E2F1-mediated human POMC expression in ectopic Cushing's syndrome

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Abstract

Cushing's syndrome is caused by excessive adrenocorticotropic hormone (ACTH) secretion derived from pituitary corticotroph tumors (Cushing disease) or from non-pituitary tumors (ectopic Cushing's syndrome). Hypercortisolemic features of ectopic Cushing's syndrome are severe, and no definitive treatment for paraneoplastic ACTH excess is available. We aimed to identify subcellular therapeutic targets by elucidating transcriptional regulation of the human ACTH precursor POMC (proopiomelanocortin) and ACTH production in non-pituitary tumor cells and in cell lines derived from patients with ectopic Cushing's syndrome. We show that ectopic *hPOMC* transcription proceeds independently of pituitary-specific Tpit/Pitx1 and demonstrate a novel E2F1-mediated transcriptional mechanism regulating *hPOMC*. We identify an E2F1 cluster binding to the proximal *hPOMC* promoter region (−42 to +68), with DNA-binding activity determined by the phosphorylation at Ser-337. *hPOMC* mRNA expression in cancer cells was upregulated (up to 40-fold) by the co-expression of E2F1 and its heterodimer partner DP1. Direct and indirect inhibitors of E2F1 activity suppressed *hPOMC* gene expression and ACTH by modifying E2F1 DNA-binding activity in ectopic Cushing's cell lines and primary tumor cells, and also suppressed paraneoplastic ACTH and cortisol levels in xenografted mice. E2F1-mediated *hPOMC* transcription is a potential target for suppressing ACTH production in ectopic Cushing's syndrome.

Introduction

ACTH-dependent Cushing's syndrome and consequent hypercortisolemia leads to critical metabolic and cardiovascular complications (Chrousos 2009, Nieman *et al*. 2015, Raff & Carroll 2015). The high ACTH levels that induce adrenal cortisol secretion may derive from pituitary corticotroph adenomas, i.e., Cushing disease, or, less commonly, from non-pituitary tumors, i.e., ectopic or paraneoplastic production (Brown 1928). Ectopic ACTH-secreting tumors can arise from a variety of sites, such as breast, lung (Ilias *et al*. 2005), colon, pancreas, thyroid, ovary and thymus. Features of hypercortisolemia are usually florid and rapidly progressive, with concomitant severe hypokalemia and higher morbidity and mortality than those observed with pituitary-derived Cushing disease. The source of excess ACTH production may be occult, and pose a challenge to identify, localize and treat. As many such tumors are already metastatic at the time of diagnosis, surgical resection is not usually an option,

and failure to control ACTH levels contributes to high morbidity and mortality. Current medical therapies, which are frequently associated with significant side effects and are often poorly tolerated, address the peripheral effects of excess ACTH by blocking adrenal steroidogenesis (Preda *et al.* 2012, Newell-Price 2014) or by antagonizing cortisol actions (Fleseriu *et al.* 2012, 2015), but do not target the ACTH source directly (Colao *et al.* 2012).

Subcellular mechanisms for ectopic Cushing's syndrome are poorly defined. ACTH produced by post-translational cleavage from the proopiomelanocortin (POMC) precursor is abundantly expressed in pituitary corticotroph cells and in the hypothalamus (Stevens & White 2010). The rodent pituitary *Pomc* promoter (*rPomc*), mostly elucidated in AtT20 mouse corticotroph adenoma cells, is regulated by Tpit/Pitx1 (Lamolet *et al.* 2001, Liu *et al.* 2001), NeuroD1 (Poulin *et al.* 1997), nuclear receptors (Maira *et al.* 1999), TR4 (Du *et al.* 2013), STAT3 (Bousquet *et al.* 2000), and cytokines and hormones such as LIF (Bousquet *et al.* 1999) and CRH (Karalis *et al.* 2004). However, regulation of the human *POMC* (*hPOMC*) gene is not well understood. In addition, whether paraneoplastic *hPOMC* in ectopic Cushing's tumors is regulated by the same factors identified in pituitary cells is not known, and pathways controlling POMC/ACTH expression in non-pituitary Cushing's tumor cells are not well defined (Picon *et al.* 1995, Ray *et al.* 1996, Newell-Price 2003). Moreover, investigation of *hPOMC* gene regulation has been hampered due to the lack of a functional human pituitary cell line.

With a view to identifying a molecular target for controlling ACTH production in patients with ectopic Cushing's syndrome, we analyzed *hPOMC* expression using primary tumor cell cultures and human cell lines derived from non-pituitary ACTH-secreting tumors. Here, we show that ectopic *hPOMC* regulation differs from pituitary regulation and report an E2F1-mediated mechanism for *hPOMC* transcription. We also show that E2F1 antagonists markedly suppress *hPOMC* expression and paraneoplastic ACTH secretion in human-derived tumor primary cultures. Our results indicate that E2F1-mediated *hPOMC* signaling is a potential target for suppressing ACTH production in ectopic Cushing's syndrome.

Materials and methods

Cell culture and transfection

After obtaining informed consent, ectopic/pituitary ACTH-secreting surgical specimens were processed using Tissue Dissociate Kits (Miltenyi Biotec, Bergisch, Gladbach, Germany) and cultured in low-glucose DMEM 10% FBS for 48 h. Then, the cell viability was monitored by trypan blue staining and culture medium was collected for RIA. DMS79 cells (human small-cell lung carcinoma) and COLO320 cells (human carcinoid-like colorectal carcinoma) were cultured in RPMI-1640 with L-glutamine and 10% FBS. Human samples are described in Supplement 6, see section on supplementary data given at the end of this article. E2F1 siRNA (107660, Thermo Fisher) transfections were performed using a 4D-Nucelofector (Lonza, Basel, Switzerland). Human ACTH and corticosterone levels were analyzed by RIA (MP Biomedicals, Santa Ana, CA, USA).

DMS79 xenografts

DMS79 cells (1.5×10^6 cells) were injected subcutaneously to 24 five-week-old male Nu/J mice (Jackson Laboratory). Seven days later, mice were treated by oral gavage with R-roscovitine (200 mg/kg, $n = 12$) or vehicle (0.5% methylcellulose, 0.5% Tween 80/PBS; 100 μL, $n = 12$) for 17 days. Animals were then killed, tumors were measured and weighed and blood was collected for ACTH and corticosterone RIA. Pre- and post-treatment ACTH and corticosterone levels are depicted in Supplement 3.

RT-PCR

POMC, PTH and *GH* mRNA levels were analyzed by RT-PCR. Total RNA was isolated using RNeasy mini kit (Qiagen), and cDNA was prepared using a QuantiTect Reverse Transcription Kit with random oligo primers (Applied Biosystems). Quantitative RT-PCR was performed by TaqMan Gene Expression Assays (*hPOMC* probe; Hs01596743_m1, *hGAPDH* probe; Hs02758991_g1, *hPTH* probe; Hs00757710_g1, *hGH* probe; Hs00792200_g1, Taqman Gene Expression Master mix; 439016, Applied Biosystems). *POMC* RNA levels were normalized by *GAPDH*. Each figure depicting RT-PCR results is compiled from at least three independent experiments, and each sample was analyzed in triplicate or quadruplicate.

Immunoblotting

Immunoblotting was performed using anti-ACTH (ab20358; Abcam), anti-E2F1 (sc-251; Santa Cruz Biotechnology), anti-phospho-Serine 364-E2F1 (ab5391; Abcam), anti-phospho-Serine337-E2F1 (ab135549;

Abcam), Lamin A/C (sc-20681; Santa Cruz Biotechnology) and GAPDH (sc-25778; Santa Cruz Biotechnology). Ab binding was detected using Mini PROTEAN electrophoresis protocol (BioRad).

