Cellular Signalling 63 (2019) 109392

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

TGF-β1 promotes hyaluronan synthesis by upregulating hyaluronan synthase 2 expression in human granulosa-lutein cells

Fuxin Wang[a,b], Hsun-Ming Chang[b], Yuyin Yi[b], Hong Li[a,*], Peter C.K. Leung[b,*]

[a] Center of Reproduction and Genetics, Affiliated Suzhou Hospital of Nanjing Medical University, Suzhou Municipal Hospital, Suzhou 215002, China
[b] Department of Obstetrics and Gynaecology, BC Children's Hospital Research Institute, University of British Columbia, Vancouver, British Columbia V6H 3V5, Canada

ARTICLE INFO

Keywords:
TGF-β1
Hyaluronan
HAS2
SNAIL
SMAD signaling
Human granulosa-lutein cells

ABSTRACT

Hyaluronan serves as a structural component of ovarian follicles, and hyaluronan-mediated signaling cascades lead to follicular development, oocyte maturation, and ovulation. Transforming growth factor-β (TGF-β1) is highly expressed in human oocytes and granulosa cells and involved in the regulation of follicular development and ovulation. Previous studies have shown the imperative role for TGF-β signaling in the regulation of hyaluronan-mediated cumulus expansion and ovulation in human granulosa-lutein (hGL) cells. However, the detailed underlying molecular mechanisms by which TGF-β regulates the synthesis of hyaluronan in hGL cells are not fully elucidated. Using both primary and immortalized hGL cells as study models, we provide the first data showing that TGF-β1 significantly promoted the synthesis of hyaluronan by upregulating the expression of hyaluronan synthase 2 in these cells. Additionally, using dual inhibition approaches, we show that the TGF-β type II (TβRII) receptor and TGF-β type I (ALK5) receptor are functional receptors that mediate stimulatory effects in response to TGF-β1. Moreover, we found that the canonical SMAD2/SMAD3-SMAD4 signaling pathway is the principal intracellular signaling pathway that upregulates the expressionhyaluronan synthase and subsequent hyaluronan synthesis. Notably, we showed that SNAIL transcription factor is a critical molecule mediating the TGF-β signaling, which contributes to the increase in hyaluronan synthesis. These results of our in vitro studies suggest that intraovarian TGF-β1 plays a functional role in the local regulation of hyaluronan synthesis in hGL cells.

1. Introduction

Transforming growth factor-β1 (TGF-β1) is a canonical member of the TGF-β superfamily. TGF-β1 and its putative receptors are highly expressed in the granulosa cells, theca cells and oocytes of both small and large follicles, indicating that this autocrine/paracrine growth factor plays functional roles in the regulation of follicular development [1]. In our previous studies, we showed that in human granulosa cells (GCs), TGF-β1 is involved in the regulation of multiple follicular functions, including GC gap junction intercellular communication, ovarian steroidogenesis, extracellular matrix remodeling, cumulus-oocyte complex (COC) formation, and prostaglandin formation [2–5]. Blocking TGF-β1 signaling by inducing the conditional knockout of the TGF-β type I receptor (*Tgfbr1,* also known as *Alk5*) in mice led to a sterile phenotype that included multiple defects in the oviduct and uterine myometrium [6]. In addition, the natural occurrence of gene mutations in *TGFB1* (which encodes TGF-β1 protein) and the dysregulation of the TGF-β1 signaling pathway have been reported in several pathological disorders in the female reproductive system [7]. For instance, serum levels of TGF-β1 were higher in women with polycystic ovary syndrome (PCOS) than in normal controls [8]. Furthermore, studies have demonstrated the involvement of TGF-β1 in the process of extracellular matrix deposition in patients with chocolate cysts or PCOS [7]. Collectively, these findings indicate that TGF-β1 is a critical intraovarian factor that is involved in the regulation of extracellular matrix remodeling during the periovulatory stage.

Approximately 36 h before ovulation, the pituitary-derived luteinizing hormone (LH) surge reactivates meiosis in the oocyte and stimulates the rapid production of a unique oocyte-embedding matrix, leading to the expansion of the COC (also known as cumulus expansion) [9]. This critical physiological process involves the rapid synthesis and accumulation of hyaluronan, a polysaccharide that belongs to the glycosaminoglycan family [10]. In mammalian dominant follicles, a substantial amount of hyaluronan is secreted by cumulus cells and can be

* Corresponding authors at: Department of Obstetrics and Gynaecology, BC Children's Hospital Research Institute, University of British Columbia, Room 317, 950 West 28th Avenue, Vancouver, British Columbia V5Z 4H4, Canada.
E-mail addresses: hongliszivf@163.com (H. Li), peter.leung@ubc.ca (P.C.K. Leung).

https://doi.org/10.1016/j.cellsig.2019.109392
Received 23 May 2019; Received in revised form 17 August 2019; Accepted 18 August 2019
Available online 19 August 2019
0898-6568/

detected in follicular fluid [11]. Hyaluronan serves as a structural component of ovarian follicles, and hyaluronan-mediated signaling cascades lead to follicular development, oocyte maturation, and ovulation [12]. Furthermore, studies have shown that the hyaluronan-rich COC matrix is essential for oocyte maturation, successful ovulation, in vivo fertilization, and early embryonic development [13]. Indeed, studies performed using clinical samples have demonstrated that the quality of cumulus expansion can be used as a selection criterion for good oocytes during in vitro fertilization [14]. The increase in the production of hyaluronan can be mediated via either an increase in its synthesis or a decrease in its degradation, which involve hyaluronan synthases and hyaluronan-degrading enzymes (also known as hyaluronidases or HYALs), respectively. At present, three hyaluronan synthases (HAS1, HAS2, and HAS3, which are encoded by three different genes) [15] and two major hyaluronidases (HYAL1 and HYAL2) [16] have been identified in mammals.

Given that the hyaluronan-rich COC matrix is critical for the female reproductive system, studies exploring the regulation of hyaluronan synthases and hyaluronidases in the follicular environment have been a focus of research in this area. During mammalian cumulus expansion, compared to HAS1 and HAS3, HAS2-mediated synthesis accounts for a much higher proportion of hyaluronan molecules [17]. The expression of HAS2 is mainly activated by the preovulatory LH surge or human chorionic gonadotropin (hCG) [18]. In the mouse COC, the cumulus cells secrete a peak level of hyaluronan at 4–10 h after the LH surge [19]. In addition to LH/hCG, the synthesis of hyaluronan and the activity of HAS2 can also be modulated by locally produced growth factors [20].

Previous studies have demonstrated that TGF-β/SMAD2/3/4 signaling is required for the regulation of hyaluronan-mediated cellular activities in various cells, including fibroblasts, epithelial cells, endothelial cells, tumour cell lines, chondrocytes, cardiomyocytes [21–23]. Moreover, our and previous studies have demonstrated that the expression of HAS2 and subsequent hyaluronan synthesis can be regulated by several intraovarian TGF-β superfamily members, including bone morphogenetic protein (BMP)4, BMP6, BMP7, and growth differentiation factor 9 (GDF9) [24–26]. These findings prompted us to hypothesize that intrafollicular TGF-β1 may be involved in the regulation of hyaluronan synthesis by targeting HAS2 expression in human GCs. In the present study, we sought to investigate the biological role of TGF-β1 in the regulation of hyaluronan synthesis and the underlying molecular mechanisms in human GCs.

2. Materials and methods

2.1. Cell culture

Primary human granulosa lutein (hGL) cells were collected from infertile patients who underwent in vitro fertilization (IVF) treatment. All participants signed an informed consent form, which was approved by the University of British Columbia Research Ethics Board. The hGL cells were purified with Ficoll Paque density centrifugation as previously described [27]. The cells were seeded at 2×10^5 cells per well in 12-well plates and cultured in a humidified atmosphere of 5% CO2 and 95% air at 37 °C. The cells were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DMEM/F-12; Sigma-Aldrich, Oakville, ON, Canada) supplemented with 10% charcoal/dextran-treated fetal bovine serum (HyClone, Logan, UT, USA), 100 U/ml penicillin (Life Technologies, Inc./BRL, Grand Island, NY, USA), 100 μg/ml streptomycin sulfate (Life Technologies), and 1 × GlutaMAX (Life Technologies). A nontumorigenic immortalized hGL cell line (SVOG), which was established by transfecting hGL cells with the SV40 large T antigen [28], was used in the present study. SVOG cells retain the physiological characteristics of hGL cells, including steroidogenic function and responsiveness to many growth factors in manner similar to that of primary hGL cells [29,30]. SVOG cells were seeded ($4–8 \times 10^5$ cells per well in 6-well plates) and cultured in DMEM/F-12 medium supplemented with 10% charcoal/dextran-treated fetal bovine serum (HyClone), 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin sulfate (Life Technologies) and 1 × GlutaMAX (Life Technologies) in a humidified incubator at 37 °C with 5% CO2. The culture medium was changed every other day for all of the experiments, and the cells were maintained in serum-free medium for 24 h before specific treatment with growth factors.

2.2. Antibodies and reagents

Anti-SMAD2, anti-phospho-SMAD2, anti-SMAD3, anti-phospho-SMAD3, anti-SMAD4, and anti-SNAIL antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-HAS2 (sc-514,737) and anti-GAPDH (sc-32,233) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant human TGF-β1, recombinant human soluble TGF-β type II receptor (TGF-β RII) Fc chimera protein (rTbRII, #341-BR-050) and 4-[6-[4-(1-Methylethoxy) phenyl] pyrazolo[1,5-a] pyrimidin-3-yl]-quinoline (DMH-1, #4126) were obtained from R&D Systems (Minneapolis, MN, USA). The TGF-β type I receptor inhibitor SB505124 (S4317) and epidermal growth factor receptor (EGFR) inhibitor AG1478 was purchased from Sigma-Aldrich.

