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Research Article

Aberrant Nuclear Translocation of E2F1 and Its Association in Cushing's Disease

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Abstract

Nonsurgical medical treatments are often performed for Cushing's disease due to high recurrence rates. However, current medical treatment that targets corticotroph adenomas are limited. To develop a treatment that specifically targets corticotrophs in Cushing's disease, it is necessary to identify corticotroph lineage–specific proteins, which are involved in the Cushing's tumor phenotype. We have previously reported that the expression of E2F transcription factor 1 (E2F1), one of the cell cycle regulatory proteins, was increased in corticotrophs in Cushing's disease model mice and was involved in the regulation of *POMC* gene expression. Phosphorylation of Ser337 of E2F1 (pS337-E2F1) facilitates its binding to the *POMC* promoter, which was suggested to contribute to elevated *POMC* expression in corticotrophs. Here, we report that E2F1 expression is specific to the corticotroph lineage in normal human pituitaries and that the E2F1 protein is localized in the cytosol in normal corticotrophs. We show that pS337-E2F1 is localized in the nucleus specifically in Cushing's tumors, while it is localized in the perinuclear cytoplasm in the normal pituitary. This observation demonstrates that pS337 is a marker for Cushing's tumors and suggests that phosphorylation of E2F1 may be a target for developing a novel pharmacological treatment for tumorigenesis and hormone dysregulation of Cushing's disease.

Key Words: Cushing's disease, POMC, E2F1, pS337-E2F1, corticotroph

Cushing's disease is caused by corticotroph tumors in the pituitary, leading to various disorders including diabetes, coronary artery disease, severe coagulopathy, osteoporosis, infection, and psychosis. If untreated, Cushing's disease can be fatal for some patients. Corticotrophs express the proopiomelanocortin (*POMC*) gene, which encodes a precursor peptide of adrenocorticotropic hormone (ACTH) that stimulates the adrenal gland to produce cortisol. Even if the tumor is small in size, elevated ACTH levels in Cushing's disease can lead to hypercortisolemia, which significantly affects the quality of life of patients. Surgical removal of the tumor is the primary treatment of the disease. Due to high rates of tumor recurrence, lifelong monitoring is needed for the postsurgical patient. The current mainstream of nonsurgical treatment options for Cushing's disease is the use of drugs that inhibit cortisol synthesis (1) or block cortisol receptors (2). These drugs control cortisol production or cortisol actions; however, they do not target Cushing's tumors: they cause persistent elevation of ACTH levels and an increased risk of tumor growth promotion. They can also increase the risk of adrenal insufficiency. Drugs that target pituitary tumors are available, such as somatostatin analogues and dopamine agonists; however, their actions are not specific to corticotrophs (3-5). Although treatments with these pituitary-directed drugs are effective in some patients with Cushing's disease, they also target the other lineages of pituitary endocrine cells (6-8). Therefore, to develop a strategy to directly and specifically target corticotroph adenomas, it is critical to identify proteins whose expression is specific to the corticotroph lineage and whose expression pattern is distinct between normal corticotrophs and corticotroph adenomas.

Epidermal growth factor receptor (EGFR) is expressed in aggressive Cushing's tumors, and the EGF signaling pathway is activated in Cushing's tumors (9). We have reported that the expression of E2F transcription factor 1 (E2F1), one of the cell cycle regulatory proteins, was increased in corticotrophs in Cushing's disease model mice that specifically express a transgene EGFR in corticotrophs (10). Moreover, blocking EGF signaling by tyrosine kinase inhibitors reduced expression of the E2F1 levels in these model mice (10). These results suggest that activation of E2F1 is downstream of EGF signaling in Cushing's tumors. Moreover, our previous study demonstrated a functional link between E2F1 and expression of the *POMC* gene. E2F1 binds to the proximal site (–42/+68) of the *POMC* promoter region by chromatin immunoprecipitation in the AtT20 cell line, a mouse ACTH-producing cell line (11). E2F1 can also upregulate transcription from the *POMC* promoter by luciferase reporter assays in ACTH-producing cell lines (AtT20 cells, DMS79 cells, and COLO320 cells). Furthermore, treating primary cultures of Cushing's tumors with HLM006474, a specific inhibitor of DNA binding of the E2F family proteins, downregulated *POMC* gene expression (10). These data collectively support the idea that E2F1 directly regulates *POMC* gene expression in corticotrophs.