Flow cytometry

After drug treatment, cells were collected and stained with annexin V and 7-AAD using FITC Annexin V detection kit with 7-AAD (Biolegend, San Diego, CA, USA). Cell viability was analyzed by flow cytomtery using a CyAn flow cytometer (Beckman Coulter).

Mapping of *hPOMC* RNA start sites

The human POMC transcription start site was determined by 5′ RACE (Frohman *et al.* 1988) in DMS79 and COLO320 cells, with minor modifications (5). Briefly, cDNA was prepared using an oligo primer (CAGTCAGCTCCCTCTTGAACTCCA) binding to POMC RNA. The G-tail was added to the 5′-end of the resulting cDNA, and 5′-ends were PCR amplified using a poly(C) primer and an antisense POMC primer (ACTCCAGGGGGAAGGCCTCGGCCGA). To concentrate the *hPOMC* cDNA fragments, a second PCR was performed using the antisense POMC primer (TTGCCCTCGCGCGGGCCCGGCT) and the poly(C) primer. Resulting PCR products were cloned and 5′-ends were determined by DNA sequencing. To avoid mismapping due to PCR artifacts, three independent PCR products were analyzed.

Construction of promoters and luciferase assays

The *hPOMC* promoter (−1200 to +83) was PCR amplified from genomic DNA (Jurkat cells) and cloned. Wild type (Wt) and 5′ deletion mutants were amplified using 5′-primers binding to −1140, −428, −303, −102 and −42, (containing *Sal* I site at the 5′-end) and 3′-primer binding to +68 (containing *Hind* III sites at the 3′-end). Resulting amplified fragments were digested with *Sal* I and *Hind* III and cloned into *Xho* I and *Hind* III sites in pGL3 basic vector. Point mutations in the −428/+68 sequence (Fig. 1C) were introduced by PCR assembly, and mutated fragments were integrated into *Xho* I and *Hind* III sites in pGL3 basic vector. DNA sequences of all the inserted fragments were determined to remove defective fragments generated by PCR errors. For minimal promoters, −42 to +68 probes were divided into seven small fragments (Fig. 2B). Sense and antisense oligonucleotides encoding these

sequences were synthesized, duplicated and ligated upstream of the minimal promoter in pGL3 as described (Ogawa *et al.* 2014). Luciferase reporter plasmids containing five direct-repeat inserts were selected by DNA sequencing (Fig. 2B). DNA sequences of inserted fragments were determined to remove the defective fragments generated by PCR errors.

Luciferase reporter assays were performed in 2.5×10^5 DMS79 cells with 0.7 µg luciferase reporter plasmids, and 50 ng pRL-CMV used as an internal control plasmid. Cells were transfected using lipofectamine 2000 (Invitrogen) and cultured in 0.5 mL medium in 24-well plates. Forty-eight hours after transfection, cells were harvested and luciferase activity analyzed by Dual-Luciferase Reporter Assay System (Promega). Luciferase assays were repeated more than three times, and luciferase activities normalized by internal renilla activity. For co-transfection experiments, 0.5 µg luciferase reporter plasmids were co-transfected with 1.5 µg E2F1, E2F3 and/or DP1 expression plasmids constructed using pMF vector carrying the EF-1α promoter. Total DNA (2.0 µg) was adjusted with the empty vector.

EMSA

Nuclear extracts were prepared as described (Tone *et al.* 2002). 5 µg nuclear extracts were incubated in 14 µL pre-incubation buffer (5 mM Tris (pH 8.0) and 14% glycerol) for 20 min; 0.5 µg poly (dI-dC), 1.2 µL of 5× EMSA standard buffer (Tris–HCl 4 mM, 12 mM HEPES (pH 7.9), 60 mM KCl, 0.2 mM EDTA, 2 mM DTT and 12% glycerol) and ^{32}P-labeled probes were added for a further 15 min. For competition assays, 100-fold excess unlabeled competitor primer was added to EMSA reaction mixtures. For super-shift assays, nuclear extracts in EMSA reaction buffer were incubated for 15 min with anti-E2F1 (sc-251X; Santa Cruz Biotechnology), anti-E2F2 (sc-633X; Santa Cruz Biotechnology), anti-E2F3 (sc-878X; Santa Cruz Biotechnology), anti-E2F4 (sc-866X; Santa Cruz Biotechnology), anti-Sp1 (07-645; Millipore) or anti-Sp3 (sc-644X; Santa Cruz Biotechnology), and then the probes were added. DNA–protein complexes were analyzed by 4% DNA retardation gel. Probe and competitor sequences (sense strand) used were # 1: CCACCAGGAGAGCTCGGCAAGTATATAAGG, # 2: GTAT ATAAGGACAGAGGAGCGCGGGACCAA, # 3: GACCAA GCGGCGGCGAAGGAGGGGAAG, # 4: AA GGAGGGGAA GAAGAGCCGCGACCGAG, #5: AAGAGCCGCGACCGA GAGAGGCCGCCGAGC, # 6: AGAGGCCGCCGAGCGTC

CCCGCCCTCA and # 7: GTCCCCGCCCTCAGAG
AGCAGCCTCCC.

EMSA was also performed using CIP-treated nuclear extract from DMS79 cells as described (Tone *et al.* 2002). Briefly, 5 µg nuclear extract were treated with 0.6 units CIP (New England BioLabs, Ipswich, MA, USA) in EMSA buffer at room temperature for 1 h, and the reaction was terminated by adding phosphatase inhibitor cocktail (Sigma-Aldrich). To prepare control nuclear extracts, phosphatase inhibitor cocktail was added before the addition of CIP and incubated for 1 h.

Chromatin immunoprecipitation assay

ChIP assay was performed using DMS79 and COLO320 cells as described (Tone *et al.* 2008). Cells were fixed in 1% formaldehyde, 4.5 mM HEPES pH 8.0, 9 mM NaCl, 0.09 mM EDTA and 0.045 mM EGTA for 10 min at room temperature and sonicated (Bioruptor, Liege, Belgium) in lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris–HCl pH 8.0) with proteinase inhibitor (Sigma-Aldrich P8340). Pre-cleared lysates were incubated overnight at 4°C with

anti-E2F1 (sc-251X; Santa Cruz Biotechnology), anti-acetyl histone H4 (06-866; Millipore) and normal rabbit IgG (sc-2027; Santa Cruz Biotechnology) polyclonal antibody. DNA fragments were isolated from immunoprecipitated chromatin and analyzed by RT-PCR analysis with SYBR Green PCR Master Mix (Applied Biosystems). PCR primers used were hPOMC 5′-CCCCTCCTACCCTTGCTGTA, 5′-GGGCACAGCTCCCATAATCA, 5′-CAAAGAGGGCTTC AGAGAACA, 5′-CACTTCAGCATCCACAACATTC, 5′-GAG AGCTCGGCAAGTATAAAG and 5′-TCGGCGCAGAAA GTTTG.

