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The role of TGF-β/Smad signaling in dopamine agonist-resistant prolactinomas

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ABSTRACT

Background: Prolactinomas are the most common secretory pituitary adenomas. The first line of treatment involves dopamine agonists (DAs); however, a subset of patients is resistant to such therapy. Recent studies suggest that dopamine can up-regulate TGF-β1 synthesis in rat pituitary lactotrophs whereas estradiol down-regulates TGF-β1. To date, the role of TGF-β/Smad signaling in DAs-resistant prolactinomas has not been explored.
Methods: High-content screening (HCS) techniques, qRT-PCR, Western blot, immunofluorescence and ELISA, were performed to determine the role of TGF-β/Smad signaling in DAs-resistant prolactinomas.
Results: We reported a significant down-regulation of TGF-β/Smad signaling cascade in DAs-resistant prolactinomas compared to normal human anterior pituitaries. Following treatment with TGF-β1, the dopamine agonist, bromocriptine, and the estrogen antagonist (ER), fulvestrant in GH3 cells, we found that TGF-β1 and fulvestrant caused significant cytotoxicity in a dose- and time-dependent manner and activated Smad3 was detected following exposure to TGF-β1 and fulvestrant. In addition, treating GH3 cells with fulvestrant increased active TGF-β1 levels and decreased PRL levels in a dose-dependent manner.
Conclusion: TGF-β/Smad signaling pathway may play an important role in DA-resistant prolactinomas and has the potential to be a viable target for the diagnosis and treatment of prolactinomas, particularly in patients who are resistant to DAs.

1. Introduction

A prolactinoma is a prolactin-secreting pituitary adenoma and accounts for approximately 40–60% of all pituitary tumors (Colao and Savastano, 2011; Daly et al., 2006). These tumors secrete prolactin (PRL) in excess which leads to various health-related complications such as galactorrhea, hypogonadism, decreased libido, infertility, and osteoporosis (Gillam et al., 2006). Additionally, prolactinomas can cause headaches, visual dysfunction, and hypopituitarism. Dopamine agonists (DAs) such as bromocriptine and cabergoline are first-line therapies for prolactinomas. DAs have a higher affinity for dopamine D2 receptors (D2R), which suppresses the secretion of prolactin, inhibits tumor growth that leads to a decrease in the size of prolactin-secreting tumors (Colao and Savastano, 2011). However, nearly 10% prolactinomas cases do not respond to DA therapy (Oh and Aghi, 2011). Therefore, new medical treatments are needed for these patients.

Although it has been reported that the growth-inhibitory effect of dopamine is mediated in part by TGF-β1 (Sarkar et al., 2005), the role of TGF-β signaling in the development of prolactinomas has not been explored until now. Depending upon tumor stage and tumor type, TGF-β signaling can act as a tumor suppressor or tumor promoter (Wakefield and Roberts, 2002). The TGF-β signaling cascade is initiated by the binding of TGF-β1, TGF-β2, and TGF-β3 ligands to the type II TGF-β receptor (TGF-β RII), followed by recruitment and phosphorylation of the type I TGF-β receptor (TGF-β RI) to form a complex. Activated TGF-β RI propagates signaling by phosphorylating Smad2 and Smad3 (p-Smad2 and p-Smad3), which forms a heteromeric complex with the signal transducer, Smad4. Smad2/3 and Smad4 translocate into the nucleus to regulate gene expression of various transcription factors (Heldin et al., 1997; Massague, 2012).

It is well documented that dopamine increases TGF-β1 synthesis in rat pituitary lactotrophs to regulate cell growth and differentiation and elicits the same effect in prolactinomas (Recouvreux et al., 2011). However, most DAs-resistant prolactinomas have decreased expression of D2R, so DAs cannot stimulate TGF-β1 secretion in these prolactinomas and this may be one of the

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Table 1
Patient characteristics.

Patient ID	Gender/ Age	PRL levels (ng/ml)		Tumor size (mm)	Clinical symptoms
		Before DA treatment	Pre-operative		
1	F/48	562	341	17	Headache
2	M/35	1863	1149	37	Headache and visual impairment
3	F/36	326	243	21	Amenorrhoea and galactorrhoea
4	M/23	383	364	31	Gonadal dysfunction
5	F/36	386	268	28	Amenorrhoea and visual impairment
6	F/47	341	282	20	Headache and menstrual disorder
7	M/33	426	325	15	Headache
8	F/21	385	202	26	Amenorrhoea
9	F/42	227	192	28	Amenorrhoea and galactorrhoea
10	F/31	1063	742	29	Headache and menstrual disorder
11	F/34	1358	1326	35	Amenorrhoea and visual impairment
12	F/48	1725	928	40	Amenorrhoea and visual impairment

PRL, prolactin; DA, dopamine agonist.

Table 2
RT-PCR primer list.

