Triiodothyronine (T_3) upregulates the expression of proto-oncogene *TGFA* independent of MAPK/ERK pathway activation in the human breast adenocarcinoma cell line, MCF7

[1] Departamento de Medicina Interna, Faculdade de Medicina de Botucatu, Universidade Estadual Paulista (UNESP), Botucatu, SP, Brasil
[2] Universidade Estadual Paulista (UNESP), Botucatu, SP, Brasil
[3] Departamento de Fisiologia e Biofísica, Instituto de Ciências Biomédicas, Universidade de São Paulo (USP), São Paulo, SP, Brasil
* Tabata M. Silva and Fernanda C. F. Moretto equally contributed to the article

Correspondence to:
Tabata M. Silva
Departamento de Medicina Interna, Faculdade de Medicina de Botucatu, Universidade Estadual Paulista (UNESP)
Av. Prof. Mário Rubens Guimarães Montenegro, s/n, Campus de Botucatu
18618687 – Botucatu, SP, Brasil
tabata.dih@gmail.com

Received on Apr/10/2018
Accepted on Dec/12/2018

DOI: 10.20945/2359-3997000000114

Tabata M. Silva[1*], Fernanda C. F. Moretto[1*], Maria T. De Sibio[1], Bianca M. Gonçalves[1], Miriane Oliveira[1], Regiane M. C. Olimpio[1], Diego A. M. Oliveira[2], Sarah M. B. Costa[1], Igor C. Deprá[1], Vickeline Namba[1], Maria T. Nunes[3], Célia R. Nogueira[1]

ABSTRACT

Objective: To verify the physiological action of triiodothyronine T_3 on the expression of transforming growth factor α (*TGFA*) mRNA in MCF7 cells by inhibition of RNA Polymerase II and the MAPK/ERK pathway. **Materials and methods:** The cell line was treated with T_3 at a physiological dose (10^{-9}M) for 10 minutes, 1 and 4 hour (h) in the presence or absence of the inhibitors, α-amanitin (RNA polymerase II inhibitor) and PD98059 (MAPK/ERK pathway inhibitor). *TGFA* mRNA expression was analyzed by RT-PCR. For data analysis, we used ANOVA, complemented with the Tukey test and Student t-test, with a minimum significance of 5%. **Results:** T_3 increases the expression of *TGFA* mRNA in MCF7 cells in 4 h of treatment. Inhibition of RNA polymerase II modulates the effect of T_3 treatment on the expression of *TGFA* in MCF7 cells. Activation of the MAPK/ERK pathway is not required for T_3 to affect the expression of *TGFA* mRNA. **Conclusion:** Treatment with a physiological concentration of T_3 after RNA polymerase II inhibition altered the expression of *TGFA*. Inhibition of the MAPK/ERK pathway after T_3 treatment does not interfere with the *TGFA* gene expression in a breast adenocarcinoma cell line.
Arch Endocrinol Metab. 2019;63(2):142-7

Keywords
Thyroid hormone; nongenomic actions; gene expression; breast cancer

INTRODUCTION

In the last few years, there has been a disturbing increase in the incidence of breast cancer (1), the second most common type of cancer and the fifth leading cause of cancer death worldwide (2). The thyroid hormone (TH) signaling pathway is complex and highly regulated by the expression of specific transporters of TH and multiple isoforms of the receptors of this hormone (TRs) present in several cells and by the interactions that occur between them (3,4). Besides its classic action via TR/elements responsive to TH (TERs), TH can act by other mechanisms (5); such mechanisms can be called non-classical or nongenomic action.

There is evidence that TH may affect the organization of the cytoskeleton, a mechanism by which it could interfere with the trafficking of vesicles to the membrane; this mechanism appears to involve αvβ3 integrin or a truncated TRα TH receptor that does not have a DNA binding domain and remains in the cytoplasm, where it can associate with other proteins/enzymes (3). From the interaction of TH with this integrin, the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway is activated, which mediates the nongenomic effects of triiodothyronine (T_3) on angiogenesis (6).

