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Original Article

Functional cross-talk between cAMP and ERK1/2 pathway promotes proliferation of somatotropinomas in a B-Raf and C-Raf dependent manner

Wei Jiao, Yimin Huang, Wei Sun, Junwen Wang, Ting Lei

Department of Neurosurgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China

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Abstract: The cross-talk between cAMP and ERK1/2 pathway has been implicated in the proliferation of somatotropinomas. Both B-Raf and C-Raf are intricately involved in the cross-talk depending on cell-type specificity, however, it has not been illustrated in somatotropinomas. This study aimed to figure out the role of B-Raf and C-Raf in the proliferation of somatotropinomas mediated by the cross-talk. By employing human sommatotroph cells and a rat cell line (GH3), selective inhibitors of B- and C-Raf and knockdown with siRNAs were used in conjunction with forskolin to investigate effects on cell proliferation. In addition, effects on phosphorylation of ERK1/2 and CREB, levels of cyclinsD1 and D3, B-Raf and C-Raf activity, together with B-Raf/C-Raf binding were determined. Elevation of cAMP by exogenously added forskolin exerts significant stimulatory effects on somatotroph proliferation. Moreover, forskolin induced phosphorylation of ERK1/2 and CREB, and increased levels of cyclinsD1 and D3. These effects were attenuated by B-Raf and C-Raf inhibitors and by specific knockdown with siRNAs. Forskolin elevated B-Raf and C-Raf activity, and both molecules are not heterodimerized in somatotrophs. Results indicate that cAMP promotes proliferation of tumorous somatotrophs via cross-talk with the ERK1/2 pathway in a B-Raf and C-Raf dependent manner. In particular, heterodimerization of B-Raf and C-Raf does not appear to be necessary in this mechanism in somatotrophs.

Keywords: cAMP, B-Raf, C-Raf, proliferation, somatotropinomas

Introduction

Growth hormone (GH)-secreting somatotropinomas represent about 9-11% of all human pituitary adenomas and lead to acromegaly in adults and acromegalic gigantism in pre-puberty [1, 2]. Although benign, the insidious nature of acromegaly often results in delayed diagnosis and increased occurrence of cardiovascular, glycometabolic and respiratory complications [2-4]. Surgical resection remains the optimal treatment but adequate clinical and biochemical remission is achieved in only 50% and 75% of patients with micro- and macroadenomas, respectively [2, 3]. It is therefore desirable to have available additional pre- and post-operative medical therapies, especially for those patients in whom full remission is not achieved following surgery. Based upon a better understanding of the mechanisms by which GH secretion is controlled, some medical therapies have been developed in the form of the long acting somatostatin analogues, octreotide and lanreotride, which, like somatostatin, inhibit GH secretion. Although these analogues normalize serum GH levels in many cases, there remains a subset of about 25-30% of acromegalics who are resistant to these modes of therapy [5]. A deeper insight into the molecular mechanism involved in GH secretion and, perhaps more importantly, abnormal somatotroph proliferation could conceivably lead to development of novel and improved medical therapies.

Gsp oncogene referring to mutations of the alpha subunit of the stimulatory G protein gene has been found in about 30-40% of somatotropinomas, these mutations result in the activation of adenylyl cyclase and ultimately higher cyclic AMP (cAMP) levels, indicating that cAMP

may be a growth factor for somatotropinomas although the finer molecular mechanisms by which this occurs remains to be determined [6-8]. Nevertheless, previous studies using a variety of different cell types show that one target of cAMP is extracellular signal regulated kinase (ERK1/2) and that there is cross-talk between the two pathways [9-12]. The ultimate effect on proliferation depends upon cell type, with stimulatory, inhibitory and absent effects observed. In somatotrophs, the cAMP and ERK1/2 systems act together via cross-talk to induce proliferation [11, 13, 14]. As one key molecule in the ERK1/2 signalling pathway, the Raf family of protein kinases are intricately involved in the cAMP-ERK1/2 cross-talk system in many cell types that have been investigated, nonetheless, to date there have been no studies of whether the proteins contribute to pituitary somatotroph proliferation. The present study was thus designed to investigate the potential role of Raf proteins on somatotroph proliferation and to further confirm a cross-talk mechanism between the cAMP and ERK1/2 systems in this cell type. Studies focused on B- and C-Raf since it appears the A isoform is restricted to urogenital cells and is not involved in pituitary function [15].

Materials and methods

Cell cultures and reagents

GH3 rat pituitary cells were purchased from the China Infrastructure of Cell Line Resources (Beijing, China) and cultured in Ham's F10 medium containing 2.5% fetal bovine serum (FBS), 15% heat-inactivated horse serum and antibiotics (Gibco, Grand Island NY, USA).

Human somatotropinomas were obtained from 19 GH-secreting adenomas, all negative for *Gsp* mutations, screened as previously reported [14]. Tissues were enzymatically dispersed with collagenase (Sigma, Saint Louis MO, USA). Collected cells were maintained in minimum essential media (MEM) supplemented with 10% FBS and antibiotics (Gibco). To eliminate fibroblasts, antifibroblast microbeads (Miltenyi Biotechnology, Paris, France) were used according to the manufacturer's instructions. Informed consent was obtained from all individual participants included in the study.

Forskolin as stimulator of cAMP, 3-isobutyl-1-methylxantine (IBMX) as inhibitor of cAMP degradation, B-Raf kinase inhibitor SB590885, and C-Raf kinase inhibitor GW5074 were from Sigma, and prepared in DMSO (Sigma). The final concentration of DMSO was no more than 0.5% in extracellular solution, which has no confounding effects on cell growth.

Cell assays

Cell proliferation was determined by cell counting kit-WST-8 (CCK-8) assay (Dojindo, Kumamoto, Japan). Briefly, cells were seeded in 96-well plates (6×10^3 cells/well), serum-starved for 24 h, pretreated with or without increasing concentrations of SB590885 or GW5074 for 30 min, and then stimulated with 20 μM forskolin containing 0.3 mM IBMX and raf kinase inhibitor at indicated concentrations for 48 h. Finally, 10 μl CCK-8 solution was added to each well and incubated at 37°C for 4 h. The absorbance was then measured using a wavelength of 450 nm with a microplate reader.