Gene name	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
Smad2	ATCCTAACAGAACTTCCGCC	CTCAGCAAAAACTTCCCCAC
Smad3	GGAGAAATGGTGCGAGAAGG	GAAGGCGAACTCACACAGC
TGF-β1	CCCTGGACACCAACTATTGC	TGCGGAAGTCAATGTACAGC
GAPDH	GAAGGTCGGAGTCAACGGATT	CGCTCCTGGAAGATGGTGAT

mechanisms of DAs resistance (Oh and Aghi, 2011). Estrogen receptor antagonists have been used in cases where surgical resection and radiotherapy have not induced remission of hyperprolactinemia (Oh and Aghi, 2011). In our previously published work, we found that fulvestrant, a novel ER antagonist, can inhibit proliferation of MMQ rat prolactinoma cells, promote apoptosis and necrosis, and inhibit prolactin secretion (Cao et al., 2014; Lv et al., 2011). However, no reports have studied the relationship between estrogen receptor antagonists and TGF-β signaling in DAs-resistant prolactinomas. Therefore, in this study, we examined the expression of Smad2, p-Smad2, Smad3, and p-Smad3 in normal human anterior pituitaries and DA-resistant prolactinomas. Furthermore, we studied the anti-tumor effect of fulvestrant on GH3 cells, and to explore whether fulvestrant could regulate cell proliferation in DAs-resistant prolactinomas via TGF-β/Smad signaling.

2. Materials and methods

2.1. Patients and prolactinoma samples

DAs-resistant prolactinomas were obtained from 12 patients who underwent endoscopic transsphenoidal surgery between January 2011 and March 2012 at Beijing Tiantan Hospital. DAs-resistant patients were defined as patients whose serum PRL levels remained abnormally high (>150 ng/ml) after at least 3 months of treatment with a daily dose of 15 mg bromocriptine. The diagnosis of a prolactinoma was confirmed by clinical manifestation, hormonal and magnetic resonance imaging (MRI) data, histopathological analysis, and immunohistochemical staining for all anterior pituitary hormones. Patients who had received previous radiation therapy or had recurrence were excluded from this study. We received six normal human anterior pituitaries from organ donors that died of non-neurological and non-endocrine diseases. All normal anterior pituitaries were histologically examined using immunocytochemistry to exclude the possibility of incidental pathologies. This study

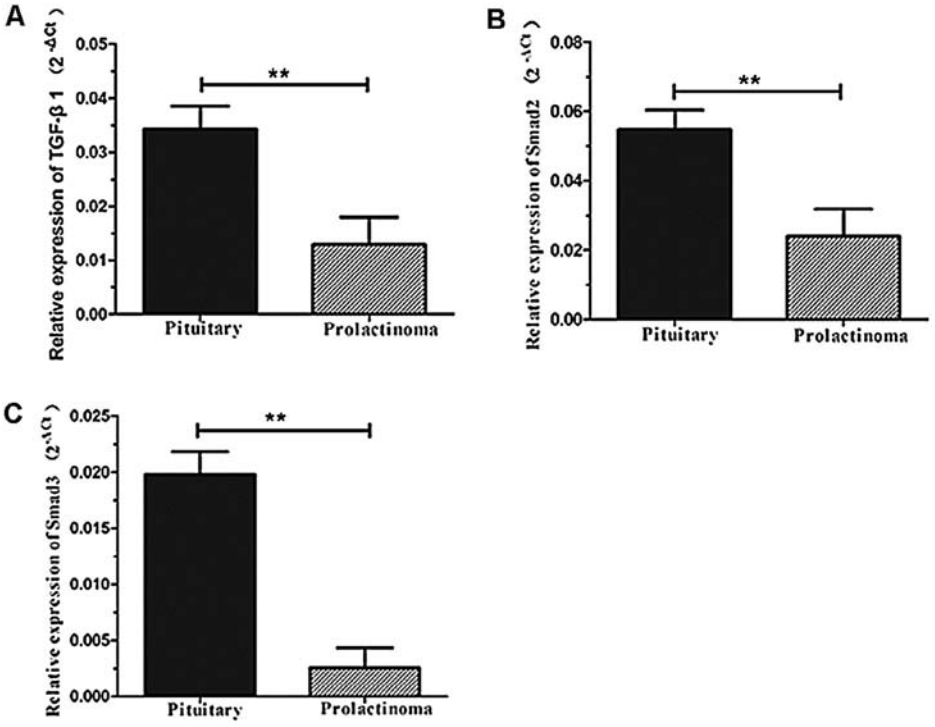

Fig. 1. TGF-β1, Smad2/3 levels are down-regulated in DA-resistant prolactinomas. TGF-β1 (panel A), Smad2 (panel B) and Smad3 (panel C) mRNA levels are significantly lower in DA-resistant prolactinomas compared to normal anterior pituitaries ($^{*}P < 0.05$; $^{**}P < 0.01$).

was approved by the Ethics Committee of Beijing Tiantan Hospital and each patient provided informed consent. The patient characteristics are summarized in Table 1.

2.2. High-content screening (HCS) assay

The GH3 cells were obtained from rat pituitary adenoma that spontaneously synthesizes and secretes both prolactin (PRL) and growth hormone (GH). These cells possess a variety of hormones, growth factors, and neurotransmitter receptors and serves as a viable model for lactotroph function. However, GH3 cells are resistant to the inhibitory action of DAs because of the absence of dopamine receptors, which are present on normal lactotrophs which allows GH3 cells to serve as useful model for examining the function of the dopamine D2 receptors (An et al., 2003; Cronin et al., 1980).

GH3 pituitary adenoma cells were cultured in phenol red-free DMEM medium supplemented with 10% (v/v) charcoal-stripped FBS and fed every 2 days. GH3 cells were plated into 96-well plates (~1×10^4 cells/well) and treated with increasing concentrations of recombinant human TGF-β1 (5, 10, 15, 50 and 100 ng/ml) (PeproTech, Rocky Hill, NJ), fulvestrant (0.01, 0.05, 0.1, 0.5 and 1 μM) (European pharmacopoeia, Strasbourg, France), bromocriptine (5, 10, 15, 50 and 100 nM) (Sigma, St. Louis, MO) that was dissolved in DMSO or in the control medium (containing the same concentration of DMSO). After 24, 48 and 72 hours of treatment, the culture medium was stored at –20 °C, cells were harvested, stained with DAPI, and imaged using the high-content screening (HCS) imaging system (Molecular Devices, Sunnyvale, CA) as before described (Quintavalle et al., 2011). Images were analyzed with ImageXpress software (Molecular Devices, Sunnyvale, CA), individual cells were identified and total cell numbers were determined to estimate cell proliferation.

