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EGFR Induces E2F1-Mediated Corticotroph Tumorigenesis

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The epidermal growth factor receptor (EGFR), expressed in adrenocorticotrophic hormone (ACTH)–secreting pituitary adenomas causing Cushing disease, regulates ACTH production and corticotroph proliferation. To elucidate the utility of EGFR as a therapeutic target for Cushing disease, we generated transgenic (Tg) mice with corticotroph-specific human EGFR expression (corti-EGFR-Tg) using a newly constructed corticotroph-specific promoter. Pituitary-specific EGFR expression was observed by 2.5 months, and aggressive ACTH-secreting pituitary adenomas with features of Crooke's cells developed by 8 months with 65% penetrance observed. Features consistent with the Cushing phenotype included elevated plasma ACTH and corticosterone levels, increased body weight, glucose intolerance, and enlarged adrenal cortex. Gefitinib, an EGFR tyrosine kinase inhibitor, suppressed tumor POMC expression and downstream EGFR tumor signaling, and ACTH and corticosterone levels were attenuated by 80% and 78%, respectively. Both E2F1 and phosphorylated Ser-337 E2F1 were increased in corti-EGFR-Tg mice and also colocalized with human POMC (hPOMC) in human pituitary corticotroph tumor samples. EGFR inhibition reversed E2F1 activity *in vivo*, whereas E2F1 inhibition suppressed POMC and ACTH in cultured human pituitary tumor cells. The corti-EGFR-Tg phenotype recapitulates ACTH-secreting pituitary adenomas and Cushing disease, validating the relevance of EGFR to corticotroph tumorigenesis. E2F1 is identified as a promising corticotroph-specific target for ACTH-dependent Cushing disease.

Freeform/Key Words: Cushing disease, pituitary growth factor, pituitary tumor

The proopiomelanocortin (*POMC*) gene encodes adrenocorticotrophic hormone (ACTH), which induces adrenal cortisol production, essential to maintain cell viability and physiological stress responses [1]. Cushing disease is caused by excessive ACTH secretion from a pituitary corticotroph adenoma, and is associated with increased morbidity and mortality due mainly to cardiovascular and musculoskeletal disorders, secondary infections, and diabetes. Although surgical resection of ACTH-secreting corticotroph adenomas is the primary therapy, high postoperative recurrence rates (up to 45%) make the disease challenging to cure [1]. Pharmacotherapy directly targeting tumorous corticotrophs is associated with substantial side effects and is only modestly effective. Pasireotide targets the corticotroph SSTR5 receptor [2], and normalizes urinary free cortisol levels in ~20% of patients, mostly with mild disease, and is commonly associated with hyperglycemia development [3, 4]. Other drugs target

Abbreviations: ACTH, adrenocorticotrophic hormone; AL, anterior lobe; cDNA, complementary DNA; corti-EGFR-Tg, corticotroph-specific human epidermal growth factor receptor expression; EGFR, epidermal growth factor receptor; GH, growth hormone; hEGFR, human epidermal growth factor receptor; HPA, hypothalamic-pituitary-adrenal; hPOMC, human proopiomelanocortin; IL, intermediate lobe; IPGTT, intraperitoneal glucose tolerance test; MRI, magnetic resonance imaging; mRNA, messenger RNA; PCR, polymerase chain reaction; PENT, *rPomc*-enhancer-neuroD1-Tpit/Pitx1; POMC, proopiomelanocortin; PRL, prolactin; PS337, phosphorylation at Ser337; RIA, radioimmunoassay; *rPomc*, rat Pomc; Tg, transgenic; USP8, ubiquitin carboxyl-terminal hydrolase 8; WT, wild-type.

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peripheral cortisol actions to reduce symptoms but do not target pituitary tumor ACTH oversecretion, or adenoma growth [5–8].

Few animal models recapitulate human Cushing disease pathogenesis with fidelity. Mice expressing a corticotrophin-releasing hormone transgene demonstrate a Cushing phenotype, including high ACTH and corticosterone levels, but do not form pituitary tumors [9]. Heterozygous $Rb^{+/-}$ mice exhibit POMC-expressing pituitary tumors in the intermediate lobe (IL), but do not demonstrate anterior lobe (AL) corticotroph tumors or the Cushing phenotype [10, 11]. A zebrafish model of *Pttg*-driven corticotroph adenoma recapitulates features of Cushing disease and enabled identification of a small molecule to suppress ACTH [12]. However, mouse models recapitulating human Cushing disease exhibiting corticotroph tumors with upregulated hypothalamic-pituitary-adrenal (HPA) axis activity have not been developed.

EGFR is expressed to varying degrees in human pituitary tissue including corticotroph adenomas [13] and EGFR regulates POMC transcription and ACTH production [14]. The relevance of EGFR to Cushing disease pathogenesis [14] has been underscored by the finding of ubiquitin carboxyl-terminal hydrolase 8 (USP8) mutations in a subset of corticotroph adenomas [15, 16]. The USP8 mutation (14-3-3 somatic mutations) inhibits EGFR degradation, enhances EGFR accumulation, and consequently, likely induces corticotroph EGFR tumor signaling. However, neither mechanisms for corticotroph-specific tumor induction by EGFR nor mechanisms for EGFR upregulation of ACTH/POMC expression have been clearly elucidated.

Recently, we demonstrated that human POMC gene expression in ectopic ACTH-producing tumors is regulated by E2F1 and its phosphorylation at Ser337 (pS337-E2F1) [17]. To investigate whether E2F1-mediated transcription is involved in development of EGFR-induced Cushing disease, we generated EGFR-transgenic (Tg) mice using an artificial promoter containing pituitary-specific binding sequences of rat Pomc (*rPomc*) transcriptional factors [18–21]. and the *rPomc* enhancer located 7 kb upstream from the transcription start site [22] to maximize corticotroph-specific human EGFR expression. The resultant EGFR-Tg mice (corti-EGFR-Tg) develop pituitary corticotroph tumors and a Cushing phenotype similar to human Cushing disease. The pituitary tumors exhibited upregulated E2F1 and pS337-E2F1, which were attenuated by EGFR inhibition. Furthermore, E2F1 inhibition by HLM006474 resulted in suppressed POMC and ACTH in primary cell cultures derived from human pituitary corticotroph adenomas.

Our findings suggest that EGFR signaling induces E2F1-mediated POMC transcription and corticotroph adenoma pathogenesis, suggesting this pathway may be a corticotroph specific target for Cushing disease therapy.

1. Materials and Methods

A. Cell Culture

AtT20 cells and MMQ cells were cultured in low glucose Dulbecco's modified Eagle medium 10% fetal bovine serum. Deidentified, surgically resected Cushing disease specimens were processed using Tissue Dissociate Kits (Miltenyi Biotec, San Diego, CA), then cultured in low-glucose Dulbecco's modified Eagle medium 10% fetal bovine serum and HLM006474 at indicated concentrations. Cell viability was monitored by trypan blue staining, cell pellets collected for RNA extraction, and culture medium collected for radioimmunoassay (RIA).

B. Generation of Transgenic Mice

The *rPomc* promoter (–480 to +63 bp) was polymerase chain reaction (PCR)–amplified from rat genomic DNA and cloned. 5′-primers binding to –480 (Spe I site at the 5′ end) and 3′-primer binding to +63 (Nhe I sites at the 3′ end) were used for PCR amplification. Resulting fragments were digested with Spe I and Nhe I, and cloned into Nhe I sites in the pGL4 basic vector (Promega, Madison, WI). Defective fragments generated by PCR errors were removed.

Response elements for Tpit/Pitx (TTTCCTCACACCAGGATGCTAAGCCTCTTT) and NeuroD1 (TTTGCAGATGGTTT) are shown in Fig. 1(a). Sense and antisense oligonucleotides encoding these sequences were annealed and ligated upstream of the *rPOMC* –480 promoter in pGL4. Copy numbers and directions of luciferase plasmids of *rPomc*-enhancer-neuroD1-Tpit/Pitx1 (PENT) promoter were determined by DNA sequencing, and structures illustrated in Fig. 1(a) and Supplemental Fig. 1.

