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TIME AND DOSE-DEPENDENT EFFECTS OF DEXAMETHASONE ON PTTG GENE EXPRESSION IN AT-T20 CELLS

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Abstract

Murine pituitary adrenocorticotropic hormone (ACTH) producing corticotroph tumor-derived AtT20 cells were treated with dexamethasone (1-100 nM), and it was cultured for 1 hour to 24 hours. Thereafter, pituitary tumor transforming gene *(Pttg)* mRNA expression was studied by quantitative real-time Polymerase Chain Reaction (RT-qPCR). *Pttg* mRNA expression were examined by dexamethasone in a dose-dependent and time-dependent manner. Dexamethasone dose-dependently and time-dependently inhibited *Pttg* mRNA expression in AtT20 cells at 100 nM after 9 hours. Besides, we have examined the effects of dexamethasone on the mouse *KLF6*, *cMyc*, *OCT1* and *SP1* mRNA expression by RT-qPCR technique. Taken together, it is suggested that dexamethasone plays an important role in the negative regulation of *Pttg* expression.

Keywords: *Pttg*, Dexamethasone, ACTH, Pituitary, RT-qPCR, AtT20 cells.

Introduction

Pituitary tumor transforming gene (*Pttg*) gene is over expressed in different types of tumors of pituitary (Kakar 1999, McCabe et al. 2003, Yu and Melmed 2001, Zhang et al. 1999), breast (Solbach et al., 2004), thyroid (Boelaert et al., 2003, Heaney et al., 2001), ovarian (Puri et al., 2001), and colon (Heaney et al., 2000). Abundant expression of PTTG in all adenoma subtypes indicates its importance in the pituitary tumorigenesis. Human *Pttg1*, a novel pituitary transforming gene is expressed in pituitary adenomas but not in normal pituitary (Tfelt-Hansen et al., 2006). Another research showed that low

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Pttg1 expression was detected in normal pituitary tissue using a much more sensitive detection method quantitative real-time Polymerase Chain Reaction (RT-qPCR) (Zhang, et al. 1999). However, *Pttg1* expression was higher in pituitary tumors than normal pituitary tissue. Therefore, it seems that PTTG plays an important role in pituitary tumorigenesis and probably in hormone-secreting tumors. Several candidate genes cause pituitary tumor genesis and tumor invasiveness. The *Pttg* plays a central role in pituitary tumorigenesis but PTTG protein expression is poorly documented and its relationship with tumor cell proliferation and the prognosis of pituitary adenomas is unclear (Filippella et al., 2006). Therefore it needs further research related *Pttg* gene and pituitary tumor due to clear understanding. Moreover siRNA expression vector targeting PTTG successfully inhibited the cell proliferation (Zhang et al., 2016).

mKLF6 (Chen et al. 2013), *m-cMyc* (Pei 2001), *mOCT1* (Zhou et al. 2008) and *mSP1* are important transcription factors for *Pttg* expression. Genetic instability or chromosomal alteration, is a classical mechanism of tumor formation, generating mutations in or over expression of oncogenes and tumor suppressor genes. Low-frequency mutations of oncogenes and tumor suppressor genes are observed in pituitary adenomas but give no specific information about tumor development (Tfelt-Hansen et al., 2006). Cloning of PTTG was also important to show that most of the pituitary adenomas over express this gene compared with normal pituitary tissue, which expresses very little PTTG (Tfelt-Hansen et al. 2006). Several reports showed that although PTTG was up-regulated in all histological subtypes, its expression is the highest in adrenocorticotropic hormone (ACTH)-secreting and non-functioning pituitary tumors. Excessive production of cortisol or ACTH causes Cushing's syndrome whereas pituitary tumour causes Cushing's disease.

Moreover dexamethasone suppression test is used to differentiate between Cushing's disease and Cushing's syndrome. Therefore dexamethasone is a very important drug in pituitary related diseases where *Pttg* gene is over expressed. It remains to be elucidated the dexamethasone-mediated effect on *Pttg* gene expression in AtT20 cells.

Materials and Methods

Reagents

Dexamethasone, a synthetic glucocorticoid, was purchased from Sigma-Aldrich (St. Louis, MO). It was dissolved in 100% ethanol at 1 mM concentration and stored at -20°C. These stocks were diluted with 100% ethanol to the desired concentration immediately before each experiment and maintained at a final concentration of ethanol at 0.1%.