2.3. qRT-PCR

Total RNA was extracted from frozen tumor samples and normal anterior pituitaries using Trizol (Invitrogen, Carlsbad, CA). First-strand cDNA synthesis was generated using the kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). qRT-PCR primer sets are listed in Table 2.

2.4. Western blot

Protein samples (30 μg) were separated by 10% SDS–PAGE, transferred to polyvinylidene membranes, and blocked. The following antibodies were applied: human anti-Smad2 (1:1000; Cell Signaling

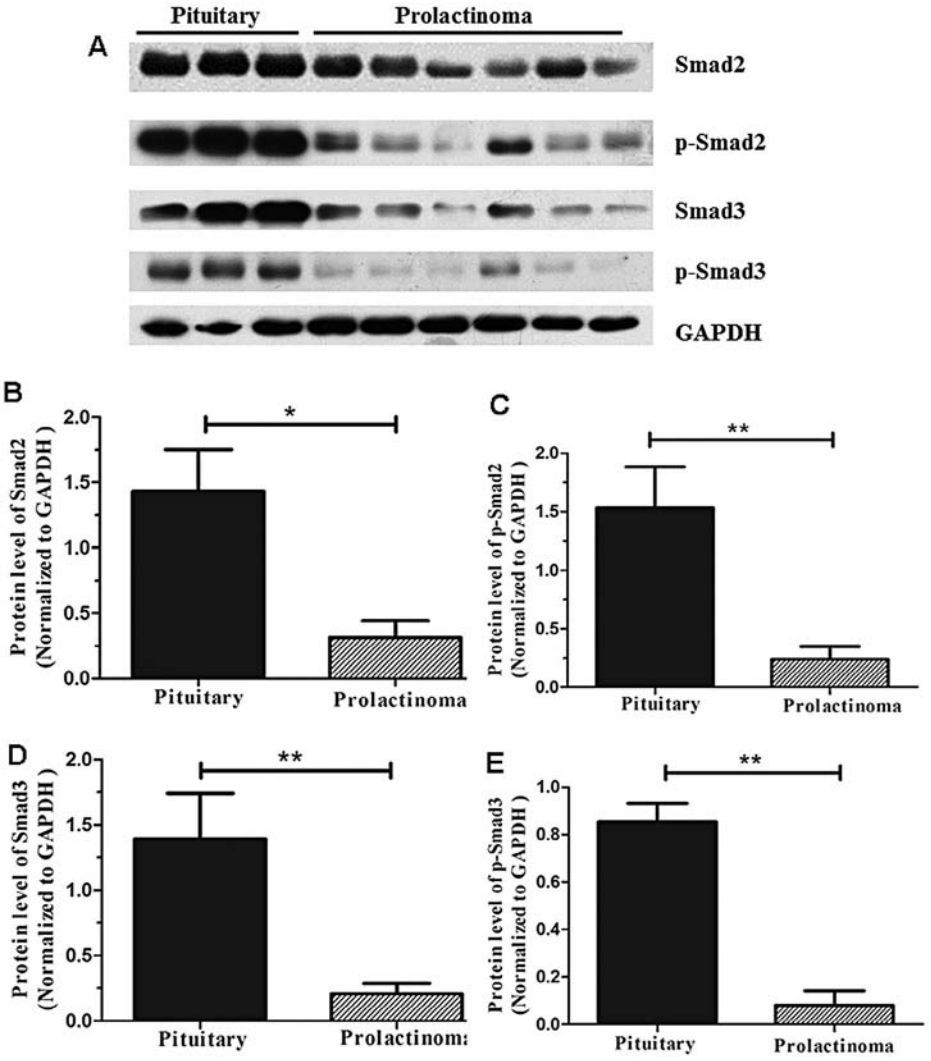

Fig. 2. TGF-β/Smad2/3 protein levels are down-regulated in human prolactinomas. Protein expression levels of Smad2, p-Smad2, Smad3, and p-Smad3 significantly decreased in DA-resistant prolactinomas compared to normal anterior pituitaries ($^*P < 0.05$; $^{**}P < 0.01$).

Technology), anti-p-Smad2 (Ser465/467) (1:1000; Cell Signaling Technology), anti-Smad3 (1:1000; Cell Signaling Technology), anti-p-Smad3 (Ser423/425) (1:1000; Cell Signaling Technology), and anti-GAPDH (1:5000; Sigma-Aldrich). The protein bands were subjected to grayscale scanning and semi-quantitative analysis using Quantity One software (Bio-Rad).

2.5. Immunofluorescence

GH3 cells were fixed with 4% paraformaldehyde and incubated with anti-p-Smad3 (1:100) followed by FITC-conjugated goat anti-mouse antibodies and DAPI counterstain. Images were captured using a Laser Scanning Confocal Microscope (Leica Microsystems).

