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# Effects of fulvestrant on biological activity and Wnt expression in rat GH3 cells☆

**Jiwei Bai[1], Yan Wang[2], Chuzhong Li[1], Yazhuo Zhang[1]**

1*Beijing Neurosurgical Institute, Capital Medical University, Beijing 100050, China*
2*Center of Clinical Genetics, Affiliated Bayi Children's Hospital, General Hospital of Beijing Military Command of Chinese PLA, Beijing 100700, China*

## Abstract

The present study investigated the influence of anti-estrogen treatment (fulvestrant) on pituitary adenoma cell line GH3 biological activity, the estrogen receptor α pathway, the WnT pathway, and mechanisms of decreased Wnt inhibitory factor-1 expression in GH3 cells. Results showed that fulvestrant suppressed GH3 cell proliferation and reduced hormone secretion in a dose-dependent manner. Estrogen receptor α and Wnt4 expression decreased, but Wnt inhibitory factor-1 expression increased in a dose-dependent manner following fulvestrant treatment, and β-catenin expression remained unchanged. Inhibitors of DNA methylation and histone modification upregulated Wnt inhibitory factor-1 expression. Results suggested that fulvestrant suppressed biological activity of GH3 cells *via* the estrogen receptor α and Wnt pathways. These results suggested that decreased Wnt inhibitory factor-1 expression in GH3 cells played a role in epigenetic mechanisms. Anti-estrogen therapies could provide novel treatments for growth hormone adenomas.

**Key Words:** β-catenin; estrogen; estrogen receptor α; GH3 cell line; Wnt inhibitory factor-1; Wnt4

Jiwei Bai☆, M.D., Beijing Neurosurgical Institute, Capital Medical University, Beijing 100050, China

Corresponding author: Yazhuo Zhang, M.D., Chief physician, Professor, Beijing Neurosurgical Institute, Capital Medical University, Beijing 100050, China
zyz2004520@126.com

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## INTRODUCTION

The pituitary gland functions as a central organ in the vertebrate endocrine system[1]. Pituitary adenomas are the most common pituitary gland-derived neoplasms, accounting for up to 25% of all intracranial tumors. More than two-thirds of these tumors manifest in clinical symptoms of excess hormonal secretion. Growth hormone (GH)-secreting pituitary adenomas (GHomas) constitute 10–15% of all pituitary adenomas[2-3]. Although GHomas are classified as benign, due to their inability to metastasize, these tumors lead to a wide range of cardiovascular, respiratory, endocrine, and metabolic morbidities due to metabolic complications and excess hormone syndromes[3-4].

Estrogen plays an important role in the regulation of anterior pituitary function and maintenance of tissue homeostasis[5]. Estrogen involvement in GH synthesis and release from the anterior pituitary is well accepted, although it remains unclear whether estrogen regulates these actions by directly targeting GH-secreting cells or *via* modulation of hypothalamic factors controlling GH secretion[6-7]. For example, changes in GH mRNA expression occur during the estrous cycle, which positively correlates with changes in circulating estrogen in rat GH-secreting cells[8]. In addition, xeno-estrogens are reported to induce GH mRNA and protein expression *via* the estrogen receptor (ER) pathway in rat GH-secreting GH3 cells[9]. Estrogen acts mainly by regulating transcription of specific genes through two genetically distinct receptors, ERα and ERβ, which function as hormone-inducible transcription factors. Although ERα and ERβ exist in GH-secreting cells, ERβ has not been established directly as a clinical mediator of *in vivo* pituitary effects[10]. Estrogen may exert its role in GH-secreting cells primarily *via* ERα. Although the relationship between estrogen and GH-secreting cells has been studied, little is known about the biological effect of anti-estrogen treatment on these cells.