Cell culture

AtT20 cells were grown with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100U/mL penicillin and 100µg/mL streptomycin. Cells were cultured in a humidified incubator at 37°C with 5% CO_2. AtT20 cells were obtained from the American Type Culture Collection (AtT20: CCL-89).

RNA isolation

AtT20 cells grown to 70% confluence in regular medium in 24-multiwell plates were incubated either without or with dexamethasone at appropriate concentrations in DMEM supplemented with 1% resin and charcoal-treated (stripped) FBS media (Parvin et al. 2017, Saito-Hakoda et al. 2015) and cultured for 9 hours. The cells were then lysed and their total RNAs were isolated using ISOGEN (NIPPONGENE, Toyama, Japan) according to the manufacturer's instructions. The RNA was quantified by a Nano-drop 2000 (Thermo Scientific, Waltham, MA). The final preparation of total RNA was free of DNA and Proteins, and had a 260/280 ratio 1.6 -1.9.

Quantitative real-time PCR

Quantitative real-time polymerase chain reaction (RT-qPCR) was performed using KAPA SYBR FAST Universal 2x qPCR Master Mix (KAPA Biosystems, Woburn, MA).One microgram from total RNA was converted to reverse transcription (RT) reaction using Prime Script Reverse transcriptase (Takara Bio, Shiga, Japan) with the oligo-dT primer and random hexamer according to the manufacturer's instructions**.** Reverse transcription mixtures were subjected to qPCR with KAPASYBR FAST Universal 2x qPCR Master Mix (KAPA Biosystems) for primers of mouse *Pttg*, *cMyc*, *KLF6*, *Oct1*, *SP1* and *GAPDH* (Glyceraldehyde-3phosphate dehydrogenase) using a CFX connect Real Time PCR thermal cycler (Bio-Rad, Hercules, CA). Reactions were incubated at 95°C for 1 min and then amplified using temperature parameters of 95°C for 15 sec; 60°C for 10 sec; 72°C for 20 sec. Amplifications were carried out for 45 cycles, followed by a 3 min extension at 72°C. After amplification, a melting-curve analysis was performed from 60°C to 95°C with a heating rate of 0.5°C/10 sec and continuous fluorescence acquisition. The signals of the samples of interest were then quantified from the standard curve, and all obtained data were normalized by *GAPDH*. To confirm the amplification specificity, the PCR products from each primer pair with SYBR green were

subjected to a melting curve analysis. Results are expressed as percentages of each control.

The following primer sequences were used for RT-qPCR:

Mouse *Pttg*	Forward	5′-CTGGGCACTGGTGTCAAG-3′
	Reverse	5′-GCTGTTTTGGTTGGAGGGG-3′
Human *Pttg*	Forward	5′-TGATCCTTGACGAGGAGAGAG-3′
	Reverse	5′-GGTGGCAATTCAACATCCAGG-3′
Mouse O*ct1*	Forward	5′-GTAAGCTCTGCCTCCTGGTG-3′
	Reverse	5′-GCTGTCGTTCTCCTGTAGCC-3′
Mouse *c-Myc*	Forward	5′-CACCCCATCTCTGCCTGCAGATCC-3′
	Reverse	5′-CATGACCAGAGTTCGAGCTGAGAA-3′
Mouse *KLF6*	Forward	5′-GAGTTCCTCCGTCAT TTCCA-3′
	Reverse	5′-CATGACCAGAGTTCGAGCTGAGAA-3′
Mouse *SP1*	Forward	5′-TCATGGATCTGGTGGTGATGGG-3′
	Reverse	5′-GCTCTTCCCTCACTGTCTTTGC-3′
Mouse *GAPDH*	Forward	5′-ACAGTCCATGCCATCACTGCC-3′
	Reverse	5′-GCCTGCTTCACCACCTTCTTG-3′
Human *GAPDH*	Forward	5′-CTGACTTCAACAGCGACACC-3′
	Reverse	5′-TAGCCAAATTCGTTGTCATACC-3′

Statistical analyses

Data are displayed as means ± standard errors of means (SEM). Statistical analysis was performed with one way ANOVA followed by Tukey's post hoc test among the groups and Paired Sample t test between the groups. *P*-value <0.05 was considered as statistically significant. Statistical details are found in figure legends.

