L for 48 h) and CRH (C, at 10 nmol/L for the last 4 h). Data represent mean $\pm$ SEM (A, n = 6; B, n = 7 at 1 nmol/L and 1 μ mol/L Am80 and n = 10 at 0 nmol/L, 10 nmol/L, and 100 nmol/L Am80; C, n = 4), percent of control. D, Effects of Am80 on AtT20 cell number. AtT20 cells were treated with at indicated concentrations for 72 h. Data represent mean $\pm$ SEM (n = 8), percent of control. *** $P < 0.001$, vs. control.

Fig. 3 RAR isoform expression and the effect of RAR α on *Pomc* gene expression. (A) Expression levels of RAR isoform mRNA in AtT20 cells. The cells were treated with Am80, HX630 (at 1 μ mol/L for 72 h), or 0.1% ethanol as vehicle control. Data represent mean $\pm$ SEM (n = 3), copies of RARs per 18S rRNA determined by quantitative RT-PCR. † $P < 0.001$, vs. vehicle. ‡ $P < 0.001$, vs. vehicle. (B) Effect of RAR isoform-specific agonists on *Pomc* transcriptional activity. AtT20 cells transiently transfected with rPomc-Luc and pCMV- β -gal were treated with Am80 or CD2019 at 1 μ mol/L for 48 h. Data represent mean $\pm$ SEM (n = 6), percent of control. *** $P < 0.001$, vs. control. (C) Effect of RAR α overexpression on *Pomc* transcriptional activity. AtT20 cells were transiently transfected with rPomc-Luc and pCMV- β -gal, and RAR α -pcDNA3 (adjust volume to 100 ng each with pcDNA3 empty vector). Data represent mean $\pm$ SEM (n = 4), percent of control. *** $P < 0.001$, vs. control. (D) Effect of RAR α knockdown efficacy by siRNA. AtT20 cells were transfected with siRAR α . RAR α mRNA expression levels determined by quantitative RT-PCR corrected with 18S rRNA. Data represent mean $\pm$ SEM (n = 3), percent of control. * $P < 0.05$, vs. control siRNA group. (E) Effect of RAR α knockdown by siRNA on *Pomc* transcriptional activity. AtT20 cells transiently transfected with rPomc-Luc and pCMV- β -gal, and siRAR α were treated with Am80 at 1 μ mol/L for 48 h or 0.1% ethanol as vehicle control. Data represent mean $\pm$ SEM (n = 4), percent of control siRNA without Am80 group. *** $P < 0.001$, vs. without Am80 group. † $P < 0.001$, vs. control siRNA group.

Pomc promoter activity. An RAR α agonist Am80 potently increased *Pomc* promoter activity than RAR β (CD2019) agonist (Fig. 3B). Overexpression of RAR α increased *Pomc* promoter activity in a dose dependent manner (Fig. 3C). We next examined effects of RAR α knockdown by small interfering RNA (siRNA) on *Pomc* promoter activity. The siRNA for RAR α strongly suppressed RAR α mRNA expression levels (Fig. 3D), and decreased both basal and Am80-induced *Pomc* promoter activity (Fig. 3E). These data suggest that RAR α positively regulates *Pomc* gene expression.

Deletion mutant analyses of the Am80-mediated *Pomc* gene transactivation

To explore the mechanism(s) for the Am80-mediated

Pomc gene transactivation, we next examined transcriptional activity of *Pomc* 5'-flanking region deletion mutants. The Am80-mediated *Pomc* transactivation gradually diminished according to the deletion from -703 to -359 and -293 bp relative to the transcription start site (Fig. 4A), suggesting the involvement of multiple elements and transcription factors. Since transcription factors NeuroD1 and Tpit were reported to bind to *Pomc* gene promoter from -703 to -293 bp relative to the transcription start site (NeuroD1 -379/-368 [23, 24], Tpit -316/-309 [25]), we next examined mutant analyses of these binding elements. The Am80-mediated transactivation was suppressed by *Pomc* gene promoter mutation of NeuroD1 binding element (rPomc-Luc-Ebox_{Neuro}-Mut), and Tpit binding element

Fig. 4 Effects of *Pomc* 5'-flanking region mutants on the Am80-mediated *Pomc* transactivation. (A, B) AtT20 cells transfected with luciferase-reporter vectors and pCMV- β -gal were treated with Am80 at 1 μ mol/L for 48 h. Data represent mean $\pm$ SEM (n = 4), percent of basal in pGL3 Basic group. *** $P < 0.001$ vs. vehicle control group in each reporter vectors.

(rPomc-Luc-Tpit-Mut) (Fig. 4B). These data suggest that both NeuroD1 and Tpit are involved in the Am80-mediated *Pomc* gene transactivation.