2.6. ELISA assay

ELISA was performed to quantify activated TGF-β1 and PRL levels using a TGF-β1 Emax ImmunoAssay-System (Promega) and rat PRL ELISA kit (R&D Systems) according to the manufacturer's instructions, respectively. GH3 were harvested following 24, 48 and 72 h exposure to fulvestrant or bromocriptine. (Infinite® M200 PRO Microplate Reader, Tecan Group AG, Männedorf, Schweiz).

2.7. Statistical analysis

Statistical analyses were carried out using SPSS (v16.0). Differences between groups were determined by one-way ANOVA and an independent two-sample t-test. Cytotoxicity data were analyzed by two-way ANOVA in combination with a Tukey post-hoc test (significance level of 5%). Data are expressed as mean ± SD and $P < 0.05$ is considered statistically significant.

3. Results

3.1. TGF-β/Smad signaling is down-regulated in human DAs-resistant prolactinomas

To assess changes of TGF-β/Smad signaling pathway in DAs-resistant prolactinomas, we evaluated TGF-β1 mRNA expression levels in DAs-resistant prolactinomas and normal anterior pituitaries. As shown in Fig. 1A, qRT-PCR data revealed that TGF-β1 levels were significantly decreased in human DAs-resistant prolactinomas compared to normal anterior pituitaries ($P < 0.01$). Next, we evaluated Smad2 and Smad3 mRNA levels, which are the signal transducers of the TGF-β cascade. Our results showed that Smad2 (Fig. 1B) and Smad3 (Fig. 1C) were significantly down-regulated in

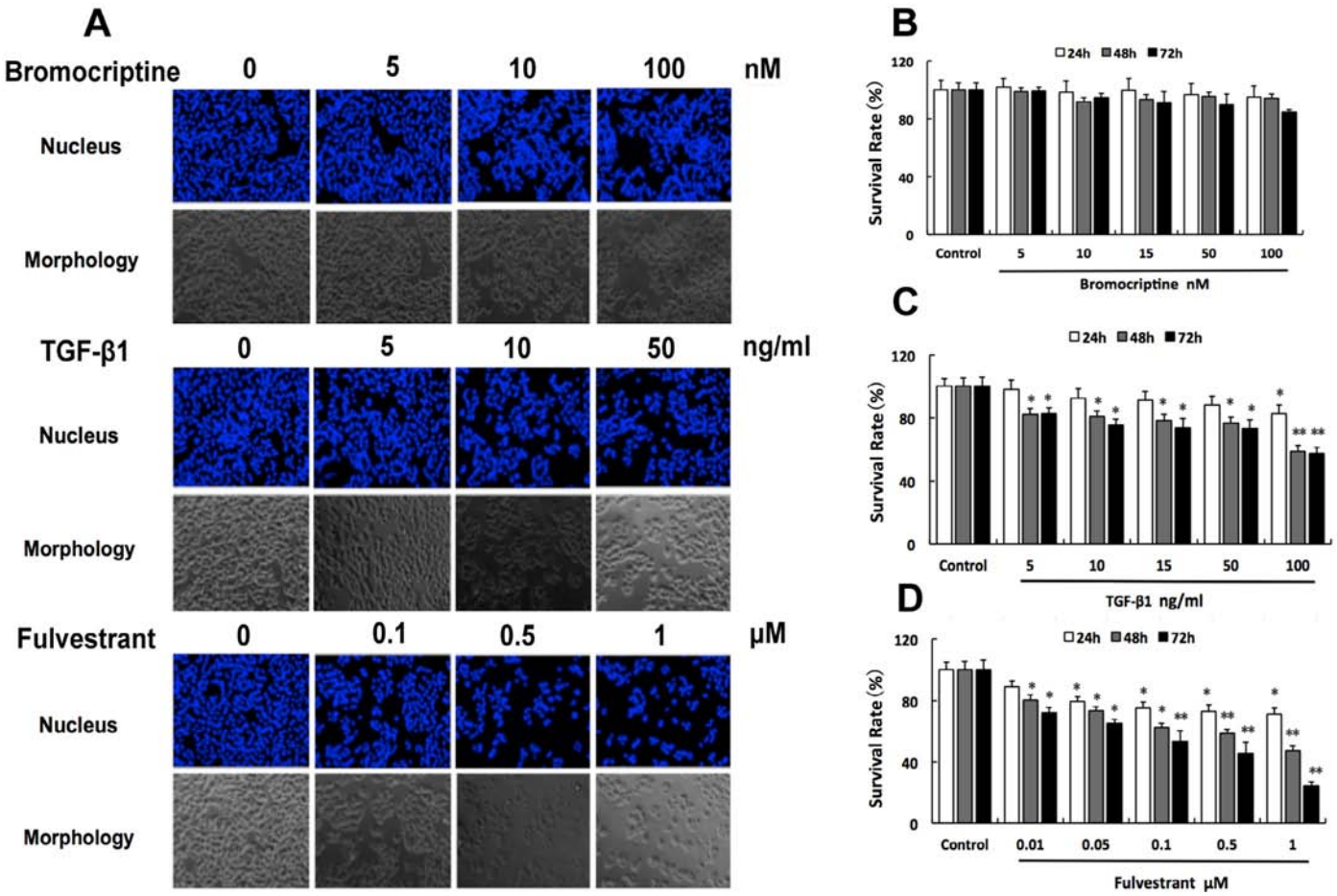

Fig. 3. GH3 cell proliferation is unaffected by bromocriptine. Panel A: After exposure to TGF-β1 (0–100 ng/ml), fulvestrant (0–1 μM) and bromocriptine (0–100 nM) for 72 h, the morphology of the GH3 cells was observed using the high-content screening techniques and total cell numbers were determined to estimate cell proliferation. Compared with the control group, the number of cells was reduced after treatment with TGF-β1 and fulvestrant (20× magnification). Panels B–D: The proliferation of GH3 cells was reduced in a dose- and time-dependent manner after treatment with TGF-β1 (C) and fulvestrant (D). In contrast, 5–100 nM bromocriptine (B) was ineffective in inhibiting the proliferation of GH3 pituitary tumor cells (*$P < 0.05$; **$P < 0.01$).