E2F1 is modified by phosphorylation at different residues. Among them, phosphorylation of 2 serine residues,

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S332 and S377, is known to be involved in DNA binding affinity in vitro (11). Specifically, phosphorylation of S332 and/or S337 prevents E2F1 binding to retinoblastoma (Rb) protein, a tumor suppressor protein, in vitro (12). E2F1 that is not bound to Rb has higher DNA binding affinity than E2F1 that is bound to Rb (13). In corticotrophs, we previously showed that phosphorylation of E2F1 at the S337 residue (pS337-E2F1) facilitates E2F1 binding to the *POMC* promoter by in vitro DNA binding assays (11). In addition, pS337-E2F1 exhibited higher transcriptional activities for the *POMC* promoter, compared to nonphosphorylated E2F1 in AtT20 cells (11).

In this study, we followed up our previous findings on E2F1 in normal corticotrophs. We observed broad expression of E2F1 in the cytoplasm of corticotrophs in normal human pituitaries. In contrast, we observed accumulation of pS337-E2F1 in the perinuclear cytoplasm in normal human corticotrophs. By analyzing 9 Cushing's tumor samples, we observed elevated expression of pS337-E2F1 in the nucleus in Cushing's tumors but not in normal corticotrophs, suggesting that pS337-E2F1 translocated into the nucleus in Cushing's tumors. Our finding provides information about a difference between normal corticotrophs and Cushing's tumors, which may be useful for developing a novel therapeutic target for Cushing's disease.

Materials and Methods

Study Approval

Study protocols were approved by the University of Minnesota Institutional Review Board (protocol no. 00005168). Informed consent was obtained from patients >18 years of age, who were diagnosed with Cushing's disease. Human autopsy pituitary tissue collections were approved by the University of Minnesota Institutional Review Board (protocol no. 00005168). Pituitary autopsy specimens used for this study were collected and processed for formalin fixation within 24 hours of the subject's death.

Immunohistochemical Staining

Normal human pituitary samples and corticotroph adenomas were fixed in 10% formalin and embedded in paraffin per the University of Minnesota Pathology Department clinical protocol. After deparaffinization and antigen retrieval, the slides were blocked with 5% donkey serum in phosphate-buffered saline (PBS) + 0.1% Triton X-100 for 60 minutes at room temperature and incubated with primary antibodies overnight at 4°C. After washing with PBS + 0.1% Triton X-100, the slides were incubated with secondary antibodies overnight at 4°C, washed with PBS + 0.1% Triton X-100, incubated with diaminopyrolylindole 4,6-diamino, 2-pyrolylindole (DAPI)/PBS solution, and mounted with DAPI Fluoromount-G. Images were acquired using a Zeiss LSM710 confocal microscope with Zen software. Primary antibodies used are goat polyclonal anti-POMC (Abcam ab32893, RRID: AB_777375, 1:100 dilution), goat polyclonal anti-growth hormone (GH; R&D AF1067, RRID: AB_354573, 1:100 dilution), goat polyclonal anti-prolactin (R&D AF682, RRID: AB_355522, 1:100 dilution), mouse monoclonal anti-thyroid-stimulating hormone (TSH; R&D MAB57941, RRID: AB_10643835, 1:50 dilution), mouse monoclonal anti-follicle-stimulating hormone (FSH; Dako M350401, RRID: AB_2079146, 1:50 dilution), mouse monoclonal anti-E2F1(Abcam ab135251, RRID: AB_2631246, 1:100 dilution), rabbit polyclonal anti-pS337-E2F1(Abcam ab135549, RRID:AB_2631245, 1:100 dilution), and mouse monoclonal anti-lamin A/C (Cell Signaling 4777, RRID: AB_10545756, 1:100 dilution). Secondary antibodies used are Alexa Fluor 488-donkey anti-goat immunoglobin G (IgG; Invitrogen A32814, RRID: AB_2762838, 1:500 dilution), Alexa Fluor 594-donkey anti-mouse IgG (Invitrogen, A32754, RRID: AB_2762827, 1:500 dilution), and Alexa Fluor 647-donkey anti-rabbit IgG (Invitrogen, A31573, RRID: AB_2536183, 1:500 dilution).

