Biochemical and Biophysical Research Communications 443 (2014) 537–542

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

FOXL2-induced follistatin attenuates activin A-stimulated cell proliferation in human granulosa cell tumors

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ARTICLE INFO

Article history:
Received 21 November 2013
Available online 8 December 2013

Keywords:
Activin
Follistatin
FOXL2
Human granulosa cell tumors

ABSTRACT

Human granulosa cell tumors (GCTs) are rare, and their etiology remains largely unknown. Recently, the *FOXL2* 402C > G (C134W) mutation was found to be specifically expressed in human adult-type GCTs; however, its function in the development of human GCTs is not fully understood. Activins are members of the transforming growth factor-beta superfamily, which has been shown to stimulate normal granulosa cell proliferation; however, little is known regarding the function of activins in human GCTs. In this study, we examined the effect of activin A on cell proliferation in the human GCT-derived cell line KGN. We show that activin A treatment stimulates KGN cell proliferation. Treatment with the activin type I receptor inhibitor SB431542 blocks activin A-stimulated cell proliferation. In addition, our results show that cyclin D2 is induced by treatment with activin A and is involved in activin A-stimulated cell proliferation. Moreover, the activation of Smad signaling is required for activin A-induced cyclin D2 expression. Finally, we show that the overexpression of the wild-type FOXL2 but not the C134W mutant FOXL2 induced follistatin production. Treatment with exogenous follistatin blocks activin A-stimulated cell proliferation, and the overexpression of wild-type FOXL2 attenuates activin A-stimulated cell proliferation. These results suggest that FOXL2 may act as a tumor suppressor in human adult-type GCTs by inducing follistatin expression, which subsequently inhibits activin-stimulated cell proliferation.

1. Introduction

Activins and inhibins belong to the transforming growth factor-beta (TGF-β) superfamily and are originally identified based on their ability to stimulate and inhibit pituitary FSH secretion, respectively [1]. Activins are produced in many tissues, including the ovary, where they regulate many important biological functions through autocrine/paracrine actions. Activins are disulfide-linked homodimers of β subunits, and the primary isoforms of activin are activin A (βA βA), activin AB (βA βB) and activin B (βB βB) [1]. The effects of activin on normal granulosa cell proliferation have been described by few studies. Exogenous treatment with recombinant activin A has been shown to increase DNA synthesis in rat granulosa cells [2]. Studies with human granulosa cells show that activin A stimulates cell proliferation *in vitro* [3].

Granulosa cell tumors (GCTs) are rare and account for approximately 2–5% of all ovarian cancers, which are the most common type of ovarian sex cord-stromal tumor [4]. To date, the pathogenesis of GCTs is poorly understood, and there is no effective treatment beyond primary surgery. The mRNA and protein expression patterns of inhibin α, βA and βB subunits have been identified in human GCTs by RT-PCR and immunohistochemistry, respectively [5,6]. It has been shown that serum levels of inhibin in women with GCTs are significantly elevated, and measurements of serum levels of inhibin can be used as a marker for early detection of the disease and monitoring its recurrence [7]. Mice deficient in the inhibin α subunit, which only results in a decrease of inhibins without causing a decrease of activins, develop gonadal stromal tumors [8]. In addition, treatment with activin A stimulates gonadal stromal tumor cell proliferation [9]. However, the effect of activin A on the formation and progression of human GCTs remains unclear.

FOXL2 is a single-exon gene encoding a member of the forkhead/winged-helix family of transcription factors, which is an early marker of ovarian differentiation [10,11]. FOXL2 is expressed in granulosa cells and plays a critical role in granulosa cell differentiation and folliculogenesis [12]. Recently, the *FOXL2* gene 402C > G (C134W) mutation has been reported to be specifically present in human adult-type GCTs (97%) [13]. These results indicate that mutation of the *FOXL2* gene may be involved in the tumorigenesis of GCTs. Follistatin is highly expressed in granulosa cells and can bind to activin with high affinity. Binding of follistatin to activin blocks the majority of activin effects by preventing activin from binding to the activin receptors [14,15]. It has been shown that

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http://dx.doi.org/10.1016/j.bbrc.2013.12.010

FOXL2 is required for follistatin expression [16]. However, whether FOXL2 regulates follistatin expression in human GCTs remains unknown.

KGN is a cell line derived from human GCTs and has been shown to harbor the somatic *FOXL2* gene 402C > G mutation in the heterozygous state [17]. In the present study, we first examined the effect of activin A on KGN cell proliferation. Our results show that treatment with recombinant human activin A stimulates KGN cell proliferation by up-regulating cyclin D2 through a Smad-dependent pathway. In addition, overexpression of wild-type FOXL2 but not the C134W mutant up-regulates follistatin mRNA levels and induces its production. Moreover, treatment with exogenous recombinant follistatin abolishes activin A-stimulated cell proliferation. Overexpression of wild-type FOXL2 attenuates activin A-stimulated cell proliferation. These results indicate that in human GCTs, FOXL2 may act as a tumor suppressor by up-regulating follistatin-mediated inhibition of activin A-stimulated cell proliferation.