Roles of transcription factors Tpit and NeuroD1 on the Am80-mediated *Pomc* transactivation

Next, we examined roles of Tpit and NeuroD1 on *Pomc* gene transactivation by Am80. As shown in Fig. 5A, Am80 increased Tpit mRNA expression in AtT20 cells, but did not change NeuroD1 mRNA expression. However, an RXR agonist HX630 did not alter mRNA expression levels of Tpit and NeuroD1 (Fig. 5A). The Am80-induced Tpit mRNA expression was strongly suppressed by an RAR antagonist LE540 (Fig. 5B). Since Tpit was abundantly expressed in AtT20 cells than other transcription factors and nuclear receptors (Fig. 5C), we next tested whether an increase of Tpit expression could enhance *Pomc* gene transcriptional activity. As shown in Fig. 5D, an overexpression of Tpit in AtT20 cells increased *Pomc* gene transcriptional activity in a dose-dependent manner. Since expression level of NeuroD1 was not altered by Am80 (Fig. 5A), we next examined whether Am80 could induce interaction between NeuroD1 and RAR α in AtT20 cells. As shown in Fig. 6A, VP16-NeuroD1 did not inter-

act with GAL4-RAR α in the absence of Am80, but strongly interacted with GAL4-RAR α in the presence of Am80. An overexpression of NeuroD1 enhanced the Am80-mediated *Pomc* gene transactivation (Fig. 6B). Am80 induced *Pomc* gene transcriptional activity in the presence of mock vector (Fig. 6C). Although Am80 increased *Pomc* transcriptional activity in the presence of 100 ng of the truncated mutant of NeuroD1 (NeuroD1-233) expression vector (NeuroD1-233-pcDNA3), the Am80-mediated *Pomc* transcriptional activation was canceled in the presence of 50 and 200 ng of the mutant NeuroD1 expression vector (Fig. 6C). These results indicate that the abundant expression of the truncated mutant of NeuroD1 suppresses *Pomc* transcriptional activity. These data suggest that RAR α elevates *Pomc* gene expression via the increase in Tpit mRNA expression and the interaction with NeuroD1.

Discussion

ATRA has been reported to inhibit ACTH secretion/*Pomc* transcription in AtT20 cells [14], and the effect has partially been suggested to mediate via the induction of bone morphogenetic protein-4 [26]. Additionally, administration of ATRA has recently been reported to

Fig. 5 Roles of Tpit on the Am80-mediated *Pomc* transactivation. (A) Effects of RAR and RXR specific agonists on Tpit and NeuroD1 mRNA expression in AtT20 cells. The cells were treated with Am80, HX630 (at 1 μ mol/L for 48 h), or 0.1% ethanol as vehicle control. Data represent mean $\pm$ SEM (n = 3), percent of vehicle group, corrected with 18S rRNA determined by quantitative RT-PCR. (B) Effects of an RAR antagonist on the Am80-mediated increase in Tpit mRNA expression in AtT20 cells. The cells were treated with Am80 (1 μ mol/L) either with or without LE540 (5 μ mol/L) for 48 h. Data represent mean $\pm$ SEM (n = 3), percent of vehicle group, corrected with 18S rRNA determined by quantitative RT-PCR. (C) Expression levels of transcription factors and retinoid receptors mRNA in AtT20 cells. Data represent mean $\pm$ SEM (n = 3), copies of RARs per 18S rRNA determined by quantitative RT-PCR. (D) Effects of Tpit overexpression on *Pomc* transcriptional activity. AtT20 cells were transiently transfected with rPomc-Luc and pCMV- β -gal, and RAR α -pcDNA3 (adjust volume to 300 ng each with pcDNA3 empty vector). Data represent mean $\pm$ SEM (n = 6), percent of control. *** $P < 0.001$, vs. control.

be therapeutic on patients with Cushing's disease [16]. 9cRA has also been reported to inhibit ACTH secretion in dogs with Cushing's disease [15]. Although ATRA is known to bind to RAR, it can intracellularly be isomerized to 9cRA that can bind both to RAR and RXR [1]. Moreover, ATRA is known to activate protein kinase C- δ independent of RAR [4]. Therefore, it is important to elucidate if RAR is involved in the ATRA-mediated effect. To solve the question, we here examined the effect of Am80, a synthetic RAR α agonist [2-4], on ACTH secretion/*Pomc* transcription in AtT20 cells. Surprisingly, Am80 induced *Pomc* transcriptional activity, *Pomc* mRNA expression, as well as augmentation of CRH-induced ACTH secretion, in contrast to the ATRA effect. Therefore, the previously observed inhibitory effect of ATRA on ACTH secretion/*Pomc* transcription may be mediated via the mechanisms independent of RAR, e.g. RXR. Consistently, we have recently observed the inhibitory effect of

RXR agonist on ACTH secretion/*Pomc* transcription in AtT20 cells [27]. Therefore, RXR agonist, rather than Am80, may possibly be the potential therapeutics for Cushing's disease in the future. RAR-RXR heterodimer binds to direct repeat (DR) 5 element and is activated primarily by RAR agonist, while RXR homodimer binds to DR1 element and is activated by RXR agonist [28]. It is therefore speculated that the target elements of RXR agonist on *Pomc* gene or its upstream genes may possibly be different from those of Am80.