DAs-resistant prolactinomas compared to normal anterior pituitaries ($P < 0.01$).

We employed Western blotting to assess Smad2/3 protein levels which were found to correlate with mRNA levels. As shown in Fig. 2, the expressions of Smad2, Smad3, p-Smad2, and p-Smad3 were significantly decreased in DA-resistant prolactinomas compared to normal anterior pituitaries ($P < 0.01$) (Fig. 2A–E).

3.2. GH3 cells are resistant to bromocriptine

To determine whether TGF-β1, bromocriptine (dopamine agonist), fulvestrant (ER antagonist) can affect prolactinoma cell growth, GH3 cells were exposed to increasing concentrations of each compound for 24, 48 and 72 hours. TGF-β1 (5–100 ng/ml) and fulvestrant (0.01–1 μM) caused significant cytotoxicity in GH3 cells in a dose- and time-dependent manner (Fig. 3A–C). In contrast, bromocriptine (5–100 nM) did not affect GH3 cell proliferation (Fig. 3D).

3.3. Fulvestrant increase active TGF-β1 levels and decrease PRL levels

Due to compelling evidence that the growth-inhibitory action of dopamine is mediated in part by TGF-β1 (Ben-Jonathan, 2005), we hypothesized that DA-resistant cells are unable to stimulate active TGF-β1 secretion. To test this hypothesis, we treated GH3 cells with increasing doses of bromocriptine (5–100 nM) for 24–72 hours and measured active TGF-β1 levels. Bromocriptine had no effect on active TGF-β1 secretion from GH3 cells (Fig. 4A). Treating GH3 cells with fulvestrant (0.1–1 μM) increased active TGF-β1 levels in a dose-dependent manner (Fig. 4B). Furthermore, the levels of PRL in the supernatant were also detected (Fig. 4C). The secretion of PRL was reduced with exposure to TGF-β1, bromocriptine and fulvestrant for 24 h, respectively.

3.4. Fulvestrant increases Smad3 phosphorylation

We compared Smad2/3 phosphorylation levels to total Smad2/3 protein levels following 24-hour exposure to bromocriptine (5–100 nM) and fulvestrant (0.1–1 μM). The results showed that fulvestrant significantly increased the expression of p-Smad3 ($P < 0.01$) (Fig. 5D and E). However, there was no significant difference in p-Smad2 protein levels after fulvestrant treatment (Fig. 5D and F). Additionally, comparable levels of total Smad2/3 were observed after bromocriptine treatment, whereas bromocriptine was ineffective in stimulating Smad2 or Smad3 phosphorylation (Fig. 5A–C).

To determine whether p-Smad3 translocates to the nucleus in response to bromocriptine or fulvestrant, we assessed their subcellular distribution in GH3 cells immunofluorescence staining. We observed no change in Smad3 phosphorylation after exposure to bromocriptine (10 and 50 nM) for 3 h (Fig. 6A) or 24 h (Fig. 6D) compared to untreated cells. However, very strong staining of p-Smad3 was identified in the cytoplasm and nucleus of the GH3 cells after TGF-β1 (10 and 50 ng/ml) (Fig. 6B) and fulvestrant (0.1 and 0.5 μM) (Fig. 6C) compared to untreated cells. Similar results were seen in GH3 cells treated with TGF-β1 (Fig. 6E) or fulvestrant (Fig. 6F) for 24 h.

4. Discussion

Prolactinomas are pituitary adenomas that over produce the hormone prolactin (Raverot et al., 2014). The first choices for treatment are dopamine agonists (DAs) such as bromocriptine and cabergoline; however, some patients are resistant to DAs. Recent studies show that the TGF-β family of growth factors plays a prominent role in regulating pituitary tumor growth and prolactin secretion from anterior pituitary lactotrophs (Bailey et al., 2004; Giacomini et al., 2009; Recouvreux et al., 2011). Recouvreux et al. (2011) demonstrated that TGF-β1 can mediate the inhibition of prolactin secretion from lactotrophs and their proliferation via the dopamine in vivo. However, the role of TGF-β/Smad signaling in DAs-resistant prolactinomas is poorly understood.