2.3. Reverse transcription quantitative real-time PCR (RT-qPCR)

Total RNA was extracted using TRIzol Reagent (Life Technologies) according to the manufacturer's instructions. RNA (3 μg) was reverse-transcribed into first-strand cDNA with random primers and Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA). RT-qPCR was performed on an Applied Biosystems 7300 Real-time PCR System in 96-well optical reaction plates. Each 20 μl RT-qPCR reaction contained 1× SYBR Green PCR Master Mix (Applied Biosystems, USA), 20 ng of cDNA and 250 nM of each specific primer. The sequences of the primers used in this study were as follows: *HAS2*, 5′-GACCAAGAGCTGAACAAGATGC-3′ (sense) and 5′- GGTGTG ATGCCAAAAAGGCA-3′ (antisense); glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5′-GAGTCAACGGATTTGGTCGT-3′ (sense) and 5′-GACAAGCTTCCCGTTCTCAG-3′ (antisense); *SNAIL*, 5′-CCCCAATCG GAAGCCTAACT-3′ (sense) and 5'-GCTGGAAGGTAAACTCTGGATT AGA-3′ (antisense); *SMAD2*, 5′-GCCTTTACAGCTTCTCTGAACAA-30 (sense) and 5′-ATGTGGCAATCCTTTTCGAT-3′ (antisense); *SMAD3*, 5'-CCCCAGCACATAATAACTTGG-3′ (sense) and 5′-AGGAGATGGAGCAC CAGAAG-3′ (antisense); and *SMAD4*, 5′-TGGCCCAGGATCAGTAGGT-3′ (sense) and 5'-CATCAACACCAATTCCAGCA-3′ (antisense). The primers used for the TaqMan gene expression assays were as follows: *ACVR1B* (ALK4, Hs00244715_m1), *TβRI* (ALK5, Hs00610320_m1), and *GAPDH* (Hs02758991_g1) (Applied Biosystems). RT-qPCR was performed in triplicate using the corresponding cDNA samples. For each 20 μl TaqMan reaction, 100 ng of cDNA was mixed with 10 ml of 2 × TaqMan gene expression master mix (Applied Biosystems), and 1 ml of 20 × TaqMan gene expression probe. The specificity of each assay was validated using dissociation curve analysis and agarose gel electrophoresis of the PCR products. Assay performance was validated by evaluating amplification efficiencies using calibration curves to ensure that the plot of the log input amount vs ΔCt had a slope $< |0.1|$. The relative quantification of the mRNA levels was performed using the comparative cycle threshold (Ct) method with the formula $2^{\Delta\Delta Ct}$, and GAPDH was used as a reference gene.

2.4. Western blot analysis

Cells were lysed in lysis buffer (Cell Signaling Technology) containing a protease inhibitor cocktail (Sigma-Aldrich). The cell lysate was centrifuged at 20,000 ×*g* for 10 min at 4 °C, and protein concentrations were determined using a DC Protein Assay (Bio-Rad

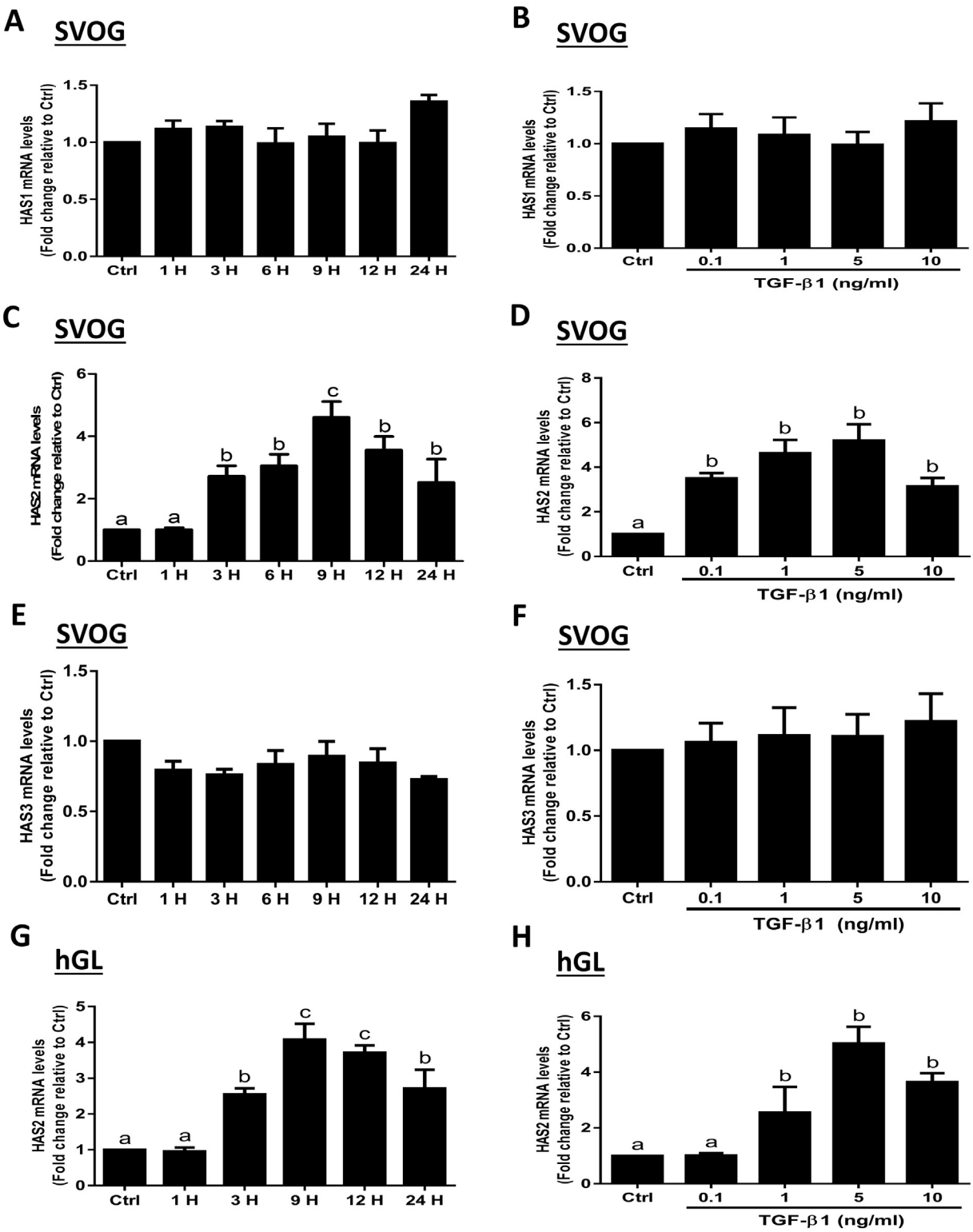

Fig. 1. Effects of TGF-β1 on the expression of HAS1, HAS2, and HAS3 in human granulosa-lutein cells. A, C, and E, SVOG cells were treated with vehicle control (PBS) or 5 ng/ml TGF-β1 for 1, 3, 6, 9, 12, or 24 h, and the mRNA levels of HAS1 (A), HAS2 (C), and HAS3 (E) were examined using RT-qPCR. B, D, and F, SVOG cells were treated with vehicle control (PBS) or different concentrations (0.1, 1, 5, or 10 ng/ml) of TGF-β1 for 9 h, and the mRNA levels of HAS1 (B), HAS2 (D), and HAS3 (F) were examined using RT-qPCR. G, Primary hGL (hGL) cells were treated with 5 ng/ml TGF-β1 for 1, 3, 9, 12, or 24 h, and the mRNA level of HAS2 was examined using RT-qPCR. H, Primary hGL cells were treated with vehicle control (PBS) or different concentrations (0.1, 1, 5, or 10 ng/ml) of TGF-β1 for 9 h, and the mRNA level of HAS2 was examined using RT-qPCR. The results are expressed as the mean ± SEM of at least three independent experiments. Values marked with different letters are significantly different ($P < 0.05$). Ctrl, Control.

Laboratories, Hercules, CA, USA). Equal amounts of protein were separated using 10% SDS-PAGE and then transferred onto polyvinylidene fluoride membranes. After 1 h of blocking in Tris-buffered saline containing 0.05% Tween 20 and 5% nonfat dried milk, the membranes were then incubated overnight at 4 °C with the relevant primary antibodies. After the membranes were washed, they were incubated with peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories) for 1 h. Immunoreactive bands were detected using enhanced chemiluminescence reagents or SuperSignal West Femto chemiluminescence substrate (Pierce, Rockford, IL, USA) followed by exposure to CL-XPosure film (Thermo Fisher). The membranes were stripped with stripping buffer (50 mM Tris–HCl pH 7.6, 10 mmol/L β-mercaptoethanol and 1% SDS) at 50 °C for 30 min and then reprobed with mouse anti-SMAD2, rabbit anti-SMAD3 or mouse anti-GAPDH antibodies as a loading control. Immunoreactive band intensities were quantified by densitometry (Scion Image software, Scion Corporation, Frederick, MD, USA). Targeted protein levels were normalized to those of GAPDH, and the results are expressed as the fold-change relative to the respective control.

2.5. Small interfering RNA transfection

We used transient knockdown assays with ON-TARGET plus Nontargeting Control Pool or separate ON-TARGET plus SMARTpools (Thermo Fisher Scientific) to knock down endogenous activin receptor-like kinase (ALK)4, ALK5, SMAD2, SMAD3, SMAD4, and SNAIL (L-004925-00-0005, L-003929-00-0005, L-003561-00-0005, L-020067-00-0005, L-003902-00-0005, and L-010847-01-0005, respectively). Cells were precultured to 50% confluence in antibiotic-free DMEM/F12 medium containing 10% charcoal/dextran-treated fetal bovine serum and then transfected with 25 nM small interfering RNA (siRNA) using Lipofectamine RNAiMAX (Life Technologies) for 48 h. The knockdown efficiency of each target was confirmed using RT-qPCR.