A previous study from our group utilized fiber-optic BeadArray to examine gene expression profiles in GHomas and the findings were compared with normal pituitaries. Results demonstrated that the Wnt signaling pathway plays an important role in promoting tumorigenesis and progression of GHomas[11]. Other microarray analyses have identified several Wnt pathway inhibitors that are frequently reduced in all subtypes of pituitary tumors, including Wnt inhibitory factor-1 (WIF1), secreted frizzled-related protein 2, and secreted frizzled-related protein[12]. The

Wnts comprise a large family of highly conserved growth factors that play crucial and diverse biological roles in the regulation of normal and pathological processes, such as cell growth, differentiation, apoptosis, migration, polarity, and oncogenesis[13-16]. To date, three major kinds of pathways have been identified in the Wnt signaling pathway: (I) the canonical Wnt/β-catenin pathway: β-catenin protein, a key effector in the Wnt signaling cascade; (II) non-canonical Wnt/c-Jun N-terminal kinase pathway; and (III) non-canonical Wnt/$Ca^{2+}$ pathway. It is thought that Wnt4 signals through a third pathway in pituitary cells[2, 12, 17]. However, the role of these pathways in GHomas tumorigenesis remains poorly understood. Recently, Kouzmenko *et al* [18] confirmed the first direct *in vivo* evidence of cross-talk between Wnt and estrogen receptor pathways by analyzing functional interactions between β-catenin and ERα in transgenic *Drosophila*. However, the influence of estrogen or anti-estrogen treatment on Wnt gene expression remains poorly understood[19]. The present study hypothesized that anti-estrogen treatment might suppress biological activity of rat GH-secreting pituitary tumor GH3 cells, and the ERα and Wnt pathways might be involved in this process. Fulvestrant, a “pure” anti-estrogen compound without estrogen-like activity (such as raloxifene or tamoxifen) was used to treat GH3 cells. The present study investigated biological changes in cells following treatment with fulvestrant, as well as the possible mechanisms of action.

## RESULTS

### Fulvestrant effects on GH3 cell proliferation

A stable human GH-secreting cell line is not currently available for use. However, GH3 cells, which secrete GH and prolactin, have been widely used to investigate biological activity of GH-secreting cells[9]. Morphological changes in GH3 cells after 72 hours of fulvestrant treatment are shown in Figures 1A–C. The treated cells appeared irregularly shaped, and floating cell clusters were smaller than in the control group (the cells were treated in the medium with the corresponding solvent). The number of viable cells decreased in a dose-dependent manner compared with the control group. These changes suggested that ERα was necessary for the survival of GH3 cells.

WST-8 cell staining analysis showed that GH3 cell proliferation was inhibited by fulvestrant at all tested concentrations (Figure 1F). The maximal inhibition rate was 63.06 ± 0.64% at 625 nM.

### Fulvestrant effects on cell secretion

Estrogen regulates synthesis and secretion of several pituitary hormones, including GH, prolactin, luteinizing hormone, and follicle-stimulating hormone[9, 20]. Therefore, the effects of anti-estrogen treatment on GH secretion were tested in GH3 cells.