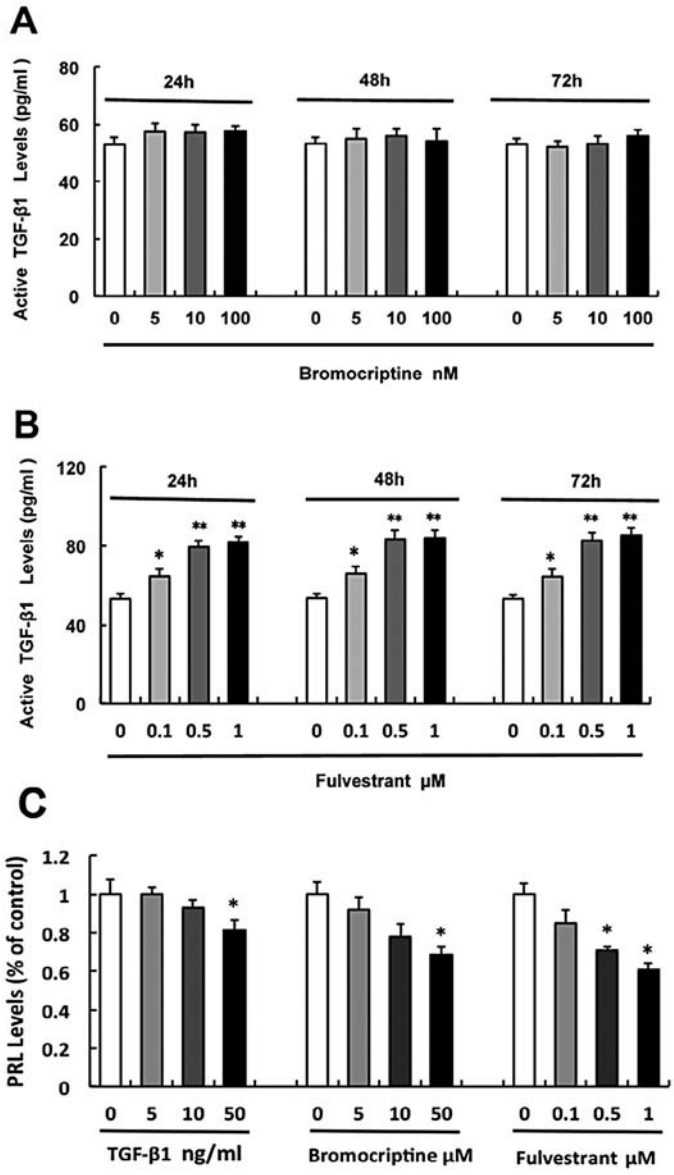

Fig. 4. Fulvestrant increase active TGF-β1 levels and decrease PRL levels. GH3 cells were treated with bromocriptine (0–100 nM) (panel A), fulvestrant (0–1 μM) (panel B) or TGF-β1 (0–50 ng/ml) (panel C) for 24, 48 or 72 h. Media samples were collected at 24 hour intervals and active TGF-β1 or PRL levels were determined by ELISA (n = 4 samples/treatment group). $^{*}P < 0.05$; $^{**}P < 0.01$, compared with untreated control cells.

In this study, we report that TGF-β/Smad signaling is down-regulated in human DAs-resistant prolactinomas. To the best of our knowledge, we are the first group to systematically investigate the differential expression of TGF-β/Smad in normal anterior pituitaries and human DA-resistant prolactinomas.

Although the involvement of TGF-β/Smad signaling pathway in regulating cellular processes is well documented (Giannelli et al., 2014; Massague, 2012; Oh and Li, 2013), TGF-β works as both a tumor promoter and a tumor suppressor. Under normal conditions, TGF-β acts as a potent tumor suppressor to prevent normal cells from transforming to malignant cells by regulating the rate of proliferation, inducing differentiation, promoting apoptosis