DNA synthesis was assessed by 5-Ethynyl-2'-deoxyuridine labeling (EdU-labeling; RiboBio, Guangzhou, China). In brief, cells were seeded in 24-well plates (4×10^4 cells/well), serum-starved for 24 h, pretreated with 1 μM (GH3 cells) or 0.01 μM (primary cells) SB590885 or 10 μM GW5074, incubated with forskolin supplemented with 0.3 mM IBMX for 48 h, then with 10 μM EdU for 24 h (primary cultures) or 2 h (cell lines). After fixing in 4% paraformaldehyde, the cells were permeabilized with 0.5% Triton X-100. Hoechst 33342 were used to stain cell nuclei. Cells were visualized and photographed with a fluorescent microscope.

In separate experiments, GH3 cells were transfected with scramble, B-Raf or C-Raf small interfering RNA (siRNA) for 48 h before stimulation of forskolin for an additional 48 h. Then CCK-8 assay and EdU incorporation assay were performed.

B-Raf and C-Raf kinase activity assays

For the determination of B-Raf and C-Raf activity, serum-starved cells were exposed to forskolin for 5 min, then cell lysates with an equal amount of protein were selectively immunoprecipitated with corresponding antibody covalently coupled to protein A/G plus agarose beads (Beyotime, Haimen, China). The immunoprecipitates were incubated with inactive MEK1

(SignalChem, Richmond BC, Canada) in the presence of ATP and kinase buffer (Cell Signaling, Beverly MA, USA) for 30 min at 37°C, which allow immunoprecipitated active B-Raf or C-Raf to phosphorylate MEK1. The activity of B-Raf and C-Raf was confirmed by phosphorylation level of MEK1, which was measured by Western blot.

B-Raf/C-Raf binding assays

In short, starved cells were stimulated with or without forskolin for 5 min, B-Raf/C-Raf heterodimers were detected by immunoprecipitation with C-Raf antibody, followed by immunoblotting for B-Raf and vice versa.

Knockdown of B-Raf or C-Raf by siRNA transfection

siRNA targeting rat B-Raf mRNA (B-Raf siRNA, 5'-GUUCCACUGAUGUGUGUUATT-3'), C-Raf mRNA (C-Raf siRNA, 5'-GUGGACUGGAGUAAUAUCATT-3') and non-specific control siRNA sequence (scramble siRNA, 5'-UUCUCCGAACGUGUCACGUTT-3') were synthesized by Genepharma (Shanghai, China) and transfected into GH3 cells with Lipofectamine 3000 (Invitrogen, Carlsbad CA, USA) according to the manufacturer's protocol. Knockdown efficiency was assessed by western blot at 48 h after transfection.

Protein extraction and western blot

Cells were plated in 6-well plates (6×10^5 cells/well), after 24 h serum starvation, and then treated with indicated reagents for the desired periods. To detect ERK1/2 and CREB phosphorylation, human somatotroph cells and GH3 cells were stimulated with 20 μM forskolin for 5 min. For cyclinD1/D3 expression analysis, cells were stimulated with 20 μM forskolin for 3 h (primary cultures) or 1 h (GH3 cells). Protein was harvested using pre-cold RIPA buffer (Beyotime) with proteinase inhibitor cocktail (Sigma) for 30 min and centrifuged at 14,000 rpm for 20 min. Concentrations of the protein were determined with a BCA protein assay kit (Beyotime). For western blot analysis, a total of 30 ug of protein was subjected to 10% SDS-PAGE for electrophoresis and transferred to nitrocellulose filter membranes. After blocking in Tween-Tris buffered saline (TTBS) supplemented with 5% non-fat milk at room temperature for 1 h, the blots were incubated overnight at 4°C with primary antibodies. The blots were then washed with TTBS four times for 15 min each and incubated with the 1:2,000 diluted corresponding secondary goat anti-rabbit or goat anti-mouse antibody (ProteinTech, Chicago IL, USA) for 1 h at room temperature. After being washed again four times for 15 min with TTBS, signals were detected using an ECL chemiluminescence system (Applygen, Beijing, China). The results were analyzed by Image J software (http://rsb.info.nih.gov/ij/, Bethesda MD, USA).

The primary antibodies were obtained from the following sources: anti-B-Raf and anti-C-Raf antibodies were from Abcam (Cambridge, UK); anti-p44/p42 ERK1/2, anti-phospho-p44/p42 ERK1/2 (thr202/tyr204), anti-phospho-MEK1 (thr286), anti-phospho-CREB (ser133), anti-CREB, anti-β-actin, anti-cyclinD1 and anti-cyclinD3 antibodies were from Cell Signaling.

Statistical analysis

All results are represented as means ± standard derivation for 3 experiments and analyzed using SPSS 13.0 statistical software package (SPSS Inc., Chicago IL, USA). Significance between control group and forskolin-treated group in the B-Raf and C-Raf activity were determined using Student's t-test. Significance of other analyses was performed by One Way Anova followed by Dunnett's multi-comparison test. $P<0.05$ was taken to be statistically significant.