TH may act through non-classical or non-genomic mechanisms by interacting with iodothyronine receptors found on the plasma membrane or in the cytoplasm. The initiation sites are proteins characterized as iodothyronine receptors (7). The so-called non-genomic mechanisms may potentially influence gene expression, the onset of the pathway is non-genomic, as they depend on alternative pathways, but the consequences include increased transcription (5).

It is known that T_3 plays a role in the development and progression of breast cancer, by inducing the expression of progesterone receptors and increasing the mRNA levels of proto-oncogenes, such as the transforming growth factor α (*TGFA*) (8,9).

TGFA can be produced by a variety of cells, primarily by cells of ectodermal origin and may interact with the Epidermal Growth Factor (EGF) receptor to induce growth and proliferation responses during cell contact (10). It is expressed in several tissues, from embryogenesis to the adult phase (11). *TGFA* has been described as a factor that is produced by tumor cells and is circumstantially implicated in the regulation of the autocrine growth of breast cancer cells (12,13); *TGFA* overexpression may occur during malignant progression (14). It has been reported that *TGFA* mRNA and protein can be detected in approximately 50-70% of breast tumors. In addition, 2-3 times higher levels of this biologically active factor can be found in the pleural effusions of patients with breast cancer (13).

Our group has shown that there is an increase in the expression of *TGFA* during T_3 treatment (15), but this expression does not occur in cellular models that do not express the estrogen receptor or when the cells are simultaneously treated with antiestrogen medications, like tamoxifen (9,16).

For this study, we selected the MCF7 lineage of breast cancer cells. Since the 1980s, this cell line has been used as a model for breast tumors expressing estrogen receptor α (ERα), which is the main estrogen receptor (ER) expressed in this cell line and estrogen receptor β (ERβ) and also thyroid hormone receptor (TR) α and β (9,17-19).

The objective of the present study was to verify if the hormone T_3, at a physiological concentration, activates the MAPK/ERK pathway (its inhibitor is PD98059) and RNA Polymerase II (its inhibitor is α-amanitin) to modulate the expression of *TGFA* gene in the MCF7 breast adenocarcinoma cell line in a short time period of 10 minutes (min), 1 and 4 hour (h) of treatment.

MATERIALS AND METHODS

Reagents

Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS) and antibiotic solution at a 1:100 dilution were purchased from Gibco BRL (Grand Island, NY, USA). PD98059 (PD), α-amanitin, triiodothyronine (T_3), dimethylsulfoxide (DMSO), sodium hydroxide (NaOH) and charcoal-stripped FBS were purchased from Sigma Aldrich (St Louis, MO, EUA).

Cell culture

This project was approved by the Research Ethics Committee of Botucatu Medical School, protocol no. 3367-2009.

The MCF7 cell line, an eternal breast cancer cell line, was initially obtained from a primary culture of this cancer, developed from a pleural effusion of a female patient showing metastasis of the disease (20). These cells express both ERα and β as well as TRα and β (9,18,19). The cell line, initially acquired from the American Type Culture Collection (ATCC), Manassas, Virginia, USA, was expanded, grown and maintained in the cell bank of the Clinical Medicine Experimental Laboratory, UNESP, Botucatu. The cells were grown in RPMI 1640 medium supplemented with 1.2 g/L $NaHCO_3$, 10 nM Hepes with pH 7.4 and 10% FBS and maintained at 37 °C in 5% CO_2. The medium was changed every two days. To deplete all hormone sources in the culture medium, the cells were incubated with phenol red-free medium, supplemented with 10% charcoal-stripped FBS. After incubation, cells were treated with a physiological concentration T_3, 10^{-9}M, for 10 min, 1 h or 4 h, with all treatments being initiated at the same time, following which the cells were collected. The inhibitor and T_3 hormone concentrations used were as follows: PD98059 (5 μM; T_3 group associated with PD) was used as an inhibitor of the MAPK/ERK pathway and α-amanitin (50 μg/mL; T_3 group associated with α-amanitin) was used as a transcription inhibitor. The untreated group received only 0.1% NaOH (T_3 diluent) and served as the control (C). The inhibitors PD98059 and α-amanitin were added to the medium 1 h prior to the T_3 treatment. The experiments were performed in triplicate.