Results

Effects of dexamethasone on m*Pttg* mRNA expression dose-dependently

We first analysed the effects of dexamethasone on mRNA expression of *Pttg* at various concentrations in AtT20 cells for 9 hours (Fig. 1). After treatment of the cells with various concentrations (1 nM, 10 nM and 100 nM) of dexamethasone on *Pttg* mRNA for 9 hours and *Pttg* mRNA was significantly decreased at 10 nM and 100 nM of dexamethasone concentrations. These results indicated that dexamethasone decreased mRNA expression of *Pttg* dose-dependently.

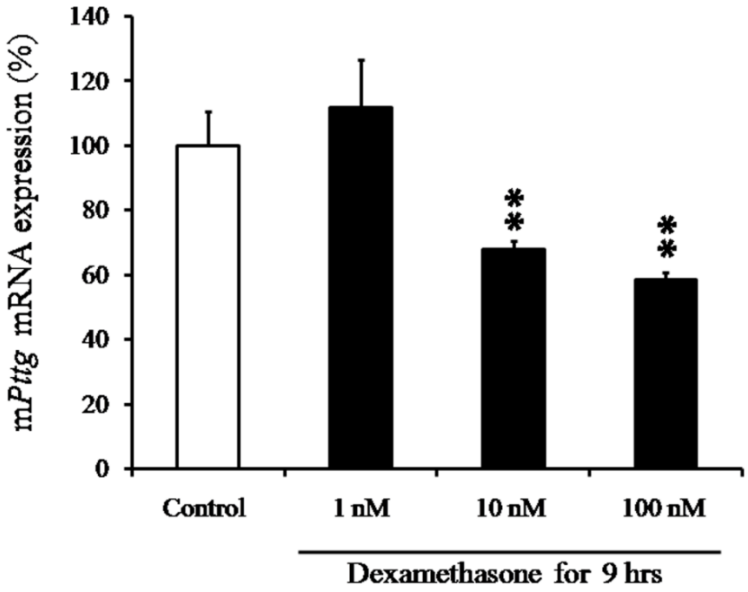


Fig.1. Effects of dexamethasone on mRNA expression of mouse *Pttg* in AtT20 cells.

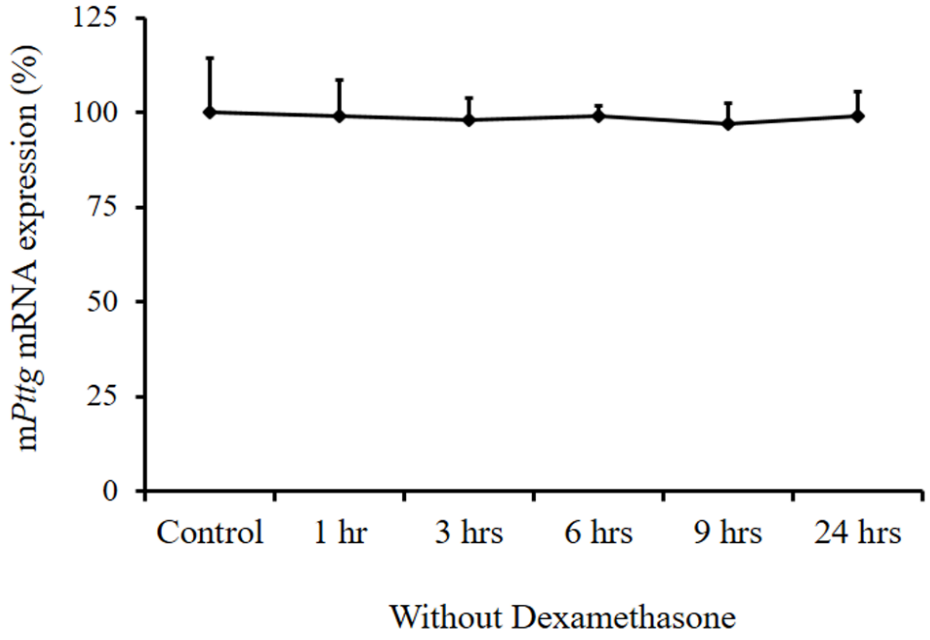
Effects of dexamethasone on mRNA expression of mouse *Pttg* dose-dependently. AtT20 cells were treated with dexamethasone at 1 nM, 10 nM, 100 nM, or 0.1% DMSO (vehicle control) for 9 hours. Data are expressed as percentages (100%) of control. Each point indicates mean ± SEM (n=4). ***P*<0.01 vs control.