Line Scan Image Analysis of the Lamin A/C Signals and the pS337-E2F1 Signals

Grayscale, single-channel images of lamin A/C and pS337-E2F1 were converted into 8-bit images and stacked together with Fiji. For 10 to 15 locations with a lamin A/C signal accompanied by overlapping or adjacent pS337-E2F1 signal, the straight-line tool was used with a line width of 10 to draw a line across the both the lamin A/C and pS337-E2F1 signals. The intensity of the lamin A/C signal and the pS337-E2F1 signal along each line was plotted with the plot profile tool, and the intensity values along the line were saved in Excel files. In Microsoft Excel, both the lamin A/C and pS337-E2F1 intensity values were normalized such that the max intensity was 1 by dividing all intensities by the maximum intensity, and the x-axis was normalized such that the lamin A/C signals started at −1 and ended at 1 by setting the center of these signals as 0 and increasing/decreasing the x values in increments of 2/n, where n is the number of points between the start and end of the lamin A/C signal. Finally, the intensities were graphed with a scatter plot.

Line Scan Image Analysis of the Nucleus and the pS337-E2F1 Signals

Grayscale, single-channel images of DAPI and pS337-E2F1 were converted into 8-bit images and stacked together with Fiji. For 10 to 17 locations with a DAPI signal accompanied by an overlapping or adjacent pS337-E2F1 signal, the straight-line tool was used with a line width of 10 to draw a line across the both the DAPI and pS337-E2F1 signals. The intensity of the DAPI signal and the pS337-E2F1 signal along each line was plotted with the plot profile tool, and the intensity values along the line were saved in Excel files. In Microsoft Excel, both the DAPI and pS337-E2F1 intensity values were normalized such that the max intensity was 1 by dividing all intensities by the maximum intensity, and the x-axis was normalized such that the DAPI signals started at −1 and ended at 1 by setting the center of the DAPI signal as 0 and increasing/decreasing the x values in increments of 2/n, where n is the number of points between the start and end of the DAPI signal. Finally, the intensities were graphed with a scatter plot.

Statistics

Differences in relative signal intensities between 2 groups (normal corticotrophs and Cushing's tumors) were analyzed using a *t*-test with the Welch correction. $P < 0.05$ was considered significant.

Results

E2F1 Is Specifically Expressed in the Corticotroph Lineage in the Human Pituitary

We previously identified that E2F1 is highly expressed in corticotrophs and rarely detected in lactotrophs [prolactin (PRL)-positive cells] and somatotrophs (GH-positive cells) in normal human pituitaries (10). We sought to determine whether E2F1 expression is specific to corticotrophs among all the 5 pituitary cell lineages and immunostained human pituitary sections using an anti-E2F1 antibody and antibodies against other pituitary hormones (POMC, GH, TSH, PRL, or FSH) (Fig. 1A and 1B). To preserve epitopes, the autopsy specimens were processed within 24 hours of death, which is a standard protocol for pathological examination of human specimens (14). We observed E2F1 in POMC-expressing corticotrophs (Fig. 1A) but not in other cell lineages (GH, TSH, PRL, and FSH) (Fig. 1B). We also noted that the E2F1 signals were localized in the cytoplasm in corticotrophs, not in the nucleus (Fig. 1A, E2F1). This localization is unexpected, given that E2F1 is localized in the nucleus in other tissues, such as the testis (15), tonsil (16), and ovary (17). Consistent with previous reports (15), we detected E2F1 in the nucleus of the human testis using the same anti-E2F1 antibody (Fig. 1C). These results demonstrate that E2F1 is specifically expressed in the cytoplasm of corticotrophs in the human pituitary. Of note, the lactotrophs in the normal autopsy pituitary seem to be arranged in discrete loci, resembling hyperplasia or microadenoma (Fig. 1B, PRL). Given that up to 20% of the autopsy pituitaries contains microadenomas (18, 19), microadenoma might exist in this pituitary specimen. Despite these morphological variations, the corticotroph lineage–specific patterns of E2F1 expression do not seem to be affected.

