Human Recombinant Thyrotropin Fails to Induce Thyroid Cell Proliferation

Georgios Kalampounias[1], Athina Varemmenou[1], Christos Aronis[1], Irene Mamali[2], Athanasios-Nasir Shaukat[3], Dionysios V Chartoumpekis[2], Panagiotis Katsoris[1†*] and Marina Michalaki[2†]

[1]Division of Genetics, Cell Biology and Development, Department of Biology, School of Natural Sciences, University of Patras, 26504 Patras, Greece

[2]Endocrine Division, Department of Internal Medicine, School of Medicine, University of Patras, 26504 Patras, Greece

[3]Department of Biochemistry, School of Medicine, University of Patras, 26504 Patras, Greece

[†] **These authors share the last authorship**

***Correspondence :**

Panagiotis Katsoris : katsopan@upatras.gr

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Abstract

Background: Iatrogenic subclinical thyrotoxicosis that leads to thyrotropin (TSH) suppression is required in the management of patients with papillary thyroid carcinoma (PTC) due to the mitogenic effects of TSH. Nevertheless, the available evidence supporting this practice is limited and weak. Our study seeks to explore the impact of human recombinant thyrotropin (rh-TSH) at clinically relevant doses on cell cultures of human normal and PTC follicular cells.

Materials and Methods: A human normal thyroid cell line (Nthy-Ori 3-1) and cancerous cell lines (K1 and TPC-1) were transformed using the Lenti-X Tet-on Advanced system to overexpress the thyrotropin receptor (TSHR). Transformed and non-transformed cells were treated with escalating doses of rh-TSH (5–1,000 μIU/mL) under various conditions, such as the presence or absence of 0.5 U/mL insulin. Cell proliferation was estimated by the crystal violet assay, migration of the cells was assessed by a scratch test, and the expression levels of TSHR and thyroglobulin (Tg) were determined using RT-PCR and Western Blot.

Results: Under the in-vitro culture conditions employed, rh-TSH was not adequate to induce either the proliferation rate or the migration potential in transformed and non-transformed cells. On the contrary, Tg expression was increased with rh-TSH under the same culture conditions.

Conclusion: Our experiments indicate that clinically relevant concentrations of rh-TSH cannot induce proliferation and migration in both normal and cancerous cell lines, even after overexpression of TSHR. Further research is warranted to dissect the molecular mechanisms underlying these effects. These results could translate into better management of PTC patients.

1 Introduction

Thyroid cancer is the most common endocrine malignancy, accounting for 88% of all endocrine carcinomas and 3% of all human cancers (1–3). Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer, accounting for 84% of all cases (4). In the management of PTC patients, a degree of serum TSH suppression is required depending on disease severity and response to therapy (1,5). Thus, a substantial number of PTC patients are subjected to iatrogenic subclinical hyperthyroidism, which is known to have adverse effects on cardiac structure and function as well as bone health (6–8) However, the beneficial effects of suppressed TSH on progression-free survival or overall survival in PTC patients are not well established. Few retrospective epidemiological studies in PTC patients suggest that complete suppression of serum TSH is reasonable in high-risk patients with advanced disease (9,10). However, in one prospective study, the degree of TSH suppression was not an independent predictor of disease progression in high-risk patients when radioiodine therapy was included in the model (4). In another prospective randomized controlled trial that included high-risk patients, disease-free survival was not inferior to that of those without TSH suppression (11). The rationale for TSH suppression in the management of PTC patients comes from the notion that TSH, via its receptor TSHR, expressed in the follicular epithelial cells of the thyroid gland, regulates thyroid function and growth (12). TSHR couples to G proteins of all four subfamilies, including the stimulatory G protein Gs, which activates adenylyl cyclase to produce cAMP; Gi, which inhibits cAMP production; G13, which activates p44/42 mitogen-activated protein kinases (MAPK), commonly known as Erk1/2; and Gq/G11, which activates phospholipase C to produce inositol-1,4,5-trisphosphate, which is rapidly degraded to inositol monophosphate (IP-1; phosphoinositide signaling). From another perspective, activating mutations of either TSHR or Ga proteins that underlie functioning thyroid nodules preclude thyroid cancer (1,13). Similarly, autoantibodies against TSHR in Graves' disease are not widely recognized as a risk factor for thyroid cancer development (13,14). However, intriguing findings from a mouse model carrying a patient-derived constitutively active *TSHR D633H* mutation reveal a different scenario (15); large papillary thyroid cancer emerged in mice carrying the mutation at around one year of age, with nearly all homozygous mice affected. Remarkably, the commonly observed driver mutations of *Braf*, *Nras*, and *Kras* were notably absent in these tumors. Thus, it seems that enhanced TSHR signaling due to some rare *TSHR* activating mutations could lead to thyroid cancer development and growth. However, in the most common thyroid cancers due to *Braf*, *Nras*, and *Kras* mutations, the effects of TSHR signaling may be variable regarding the development and/or growth of these tumors. Theoretically, the impact of TSHR downstream signaling on cell proliferation in these thyroid tumors could be dependent on the presence of other mutations in *TSHR per se*, or in other components of the signaling pathway downstream of TSHR, or due to the crosstalk with other pathways. Hence, the role of TSH suppression in all these cases warrants investigation.

Few *in vitro* studies of various thyroid cell culture systems treated with different doses of bovine (b-TSH) or cattle TSH give inconclusive results regarding the proliferation and function of thyroid cells (16–21). The doses of TSH used in these studies were extremely high compared to circulating concentrations in humans. Recently, human recombinant thyrotropin (rh-TSH) became available on the market. Human TSH has 88% homology with bovine (NCBI Homologene). Bovine TSH contains positively charged residues at the α-subunit that are known to increase hormone-binding properties compared to human TSH (22). The bio- and immune activities of rh-TSH were examined in human fetal thyroid cells (23). Its biopotency was estimated to be 4.3±0.7 IU/mg and the immunopotency to 10.2±0.7 IU/mg whereas the immunopotency of b-TSH is 20-40 IU/mg depending on purification (24).

Herein, we investigated for the first time the effects of rh-TSH in physiologically relevant doses resembling the TSH concentrations in human plasma on the function and proliferation of normal and cancerous human thyroid cell lines.

2 Materials and Methods

2.1 Cell Lines

The Nthy-Ori 3-1 human non-cancerous thyroid follicular epithelial cell line and the K1 and TPC-1 papillary thyroid carcinoma cell lines were used as models for our study (source: ECACC, UK). K1 were cultured in high-glucose DMEM (Biowest, Nuaille, France), while TPC-1 and Nthy-ori-3-1 lines were grown on RPMI 1640 (Biowest, Nuaille, France), respectively, supplemented with 2.5% fetal bovine serum (FBS) (Biowest, Nuaille, France) and 1% penicillin/streptomycin (Biosera, Nuaille, France) in a 37°C incubator with 5% CO_2. The 2.5% FBS concentration was chosen based on preliminary experiments as the minimum (from a range of 1–10%) that allows the cells to survive and replicate as expected. All cell culture expendables were purchased from Greiner Bio-One (Kremsmünster, Austria).

2.2 Transformation of Cells to Overexpress the TSHR Using the Lenti-X Tet-on Advanced System

Wild-type human TSHR cDNA was subcloned from a pcDNA 3.1 hygro (-) plasmid (kind gift from Dr Holger Jaeschke from University Hospital Essen, Germany to a pLVX-Tight-Puro plasmid (25). This plasmid was used to transfect HEK293T cells (ECACC, UK) for the production of lentivirus. The culture supernatant containing the viruses produced in the HEK293T cells was used to transfect Nthy-Ori 3-1, K1, and TPC-1 cells. Following a 24-hour incubation with the lentiviruses, the medium was replaced with puromycin- and G418-containing medium at concentrations of 150 µg/mL. After 48 hours, only the transformed cells survived and were still attached to the plate.
Plasmids containing the Lenti-X Tet-on (pLVX-Tight-Puro) advanced system and the embedded cDNA TSHR sequence were constructed using the restriction enzymes BamHI and EcoRI. Subsequently, the transformed plasmids were analyzed using agarose electrophoresis and the constructed plasmid was isolated and transferred to DH5a E. coli bacteria. The bacteria were cultured in an LB growth medium containing 100 µg/mL ampicillin thus only the transformed clones survived. Plasmids were then isolated using the Monarch® Plasmid Miniprep Kit (New England Biolabs, Ipswich, MA, USA) and used to transfect HEK293T cells (ECACC, UK). The culture supernatant containing the viruses produced in the HEK293T cells was used to transfect Nthy-Ori 3-1, K1, and TPC-1 cells. Following a 24-hour incubation with the Lentiviruses, the medium was replaced with puromycin- and G418-containing medium at concentrations of 150 µg/mL. After 48 hours, only the transformed cells had survived and were still attached.