Effects of dexamethasone on m*Pttg* mRNA expression time-dependently

Next we examined the dexamethasone-mediated effect on *Pttg* mRNA expression using different durations of drug incubation in the cells. After treatment of the cells without and with dexamethasone (100 nM) for 1 hour, 3 hours, 6 hours, 9 hours and 24 hours, where the *Pttg* mRNA expression was not suppressed at all without drug (Fig. 2A) and the mRNA expression was significantly decreased from 6 hours to 24 hours in a time-

dependent manner (Fig. 2B). These results indicated that dexamethasone decreased mRNA expression of *Pttg* time-dependently, but at 9 hours the drug effect was most significant.

A



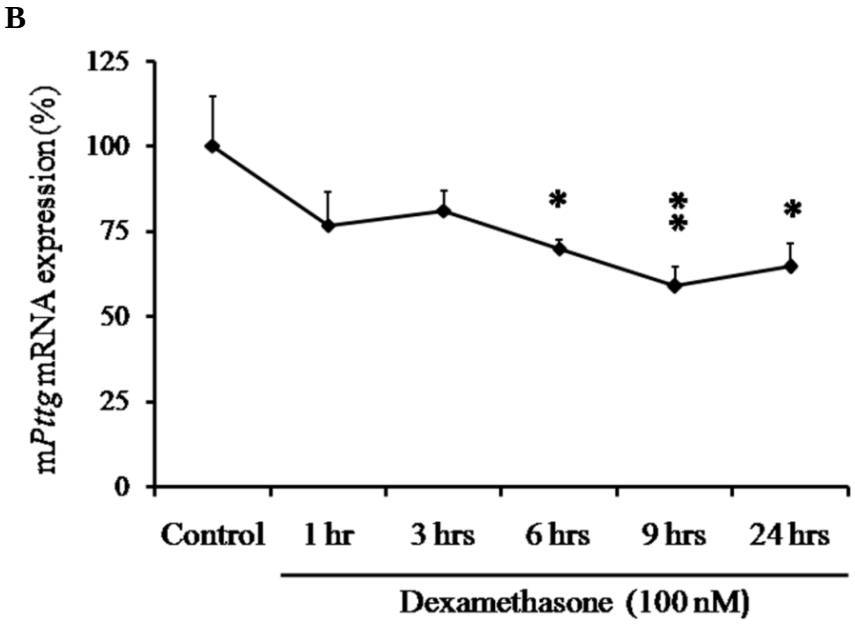


Fig. 2. Effects of dexamethasone on mRNA expression of *Pttg* mRNA in AtT20 cells.

(A). Effects of 0.1% DMSO on *Pttg* mRNA expression time-dependently. AtT20 cells were treated with 0.1% DMSO for 0 hour (control) 1 hour, 3 hours, 6 hours, 9 hours and 24 hours. Data are expressed as percentages (100%) of control. Each point indicates mean ± SEM (n=4). (B). Effects of dexamethasone on *Pttg* mRNA expression time-dependently. AtT20 cells were treated with 100 nM of dexamethasone for 1 hour, 3 hours, 6 hours, 9 hours and 24 hours. Vehicle control, 0.1% DMSO. Data are expressed as percentages (100%) of control. Each point indicates mean ± SEM (n=4), *P<0.05 vs control. ***P*<0.01vs control.

Effects of dexamethasone on *Pttg* mRNA expression in HepG2 cells

As *Pttg* was over expressed in various tumors, we analysed the effects of dexamethasone on mRNA expression of *Pttg* at various concentrations in HepG2 cells for 9 hours (Fig. 3). After treatment of the cells with various concentrations at 1 nM, 10 nM and 100 nM of the drug on *Pttg* mRNA, but no significant effects were observed in HepG2 cells. This result indicated that dexamethasone has no effect on mRNA expression of *Pttg* in HepG2 cells.

