Effects of sorafenib and an adenylyl cyclase activator on in vitro growth of well-differentiated thyroid cancer cells

Aya Sawa¹, ², Tomohiro Chiba¹, Jun Ishii¹, Hiroyuki Yamamoto¹, ⁴, Hisato Hara³ and Hiroshi Kamma¹

¹ Department of Pathology, School of Medicine, Kyorin University, Mitaka-shi, 181-8611, Japan
² Department of Breast and Endocrine Surgery, University of Tsukuba Hospital, Tsukuba 305-8576, Japan
³ Department of Breast and Endocrine Surgery, Faculty of Medicine, University of Tsukuba, Tsukuba 305-8575, Japan
⁴ Division of Nephrology and Endocrinology, The University of Tokyo, Tokyo 113-8655, Japan

Abstract. Well-differentiated thyroid carcinomas have driver mutations involving growth factor receptor-tyrosine kinases (RTKs) or their intracellular signaling pathway, that is, the mitogen-activated protein kinase (MAPK) pathway. Sorafenib is a multikinase inhibitor of RTKs and the MAPK pathway and has recently been used for the treatment of unresectable well-differentiated thyroid carcinoma. In normal thyroid follicular cells, stimulation of the thyroid-stimulating hormone (TSH) receptor activates the cyclic adenosine monophosphate (cAMP) pathway and promotes cell growth as well as hormonal secretion. However, an adenylyl cyclase (AC) activator, forskolin, has been reported to suppress the growth of thyroid carcinoma cells. To clarify the roles of the MAPK and cAMP pathways in proliferation of well-differentiated thyroid carcinoma cells, we compared the effects of sorafenib and forskolin in in vitro models. Sorafenib inhibited constitutive activation of the MAPK pathway, cyclin-dependent kinase 4 (CDK4), and phosphorylated retinoblastoma protein (RB) in 3 well-differentiated carcinoma cell lines, but it did not show sufficiently effective suppression of cell growth. forskolin significantly suppressed the growth of all 3 cell lines and also activated the cAMP pathway and inhibited expression of cyclin D1. Our results suggest that activation of the cAMP pathway could be more potent than activation of the MAPK pathway in suppressing proliferation of well-differentiated thyroid cancer cells. We postulate that the AC activator suppresses growth of thyroid carcinoma cells through undetermined mechanisms.

Key words: Well-differentiated thyroid cancer, Sorafenib, Forskolin, cAMP pathway, MAPK pathway

THYROID CARCINOMA derived from the follicular epithelium is histopathologically classified into 4 types: papillary, follicular, poorly differentiated, and undifferentiated (anaplastic). The former two are well-differentiated carcinomas [1]. It is well known that genetic alterations play a role in the development of papillary and follicular carcinomas: BRAF mutations, RET/PTC rearrangements, and RAS mutations have been reported in papillary carcinoma [1-3], and RAS mutations and PAX8-PPAR gamma rearrangements have been reported in follicular carcinoma [1]. These genetic mutations are often found in cancers of other organs; for example, the BRAF V600E mutation is frequently found in malignant melanoma, colorectal cancer, and lung cancer [4-6]. These mutations drive constitutive activation of receptor tyrosine kinases (RTKs) and their downstream mitogen-activated protein kinase (MAPK) pathway. The MAPK pathway is a transphosphorylation cascade of RAS-RAF-MEK-ERK1/2 (MAPK). Despite genetic alterations involved in RTKs and the MAPK pathway, well-differentiated thyroid carcinomas show lower mitogenic activity than do cancers of other organs bearing identical genetic mutations. Consistently, well-differentiated thyroid cancer has an exceptionally better prognosis than those of other cancers: the 10-year survival rate for papillary thyroid carcinoma is higher than 90% [1].