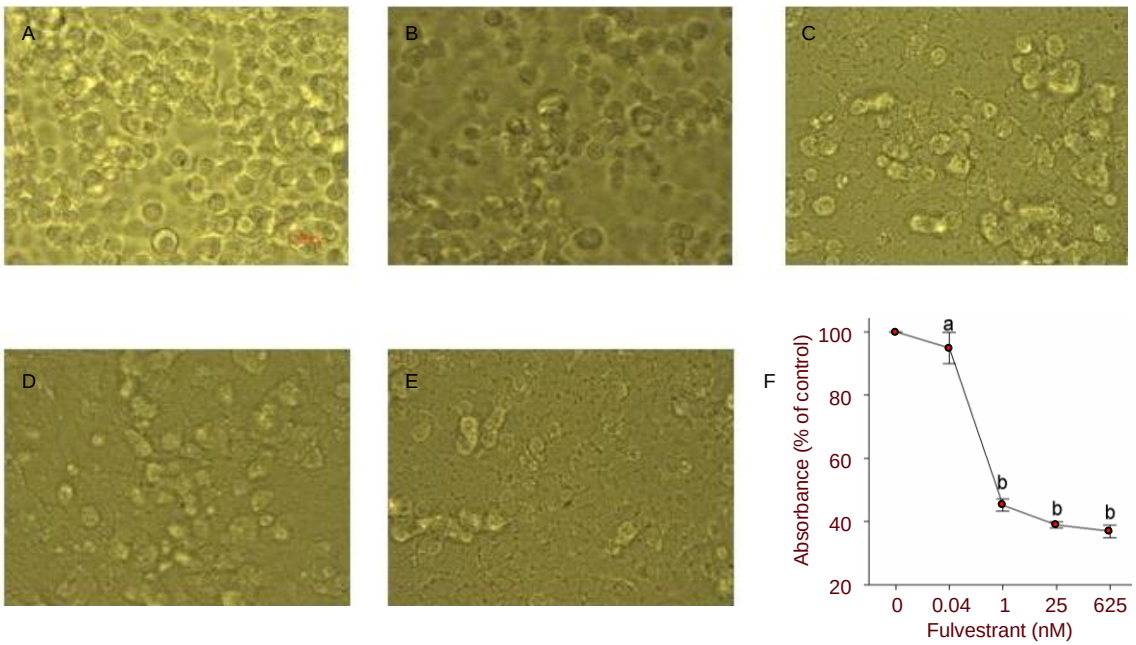


Figure 1 Morphological changes and proliferation of GH3 cells following fulvestrant treatment for 72 hours. The shape of the cells treated with fulvestrant was not as regular as control cells, and floating cell clusters are smaller than controls. The number of viable cells decreased in a dose-dependent manner compared with the control group.

(A–E) Control, 0.04, 1, 25, and 625 nM fulvestrant groups, respectively. Phase contrast microscopy (× 400) was used to visualize changes in cell morphology.

(F) Results of WST-8 assay. The assay was performed three times in quadruplicate. Data were shown as percentage of control. $^{a}P < 0.05$, $^{b}P < 0.001$, *vs.* 0 nM group (one-way analysis of variance).

In addition, prolactin is a well-known biomarker gene for the induction of transcription, and levels of prolactin mRNA and estrogen-induced secretion are useful indicators of estrogen bioactivity *in vitro*[9]. Therefore, prolactin levels in culture medium were utilized as an internal control for antiestrogen activity throughout the study. Results showed that fulvestrant decreased secretion of GH and prolactin in all tested concentrations (Figure 2).

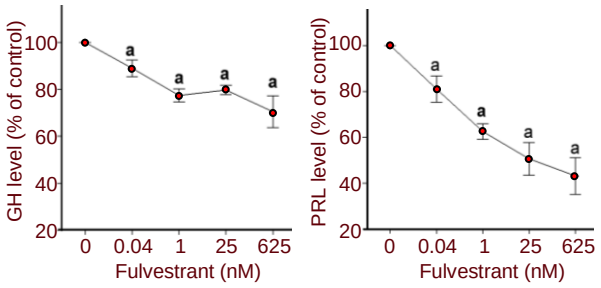


Figure 2 Growth hormone (GH) and prolactin (PRL) levels in culture medium supernatant at different fulvestrant concentrations after 24 hours of treatment.

Enzyme-linked immunosorbent assays were performed three times in quadruplicate. Data were shown as percentage of control. [a]*P* < 0.05, *vs.* 0 nM group (one-way analysis of variance).

## Fulvestrant effects on ERα, β-catenin, WIF1, and Wnt4 expression in GH3 cells

Figure 3 shows mRNA expression of *ERα, β-catenin, WIF1,* and *Wnt4* in GH3 cells after 72 hours of fulvestrant treatment (lower rows). *ERα* and *Wnt4* mRNA expression levels decreased in a dose-dependent manner when fulvestrant concentrations were > 1 nM ($P < 0.05$), although *β-catenin* mRNA levels remained unchanged ($P > 0.05$). In addition, *WIF1* mRNA expression increased in a dose-dependent manner when the fulvestrant concentration was > 1 nM ($P < 0.05$). Western blot analysis was utilized to determine protein expression in fulvestrant-treated GH3 cells to confirm qPCR results (Table 1; Figure 3, upper rows). As expected, ERα and WNT4 protein expression decreased following fulvestrant treatment in a dose-dependent manner, while β-catenin protein expression remained unchanged. In addition, WIF1 protein expression decreased in a dose-dependent manner following fulvestrant treatment.