Tg constructs were generated using the previously-constructed PENT-pGL4 vector, with inserted IRES sequences carrying IRES/mCherry complementary DNA (cDNA)/BDH polyA [23]. cDNAs encoding human EGFR were kindly gifted by Dr. Mark Greene, University of Pennsylvania. *Xho*I (5′ end) and *Eco*RV (3′ end) were introduced by PCR, and the human

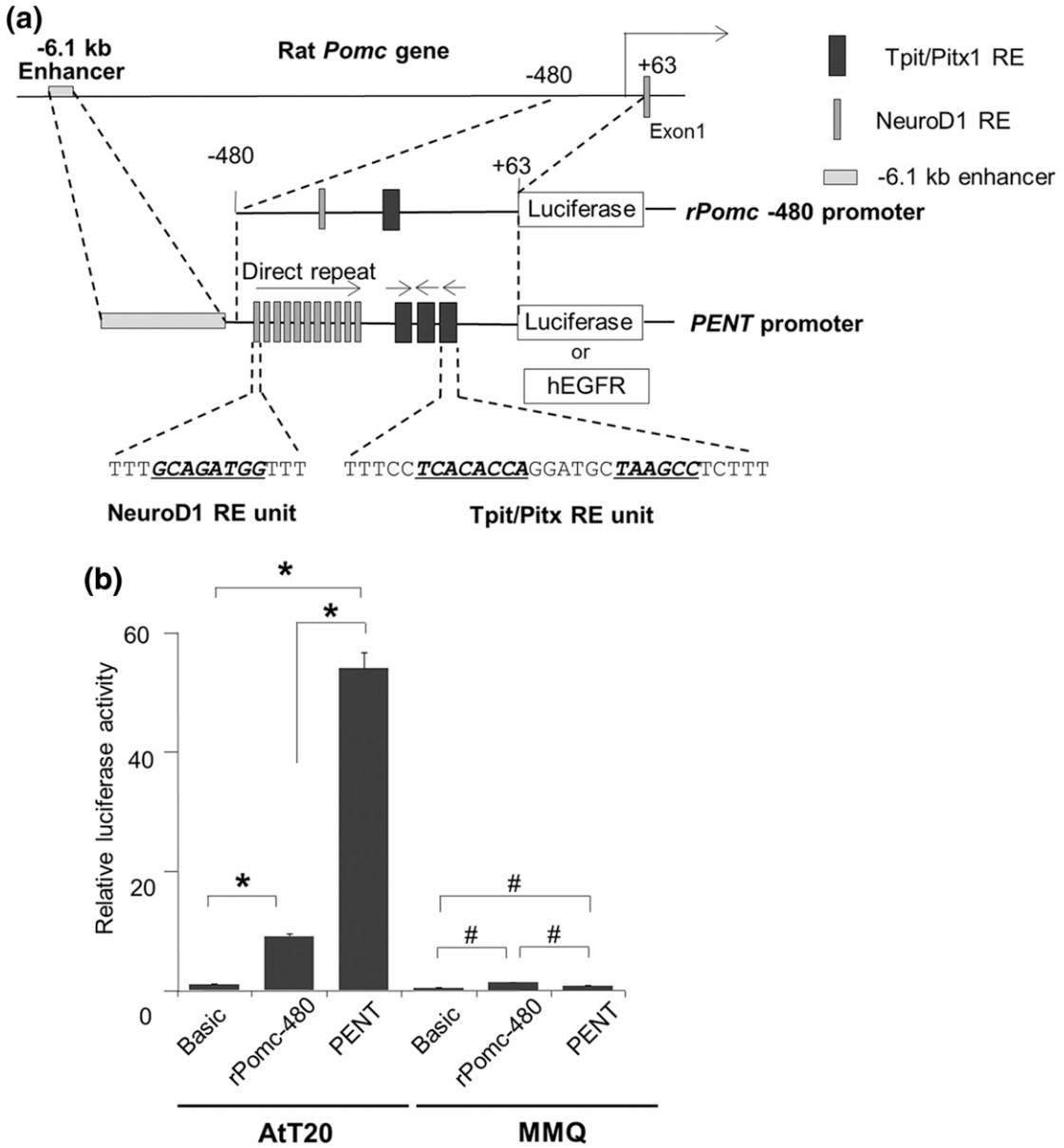

Figure 1. Construct of rPOMC enhancer/promoter (PENT). (a) Depicted structure of PENT reporter plasmids. (b) Luciferase activity of PENT promoter compared with *rPomc* –480 promoter (–480/+63) in AtT20 and MMQ cells. **P* < 0.05; #no significant difference. Results are representative of three independent experiments depicted as mean ± SE of triplicate samples.

EGFR (hEGFR) fragment joined upstream of IRES sequences. The PENT promoter fragment in pGL4 vector was digested with Nhe I and Xho I, and inserted into the IRES sequence containing hEGFR cDNA vector. PENT-hEGFR expression constructs were microinjected into male pronuclei of fertilized eggs, and injected eggs transplanted to pseudopregnant foster mothers (FVB/NJ background) following standard procedures.

To identify Tg animals, genomic DNA was isolated from 10- to 14-day-old offspring using a KAPA Mouse Genotyping Kit (KAPABIOSYSTEM, Boston, MA), and analyzed by PCR using two sets of primers. Primers binding the POMC promoter (POMC-F1 and -F2), EGFR cDNA (EGFR-R), and mCherry sequence (mCherry-F and mCherry-R) were as follows: F1 (POMC): TGCAGATGAGAAAGCAGTGG; R1 (hEGFR): TGCCTTGGCAA ACTTTCTTT; F2 (mCherry): TACGAGGGCACCCAGACCGC; R2 (mCherry): AAGTTGGTGCCGCGCAGCTT.

C. Human Tissue Collection

Protocols to collect human pituitary samples were approved by the Cedars-Sinai Institutional Review Board (see the Study Approval section). Five human pituitary specimens were collected: three normal pituitaries from autopsies and two pituitary ACTH-secreting human surgical specimens. Autopsy specimens were obtained from subjects who died due to non-endocrine causes, and tissue harvested within 24 hours of death, fixed in 10% formalin, and embedded in paraffin until use. Surgical specimens were obtained for primary culture, as well as fixed in 10% formalin and paraffin embedded.

D. Luciferase Assays

Luciferase reporter assays were performed in 0.8×10^4 AtT20 or MMQ cells with 0.5 μg luciferase reporter plasmids and 50 ng pRL-Renilla as an internal control plasmid. Cells were transfected using lipofectamine 2000 (Invitrogen, Carlsbad, CA) and cultured in 0.5 mL medium in 24-well plates. Twenty-four hours posttransfection, 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.

E. Immunoblotting

Immunoblotting was performed using anti-ACTH (ab20358; Abcam, Cambridge, MA; RRID: AB_445534), anti-pErk1/2 (Thr202/Tyr204; 9101; Cell Signaling Technology, Boston, MA; RRID:AB_331646), anti-Erk1/2 (4695; Cell Signaling Technology; RRID:AB_390779), anti-pAkt (Ser473; 4060; Cell Signaling Technology; RRID:AB_2315049), anti-Akt (4685; Cell Signaling Technology; RRID:AB_2225340), anti-EGFR (ab2430; Abcam, RRID:AB_303065), antipEGFR (Tyr1068; 2234; Cell Signaling Technology; RRID:AB_331701), anti-E2F1 (sc-251; Santa Cruz Biotechnology, Santa Cruz, CA; RRID:AB_627476), anti-pS337-E2F1 (ab135549; Abcam; RRID:AB_2631245), antidopamine D2 receptor (AB5084P; Millipore, Billerica, MA; RRID:AB_2094980), anti-PC1/3 (11914, Cell Signaling Technology; RRID: AB_2631284), anti-PC2 (14013, Cell Signaling Technology; RRID:AB_2631285), anti-glucocorticoid receptor (12041, Cell Signaling Technology; RRID:AB_2631286), and anti-β-actin (A4700, Sigma-Aldrich, St. Louis, MO; RRID:AB_476730). Antibody binding was detected using Mini PROTEAN electrophoresis protocol (Bio-Rad, Hercules, CA). Band densities were quantified using Photoshop and normalized to β-actin to correct for variations in protein loading.