2.6. Immunofluorescence staining

Cells were plated on glass cover slips, fixed with 4% paraformaldehyde in PBS for 20 min, and then permeabilized with 0.1% Triton X-100 in PBS for 5 min. After the cells were washed with PBS, the cover slips were mounted on microscope slides and blocked with Dako Protein Block (Dako, Mississauga, ON, Canada) for 1 h followed by an overnight incubation with HAS2 antibodies (1:50 diluted in Dako Protein Block). Alexa Fluor 555 donkey antirat IgG (Life Technologies) was used as a secondary antibody. Finally, the cells were counterstained with the chromosomal dye DAPI (Sigma-Aldrich), rinsed with PBS, mounted in Gelvatol and imaged under a Zeiss Axiophot fluorescence microscope equipped with a digital camera (Q Imaging, Burnaby, BC, Canada).

2.7. Measurement of hyaluronan

After the specified treatment, the culture medium was obtained and stored at −20 °C until analyzed. The accumulated concentrations of hyaluronan in the conditioned media were measured using a quantitative sandwich enzyme immunoassay Quantikine kit (R&D Systems) according to the manufacturer's instructions. The inter- and intra-assay coefficients of variation for these assays were 7.7% and 8.9%, respectively. The detection limit of hyaluronan was 0.068 ng/ml. Each sample was measured in triplicate, and the secreted hyaluronan levels were normalized to the total cellular protein content.

2.8. Statistical analysis

The results are presented as the mean ± SEM of at least three independent experiments performed using three separate cultures. The results were analyzed by one-way analysis of variance followed by Tukey's multiple comparison tests in PRISM software (GraphPad Software, Inc., San Diego, CA, USA). Data were considered significantly different if $P < 0.05$.

3. Results

3.1. TGF-β1 increased the mRNA levels of HAS2 but not HAS1 or HAS3 in hGL cells

To investigate the functional role of TGF-β1 in the regulation of hyaluronan production, we first examined the effects of TGF-β1 on related enzymes (hyaluronan synthases, HAS1, HAS2, and HAS3) involved in the synthesis of hyaluronan. The time-course studies showed that in SVOG cells, treatment with 5 ng/ml TGF-β1 for different durations (1, 3, 6, 9, 12, or 24 h) led to increases in the levels of HAS2 mRNA starting at 3 h after treatment (Fig. 1C). However, the same treatment course did not have these effects on the mRNA levels of HAS1 or HAS3 (Fig. 1A and E). Additionally, the concentration-dependent studies showed that treatment with different concentrations (0.1, 1. 5, or 10 ng/ml) of TGF-β1 for 9 h significantly increased the mRNA levels of HAS2 but not HAS1 or HAS3 in SVOG cells (Fig. 1B, D, and F). To increase the physiological relevance of these results, we used primary hGL cells isolated directly from the follicular fluid of IVF patients to confirm the regulatory effect of TGF-β1 on the expression of HAS2. Similar to the results obtained in SVOG cells, treatment with 5 ng/ml TGF-β1 for different durations increased the mRNA level of HAS2 beginning at 3 h and lasting until 24 h (Fig. 1G). Additionally, in primary hGL cells, treatment with different concentrations (0.1, 1. 5, or 10 ng/ml) of TGF-β1 for 9 h increased the mRNA level of HAS2 (effects were significant beginning at a concentration of 1 ng/ml) (Fig. 1H).

Because increases in hyaluronan production can be mediated by either an increase in the synthesis or a decreased in the degradation of the protein, we next investigated the effects of TGF-β1 on the expression of hyaluronidases, including HYAL1 and HYAL2. As shown in Supplemental Fig. 1, treatment with 5 ng/ml TGF-β1 for different durations (1, 3, 6, 9, 12, or 24 h) (Supplemental Fig. 1A and C) and treatment with different concentrations (0.1, 1. 5, or 10 ng/ml) of TGF-β1 for 9 h (Supplemental Fig. 1B and D) did not affect the mRNA levels of HYAL1 and HYAL2 in SVOG cells.

3.2. TGF-β1 increased the protein levels of HAS2 in SVOG cells

Because TGF-β1 treatment increased the mRNA level of HAS2 in hGL cells, we next examined the effect of TGF-β1 on the localization and expression of the HAS2 protein in SVOG cells. To ensure the application of the antibodies used in this study, we first chose a targeted siRNA-mediated approach to validate the specificity of the HAS2 antibodies. As shown in Fig. 2A, knocking down HAS2 for 24 h using an siRNA targeting HAS2 significantly decreased the protein level of HAS2 in SVOG cells. To determine the subcellular localization of the HAS2 protein, we immunolabelled and probed SVOG cells with HAS2 antibodies. The immunofluorescence staining in SVOG cells showed cytoplasmic immunoreactivity for HAS2, which was not localized in nuclei (Fig. 2B). Additionally, the expression of HAS2 increased following 12-h of TGF-β1 stimulation (Fig. 2B). Notably, western blot analysis showed that treatment with 5 ng/ml TGF-β1 for 12 or 24 h but not 6 h increased the protein levels of HAS2 in SVOG cells (Fig. 2C). Concentration-dependent studies showed that treating cells with TGF-β1 (1 ng/ml to 10 ng/ml) for 12 h increased the protein level of HAS2 (Fig. 2D).

3.3. TβRII and TβRI are involved in the TGF-β1-induced upregulation of HAS2 expression in SVOG cells

In many mammalian cells, TGF-β1 initiates its cellular activities by binding to the TGF-β type II receptor (TβRII); it then recruits and