Perinuclear Localization of pS337-E2F1 in Normal Human Corticotrophs

Phosphorylation of E2F1 at S337 (pS337-E2F1) enhances its DNA binding ability in vitro (12). To examine whether E2F1 is phosphorylated at S337 in corticotrophs, we stained normal human pituitary sections with an antibody against pS337-E2F1 (Fig. 2A). We observed pS337-E2F1 in the cytoplasm of corticotrophs; however, its localization was different from that of E2F1. Specifically, the signals were accumulated around the nucleus in some corticotrophs (Fig. 2A, pS337-E2F1), compared to the broad cytoplasmic expression of E2F1 (Figs. 1A and 2A, E2F1). To further characterize the subcellular localization of pS337-E2F1 in relation to the nuclear envelope, we immunostained pituitary sections with antibodies against pS337-E2F1 and lamin A/C, a protein localized at the nuclear envelope (Fig. 2B) and performed a line-scanning analysis of each cell (see Material and Methods). Figure 2B shows the results of the line-scanning analysis of 15 cells (Fig. 2B, right). We normalized the position of the lamin A/C signals, such that the nuclear envelope positions of each cell are plotted at the same position in the x-axis. Signal intensity of each cell is normalized; the highest signal intensity of each cell was set as 1.0 and plotted on the y-axis. This analysis shows that the pS337-E2F1 signals are localized immediately outside of the nuclear envelope. As a control, we also stained the human testis with both E2F1 and pS337-E2F1. In the human testis, pS337-E2F1 signals are localized within the nucleus together with the E2F1 signals (Fig. 2C). These results

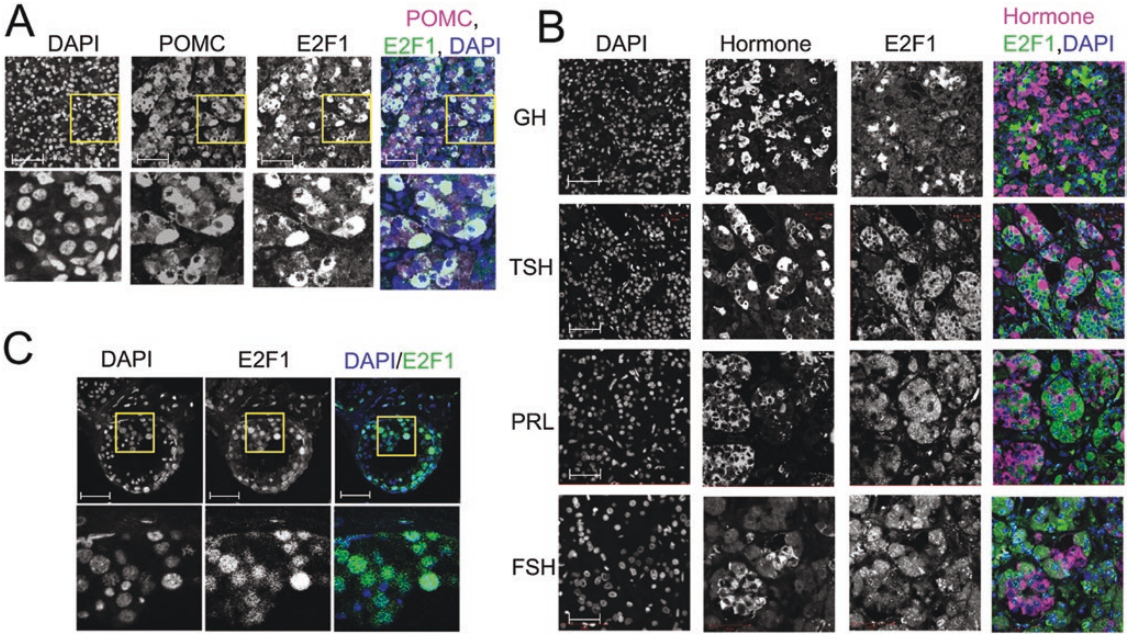

Figure 1. E2F1 corticotroph-specific expressions. (A) Autopsy-derived normal human pituitaries were immunostained with E2F transcription factor 1 (E2F1), proopiomelanocortin, and diaminopyrolylindole 4,6-diamino, 2-pyrolylindole (DAPI). E2F1 and POMC cytoplasmic colocalization was shown in merged images. (B) Autopsy derived normal human pituitaries were immunostained with E2F1 and pituitary hormones (growth hormone, thyroid-stimulating hormone, prolactin, and follicle-stimulating hormone) and DAPI. E2F1 and pituitary hormones were not colocalized. (C) Autopsy derived normal human testis were immunostained with E2F1 and DAPI. Colocalization of E2F1 and DAPI is shown. Scale bar, 50 μm.