F. Radioimmunoassay

Plasma ACTH and corticosterone levels were analyzed by RIA (MP Biomedicals, Santa Ana, CA).

G. Micro-MRI

Micro–magnetic resonance imaging (MRI) was acquired on a 9.4-T small animal magnet (94/20 Bruker BioSpec, Bruker Biospin Corporation, Billerica, MA), with a gradient strength of 440 mT/m and a slew rate of 3440 mT/m/s; a whole-body transmission coil (T10325V3, Bruker Biospin MRI GmbH, Ettlingen, Germany) with an inner diameter of 6 cm was used for RF excitation. A four-channel mouse receive-only brain array coil (T11071V3, Bruker Biospin MRI GmbH) was used to achieve a high signal-to-noise ratio. Mice were sedated with 1.6% isoflurane dissolved in 100% oxygen and placed in a custom-made holder to keep the head steady. Respiration was monitored to maintain a breathing rate between 45 and 65 breaths/min by slightly increasing or decreasing the isoflurane concentration. MRI spin echo images were obtained (echo time = 8 ms; repetition time = 900 ms; average data acquisition = 4 times), with a field of view of 1.8 × 1.8 cm and a slice thickness of 500 μm was used to follow tumor development. Thirty-two axial slices were obtained to cover the extent of the brain including the pituitary. Axial images had a matrix size of 196 × 196 resulting in a 92-μm isometric in-plane resolution.

H. Glucose Tolerance Test

While mice were anesthetized, tail snipping was performed to collect blood for glucose (after 5 hours of fasting) measurement using a glucose analyzer (Accucheck Instruments, Lunenburg, MA). Intraperitoneal glucose tolerance test (IPGTT) was performed with 2.0 g glucose per kg body weight in both wild-type (WT) and corti-EGFR-Tg mice at 8 months of age (n = 6 for each group), as well as in corti-EGFR-Tg mice treated with gefitinib or vehicle pre-/posttreatment (n = 5 for each group). Blood glucose levels were determined at 0, 30, 60, and 120 minutes.

I. Immunohistochemical Staining

Murine pituitary and adrenal glands and human corticotroph adenomas and normal autopsy-derived pituitary tissues (see the Human Tissue Collection section) were fixed in 10% formalin and embedded in paraffin. After deparaffinization and antigen retrieval, slides were blocked in animal free blocker (Vector Laboratories, Burlingame, CA), incubated overnight with rabbit polyclonal anti-EGFR (4267; Cell Signaling Technology; RRID:AB_2246311), goat monoclonal anti-POMC (ab32893; Abcam; RRID:AB_777375), mouse monoclonal anti-E2F1 (ab135251; Abcam; RRID:AB_2631246), rabbit polyclonal anti-pS337-E2F1 Ab (ab135549; Abcam; RRID:AB_2631245), goat monoclonal anti-growth-hormone (anti-GH; sc-10365; Santa Cruz Biotechnology; RRID:AB_2111154), or goat monoclonal antiprolactin (anti-PRL; sc-7805; Santa Cruz Biotechnology; RRID:AB_2170961). After washing, samples were incubated with Alexa Fluor donkey anti-mouse 488 (H+L, 1:500 dilution; Invitrogen), Alexa Fluor donkey anti-goat 568 (Invitrogen), and Alexa Flour donkey anti-rabbit 647 (Invitrogen) mounted with Prolong Gold antifade reagent (Invitrogen). Confocal microscope images were obtained using True Confocal Scanner (Leica Microsystems, Buffalo Grove, IL) in a dual-emission mode to separate autofluorescence from specific staining.

J. Gefitinib Treatment

Female corti-EGFR-Tg mice were divided into two groups for gefitinib (150 mg/kg; n = 10) and vehicle (0.5% methylcellose, 0.5% Tween 80/PBS; 100 μL; n = 10) by oral gavage daily for 3 weeks. Blood (200 μL) was collected twice for hormone assessment before treatment by retroorbital bleeding under isoflurane inhalational anesthesia. After the last treatment day (Day 21), mice were euthanized within 3 hours of drug administration, cardiac blood collected, and pituitary glands or tumors harvested. Fragments of each pituitary or tumor were fixed in 10% formalin, embedded in paraffin for immunohistochemical staining, or preserved in liquid nitrogen for subsequent protein extraction.

K. Study Approval

Protocols to collect human pituitary Cushing tumors were approved by the Cedars-Sinai Institutional Review Board (Protocol #2873). Male and female patients over 18 years of age with a clinical diagnosis of pituitary Cushing disease were screened and informed consent obtained. Human autopsy pituitary tissue collections were approved by the Cedars-Sinai Institutional Review Board (Protocol # Pro000375). Animal protocol (Protocol #2603/5652) was approved by the Cedars-Sinai Institutional Animal Care and Use Committee.

L. Statistics

Differences between vehicle and gefitinib groups were analyzed using one-way analysis of variance followed by nonparametric *t* test (Mann–Whitney) or Student's *t* test. Probability of $P < 0.05$ was considered significant.

2. Results

A. Generation of Corti-EGFR-Tg

We generated EGFR-Tg mice using a corticotroph-specific artificial promoter. *Pomc* activity in mouse AtT20 pituitary corticotroph cells is well established, with corticotroph-specific *rPomc* gene expression regulated by NeuroD1 and Tpit/Pitx binding sequences [18–21] [Fig. 1(a)]. We therefore used the 480 bp *rPomc* promoter as a basal promoter. To enhance corticotroph specificity and expression levels, the 832 bp *rPomc* enhancer (–7733 to –6902) [22] was joined at the 5′ end of the 480 bp promoter, and multiple copies of NeuroD1 (11 copies) and Tpit/Pitx (three copies) binding sequences inserted [Fig. 1(a)]. Sequence copy numbers were determined by preliminary experiments shown in Supplemental Fig. 1(a) and 1(b).

The resultant PENT promoter activity and cell type specific expression were assessed by luciferase assay in AtT20 and in rat lactotroph MMQ cells secreting ACTH or PRL, respectively [Fig. 1(b)]. The PENT promoter generated fivefold enhanced luciferase activity in AtT20 cells compared with the original *rPomc* 480 bp promoter and enhanced luciferase activity was not observed in MMQ cells secreting PRL, indicating corticotroph-specific PENT promoter activity [Fig. 1(b)].

A total of 26 corti-EGFR-Tg mice were used in this study. Pituitary hEGFR expression was detected at 2.5 months by immunoblotting [Fig. 2(a, left], and was significantly increased after 8 months in PENT-driven human EGFR-Tg mice [corti-EGFR-Tg; Fig. 2(a, right)]. hEGFR was detected in whole-cell pituitary corti-EGFR-Tg extracts, but not in WT pituitaries, suggesting transgene tissue-specific expression [Fig. 2(b, pituitary)]. By contrast, EGFR expression was similar in both Tg and WT liver and ovary, suggesting endogenous expression, not an effect of the transgene [Fig. 2(b, liver, ovary)]. Colocalization of corticotroph hEGFR and mPomc was confirmed by confocal immunofluorescence microscopy [Fig. 2(c) and 2(d)]. Pituitary hEGFR and Pomc were expressed in both the Tg pituitary ILs and ALs [Fig. 2(d)].