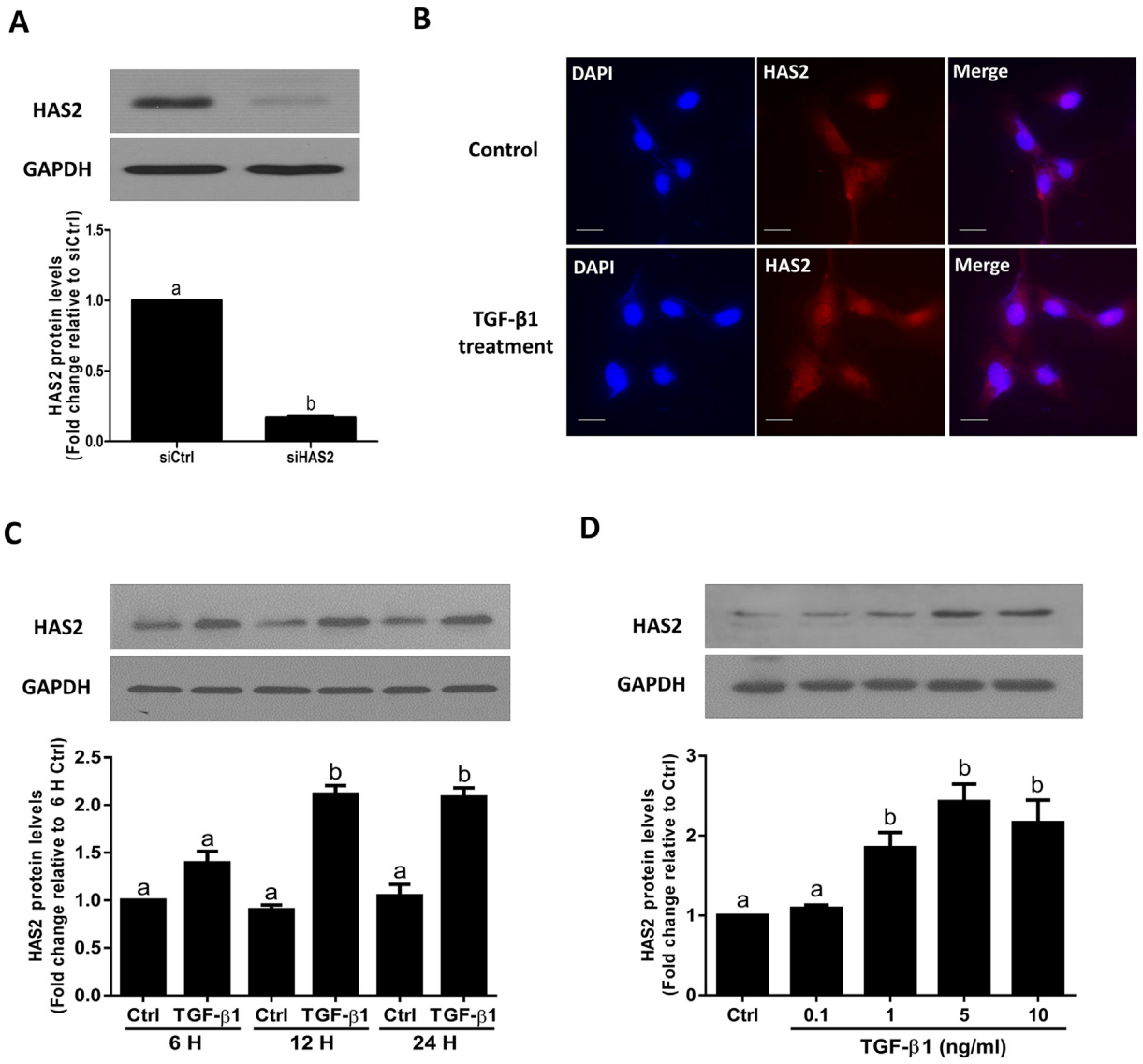

Fig. 2. Effects of TGF-β1 on the localization and expression of HAS2 in SVOG cells. A, SVOG cells were transfected with 25 nM control siRNA (siCtrl) or 25 nM siRNA targeting HAS2 (siHAS2) for 24 h, and the protein level of HAS2 was then examined using western blot analysis. B, SVOG cells were treated with 5 ng/ml TGF-β1 for 12 h, fixed in 4% paraformaldehyde in PBS, and examined for HAS2 (red) and nuclear (DAPI in blue) immunofluorescence. C, SVOG cells were treated with 5 ng/ml TGF-β1 for 6, 12, or 24 h, and the protein level of HAS2 was examined using western blot analysis. D, SVOG cells were treated with vehicle control (PBS) or different concentrations (0.1, 1, 5, or 10 ng/ml) of TGF-β1 for 12 h, and the protein level of HAS2 was examined using western blot analysis. The results are expressed as the mean ± SEM of at least three independent experiments. Values marked with different letters are significantly different ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dimerizes with TGF-β type I receptor [31]. Recombinant human TGF-β type II receptor (rTβRII) is a soluble TGF-β type II receptor that is capable of binding to TGF-β1 and TGF-β3 with sufficient affinity to act as an inhibitor when present at a high concentration [32]. To evaluate the involvement of TβRII receptor in the TGF-β1-induced upregulation of HAS2 expression, we used rTβRII as a competitive inhibitor of endogenous TβRII. The results showed that preincubating 5 ng/ml TGF-β1 with 5 mg/ml rTβRII at room temperature for 1 h completely abolished the TGF-β1-induced increases in the mRNA and protein levels of HAS2 (Fig. 3A and B). Currently, seven distinct TGF-β type I receptors (also known as activin receptor like-kinase 1–7, ALK1–7) have been identified and shown to mediate cellular activities in response to TGF-β superfamily members [33]. Next, we used two receptor kinase inhibitors, SB505124 (a potent and specific inhibitor of ALK4/5/7) [34], and DMH-1 (an inhibitor of ALK2/3) [35], to investigate the involvement of TGF-β type I receptors in TGF-β1-induced cellular activity. The results showed that pretreatment with 10 μM SB505124 completely abolished the stimulatory effects of TGF-β1 on the expression of HAS2 at both the mRNA and protein levels (Fig. 3C and D). However, pretreatment with 10 μM DMH-1 did not have these effects (Fig. 3E and F).

3.4. ALK5 is required for the TGF-β1-induced upregulation of HAS2 expression in SVOG cells

Considering the limitations and off-target effects of these inhibitors, we next used a targeted siRNA-mediated approach to determine which specific ALK mediates the TGF-β1-induced increase in HAS2 expression. The results showed that knocking down ALK4 did not alter the TGF-β1-induced increases in the mRNA and protein levels of HAS2 in SVOG cells (Fig. 4A and B). In contrast, knocking down ALK5 completely abolished the TGF-β1-induced increases in the mRNA and protein levels of HAS2 in SVOG cells (Fig. 4C and D).

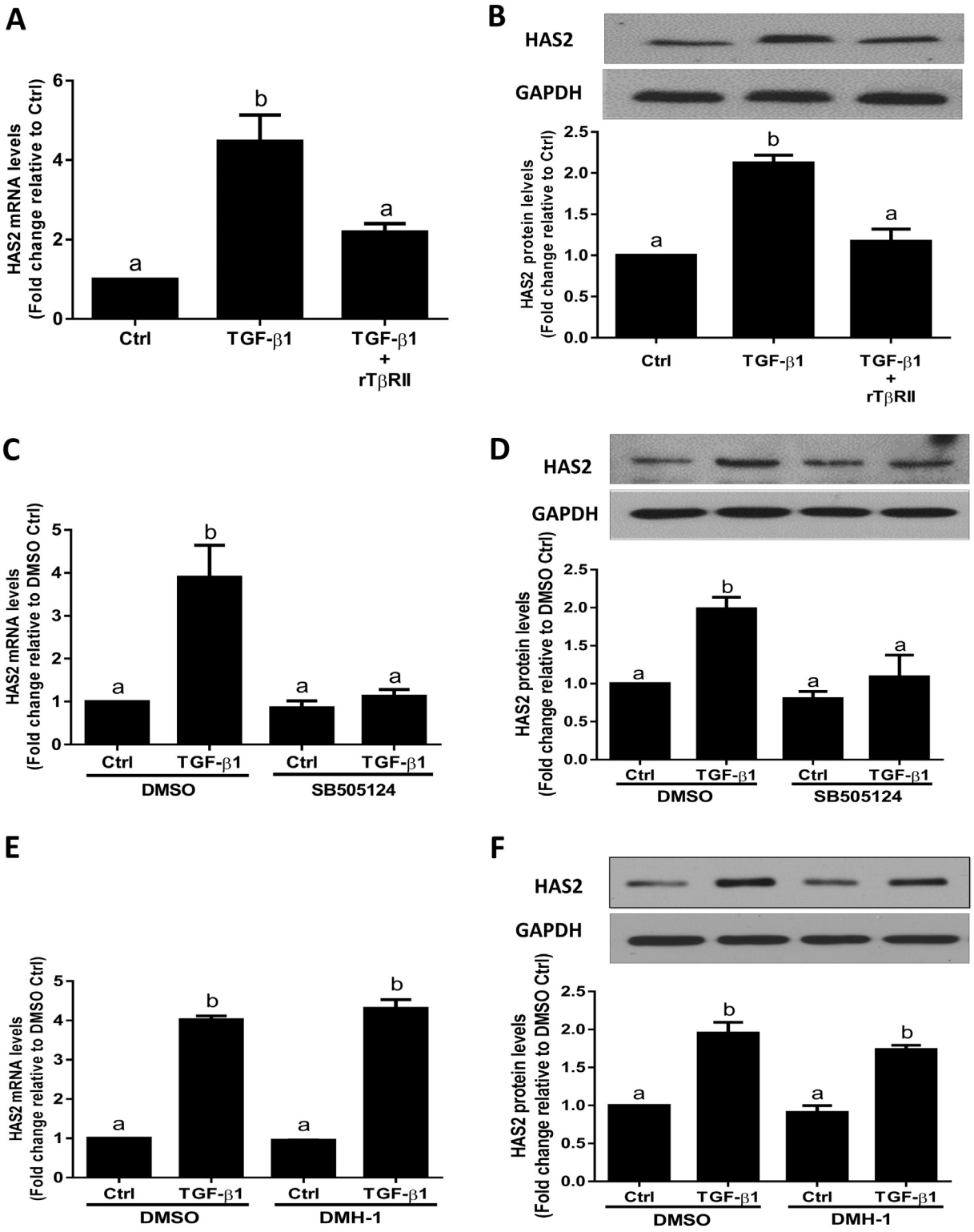

Fig. 3. The involvement of TβRII and TβRI in the TGF-β1-induced upregulation of HAS2 expression. A and B, A total of 5 mg/ml rTβRII was preincubated with 5 ng/ml TGF-β1 at room temperature for 1 h, and the mixture was then added to SVOG cells for 9 h (A) or 12 h (B). The mRNA (A) or protein (B) level of HAS2 was examined using RT-PCR (A) or western blot analysis (B), respectively. C and D, SVOG cells were treated with 5 ng/ml TGF-β1 for 9 h (C) or 12 h (D) in the presence of vehicle control [dimethyl sulfoxide (DMSO)] or 10 μM SB505124. The mRNA (C) or protein (D) level of HAS2 was examined using RT-qPCR (C) or western blot analysis (D), respectively. E and F, SVOG cells were treated with 5 ng/ml TGF-β1 for 9 h (E) and 12 h (F) in the presence of vehicle control (DMSO) or 10 μM DMH-1(d), the mRNA (E) or protein (F) level of HAS2 was examined using RT-qPCR (E) or western blot analysis (F), respectively. The results are expressed as the mean ± SEM of at least three independent experiments. Values marked with different letters are significantly different ($P < 0.05$).