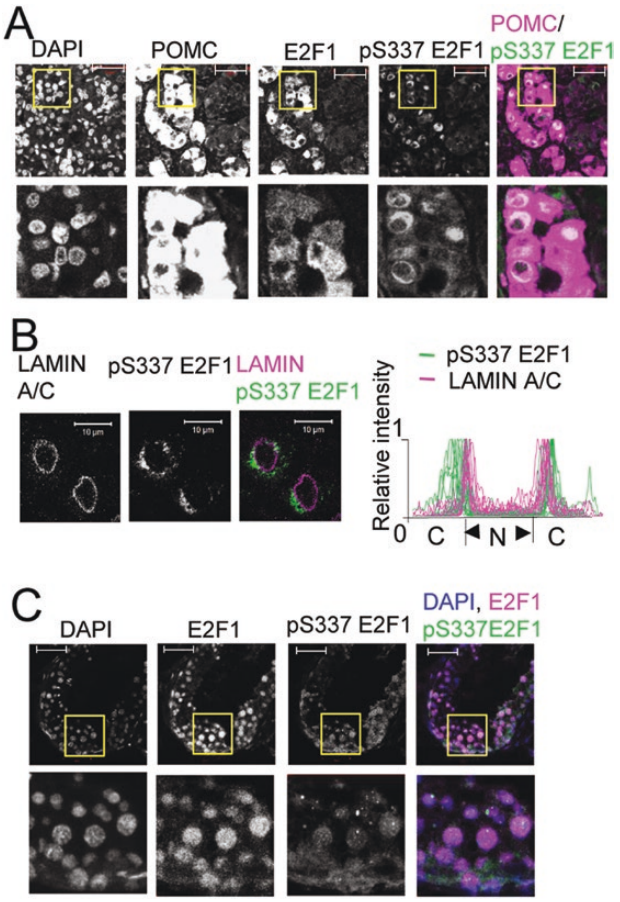

Figure 2. Perinuclear expression of pS337-E2F transcription factor 1 (E2F1) in human corticotrophs. (A) Autopsy derived normal human pituitaries were immunostained with E2F1, pS337-E2F1, proopiomelanocortin, and diaminopyrolylindole 4,6-diamino, 2-pyrolylindole (DAPI). Scale bar, 20 μm. (B) Autopsy derived normal human pituitaries were immunostained with pS337-E2F1 and lamin A/C (left), and plotted line scans of pS337-E2F1 and lamin A/C were shown on the right. Width of the nucleus were normalized in all cells analyzed, and the highest intensity of fluorescence was described as relative intensity 1.0 (see Materials and Methods). Area within the 2 arrows on the x-axis represents nucleus (N) area, and the area outside of the 2 arrows in the x-axis represents cytoplasm (C) area. Scale bar, 10 μm. (C) Autopsy-derived normal human testis were immunostained with pS337-E2F1, E2F1, and DAPI. Scale bar, 50 μm.

demonstrate that the perinuclear localization of pS337-E2F1 is unique to human corticotrophs.

Nuclear Translocation of pS337-E2F1 in Cushing's Tumors

Next, we asked whether subcellular localization of pS337-E2F1 is altered in human corticotroph adenomas (Cushing's tumors). We immunostained Cushing's tumor sections with antibodies against pS337-E2F1 and lamin A/C and performed a line-scanning analysis (Fig. 3A). Figure 3A shows results of the analysis of 15 cells, plotted in a graph, using the same method in Figure 2B (Fig. 3A, right, line scan). This analysis shows that the pS337-E2F1 signals are detected both in the nucleus and at the perinuclear region of the cytoplasm. This localization pattern is different from that of normal human pituitary corticotrophs, where pS337-E2F1 is localized only in the perinuclear cytoplasm and not in the nucleus (Fig. 2B). To further confirm our finding, we immunostained and analyzed 9 additional Cushing's tumor specimens (Table 1) and 3 normal human pituitary samples (Fig. 3B). We detected nuclear-localized signals of pS337-E2F1 in all Cushing's tumor samples (Fig. 3C, Cs1-Cs9).