Table 1 Semi-quantitative measurement of ERα, β-catenin, WIFI, and WNT4 proteins by grayscale value (/control, western blot)

| Protein | Fulvestrant (nM) | | | | |
|---|---|---|---|---|---|
| | 0 | 0.04 | 1 | 25 | 625 |
| ERα | 1 | 0.88±0.04[a] | 0.81±0.07[b] | 0.79±0.09[b] | 0.55±0.06[b] |
| β-catenin | 1 | 0.64±0.12 | 1.17±0.55 | 1.41±0.10 | 1.20±0.16 |
| WIF1 | 1 | 1.42±0.05[b] | 1.84±0.03[b] | 2.79±0.16[b] | 4.15±0.20[b] |
| WNT4 | 1 | 0.73±0.11 | 0.71±0.12[a] | 0.51±0.02[b] | 0.42±0.23[b] |

[a]*P* < 0.05, [b]*P* < 0.01, *vs.* 0 nM group (Student *t*-test or non- parametric Mann-Whitney *U* analysis). Data were expressed as mean ± SEM. ERα: Estrogen receptor α; WIF1: Wnt inhibitory factor-1.

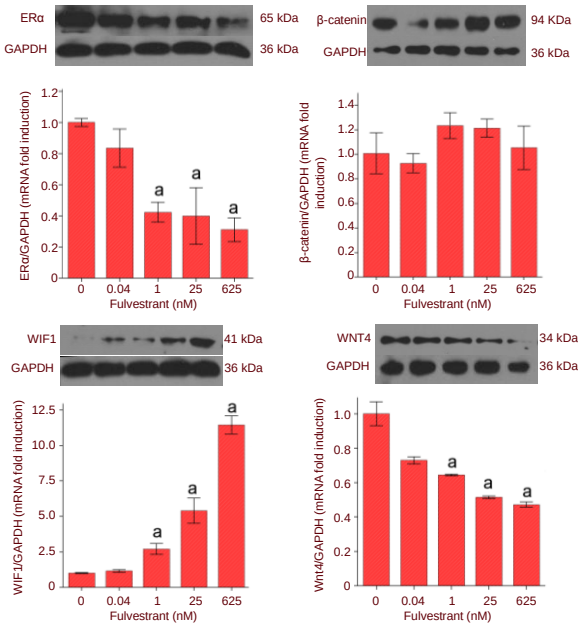


Figure 3 Effects of fulvestrant on expression of estrogen receptor α (ERα), β-catenin, Wnt inhibitory factor-1 (WIF1), and WNT4 in GH3 cells (real-time PCR analysis).

GH3 cells were treated with different concentrations of fulvestrant, as described above. Protein expression was detected by western blot, and mRNA levels were determined by real-time PCR analysis and were normalized to GAPDH mRNA levels in the same samples.

Results were obtained from experiments in triplicate. Data were expressed as mean ± SEM. [a]*P* < 0.05, *vs.* 0 nM group (one-way analysis of variance).

## 5-aza-2'-deoxycytidine (DCA) and trichostatin A (TSA) effects on *WIF1* mRNA expression in GH3 cells

To determine the mechanisms of decreased WIF1 expression in GH3 cells, the cells were treated with DCA (histone deacetylase inhibitor) and TSA (DNA methylation inhibitor) to inhibit DNA methylation and histone deacetylase, respectively. *WIF1* mRNA expression increased following treatment with DCA and TSA ($P < 0.05$, respectively; Figure 4).

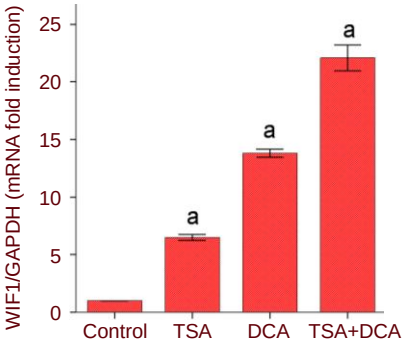


Figure 4 *WIF1* mRNA expression is affected by epigenetic mechanisms (real-time PCR analysis).

TSA: Trichostatin A (DNA methylation inhibitor); DCA: 5-aza-2'-deoxycytidine (histone deacetylase inhibitor). Results were obtained from experiments in triplicate. Data were expressed as mean ± SEM. [a]*P* < 0.05, *vs.* 0 nM group (one-way analysis of variance).