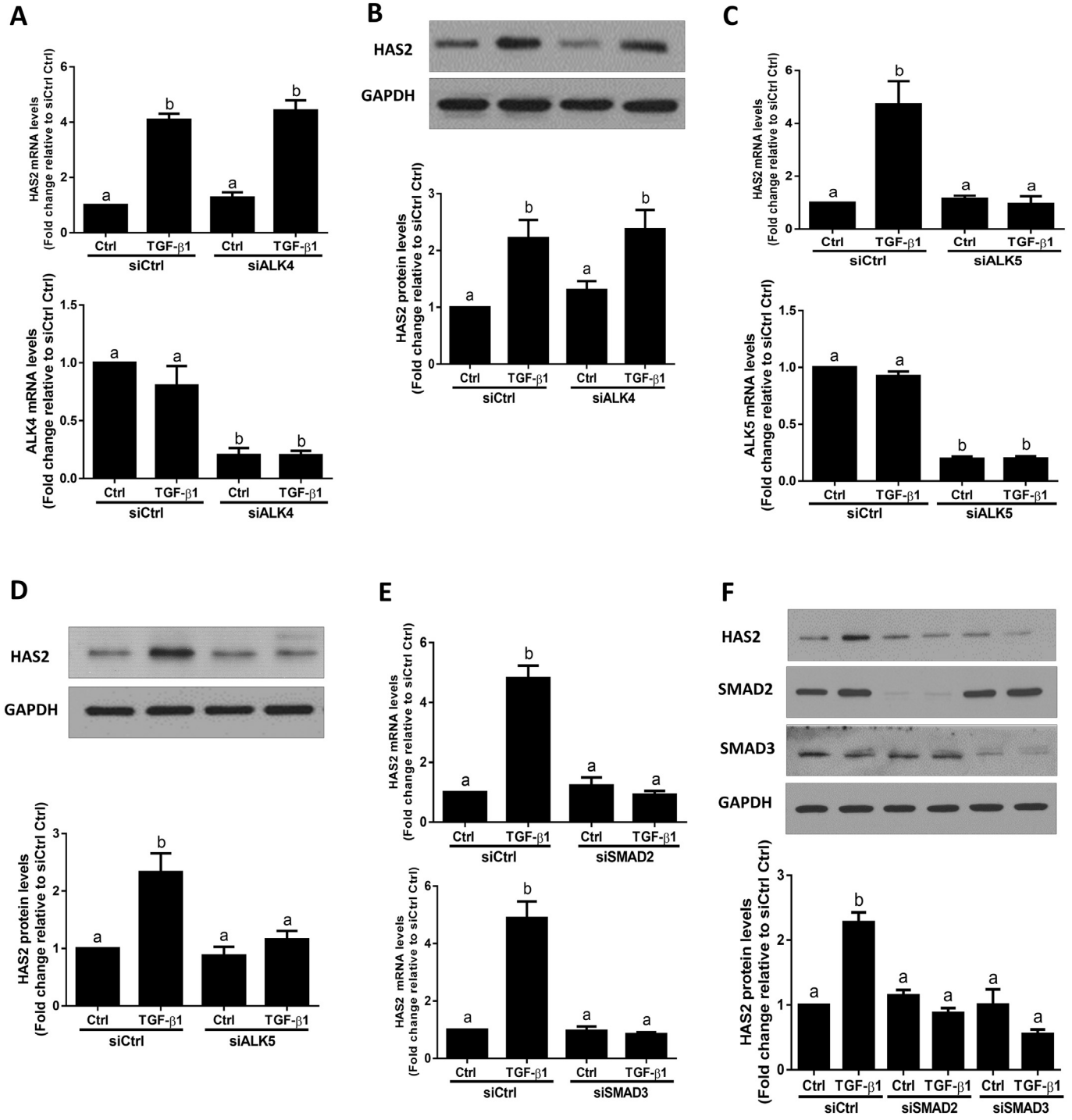

Fig. 4. The involvement of the TGF-β type I receptors ALK4, ALK5, SMAD2 and SMAD3 in the TGF-β1-induced upregulation of HAS2 in SVOG cells. A and B, SVOG cells were transfected with 25 nM control siRNA (siCtrl) or 25 nM siRNA targeting ALK4 (siALK4) for 24 h, and then treated with vehicle control (PBS) or 5 ng/ml TGF-β1 for 9 h (A) or 12 h (B). The mRNA (A) or protein (B) level of HAS2 was examined using RT-qPCR (A) or western blot analysis (B), respectively. C and D, SVOG cells were transfected with 25 nM siCtrl or 25 nM siRNA targeting ALK 5 (siALK5) for 24 h, and then treated with vehicle control (PBS) or 5 ng/ml TGF-β1 for 9 h (C) or 12 h (D). The mRNA (C) or protein (D) level of HAS2 was examined using RT-qPCR (C) or western blot analysis (D), respectively. E, SVOG cells were transfected with 25 nM siCtrl, 25 nM siRNA targeting SMAD2 (siSMAD2), or 25 nM siRNA targeting SMAD3 (siSMAD3) for 24 h, and then treated with vehicle control (PBS) or 5 ng/ml TGF-β1 for an additional 9 h. The mRNA levels of HAS2 were examined using RT-qPCR. F, SVOG cells were transfected with 25 nM siCtrl, 25 nM siSMAD2, or 25 nM siSMAD3 for 24 h, and then treated with vehicle control (PBS) or 5 ng/ml TGF-β1 for an additional 12 h. The protein levels of HAS2, SMAD2, and SMAD3 were examined using western blot analysis. The results are expressed as the mean ± SEM of at least three independent experiments. Values marked with different letters are significantly different ($P < 0.05$).

3.5. Both SMAD2 and SMAD3 are required for the TGF-β1-induced upregulation of HAS2 in SVOG cells

Upon TGF-β1 ligand binding, activated TGF-β receptors are able to induce the phosphorylation of the receptor-regulated SMAD (R-SMAD) proteins SMAD2 and SMAD3. Phosphorylated SMAD2 or SMAD3 then binds to the common mediator SMAD, SMAD4, which then translocates to the nucleus to regulate gene expression [36]. To determine which specific R-SMAD was involved in the TGF-β1-induced upregulation of HAS2 expression, we induced the siRNA-mediated knockdown of endogenous SMAD2 and SMAD3. The results showed that knocking down SMAD2 completely abolished the increase in the mRNA level of HAS2 induced by TGF-β1 (Fig. 4E). Similarly, knocking down SMAD3 completely abolished the increases in the mRNA level of HAS2 induced by TGF-β1 (Fig. 4E). Notably, western blot analysis further confirmed the results obtained from RT-qPCR as knocking down either SMAD2 or SMAD3 completely abolished the TGF-β1-mediated increase in the protein level of HAS2 (Fig. 4F).

3.6. SMAD4 is required for the TGF-β1-induced upregulation of HAS2 and SNAIL in SVOG cells

To further confirm the regulatory role of the SMAD-dependent pathway, we used a targeted siRNA-mediated approach to knock down the endogenous principal mediator of this signaling pathway, SMAD4. As shown in Fig. 5A and B, knocking down SMAD4 completely abolished the TGF-β1-induced upregulation of HAS2 expression at both the mRNA and protein levels. Because the transcription factor SNAIL has been identified to mediate TGF-β1-induced cellular activities in human cells [5,37], we next investigated the involvement of the SMAD-dependent signaling pathway in the induction of this transcription factor. Interestingly, the results showed that treatment with TGF-β1 significantly increased the mRNA and protein levels of SNAIL, and these stimulatory effects were completely abolished by knocking down endogenous SMAD4 (Fig. 5C and D).

3.7. SNAIL is involved in the TGF-β1-induced upregulation of HAS2 expression in SVOG cells

Our recent studies demonstrated that SNAIL mediated the TGF-β1-induced downregulation of pentraxin 3 expression in hGL cells [5]. Given that SNAIL is a critical transcription factor that is essential for the formation of extracellular matrix [38], we sought to investigate the involvement of SNAIL on the TGF-β1-induced upregulation of HAS2 expression in SVOG cells. Using a targeted siRNA-mediated inhibition approach, the results showed that knocking down SNAIL completely abolished the TGF-β1-induced increase in the mRNA level of HAS2 in SVOG cells (Fig. 6A). Similarly, the western blot analysis results showed that knocking down SNAIL completely abolished the TGF-β1-induced increase in the protein level of HAS2 in SVOG cells (Fig. 6B).

3.8. TGF-β1 increased the accumulation of hyaluronan via the upregulation of SNAIL in SVOG cells

Finally, we sought to investigate whether the TGF-β1-induced upregulation of HAS2 expression contributes to the increased levels of accumulated hyaluronan in SVOG cells. An enzyme immunoassay (ELISA) was used to examine the accumulated levels of hyaluronan in conditioned medium obtained from cultured hGL cells, and the results showed that TGF-β1 treatment significantly increased the concentrations of hyaluronan produced by SVOG cells (Fig. 6C). Consistent with the findings obtained in SVOG cells, TGF-β1 also significantly increased the accumulated levels of hyaluronan in primary hGL cells (Fig. 6C). Additionally, the stimulatory effect of TGF-β1 on the accumulated levels of hyaluronan was abolished by knocking down SNAIL in SVOG cells (Fig. 6D).

Previous studies have shown that hyaluronan can promote CD44/EGFR interaction and EGFR-mediated oncogenic signaling in head and neck squamous cell carcinomas [39]. To investigate whether SNAIL expression is a direct consequence of the canonical TGF-β signaling, or the non-canonical coactivation of EGFR signaling, we thus used an EGFR chemical inhibitor to examine whether EGFR plays any role in hGL cell HA synthesis. As shown in Fig. 6E, the TGF-β1-induced increase in hyaluronan synthesis was reversed by the addition of an EGFR inhibitor AG1478 (10 μM).

4. Discussion

In the present study, we have demonstrated that TGF-β1 treatment significantly increased the synthesis of hyaluronan in both primary and immortalized hGL cells. Consistent with the results obtained in this study, studies performed using mouse GCs have also shown that oocyte- and GC-derived TGF-β1 induced the synthesis of hyaluronic acid [40]. These findings suggest that TGF-β1 is an essential intraovarian factor that promotes the synthesis of hyaluronan in GCs and cumulus cells. Due to studies indicating that the accumulated levels of hyaluronan can be influenced by either an increase in the synthesis or a decrease in the degradation of the protein, we examined the effects of TGF-β1 on the expression of several forms of HAS (HAS1, HAS2, and HAS3) and HYAL (HYAL1 and HYAL2) in hGL cells. Our results showed that TGF-β1 treatment did not affect the expression of HYAL1 and HYAL2. However, TGF-β1 treatment significantly upregulated the expression level of HAS2 but not HAS1 and HAS3, indicating that HAS2 is the principal enzyme that contributes to the TGF-β1-induced increase in the accumulated level of hyaluronan. Consistent with the results obtained in the present study, our previous data also showed that the expression levels of HAS1 or HAS3 were significantly lower in primary hGL and SVOG cells compared to that of HAS2 [24]. In the bovine ovary, the mRNA levels of HAS2 were significantly increased, but the mRNA levels of HAS3 were only slightly increased (mRNA of HAS1 was not detectable) in response to stimulation with gonadotropins [41]. Similarly, the mRNA level of HAS2 was substantially upregulated by FSH/eCG in cumulus cells during cumulus expansion in several mammals [42].