2.3 Successful Cell Transformation and TSHR Overexpression Verification

Polymerase Chain Reaction

DNA was extracted from lentiviral transduced cells using the Monarch® Genomic DNA Purification Kit (New England Biolabs, Ipswich, MA, USA), and its quality was determined using the NanoDrop Q500 spectrophotometer *(*Quawell CF, USA*)*, and PCR to detect the TSHR cDNA was performed using the primers:
TSHR forward 5’-AGCAGCTGGAATCACACTCC-3’
TSHR reverse 5’- GGGAGCTCTTTGAGGGCATC-3’
β-actin forward 5´-GCACAGAGCCTCGCCTT-3´
β-actin reverse 5´-GTTGTCGACGACGAGCG-3´

All primers were purchased from IDT (Leuven, Belgium). The cycling conditions consisted of 3 min denaturation at 95 °C followed by 30 cycles of 95 °C for 3 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds. The DNA polymerase FirePol and the mastermix were from Solis BioDyne, Estonia.

Quantitative Polymerase Chain Reaction

To assess the induction of TSHR expression, a dose of 3 μg/mL doxycycline was administered to the relevant cell lines. 24 hours later, total RNA was extracted using the Monarch® Total RNA Miniprep Kit (New England Biolabs, Ipswich, MA, USA). The extracted RNA was eluted in 50 μL of nuclease-free water and treated with Qiagen DNAse I (Germantown, MD, USA), following the manufacturer's procedure. The RNA quantity was assessed using the NanoDrop Q500 spectrophotometer *(*Quawell CF, USA*)*. RNA quality was assessed by determination of the ratio for absorbance at 260 nm vs. absorbance at 280 nm (A260 nm/A280 nm) using the NanoDrop Q500 spectrophotometer (which was assessed by calculating the ratios of absorbance A260nm/A280nm and A260nm/A230nm). RNA integrity was roughly verified by electrophoresis on a 1.2% denaturing agarose gel stained with ethidium bromide. 1μg of total RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Life Sciences, Penzberg, Germany), according to the manufacturer's instructions. The cDNAs were subjected to quantitative PCR (qPCR), which was performed with the LightCycler® 2.0 Real-Time PCR System (Roche Life Sciences, Penzberg, Germany) using LightCycler® FastStart DNA Master SYBR® Green I (Roche Life Sciences, Penzberg, Germany). The cycling conditions consisted of 15 minutes of denaturation at 95 °C, followed by 45 cycles of 95 °C for 10 seconds, 60 °C for 30 seconds, and 72 °C for 20 seconds. The primers used in qPCR were:
Tg forward 5´-CACCAACTCCCAACTTTTCC-3´
Tg reverse 5´-CAACTGACCTCCTTTGCCA-3´
The same pairs of primers as in the regular PCR for the *TSHR* gene and the reference gene, *β-actin*, were used for the qPCR as well. The specificity of the PCR product was verified by melting curve analysis (single-peak). For qPCR analysis, the data were analyzed using the relative quantification method. The efficiency of each PCR reaction was calculated by serial dilutions of pooled cDNA samples (26).

Western Immunoblotting

To assess the TSHR protein levels, cells were lysed using LaemmLi Sample Buffer, and the lysates were loaded onto 12% polyacrylamide gels for SDS-PAGE analysis. The proteins were then transferred onto the 0.20 μm PVDF Porablot® membrane (MN, Nordrhein-Westfalen, Germany), and Western blot analysis was performed using the Anti-TSHR rabbit IgG antibody (Biorad, Hertfordshire, UK). The secondary anti-IgG rabbit antibody and the chemiluminescence system SignalFire™ ECL were from Cell Signaling Technology (Danvers, MA, USA). To normalize the total protein amount, the cytoskeletal protein beta-tubulin was used, which was detected using an anti-tubulin mouse IgG antibody (#T6793, Sigma-Aldrich, Darmstadt, Germany) and a secondary antibody from Cell Signaling Technology (Danvers, MA, USA).

2.4 Treatment with Recombinant Human TSH, Insulin, or Both

25,000 K1, TPC-1, or Nthy-ori-3-1 cells were plated on 48-well plates (Greiner Bio-One, Kremsmünster**,** Austria) and left overnight to settle. Then, they were treated with recombinant human TSH (Thyrogen®, Sanofi Genzyme, Cambridge, MA, USA) and/or insulin (Humalog, Eli Lilly and Company, Indianapolis, IN, USA) at increasing concentrations indicated in the relevant figures in the presence of 2.5% FBS for 48 or 72 hours. (5-1000 μIU/mL). A negative control was included in each experiment without rh-TSH/FBS as well as a positive control with an elevated FBS concentration of 5%. The synthesis and excretion of T3 and T4 hormones as a result of TSHR stimulation were verified using the Alinity analyzer (Abbot, Abbott Park, IL, USA). We performed three independent experiments in triplicate.

2.5 Assessment of the Cell Number

After the incubation period, the cell number was estimated by the crystal violet assay. Specifically, cells were washed twice with PBS and then fixed with 3.7% formaldehyde in PBS (Phosphate Buffer Saline) (pH = 7.4) for 10 minutes at 25°C. Then, formaldehyde solution was aspirated, and cells were stained with crystal violet solution (Sigma-Aldrich, Burlington, MA, USA) for 20 minutes at 25°C. Subsequently, the cells were washed three times with distilled water. Then, water was carefully aspirated, and a 30% acetic acid solution was added to extract the crystal violet dye by light shaking for 20 minutes at 25°C. Finally, the extracted solution was transferred to a 96-well flat bottom plate (Greiner Bio-One, Kremsmünster, Austria) for spectrophotometry at 590 nm using a microplate reader. The equation used to convert the optical density (OD) to a number of cells (N) was the following: $N = 0.5 \times 10^5 \times OD$. This equation was based on preliminary experiments with an increasing number of cells.

2.6 The Effect of rh-TSH in Wound Healing of Nthy-Ori 3-1, K1 and TPC-1 Cells

Cells were cultured in a 6-well plate (Greiner Bio-One, Kremsmünster, Austria) until they reached maximum confluency. A scratch was then introduced in the cells' monolayer, and after washing away the scraped cells with a PBS solution, the cells were incubated with rh-TSH concentrations of 5, 10, 20, 50, and 100 μIU/mL and 2.5% FBS for 72 hours. Consequently, the healing rate was estimated by capturing images of the wounds at key time points.

2.7 Statistical Analysis

To compare the number of cells among the different concentrations of rh-TSH or insulin, a one-way ANOVA was used, followed by Sidak's multiple comparisons test. The data were expressed as means ± SEM. Prism 8 (Graphpad, Boston, MA, USA) for Windows was used for the statistics test and the generation of graphs. Statistical significance was set at $p<0.05$.

3 Results

3.1 Effects of escalating rh-TSH concentrations, on Thyroglobulin Expression, T3/T4 Excretion, Proliferation, and Migration of Nthy-ori-3-1, K1 and TPC-1 Cells

Under basal conditions, all cell lines expressed Tg mRNA. K1 basal Tg expression level was four and a half times higher than that of Nthy-Ori 3-1 cells (**Figure 1a**). Stimulation with rh-TSH (10 μIU/mL, 100 μIU/mL) led to an increase in Tg mRNA expression of both Nthy-Ori 3-1(**Figure 1b**) and K1 cells (**Figure 1c**). Cells treated with FBS, excreted T3 and T4 in the medium apparently because of endogenous b-TSH as well as iodine salts dissolved in the serum. The addition of rh-TSH did not alter T3/T4 levels.