Recently, targeted therapies are being used for the treatment of thyroid cancer. The Japanese medical insurance system approved coverage of sorafenib for treatment of unresectable well-differentiated thyroid carcinoma in 2014, and of lenvatinib for treatment of all unresectable thyroid carcinomas, in 2015. Most molecularly targeted drugs suppress tumor cell
proliferation and neovascularization by inhibiting both RTKs, such as RET, vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF), and their downstream signaling pathway, that is, the MAPK pathway [7–11]. Especially, sorafenib is a multitarget inhibitor that suppresses various types of RTKs and the following MAPK pathway [7, 9].

The proliferation and function of normal follicular cells are physiologically regulated by thyroid-stimulating hormone (TSH), which works in homeostatic control of thyroid hormone secretion. In Graves disease, continuous stimulation of TSH receptors with autoantibodies induces proliferation of follicular cells and causes thyroid swelling (“goiter”). The TSH receptor is a G protein-coupled receptor that activates AC. AC synthesizes cyclic AMP (cAMP), which is involved in various steps of intracellular signaling pathways (cAMP pathway). Clinically, the activation of the cAMP pathway by TSH is considered to induce cell proliferation, and therefore, TSH-suppressive therapy is applied for thyroid carcinomas [12, 13]. However, in vitro studies have shown that the AC activator forskolin suppresses proliferation of thyroid cancer cell lines. As regards the mechanism of growth suppression, the activated cAMP pathway has been reported to cause reduced phosphorylation of the MAPK pathway [14–16]. On the other hand, it has also been reported that the activated cAMP pathway directly inhibits the cell cycle by suppressing the phosphorylation of cyclin-dependent kinase 4 (CDK4) [17], although this is still a matter of dispute.

The purpose of this study was to clarify the relationship between the MAPK pathway and the cAMP pathway in in vitro growth of well-differentiated thyroid carcinoma cells. We compared the inhibitory effects of sorafenib and forskolin on proliferation of well-differentiated thyroid cancer cell lines and attempted to reveal the differences in their mechanisms.

Materials and Methods

Cell lines, cell culture, and cell growth assays

We used 3 human differentiated thyroid cancer cell lines, TPC-1, KTC1, and WRO [18, 19]. The TPC-1 and KTC1 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA), and the WRO cells, in RPMI-1640 (Sigma-Aldrich) with 10% FBS, supplemented with 50 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich) at 37°C and 5% CO2.

For the cell growth assays, 1,500 cells per well were seeded into 96-well plates and cultured for 4 to 5 hours. The cells were then treated with sorafenib (ChemScene, Monmouth Junction, NJ, USA) and forskolin (Wako Pure Chemical Industries, Osaka, Japan) at the indicated concentrations in appropriate cell culture media supplemented with 5% FBS for 24, 48, 72, and 96 hours [14]. Cell viability assays were performed with WST-8 as described previously (Cell Counting Kit 8; Dojindo Laboratories, Kumamoto, Japan) [20–22]. Absorptions at a wavelength of 450 nm were measured with a microplate reader (Bio-Rad Model 680 Microplate Reader; Bio-Rad Laboratories, Hercules, CA, USA).

It has been reported that a 3-dimensional (3-D) cell culture system can simulate in vivo conditions better than can a 2-D culture system [23–25]. TPC-1, KTC1, and WRO cells were seeded into a 96-well spheroid culture plate (EZSPHERE SP microplate, 96-wells, 4,860-900SP; AGC Techno Glass, Shizuoka, Japan) at 4 × 10³ cells/well. The cells were seeded in the plate for 6 hours. The cells were then treated with DMSO only (control), 10 μM sorafenib, 10 μM forskolin, and a combination of 10 μM sorafenib and 10 μM forskolin. After 96 hours of treatment, cell proliferation was evaluated using the WST-8 assay. The cultured cells were photographed using a light-microscope (IX71; Olympus, Tokyo, Japan), and the taken photographs were analyzed using an image analysis system (cellSens Standard 1.3; Olympus). The cell proliferation of each group was compared with that of the control.