TSA led to a 6.5-fold increase in *WIF1* mRNA expression after 24 hours of treatment, and DCA increased *WIF1* mRNA expression by 13.8-fold. The combination of DCA and TSA treatment produced a synergistic 22.1-fold increase in *WIF1* mRNA expression. Results suggested that *WIF1* is partially epigenetically silenced in GH3 cells.

## DISCUSSION

GH plays an important role in the regulation of growth, development, and body composition[21]. However, high serum GH levels often present signs and symptoms of acromegaly or gigantism, and the vast majority of patients with acromegaly also present with pituitary GHoma. The first-line and only curative treatment for acromegaly is complete surgical excision of the GHoma. However, surgery alone rarely cures invasive GHomas with high secretory activity, suggesting that further adjuvant treatments, such as medical therapy, should be considered[22-23]. Currently, three classes of drugs are available for treating acromegaly: dopamine agonists, somatostatin analogs, and GH receptor antagonists[4]. However, some remain GHomas resistant to these drugs.

Estrogen has been shown to affect GH secretion[6, 10], and recent evidence suggests that estrogenic endocrine disruptors induce GH mRNA and protein expression *via* the ERα-mediated signaling pathway in the rat pituitary gland and GH3 cells[5, 9, 21]. However, little is known about the effect of anti-estrogen treatment on GH3 cells in the absence of estrogen. Fulvestrant is a selective, noncompetitive, non-central nervous system permeable ERα antagonist[10], and has been approved for the treatment of hormone receptor-positive metastatic breast cancer in postmenopausal women with disease progression after anti-estrogen therapy[10, 24]. The present study observed the effects of fulvestrant on ERα expression and GH3 cells.

ERα has been shown to be associated with GHoma tumorigenesis, and anti-estrogen treatment could provide promising medical treatment strategies for certain GHomas[22, 25-26]. For instance, raloxifene might be useful for treating male patients with active acromegaly[27], and tamoxifen has positive treatment effects in certain female patients with acromegaly[22]. In the present study, GH3 cell proliferation was suppressed by fulvestrant in the absence of estrogen, and ERα expression decreased in a dose-dependent manner following fulvestrant treatment when the fulvestrant concentration was > 1 nM. In addition, *in vivo* exposure to estrogens induces GH mRNA and protein expression in the rat pituitary gland, and these activities are reduced by fulvestrant[21]. Similarly, results from the present study demonstrated that GH secretion significantly decreased when GH3 cells were treated with fulvestrant, which suggested that anti-estrogen treatment could exert a suppressing role *via* the ERα-mediated signaling pathway in GH-secreting GH3 cells. This could explain why anti-estrogen treatment results in significant effects in certain GHoma patients.

Molecular mechanisms of estrogen action in the pituitary are complex and involve interactions between multiple signaling pathways[5]. Wnt signaling plays an important role in pituitary gland development, as well as in pituitary adenomas[2, 16, 28]. Cross-talk might exist between Wnt and ERα through functional interactions between β-catenin and ERα[18]. These results suggested that suppression of GH3 cell proliferation by fulvestrant could take place *via* the Wnt signal pathway, as well as the ERα pathway.

The Wnt/β-catenin pathway, also known as the canonical Wnt pathway, is the most understood Wnt pathway. β-catenin is a major component in the Wnt/β-catenin pathway and functions as an important oncogene in many malignant tumors[2]. In the absence of Wnt signals, free β-catenin is phosphorylated by glycogen synthase kinase-3β in the cytosol[19]. Following Wnt ligand binding to its receptor, hypophosphorylated β-catenin translocates to the nucleus, where it binds to transcription factors of the lymphocyte enhancement factor/T cell factor family, subsequently activating downstream target genes. Therefore, activation of the canonical Wnt pathway should be detectable by abnormal β-catenin nuclear expression. However, studies on nuclear expression of β-catenin in pituitary tumors resulted in contradictory findings. Abnormal nuclear accumulation of β-catenin was shown in 57% of investigated pituitary adenomas[29], but another study demonstrated that pituitary adenomas and normal pituitary glands did not exhibit nuclear or cytoplasmic expression, but rather membranous localization, of β-catenin[16]. In the present study, β-catenin real-time PCR and western blot results revealed unchanged β-catenin expression following anti-estrogen treatment in GH3 cells. Similarly, β-catenin expression in GH3 cells has been previously shown to be restricted to the cell membrane and remains unaltered by treatment with estradiol[17]. These results suggested that, even if the Wnt pathway was involved, activation or inactivation of the Wnt pathway by anti-estrogen treatment does not take place through the canonical Wnt/β-catenin pathway, but rather through the other non-canonical pathways.