DNA pull-down assay

DNA pull-down assay was performed as described (Ogawa *et al.* 2014). A 74 bp DNA fragment containing the E2F1-binding site (−24/+50) (Fig. 2B) was amplified by PCR using biotinylated primers. 30 µL nuclear extract (100 µg) were added to 180 µL of 5 mM Tris (pH 8.0)–14% glycerol buffer and pre-incubated on ice. 15 µL poly(dI-dC)(dI-dC) (7.5 µg) and 18 µL of 5× binding buffer (300 mM KCl, 60 mM HEPES (pH 7.9), 20 mM Tris–HCl (pH 8), 0.5 mM
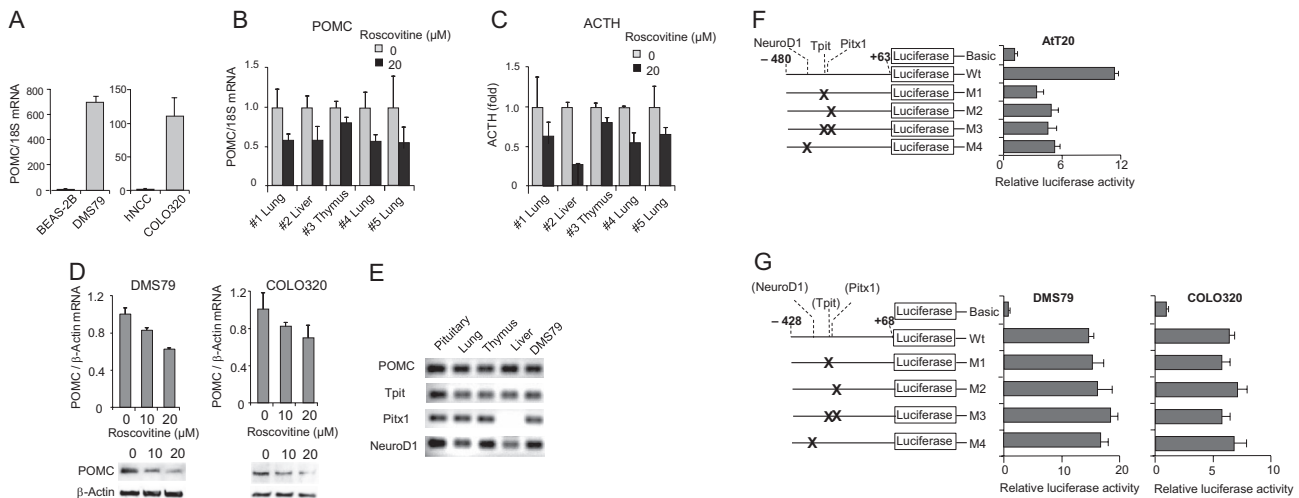

Figure 1

hPOMC promoter activity in ectopic Cushing's cell lines. (A) POMC expression in lung (DMS79) and colon (COLO320) cancer cell lines were analyzed by RT-PCR and compared with those in normal lung (BEAS-2B) and normal colon (hNCC) cells. Expression levels were normalized with 18S rRNA. (B, C) Primary cultures of cells from five human ectopic ACTH producing tumors (#1, #4, #5 lung; #2 liver; #3 thymus) treated with or without R-roscovitine for 48 h. *POMC* mRNA were measured by RT-PCR (B) and medium ACTH concentrations in primary cultures were measured by RIA (normalized for viable cell numbers; $n = 5$ tumors, mean $\pm$ s.e.) (C). (D) POMC mRNA levels were analyzed by RT-PCR using RNA derived from DMS79 and COLO320 cells treated with or without indicated doses of R-roscovitine. POMC protein levels in derived whole cell extracts were also analyzed by immunoblotting with anti-POMC. (E) POMC, Tpit, Pitx1, and NeuroD1 expression in pituitary Cushing's tumor, ectopic Cushing's tumors (lung, thymus, and liver), and DMS79 cells were analyzed by RT-PCR. (F) Structure of point mutated luciferase reporter plasmids of the *rPomc* promoter (−480/+63) (left). Luciferase assays performed using point mutations in AtT20 cells (right) were compared with those generated using negative control plasmid basic (no promoter). (G) Structure of point mutated luciferase reporter plasmids of the *hPOMC* promoter (−428/+68) (left). Luciferase assays performed using point mutants in DMS79 cells and COLO320 cells (right) were compared with those generated using negative control plasmid basic (no promoter). Luciferase (F, G) and RT-PCR (A, B, D) results are representative of four independent experiments, RIA was performed in duplicate tubes; values are mean $\pm$ s.e. (C), and immunoblotting results are representative of three independent experiments (D).

EDTA, 25% glycerol and 5 mM DTT) and 6 µL probe (1 µg) were incubated at room temperature for 15 min. DNA–protein complexes were collected with 10 µL Dynabeads M-280 Streptavidin magnetic beads (Life Technologies) and analyzed by immunoblotting.

Statistics

Differences between vehicle and R-roscovitine groups were analyzed using ANOVA followed by nonparametric t test (Mann–Whitney) or Student t test. Probability of $P < 0.05$ was considered significant. In bronchial carcinoid tumors, the correlation of $POMC$ and $E2F1$ expression were assessed by Spearman's correlation.

Study approval

Protocols were approved by the Cedars-Sinai Institutional Review Board (protocol #30753). Male and female patients aged more than 18 years with a clinical diagnosis of ectopic or pituitary ACTH-producing tumors and lung carcinoid tumors were screened, and informed consent was obtained. Human samples (age, gender and diagnosis) are described in Supplement 6. Animal protocol (protocol #2603) was approved by the Cedars-Sinai Institutional Animal Care and Use Committee.

Results

hPOMC promoter activity in non-pituitary cells

We used transfection-based luciferase assays to analyze $hPOMC$ promoter activity and investigate transcriptional regulation in non-pituitary human cells. As primary tumor cells are not suitable for these experiments, we used human small-cell lung cancer DMS79 cells that are widely used in studies of ectopic Cushing's syndrome (Pettengill *et al.* 1980, White *et al.* 1989, Picon *et al.* 1999, Newell-Price *et al.* 2006). We also found that ACTH-positive human colon carcinoid-like cancer cells COLO320 (Quinn *et al.* 1979) express $POMC$ mRNA. Therefore, we analyzed the expression of $hPOMC$ mRNA in these two
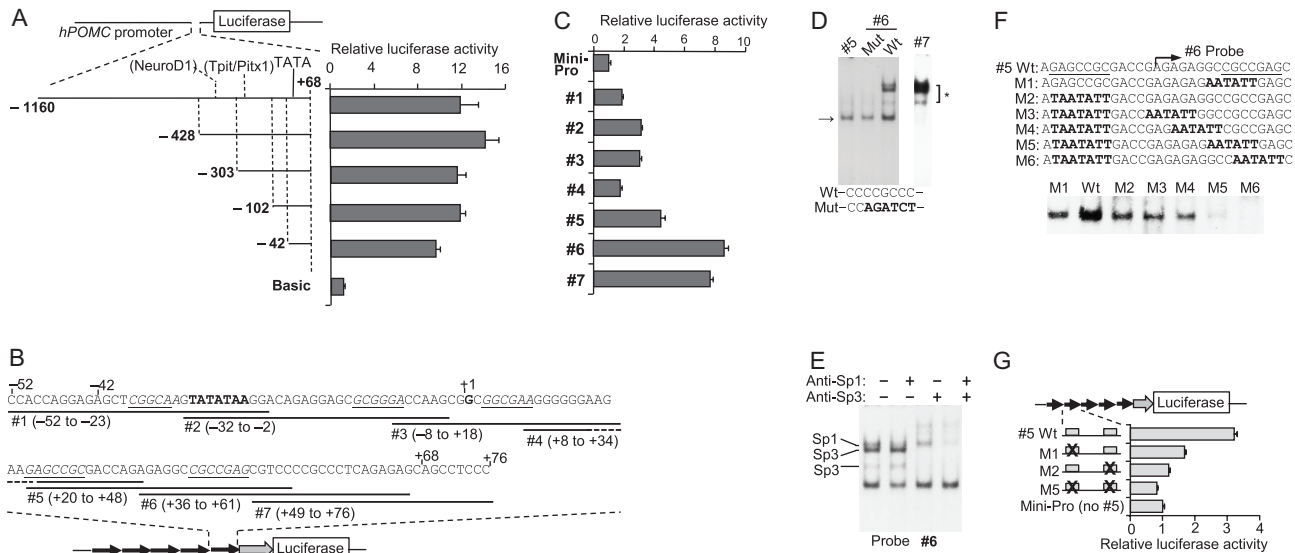