Gene expression

Total RNA was extracted from MCF7 cells using the Trizol method (Invitrogen, São Paulo, Brazil), according to the manufacturer's instructions. The High-Capacity cDNA Reverse Transcription RT-PCR kit (Invitrogen, São Paulo, Brazil) was used for the synthesis of 20 μL of complementary DNA (cDNA) from 1000 ng of total RNA. *TGFA* levels (Hs00608187_m1, Applied

Biosystems, Foster City, CA) were determined by Real-Time Quantitative Reverse Transcription PCR (qRT-PCR). The quantitative measurements were performed with the Applied Biosystems StepOne Plus detection system using the commercial TaqMan kit for qPCR (Applied Biosystems), according to the manufacturer's instructions. The amplification conditions were activation of the enzyme at 50°C for 2 min, denaturation at 95°C for 10 min and amplification for 40 cycles of denaturation at 95°C for 15 s and extension at 60°C for 1 min. The analyses were performed in duplicate. Gene expression was quantified relative to the values of the control group, after normalization of the expression to that of an internal control, GAPDH (Hs02758991_g1), by the method of $2^{-\Delta\Delta Ct}$, as previously described (21).

Statistical analysis

For statistical analysis, ANOVA was used in conjunction with the Tukey test and Student's t-test and a minimum significance of 5% was assumed. Data were expressed as the mean ± standard deviation.

RESULTS

T_3 increases the expression of *TGFA mRNA* in MCF7 cells in 4 h

There was a significant increase in the gene expression of *TGFA* after the treatment of MCF7 cells with T_3 for 4 h, but the increase was not observed after the treatment time-points of 10 min and 1 h (Figure 1). Comparison CxT_3.

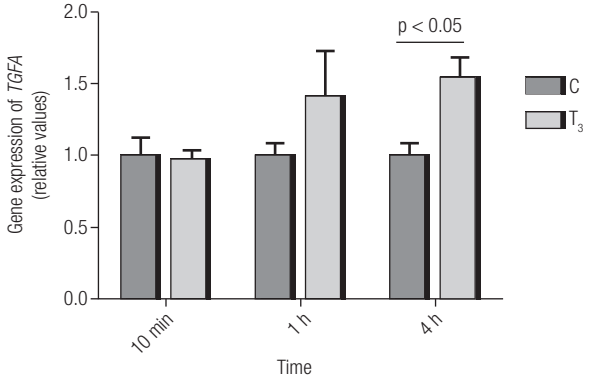

Figure 1. Effects of T_3 treatment length on *TGFA* mRNA levels in MCF7 cells. C: Control; T_3: Triiodothyronine. The treatment times were 10 min, 1 h and 4 h. Relative expression levels represent the mean of 3 replicates. Statistical analysis was performed using a Student's t-test; $p<0.05$ is considered significant.

Effect of T_3 on the maintenance of *TGFA* gene expression after inhibition of RNA polymerase II in MCF7 cells

After 4 h of treatment, the T_3 group associated with α-amanitin showed a significant decrease in *TGFA* gene expression, compared to the T_3 group. However, there was no change in the expression when comparing the *TGFA* expression in the α-amanitin group with that in the control group and in the T_3 group associated with α-amanitin (Figure 2). Comparison CxT_3, CxAM, T_3xAM+T_3 and $AMxAM+T_3$.