WIF1 belongs to the secreted Frizzled-related protein class of Wnt antagonists and is detected throughout pituitary gland embryogenesis, as well as in the adult mouse pituitary[1]. WIF1 exhibits consistent growth-inhibitory effects on various cancer cell lines, including pituitary tumors[28, 30-31]. A previous study[28] showed that *WIF1* expression decreases in nonfunctioning and clinically functioning pituitary tumors, compared with normal pituitary controls, using microarray analysis. In addition, decreased cell proliferation and colony formation were observed following transfection of GH3 cells with *WIF1,* compared with empty vector controls. Results from the present study showed, for the first time, increased *WIF1* mRNA

and protein expression when GH3 cells were treated with fulvestrant, which suggested that fulvestrant suppressed pituitary proliferation *via* the ERα pathway, as well as the Wnt pathway. It has been reported that WIF1 suppresses β-catenin levels in human osteosarcoma cell lines[31], but these results were not observed in the present study. Although WIF1 was upregulated in GH3 cells by fulvestrant treatment, β-catenin was not suppressed by the increased WIF1 expression. This suggested that WIF1 suppression of β-catenin might not be a general mechanism in benign pituitary adenoma and malignant tumors, such as osteosarcoma. WIF1 plays binds directly to Wnts, thereby altering binding to the Wnt receptor complex. Therefore, in theory, WIF1 could inhibit both canonical and noncanonical Wnt pathways[32]. Results from the present study suggested WIF1 exerted its effect through noncanonical Wnt pathways in GH3 cells treated with fulvestrant. Recent evidence has shown that *WIF1* is epigenetically silenced in some malignant tumors, such as osteosarcomas[31], and all investigated non-functioning pituitary tumors exhibited *WIF1* promoter methylation[28]. This suggests that *WIF1* could be epigenetically silenced in the rat GH3 cell line. Following DCA treatment for 3 days, the GH3 cells expressed *WIF1* mRNA than the control group, which suggested that decreased *WIF1* expression was due to promoter hypermethylation in the GH3 cells. In addition, to determine whether histone modification was involved in epigenetic mechanisms of *WIF1* mRNA, cells were treated with TSA alone or DCA and TSA together. Results showed that TSA also increased *WIF1* mRNA expression, and the combination of TSA and DCA produced a synergistic effect on *WIF1* mRNA expression. DCA was more effective in up-regulating *WIF1* expression than TSA. These results suggested that anti-estrogen treatment increased *WIF1* expression, and decreased *WIF1* expression in GH3 cells was partially due to epigenetic mechanisms, although the exact mechanisms require further investigation.

The growth factor WNT4 is expressed throughout pituitary gland development during the embryogenesis, which is frequently associated with the noncanonical class of Wnt molecules[1, 32-33]. A previous study showed that WNT4 expression is greater in human GHomas than in normal pituitary glands, and WNT4 activates the β-catenin-independent “noncanonical” pathway[16]. In addition, *Wnt4* mRNA has been shown to be upregulated in the female Fischer-344 rat pituitary following treatment with estrogen for three weeks, and *Wnt4* mRNA and protein expression increases in estradiol-treated GH3 cells[17]. Because *Wnt4* mRNA and protein expression have been shown to be downregulated by fulvestrant treatment under estrogen-free conditions, we concluded that *Wnt4* mRNA and protein expression in rat pituitary GH3 cells decreased following anti-estrogen treatment. This reduction could be due to ERα-dependence, even without estrogen. Therefore, these results suggested that WNT4 could act as a growth factor for tumor progression in GHomas.