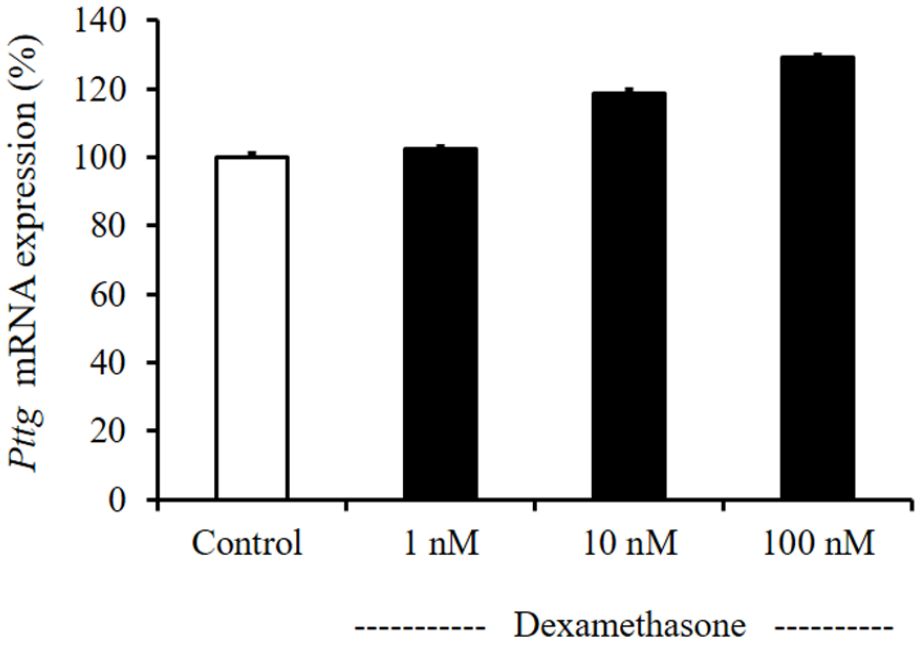
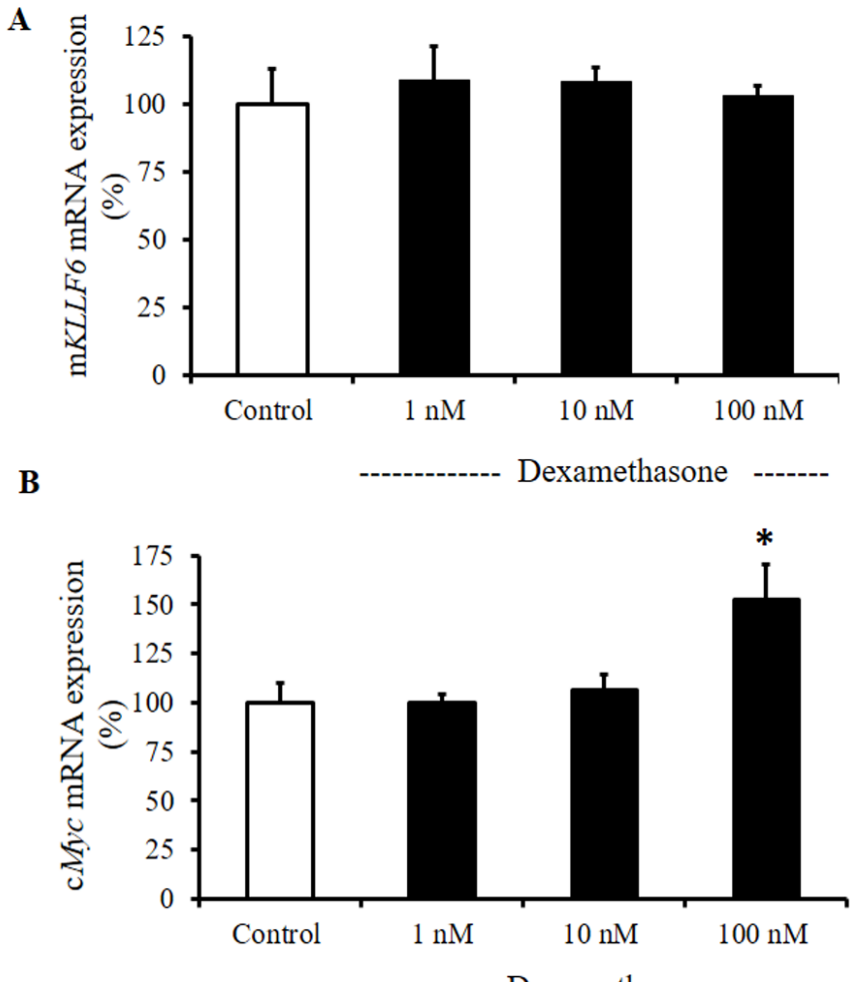


Fig. 3. Effects of dexamethasone on mRNA expression of *Pttg* in HepG2 cells.