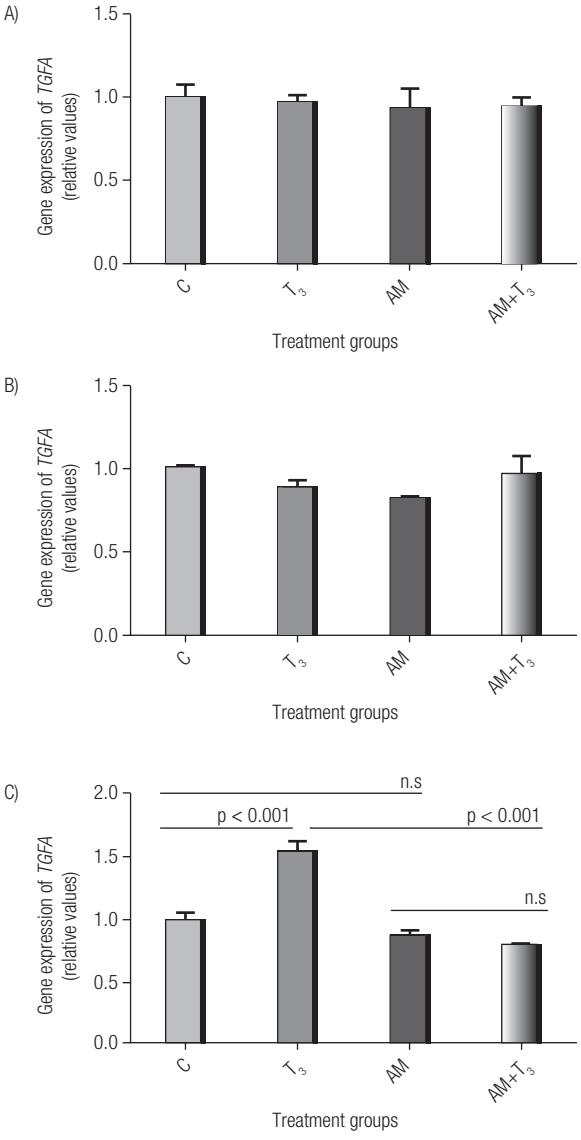

Figure 2. Effect of T_3 in association with α-amanitin (AM) on the gene expression of *TGFA* in MCF7 cells. C: Control; T_3: Triiodothyronine; AM: α-amanitin; $AM+T_3$: α-amanitin associated with triiodothyronine. Triiodothyronine treatment was performed for 10 min (A), 1 h (B) and 4 h (C). Relative expression levels represent the mean of 3 replicates. Statistical analysis was performed using an ANOVA, supplemented with the Tukey test; $p<0.05$ is considered significant.

Activation of the MAPK/ERK pathway is not required for T_3 to affect the expression of *TGFA mRNA*

After 4 h of treatment, the T_3 group associated with PD showed a significant increase in *TGFA* gene expression, compared to the T_3 group and the PD group. However, there was no change in this expression when we compared the *TGFA* expression in the PD and control groups (Figure 3). Comparison CxT_3, CxPD, T_3xPD+T_3 and PDxPD+T_3.