Results

Suppression of B-Raf and C-Raf attenuates forskolin-induced cell proliferation and DNA synthesis

Both human tumorous somatotrophs and rat GH3 cells were seeded in 96-well plates for CCK-8 assay and in 24-well plates for EdU incorporation assay. In this study, forskolin at the concentration of 20 μM was used as stimulator of cAMP. First of all, we identified whether 20 μM forskolin could exert proliferative effect on human tumorous somatotrophs and rat GH3 cells. Long-term treatment of both human (**Figure 1A**) and GH3 (**Figure 1B**) somatotrophs with forskolin for 48 hours significantly increased cell proliferation as assessed by

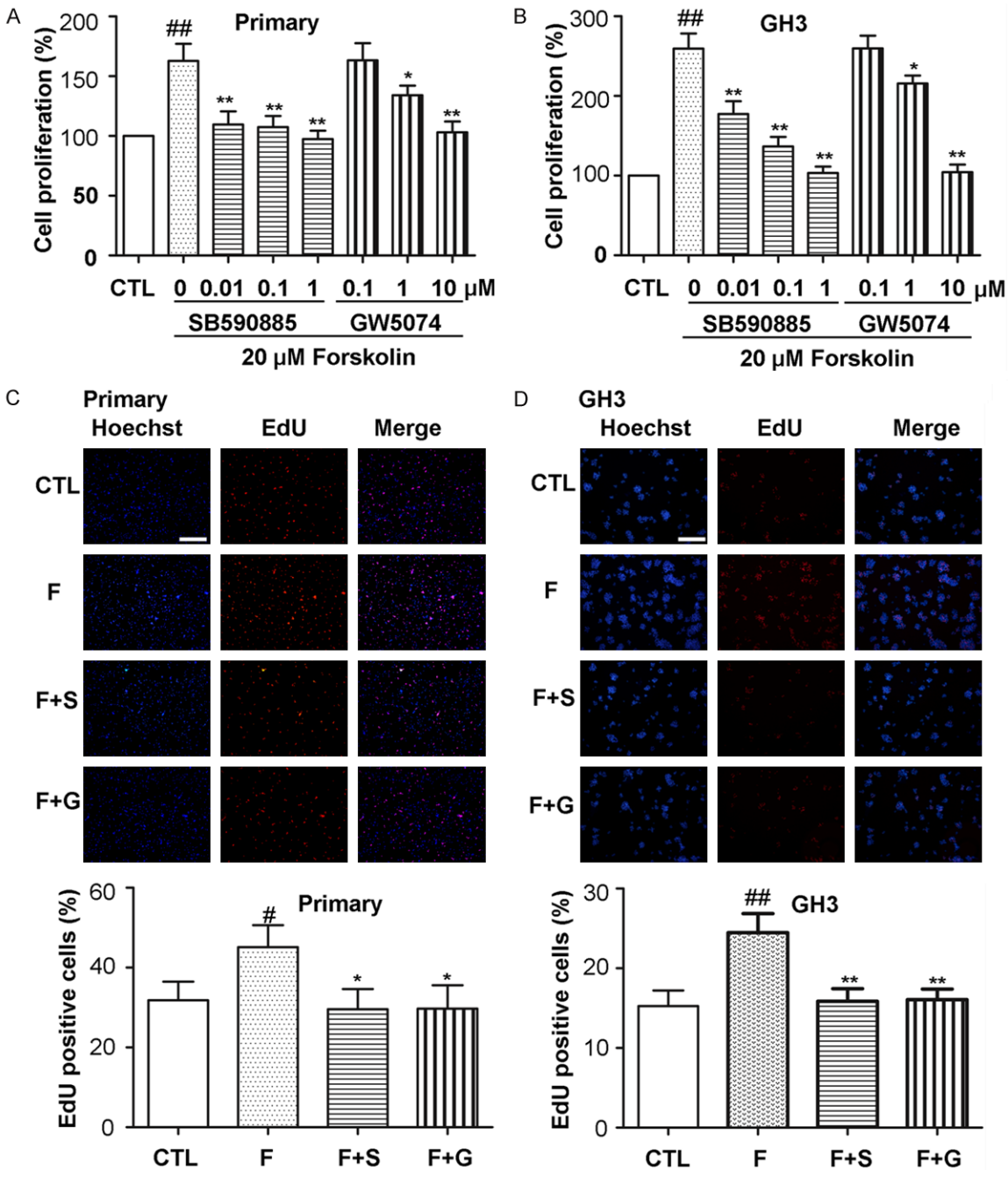

Figure 1. (A, B) Cell proliferation of human somatotroph primary cells (A) and GH3 cells (B), respectively, treated with 20 μM forskolin in the presence and absence of SB590885 (B-Raf inhibitor) or GW5074 (C-Raf inhibitor) at various concentrations for 48 h. Data were normalized to control values and expressed as percentage of control; (C, D) DNA synthesis of human somatotroph primary cells (C) and GH3 cells (D) treated with 20 μM forskolin combined with 0.01 μM SB590885 (primary cells), 1 μM SB590885 (GH3 cells) and 10 μM GW5074 for 48 h. *Upper:* Representative images of Hoechst 33342-stained nuclei (blue) and EdU immunofluorescence (red); *Lower:* Bar chart quantification of the percentage of EdU positive cells. Scale bars are 20 μm. *CTL:* Control; *F:* Forskolin; *S:* SB590885; *G:* GW5074. $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs.* control group; $^{*}P<0.01$, $^{**}P<0.01$ *vs.* forskolin-treated group.

CCK-8 tests by 163 ± 15% and 259 ± 20%, respectively. The B-Raf and C-Raf inhibitors, SB590885 (0.01, 0.1, 1 μM) and GW5074 (0.1, 1, 10 μM), respectively, reduced these stimulatory effects of forskolin in a concentration-dependent manner. It was noted that 0.01 μM