Effects of dexamethasone on mRNA expression of *Pttg* dose-dependently. HepG2 cells were treated with dexamethasone at concentration 1 nM, 10 nM, 100 nM, or 0.1% DMSO (vehicle control) for 9 hours. Each point indicates mean ± SEM (n=4). Data are expressed as percentages (100%) of control.

mKLF6, m-cMyc, mOCT1 and *mSP1* mRNA levels in dexamethasone-treated AtT-20 cells

We next examined the effect of dexamethsone on *mKLF6*, *m-cMyc*, *mOCT1* and *mSP1* mRNA expression for 9 hours in At T20 cells. As shown in Figs 4A, 4B, 4C and 4D, the drug did not suppress the mRNA expression of *mKLF6*, *mOCT*1, *m-cMyc* and *mSP1* at concentration of 1 nM, 10 nM and 100 nM.


A
mKLF6 mRNA expression (%)
125
100
75
50
25
0
Control
1 nM
10 nM
100 nM
---------- Dexamethasone -------
B
cMyc mRNA expression (%)
175
150
125
100
75
50
25
0
*
Control
1 nM
10 nM
100 nM

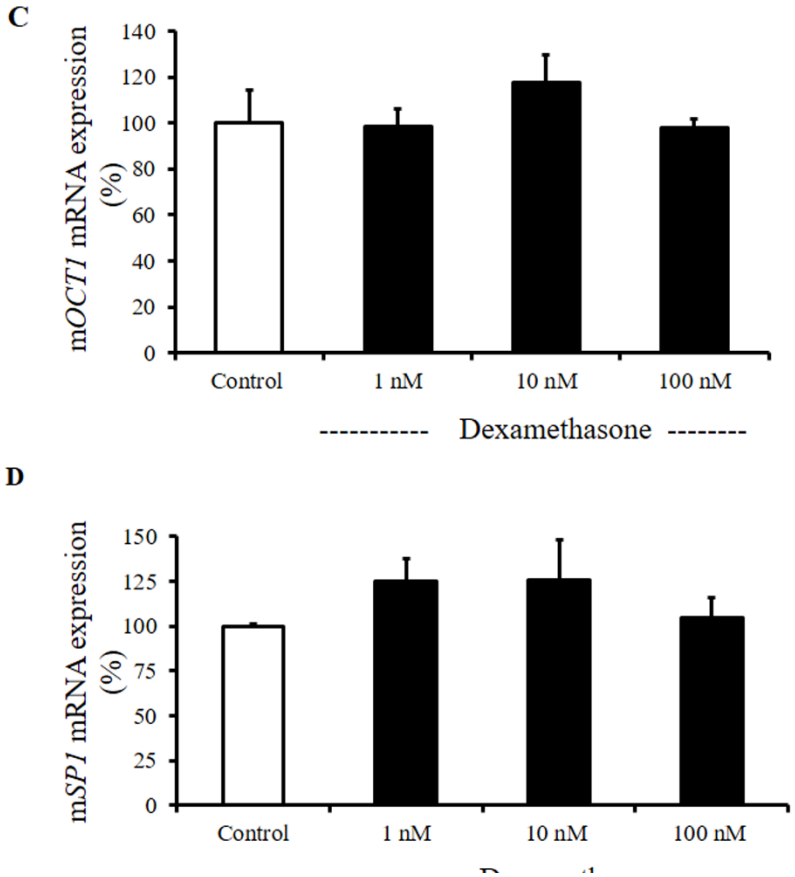


Fig. 4. Dexamethasone-mediated effects on the mRNA expression of mouse *KLF6*, *cMyc*, *OCT1* and *SP1* in AtT20 cells.

At T20 cells were treated with dexamethasone at the concentration of 1 nM, 10 nM, 100 nM or 0.1% DMSO (vehicle control) for 9 hours. (A) *mKLF6* mRNA expression, (B) *m-cMyc* mRNA expression, (C) *mOCT1* mRNA expression and (D) *mSP1* mRNA expression. Data are expressed as percentages (100%) of control. Data represent mean ± SEM (n=4).*P<0.05 vs control.