Next, we performed a line-scanning analysis of 15 cells in each tumor specimen (Fig. 3C). We used the DAPI signal to locate the position of the nucleus of each cell and normalized the position of the nucleus at the same position on the x-axis. Signal intensities were normalized with the same protocol used for analysis in Figure 2. Line-scanning analysis showed that pS337-E2F1 signals were present both in the nucleus and the cytoplasm in all 9 Cushing's tumors. To compare relative signal intensity in the nucleus, we first averaged intensities of signals in each nucleus, and then we averaged the 15 cells' average nuclear signal intensities. A purple solid arrow in Figure 3B (right panel) points to the average intensity of the nuclear signals of 15 normal corticotrophs. Similarly, red dotted arrows in Figure 3C (right panels) point to the average signal intensity of 15 nuclei in each Cushing's tumor sample (Cs1-Cs9). The average nuclear intensities of normal corticotrophs in 3 normal pituitaries and 9 Cushing's tumors were plotted in Figure 3D, which demonstrates significantly elevated nuclear signals in Cushing's tumors compared to normal corticotrophs. These results suggest nuclear translocation of pS337-E2F1 in all the Cushing's tumors examined in this study. The results also suggest that pS337-E2F1 nuclear translocation is a specific characteristic of Cushing's tumors.

Discussion

Nonsurgical medical treatments are often used for Cushing's disease, because recurrence of the Cushing's tumor is high. Currently available medical treatments mainly target cortisol, which acts downstream of ACTH. Such treatments cause persistent elevation of ACTH levels or a risk of adrenal insufficiency. Medications that target the pituitary are also available, including a somatostatin analogue (pasireotide) (5) and a dopamine agonist (cabergoline) (20). These medications have been associated with tumor size reduction in approximately 40% of responders (5, 20). Temozolomide, an alkylating agent, is also effective for treatment of some of the more aggressive tumors (21). However, actions of all of these medications are not specific to corticotrophs and may affect other endocrine lineages. Medical treatments that specifically target corticotroph adenomas are not available. To develop a specific treatment that targets corticotroph tumors in Cushing's disease, it is necessary to identify corticotroph lineage–specific proteins that are specific to corticotroph tumors in Cushing's disease. Here, we report that E2F1 expression is specific to the corticotroph lineage in normal human pituitaries and that nuclear translocation of pS337-E2F1 is specific in Cushing's tumors.

Our present and previous studies (10, 11) support the idea that E2F1 is involved in Cushing's tumor pathogenesis. We previously showed that E2F1 is a downstream factor of EGFR signaling, which is activated in aggressive Cushing's tumors (10). Moreover, E2F1 binds to the *POMC* promoter and upregulates *POMC* transcription in ACTH-producing cell lines (11). Phosphorylation of S337 enhanced the DNA binding affinity of E2F1 to the *POMC* promoter and