Regarding proliferation, Nthy-ori-3-1 cells were incubated for 48 hours with increasing concentrations of rh-TSH of 0, 1, 5, 10, 20, 50, and 100 μIU/mL. A concentration of 2.5% FBS was used in all the proliferation experiments, as it was determined to be the minimum dose needed for sustaining cell survival and proliferation, therefore avoiding possible masking effects of the rh-TSH action (**Supplementary Figure 1**). Neither the cell number nor the migration of the cells were significantly impacted (**Figure 2a**). The action of rhTSH was examined under various conditions, such as the presence or absence of insulin and the replenishment of the cell medium every 24 hours (to supply the cells with an abundance of rh-TSH) (**Supplementary Figure 2a, 2b**). No discernible differences in cell number were documented between the various rh-TSH concentrations; therefore, a range of higher concentrations between 100 and 1,000 μIU/mL was tested (**Figure 2d**). Even under those conditions, rh-TSH was incapable of significantly inducing proliferation compared to the control group, which consisted of cells cultured with 5% FBS. The same conditions were used to monitor the cancerous cell lines K1 (**Figure 2b, 2e**) and TPC-1 (**Figure 2c, 2e**), and the results

showed that rh-TSH failed to induce cell proliferation in any tested concentration (0–1,000 μIU/mL). Modifying the medium to contain 0.5 U/mL of insulin, all treated samples showed significantly elevated cell numbers compared to the insulin-untreated group, whereas the rh-TSH concentration did not show an additive effect on cell number (**Figure 2**). Moreover, assessment of the proliferation after 72 hours of incubation with rh-TSH did not indicate any significant changes in either (**Supplementary Figure 2c**) Nthy-Ori 3-1, or (**Supplementary Figure 3d**) K1 cells.
The wound healing assay was used to study cell migration, and the results showed that rh-TSH was incapable of significantly inducing migratory effects on any of the three cell lines, whether insulin was present or not (**Figure 3a-c).**

3.2 Cell Transformation and TSHR Overexpression Verification

Under basal conditions, assessment of TSHR mRNA expression by qRT-PCR showed that Nthy-ori-3-1 cells express low levels of TSHR but higher than K1 cells (**Figure 1d**). To eliminate the problem of low and fluctuating TSHR levels, we generated clones of Nthy-Ori 3-1, K1, and TPC-1 cells with controllable WT TSHR expression using the Lenti-X Tet-on Advanced system. Plasmids were constructed containing the wild-type TSHR gene, and the successful construction was verified with DNA agarose gel electrophoresis (**Suppl. Figure 2a**). The successful transfection and subsequent transformation were verified using PCR to detect copies of the TSHR cDNA insert. All three cell lines were observed to have been successfully transformed, and thus the insert was successfully embedded inside the genome (**Suppl. Figure 2b**). To confirm the induced expression of TSHR after 24 hours of doxycycline stimulation TSHR mRNA levels were assessed by qPCR. In the transformed thyroid cell line Nthy-Ori 3-1, a 17-fold increased expression was achieved, and this effect was more intense in the cancerous cell lines. The transformed K1 cells reached an almost 25-fold overexpression, and the transformed TPC-1 cell line increased its mRNA TSHR levels 60 times more than the naïve one (**Figure 4a**). To confirm that the observed changes at the mRNA level of TSHR are translated into differential protein expression, TSHR protein expression after stimulation of cell lines with doxycycline was analyzed by western immunoblotting. The Nthy-Ori 3-1 cells increased their TSH receptor levels two times compared to the basal levels, while the cancerous K1 and TPC-1 cells overexpressed the receptor by four and eight times, respectively (**Figure 4b-c**).

3.3 Effects of rh-TSH Concentrations, on Proliferation and Migration of Nthy-Ori 3-1, K1 and TPC-1 Cells that Overexpress the TSH Receptor

The same protocol of proliferation and migration experiments was carried out on transformed Nthy-Ori 3-1, K1, and TPC-1 cells after pre-treatment with 3 g/mL doxycycline. Comparing the doxycycline-treated cells to the untreated group, which served as the control group, overall slightly lower cell numbers in the doxycycline-treated cells were observed, which were justified as a cytostatic result of doxycycline (27). The same cytostatic effect was observed on the non-transformed cells (**Suppl. Figure 4a-c**). Although the dose used during transduction experiments is far lower than cytotoxic levels, the presence of doxycycline was expected to affect the proliferation rate which would be evident due to low FBS concentrations. However, the positive control (5% FBS) successfully induced proliferation compared to the starved sample; therefore, the presence of doxycycline was not theorized as responsible for rh-TSH ineffectiveness in promoting mitosis. Independently of the TSHR overexpression, treatment with a wide range of rh-TSH concentrations (with or without insulin) did not affect the rate of cell proliferation (**Figure 5**).
The scratch tests used to examine cell migration revealed that rh-TSH stimulation (with or without insulin) did not cause any changes (**Figure 3d-f**). A complete matrix of all concentrations monitored, and all time points can be found in the supplement section (**Supplementary Figures 5-7**).

4 Discussion

Herein, we examined, for the first time, the effects of clinically relevant concentrations of rh-TSH on proliferation, migration, and function in cultures of normal and cancerous thyroid cells. We found that the administration of escalating doses of rh-TSH, similar to those found in plasma, did not increase cell number or migration rate in the normal thyroid cell line Nthy-Ori-3-1, as well as in K1 and TPC-1 papillary thyroid carcinoma cell lines. However, it tended to increase Tg mRNA levels. Due to the low expression of TSHR in these cell lines (**Figure 4a**), we successfully transformed these cells to overexpress TSHR (**Figure 5a**). Once again, the same escalating doses of rh-TSH were unable to induce proliferation or migration of the cells. Moreover, all experiments were conducted under various conditions, such as the presence or absence of insulin, and with the cell medium replenished every 24 hours.

Although there is a prevailing perception that TSH induces the growth and proliferation of follicular thyroid cells, there is limited evidence supporting this knowledge. *In vitro* studies have primarily been conducted using cultures derived from animal cell lines, with only a few studies focusing on human cell cultures that include both normal and malignant thyrocytes. These studies have investigated the effects of various doses of bovine or cattle TSH mainly on cellular responses, particularly regarding cell proliferation. However, the results obtained from these studies have been inconsistent, suggesting that the responses of thyroid cells to TSH treatment can vary widely across different experimental conditions and cell types (17–19,21,24,28–30). In one study, researchers investigated the effects of escalating doses of b-TSH (1, 10, and 100 mU/mL) on growth and invasion in primary cell cultures of follicular and papillary thyroid carcinomas (28). Specifically, they analyzed three follicular thyroid carcinoma (FTC) cell lines derived from one patient, comprising one from a primary tumor and two from metachronous metastases originating from a lymph node and lung metastasis. Additionally, they examined two papillary thyroid carcinoma (PTC) cell lines from two patients who had not experienced recurrence. Notably, the maximal effect on proliferation and invasion was observed at a dose of 10 mU/mL (equivalent to 1000x of that found in human plasma), while the dose of 100 mU/mL was found to inhibit proliferation. Intriguingly, the response of the five cell lines varied in terms of growth and invasion at the same b-TSH dose, with PTC lines exhibiting maximal response, followed by FTC lines, and even smaller responses observed in metastatic lines, while inhibition remained consistent (28). In another *in vitro* study, the authors found that the growth of papillary thyroid cancer cells and adenomatous goiter cells was differentially regulated. Both cell lines were found to express functional TSH receptors. When treated with bovine-TSH at a dose of 10 μU/mL, a dosage similar to that used in our study, with or without IGF-1, the proliferation of cancerous thyrocytes was inhibited, while that of human adenomatous goiter cells was stimulated. Intriguingly, the activation of TK receptors by various growth factors, such as EGF, bFGF, and IGF-1, along with stimulation by E2 (at subclinical concentrations), promoted proliferation in both cancerous and goitrous cells (19). Morgan S et al. investigated the effects as well of 10 mU/mL b-TSH on primary cultures of normal human thyrocytes (18). They observed that b-TSH in combination with IGF-1 did not induce thyrocyte proliferation. However, it did upregulate the expression of thyroid-specific genes, including thyroglobulin (TG), thyroperoxidase, sodium-iodide symporter (NIS), deiodinase type 2, and TSHR. These discrepancies became the subject of a published commentary that mainly raised methodological issues (18). On the contrary, Roger et al. have demonstrated that b-TSH serves as a mitogenic factor for normal human thyroid cells (24). Employing low concentrations of b-TSH (8-1000 μU/mL) and cAMP activators, they conducted experiments using primary thyroid cell cultures derived from seven healthy subjects and two subjects with non-functioning thyroid nodules, albeit utilizing normal thyroid tissue. Their findings revealed that iodide uptake reached its maximum efficiency at 100 μU/mL b-TSH while being inhibited at 1000 μU/mL. Additionally, purified b-TSH, starting from a dose of 20 μU/mL, induced DNA synthesis, with maximal stimulation observed between 100-300 μU/mL, leading to the conclusion that TSH serves as a potent goiterogenic factor acting via cAMP signaling. Furthermore, the researchers explored the effects of EGF and an activator of calcium phospholipid-dependent protein kinase-C, finding that these growth factors induced proliferation and de-differentiation of thyroid cells by impairing their iodide-trapping capacity. A