Although the transsphenoidal adenomectomy is the first choice for the treatment of Cushing's disease, its remission rate after the initial surgery is reported to be 69-93%, and 3-22% of patients who achieved remission are reported to recur [29]. The remission rate for recurrent or refractory Cushing's disease is 20-77% by the repeated transsphenoidal surgery and 17-54% by radiotherapy [29]. For the patients who were refractory both to surgery and radiotherapy, effective med-

Fig. 6 Roles of NeuroD1 on the Am80-mediated *Pomc* transactivation. (A) Protein-protein interaction of NeuroD1 and RARα determined by mammalian two-hybrid system. AtT20 cells transiently transfected with GAL4-RARα or GAL4, and VP-16-NeuroD1, VP16-Tpit, or VP16 and pCMV-β-gal were treated with Am80 at 1 μmol/L for 48 h. Data represent mean ± SEM (n = 4), percent of control. *** $P < 0.001$. (B) Effects of NeuroD1 overexpression on *Pomc* transcriptional activity. AtT20 cells transiently transfected with rPomc-Luc and pCMV-β-gal, and NeuroD1-pCMV-HA were treated with Am80 at 1 μmol/L for 48 h. Data represent mean ± SEM (n = 4), percent of control. *** $P < 0.001$ and ** $P < 0.01$. (C) Effects of truncated mutant of NeuroD1 overexpression on *Pomc* transcriptional activity. AtT20 cells were transiently transfected with rPomc-Luc and pCMV-β-gal, and NeuroD1-233-pcDNA3 (adjust volume to 200 ng each with pcDNA3 empty vector). Data represent mean ± SEM (n = 4), percent of control. *** $P < 0.001$ and * $P < 0.05$. n.s., not significant.

ical treatment is necessary [10, 11]. Regarding the medical treatment, inhibitors of steroidogenesis including ketoconazole and metyrapone are widely used. However, due to their side effects, drugs that directly act on pituitary adenoma to inhibit ACTH secretion are more ideal for the long-term treatment [11]. So far, somatostatin analogs including octreotide/lantreotide/pasireotide and dopamine agonists have been utilized, although their effects are limited [11]. Interestingly, therapeutic effects of peroxisome proliferator-activated receptor (PPAR)-γ ligands on Cushing's disease have been focused [30]. However, treatment of Cushing's disease patients with pioglitazone for one month has recently been demonstrated to be ineffective both at serum ACTH/cortisol levels and urinary

free cortisol levels [31]. Moreover, rosiglitazone treatment failed to lower plasma ACTH levels in patients with Nelson's syndrome [32], indicating the limitation of PPAR-γ ligands as the therapeutics for Cushing's disease. Although ATRA has been shown to be effective on patients with Cushing's disease [16], our observation indicates that Am80 cannot be used as a therapeutic for the Cushing's disease. Since the effects of ATRA on serum ACTH/cortisol levels and urinary free cortisol levels significantly varied among patients [16], the difference of intracellular isomerization levels of ATRA to 9cRA among individuals may have induced these variations.

Our data demonstrated that both Tpit and NeuroD1 are involved in the Am80-mediated *Pomc* gene trans-

Fig. 7 Schematic illustration of the RAR-mediated *Pomc* gene transactivation. The numbers indicate nucleotide position of NeuroD1 and T-Pit binding elements.

activation (Fig. 7). Tpit is a positive regulator for late *Pomc* cell differentiation [33] and transactivates *Pomc* gene expression in cooperation with Pitx homeoproteins [25]. Tpit mRNA has been reported to be abundantly expressed in ACTH-secreting human pituitary adenomas causing Cushing's disease [34]. On the other hand, NeuroD1 is important for early *Pomc* cell differentiation [35]. NeuroD1 is also involved in cell-specific transcription of *Pomc* gene [23], and its expression has also been observed in ACTH-secreting pituitary tumors causing Cushing's disease as well as in normal human pituitaries [36, 37]. Further studies are needed to elucidate the mechanisms regarding the RAR α -mediated Tpit mRNA increase and the protein-protein interaction between RAR α and NeuroD1.

In conclusion, we here have demonstrated the stim-

ulative effect of RAR α agonist Am80 on *Pomc* gene expression mediated via NeuroD1 and Tpit. Since the introduction of novel drugs for Cushing's disease is urgently necessary, our observation regarding the ineffectiveness of Am80 for Cushing's disease will be useful information for the future innovation of the RA/retinoid-derived therapeutics of the disease.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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