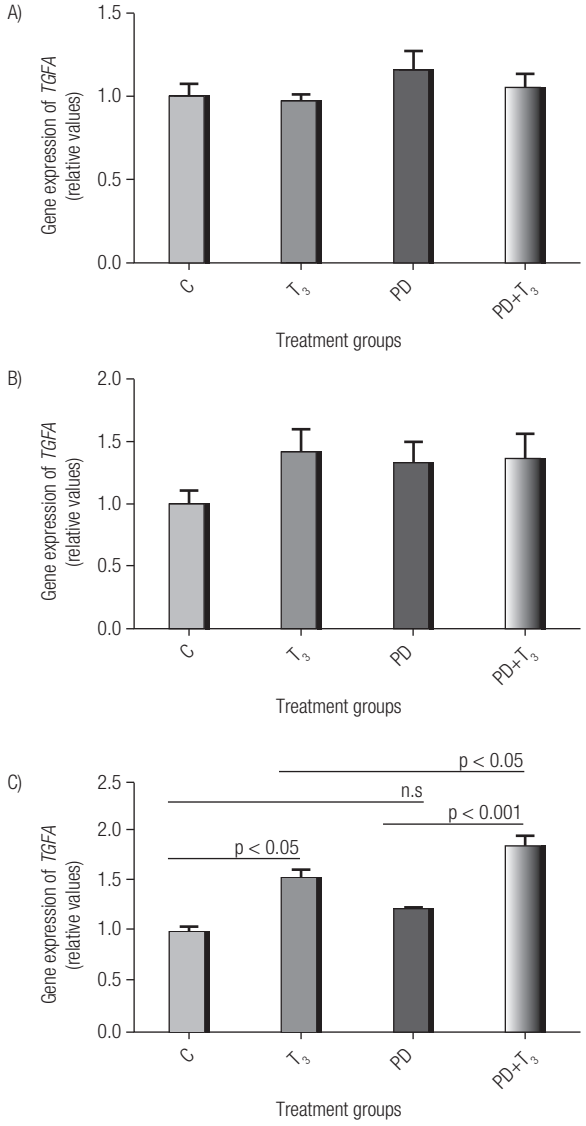

Figure 3. Effect of T_3 in association with PD98059 (PD) on the gene expression of *TGFA* in MCF7 cells. C: Control; T_3: Triiodothyronine; PD: PD98059; PD+T_3: PD98059 associated with triiodothyronine. Triiodothyronine treatment was performed for 10 min (A), 1 h (B) and 4 h (C). Relative expression levels represent the mean of 3 replicates. Statistical analysis was performed using an ANOVA, supplemented with the Tukey test; $p<0.05$ is considered significant.

DISCUSSION

In the breast cancer cell line MCF7, T_3 regulates gene expression either by binding to specific TRs that bind to TREs in the promoter region of target genes (22-25), or by activating alternative pathways, such as the MAPK/ERK pathway, via their initiation sites, which are present in the plasma membrane or in the cytoplasm (7).

Our group identified an increase in the expression of *TGFA* (indicative of increased proliferative activity) in primary breast cancer cultures following treatment with estrogen and T_3 (16). Moretto and cols. (15) demonstrated an increased *TGFA* expression in MCF7 cells after treatment with supraphysiological concentrations of T_3 (10^{-8}M) over periods of 10 min, 30 min, 1 h, and 4 h. In the present study, we show that treatment with physiological concentrations of T_3 (10^{-9}M) also increases the expression of *TGFA*, corroborating the results obtained by Moretto and cols.; however, at this lower dosage we were unable to detect expression changes in treatments under 4 h.

Fernandez and cols. (26) studied the effects of triiodothyronine on MDA-468 breast cancer cells, showing that at physiological doses, the hormone exerts control over the actions of *TGFA*. Unlike these studies, we show that short treatments with physiological concentrations of T_3 do not affect *TGFA* expression. Brito and cols. (27) demonstrated that *TGFA* expression is increased in esophageal cancer, indicating differential regulation of the gene in esophageal tumorigenesis.

α-amanitin is a general transcription inhibitor that is selective for RNA polymerase II and is approved for the treatment of some types of sarcomas (28,29). We have shown that on exposure to physiological T_3 concentrations, there was an increase in the *TGFA* expression in a breast cancer cell line after 4 h, which suggests that further studies should be done to determine the effects of transcription blocking drugs on breast adenocarcinoma cell lines. Our data suggest that α-amanitin may provide additional therapeutic benefits when used in combination with other anticancer agents. This could allow for the development of more specific and effective drugs in the treatment of breast adenocarcinomas.

We determined that T_3-mediated inhibition of the MAPK/ERK pathway leads to overexpression of *TGFA*. Studies have reported that increased expression of EGFR family proteins are a poor prognostic factor for cancer patients (30-32); is also associated with tumor

aggressiveness (32) and resistance to chemotherapy (30) and their levels are increased in 30% of solid tumors (31). Hence, we can infer that as the MAPK/ERK pathway is not modulated by T_3 treatment, this hormone can use some precursor that induces increased tumorigenesis in MCF7 cells. This situation was previously described in a report by Boldt and cols. (33), who demonstrated that the modulation of the MAPK/ERK pathway, as an adjuvant treatment for breast cancer, is of limited value, as it may increase or decrease the efficacy of other chemotherapeutic drugs.