Figure 2

Nuclear factor binding to the proximal $hPOMC$ promoter (−42 to +68). (A) Structures of luciferase reporter plasmids using human $POMC$ promoter 5′-deletion mutants. Positions of 5′-ends are indicated (all deletion mutants contain the same 3′-end, +68). Luciferase assays using 5′ deletion mutants in DMS79 cells (right), and activities compared with negative control plasmid basic (no promoter). (B) $hPOMC$ DNA sequence (−52/+76). The transcription start site is defined as position +1. Minimal promoter plasmids contain five direct repeat copies of the indicated fragments (#1 to #7) and oligonucleotide orientation is indicated by an arrow (direct repeat). (C) Luciferase assays in DMS79 cells using minimal promoter plasmids shown in B, compared with mini-Pro (no inserted fragment). (D) EMSA performed using probes #5, #6, mutant probe #6, and #7 in DMS79 cells. The mutated sequence in probe #6 is shown in bold. The three upper bands are indicated by * (the top thick band consists of two bands) and the common band from probes #5 and #6 is indicated by the arrow. (E) Super-shift EMSA was performed using probe #6 with nuclear extracts from DMS79 cells, and anti-Sp1, and anti-Sp3. Positions of Sp1 and two different Sp3 sizes are shown. (F) EMSA was performed with Wt and M1-6 probes with nuclear extracts from DMS79 cells. Mutant sequences are in bold; bands are shown under the probe sequences. (G) Luciferase assays were performed in DMS79 cells using the indicated reporter plasmids. Inserted fragments (five direct repeat copies) are shown, and mutated sequences (shown in F) are indicated by ×. Luciferase activities were compared with those without inserted fragment. EMSA results are representative of three independent experiments (D, E, F) and luciferase results are representative of four independent experiments, each depicted as mean ± s.e. of triplicate samples (A, C, G).

cell lines by RT-PCR. Compared with normal lung BEAS-2B cells (Lechner *et al*. 1983) and normal colon hNCC cells (Chesnokova *et al*. 2013), DMS79 and COLO320 cells expressed hPOMC mRNA at high and intermediate levels, respectively (Fig. 1A).

Previously, we showed that *POMC* gene expression in pituitary cells is suppressed by the cyclin-dependent kinase/cyclin E inhibitor R-roscovitine (Liu *et al*. 2011, 2015) We therefore analyzed *hPOMC* gene expression in human tumor cells derived from five patients with ectopic Cushing's syndrome and in DMS79 and COLO320 cells. Similar to what we found in pituitary cells, hPOMC mRNA expression was suppressed with R-roscovitine treatment (Fig. 1B, C, D), suggesting that *hPOMC* gene expression is, at least in part, regulated by mechanisms similar to those in human ectopic primary cells. We therefore used these cell lines for subsequent *hPOMC* gene analysis.

We next considered whether the transcription factors Tpit, Pitx1, and NeuroD1 regulating POMC expression in pituitary corticotrophs are similarly involved in ectopic Cushing's syndrome. We found the mRNA expression of all three transcription factors in patient-derived ectopic Cushing's tumors arising from the lung, thymus and liver as well as in DMS79 cells (Fig. 1E), although Pitx1 mRNA was undetectable in the liver tumor cells. We therefore analyzed whether these transcription factors regulate

hPOMC promoter activity in non-pituitary cells, as they do in pituitary cells.

To map the *hPOMC* promoter region, we determined the major transcription start site in non-pituitary tumor cells by 5′-RACE (rapid amplifications of cDNA ends) (Frohman *et al*. 1988). This start site, which we defined as position +1, is 4-bp downstream from the site previously defined by primer extension (Takahashi *et al*. 1983) (Fig. 2A). A TATA-like sequence was identified 32-bp upstream of the start site, similar to the previously identified rat promoter (Drouin *et al*. 1985). We then analyzed *hPOMC* promoter activity in non-pituitary tumor cells by performing luciferase reporter assays with point mutations within the Tpit/Pitx1 and NeuroD1 sites (Fig. 1F). These sequences identified in the *rPomc* promoter are conserved in the *hPOMC* promoter (Supplement 1). When similar point mutations were introduced in the *rPomc* promoter in AtT20 pituitary cells, promoter activity was attenuated (Fig. 1F), but mutated Tpit/Pitx1 and NeuroD1 sites did not reduce *hPOMC* promoter activity in ectopic cell lines (Fig. 1G). Next, we performed *hPOMC* promoter analysis using a series of 5′-deletion mutants. Previously, the *hPOMC* promoter was analyzed up to –428bp (Picon *et al*. 1995); we now extended the analysis to –1160/+68 (Fig. 2A). Similar promoter activity was observed with all deletion mutants. Seventy percent of maximum promoter
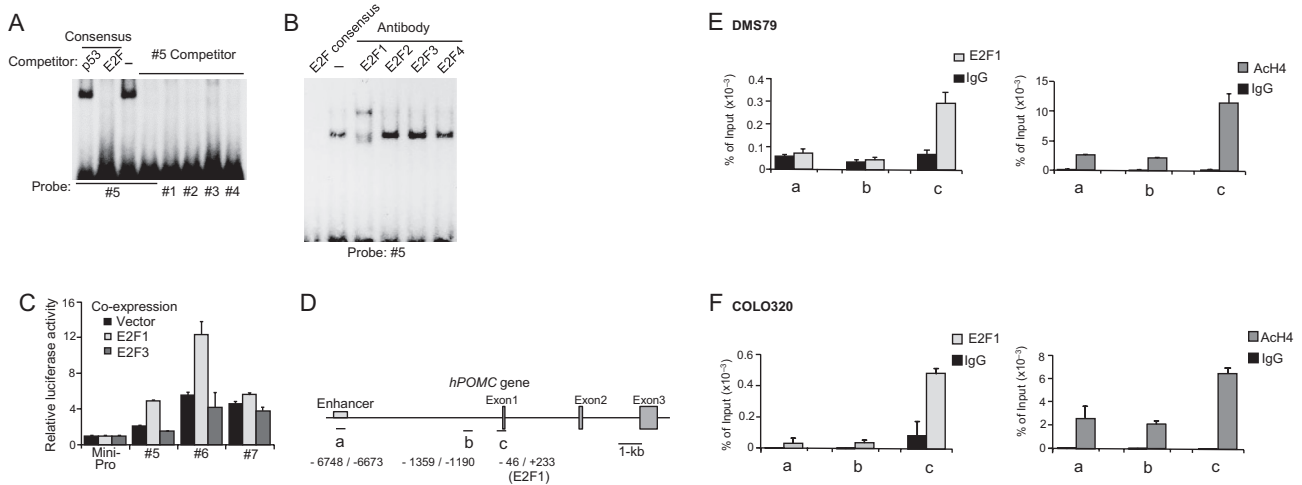

Figure 3

E2F1 binding to the *hPOMC* promoter. (A) Results of EMSA performed with indicated probes and consensus competitors. No competitor is indicated by –. (B) EMSA and supershift-EMSA were performed with nuclear extracts prepared from DMS79 cells using probe #5, E2F consensus competitor, and indicated antibodies to E2F molecules. (C) Results of luciferase assays performed using the indicated reporter plasmids constructed with *hPOMC* promoter fragments (#5, #6, and #7) and the minimal promoter shown in Fig. 2B. Reporter plasmids were co-transfected with E2F1 or E2F3 expression plasmids or negative control empty vector (Vector). (D, E, F) ChIP assays were performed with anti-E2F1 (E2F1), anti-acetyl histone H4 (AcH4), or negative control (IgG) using primer sets shown in D (a, b, c). The analyzed ChIP regions are from DMS79 (E) and COLO320 cells (F). EMSA and ChIP results are each representative of triplicate independent experiments (A, B, E, F) and luciferase results are representative of four independent experiments, each depicted as mean±s.e. of triplicate samples (C).

activity was maintained in the shortest –42/+68 promoter, suggesting this is a key proximal regulatory region.