Immunoblot analysis

Immunoblot analyses were carried out according to previously reported methods [14, 15, 26]. Briefly, TPC-1, KTC1, and WRO cells in cell culture media with 10% FBS were treated with vehicle (dimethyl sulfoxide, DMSO) (Sigma-Aldrich), 10 μM forskolin, and/or 10 μM sorafenib dissolved in DMSO for 30 minutes or 24 hours. The cells were harvested in a lysis buffer (50 mM Tris/HCl [pH 6.8], 150 mM NaCl, 1% Triton-X100, PhosStop [Roche, Mannheim, Germany], and cOmplete ULTRA tablets EDTA-free [Roche]) as described previously. Then, 5 μg proteins from each treatment were subjected to normal SDS-PAGE with 8% or 12% polyacrylamide gels. After separation by SDS-PAGE, the protein samples were transferred onto
a polyvinylidene difluoride (PVDF) membrane (Pall Corporation, East Hills, NY, USA) or nitrocellulose membrane (Amersham Protran 0.2 um NC, 10600001; GE Healthcare Life Sciences, Pittsburgh, PA, USA). The membranes were blocked in 5% or 10% skim milk powder in Tris-buffered saline (TBS) with 0.5% Tween-20 (TBS/T) for 30 minutes. The membranes were soaked with the appropriate primary antibodies at 4°C overnight: anti-phospho-p44/42 ERK1/2 (MAPK) (Thr202/Tyr204) (D13.14.4E) XP rabbit monoclonal antibody (1:1,000 dilution; Cell Signaling Technology, Beverly, MA, USA); anti-ERK1 (K-23) rabbit polyclonal antibodies (sc-94, 1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-cyclin D1 rabbit monoclonal antibodies (413531, 1:200 dilution; Nichirei Biosciences, Tokyo, Japan); anti-phospho-AKT (Ser 473) (D9E) XP rabbit monoclonal antibodies (9271, 1:2,000 dilution; Cell Signaling Technology); anti-AKT antibodies (9272, 1:1,000 dilution; Cell Signaling Technology); anti-CDK4 (D9G3E) rabbit monoclonal antibodies (12790, 1:1,000 dilution; Cell Signaling Technology); anti-phospho-human retinoblastoma protein (RB) (T826) (EPR5351) rabbit monoclonal antibody (ab133446, 1:2,000 dilution; Abcam, Cambridge, MA, USA); anti-RB mouse monoclonal antibodies (554136, 1:250 dilution; BD Pharmingen, San Diego, CA, USA); and mouse monoclonal anti-β-actin (1:3,000 dilution; Sigma-Aldrich). The membranes were further incubated at room temperature for 1 hour with peroxidase-labeled secondary antibodies (anti-rabbit IgG, HRP-linked whole Ab [from sheep] and anti-mouse IgG, HRP-linked whole Ab [from sheep], diluted 1:5,000; GE Healthcare Life Sciences). Immunogenic bands were visualized using an enhanced chemiluminescence reagent (Western Lightning Plus-ECL; Perkin-Elmer, Waltham, MA, USA) and an ImageQuant LAS-4000 mini imaging system (GE Healthcare Life Sciences).

Cell cycle analysis
For the cell cycle analysis, 2 × 10^5 cells per well were seeded into 6-well plates and cultured for 4 hours. The cells were treated with 10 μM forskolin, and/or 10 μM sorafenib dissolved in DMSO in appropriate cell culture media supplemented with 5% FBS for 96 hours and then harvested and fixed with ice-cold 70% ethanol. The cells were incubated with RNase A and propidium iodine (Muse Cell Cycle Kit, MCH100106; Merck Millipore, Frankfurter Strabe, Darmstadt, Germany) for 30 minutes at room temperature in the dark and examined for the cell cycle distribution by means of flow cytometry (Muse Cell Cycle Analyzer; Merck Millipore).

Statistical analysis
For the cell proliferation study, in vitro experiments for each drug treatment were performed in 3 or 4 wells, and the results were expressed as means and standard deviations (SD). Two-way ANOVA was used for the statistical analysis using a statistical package (GraphPad Prism 6; GraphPad Software, La Jolla, CA, USA). For all statistical tests, probability values of less than 0.05 were considered to be significant.