We conclude that the treatment of MCF7 cells with physiological doses of the hormone T_3, over a period of 4 h, leads to an increased expression of *TGFA* via RNA Polymerase II. We also conclude that T_3 does not increase *TGFA* expression by acting on the MAPK/ERK pathway.

The results obtained in this study, together with previous results from our research group, suggest that T_3 acts via nuclear pathways when present at physiological doses and via extra-nuclear pathways when present at supraphysiological doses.

Further studies are required to explore the effects of the modulation of the *TGFA* proto-oncogene in the treatment of breast adenocarcinomas.

Funding: this work was supported by FAPESP: scientific initiation process no. 2015/09686-8, thematic project process no. 2013/05629-4 and doctoral project process no. 2014/15209-5.

Disclosure: no potential conflict of interest relevant to this article was reported.

REFERENCES

1. Porter PMD. "Westernizing" Women's Risks? Breast cancer in lower-income countries. N Engl J Med. 2008;358(3):213-6.
2. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer. 2015;136(5):359-86.
3. Cheng SY. Thyroid hormone receptor mutations and disease: Beyond thyroid hormone resistance. Trends Endocrinol Metab. 2005;16(4):176-82.
4. Williams GR. Neurodevelopmental and neurophysiological actions of thyroid hormone. J Neuroendocrinol. 2008;20(6):784-94.
5. Moeller LC, Broecker-Preuss M. Transcriptional regulation by nonclassical action of thyroid hormone. Thyroid Res. 2011;4(Suppl 1):S6.
6. Bergh JJ, Lin H-Y, Lansing L, Mohamed SN, Davis FB, Mousa S, et al. Integrin AVB3 contains a cell surface receptor site for thyroid hormone that is linked to activation of mitogen-activated protein kinase and induction of angiogenesis. Endocrinology. 2005;146(7):2864-71.
7. Cheng SY, Leonard JL, Davis PJ. Molecular aspects of thyroid hormone actions. Endocr Rev. 2010;31(2):139-70.
8. Hall LC, Salazar EP, Kane SR, Liu N. Effects of thyroid hormones on human breast cancer cell proliferation. J Steroid Biochem Mol Biol. 2008;109(1-2):57-66.
9. Nogueira CR, Bentani MM. Triiodothyronine mimics the effects of estrogen in breast cancer cell lines. Molec Biol J Steroid Biochem Moh'c Biol. 1996;59(3-4):271-9.
10. Wilcox JN, Derynck R. Developmental expression of transforming growth factors alpha and beta in mouse fetus. Mol Cell Biol. 1988;8(8):3415-22.
11. Rappolee DA, Brenner CA, Schultz R, Mark D, Rappolee DA, Brenner CA, et al. Developmental expression of PDGF, TGF-alpha, and TGF-beta genes in preimplantation mouse embryos. Nature. 1988;241(4874):1823-5.
12. Verheul HMW, Voest EE, Schlingemann RO. Are tumours angiogenesis-dependent? J Pathol. 2004;202(1):5-13.
13. Ciardiello F, Kim N, McGeady ML, Liscia DS, Saeki T, Bianco C, et al. Expression of transforming growth factor alpha (TGF alpha) in breast cancer. Ann Oncol. 1991;2(3):169-82.
14. Pavelic K, Hrascan R, Kapitanovica S, Karapandza N, Vranes Z, Belicza M, et al. Multiple genetic alterations in malignant metastatic insulinomas. J Pathol. 1995;177(4):395-400.
15. Moretto FCF, De Sibio MT, Luvizon AC, Olimpio RMC, De Oliveira M, Alves CAB, et al. Triiodothyronine (T3) induces HIF1A and TGFA expression in MCF7 cells by activating PI3K. Life Sci. 2016;154:52-7.
16. Conde SJ, Luvizotto RAM, Sibio MT, Katayama MLH, Brentani MM, Nogueira CR. Tamoxifen inhibits transforming growth factor-alpha gene expression in human breast carcinoma samples treated with triiodothyronine. J Endocrinol Invest. 2008;31(12):1047-51.
17. Brandes LJ, Hermonat MW. Receptor status and subsequent sensitivity of subclones of MCF-7 human breast cancer cells surviving exposure to diethylstilbestrol. Cancer Res. 1983;43(6):2831-5.
18. Razandi M, Pedram A, Merchenthaler I, Greene GL, Levin ER. Plasma membrane estrogen receptors exist and functions as dimers. Mol Endocrinol. 2004;18(12):2854-65.
19. McGuire WL, Chamness GC, Costlow ME, Shepherd RE. Hormone dependence in breast cancer. Metabolism. 1974;23(1):75-100.
20. Brooks S, Locke E, Soule H. Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. J Biol Chem. 1973;248(17):6251-3.
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2 (- delta delta C (T)) method. Methods. 2001;25(4):402-8.
22. Larsen PR, Harney JW, Moore DD. Sequences required for cell-type specific thyroid hormone regulation of rat growth hormone promoter activity. J Biol Chem. 1986;261(31):14373-6.
23. Catanzaro DF, West BL, Baxter JD, Reudelhuber TL. A pituitary-specific factor interacts with an upstream promoter element in the rat growth hormone gene. Mol Endocrinol. 1987;1(1):90-6.
24. Ye, ZS, Samuels H. Cell-and sequence-specific binding of nuclear proteins to 5'-flanking DNA of the rat growth hormone gene. 1987;262(13):6313-7.
25. Gustafson TA, Markhamt BE, Bahlt JJ. Thyroid hormone regulates expression of a transfected a-myosin heavy-chain fusion gene in fetal heart cells. 1987;84(5):3122-6.
26. Fernandez PJ, Klos DJ, Hamilton PD. Modulation of transforming growth factor alpha-dependent expression of epidermal growth factor receptor gene by transforming growth factor beta, triiodothyronine, and retinoic acid. J Cell Biochem. 1989;41(3):159-70.
27. Brito MJ, Filipe MI, Linehan J, Jankowski J. Association of transforming growth factor alpha (TGFA) and its precursors with