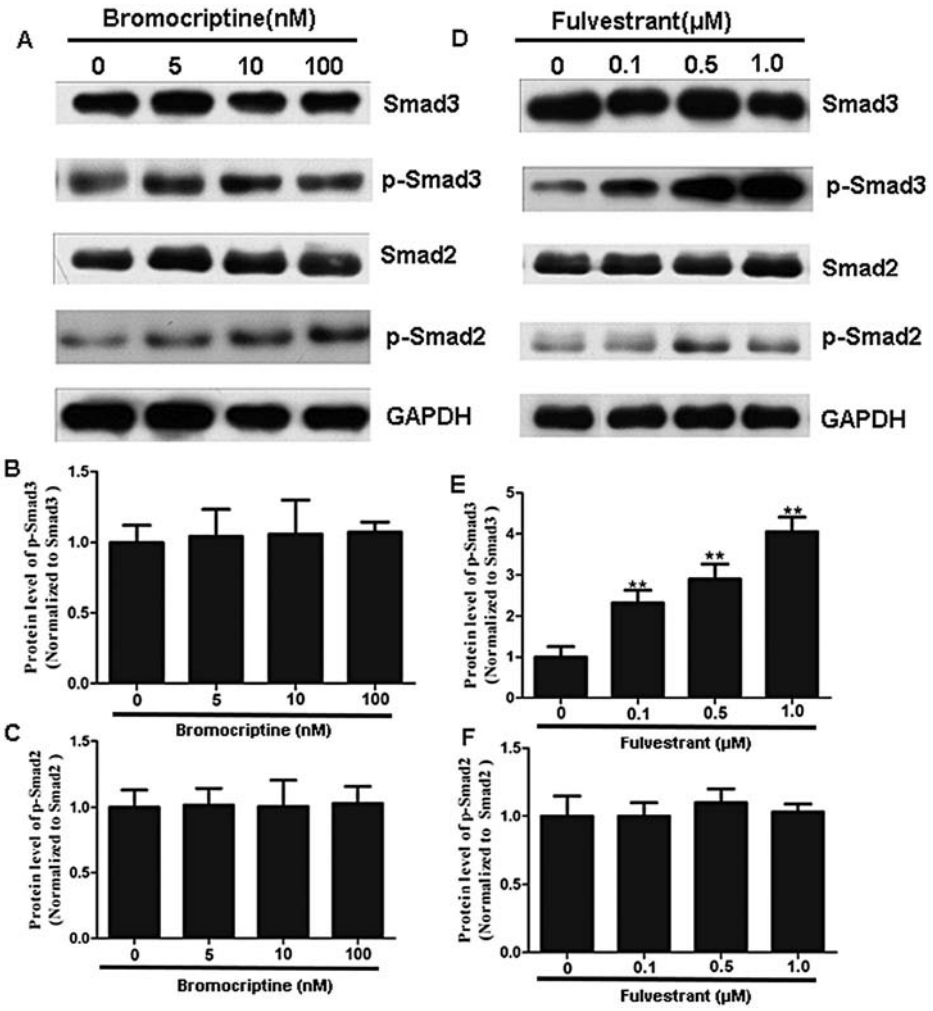

Fig. 5. Exposure to fulvestrant increases Smad3 phosphorylation. GH3 cells were treated with bromocriptine (5, 10, and 100 nM) (panels A–C) or fulvestrant (0.1, 0.5, and 1 μM) (panels D–F) for 24 h. Whole cell lysates were harvested and separated. Fulvestrant can up-regulate phosphorylation of Smad3, while bromocriptine causes a slight increase Smad2 phosphorylation as shown by Western blot. $^{*}P < 0.05$; $^{**}P < 0.01$, versus respective control.

(Massague, 2008). When TGF-β becomes mutated it no longer regulates cell proliferation thus a cell can grow at an uncontrollable rate and cancer develops (Zhenye et al., 2014).

It has been reported that dopamine and TGF-β1 act in a synergistic manner to inhibit lactotroph proliferation (Ben-Jonathan, 2005). Dopamine binds to dopamine receptor 2 (DR2), reduces the rate of lactotroph proliferation, and up-regulates TGF-β1 expression and secretion (Sarkar et al., 2005). In this study, the data suggest that GH3 cells are sensitive to the effects of TGF-β1 and the ER antagonists, fulvestrant. On the other hand, GH3 cells are resistant to the dopamine agonist, bromocriptine.

Several studies have shown that a mutated or deficient form of Smad3 leads to tumor development (Millet and Zhang, 2007; Roberts et al., 2006). In this study, we observed no change in Smad3 phosphorylation after exposure to bromocriptine. However, very strong staining of p-Smad3 was identified in the cytoplasm and nucleus of the GH3 cells after exposure to TGF-β1 and fulvestrant. These results suggest that Smad3 becomes phosphorylated and thus activated after stimulation with TGF-β1 and fulvestrant. In contrast, bromocriptine is unable to initiate TGF-β1-mediated Smad3 activation, may be due to the absence of functional dopamine receptors in GH3 cells.

Several studies have reported that estradiol inhibits the release of dopamine from the hypothalamus thereby reducing the antiproliferative action of dopamine on lactotrophs resulting in tumor (Grubisha et al., 2012; Maurya et al., 2013; Sarkar and Boyadjieva, 2007). These results correlate with our previous studies that showed that TGF-β1 plays a role in the progression of estrogen-induced prolactinomas (Lv et al., 2012) (Fig. 7).

We recognized that the lack of tissue samples from patients with non-resistant prolactinomas was a limitation of our study, which is the key to further validate our conclusions. However, it is difficult to collect prolactinoma tissue from patients that respond well to dopamine agonist therapy. Consequently, further experiments may be needed to confirm our findings. In addition, the effects on growth hormone (GH) secretion in GH3 cells with different drug treatments are worthy of further investigation. Furthermore, we found that bromocriptine produced a statistically significant, dose-dependent reduction in PRL secretion. However, bromocriptine was ineffective in stimulating TGF-β/Smad signaling and did not significantly affect GH3 cell proliferation. Consequently, we considered that other mechanisms may be involved in the process of bromocriptine suppressing PRL secretion in GH3 cells and need further investigation.

In summary, as a mediator of lactotroph viability via dopamine, TGF-β1 and local activators such as the ER antagonist, fulvestrant, may provide a new target for treating prolactinomas, particularly for patients who are resistant to dopamine agonists such as bromocriptine and cabergoline.