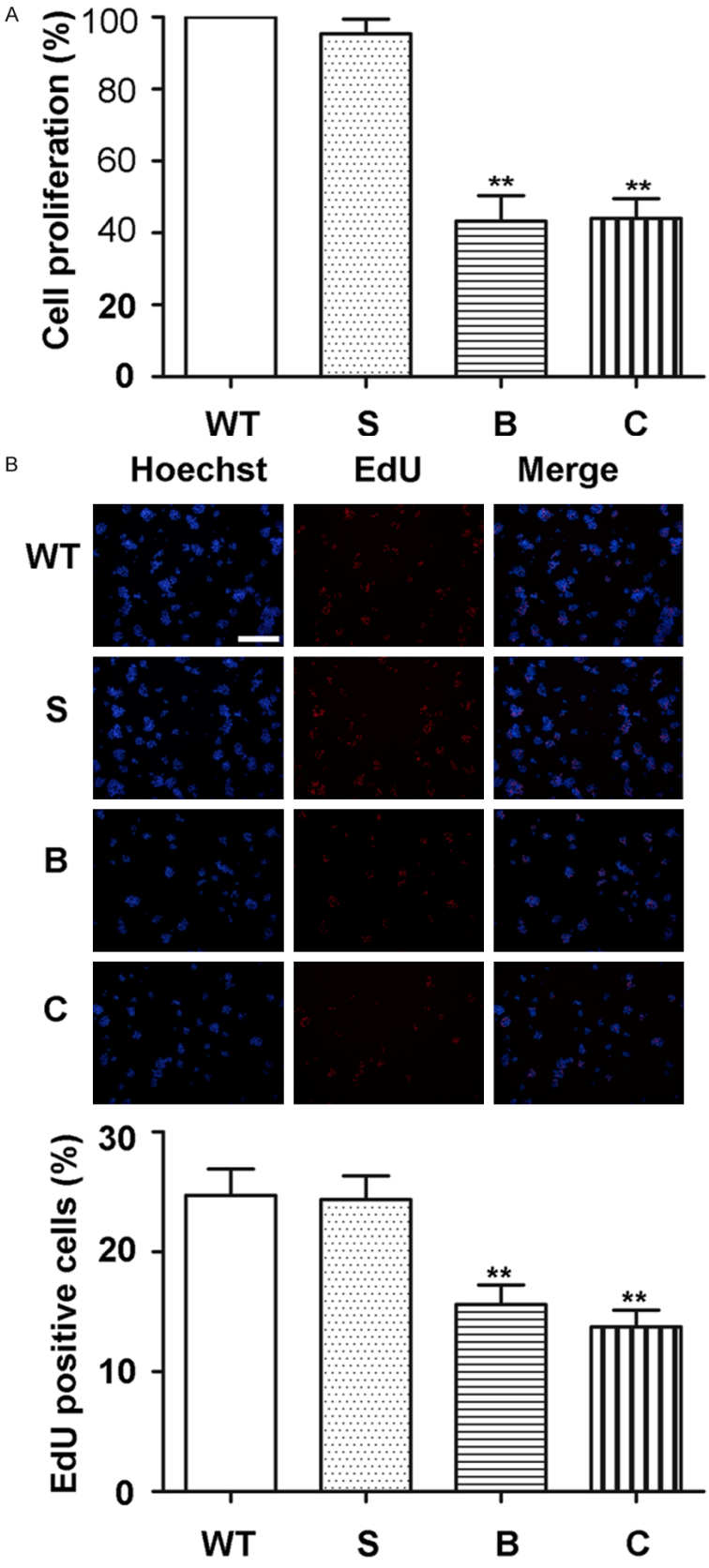

Figure 2. A. Cell proliferation, B. DNA synthesis of GH3 cells treated with 20 μM forskolin for 48 h following transfection with scramble, B-Raf or C-Raf siRNA. Data were normalized to wild type group values and expressed as percentage of wild type group in proliferation experiments. B. *Upper:* Representative images of Hoechst 33342-stained nuclei (blue) and EdU immunofluorescence (red); *Lower:* Quantification of the EdU positive cells. Scale bars are 20 μm. *WT:* Wild type (no RNA added); *S:* Scramble siRNA; *B:* B-Raf siRNA; *C:* C-Raf siRNA. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* wild type group.

SB590885 and 10 μM GW-5074 had similar effects to that of control group in human somatotrophs. For GH3 cells, the similar inhibitory effect of SB590885 and GW5074 commenced at 1 μM and 10 μM, respectively.

In addition, EdU as an indicator of DNA synthesis was used to test the effects of both inhibitors on the proliferation. Consistent with the findings of the CCK-8 assays, EdU uptake was increased by forskolin treatment from 30.8 ± 4.6% to 45.1 ± 5.5% in human somatotrophs (**Figure 1C**) and from 15.2 ± 1.9% to 24.5 ± 2.4% in GH3 cells (**Figure 1D**). Similarly, the forskolin-induced DNA synthesis was abrogated by 0.01 μM SB590885 and 10 μM GW5074 in primary cells, and by 1 μM SB590885 and 10 μM GW5074 in GH3 cells.

To get a better understanding of the role of B-Raf and C-Raf in the stimulatory effects mediated by forskolin, B-Raf and C-Raf knockdown cell line were generated by siRNA