Taken together, these results suggest that *hPOMC* promoter activity is not controlled by the same pituitary-specific factors that regulate promoter activity in Cushing's disease. Rather, it is mainly controlled by a proximal regulatory region located between –42/+68 bp in non-pituitary cells.

As this short proximal promoter comprises only a 42-bp sequence upstream of the transcription start site, including the TATA (–31 to –25 bp), we could not generate further 5′-deletion mutants. To identify the binding of transcription factors, we constructed a luciferase reporter system with a minimal promoter containing the TATA sequence and transcription start site (Tone *et al*. 2008) (Fig. 2B). This method was used previously to identify other transcription factors (Tone *et al*. 2000, 2008, Ogawa *et al*. 2014). The putative regulatory region (–52 (–42 plus additional 10-bp) to +76 (+68 plus additional 8-bp)) was systematically divided into seven fragments with partially overlapping sequences (Fig. 2B). These short (24–30 bp) DNA fragments (#1 to #7) were integrated upstream of the minimal promoter,

and five sequence copies were added as direct repeats (Fig. 2B). When the resulting luciferase reporter plasmids were transfected into DMS79 cells (Fig. 2C), moderate-to-strong luciferase activity was detected with fragments #5 to #7, and mild increments were detected with fragments #1 to #4. We then performed electrophoretic mobility shift assays (EMSA) with fragments #5 to #7 (Fig. 2D). Several bands were detected; the lower bands were consistently observed with #5 and #6 fragments, and the three upper bands (the top thick band consists of two bands) were detected in #6 and #7 fragments. Overlapping regions within #6 and #7 include a CCCCGCCC sequence similar to the Sp1 consensus, and mobility patterns resemble those identified for Sp1/Sp3 binding (Tone *et al*. 2002). Mutating this sequence resulted in dissipation of the top three bands (Fig. 2D). Sp1/Sp3 binding was also confirmed by super-shift EMSA using anti-Sp1 and anti-Sp3 (Fig. 2E). The lower single band detected with #5 and #6 regions (Fig. 2D, E, arrow) was also seen with the #6 Sp1 mutant probe. We therefore reasoned that a common factor, different from SP1/Sp3, binds this overlapping #5 and #6 sequence region (AGAGAGGCCGCCGAG).
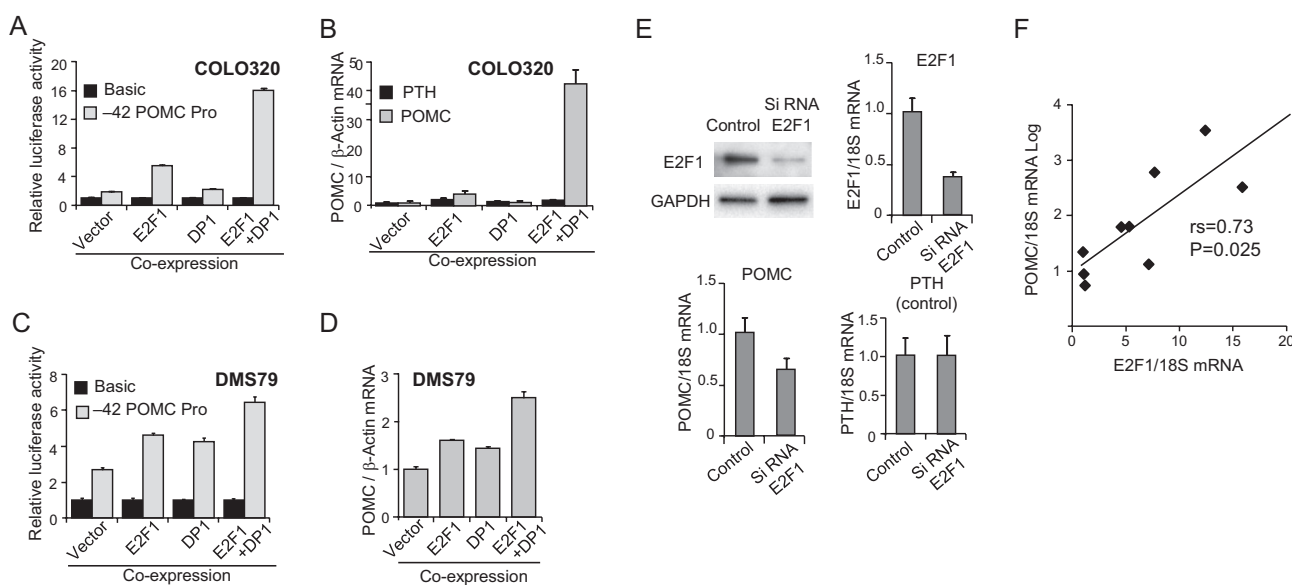

Figure 4

E2F1-mediated *hPOMC* transcription in ectopic Cushing's tumor cells. (A) Results of luciferase assays performed in COLO320 cells using the –42 *hPOMC* promoter (–42/+68) reporter plasmid (Fig. 2A) or negative control basic (no promoter). E2F1 and/or DP1 expression plasmids were co-transfected, and total DNA abundance adjusted with empty vector. (B) *POMC* and *PTH* mRNA levels were analyzed by RT-PCR using RNA derived from COLO320 cells transfected with E2F1 and/or DP1 expression plasmids. (C) Luciferase assays were performed using the –42 *hPOMC* promoter in DMS79 cells as described in A. (D) *POMC* mRNA levels were analyzed in DMS79 cells as described in B. (E) Immunoblotting of protein extracts derived from DMS79 cells transfected with control siRNA (control) or E2F1 siRNA. E2F1, *POMC*, and *PTH* expression measured by RT-PCR of cDNA derived from DMS79 cells transfected with control siRNA (control) or E2F1 siRNA. Expression levels were normalized with 18S rRNA. (F) *POMC* and *E2F1* mRNA levels were analyzed by RT-PCR using human lung carcinoid tumors. Expression levels were normalized with 18S rRNA. Immunoblotting, RT-PCR, and luciferase results are representative of three independent experiments (A, B, C, D, E, F), each depicted as mean ± s.e. of triplicate samples (A, B, C, D, E).

hPOMC expression is regulated by E2F1-mediated transcriptional mechanisms

We introduced a mutation (GCCGCC to AATATT) in the middle of the overlapping #5 and #6 sequence, and, using EMSA, showed partially attenuated binding (about 50%) (Fig. 2F), suggesting a nuclear factor binding to GCCGCC and also indicating the likely presence of another binding site for this probe. Indeed, we observed a similar GCCGC sequence near the 5′-end of the probe (Fig. 2F), with binding reduced to half by mutating GAGCCGC to TAATATT. Using EMSA with additional mutant probes (Fig. 2F), we observed that binding was abolished with M5 and M6 probes, suggesting the presence of two core binding motifs: CGCCGAG in the 3′-site and GAGCCGC (mirror image to the 3′-site core sequence) in the 5′ region of the probe (Fig. 2F). Functions of these two core binding sequences were further confirmed by luciferase reporter assays, which showed that mutating these sequences led to reduced promoter activity of probe #5 (Fig. 2G).