commentary publication from the same research group further corroborates these findings by compiling published data demonstrating that TSH, acting through cAMP, stimulates the proliferation of human thyrocytes in primary culture (31). More recently, a single application of 100, 1000, and 10^4 μIU/mL of cattle TSH in human PTC cells (TPC-1) increased the proliferation rate in a dose-dependent manner by promoting the transition of TPC-1 cells from the G_1 phase to the S phase and by remarkably increasing the mRNA and protein expression of cyclin D1 (21). The contradictory results observed in these various *in vitro* studies could be attributed to several factors, such as the TSH dose used, discrepancies in the specific culture systems used, including variations in cell culture media, supplements, and culture conditions, incubation time, context (normal or cancer cell line), and the method of growth assessment. As FBS contains endogenous TSH (b-TSH), insulin, dissolved iodine salts, and related cofactors that are required for thyroid cell survival and the FBS batches vary significantly; herein, we tried to minimize the effects of these factors by using the minimum required concentration of FBS in our experiments.

It is also noteworthy that most of the published studies have not evaluated the expression of TSHR in the various cell line models. Differences in the media used, multiple passages of cell lines can sometimes have deleterious effects on the thyroid-like phenotype and the expression of TSHR. It is known that the expression of TSHR in primary cultures is 100-fold lower than in intact thyroid cells *in vivo* (32). To overcome, at least partially, this obstacle, we transformed all three cell lines to overexpress TSHR. The overexpression was controlled by the presence of doxycycline, and the transformed cells were assessed for the same functions as previously noted. Interestingly, no significant changes were observed following stimulation with rh-TSH and insulin, exactly like the non-transformed clones. The migration rates were also unchanged, and the pro-proliferative effects of insulin (once administered alone) were also documented. Our findings are consistent with a prior study wherein 10 mU/mL of b-TSH failed to induce proliferation in human thyroid follicular carcinoma cells that were transfected to overexpress functional TSHR (33).

Besides the expression of TSHR *per se*, it is also important to note the functionality and relevant mutations of the receptor that may affect signal transduction and ultimately cell proliferation. The cell lines that were used in the present work are not known to carry any TSHR mutations. The Nthy-Ori-3.1 comes from normal thyroid tissue, while the K1 and TPC-1 ones come from papillary thyroid carcinoma and carry correspondingly the following genetic aberrations: *BRAF p.V600E* and *CCDC6-RET* fusion (34). These lines are not known to carry *TSHR* mutations. At baseline and overexpressed levels of wild-type TSHR, we did not see practically any effect on the proliferation of cells, regardless of the conditions used herein. Hence, it would be plausible to investigate further the effect of various TSH concentrations not only in WT TSHR but also in various mutated forms of TSHR that may affect intracellular signaling and thus the proliferation rate. Further assessment of the signaling pathways downstream of the TSHR is also warranted under these conditions and can be the subject of future studies. The addition of insulin at a concentration of 0.5 U/mL in the medium on top of rh-TSH increases the cell number, but not in a rh-TSH dose-dependent manner. The insulin-like growth factor 1 (IGF-1) receptor (IGF-1R) is a transmembrane tyrosine kinase receptor involved in growth and development. It seems that there is a cross-talk between the TSH/TSHR and insulin/IGF-I/IGF-1R pathways in thyroid cell proliferation and differentiation (35–42). Thus, in agreement with our data, in most of the *in vitro* studies, IGF-1 or high insulin levels are required for the culture (24,31).

TSH, besides proliferation, modulates thyroid hormone synthesis and secretion by regulating the expression of various thyroid-specific genes such as TSHR, Tg, thyroid peroxidase (TPO), and sodium-iodide-symporter (NIS). Our findings indicated that treatment of both Nthy-Ori-3-1 normal thyroid follicular epithelial cells and K1 papillary thyroid cancer cells with increasing concentrations of rh-TSH led to a gradual increase in expression of the *Tg* gene, with peak mRNA expression levels at the highest dose. Our results seem to be consistent with previous studies where treatment of cultured thyroid cells with b-TSH stimulated the TPO and Tg mRNA levels in a dose- and time-dependent manner (43,44).

A limitation of our study is that it is based on *in vitro* experiments and employs cell lines that may gradually lose some of the properties of the original thyroid tissue they come from due to mutations

and selection pressure during culture (45,46). To at least partially address this concern, we evaluated our cell lines for the preservation of thyroidal phenotype by measuring T3/T4 secretion and assessing Tg mRNA expression, observing that the cells retained some features of their original phenotype. However, it is noteworthy that under basal conditions, TSHR mRNA expression was low in both normal and cancerous cell lines. To overcome this limitation, we engineered all three cell lines to overexpress TSHR. To strengthen our findings, further investigations are warranted using additional cell lines that overexpress wild-type as well as mutant TSHR variants (various versions of constitutively active or inactive, as aforementioned) (25) or utilizing more physiologically-relevant cell systems such as primary cultures of thyroid gland organoids (47) from normal and cancerous thyroid tissue. Additionally, it is imperative in future studies to assess the functionality of the overexpressed TSHR by assessing the activation status of downstream signaling pathways that were not assessed in this study, given that no effect was seen in proliferation.

In conclusion, we have shown that rh-TSH in clinically relevant doses cannot induce proliferation and migration in both normal and cancerous cell lines under various conditions. Further research is warranted to dissect the molecular mechanisms underlying these effects. Our results could translate into better management of PTC patients in the future.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

GK: Investigation, Methodology, Formal Analysis, Validation, Visualization, Writing – Original Draft. AV: Investigation. Methodology. CA: Investigation, Methodology. IM: Investigation, Methodology, Formal Analysis. AS: Methodology, Software, Formal Analysis. DC: Resources, Visualization, Writing – Original Draft, Writing – Review & Editing. PK: Conceptualization, Formal Analysis, Project Administration, Resources, Supervision, Visualization, Writing – Review & Editing. MM: Conceptualization, Funding Acquisition, Investigation, Project Administration, Resources, Supervision, Writing – Original Draft, Writing – Review & Editing.

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10 Data Availability Statement

We confirm that the data supporting the findings of this research are contained within the article and its supporting material.

11 Figures

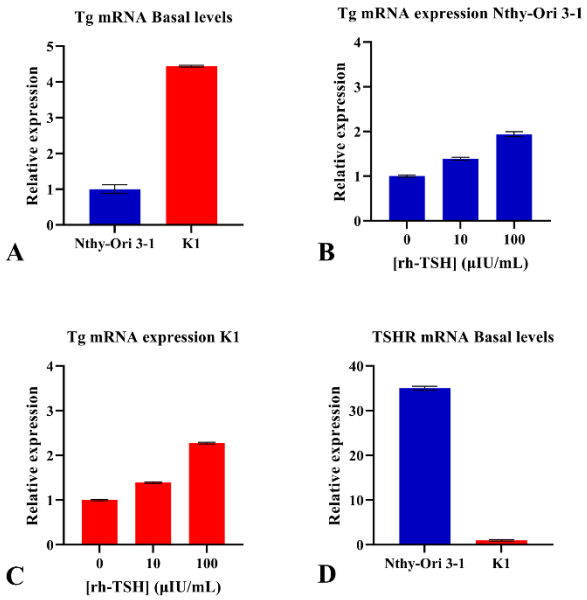


Figure 1: **Thyroglobulin Thyroid Stimulation Hormone Receptor Expression Levels Determination**. (**A**) Using q-RT-PCR, the basal expression levels of the Thyroglobulin (*Tg*) mRNA of Nthy-Ori 3-1 and K1 cells were assessed. Using q-RT-PCR, the effects of rh-TSH on *Tg* mRNA expression of (**B**) Nthy-Ori 3-1 and (**C**) K1 cells were studied. Prior to RNA synthesis, the cells were incubated for 48 hours with the designated hormone dose. (**D**) Using q-RT-PCR, the basal expression levels of thyroid stimulation receptor (TSHR) mRNA in Nthy-Ori 3-1 and K1 cells were assessed. All the samples were analyzed using Livak's model. Beta-actin was used as a reference gene to normalize the data. Each bar represents the average of three experimental values, and the error bars refer to the standard error of the mean (SEM).