In conclusion, anti-estrogen treatment suppressed proliferation and reduced GH and prolactin secretion in GH-secreting rat pituitary GH3 cells *via* ERα and Wnt pathways in the absence of estrogen. ERα may play an essential role in cross-talk between ERα and Wnt pathway in GH3 cells. Anti-estrogen treatment reduced ERα and WNT4 expression and increased WIF1 expression, while β-catenin expression remained unchanged. This suggested that the non-canonical Wnt pathway was involved in this process. This is the first evidence that anti-estrogen treatment increased WIF1 expression and that WNT4 could act as an oncogene in GH3 cells. Furthermore, decreased *WIF1* expression in GH3 cells was partially due to epigenetic mechanisms. These results suggested that anti-estrogen treatment could provide promising medical strategies for certain ERα-positive GHomas.

## MATERIALS AND METHODS

### Design

An *in vitro*, neurological, molecular biological study.

### Time and setting

The experiment was performed at the Cellular and Molecular Laboratory of Beijing Neurosurgical Institute, China, from April 2010 to January 2011.

### Materials

The pituitary tumor-derived rat somatolactotroph GH3 cell line was provided by the Cell Center of the School of Basic Medicine, Peking Union Medical College, China.

### Methods

#### *Cell culture and fulvestrant treatment*

GH3 cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and were incubated at 37°C with a humidified atmosphere of 5% $CO_2$. The cell culture medium was replaced every 2 days. Prior to the experiment, the medium was replaced with DMEM/F12 phenol red-free culture medium containing 10% charcoal-stripped serum (Biological Industries, Kibbutz Bet Haemek, Israel) without antibiotics. After 24 hours, the cells were treated with different concentrations of fulvestrant (625, 25, 1, and 0.04 nM; Sigma, St. Louis, MO, USA) or the above medium with the corresponding solvent as a control. The cells were cultured for an additional 72 hours and harvested for following experiments.

#### *DAC and TSA treatment*

WIF1 is epigenetically silenced by promoter hypermethylation in human osteosarcomas[31], which suggested that WIF1 could also be epigenetically silenced in GH3 cells. GH3 cells were treated with DAC, an inhibitor of DNA methylation, and TSA, an inhibitor of histone deacetylase (Sigma). Briefly, GH3 cells at 15% confluency were treated. The cell lines were treated with 5 μM DAC for 96 hours or 200 ng/mL TSA for 24 hours, respectively. For combined treatment of DAC and TSA,

the cells were first treated with 5 μM DAC for 72 hours, followed by 200 ng/mL TSA for an additional 24 hours. Culture medium containing DAC or TSA was replaced every 24 hours. At the end of treatment, the medium was removed and total RNA was isolated for subsequent real-time PCR.

### WST-8 measurements of cell inhibition rate

GH3 cells in log phase were washed twice with PBS and plated (5 000 cells/well) onto 96-well plates in 200 μL phenol red-free DMEM/F12 medium containing 10% charcoal-stripped fetal bovine serum for 24 hours. The cells were then treated with fulvestrant or control medium. After incubation for 72 hours, cell proliferation was assessed using the WST-8 cell staining kit (Neuronbc, Beijing, China). Briefly, a 20 μL WST-8 solution was added to each well, and incubation was continued for 4 hours. Absorbance at 450 nm was measured using a SpectraMAX®M5 multidetection microplate reader (Molecular Devices, Sunnyvale, CA, USA). The cell inhibition rate was calculated according to the following formula: inhibition rate (%) = (absorbance of control group −absorbance of drug group)/absorbance of control group × 100%.

### Enzyme immunoassay of GH and prolactin secretion

Log-phase cells were inoculated into 6-well culture plates ($1 \times 10^4$ cells/well) and treated with the above-mentioned procedures. After 24 hours of treatment, 1 mL of medium was collected and stored at −20°C. *In vitro* GH release was measured using the rat GH enzyme-linked immunosorbent assay kit (RapidBio, West Hills, CA, USA) following the manufacture protocol. Color intensity of the reaction product (proportional to GH concentration) was measured using the SpectraMAX®M5 multidetection microplate reader. Sample density was calculated using the straight-line regression equation of the standard curve with the standard density and absorbance value. Prolactin levels in culture medium is a useful indicator of estrogen activity *in vitro*[9], and prolactin secretion of GH3 cells served as an internal control for anti-estrogen activity.