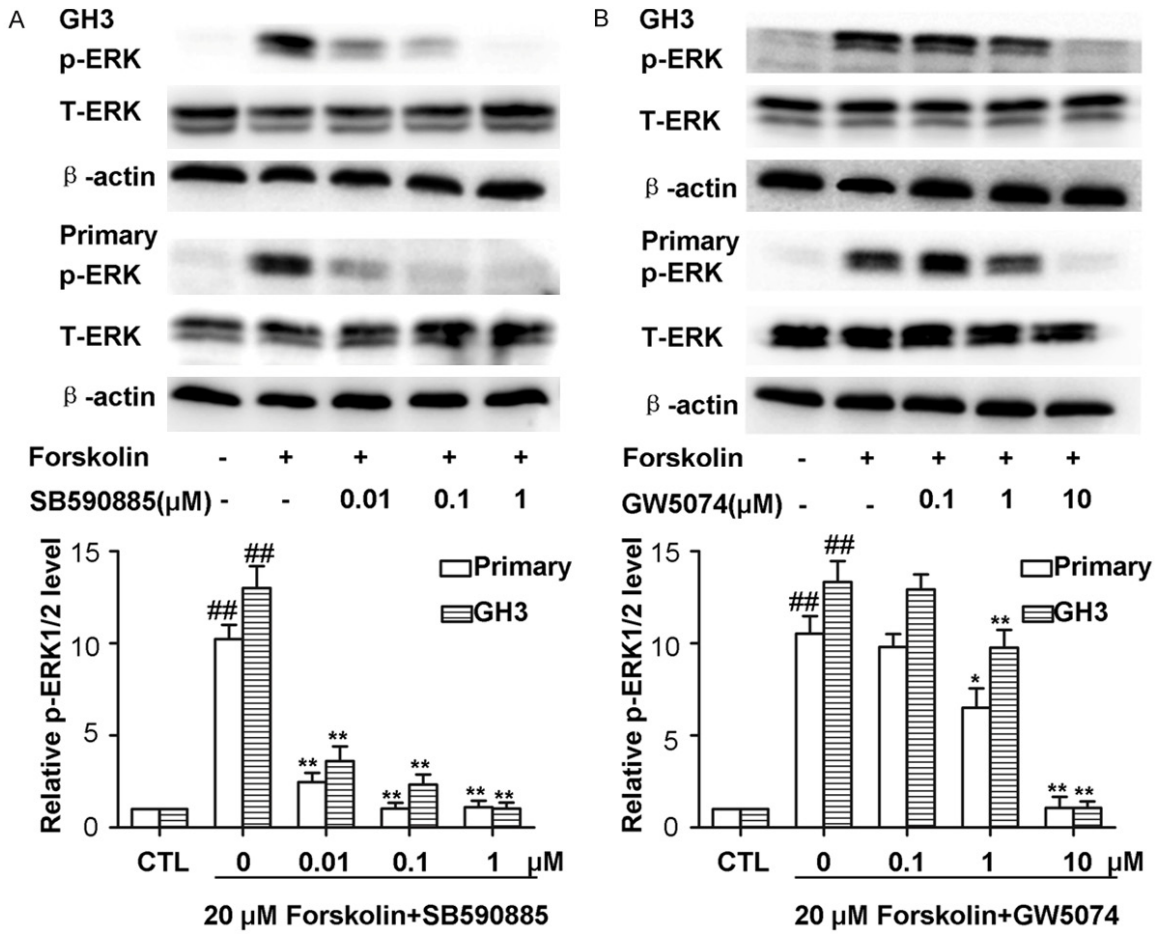

Figure 3. (A, B) Western blot showing the changes of phosphorylation levels of ERK1/2 (p-ERK1/2) in GH3 cells and human somatotroph primary cells treated with 20 μM forskolin alone or combined with SB590885 (A) and GW5074 (B) at various concentrations for 5 min. Data were normalized to control values and expressed as fold-increase. *Upper:* Western blots; *Lower:* Bar charts representing relative band intensity. *CTL:* Control; *F:* Forskolin; *S:* SB590885; *G:* GW5074. $^{\#\#}P<0.01$ *vs.* control group; $^{*}P<0.01$, $^{**}P<0.01$ *vs.* forskolin-treated group.

transfection. Surprisingly, individual knockdown of B-Raf and C-Raf with appropriately designed siRNA completely abolished rather than attenuated forskolin-induced elevation of cell proliferation (**Figure 2A**) and DNA synthesis (**Figure 2B**).

Suppression of B-Raf and C-Raf attenuates forskolin-induced phosphorylation of ERK1/2 and CREB, together with expression of cyclinsD1 or D3

Next, we examined the role of B-Raf and C-Raf in the ERK1/2 signaling mediated by forskolin. Phosphorylation state is the activated form of ERK1/2. Since phosphorylation and dephosphorylation is a fast reversible process, we examined the phosphorylation levels of ERK1/2 together with the downstream CREB after treatment of forskolin for 5 min. For the important role of cyclinD1 in the proliferation induced by ERK1/2, we assessed the change of cyclinD1 levels after stimulation of forskolin for 3 h in primary cells. On account of that cyclinD1 is undetectable in GH3 cells, we detected cyclinD3 levels after treatment of forskolin for 1 h. As clearly indicated, the elevation of p-ERK1/2 induced by forskolin in human somatotrophs and GH3 cells was attenuated by SB590885 (**Figure 3A**) and GW5074 (**Figure 3B**) dose-dependently. Similarly, the forskolin-induced phosphorylation of CREB and expression of cyclinD1 or D3 was abolished by the Raf inhibitors at higher concentrations in human somatotrophs (**Figure 4A**) and GH3 cells (**Figure 4B**).