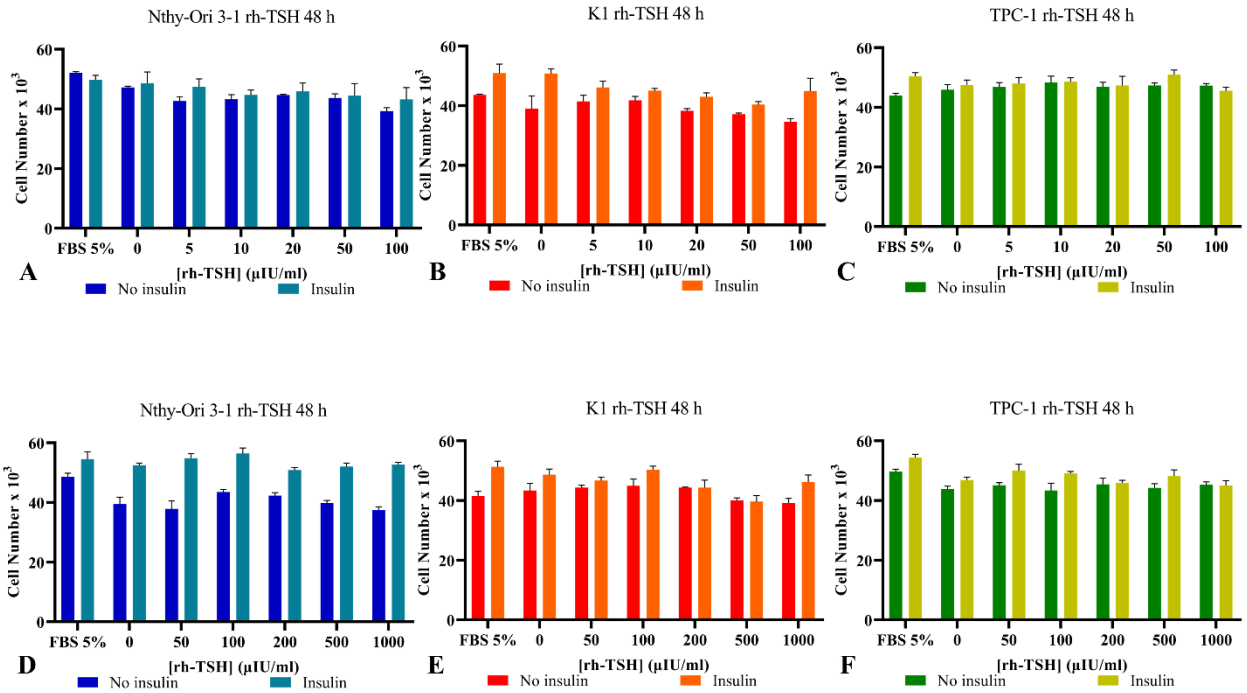


Figure 2: **Proliferation Assay of Nthy-Ori 3-1, K1 and TPC-1 Cells Treated With rh-TSH and/or Insulin.** Cells were seeded into microplates, and after 24 hours of settling and attachment, the old medium was aspirated and replaced with a new one containing 2.5% FBS and the designated doses of the hormones. A range of rh-TSH concentrations from 5 µIU/mL to 100 µIU/mL was initially tested in (**A**) Nthy-Ori 3-1, (**B**) K1, and (**C**) TPC-1 cells. The same set of experiments was designed with higher rh-TSH doses, ranging from 50 µIU/mL to 1000 µIU/mL in all three cell lines (**D-F**). Wherever insulin is annotated, a concentration of 0.5 U/mL is used. As a positive control for elevated cell proliferation rates, a 5% FBS-containing medium was used. The experiments were conducted in triplicate, and the error bars represent the standard error of the mean (SEM). The data analysis was performed using multiple t-tests, and no significant differences between the different rh-TSH concentrations were detected. Insulin-treated cells exhibited an overall increased cell number, which was statistically significant as shown by a paired t-test ($P<0.0001$).

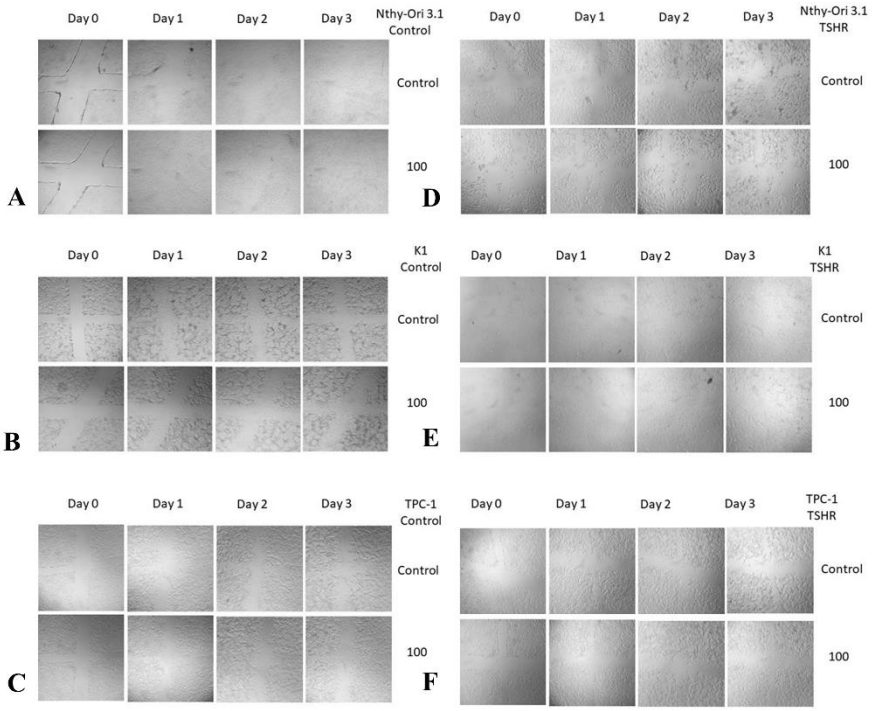


Figure 3: **Wound Healing Assay of Transformed and Non-Transformed Nthy-Ori 3-1, K1 and TPC-1 Cells Treated With rh-TSH**. Cells were seeded into six-well microplates, and after the formation of a confluent monolayer, scratches of equal dimensions were made using a pipette tip. Subsequently, the old medium and the debris were aspirated and replaced with a new one containing 2.5% FBS and the designated doses of the hormones. Photographs were taken every 24 hours using a camera mounted on a microscope (magnification X100). Changes were observed in either non-transformed cells (**A**, **B**, and **C**) or transformed cells (**D**, **E**, and **F**).