Results
Effects of sorafenib and forskolin on the growth of 3 thyroid cancer cell lines
The effects of sorafenib, forskolin, and their combination treatment were compared among the 3 well-differentiated thyroid cancer cell lines in a time-course-dependent manner (Fig. 1). TPC-1, KTC1, and WRO cells were seeded in 96-well plates and cultured for 24, 48, 72, and 96 hours with 10 μM sorafenib and/or 10 μM forskolin. Their proliferation activities were estimated with the WST-8 assay and expressed in values relative to the starting value (0 hour) (Fig.1). Sorafenib treatment tended to suppress the proliferation of the TPC-1, KTC1 and WRO cells within 96 hours (P < 0.05). Forskolin treatment more strongly suppressed the proliferation of the 3 cell lines within 72 and 96 hours than did sorafenib alone (P < 0.05). The effect of the combination treatment with sorafenib and forskolin was almost the same as that of forskolin alone and suppressed the proliferation of the 3 cell lines.

Effect of sorafenib and forskolin on 3-D cell growth
We seeded the 3 thyroid carcinoma cell lines in low-attachment spheroid-forming culture plates. The WRO cells could successfully produce spheroids within 96 hours of culture, whereas the TPC-1 and KTC1 cells failed to do so, because the proliferation of the TPC-1 and KTC1 cells was very slow. After spheroid formation, we treated the WRO cells with or without 10 μM sorafenib and 10 μM forskolin. Sorafenib treatment showed mild, but statistically insignificant, suppression of the WRO cells (Fig. 2). Forskolin treatment significantly suppressed the spheroid growth.
Fig. 1  Effects of sorafenib with forskolin

(A) TPC-1, (B) KTC1, and (C) WRO cells were treated with 10 µM sorafenib, 10 µM forskolin, or combination treatment of 10 µM sorafenib with 10 µM forskolin for 24, 48, 72, and 96 hours. The proliferation of the cells was assessed using the WST-8 assay. The cell proliferation indicates a ratio to that of the DMSO values at 24 hours. Values are expressed as means ± SDs. * indicates significant decrease vs the control and ** indicates significant decrease both vs the control and vs 10 µM sorafenib (P < 0.05, 2-way ANOVA).
< 0.05), and the combination treatment with sorafenib and forskolin elicited more potent suppression of the growth of the WRO spheroids than did forskolin alone ($P < 0.05$) (Fig. 2E).

**Effect of sorafenib and forskolin on cell growth signaling and the cell cycle**

Immunoblot analysis using antibodies against phosphorylated ERK (p-ERK) and phosphorylated AKT (p-AKT) demonstrated differences between the effects of sorafenib and of forskolin on cell growth signaling (Fig. 3). In the untreated TPC-1, KTC1, and WRO cells, we detected similar intensities of the p-ERK bands and total ERK bands among the 3 cell lines. We also detected similar expression of the p-AKT and total AKT bands. The intensity of the p-ERK bands was the same as those of the total ERK bands as well as those between the p-AKT bands and the total AKT bands.

![Fig. 2](image_url)

**Fig. 2** Effects of sorafenib and forskolin on WRO in 3-D culture

(A - D) represent microscopic images of WRO spheroids in 3-D culture. Scale bars: 500 µm. The WRO cells were seeded at 4000 cells per well and incubated for 4 hours in EZSPHERE plates and treated with (A) DMSO, (B) 10 µM sorafenib, (C) 10 µM forskolin, and (D) combination treatment of 10 µM sorafenib with 10 µM forskolin. (E) The WRO cells were treated for 96 hours with 10 µM sorafenib, 10 µM forskolin, and combination treatment of 10 µM sorafenib with 10 µM forskolin. The cell proliferation was assessed using the WST-8 assay. The cell proliferation indicates a ratio to that of the control values. **indicates significant decreases both vs the control and vs 10 µM sorafenib ($P < 0.05$, 2-way ANOVA).
bands (except for TPC-1 cells). Sorafenib treatment with or without forskolin decreased levels of p-ERK in all 3 cell lines, although forskolin treatment alone did not suppress p-ERK. On the other hand, the intensity of the detected p-AKT levels were slightly increased in TPC-1 and WRO cells treated with sorafenib. Expression of p-AKT and p-ERK was not affected by forskolin monotherapy in any of the 3 cell lines.