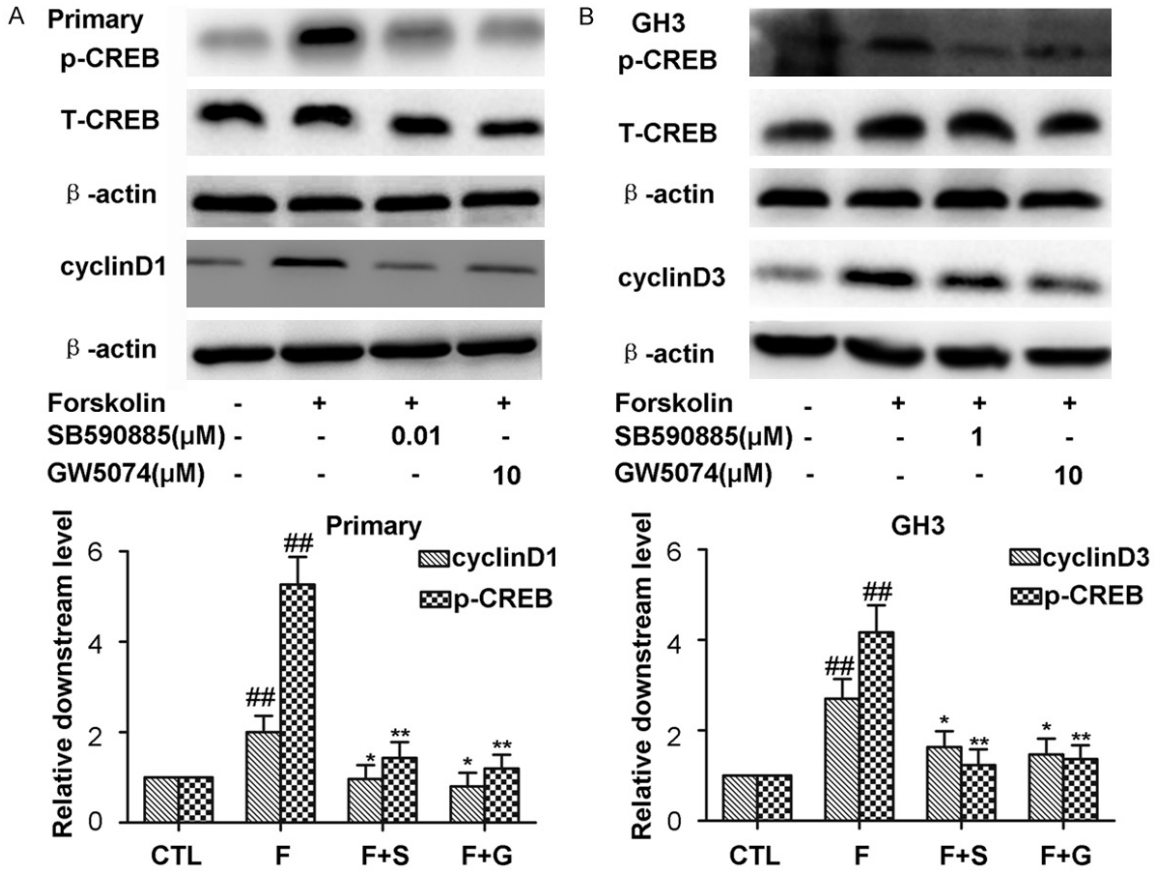

Figure 4. (A, B) Western blot showing the changes of phosphorylation levels of CREB (p-Creb) and the levels of cyclinsD1 or D3 in human somatotroph primary cells (A) and GH3 cells (B) treated with 20 μM forskolin alone or combined with 0.01 μM SB590885 (primary cells), 1 μM SB590885 (GH3 cells) and 10 μM GW5074 for 5 min. Data were normalized to control values and expressed as fold-increase. *Upper:* Western blots; *Lower:* Bar charts representing relative band intensity. *CTL:* Control; *F:* Forskolin; *S:* SB590885; *G:* GW5074. $^{\#\#}P<0.01$ *vs.* control group; $^{*}P<0.01$, $^{**}P<0.01$ *vs.* forskolin-treated group.

In B-Raf and C-Raf knockdown cell line, western blot analysis was performed to confirm that each siRNA oligonucleotide was specific for its target protein, the B-Raf siRNA only depleted B-Raf and C-Raf siRNA only targeted C-Raf. Individual knockdown of B-Raf (**Figure 5A**) and C-Raf (**Figure 5B**) significantly reduced forskolin-induced levels of cyclinD3 and phosphorylation of ERK1/2 and CREB in GH3 cells.

Forskolin increased B-Raf and C-Raf activity, both molecules do not heterodimerize

Finally, we measured B-Raf and C-Raf kinase activity after treatment with or without forskolin for 5 min, using MEK1 as Raf substrate. Forskolin increased B-Raf and C-Raf kinase activity in human somatotroph primary cells (**Figure 6A**) and GH3 cells (**Figure 6B**). To determine whether B- and C-Raf heterodimerize or act individually, immunoprecipitation of cell lysates was carried out. The immunoprecipitates extracted from human somatotrophs (**Figure 6C**) and GH3 cells (**Figure 6D**) were shown by Western blot analysis to contain only B-Raf and only C-Raf in B-Raf antibody and C-Raf antibody treated lysates, respectively. This demonstrates that B- and C-Raf function alone rather than in a heterodimer.

Discussion

cAMP, an ubiquitous intracellular second messenger, plays an pivotal role in the regulation of a series of cell functions including cell proliferation [16]. Both inhibitory and stimulatory effects of cAMP on cell proliferation may occur, depending upon cell type [17-19]. So far as pituitary adenomas are concerned, the growth promoting effect of cAMP seems to occur exclusively in