In many mammalian cells, TGF-β1 initiates cellular activities by binding to the TGF-β Type II receptor, which then recruits and activates the TGF-β type I receptor. Our data, in which soluble TβRII (acting as a competitive inhibitor) completely abolished the TGF-β1-induced upregulation of HAS2 expression, confirm that TβRII is the principal type II receptor that mediates TGF-β1-induced cellular activity in hGL cells. We further used dual inhibitory approaches (pharmacological inhibitors and targeted siRNA-based knock down) to confirm that ALK5 is the specific type I receptor that primarily determines the biological responses to TGF-β1 in hGL cells. Similarly, we confirmed that the SMAD2/3–SMAD4 signaling pathway is the downstream effector that promotes the upregulation of HAS2 expression as knocking down any of these SMADs (SMAD2, SMAD3, or SMAD4) completely abolished this effect. Interestingly, our previous studies showed that BMP4 can regulate the expression of HAS2 through noncanonical SMAD2/3 signaling [24]. Notably, the SMAD2/3 signaling pathway is the canonical pathway induced by TGF-β1, and this supports our finding that TGF-β1 is involved in the regulation of HAS2 expression via the canonical SMAD-dependent signaling pathway in hGL cells. Consistent with these results, previous studies have demonstrated that the TGF-β/SMAD2/3/4 signaling is required for the regulation of hyaluronan-mediated cellular activities in other cells [21–23].

Based on our data showing that the depletion of SNAIL completely abolished the TGF-β1-induced upregulation of HAS2 expression, we demonstrated that SNAIL is the immediately downstream target gene that is required for this stimulatory effect in hGL cells. SNAIL belongs to the SNAIL superfamily of zinc-finger-type transcription factors, which regulate various physiological functions, including mesoderm formation, cell survival and cell division [38]. Animal studies have

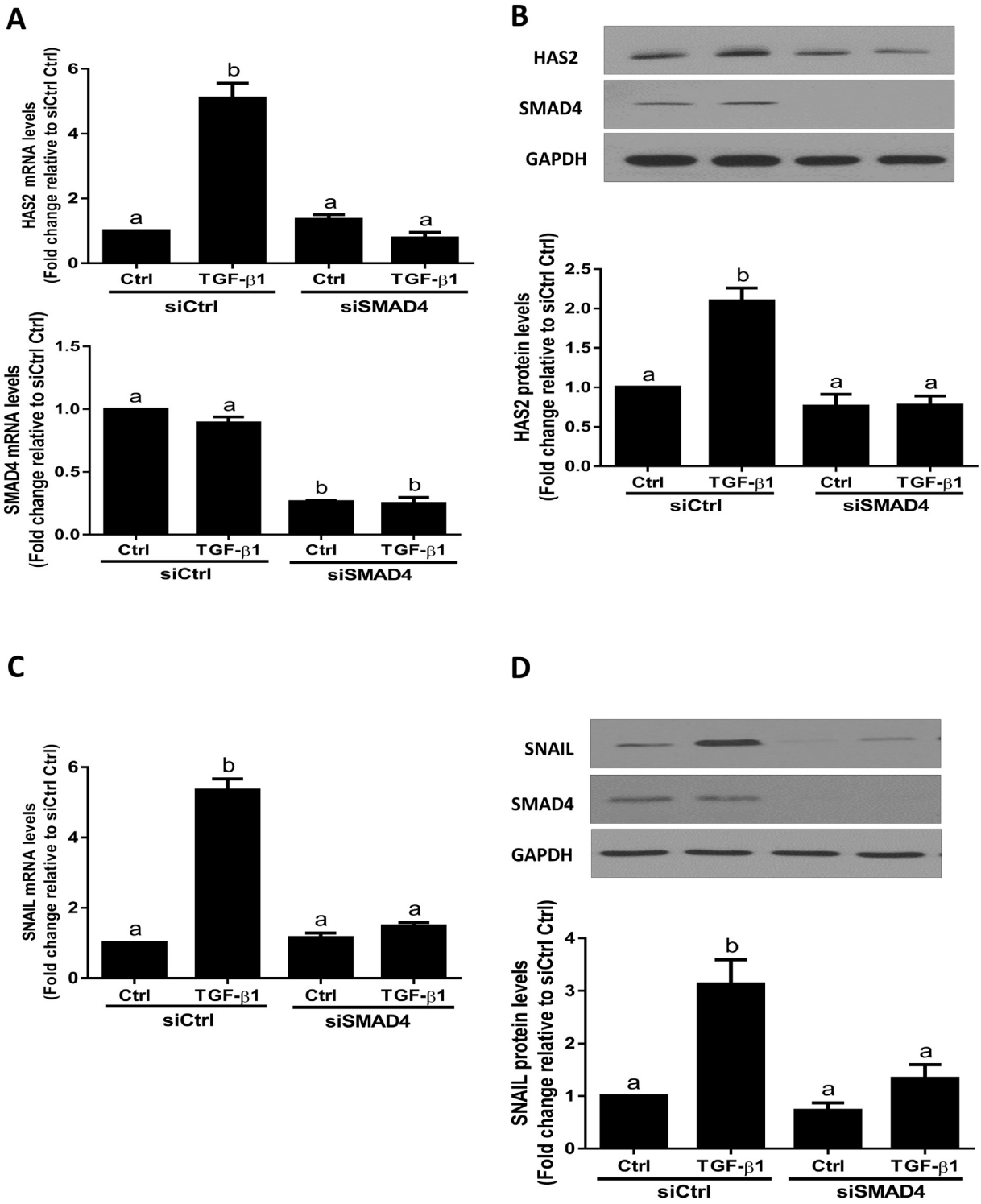

Fig. 5. The involvement of SMAD4 in the TGF-β1-induced upregulation of HAS2 and SNAIL expression in SVOG cells. A and C, SVOG cells were transfected with 25 nM siCtrl or 25 nM siRNA targeting SMAD4 (siSMAD4) for 24 h, and then treated with vehicle control (PBS) or 5 ng/ml TGF-β1 for an additional 9 h. The mRNA levels of HAS2 (A) and SNAIL (C) were examined using RT-qPCR. B and D, SVOG cells were transfected with 25 nM siCtrl or 25 nM siSMAD4 for 24 h, and then treated with vehicle control (PBS) or 5 ng/ml TGF-β1 for an additional 12 h. The protein levels of HAS2 (B) and SNAIL (D) were examined using western blot analysis. The results are expressed as the mean ± SEM of at least three independent experiments. Values marked with different letters are significantly different ($P < 0.05$).

demonstrated that SNAIL is highly expressed in mouse oocytes, follicle cells and the corpus luteum, indicating that this transcription factor plays potential functional roles during follicular development [43]. SNAIL is a well-established zinc finger protein that recognizes the canonical *E*-box sequences: CANNTG basic helix-loop-helix (HLH) motifs [44]. Indeed, increasing evidence has indicated that several target genes are modulated by the TGF-β1-induced upregulation of SNAIL via binding to their promoter sequences [45]. Consistent with these data, our recent studies showed that SNAIL is involved in the TGF-β1-induced downregulation of PTX3 in hGL cells [5]. Using an EGFR inhibitor AG1478, we also showed that the inhibition of EGF signaling reversed the TGF-β1-induced increase in hyaluronan synthesis, indicating that