malignant change in Barrett's epithelium: Biological and clinical variables. Int J Cancer. 1995;60(1):27-32.

28. Tattersall MHN, Sodergreen JE, Sengupta SK, Trites DH, Modest EJ, Frei E. Pharmacokinetics of actinomycin D in patients with malignant melanoma. Clin Pharmacol Ther. 1975;17(6):701-7.
29. Estlin EJ, Veal GJ. Clinical and cellular pharmacology in relation to solid tumours of childhood. Cancer Treat Rev. 2003;29(4): 253-73.
30. Gao J, Ulekleiv CH, Halstensen TS. Epidermal growth factor (EGF) receptor-ligand based molecular staging predicts prognosis in head and neck squamous cell carcinoma partly due to deregulated EGF- induced amphiregulin expression. J Exp Clin Cancer Res. Journal of Experimental & Clinical Cancer Research; 2016;35(1):1-12.
31. Wykosky J, Fenton T, Furnari F, Cavenee WK. Therapeutic targeting of epidermal growth factor receptor in human cancer: Successes and limitations. Chin J Cancer. 2011;30(1):5-12.
32. Ferreira GM, Martinez M, Camargo ICC, Domeniconi RF, Martinez FE, de Almeida Chuffa LG. Melatonin attenuates Her-2, p38 MAPK, p-AKT, and mTOR levels in ovarian carcinoma of ethanol-preferring rats. J Cancer. 2014;5(9):728-35.
33. Boldt S, Weidle UH, Kolch W. The role of MAPK pathways in the action of chemotherapeutic drugs. Carcinogenesis. 2002;23(11):1831-8.