### SYBR green-based semi-quantitative real-time PCR analysis for mRNA expression of ERα, β-catenin, WIF1, and WNT4 in GH3 cells

After treatment for 72 hours, the cells were harvested. Total RNA was isolated from the cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). RNA was reverse-transcribed into cDNA according to the protocol from the Quantscript RT Kit (Tiangen Biotech, Beijing, China) supplied by the manufacturer. Primer (AuGCT Biotechnology, Beijing, China) sequences used in real-time PCR are listed in Table 2. The real-time PCR reaction system was performed in a 20-μL reaction containing 2× Master Mix (10 μL), Primer F/R (0.5 μL each, 10 μM), sample cDNA (2 μL), and MilliQ $H_2O$ (7 μL). Amplification conditions were 95°C for 15 minutes, 40 cycles of 94°C for 15 seconds, 60°C or 62°C for 30 seconds, and 72°C for 30 seconds. Fluorescence of PCR products was measured following completion of the extension step. The PCR products were then separated by electrophoresis on 2.0% agarose gels and detected under ultraviolet illumination. Relative expression levels of the genes were normalized relative to glyceraldehyde-3-phosphate dehydrogenase gene expression and calculated from the cycle threshold value using the $2^{-\Delta\Delta CT}$ method for quantification[34].

Table 2 Primer design of rat genes

| Gene | Forward sequence 5'–3' | Reverse sequence 5'–3' | Temp (°C) | Size (bp) |
|---|---|---|---|---|
| ERα | CAT CGA TAA GAA CCG GAG GA | CAT CTC TCT GAC GCT TGT GC | 60 | 134 |
| β-catenin | AAC GGC TTT CGG TTG AGC TG | TGG CGA TAT CCA AGG GCT TC | 60 | 118 |
| Wnt4 | TCG CCT ATG GCG TAG CCT TC | ACC CGC ATG TGT GTC AGG A | 62 | 136 |
| WIF1 | TCG CTG GAT AAA GGC ATC ATG G | TGA CAA TCA CAT TCA CTT CAA ATG C | 60 | 148 |
| GAPDH | ATG ACT CTA CCC ACG GCA AG | TAC TCA GCA CCA GCA TCA CC | 60–62 | 136 |

ERα: Estrogen receptor α; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Temp: temperature.

### Western blot analysis for protein expression of ERα, β-catenin, WIF1 and WNT4 in GH3 cells

The GH3 cells were treated according to the above-mentioned procedures, and protein expression was analyzed using the western blot method. Briefly, experimental cells were lysed with cell lysis buffer, and protein concentrations were determined using the bicinchoninic acid protein assay (Biyuntian, Shanghai, China) prior to gel electrophoresis separation. Equal amounts of proteins were subjected to electrophoresis and then transferred onto polyvinylidene fluoride membranes. Following transfer, membranes were rinsed and incubated in blocking buffer (5% nonfat milk in TBST) for 1–2 hours at room temperature. Membranes were then incubated overnight with primary antibodies (glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (Sigma); ERα monoclonal antibodies, WNT4 and β-catenin polyclonal antibodies (Abcam, Cambridge, UK) and WIF1 polyclonal antibody (Santa Cruz Biotechnology (Santa Cruz, CA, USA) at a dilution of 1: 2 000) at 4°C, followed by three 10-minute washes with Tris-buffered saline/Tween-20, and then incubation with goat anti-rabbit horseradish peroxidase-labeled secondary antibodies (1: 2 000; Jackson Immuno Research, West Grove, PA, USA) at room temperature for 1 hour. After three 10-minute washes with Tris-buffered saline/Tween-20, the antibody-antigen complex was detected using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The final data were subjected to grayscale scanning and semi-quantitative analysis using Quantity One software (Bio-Rad, CA, USA).

### Statistical analysis

Measurement data were expressed as mean ± SEM.

WST-8 and enzyme-linked immunosorbent assay tests were performed three times in quadruplicate. All other tests were performed in triplicate. Statistical analysis was performed using one-way analysis of variance, Student *t*-test, or non-parametric Mann-Whitney *U* analysis (SPSS 13.0 for Windows software; SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

**Author contributions:** Jiwei Bai provided and processed data and images, as well as drafted the manuscript. Yan Wang provided technical support. Jiwei Bai and Chuzhong Li conducted the experiments. Yazhuo Zhang was responsible for funding and designed the study.

**Conflicts of interest:** None declared.

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