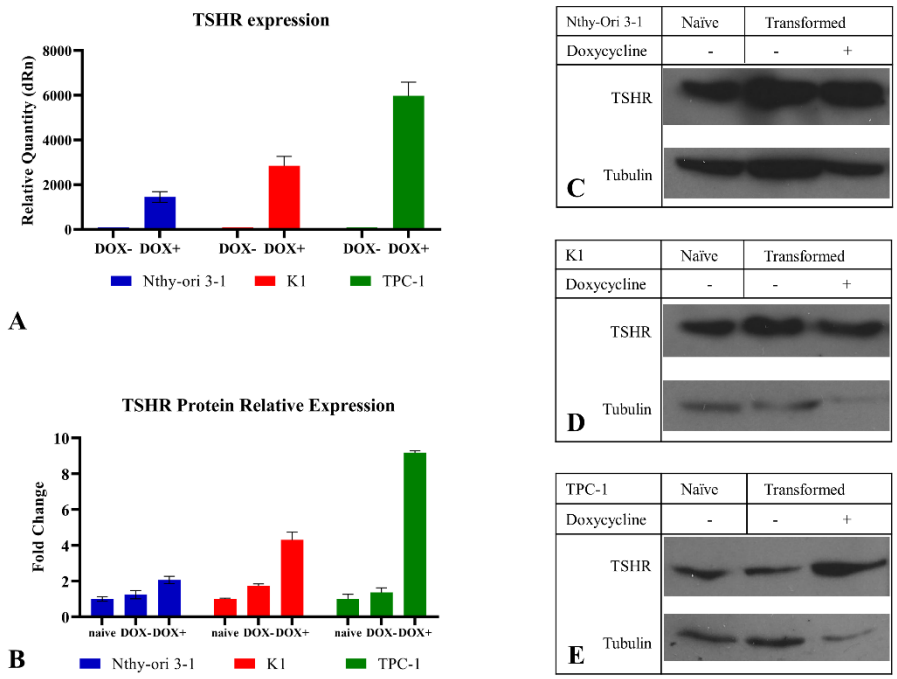


Figure 4: **TSHR mRNA and Protein Expression Levels Following Transformation with the Lenti-X Tet-on Advanced System**. After the selection of the transformed clones using the appropriate antibiotics, incubation with a dose of 3 g/mL doxycycline was introduced to activate the Tet-On Advanced system. (**A**) Following 24 h of incubation, total RNA was isolated, cDNA was constructed, and qPCR was performed. Relative expression was assessed using Livak's model. Each bar represents the average of three experimental values, and the error bars refer to the standard error (SEM). Beta-actin was used as a reference gene to normalize the data (**B**) Following 24 h of incubation, the cells were lyzed the equal amounts of proteins were analyzed using SDS-PAGE on 12% polyacrylamide gels. Western Blot (WB) analysis was performed to detect the TSHR protein band. Using ImageJ, the WB results were quantified (n=3). A representative western blot image is shown: (**C**) Nthy-Ori 3-1, (**D**) K1, and (**E**) TPC-1 cells (non-transformed and transformed). Beta-tubulin was used as a loading control to normalize the results.

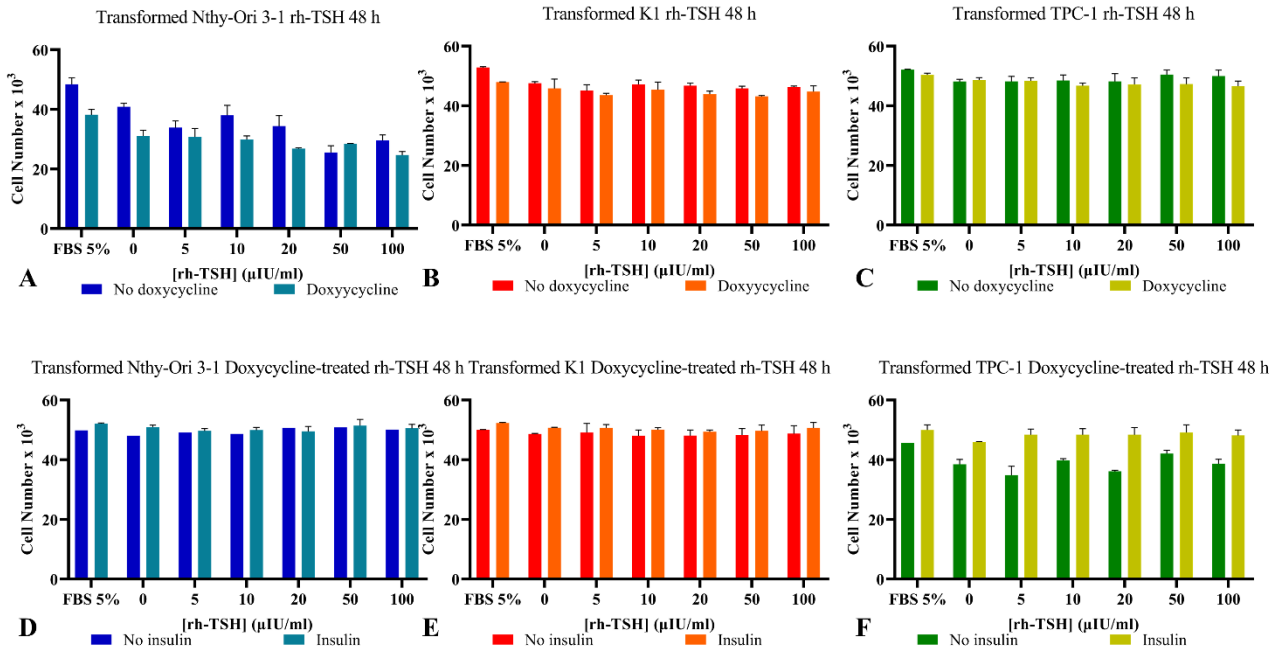
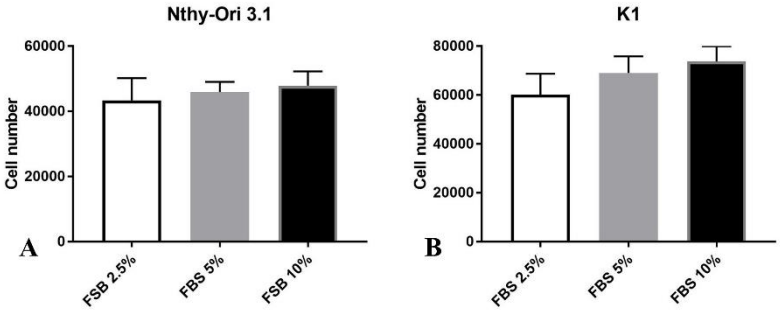
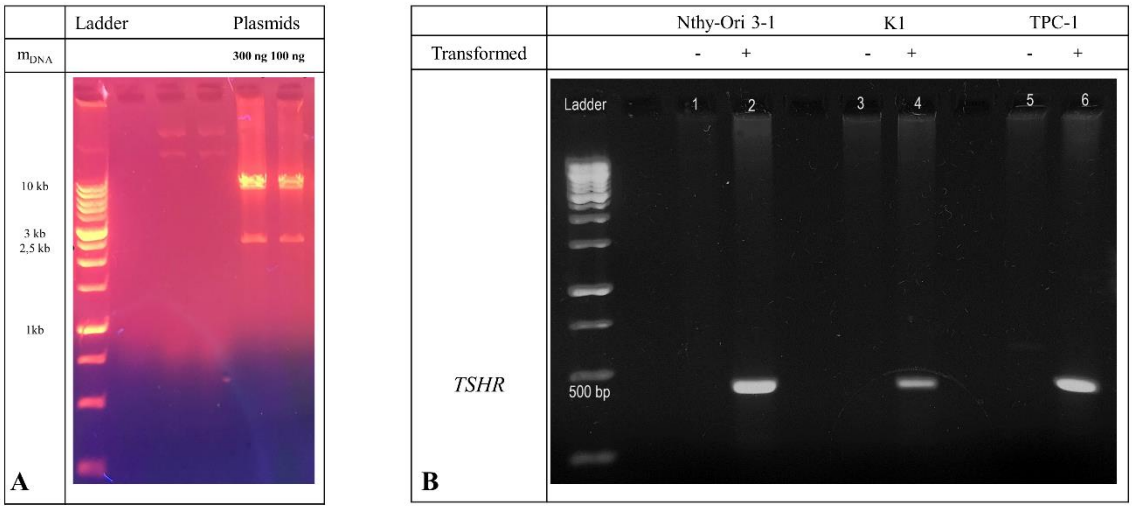


Figure 5: **Proliferation Assay of transformed Nthy-Ori 3-1, K1 and TPC-1 Cells Treated With rh-TSH and/or Insulin.** Cells were seeded into microplates, and after 24 hours of settling and attachment, the old medium was aspirated and replaced with a new one containing 2.5% FBS, 3 g/mL doxycycline, and the designated doses of the hormones. A range of rh-TSH concentrations from 5 µIU/mL to 100 µIU/mL was tested in transformed (**A**) Nthy-Ori 3-1, (**B**) K1, and (**C**) TPC-1 cells. As a control group, cells lacking doxycycline stimulation were used, which exhibited overall increased cell numbers because doxycycline had mild cytostatic effects on the experimental group. The same set of experiments was performed using a dose of 0.5 U/mL insulin in doxycycline-treated transformed cells (**D-F**). As a positive control for elevated cell proliferation rates, a 5% FBS-containing medium was used. The experiments were conducted in triplicate, and the error bars represent the standard error (SEM). The data analysis was performed using multiple t-tests. significant as shown by paired t-tests ($P<0.0001$).