B. Corti-EGFR-Tg–Induced Pituitary Tumorigenesis

Pituitary tumor development in corti-EGFR-Tg was detected by micro-MRI by 6.5 months, and by 8 months, 65% of mice had developed pituitary tumors and/or hyperplasia (Supplemental Fig. 2), and tumor sizes had markedly increased by 10 months (Fig. 3(a–c)]. Tumorigenesis was confirmed by 8 months by histologic tissue immunostaining [Fig. 3(d) and 3(e)], evidenced by typical corti-EGFR-Tg pituitary adenoma formation with disrupted reticulin fiber network [Fig. 3(d, middle)]. Morphological observation of tumors suggested an AL origin [Supplemental Fig. 4(b)], whereas variable ratios of PC1/3 and PC2 [Supplemental Fig. 4(c)] suggest that tumors originate in both AL and IL. Apparent multifocal tumor growth [Supplemental Fig. 4(a)] is consistent with our observation that both lobes

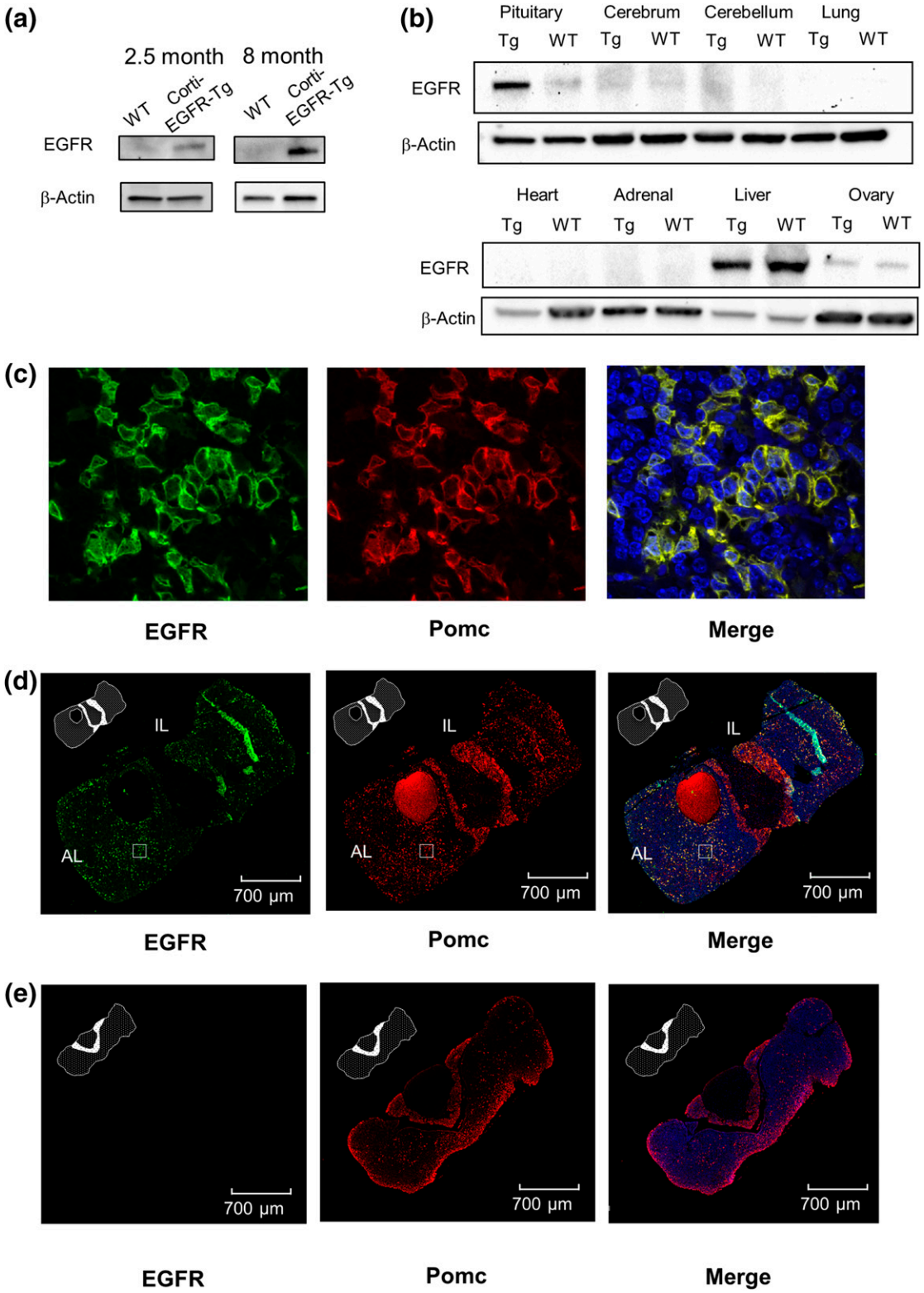

Figure 2. Expression of hEGFR in corti-EGFR-Tg mice. (a) Whole cell extracts were prepared using pituitary glands derived from WT and corti-EGFR-Tg mice (2.5 months and 8 months) and immunoblotted using anti-EGFR Ab anti-β-actin Ab. (b) Immunoblotting of whole-cell extracts derived from indicated Tg and control (WT) tissues with anti-EGFR and anti-β-actin. (c) Histochemical staining of pituitary glands from corti-EGFR-Tg mice using anti-EGFR conjugated with Alexa Fluor goat anti-rabbit 488 (green) and anti-Pomc

conjugated with Alexa Fluor donkey anti-mouse 568 (red). Staining was analyzed by confocal microscopy. To confirm EGFR and POMC colocalization, a merged image is also depicted. (d) Histochemical staining of a single whole corti-EGFR-Tg pituitary gland was analyzed by confocal microscopy to show localization of hEGFR (left), Pomc (middle), and merged image (right). The oval shape in the AL showing localized POMC staining is likely a fixation artifact. Line drawing of the pituitary and microtumors in the top left corner is depicted for orientation purposes. (e) Histochemical staining of a whole WT pituitary gland, hEGFR (left), Pomc (middle), and merged image (right).

express hEGFR and Pomc [Fig. 2(d)]. Of note, most tumor cells showed accumulation of cytoplasmic hyaline, round perinuclear dense bands by hematoxylin and eosin staining [Fig. 3(e)], and strong keratin staining with CAM5.2 [Fig. 3(d, bottom)], suggestive of Crooke's hyaline changes, characteristic of aggressive human Cushing disease [24].

C. Cushing Phenotype of Corti-EGFR-Tg

Increased body size and abdominal fat deposition were observed from about 6 months of age [Fig. 4(a–c)]. Average body mass index in corti-EGFR-Tg was higher than in WT at 10 months [$P = 0.044$; Fig. 4(d)]. At 8 months, IPGTT showed significantly elevated glucose levels in corti-EGFR-Tg mice compared with WT after 30 minutes of glucose loading, with a significant change in area under the glucose curve [Fig. 4(e)]. Plasma ACTH levels in corti-EGFR-Tg mice were significantly increased by 8 months compared with WT [483 ± 160 pg/mL vs 84 ± 62 pg/mL, $P < 0.001$; Fig. 4(f, left)], as were plasma corticosterone levels [428 ± 309 ng/mL vs 126 ± 108 ng/mL, $P < 0.01$; Fig. 4(f, right)]. Approximately 30% of Tg mice had mild bilateral adrenal gland enlargement [Fig. 4(g, left) and 4(h), Supplemental Fig. 3], with thickening of the adrenal cortex, increased cell density, and balloon-shaped morphology [Fig. 4(g, right)], consistent with adrenal hypertrophy. Thus, corti-EGFR-Tg mice developed aggressive corticotroph adenomas and demonstrated a Cushing metabolic phenotype, consistent with these Tg mice being appropriate for studying EGFR contributions to corticotroph tumor development and function.