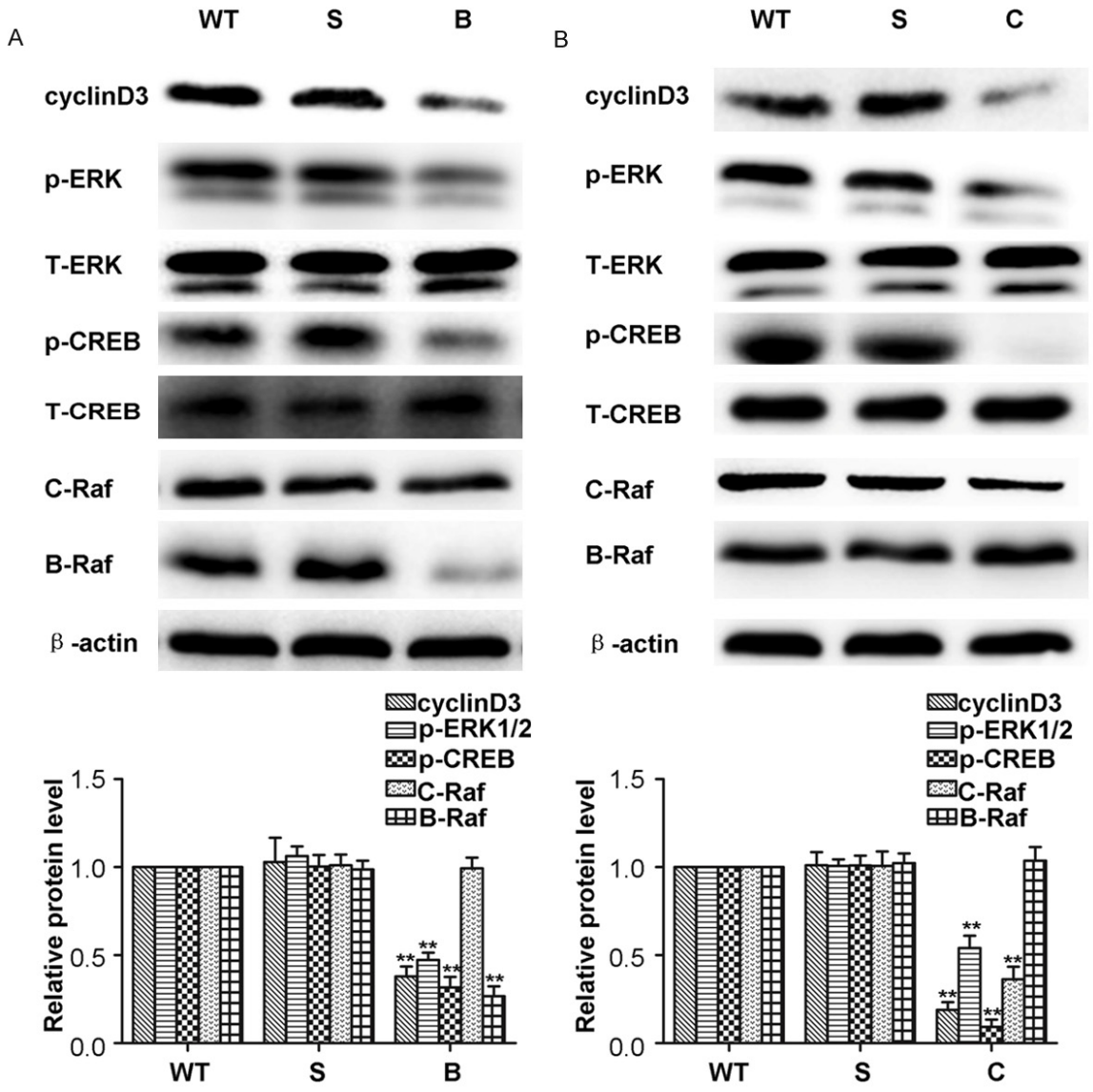

Figure 5. (A, B) Western blot showing the changes of phosphorylation of ERK1/2 (p-ERK1/2), CREB (p-Creb), and levels of cyclinD3, B-Raf and C-Raf in GH3 cells treated with 20 μM forskolin following transfection with scramble siRNA, B-Raf siRNA (A) or C-Raf siRaf (B). Data were normalized to wild type values and expressed as fold-increase. *Upper:* Western blots; *Lower:* Bar charts representing relative band intensities. *WT:* Wild type (no RNA added); *S:* Scramble siRNA; *B:* B-Raf siRNA; *C:* C-Raf siRNA. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* wild type group.

somatotropinomas relying on the phenomena that mutations triggering cAMP formation or signaling are associated with the occurrence of sporadic or familial acromegaly, rather than with other pituitary adenomas [8, 11, 16, 20]. Moreover, mice transgenic for the cholera toxin gene spliced to the GH gene promoter develop GH-secreting pituitary tumors [21].

Much progress is being made into elucidating the precise intracellular mechanisms by which cAMP can result in cellular proliferation. Most importantly, an interconnection with other transduction pathways, including ERK1/2, is becoming increasingly evident and it is clear that the multiple effects of cAMP is largely dependent upon cross-talk mechanisms [22-24]. To assess the effects of cAMP on ERK1/2 pathway, many kinds of stimulus of cAMP including analogs that selectively activate Epac or PKA were chosen [25-28]. In this study we chose forskolin as cAMP stimulator, in accordance with previous studies. The results of the present study provide strong evidence that