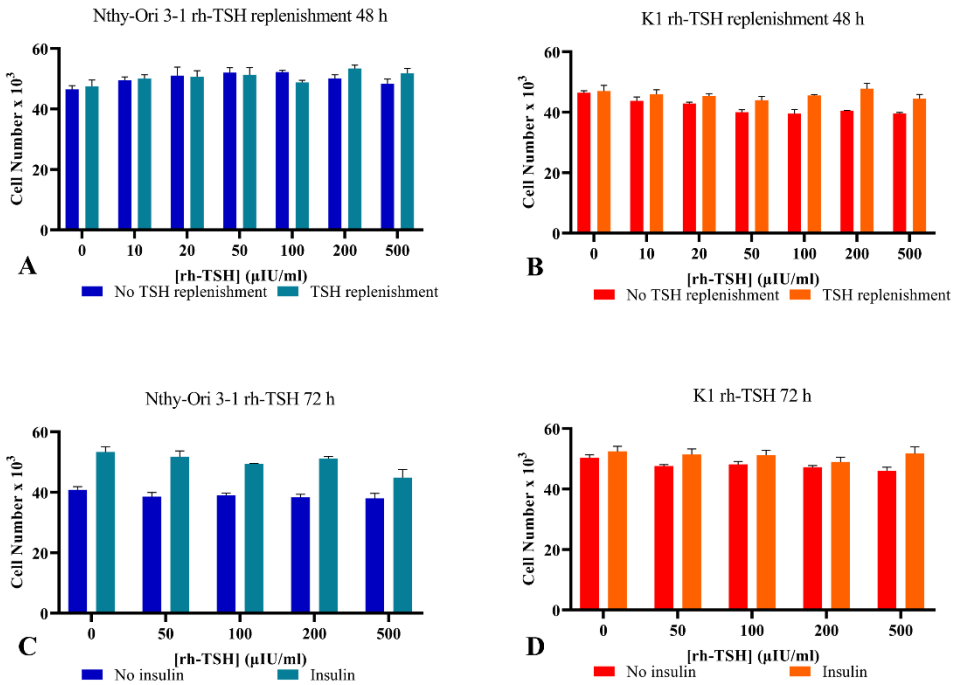
12 Supplementary Figures



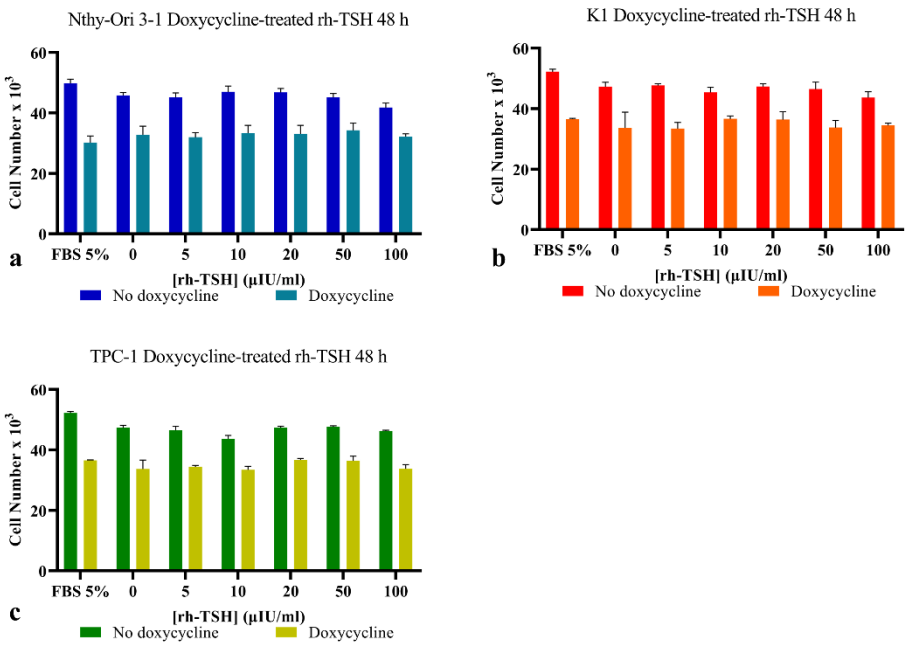
Supplementary Figure 1. Minimal FBS Concentration Optimization. (**A**) Nthy-Ori 3-1, and (**B**) K1 cells were seeded into microplates, and after 24 hours of settling and attachment, the old medium was aspirated and replaced with a new one containing different doses of FBS (2.5%, 5%, and 10%). The 2.5% FBS concentration allowed the cells to survive for 72 hours, while the final cell number was significantly lower than that observed when greater doses were used. Therefore, it was designated as the standard medium for all the experiments. The experiments were conducted in triplicate, and the error bars represent the standard error (SEM).



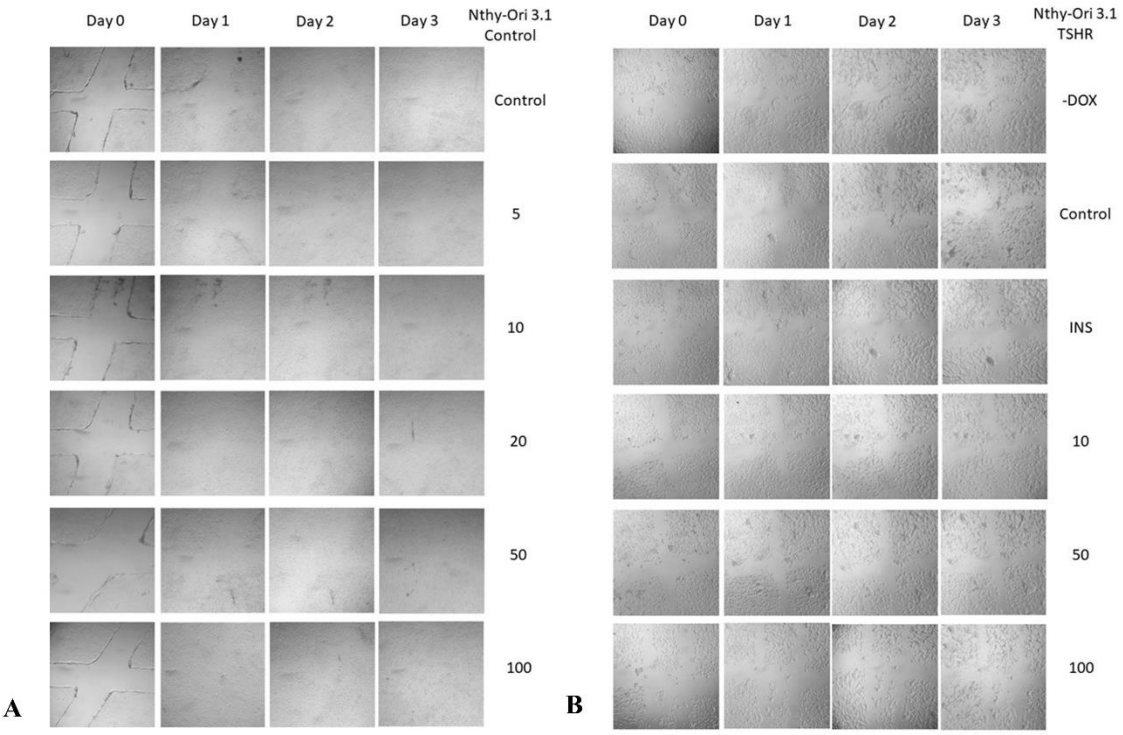
Supplementary Figure 2. Successful Plasmid Construction and Cell Transfection Verification. (**A**) TSHR cDNA was inserted into pLVX-Tight-Puro plasmid in EcoRI and BamHI positions. The expected plasmid had a predicted size of 2.5 kbp and was successfully detected. Different quantities are displayed (300 µg and 100 µg of DNA at lane five and lane 6, respectively); (**B**) After the transduction of the Nthy-Ori 3-1 (lane 2), K1 (lane 4), and TPC-1 cells (lane 6) with lentiviruses and the selection using the antibiotics, the survived cells were lysed, and total DNA was isolated using the Monarch® Genomic DNA Purification Kit by NEB. PCR was conducted and revealed that the transformed clones had successfully incorporated the *TSHR* cDNA. The expected PCR product had an expected size of 439 bp. As a control sample, the non-transformed cells were used (lanes 1, 3, and 5 respectively).