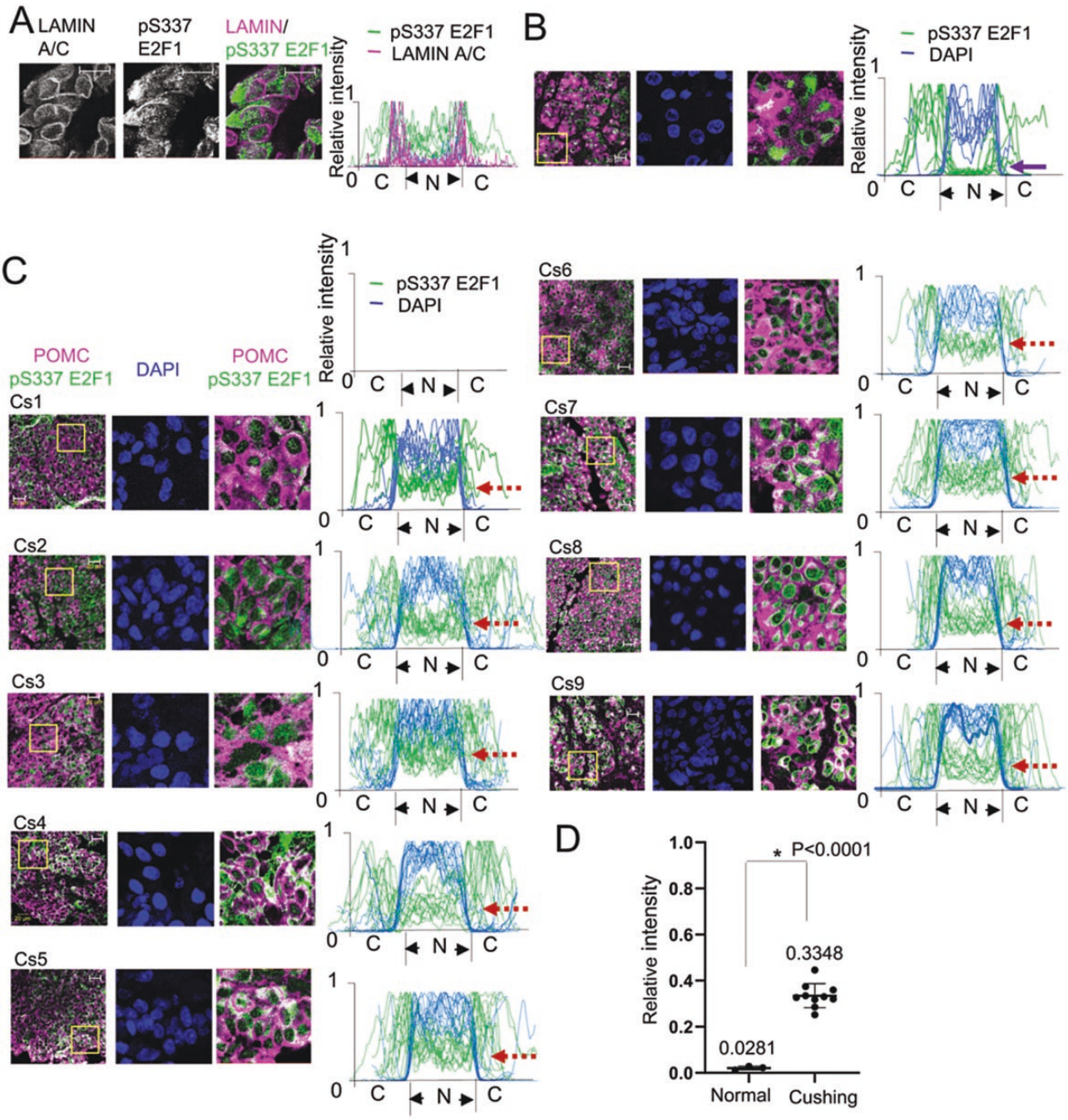

Figure 3. Nuclear translocation of pS337-E2F transcription factor 1 (E2F1) in corticotroph adenomas. (A) Cushing's tumors were immunostained with pS337-E2F1 and lamin A/C (left), and plotted line scans of pS337-E2F1 and lamin A/C were shown on the right. Scale bar, 10 μm. (B) Autopsy-derived normal pituitaries were immunostained with proopiomelanocortin, pS337-E2F1, and diaminopyrolylindole 4,6-diamino, 2-pyrolylindole (DAPI; left), and plotted line scans of pS337-E2F1 and DAPI are shown (right). Width of the nucleus were normalized in all cells analyzed, and highest intensity of fluorescence was described as relative intensity 1.0 (see Materials and Methods). Scale bar, 20 μm. (C) Nine samples of Cushing's tumors (Cs 1-9) were immunostained with proopiomelanocortin, pS337-E2F1, and DAPI (left), and plotted line scans of pS337-E2F1 and DAPI are shown (right). Width of the nucleus were normalized in all cells analyzed, and the highest intensity of fluorescence was described as relative intensity 1.0 (see Materials and Methods). Scale bar, 20 μm. Characterizations of Cushing's tumors are shown in Table 1. (D) The average of nuclear signal intensities of 3 samples of normal corticotrophs and 9 samples of Cushing's tumors were plotted. The average of each group is shown.

upregulated *POMC* transcription (11). In the current study, we observed nuclear translocation of pS337-E2F1 specifically in Cushing's tumors (Figs. 3C and 4A). Increased levels of pS337-E2F1 in the nucleus would cause increased levels of E2F1 bound to the *POMC* gene. This condition could induce elevated *POMC* expression and ACTH secretion in Cushing's tumors (Fig. 4B). Given that EGF signaling causes phosphorylation of S337-E2F1 (10) and our observation of nuclear translocation of pS337-E2F1, it is conceivable that pS337-E2F1 plays a role in Cushing's disease pathogenesis by overexpressing *POMC*. If so, phosphorylation of E2F1 at S337 or its nuclear translocation might serve as a new therapeutic target for Cushing's disease (Fig. 4). In this regard, it is of interest to investigate in the future whether pS337-E2F1