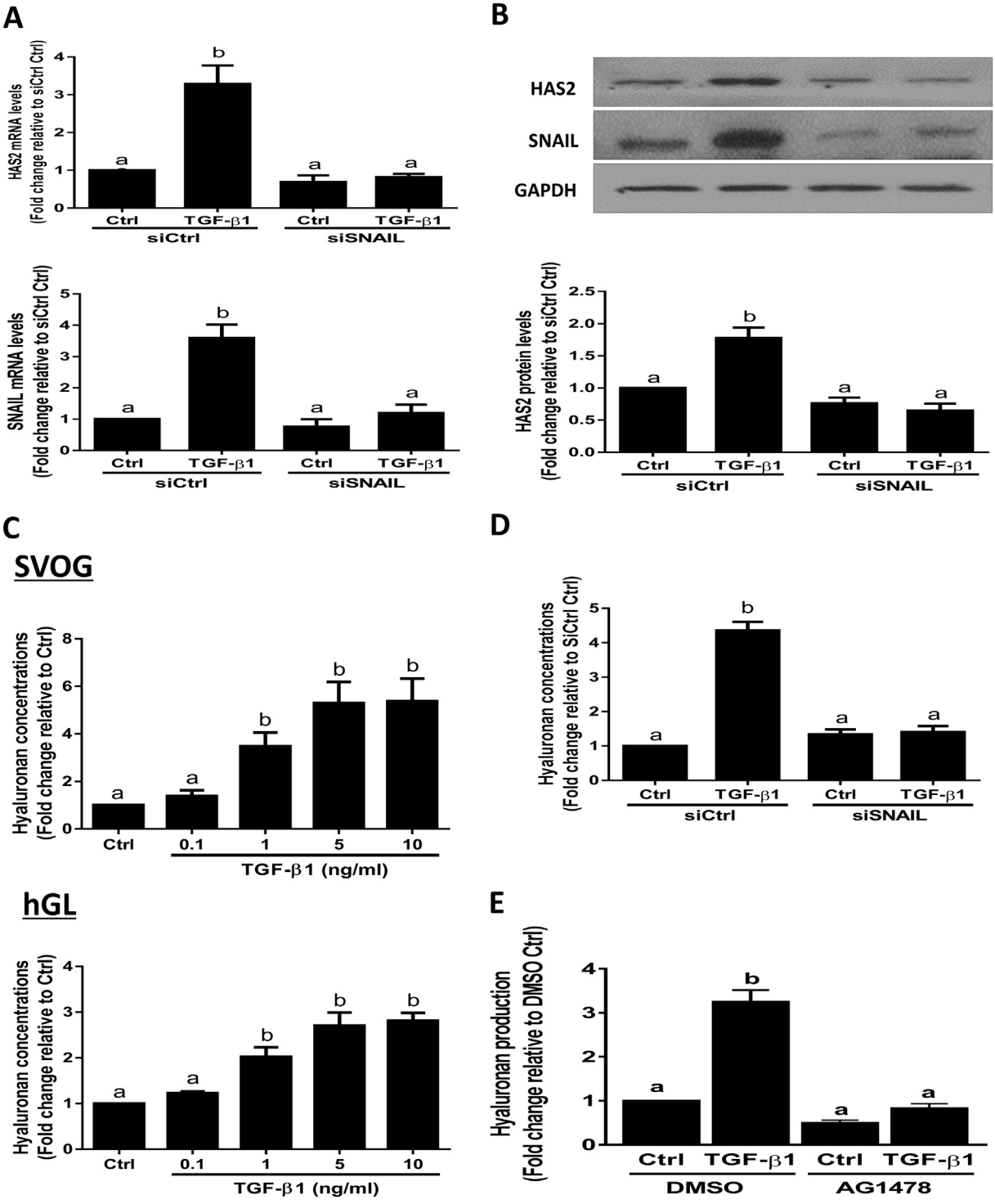

Fig. 6. The involvement of SNAIL in the TGF-β1-induced upregulation of HAS2 expression and increase in hyaluronan synthesis in SVOG cells. A and B, SVOG cells were transfected with 25 nM siCtrl or 25 nM siRNA targeting SNAIL (siSNAIL) for 24 h, and then treated with vehicle control (PBS) or 5 ng/ml TGF-β1 for an additional 9 (A) or 12 h (B), the mRNA (A) and protein (B) levels of HAS2 were examined using RT-qPCR (A) and western blot analysis (B), respectively. C, SVOG or primary hGL cells were treated with vehicle control (PBS) or different concentrations (0.1, 1, 5, 10 ng/ml) of TGF-β1 for 12 h, and the accumulated level of hyaluronan was examined using an enzyme immunoassay (ELISA). D, SVOG cells were transfected with 25 nM siCtrl or 25 nM siSNAIL for 24 h, and then treated with vehicle control (PBS) or 5 ng/ml TGF-β1 for an additional 12 h. The accumulated level of hyaluronan was examined using an enzyme immunoassay. E, SVOG cells were treated with 5 ng/ml TGF-β1 for 12 h in the presence of vehicle control [dimethyl sulfoxide (DMSO)] or 10 μM AG1478. The accumulated level of hyaluronan was examined using an enzyme immunoassay. The results are expressed as the mean ± SEM of at least three independent experiments. Values marked with different letters are significantly different ($P < 0.05$).

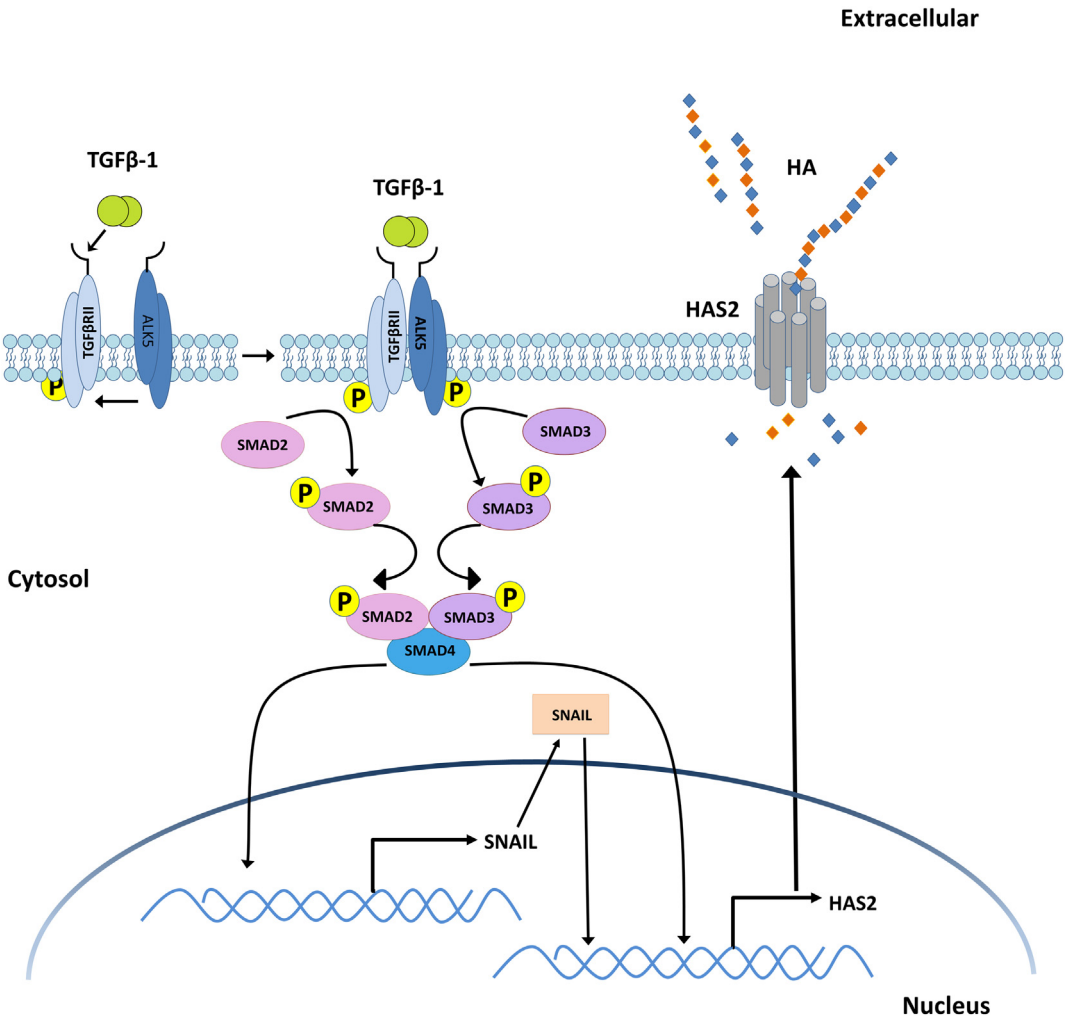

Fig. 7. Proposed model for the effect of TGF-β1 on the expression levels SNAL and HAS2 as well as the production of hyaluronan. TGF-β1 binds to a complex containing type I and II receptors leading to the phosphorylation/activation of receptor-regulated SMAD (SMAD2/3), which binds to the common SMAD (SMAD4). This complex then translocates into the nucleus to promote the transcription of SNAIL. The upregulation of SNAIL subsequently contributes to the increase in HAS2 expression, which further promote the synthesis of hyaluronan in human granulosa cells. HA, hyaluronan; HAS2, hyaluronan synthase 2.

EGFR interaction and EGFR-mediated cellular signaling are required for TGF-β1-induced downstream signaling in hGL cells. These results are consistent with previous study showing that hyaluronan can promote CD44/EGFR interaction and EGFR-mediated oncogenic signaling in head and neck squamous cell carcinomas [39]. Future studies aimed at addressing the interactions between SNAIL transcription factors and CD44/EGFR will be of great interest.

In conclusion, we provided the first data showing that TGF-β1 promoted the synthesis of hyaluronan by upregulating the HAS2 synthesis in hGL cells (Fig. 7). Additionally, using dual inhibitory approaches, we demonstrated that the TβRII type II receptor and ALK5 type I receptor are functional receptors that mediate the stimulatory effects induced in response to TGF-β1. Moreover, the canonical SMAD2/SMAD3-SMAD4 signaling pathway is the principal intracellular signaling pathway that upregulates the expression of SNAIL, which subsequently contributes to TGF-β1-induced increases in HAS2 expression and hyaluronan synthesis (Fig. 7). The results of our in vitro studies suggest that intraovarian TGF-β1 plays a functional role in the local regulation of hyaluronan synthesis in hGL cells.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.109392.

Funding

This work was supported by the Canadian Institutes of Health Research Foundation Scheme Grant (#143317) to P.C.K.L. This work was also supported by the Suzhou Introduced Project of Clinical Medical Expert Team (Grant number SZYJTD201708) and Jiangsu Provincial Medical Innovation Team (Grant number CXTDB2017013) to H.L.

Disclosure statement

The authors have nothing to disclose.

References

[1] S.G. Kristensen, K. Andersen, C.A. Clement, S. Franks, K. Hardy, C.Y. Andersen, Expression of TGF-beta superfamily growth factors, their receptors, the associated SMADs and antagonists in five isolated size-matched populations of pre-antral follicles from normal human ovaries, Mol. Hum. Reprod. 20 (4) (2014) 293–308.
[2] Y.C. Chen, H.M. Chang, J.C. Cheng, H.D. Tsai, C.H. Wu, P.C. Leung, Transforming growth factor-beta1 up-regulates connexin43 expression in human granulosa cells, Hum. Reprod. 30 (9) (2015) 2190–2201.
[3] Y. Fang, H.M. Chang, J.C. Cheng, C. Klausen, P.C. Leung, X. Yang, Transforming growth factor-β1 increases lysyl oxidase expression by downregulating MIR29A in human granulosa lutein cells, Reproduction 152 (3) (2016) 205–213.
[4] J.C. Cheng, H.M. Chang, L. Fang, Y.P. Sun, P.C. Leung, TGF-β1 up-regulates