Although no potential transcription factor binding to the identified sequences (GAGCCGC and CGCCGAG) was suggested using databases, we noted that these sequences were similar to the 3′-half of the E2F transcription family consensus motif, i.e., AGTGCCGC (Tao et al. 1997). Using EMSA competition assays with a labeled #5 probe and an unlabeled E2F consensus sequence as competitor, transcription factor binding was abolished by both the #5 probe as well as by E2F consensus competitors, but not by unrelated p53 consensus competitors (Fig. 3A left), suggesting that E2F member(s) bind the #5 probe. Using super-shift EMSA assays with antibodies to E2Fs (E2F1 to E2F4), we detected a super-shifted band with anti-E2F1 (Fig. 3B), but not with other antibodies. E2F1-mediated hPOMC promoter activity was also confirmed by luciferase reporter assay using the #5, #6 and #7 promoter plasmids with co-transfection of either E2F1 or E2F3 expression plasmid. Both promoters #5 and #6 were upregulated by co-expression of E2F1 but not by E2F3, whereas promoter #7 (as a negative control with no E2F1 binding site) was not regulated by either E2F1 or E2F3 co-expression (Fig. 3C).
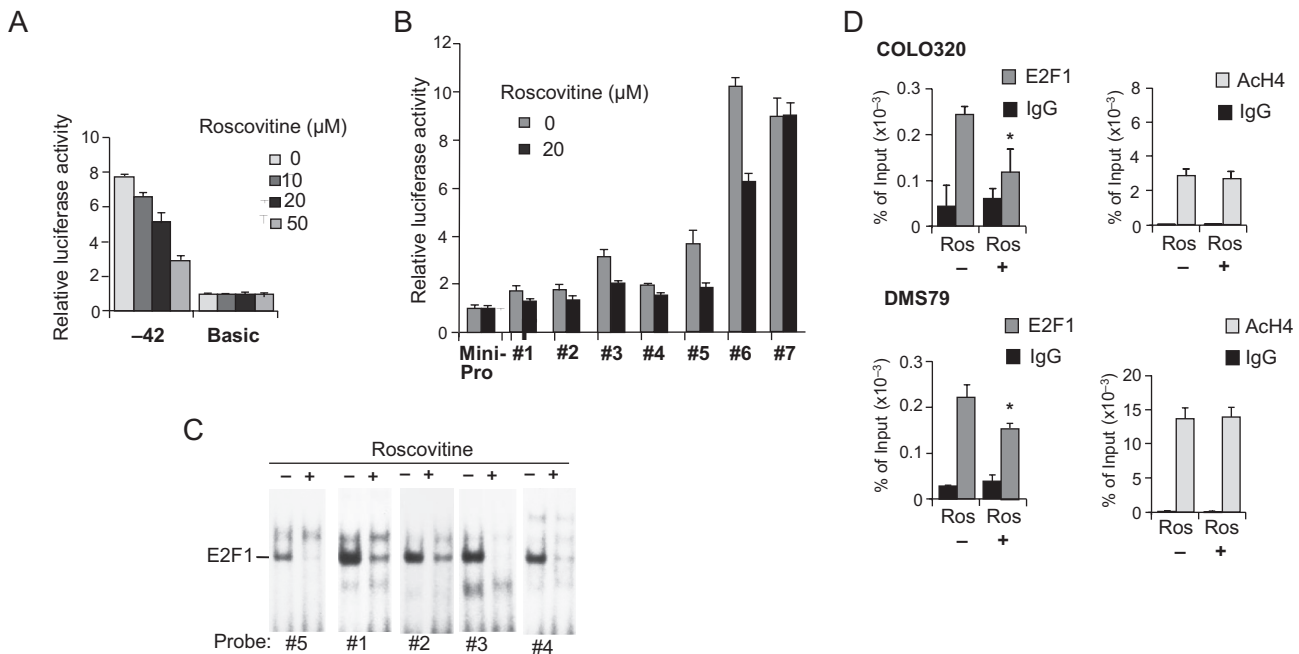

Figure 5

hPOMC is suppressed by R-roscovitine treatment through E2F1 DNA binding. (A) Luciferase assays were performed using −42 hPOMC promoter in DMS79 cells treated with (+) or without (−) R-roscovitine at the indicated concentrations, and luciferase activities compared to negative control plasmid basic (no promoter). (B) Luciferase assays using the minimal promoter (Fig. 2B) were performed in DMS79 cells treated with (+) or without (−) R-roscovitine at the indicated concentrations. (C) Results of EMSA performed using indicated probes (#1 through #5, shown in Fig. 2B) with nuclear extracts derived from DMS79 cells treated with (+) or without (−) R-roscovitine (50 μM). (D) ChIP assays were performed with anti-E2F1 (E2F1), anti-acetyl histone H4 (AcH4), or negative control (IgG) using the primers −46/+233 (Fig. 3D). Analyzed ChIP regions from R-roscovitine (50 μM) treated (Ros+) or non-treated (Ros−) DMS79 cells and COLO320 cells are shown. Luciferase results are representative of four independent experiments (A, B), EMSA results are representative of three independent experiments (C), and ChIP assays results are representative of three independent experiments (D).

E2F1 binding to the proximal promoter region was further analyzed (Fig. 2B). Similar sequences of the 3′-half of the E2F consensus (CGGCAA in #1, GCGGGA in #2, GGCGAA in #3, and GAGCCGC in #4) were identified in the −42/+68 *hPOMC* region, and minimal promoter activities were also moderately upregulated in these fragments (Fig. 2C). We therefore performed EMSA competition assays using the labeled probe #5 with unlabeled competitor (#1 to #4) (Fig. 3A right), and observed that E2F1 binding to probe #5 was blocked by #1 to #4 competitors, suggesting that the E2F1 cluster binds the −42/+68 region.

E2F1 binding to the proximal *hPOMC in vivo* was confirmed by ChIP assay using DMS79 and COLO320 cells (Fig. 3D, E, F), and histone H4 was highly acetylated in this region, but not in upstream control regions (−1.2 kb or −6.7 kb) identified as an *rPomc* Tpit palindrome enhancer (Langlais *et al.* 2011) (Fig. 3E, F).

E2F1 determines *hPOMC* expression in non-pituitary ACTH-producing tumor cells

To define the functional role of E2F1, we performed luciferase assays and RT-PCR after co-expressing E2F1 and its heterodimer DP1 (Girling *et al.* 1993). *hPOMC* promoter activity (−42/+68) and *POMC* mRNA levels in COLO320 cells were markedly upregulated by co-expression of E2F1 and DP1, but parathyroid hormone, which served as a control gene, was not upregulated (Fig. 4A, B). In DMS79 cells, *hPOMC* promoter activity and mRNA expression were also upregulated by E2F1 and DP1 co-expression (Fig. 4C, D). Gene-specific *POMC* knockdown was performed by using Si-RNA E2F1, whereas the control gene (PTH) was unchanged, further suggesting a specific contribution of E2F1 to *POMC* gene regulation (Fig. 4E).

We next confirmed the role of E2F1 in the regulation of *hPOMC* gene expression using 12 human bronchial carcinoid tumor samples, known to exhibit a propensity for ectopic ACTH production (Aniszewski *et al.* 2001, Isidori *et al.* 2006). *hPOMC* expression was detected in 9 of 12 tumors and, importantly, *E2F1* and *POMC* expression levels correlated strongly (P=0.025) (Fig. 4F).

Taken together, the results suggest that *hPOMC* expression levels are determined by E2F1, which mediates transcription through the −42/+68 proximal promoter.