Table 1. Characteristics of tissue samples used for immunostaining

Sample number	Nuclear signal intensity	Maximum tumor diameter, mm	Sex	Age	ACTH, pg/mL	24-hour UFC, μg/day	Treatment prior to surgery
Cs1	0.25	6	F	36	198	100	None
Cs2	0.33	7	F	48	115	183	Mifepristone
Cs3	0.45	25	F	26	95	69.8	None
Cs4	0.32	23	F	42	157	2033.5	Ketoconazole
Cs5	0.33	7	F	64	30	62.1	None
Cs6	0.36	16	F	44	>1250	N/A	Adrenalectomy
Cs7	0.32	8	F	37	75	170.4	None
Cs8	0.29	25	M	58	26	N/A	None
Cs9	0.33	24	F	43	33	1610	None

ACTH levels were measured using patients' serum samples. Cortisol levels were measured using 24-hour collected urine samples. Both ACTH and 24-hour UFC are shown as preoperative values.
Abbreviations: ACTH, adrenocorticotropin; Cs, Cushing's disease; F, female; M, male; N/A, not available; UFC, urinary free cortisol.

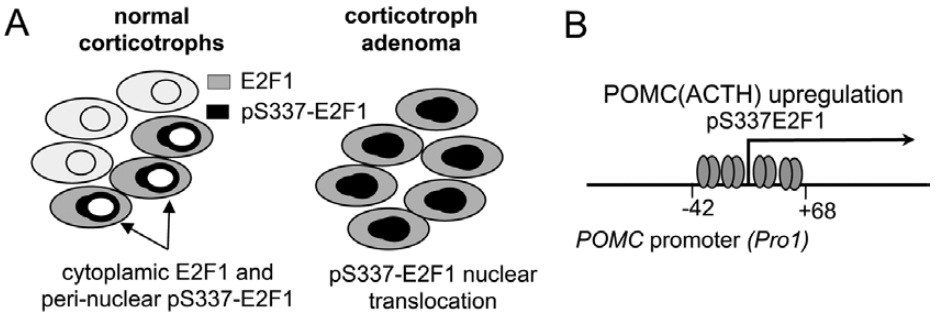

Figure 4. Schematic of E2F transcription factor 1 (E2F1) and pS337-E2F1 expressions in normal pituitary, corticotroph adenomas, and E2F1-medicated proopiomelanocortin gene regulations. (A) Schematic image of subcellular localizations of E2F1 and pS337-E2F1 expression in normal human corticotrophs (left) and in Cushing's tumors (right). (B) Schematic image of pS337-E2F1 binding to the *POMC* promoter.

also translocates into the nucleus in silent corticotroph adenomas, pituitary tumors that produce but do not secrete ACTH.

The human *POMC* gene has 2 promoters (22). While the proximal promoter or *Pro1* (–428/+68) has been well-characterized (23-25), our recent study identified an alternative promoter, *Pro2*, in the intron2/exon 3 junction (+6657/+7136). Our recent study showed that the methylation status of *Pro2* was associated with the phenotypes of Cushing's tumors (tumor size, Crooke's cell changes, recurrence, age, sex); however, the methylation status of *Pro1* did not show any correlation with tumor phenotypes (22). Consistent with these findings, the phosphorylation status of E2F1, which binds to *Pro1*, was not associated with any of these tumor phenotypes (Fig. 4, Table 1).

Association between phosphorylation and nuclear translocation is known in other proteins. For example, phosphorylation of STAT1, ERK1/2, and ERK5 is associated with nuclear translocation, while phosphorylation of NFATc1, Pho4, and PTHrP is associated with nuclear exclusion (or cytoplasmic translocation) (26). The mechanism of E2F1 nuclear translocation in corticotrophs is unknown. However, previous studies in other cell types suggested possible involvement of cargo proteins, which are known to assist nuclear translocation of cargo-targeted proteins. Karyopherine α2 is a cargo protein that enhances E2F1 nuclear translocation in hepatocellular carcinoma (27). Exportin 1 (XPO1), another cargo protein, is also involved in E2F4 nuclear translocation in osteosarcoma cells (28). Further investigation is warranted to determine the mechanism of nuclear translocation of pS337-E2F1 in corticotrophs and its significance in Cushing's disease pathogenesis.

In conclusion, we identified nuclear translocation of pS337-E2F1 in Cushing's tumors, which may be a target for developing a novel pharmacological treatment for tumorigenesis and hormone dysregulation of Cushing's disease.

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Disclosures

The authors have no multiplicity of interest to disclose.

Data Availability

Some or all data sets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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