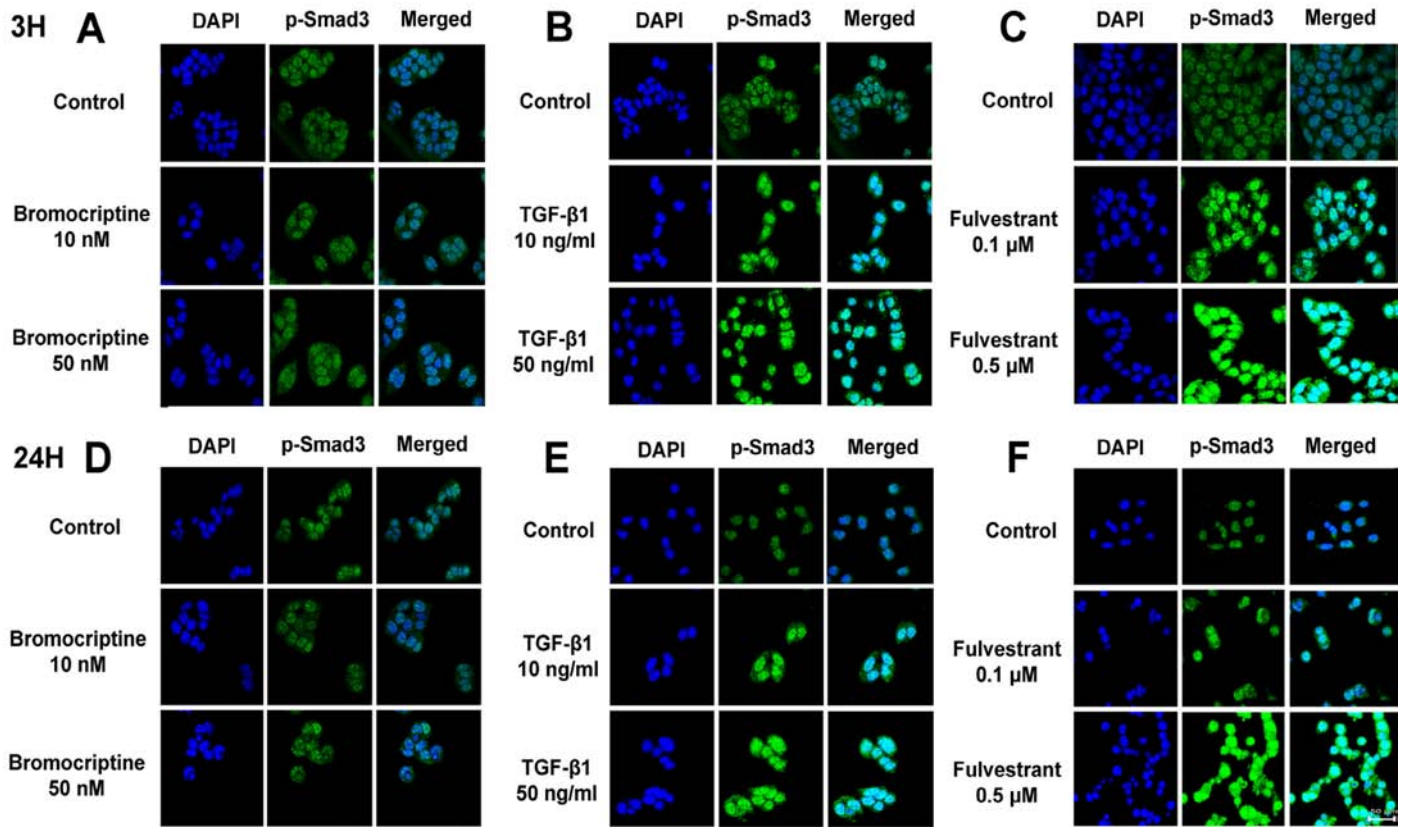

Fig. 6. Fulvestrant increases staining of p-Smad3 in GH3 cells. Serum-starved GH3 were exposed to 10 and 50 ng/ml TGF-β1 for 3 hours (panels A and D) followed by fulvestrant (0.1 and 1 μM) (panels B and E), bromocriptine (10 and 50 nM) (panels C and F) for 24 hours to assess Smad3 phosphorylation (green). Nuclei were stained with DAPI (blue). Scale bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

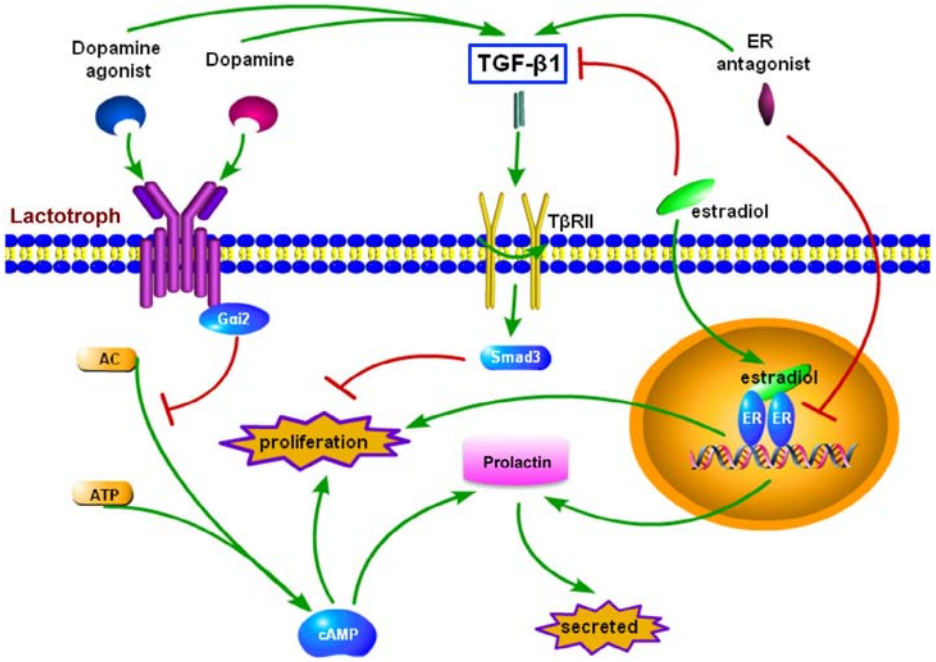

Fig. 7. Schematic of TGF-β1 secretion following bromocriptine or fulvestrant treatment. Under normal conditions, dopamine binds to dopamine receptor 2 (DR2) and suppresses the secretion of prolactin from lactotrophs, which inhibits lactotroph proliferation. Fulvestrant is an estrogen receptor antagonist that is used as an alternative for treating prolactinomas that are resistant to the dopamine agonist, bromocriptine. As a form of therapy, fulvestrant blocks the action of estrogen for tumors that depend on estrogen for growth. In our study, fulvestrant increased TGFβ1/Smad3 signaling which inhibited lactotroph proliferation. AC, adenylate cyclase; cAMP, cyclic AMP; TβRII, type II TGF-β receptor; ER, estrogen receptor; Gαi, inhibitory G protein.

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