E2F1 binding to *hPOMC* proximal promoter decreased by R-roscovitine treatment

Given the role of E2F1 in regulating *hPOMC* expression, we considered whether E2F1 could serve as a target in

ectopic Cushing's tumors. As shown in Fig. 1B, C, D, we used R-roscovitine, known to suppress *mPomc* in pituitary corticotrophs (Liu *et al.* 2015), to elucidate the mechanism. Cell viability assays were used to determine the doses for *in vitro* experiments (Supplement 2). We confirmed the suppression of paraneoplastic *hPOMC* mRNA and ACTH production in surgically excised ectopic ACTH-producing samples derived from lung, liver and thymus tumors (n=5) treated with R-roscovitine (Fig. 1B, C). Dose-dependent *hPOMC* expression was seen in DMS79 and COLO320 cells treated with R-roscovitine for 48 h (Fig. 1D). We also confirmed the *in vivo* efficacy of this drug in DMS79 cell
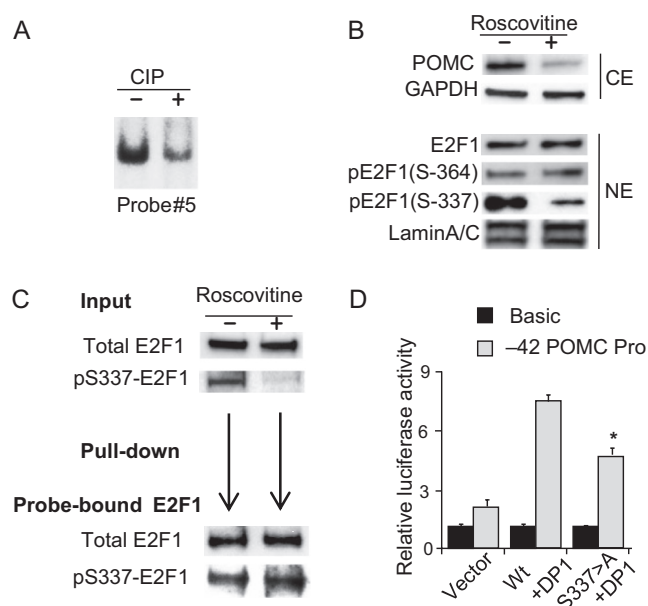

Figure 6

E2F1 phosphorylation is required for E2F1 binding to the *hPOMC* promoter. (A) Results of EMSA performed using probe #5 and nuclear extracts treated with CIP (+) or control nuclear extracts (−) incubated with CIP and CIP inhibitor (see 'Methods' section). The E2F1 complex band is shown. (B) Immunoblotting using indicated antibodies (anti-POMC, anti-GAPDH, anti-E2F1, anti-pE2F1-Ser364, anti-pE2F1-Ser337, and anti-Lamin A/C) performed using whole cell (CE) or nuclear (NE) extracts derived from DMS79 cells treated with (+) or without (−) R-roscovitine (50 μM). (C) Results of immunoblotting using anti-total E2F1 and anti-pSer337-E2F1 performed with nuclear extracts (input) derived from DMS79 cells treated with (+) or without (−) R-roscovitine (50 μM). Nuclear extracts from R-roscovitine treated cells were used for DNA pull-down assay, and proteins precipitated with biotinylated probe containing E2F1 binding sites (−24/+50) (shown in Fig. 2B) were analyzed by anti-total E2F1 and anti-pSer337-E2F1. (D) Results of luciferase assays performed in COLO320 cells using the −42 *hPOMC* promoter (−42/+68) reporter plasmid or negative control. Wild type E2F1 or S337 > A E2F1 and DP1 expression plasmids were co-transfected, and total DNA abundance adjusted with empty vector. EMSA, immunoblotting, pull-down assay, and luciferase assay results are representative of three independent experiments (A, B, C, D).

xenografted mice, showing decreased plasma ACTH and corticosterone levels (Supplement 3).

Next, using minimal promoters and the proximal –42/+68 hPOMC promoter, we showed that R-roscovitine suppressed –42/+68 hPOMC promoter activity dose dependently (Fig. 5A). Marked inhibition of promoter activity by R-roscovitine was observed, especially with fragments #5 and #6 (E2F1 sites), whereas promoter activity was unaltered with fragment # 7 (Sp1/Sp3 site) (Fig. 5B), confirming that R-roscovitine acts on E2F1-binding sites, not via SP1/SP3. To elucidate a mechanism for the observed inhibition, we performed EMSA using the same minimal promoter fragments with or without R-roscovitine treatment. E2F1 binding to probe #1 to #5 (E2F1 cluster) (Fig. 3A) was dissipated by R-roscovitine (Fig. 5C). Reduction of *in vivo* E2F1 binding to this region was also observed using ChIP assays (Fig. 5D) in DMS79 and COLO320 cells, whereas highly acetylated histone H4 was unaltered by R-roscovitine, suggesting that E2F1 determines *hPOMC* transcriptional levels without affecting chromatin remodeling. Taken together, these results provide evidence that E2F1 is an appropriate molecular target for suppressing *hPOMC*.

hPOMC transcription is regulated by E2F1 Ser-337 phosphorylation

As E2F1 DNA binding was modulated by R-roscovitine, we assessed whether E2F1 phosphorylation status affects E2F1 DNA binding. EMSA with nuclear extracts dephosphorylated by CIP (Tone *et al*. 2002) reduced E2F1 DNA binding to the *hPOMC* promoter after dephosphorylation (Fig. 6A). To test the site specificity of E2F1 dephosphorylation, we performed a series of

immunoblotting experiments in DMS79 cells. Total nuclear E2F1 and phosphorylated Ser-364 E2F1 (pSer364-E2F1) were not altered by R-roscovitine treatment. However, R-roscovitine reduced the nuclear phosphorylated Ser-337 E2F1 (pSer337-E2F1) (Fig. 6B). We further assessed the role of phosphorylated Ser337-E2F1 to DNA binding activity by pull-down assay using excess nuclear extract to saturate the *hPOMC* probe (–24/+50) (Fig. 6C). Although pSer337-E2F1 input was markedly reduced by R-roscovitine, DNA-bound pSer337-E2F1 was still clearly detected (Fig. 6C, Supplement 4), suggesting highly selective binding of pSer337-E2F1 to the proximal *hPOMC* region. Point-mutated E2F1 (Serine 337 > Alanine) also decreased *hPOMC* activity by luciferase assays (Fig. 6D).

Suppression of POMC expression by targeting E2F-binding inhibitor HLM006474

As HLM006474, a direct inhibitor of the E2F family, interrupts DNA binding in melanoma cells (Ma *et al*. 2008), and also decreases cell proliferation in DMS79 cells (Kurtyka *et al*. 2014), we investigated the effects of this small molecule on *hPOMC*. After testing cell viability to determine the appropriate dose for *in vitro* experiments (Supplement 2), similar to our results with R-roscovitine, we found that HLM006474 strongly suppressed *POMC* mRNA levels in a dose-dependent manner in DMS79 cells analyzed by RT-PCR (Fig. 7A). mRNA expression of PTH, used as a negative control, was unaltered (Fig. 7B), suggesting that E2F1-mediated antagonists elicit gene-specific *hPOMC* actions. POMC reduction was also confirmed by immunoblotting, and total E2F1 and pSer337-E2F1 were also both dose-dependently reduced (Fig. 7C). Finally, HLM006474 also suppressed *hPOMC*
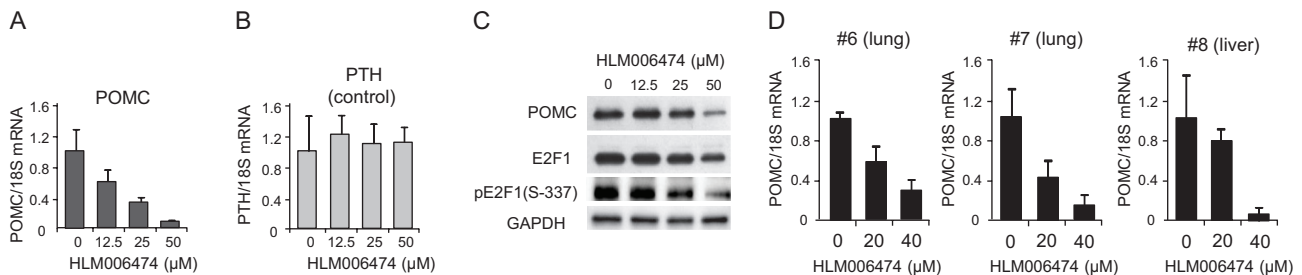

Figure 7
E2F1 inhibitors HLM006474 suppress *hPOMC* expression. (A, B, C) DMS79 cells were treated with HLM006474 at the indicated concentrations and *hPOMC* (A) and *hPTH* (B) mRNA levels analyzed by RT-PCR. Expression levels are normalized with 18S rRNA. Immunoblotting using indicated antibodies (anti-POMC, anti-E2F1, anti-pE2F1-Ser337, and anti-GAPDH) was performed using protein extracts derived from DMS79 cells treated with HLM006474 at indicated concentrations (C). (D) Primary cultures derived from non-pituitary ACTH secreting tumors (#1, 2, 3) treated with HLM006474. *hPOMC* and *hPTH* mRNA levels were analyzed by RT-PCR and normalized with 18S rRNA. RT-PCR results from DMS79 cells are representative of four independent experiments and are depicted as mean±s.e. of triplicate samples (A, B). For patient samples, RT-PCR and immunoblotting were performed in triplicate.

mRNA expression in three ACTH-secreting tumor primary cultures derived from liver and lung (Fig. 7D), whereas the expression of PTH remained unaltered (Supplement 5).