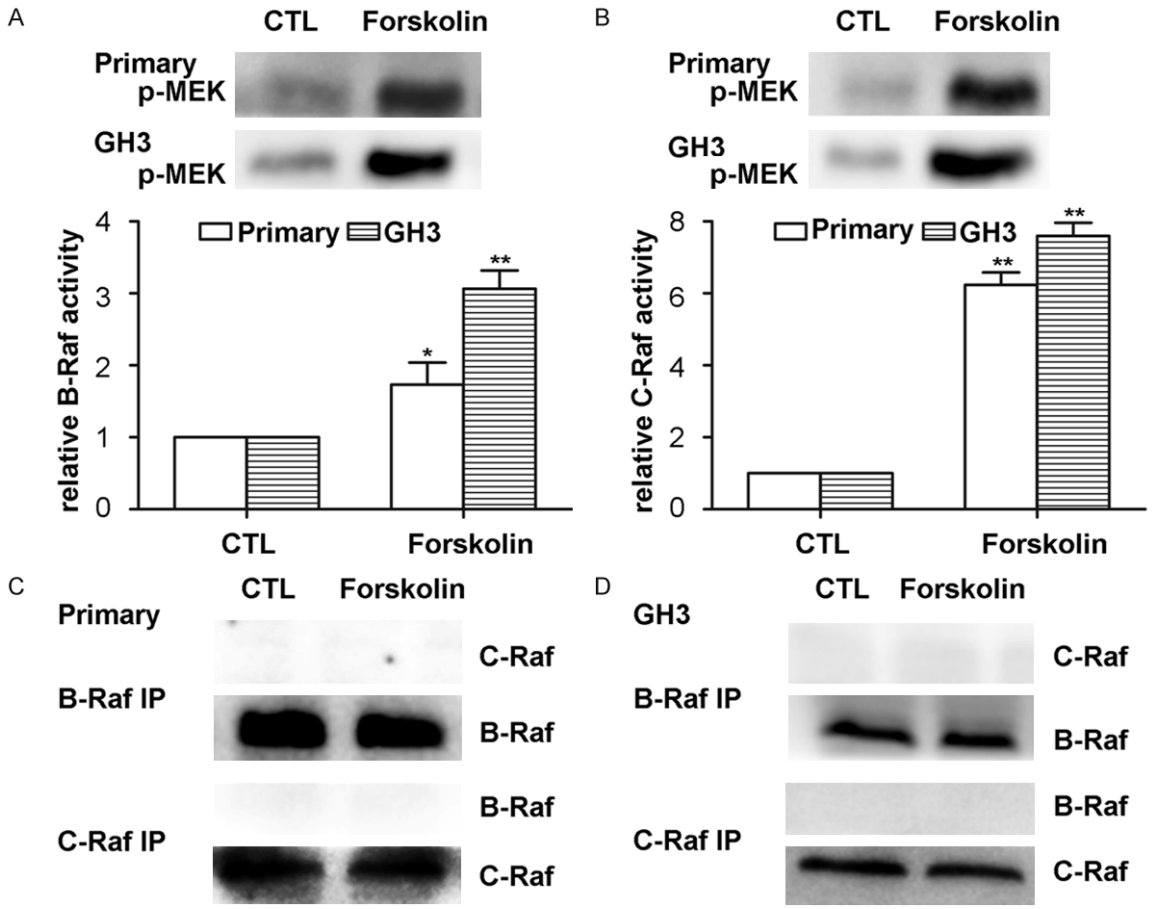

Figure 6. (A, B) Western blots showing the changes of B-Raf activity and C-Raf activity in human somatotroph primary cells (A) and GH3 cells (B) treated with 20 μM forskolin for 5 min. Raf activity was measured as MEK phosphorylation. Data were normalized to control values and expressed as fold-increase. *Upper:* Western blots; *Lower:* Bar charts representing relative band intensities. (C, D) Western blots demonstrating B-Raf/C-Raf binding assay in human somatotroph primary cells (C) and GH3 cells (D) treated with 20 μM forskolin for 5 min. $^{*}P<0.05$, $^{**}P<0.01$ vs. control group.

cAMP exerts proliferative effects in tumorous somatotrophs (both human and rat) via the ERK1/2 pathway.

Raf kinases are vital members of ERK1/2 pathway and comprise the members A-, B- and C-Raf [29]. Although with high sequence homology, the isoforms regularly exert non-overlapping functions. Raf proteins differ in tissue distribution, A-Raf is predominantly expressed in urogenital tissues, B-Raf is extensively abundant in neural tissues and C-Raf is ubiquitously expressed [15, 22]. A large variety of differing mechanism and ultimate effects of cAMP-ERK1/2 systems has been described, depending upon the cell type. Some research studies suggest that cAMP triggers inhibition of ERK1/2 via C-Raf, whilst inducing ERK1/2 activation via B-Raf [30, 31]. In PC12 and thyroid cells, cAMP activates ERK1/2 through B-Raf [32], conversely, cAMP induces activation of ERK1/2 but inhibits B-Raf kinase in HL-60 cells [33]. In neonatal rat cardiomyocytes, cAMP evokes activation of the ERK1/2 through C-Raf [34], yet exerts a negative effect on ERK1/2 pathway via C-Raf in vascular smooth muscle cell [19]. Because of this great variety of differing mechanisms, the present study was designed to examine in depth the potential role and mode of action of B- and C-Raf kinases specifically in tumorous human and rat somatotrophs. It appears that in this cell type, as shown using two different methods of inhibiting Raf activity, that both B- and C-Raf play a stimulatory role in the cAMP-ERK1/2 system. The effects of forskolin on cell proliferation and ERK1/2 signal-

ing were attenuated by pretreatment with SB590885 and GW5074. CyclinsD1 or D3, as a key regulator of G1 phase progression, has been proved to be cAMP dependent and correlated with ERK1/2 activity [11, 35]. CREB, as a central substrate of ERK1/2, plays an important role in the initiation and regulation of stimulated cellular proliferation [36, 37]. The role of B-Raf and C-Raf was further confirmed by the changes of the protein levels of phospho-CREB, cyclinsD1 or D3, which was increased by cAMP and decreased when pretreated with corresponding inhibitors and siRNA transfection.

At the same time, we detected the effects of cAMP on B-Raf and C-Raf kinase activity, the results showed that activity were elevated by cAMP. The activation of C-Raf needs a complex series of changes in phosphorylation of different sites which can lead to either activation or deactivation of its kinase activity. Activation is achieved by phosphorylation at sites including Ser338, Ser494, Tyr341 and Thr491, or dephosphorylation at Ser259 [15, 19]. These aspects of C-Raf activity were not investigated in the present reported experiments and further studies are required to determine if the same phosphorylations occur in somatotrophs leading to activation of C-Raf.

In view that B-Raf and C-Raf in some types of cell can form heterodimers which possess highly increased kinase activity in comparison with the individual subunits alone [38, 39], we further assayed B-Raf/C-Raf binding with immunoprecipitation. The results showed, surprisingly, that B-Raf and C-Raf did not co-precipitate showing that dimerization does not occur in somatotrophs. This is in contrast to that shown in Hela cells and for HEK293 cells [40, 41]. Nevertheless, the results are in agreement with the system shown to exist in liver cysts [17].

The present study may have implications for development of novel modes of medical therapy for acromegaly since they reveal new potential targets such as B-Raf and C-Raf. To date, the long-acting somatostatin analogues, octreotide and lanreotide, are available. By binding to somatostatin receptors subtypes 2 and 5, these somatostatin analogues inhibit adenylyl cyclase activity and ERK1/2 phosphorylation, thus mediating their antiproliferative effects [5, 42]. For patients with incomplete resection by surgery, somatostatin analogues are an important choice of therapy. Unfortunately, for unknown reasons, somatostatin analogues normalizes serum GH levels in only about 38% of patients, whilst 30% of acromegalic patients are completely refractory to the medical therapy [43]. With the respect of the role of B-Raf and C-Raf in the ERK1/2 pathway, as shown by the present study, therapeutic targeting both kinases may prove to be a potential additional strategy for clinical management of somatotropinomas, especially since it would be theoretically possible to block the mitotic-inducing activity of B- and C-Raf. Indeed, it has been proposed that Raf inhibitors, including SB5900885 and vemurafenib, could be used to treat papillary thyroid cancer and papillary craniopharyngioma respectively [44, 45]. And there is a theoretical role of siRNAs in gene therapy [46, 47]. It is conceivable that such novel therapeutic approaches could be combined with localized delivery systems using nanocarriers consisting of biodegradable materials [48].

In conclusion, we show that selective inhibition of B-Raf and C-Raf using kinase inhibitors and corresponding siRNA transfection significantly attenuates proliferative effects and ERK1/2 activation mediated by cAMP. These results demonstrate that cAMP exerts stimulatory effects on human and rat somatotroph proliferation in a B- and C-Raf dependent manner.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Ting Lei, Department of Neurosurgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Avenue, Wuhan, Hubei, China. Tel: +86-27-8366-5262; Fax: +86-27-8366-5262; E-mail: neurotl@163.com

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