connective tissue growth factor expression in human granulosa cells through Smad and ERK1/2 signaling pathways, PLoS One 10 (5) (2015) e0126532.
[5] H. Li, H.M. Chang, Z. Shi, P.C.K. Leung, SNAIL mediates TGF-β1-induced down-regulation of Pentraxin 3 expression in human granulosa cells, Endocrinology 159 (4) (2018) 1644–1657.
[6] Q. Li, J.E. Agno, M.A. Edson, A.K. Nagaraja, T. Nagashima, M.M. Matzuk, Transforming growth factor β receptor type 1 is essential for female reproductive tract integrity and function, PLoS Genet. 7 (10) (2011) e1002320.
[7] V.J. Young, S.F. Ahmad, W.C. Duncan, A.W. Horne, The role of TGF-β in the pathophysiology of peritoneal endometriosis, Hum. Reprod. Update 23 (5) (2017) 548–559.
[8] M. Liu, J. Gao, Y. Zhang, P. Li, H. Wang, X. Ren, C. Li, Serum levels of TSP-1, NF-kappaB and TGF-beta1 in polycystic ovarian syndrome (PCOS) patients in northern China suggest PCOS is associated with chronic inflammation, Clin. Endocrinol. (Oxf) 83 (6) (2015) 913–922.
[9] D.L. Russell, R.L. Robker, Molecular mechanisms of ovulation: co-ordination through the cumulus complex, Hum. Reprod. Update 13 (3) (2007) 289–312.
[10] A. Salustri, M. Yanagishita, C.B. Underhill, T.C. Laurent, V.C. Hascall, Localization and synthesis of hyaluronic acid in the cumulus cells and mural granulosa cells of the preovulatory follicle, Dev. Biol. 151 (2) (1992) 541–551.
[11] R.J. Rodgers, H.F. Irving-Rodgers, Formation of the ovarian follicular antrum and follicular Fluid1, Biol. Reprod. 82 (6) (2010) 1021–1029.
[12] R.J. Rodgers, H.F. Irving-Rodgers, D.L. Russell, Extracellular matrix of the developing ovarian follicle, Reproduction 126 (4) (2003) 415–424.
[13] F. Husseini-Akram, S. Haroun, S. Altmäe, L. Skjöldebrand-Sparre, H. Åkerud, I.S. Poromaa, B.-M. Landgren, A. Stavreus-Evers, Hyaluronan–binding protein 2 (HABP2) gene variation in women with recurrent miscarriage, BMC Womens Health 18 (1) (2018) 2143.
[14] L. Rienzi, G. Vajta, F. Ubaldi, Predictive value of oocyte morphology in human IVF: a systematic review of the literature, Hum. Reprod. Update 17 (1) (2011) 34–45.
[15] F.J. Diaz, K. Wigglesworth, J.J. Eppig, Oocytes determine cumulus cell lineage in mouse ovarian follicles, J. Cell Sci. 120 (Pt 8) (2007) 1330–1340.
[16] R. Stern, M.J. Jedrzejas, Hyaluronidases: their genomics, structures, and mechanisms of action, Chem. Rev. 106 (3) (2006) 818–839.
[17] N. Itano, K. Kimata, Mammalian hyaluronan synthases, IUBMB Life 54 (4) (2002) 195–199.
[18] A.E. Stock, N. Bouchard, K. Brown, A.P. Spicer, C.B. Underhill, M. Doré, J. Sirois, Induction of hyaluronan synthase 2 by human chorionic gonadotropin in mural granulosa cells of equine preovulatory follicles, Endocrinology 143 (11) (2002) 4375–4384.
[19] E. Tirone, C. D'Alessandris, V.C. Hascall, G. Siracusa, A. Salustri, Hyaluronan synthesis by mouse cumulus cells is regulated by interactions between follicle-stimulating hormone (or epidermal growth factor) and a soluble oocyte factor (or transforming growth factor beta1), J. Biol. Chem. 272 (8) (1997) 4787–4794.
[20] J.P. Pienimaki, K. Rilla, C. Fulop, R.K. Sironen, S. Karvinen, S. Pasonen, M.J. Lammi, R. Tammi, V.C. Hascall, M.I. Tammi, Epidermal growth factor activates hyaluronan synthase 2 in epidermal keratinocytes and increases pericellular and intracellular hyaluronan, J. Biol. Chem. 276 (23) (2001) 20428–20435.
[21] T. Ito, J.D. Williams, D. Fraser, A.O. Phillips, Hyaluronan attenuates transforming growth factor-beta1-mediated signaling in renal proximal tubular epithelial cells, Am. J. Pathol. 164 (6) (2004) 1979–1988.
[22] T. Ito, J.D. Williams, D.J. Fraser, A.O. Phillips, Hyaluronan regulates transforming growth factor-beta1 receptor compartmentalization, J. Biol. Chem. 279 (24) (2004) 25326–25332.
[23] T. Ito, J.D. Williams, S. Al-Assaf, G.O. Phillips, A.O. Phillips, Hyaluronan and proximal tubular cell migration, Kidney Int. 65 (3) (2004) 823–833.
[24] H. Zhang, S. Tian, C. Klausen, H. Zhu, R. Liu, P.C. Leung, Differential activation of noncanonical SMAD2/SMAD3 signaling by bone morphogenetic proteins causes disproportionate induction of hyaluronan production in immortalized human granulosa cells, Mol. Cell. Endocrinol. 428 (2016) 17–27.
[25] J.A. Elvin, A.T. Clark, P. Wang, N.M. Wolfman, M.M. Matzuk, Paracrine actions of growth differentiation factor-9 in the mammalian ovary, Mol. Endocrinol. 13 (6) (1999) 1035–1048.
[26] H.M. Chang, J. Qiao, P.C. Leung, Oocyte-somatic cell interactions in the human ovary-novel role of bone morphogenetic proteins and growth differentiation factors, Hum. Reprod. Update 23 (1) (2016) 1–18.
[27] H.M. Chang, L. Fang, J.C. Cheng, E.L. Taylor, Y.P. Sun, P.C. Leung, Effects of growth differentiation factor 8 on steroidogenesis in human granulosa-lutein cells, Fertil. Steril. 105 (2) (2016) 520–528.
[28] B.L. Lie, E. Leung, P.C. Leung, N. Auersperg, Long-term growth and steroidogenic potential of human granulosa-lutein cells immortalized with SV40 large T antigen, Mol. Cell. Endocrinol. 120 (2) (1996) 169–176.
[29] L. Fang, H.M. Chang, J.C. Cheng, P.C. Leung, Y.P. Sun, TGF-beta1 downregulates StAR expression and decreases progesterone production through Smad3 and ERK1/2 signaling pathways in human granulosa cells, J. Clin. Endocrinol. Metab. 99 (11) (2014) E2234–E2243.
[30] L. Fang, H.M. Chang, J.C. Cheng, P.C. Leung, Y.P. Sun, TGF-beta1 induces COX-2 expression and PGE2 production in human granulosa cells through Smad signaling pathways, J. Clin. Endocrinol. Metab. 99 (7) (2014) E1217–E1226.
[31] H.J. Zhu, A.W. Burgess, Regulation of transforming growth factor-beta signaling, Mol. Cell Biol. Res. Commun. 4 (6) (2001) 321–330.
[32] T. Huang, A.P. Hinck, Production, isolation, and structural analysis of ligands and receptors of the TGF-beta superfamily, Methods Mol. Biol. (Clifton, N.J.) 1344 (2016) 63–92.
[33] P.G. Knight, C. Glister, TGF-beta superfamily members and ovarian follicle development, Reproduction 132 (2) (2006) 191–206.
[34] A. van Caam, W. Madej, A. Garcia de Vinuesa, M.J. Goumans, P. Ten Dijke, E. Blaney Davidson, P. van der Kraan, TGFbeta1-induced SMAD2/3 and SMAD1/5 phosphorylation are both ALK5-kinase-dependent in primary chondrocytes and mediated by TAK1 kinase activity, Arthritis Res. Ther. 19 (1) (2017) 112.
[35] J. Hao, J.N. Ho, J.A. Lewis, K.A. Karim, R.N. Daniels, P.R. Gentry, C.R. Hopkins, C.W. Lindsley, C.C. Hong, In vivo structure-activity relationship study of dorsomorphin analogues identifies selective VEGF and BMP inhibitors, ACS Chem. Biol. 5 (2) (2010) 245–253.
[36] R. Derynck, Y.E. Zhang, Smad-dependent and Smad-independent pathways in TGF-beta family signalling, Nature 425 (6958) (2003) 577–584.
[37] J.C. Cheng, Y. Yi, H.M. Chang, P.C.K. Leung, TGF-β1 up-regulates cadherin-11 expression through snail: a potential mechanism for human trophoblast cell differentiation, Cell. Signal. 43 (2018) 55–61.
[38] M.A. Nieto, The snail superfamily of zinc-finger transcription factors, Nat. Rev. Mol. Cell Biol. 3 (3) (2002) 155–166.
[39] S.J. Wang, L.Y. Bourguignon, Hyaluronan and the interaction between CD44 and epidermal growth factor receptor in oncogenic signaling and chemotherapy resistance in head and neck cancer, Arch. Otolaryngol. Head Neck Surg. 132 (7) (2006) 771–778.
[40] A. Salustri, S. Ulisse, M. Yanagishita, V.C. Hascall, Hyaluronic acid synthesis by mural granulosa cells and cumulus cells in vitro is selectively stimulated by a factor produced by oocytes and by transforming growth factor-beta, J. Biol. Chem. 265 (32) (1990) 19517–19523.
[41] M. Schoenfelder, R. Einspanier, Expression of hyaluronan synthases and corresponding hyaluronan receptors is differentially regulated during oocyte maturation in cattle, Biol. Reprod. 69 (1) (2003) 269–277.
[42] E. Nagyova, Regulation of cumulus expansion and hyaluronan synthesis in porcine oocyte-cumulus complexes during in vitro maturation, Endocr. Regul. 46 (4) (2012) 225–235.
[43] C. Guo, X. Meng, J. Bai, C. Chen, T. Liu, S. Liu, C. Zhang, W.P. Li, Expression and localization of transcription factors SNAIL and SLUG in mouse ovaries and pre-implantation embryos, Cell Tissue Res. 358 (2) (2014) 585–595.
[44] V. Mauhin, Y. Lutz, C. Dennefeld, A. Alberga, Definition of the DNA-binding site repertoire for the Drosophila transcription factor SNAIL, Nucleic Acids Res. 21 (17) (1993) 3951–3957.
[45] J. Wu, X.J. Zhou, X. Sun, T.S. Xia, X.X. Li, L. Shi, L. Zhu, W.B. Zhou, J.F. Wei, Q. Ding, RBM38 is involved in TGF-beta-induced epithelial-to-mesenchymal transition by stabilising zonula occludens-1 mRNA in breast cancer, Br. J. Cancer 117 (5) (2017) 675–684.