D. Gefitinib Inhibition of EGFR Signal Transduction in Corti-EGFR-Tg Mice

We used the EGFR tyrosine kinase inhibitor gefitinib to confirm reversibility of EGFR effects on ACTH. Corti-EGFR-Tg mice were divided into two groups (n = 10) and treated with gefitinib (150 mg/kg), or vehicle (0.5% methylcellulose, 0.5% Tween 80/PBS; 100 μL) by daily oral gavage. After 3 weeks of treatment, plasma ACTH levels in the gefitinib group were significantly decreased compared with pretreatment levels [480 ± 104 pg/mL vs 93 ± 18 pg/mL; $P < 0.01$; Fig. 5(a)] and remained unchanged in the vehicle group [547 ± 207 pg/mL vs 689 ± 186 pg/mL, $P = 0.3$; Fig. 5(a)]. Similar inhibitory effects on plasma corticosterone levels were observed, with 78% reduction seen in the gefitinib-treated group ($P < 0.01$) vs no changes in the vehicle group [Fig. 5(b)]. Pituitary tumor size in corti-EGFR-Tg mice in the gefitinib-treated group was also significantly decreased [Fig. 5(c) and 5(d)]. IPGTT showed 40% suppressed glucose levels at 30 minutes post glucose loading, with no significant difference at 60 or 120 minutes, suggesting improved glucose tolerance [Fig. 5(f)].

Downstream EGFR signaling effects of gefitinib assessed by immunoblotting of drug- or vehicle-treated pituitary tumor extracts showed that gefitinib-treated mice exhibited decreased *Pomc* expression [Fig. 5(e)]. Phosphorylated EGFR was reduced, and phosphorylated Akt and Erk, EGFR signal-transducing molecules, were also suppressed in tumors treated with oral gefitinib. Total Erk and total Akt levels were unchanged [Fig. 5(e)].

E. EGFR Signaling Mediated by E2F1

Next, we investigated how signaling through EGFR upregulates *POMC* gene expression. Recently, we demonstrated in ectopic ACTH-secreting tumors that E2F1 is required to regulate human *POMC (hPOMC)* gene expression and that E2F1 binding to the *POMC*

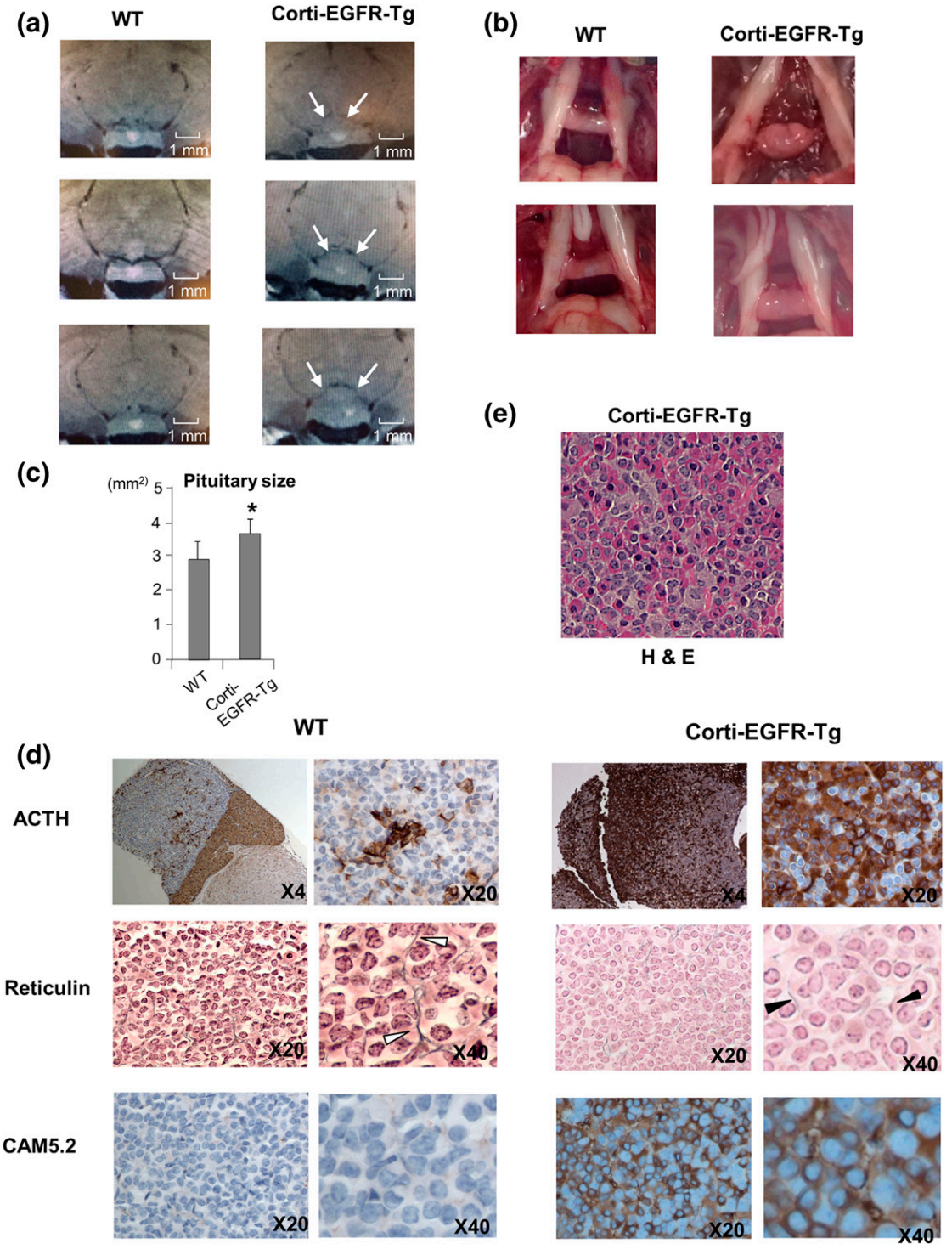

Figure 3. ACTH-secreting pituitary tumorigenesis in corti-EGFR-Tg mice. (a) Micro-MRI images of pituitary in WT (left) and corti-EGFR-Tg mice (right) at 10 months. Scale of original size depicted as 1 mm. (b) Gross images of pituitary tissue in WT (left) and pituitary tumors of corti-EGFR-Tg (right). (c) Comparison of pituitary size measured by micro-MRI at 10 months. (d) Microscopic images of WT pituitary tissue at 8 months (left) vs corti-EGFR-Tg pituitary adenoma (right) stained with ACTH (top), reticulin (middle), and CAM5.2 (bottom). Intact reticulin structures indicated by empty arrows, disrupted reticulin structures indicated by filled arrows. (e) Hematoxylin and eosin staining of corti-EGFR-Tg pituitary adenoma at 8 months.