Discussion

Ectopic Cushing's syndrome is as yet an incurable disease. We elucidated mechanisms underlying *hPOMC* transcription and ACTH production in non-pituitary tumor cells.

Unlike the rat pituitary *Pomc* promoter, paraneoplastic *hPOMC* expression does not seem to be regulated by major pituitary-specific transcription factors (Lamonerie *et al.* 1996, Poulin *et al.* 1997, Lamolet *et al.* 2001, Liu *et al.* 2001). Rather, we show that *hPOMC* expression in non-pituitary tumors is regulated by E2F1 and Sp1/Sp3 binding to the proximal *hPOMC* promoter region (−42 to +68). Since these binding sites are closely located, these two transcription factors may mutually interact (Lin *et al.* 1996). As *hPOMC* expression is regulated by E2F1 expression levels and activity, E2F1-mediated POMC transcription is a potential target for ectopic Cushing's tumors.

In earlier reports, an E2F1 binding site at position −376/−417 in the POMC promoter had been identified by luciferase assays, showing a 1.2-fold to 1.5-fold decrease in activity with deletion of the site (Picon *et al.* 1995, 1999). We found a similar mild reduction of luciferase activity by deletion of the promoter from −303 to −428 (Fig. 2A). However, we also detected much stronger activity (70% of all activity) at our newly identified proximal E2F1 cluster binding site (Fig. 2A), which was not analyzed in previous studies. We therefore focused on that proximal promoter site as being a major site affecting *hPOMC* expression in ectopic Cushing's tumors, rather than the weaker upstream E2F family binding site.

We previously found that R-roscovitine targets pituitary-specific Tpit/Pitx1-mediated rat *Pomc* transcription and suppresses Tpit mRNA expression in human pituitary corticotroph tumors (Liu *et al.* 2015). Pituitary-specific rat *Pomc* gene expression is also regulated by Tpit/Pitx1 and NeuroD1 (Poulin *et al.* 1997), binding at −316/−297 and −377/−370 (Poulin *et al.* 1997). Yet, despite these sequences being conserved in the human *POMC* promoter (located at −375/−369 and −287/−281), our current data indicate that *hPOMC* transcription is not altered by deletions or by point mutations of these potential sites in two ectopic tumor cell lines. Therefore, *hPOMC* promoter activity in ectopic Cushing's tumors seems not to be similarly regulated as

pituitary corticotrophs. Alternatively, *hPOMC* promoter activity in human pituitary corticotrophs may differ from rodent pituitary expression. Indeed, clustered E2F1-binding sequences in the human promoter region are only partially conserved in the rodent *Pomc* promoter, even though E2F1 binds rodent *Pomc* in AtT20 cells (Liu *et al.* 2015). Epigenetic modifications may also contribute to the species differences and tissue-specific regulation of *POMC*. For example, CpG sites are more abundant in the *hPOMC* compared with those in the *rPomc* promoter, and differences in tissue-specific methylation status were also reported for *hPOMC* (Newell-Price *et al.* 2001). However, further elucidation of tissue specificity (i.e., pituitary vs non-pituitary) and species specificity (i.e., human vs rodent) of *POMC* regulation will be challenging to distinguish because of the lack of functional human pituitary corticotroph cell lines.

We show the functional phenotypes of E2F1 on *hPOMC* using both over-expression and knock-down studies in DMS79 and in COLO320 cells, and also show a strong positive correlation between E2F1 and POMC expression in human bronchial carcinoid tumors, confirming a role for E2F1 on POMC expression in non-pituitary cells and supporting our *in vitro* results in human tumors using primary cell cultures. These results, together with our ChIP results showing that E2F1 controls *hPOMC* transcriptional levels without altering AcH4 levels (chromatin remodeling), further support the rationale for E2F1 as a potential molecular target.

Our observation of ACTH suppression by R-roscovitine in both patient tumors and in mouse models elucidates a novel mechanism that E2F1 DNA binding and *POMC* expression are regulated by pSer337-E2F1. E2F1 Ser-337 phosphorylation may also directly modify E2F1 structure, including the DNA binding domain, or it may prevent Rb/E2F1 binding (Fagan *et al.* 1994), and therefore increase E2F1 DNA binding (Robertson *et al.* 2000). As DMS79 and COLO320 cells also express several Rb family members (e.g., p130 and p107) (Helin *et al.* 1997), these may also be associated with E2F1 Ser-337 phosphorylation status in ectopic Cushing's tumors.

Controlling E2F1 activity may be a promising therapeutic strategy for ectopic Cushing's syndrome, because expression levels of POMC seem to be determined by E2F1 activity in POMC-positive tumor cells. However, E2F1 is a ubiquitously expressed transcription factor, and inhibition of E2F1 binding to the −42/+68 promoter region did not affect the acetylation of histone H4 in this region (Fig. 5D), suggesting E2F1 does not regulate open chromatin status in the proximal promoter region.

Induction of POMC may be cell type dependent and regulated by other transcription factors, including STAT3 (Bousquet *et al.* 2000), through chromatin remodeling and epigenetic modifications such as DNA methylation (Newell-Price *et al.* 2001). Thus, although we cannot exclude the involvement of other transcription factors, E2F1 activity seems to be a common mechanism controlling POMC expression, and E2F1 over-activity may be integral to the induction of ectopic Cushing's syndrome. Indeed, E2F1 is highly expressed in small-cell lung cancer (Eymin *et al.* 2001), which may explain the relatively high occurrence of ectopic Cushing's syndrome associated with these tumors, and may also underlie suppression of cell proliferation seen with the E2F inhibitor HLM006474 in small-cell lung cancer (Kurtyka *et al.* 2014).

POMC expression strongly correlates with E2F1 in a variety of POMC-expressing cells. The direct E2F inhibitor HLM006474 robustly suppressed POMC/ACTH levels in DMS79 cells, and also reduced total E2F1 and pSer337-E2F1, suggesting that both E2F1 expression and phosphorylation status are important to *hPOMC* gene regulation. We recognize that our study used a limited number of human samples to support mechanistic findings derived from POMC-expressing cell lines. Ectopic Cushing's syndrome is rare and up to 50% are occult (Ilias *et al.* 2005); therefore, it is often treated based on signs and symptoms rather than definitive localized pathological diagnosis, making tissue samples scarce. It is also likely that such tumors are under-diagnosed, as screening for ACTH oversecretion is typically not performed until fulminant signs of disease develop, further limiting the availability of tissue samples. Confirmation for the role of E2F1 in regulating POMC expression and ACTH secretion in paraneoplastic tumors may best be demonstrated clinically by targeting kinases involved in E2F1 regulation. Our results represent a unique mechanism for E2F1 action on paraneoplastic hPOMC/ACTH hormonal production. Elucidation of subcellular mechanisms for ectopic Cushing's syndrome and our finding of E2F1 DNA binding as a potential target for excess *hPOMC* expression provide new insights into this challenging disorder.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-16-0206.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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