Immunoblot analysis demonstrated differences between the effects of sorafenib and forskolin on the cell cycle modulators cyclin D1, CDK4, and phosphorylated RB (p-RB) (Fig. 4). Cyclin D1, CDK4, and p-RB were sufficiently expressed in untreated TPC-1, KTC1, and WRO cells. Forskolin treatment suppressed the expression of cyclin D1 in the TPC-1 and WRO cells. Forskolin treatment combined with sorafenib more obviously suppressed cyclin D1 in all 3 cell lines. However, forskolin treatment did not suppress the expression levels of p-RB (KTC1 and WRO) and CDK4. Unlike forskolin treatment, sorafenib treatment with or without forskolin suppressed the expression levels of p-RB and CDK4 in all 3 cell lines.

The effects of sorafenib and forskolin on the cell cycle differed among the 3 cell lines (Fig. 5). Sorafenib treatment tended to increase the ratio of S phase cells, whereas forskolin treatment tended to increase the ratio of G2/M phase cells.

**Discussion**

The 3 examined culture cell lines, TPC-1, KTC1, and WRO, are derived from well-differentiated thyroid carcinomas and have typical genetic mutations: TPC-1 has RET/PTC rearrangement, and KTC1 and WRO have the BRAF V600E point mutation [1-3]. These genetic alterations are thought to induce constitutive activation of the MAPK pathway, which is pivotally involved in cancer cell proliferation. Immunoblot analysis consistently demonstrated abundant p-ERK levels in the 3 cell lines. Sorafenib, a multikinase inhibitor, significantly suppressed p-ERK1/2 in the 3 cell lines, regardless of treatment with or without forskolin. Unexpectedly, however, sorafenib treatment showed only marginal growth suppression in the 3 cell lines, suggesting that the MAPK pathway might not play a pivotal role in growth of well-differentiated thyroid cancer cells.

In clear contrast, forskolin, an AC activator, showed significant growth suppression in the 3 cell lines, and the effects were almost equivalent to those of the combination treatment with sorafenib and forskolin. Forskolin is a member of the diterpenes. It pharmacologically activates AC [27] and upregulates
downstream molecules including cAMP, which activates protein kinase A (PKA) and PKA substrates. Combined with our current finding that forskolin did not suppress p-ERK levels in 3 well-differentiated thyroid carcinoma cell lines, the growth suppressing effect of forskolin is likely to be independent of the MAPK pathway. As regards this notion, a 3-D cell culture of WRO cells, simulating in vivo conditions, clearly demonstrated that the growth inhibitory effect of forskolin was stronger than that of sorafenib and weaker than their combination. This finding suggests that the effects of sorafenib and forskolin are additive and that their working mechanisms are distinct.

Various studies demonstrated important roles of the phosphoinositide 3-kinase (PI3K)/AKT pathway in proliferation of cancer cells with genetic mutations in RTKs. A recent study has reported that mammalian target of rapamycin (mTOR), a downstream regulator
of the PI3K/AKT pathway, is compensatorily activated when the MAPK pathway is inhibited [28]. In our study, sorafenib slightly increased levels of p-AKT in TPC-1 and WRO cells. This finding may indicate that the mTOR pathway is compensatorily activated instead of the MAPK pathway. On the other hand, forskolin did not affect the levels of p-AKT in all 3 cell lines, suggesting that the mTOR pathway is not compensatorily activated by forskolin.