Discussion

Pttg, the best available marker of pituitary adenomas is a pituitary tumor-specific oncogene that was discovered a decade ago. PTTG over expression with malignancy grades in thyroid (Boelaert et al. 2003, Heaney et al. 2001), colon (Heaney et al. 2000) and astroglial cancers are already proved. Moreover, there are many evidences suggesting

that PTTG, and its binding protein participate in the genesis of some cancers (Zhang et al. 1999) which is involved in transcriptional and cell cycle regulation. In this research, dexamethasone suppresses *Pttg* gene expression (Fig. 1, Fig. 2B) in AtT20 cells whereas there is no significant effect of the drug on the gene (Fig. 3) in HepG2 cells due to different factors may be involved in different cells in the same gene regulation or due to gene sequence variation in different species. Human *Pttg* is located on chromosome 5 and encodes a protein PTTG. PTTG is localized to both nucleus and cytoplasm and interacts with several protein partners (Yu and Melmed 2001). Approximately four hundred targets were identified and categorized into three major functional groups involved in cell cycle, metabolic control, or signal transduction pathways (Tong et al. 2007).

Dexamethasone is a synthetic glucocorticoid that is a ligand of glucocorticoid receptor. It down-regulates the rat Organic Cationic transporter1 (OCT1) at high concentration in rat liver (Maeda et al., 2008) whereas significantly up-regulates *Oct1* mRNA and protein in normal primary human hepatocytes, but not in hepatocyte-derived tumor cell lines HepG2 (Rulcova et al., 2013). In this research there was no inhibitory effect of dexamethasone at 100 nM on mouse *Oct1* (Fig. 4C). Previously showed that PTTG1 binds to the *c-Myc* promoter identifying a PTTG1-DNAbinding domain (Pei 2001).

The alternate name of *Pttg* in human is securin, which is a mitotic checkpoint gene involved at the metaphase-anaphase interface. *Pttg* is extensively revealed in a few neoplasms. Its over expression in human normal and cancer cells impels aneuploidy with micronuclei and multiple nuclei (Tfelt-Hansen et al., 2006). PTTG also shows transforming activity and its over expression probably leads to tumorigenesis (Tfelt-Hansen et al., 2006). Different mechanisms including intrinsic alterations in pituicytes, hypothalamic dysregulation and locally produced growth factors, seem to underlie tumorigenesis. This transforming event provides selective growth advantage to the mutant cell, resulting in secondary mutations and/or alterations in the growth factors favorable to clonal expansion and tumor progression. Due to their clonal composition, most cases of pituitary tumor exhibit excessive secretion of any given pituitary hormone which causes in endocrine disorders such as Cushing's syndrome for adrenocorticotropic hormone (ACTH), acromegaly for growth hormone, hyper-prolactinemia for prolactin and thyrotoxicosis for thyroid-stimulating hormone (TSH) (Tfelt-Hansen et al., 2006). Recently it was identified that another one drug trichostatin A (TSA) decreased the synthesis and secretion of ACTH by decreasing *Pttg* gene expression in AtT20 cells (Nakada et al., 2015) though the mechanism of dexamethasone (synthetic glucocorticoid)-mediated effect of *Pttg* expression is not examined yet.

Usually glucocorticoids show their biological activities by binding to a glucocorticoid receptors (Dostert and Heinzel 2004). Glucocorticoid receptor resides in the cytoplasm

before the presence of glucocorticoids (Cadepond et al., 1991).When glucocorticoids is added in any cells, it binds to glucocorticoid receptor, which translocates to the nucleus with glucocorticoids (Picard and Yamamoto 1987). Glucocorticoids bound glucocorticoid receptor form as a homodimer that binds to Gc-response element (GRE), then activates target gene transcription with transcription machinery (Kumar and Thompson 2005). *Pttg* is an important gene which is over-expressed in Cushing's disease and transcription factors named KLF6, cMyc, OCT1and SP1which are salient for *Pttg* gene expression and regulation. When dexamethasone is added, we found that there is no decrease of these transcription factors at mentioned concentration though there is a possibility to inhibit the interaction of these transcription factors and their respective DNA binding elements of *Pttg* resulting the suppression of *Pttg* gene expression by dexamethasone. To elucidate the detail mechanism of dexamethasone-mediated effect of *Pttg* expression, another research is needed.

Conclusion

From this research it can be concluded that glucocorticoids may play an important role in the negative regulation of *Pttg* expression in AtT20 cells. Furthermore, it can be speculated that glucocorticoid can suppress another factor which is more influential for the suppression of *Pttg* expression. Therefore, this research can be helpful for novel thinking in therapeutic medication for the treatment of Cushing's disease.

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