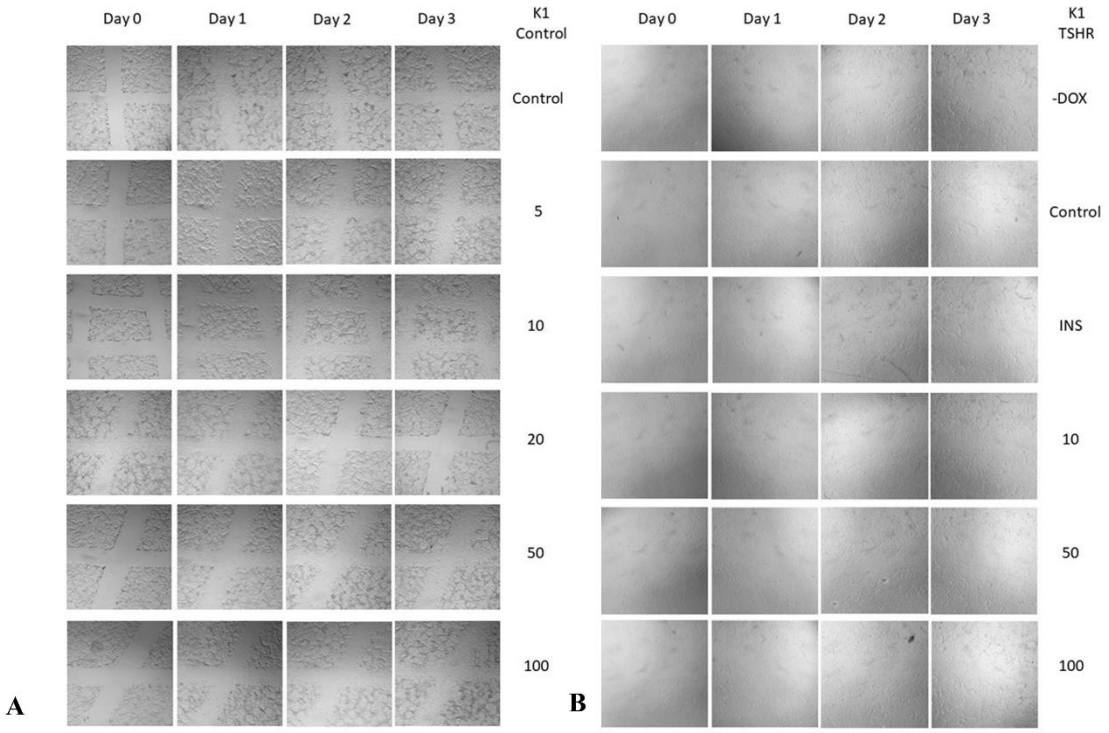
Supplementary Figure 3. Modified Proliferation Assay of Nthy-Ori 3-1 and K1 Cells Treated With rh-TSH and/or Insulin. (**A**, **B**) Cells were seeded into microplates, and after 24 hours of settling and attachment, the old medium was aspirated and replaced with a new one containing 2.5% FBS and the designated doses of the hormones. A range of rh-TSH concentrations from 5 µIU/mL to 500 µIU/mL was initially administered, and after 24 h of incubation, the medium was replenished with a new one containing the same amounts of FBS and the hormones. Wherever insulin is annotated, a dose of 0.5 U/mL is used. (**C**, **D**) Following the same seeding protocol as previously noted for Nthy-Ori 3-1 and K1 cells, a range of rh-TSH doses (with or without insulin present) were left inside the cell medium for 72 h, and the final cell numbers were subsequently determined using the crystal violet assay. Both experiment sets (**A-D**) were conducted in triplicate, and the error bars represent the standard error (SEM). The data analysis was performed using multiple t-tests, and no significant differences between the different rh-TSH concentrations were detected. Insulin-treated cells exhibited an overall increased cell number, which was statistically significant as shown by a paired t-test ($P<0.0001$).



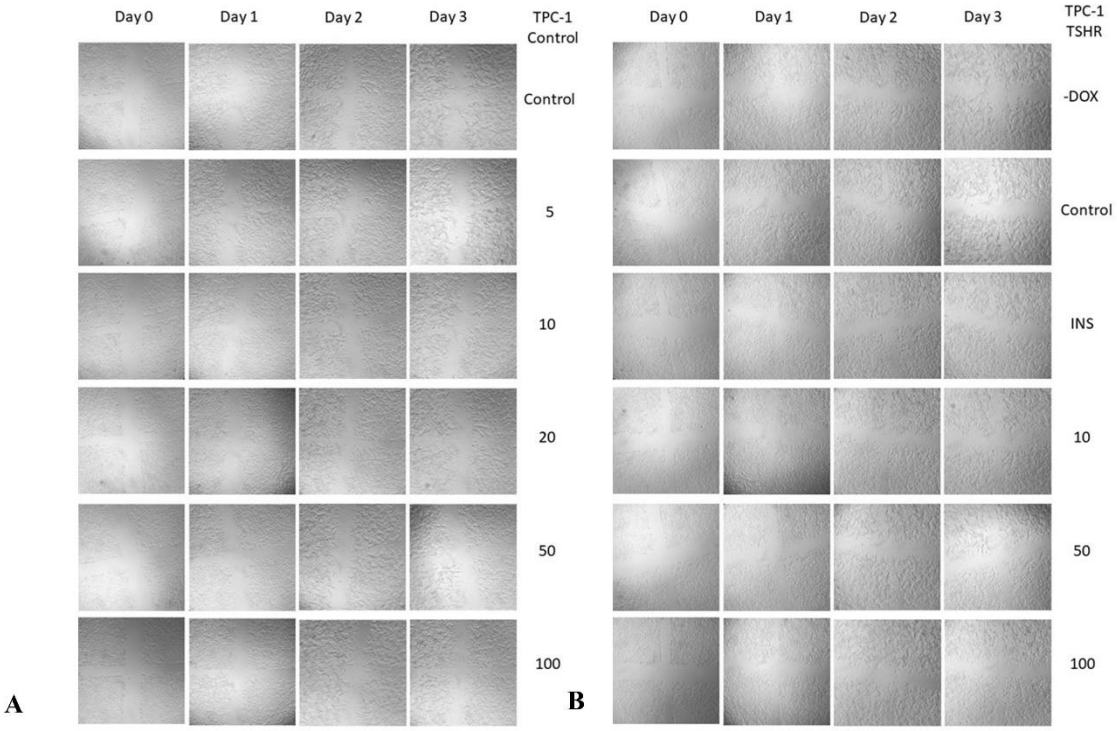
Supplementary Figure 4. Proliferation Assay of Non-transformed Nthy-Ori 3-1, K1 and TPC-1 Cells Treated With rh-TSH and doxycycline. Cells were seeded into microplates, and after 24 hours of settling and attachment, the old medium was aspirated and replaced with a new one containing 2.5% FBS, 3 g/mL doxycycline, and the designated doses of the hormones. A range of rh-TSH concentrations from 5 µIU/mL to 100 µIU/mL was tested in transformed (**A**) Nthy-Ori 3-1, (**B**) K1, and (**C**) TPC-1 cells. As a control group, cells lacking doxycycline stimulation were used.



Supplementary Figure 5. Wound Healing Assay of Transformed and Non-Transformed Nthy-Ori 3-1 Cells Treated With rh-TSH. Cells were seeded into six-well microplates, and after the formation of a confluent monolayer, scratches of equal dimensions were made using a pipette tip. Subsequently, the old medium and the debris were aspirated and replaced with a new one containing 2.5% FBS and a range of rh-TSH doses from 5 μIU/mL to 100 μIU/mL. Photographs were taken every 24 hours using a camera mounted on a microscope (100X magnification). Changes were observed in either non-transformed cells (**A**) or transformed cells (**B**).



Supplementary Figure 6. Wound Healing Assay of Transformed and Non-Transformed K1 Cells Treated With rh-TSH. Cells were seeded into six-well microplates, and after the formation of a confluent monolayer, scratches of equal dimensions were made using a pipette tip. Subsequently, the old medium and the debris were aspirated and replaced with a new one containing 2.5% FBS and a range of rh-TSH doses from 5 μIU/mL to 100 μIU/mL. Photographs were taken every 24 hours using a camera mounted on a microscope (100X magnification). Changes were observed in either non-transformed cells (**A**) or transformed cells (**B**).



Supplementary Figure 7. Wound Healing Assay of Transformed and Non-Transformed TPC-1 Cells Treated With rh-TSH. Cells were seeded into six-well microplates, and after the formation of a confluent monolayer, scratches of equal dimensions were made using a pipette tip. Subsequently, the old medium and the debris were aspirated and replaced with a new one containing 2.5% FBS and a range of rh-TSH doses from 5 µIU/mL to 100 µIU/mL (100X magnification). Photographs were taken every 24 hours using a camera mounted on a microscope. Significant changes were observed in either non-transformed cells (**A**) or transformed cells (**B**).