The present study further demonstrated that forskolin and sorafenib suppressed cell growth by regulating the cell cycle. Forskolin decreased the expression level of the cell cycle modulator cyclin D1, while sorafenib decreased CDK4 and p-RB in the 3 examined cell lines. A previous report suggested that forskolin induces G1 phase arrest [14] although sorafenib affects the cell cycle differently depending on the cell lines [9]. We postulate that the cAMP pathway activated by forskolin directly inhibits progression through the G1 phase of the cell cycle, resulting in growth suppression of well-differentiated thyroid carcinoma cells. However, the cell cycle analysis suggested that forskolin increased the ratio of G2/M phase cells in TPC-1 and WRO cells and that sorafenib increased the ratio of S phase cells. Our study thus indicated that there are differential effects on the cell cycle between forskolin and sorafenib, which might explain the differential effects of the MAPK pathway and the cAMP pathway on cancer cell proliferation. Further study is needed to clarify the detailed mechanisms.

In conclusion, the AC activator forskolin showed a more potent inhibitory effect on the cell proliferation of 3 well-differentiated thyroid cancer cell lines than did sorafenib, which is a multikinase inhibitor that suppresses the MAPK pathway. The cAMP pathway suppresses the growth of thyroid carcinoma cell lines through undetermined mechanisms independent of the well-known MAPK pathway. We postulate that the AC activator could play an important role in growth suppression of well-differentiated thyroid cancer.

Acknowledgements

We wish to thank Ms A. Sumiishi for her excellent technical assistance and Ms F. Miyamasu (Medical English Communications Center, University of Tsukuba) for grammatical review and advice.

Disclosure

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

References

1. Pathology and Genetics of Tumours of Endocrine Organs, WHO/IARC Classification of Tumours, 3rd Edition, Volume 8 (2004) DeLellis RA, Lloyd RV, Heitz PU, Eng C (eds.)
2. Santoro M, Carlomagno F, Hay ID, Herrmann MA, Greco M, et al. (1992) Ret oncogene activation in human thyroid neoplasms is restricted to the papillary cancer subtype. J Clin Invest 89: 1517-1522.
3. Kimura ET, Nikiforova MN, Zhu Z, Knauf JA, Nikiforov YE, et al. (2003) High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. Cancer Res 63: 1454-1457.
4. Kim SY, Kim SN, Hahn HJ, Lee YW, Choe YB, et al. (2015) Metaanalysis of BRAF mutations and clinicopathologic characteristics in primary melanoma. J Am Acad Dermatol 72: 1036-1046.e2.
5. Joyce T, Oikonomou E, Kosmidou V, Makrodouli E, Bantounas I, et al. (2012) A molecular signature for oncogenic BRAF in human colon cancer cells is revealed by microarray analysis. Curr Cancer Drug Targets 12: 873-898.
6. Cancer Genome Atlas Research Network (2004) Comprehensive molecular profiling of lung adenocarcinoma. Nature 511: 543-550.
7. Carlomagno F, Anaganti S, Guida T, Salvatore G, Troncone G, et al. (2006) BAY 43-9006 inhibition of oncogenic RET mutants. J Natl Cancer Inst 98: 326-334.
8. Preto A, Goncalves J, Rebocho AP, Figueiredo J, Meireles AM, et al. (2009) Proliferation and survival molecules implicated in the inhibition of BRAF pathway in thyroid cancer cells harbouring different genetic mutations. BMC Cancer 9: 387.
9. Broecker-Preuss M, Muller S, Britten M, Worm K, Schmid KW, et al. (2015) Sorafenib inhibits intracellular signaling pathways and induces cell cycle arrest and cell death in thyroid carcinoma cells irrespective of histological origin or BRAF mutational status. BMC Cancer 15: 184.
10. Takami H, Ito K, Sugino K (2014) Development of molecular targeted drugs for advanced thyroid cancer in Japan. *Endocr J* 61: 833-839.

11. Schlumberger M, Tahara M, Wirth LJ, Robinson B, Brose MS, *et al.* (2015) Lenvatinib versus placebo in radioiodine-refractory thyroid cancer. *N Engl J Med* 372: 621-630.