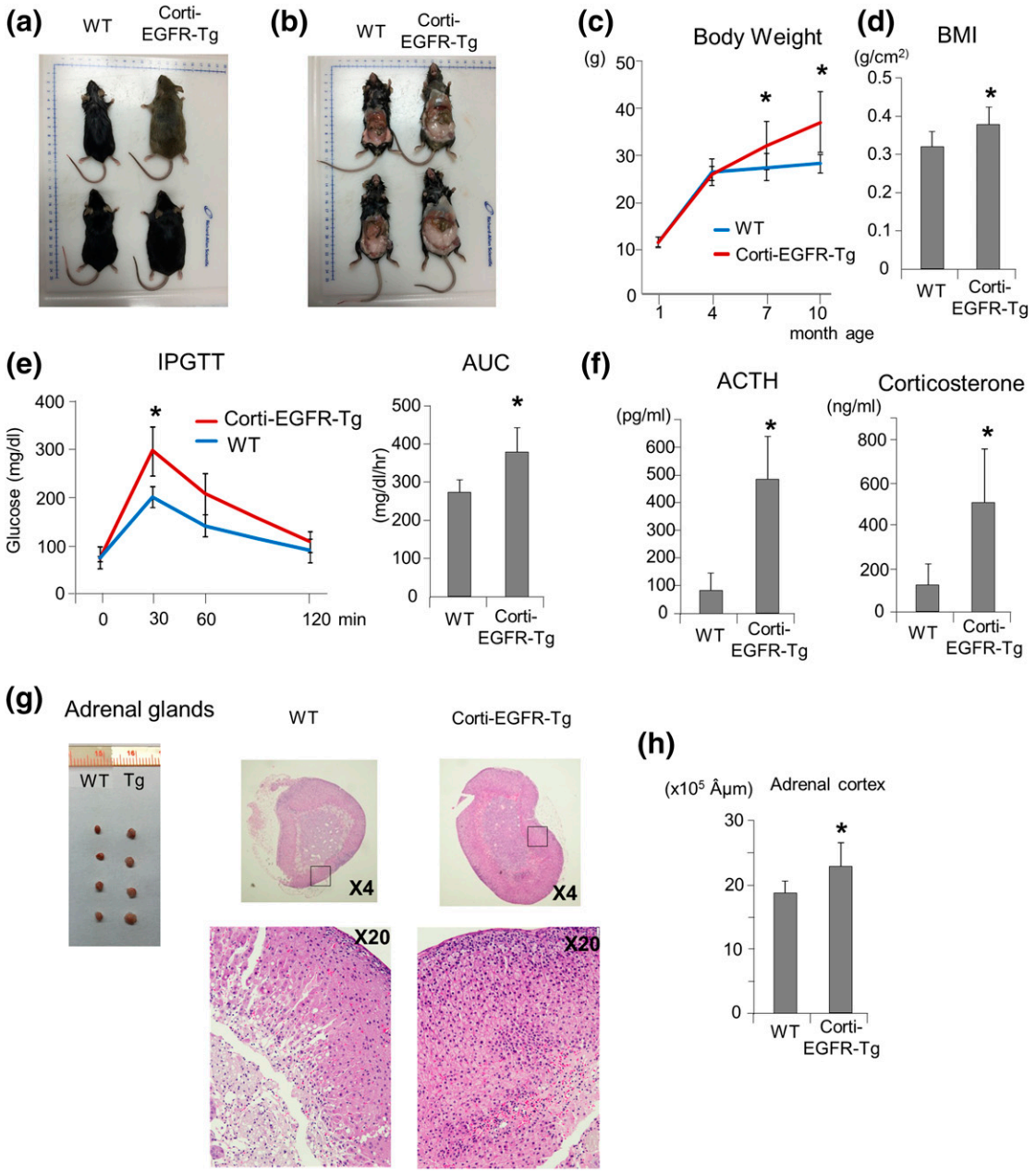

Figure 4. Tumor phenotypes of corti-EGFR-Tg mice. (a–b) Gross images of body size of WT and corti-EGFR-Tg (a) and gross images of abdominal fat of WT and corti-EGFR-Tg (b). (c–d) Body weight at 4, 7, 10 months [blue; WT (n = 15), red: corti-EGFR-Tg (n = 15)] (c), and comparison of body mass index at 10 months (d). (e) Glucose levels during IPGTT at 0, 30, 60, and 120 min in WT (n = 6) and corti-EGFR-Tg (n = 6) performed at 8 months. Blue indicates WT and red indicates corti-EGFR-Tg. (f) Comparison of plasma ACTH (left) and corticosterone levels (right) in corti-EGFR-Tg and WT mice. (g–h) Gross images of WT (left) and corti-EGFR-Tg (right) adrenal glands (g, left), and microscopic images (g, right), and average adrenal cortex depth ($\times 10^5$Âμm) (h).

promoter is enhanced by S337-E2F1 phosphorylation [17]. We therefore analyzed expression of E2F1 and pS337-E2F1 in pituitary-derived corti-EGFR-Tg mice [Fig. 5(g)] and found slightly increased total E2F1 levels and significantly increased pS337-E2F1 levels. Importantly, these changes were reversed by blocking EGFR signaling *in vivo* with gefitinib [Fig. 5(h)], suggesting that downstream EGFR signaling regulates pS337-E2F1 to induce POMC.

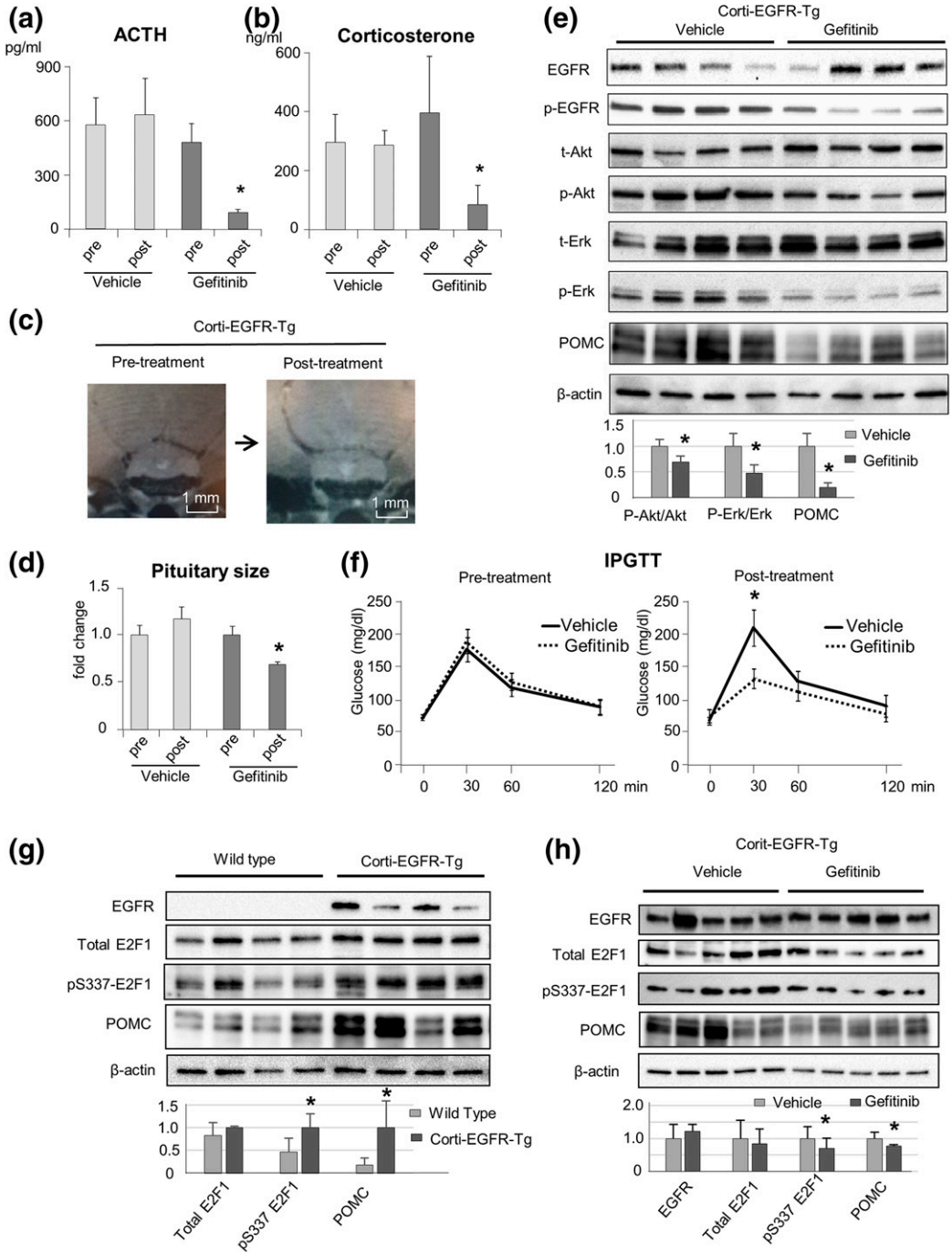

Figure 5. Effect of gefitinib treatment in corti-EGFR-Tg mice and detection of E2F1. (a–b) Plasma ACTH and corticosterone levels in corti-EGFR-Tg mice before (left) and after (right) gefitinib or vehicle treatment. (c) Micro-MRI of corti-EGFR-Tg pituitary of pre- (left) and post- (right) gefitinib treatment. (d) Comparison of pituitary sizes pre- and post- gefitinib or vehicle treatment. (e) Pomc, EGFR, *p*-EGFR, *p*-Akt, total (t)-Akt, *p*-Erk, and *t*-Erk expression analyzed by immunoblotting using pituitary tumors derived from corti-EGFR-Tg treated with vehicle (left) or gefitinib (right). Quantifications are shown in the graph. (f) Glucose levels during IPGTT at 0, 30, 60, and 120 min in corti-EGFR-Tg pre- and posttreatment with vehicle (n = 5) or gefitinib (n = 5). (g) Pomc, EGFR, total E2F1, and pS337-E2F1 expression analyzed by immunoblotting using pituitary tissues derived from WT (left) and pituitary tumors derived from corti-EGFR-Tg (right). Quantifications are shown in the graph. (h) Pomc, EGFR, total E2F1, and pS337-E2F1 expression analyzed by immunoblotting in corti-EGFR-Tg pituitary tumors after vehicle (left) or gefitinib treatment (right). Quantifications are shown in the graph. $*P < 0.05$.