12. Haugen BR, Alexander EK, Bible KC, Doherty GM, Mandel SJ, *et al.* (2016) 2015 American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer: The American Thyroid Association Guidelines Task Force on Thyroid Nodules and Differentiated Thyroid Cancer. *Thyroid* 26: 1-133.

13. Yoo JY, Stang MT (2016) Current Guidelines for Postoperative Treatment and Follow-Up of Well-Differentiated Thyroid Cancer. *Surg Oncol Clin N Am* 25: 41-59.

14. Yano Y, Kamma H, Matsumoto H, Fujiwara M, Bando H, *et al.* (2007) Growth suppression of thyroid cancer cells by adenylcyclase activator. *Oncol Rep* 18: 441-445.

15. Matsumoto H, Sakamoto A, Fujiwara M, Yano Y, Shishido-Hara Y, *et al.* (2008) Cyclic AMP-mediated growth suppression and MAPK phosphorylation in thyroid papillary carcinoma cells. *Mol Med Rep* 1: 245-249.

16. Stork PJ, Schmitt JM (2002) Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. *Trends Cell Biol* 12: 258-266.

17. Rocha AS, Paternot S, Coulonval K, Dumont JE, Soares P, *et al.* (2008) Cyclic AMP inhibits the proliferation of thyroid carcinoma cell lines through regulation of CDK4 phosphorylation. *Mol Biol Cell* 19: 4814-4825.

18. Schwepepe RE, Klooker JP, Korch C, Pugazenthii U, Benezra M, *et al.* (2008) Deoxiribonucleic acid profiling analysis of 40 human thyroid cancer cell lines reveals cross-contamination resulting in cell line redundancy and misidentification. *J Clin Endocrinol Metab* 93: 4331-4341.

19. Saiselet M, Floor S, Tarabichi M, Dom G, Hebrant A, *et al.* (2012) Thyroid cancer cell lines: an overview. *Front Endocrinol (Lausanne)* 3: 133.

20. Ishiyama M, Miyazono Y, Sasamoto K, Ohkura Y, Ueno K (1997) A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability. *Talanta* 44: 1299-1305.

21. Tominaga H, Ishiyama M, Ohse F, Sasamoto K, Hamamoto T, *et al.* (1999) A water-soluble tetrazolium salt useful for colorimetric cell viability assay. *Anl Commun* 36: 47-50.

22. Chiba T, Yamada M, Hashimoto Y, Sato M, Sasabe J, *et al.* (2005) Development of a femtomolar-acting human derivative named colivelin by attaching activity-dependent neurotrophic factor to its N terminus: characterization of colivelin-mediated neuroprotection against Alzheimer's disease-relevant insults in vitro and in vivo. *J Neurosci* 25: 10252-10261.

23. Kimlin LC, Casagrande G, Virador VM (2013) In vitro three-dimensional (3D) models in cancer research: an update. *Mol Carcinog* 52:167-182.

24. Vinci M, Gowen S, Boxall F, Patterson L, Zimmermann M, *et al.* (2012) Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. *BMC Biol* 10: 29.

25. Kunz-Schughart LA, Freyer JP, Hofstaedter F, Ebner R (2004) The use of 3-D cultures for high-throughput screening: the multicellular spheroid model. *J Biomol Screen* 9: 273-285.

26. Chiba T, Yamada M, Sasabe J, Terashita K, Shimoda M, *et al.* (2009) Amyloid-beta causes memory impairment by disturbing the JAK2/STAT3 axis in hippocampal neurons. *Mol Psychiatry* 14: 206-222.

27. Seamon KB, Padgett W, Daly JW (1981) Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proc Natl Acad Sci U S A* 78: 3363-3367.

28. Boussemart L, Malka-Mahieu H, Girault I, Allard D, Hemmingsson O, *et al.* (2014) eIF4F is a nexus of resistance to anti-BRAF and anti-MEK cancer therapies. *Nature* 513: 105-109.