To confirm the role of E2F1 in human pituitary tissue, we analyzed E2F1 expression in samples derived from pituitary ACTH-secreting tumors resected from Cushing disease patients. Cellular colocalization of pS337-E2F1 was detected with hPOMC [Fig. 6(a)]. Moreover, ACTH-secreting tumor cell cultures treated with the E2F1 inhibitor HLM006474 [25, 26] showed dose-dependent reduction in hPOMC messenger RNA (mRNA) and ACTH levels, but GH mRNA used as a negative control, was unaltered [Fig. 6(b)].

As corticotrophs are sensitive to cell-cycle changes [27], we also analyzed other cell-cycle factors related to pituitary tumorigenesis (Supplemental Fig. 4). Although no significant changes were seen in p27 or CDK2 in corti-EGFR-Tg mice, expression of cyclin E and cyclin A were slightly enhanced, and cyclin D was suppressed.

We next coimmunostained three normal human pituitary glands freshly obtained at autopsy with antibodies to POMC, E2F1, and pS337-E2F1 [Fig. 7(a)]. pS337-E2F1 was similarly detected and colocalized with hPOMC in normal pituitary glands [Fig. 7(a)] and total E2F1 also strongly colocalized with hPOMC [Fig. 7(b)]. We further coimmunostained serial sections with anti-POMC/anti-E2F1 [Fig. 7(c, left], antiprolactin/anti-E2F1 [Fig. 7(c, middle)], and anti-GH/anti-E2F1 sera [Fig. 7(c, right)]. Total E2F1 was highly expressed almost exclusively in POMC-expressing corticotrophs [Fig. 7(c, left)] but not in PRL-expressing lactotrophs [Fig. 7(c, middle)] or GH-expressing somatotrophs [Fig. 7(c, right)], further indicating that E2F1 is a potential corticotroph-specific target for pituitary Cushing disease.

3. Discussion

Here, we demonstrate EGFR/E2F1-induced corticotroph adenomas *in vivo* using an EGFR-Tg mouse model that recapitulates biochemical and pathologic features of human Cushing disease. Corti-EGFR-Tg mice show increased body weight, abdominal fat accumulation, glucose intolerance, and adrenal hypertrophy, all hallmarks of human Cushing disease. Of note, corticotroph adenoma growth was associated with hyaline and intense perinuclear keratin staining, histologically similar to Crooke's cell adenoma, a clinically aggressive pituitary Cushing disease subtype distinct from nonaggressive Cushing disease that manifests with higher mortality and recurrence [24].

Previously generated *rPomc* (–706/+64)–derived Tg mice tumors were of IL origin [28]. We found tumors with features suggestive of both an AL and IL origin. Modifying the *rPomc* promoter by adding the tandemized corticotroph specific factor NeuroD1 likely drove more corticotroph expression, enabling us to obtain tumors of AL origin in addition to those expected to be seen in the IL. Interestingly, IL-originating tumors also upregulate corticosterone and the HPA axis [28]. Both AL and IL upregulate the HPA axis, and corti-EGFR-Tg tumors therefore appear to be a suitable mouse model for hypercortisolism and Cushing disease.

We previously showed that EGFR transducers MAPK and Erk upregulate *rPomc* and that blocking EGFR signaling with gefitinib in AtT20 cells in xenografted mice but not in normal canine pituitary tissue suppresses ACTH/POMC [14], suggesting tumor-specific effects of gefitinib. Somatic USP8 14-3-3 mutations contributing to constitutive EGFR activity prevent ubiquitination-mediated EGFR degradation, leading to induced POMC in the presence of accumulated EGFR [15, 16]. Our findings suggest that EGFR activity is mediated by corticotroph-specific E2F1, and its association with aggressive tumors is consistent with previous findings of EGFR expression and abundance in aggressive pituitary tumors [29, 30].

How E2F1 pathways are induced through EGFR in corticotrophs is unclear. MAPK, JNK1, and p38 pathways are induced through EGFR [31] and regulate E2F1 activity in osteosarcoma [32] and keratinocytes [33]. EGFR may enhance pituitary E2F1-mediated transcription in pituitary cells, similar to that observed in melanoma [31–34].

E2F-mediated transcription is regulated by several cell-cycle proteins, and changes in cell-cycle regulation may be associated with pituitary corticotroph adenoma development [27]. The observed decrease in cyclin D and slight increase of cyclin E and cyclin A may be

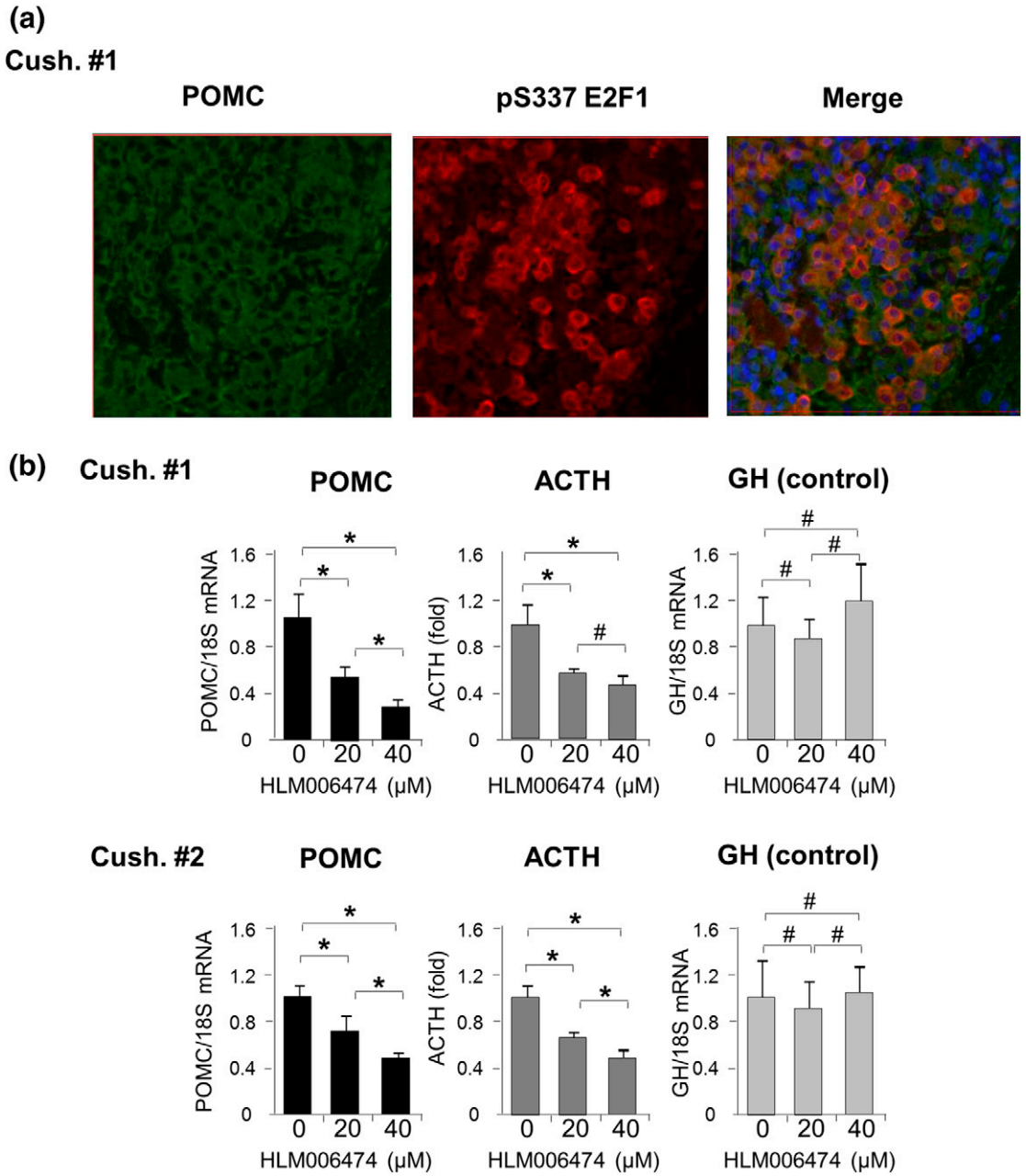

Figure 6. E2F1-mediated hPOMC regulation in human Cushing disease specimens. (a) Confocal images of human pituitary Cushing tumor specimen (Cush #1) immunostained with hPOMC (green), pS337-E2F1 (red), and 4′,6-diamidino-2-phenylindole (blue). To confirm POMC and pS337-E2F1 colocalization, a merged image is also depicted. (b) Primary cultures derived from two cultured pituitary Cushing tumor cells (Cush #1, 2) treated with HLM006474. hPOMC and human GH mRNA levels were analyzed by reverse transcription PCR and normalized with 18S ribosomal RNA. ACTH levels in culture medium were analyzed by RIA and reverse transcription PCR performed in triplicate. RIA values are from three sets of experiments, each assayed in duplicate tubes; values are mean ± SE. *$P < 0.05$; #no significant difference.

attributed to E2F1 activity, and previous reports have shown that E2F1 binds to both cyclin D and E promoters and upregulates cyclin E mRNA but not cyclin D [35]. By contrast, our Tg mice showed unchanged p27 and CDK2, whose role in corticotroph tumorigenesis has been suggested [36, 37].

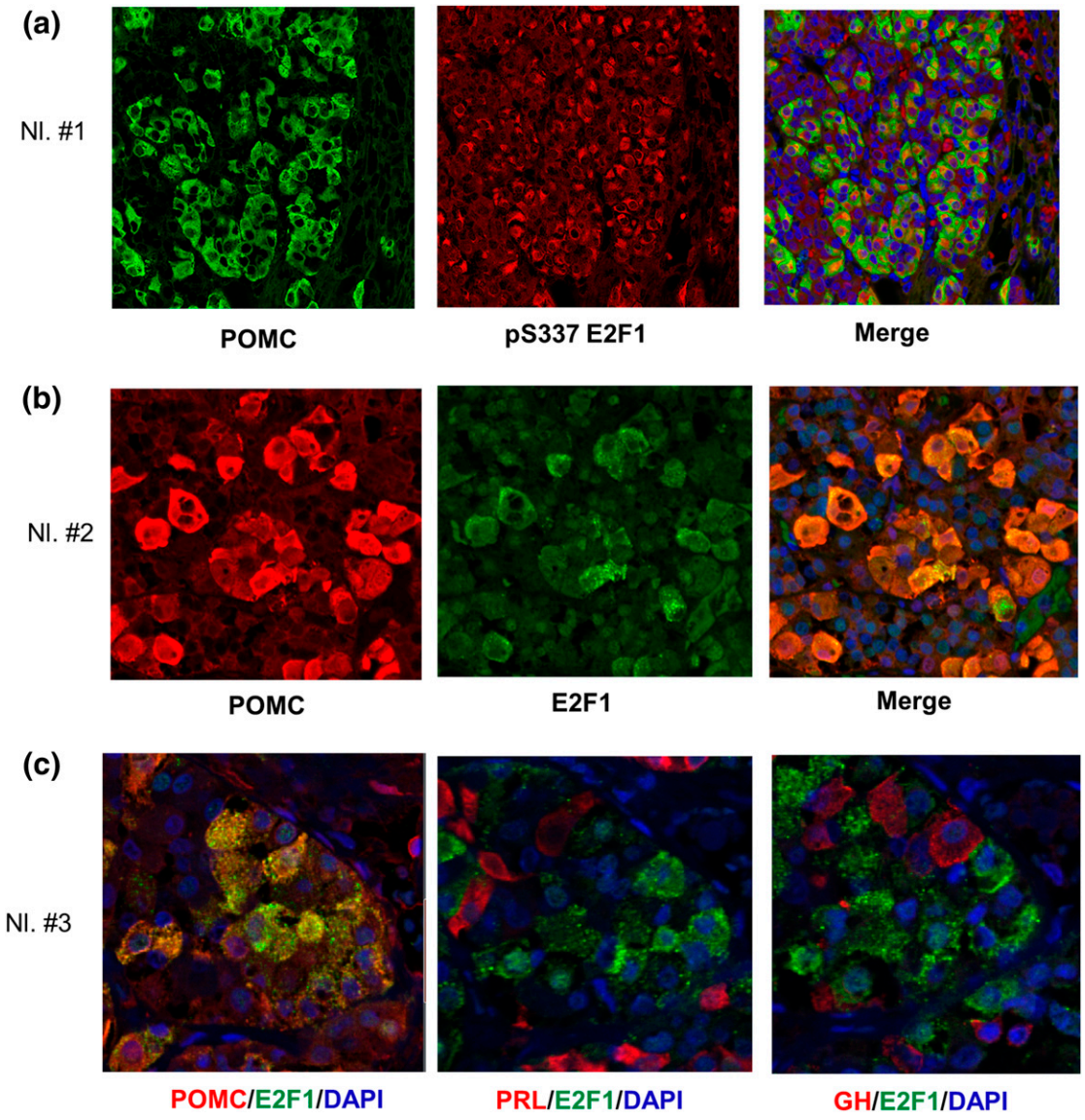

Figure 7. Corticotroph specific E2F1 expression in normal human pituitary tissue. (a) Confocal images of normal human pituitary specimen (Nl. #1) immunostained with hPOMC (green) and pS337-E2F1 (red). Nuclei are counterstained with DNA-specific dye 4′,6-diamidino-2-phenylindole (DAPI; blue). Representative pituitary gland depicted. (b) Confocal images of normal human pituitary specimen (Nl. #2) immunostained with hPOMC (red) and E2F1 (green). Nuclei are counterstained with DNA-specific dye DAPI (blue). A representative pituitary gland is depicted. (C) Confocal images of normal human pituitary specimen (Nl. #3) immunostained with E2F1 merged with hPOMC (left), human PRL (middle), or human GH (right), and DAPI. Three consecutive slide sections were assessed and representative pituitary sections depicted. Nl, normal.

In ectopic Cushing tumors, *hPOMC* promoter activity is also regulated by E2F1 [17], and E2F1 binding to the *hPOMC* promoter is enhanced by S337-E2F1 phosphorylation, whereas E2F inhibition suppresses POMC expression. Here, we show that E2F1 and pS337-E2F1 upregulate *POMC* gene expression in pituitary cells, and that E2F1 and pS337-E2F1 expression are, in turn, induced by EGFR.

Interestingly, E2F1 was preferentially expressed in corticotrophs rather than in somatotrophs or lactotrophs in normal human pituitary tissue. The role of selective E2F1 expression in nontumorous normal pituitary is not known. However, corticotroph cell differentiation is distinct from other

pituitary lineages [27], and cyclin E is expressed in nonproliferating, nondifferentiated pituitary precursor cells in the embryonic AL and is involved in pituitary development [38].

The evidence presented here shows that corticotroph EGFR signaling upregulates E2F1-mediated induction of the POMC and ACTH seen in Cushing disease and may play a role in corticotroph tumorigenesis. Blocking EGFR signaling leading to disrupted E2F1 regulation of POMC expression may be a potential therapeutic target for Cushing disease.

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