2. Materials and methods

2.1. Cell culture

The human granulosa-like tumor cell line KGN (stocked in the RIKEN CELL Bank) was kindly provided by Dr. Toshihiko Yanase (Department of Medicine and Bioregulatory Science, Kyushu University, Japan). This cell line was derived from a patient with invasive ovarian granulosa cell carcinoma and retains the physiological characteristics of normal granulosa cells, including the expression of functional FSH receptor and the expression of aromatase [18]. KGN cells were grown in DMEM/F12 medium (Sigma–Aldrich, Oakville, ON) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT). The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.2. Antibodies and reagents

Polyclonal anti-Smad4, anti-phospho-Smad2, monoclonal anti-Smad2, anti-phospho-Smad3, anti-Smad3 and anti-cyclin D2 antibodies were obtained from Cell Signaling (Danvers, MA). A monoclonal anti-α-tubulin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Bio-Rad Laboratories (Hercules, CA). The recombinant human activin A was obtained from R&D Systems (Minneapolis, MN). The recombinant human follistatin was obtained from Peprotech (Rocky Hill, NJ).

2.3. Plasmid constructs and FOXL2 overexpression

The pcDNA3 expression vector encoding the full-length wild-type human FOXL2 was kindly provided by Dr. Aaron Hsueh (Department of Obstetrics and Gynecology, Stanford University) [19]. The generation of *FOXL2* gene 402C > G (C134W) construct and FOXL2 overexpression were the same as described in our previous study [20].

2.4. Small interfering RNA (siRNA) transfection

To knock down endogenous cyclin D2 and Smad4, the cells were transfected with 50 nM ON-TARGET*plus* SMART*pool* siRNA targeting cyclin D2 and Smad4 (Dharmacon, Lafayette, CO) using Lipofectamine RNAiMAX (Invitrogen, Life Technologies). The siCONTROL NON-TARGETING *pool* siRNA (Dharmacon) was used as the transfection control.

2.5. Western blot analysis

Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes. Following blocking with Tris-buffered saline containing 5% non-fat dry milk for 1 h, the membranes were incubated overnight at 4 °C with primary antibodies followed by incubation with HRP-conjugated secondary antibodies. The immunoreactive bands were detected with enhanced chemiluminescent substrate. The membranes were stripped with stripping buffer at 50 °C for 30 min and reprobed with anti-α-tubulin as a loading control.

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

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed with 3 μg RNA, random primers and M-MLV reverse transcriptase (Promega, Madison, WI). The primers used for the SYBR green reverse transcription-qPCR (RT-qPCR) were as follows: FOXL2, 5′-CAT GTT CGA GAA GGG CAA CT-3′ (sense) and 5′-AGG AAG CCA GAC TGC AGG TA-3′ (antisense); follistatin, 5′-TGC TCT GCC AGT TCA TGG-3′ (sense) and 5′-CTT GAC GGA GCC AGC AGT-3′ (antisense); and GAPDH, 5′-GAG TCA ACG GAT TTG GTC GT-3′ (sense) and 5′-GAC AAG CTT CCC GTT CTC AG-3′ (antisense). RT-qPCR was performed on an Applied Biosystems 7300 Real-Time PCR System equipped with a 96-well optical reaction plate. Relative quantification of the mRNA levels was performed using the comparative Ct method with GAPDH as the reference gene using the formula $2^{-\Delta\Delta Ct}$.

2.7. Enzyme-linked immunosorbent assay (ELISA)

A human follistatin ELISA was used according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). Briefly, the cells were transfected with empty vector or vector encoding wild-type FOXL2 or FOXL2 C134W mutant for 48 h. The culture media were collected, and ELISA was used to measure the follistatin levels in the culture media. The follistatin levels were normalized to the protein concentrations from the cell lysates.

2.8. Statistical analysis

The results are presented as the mean ± SEM of at least three independent experiments. Multiple comparisons were analyzed by one-way ANOVA followed by Tukey's multiple comparison test using the PRISM software. Significant differences were defined as $p < 0.05$.

3. Results

3.1. Activin A stimulates KGN cell proliferation

Because of the mitogenic effect of activin A in normal human granulosa cells [3], we first tested the effect of activin A on the proliferation of the human GCT-derived cell line KGN. KGN cells were treated with or without different concentrations of recombinant human activin A (1, 10, 25, 50 and 100 ng/ml) every 24 h for 72 h. The effects of activin A on cell proliferation were determined by MTT assay. As shown in Fig. 1A, treatment with 50 and 100 ng/ml activin A significantly stimulated cell proliferation. Moreover, the effects of activin A on KGN cell proliferation were further confirmed by trypan blue exclusion assay (Fig. 1B). To further evaluate the pro-proliferative effect of activin A on KGN cells, a selective inhibitor for activin type I receptor, SB431542, was used [21]. As shown in Fig. 1C, treatment with SB431542 did not significantly affect the basal proliferation rate. However, activin A-stimulated cell proliferation was blocked by SB431542.

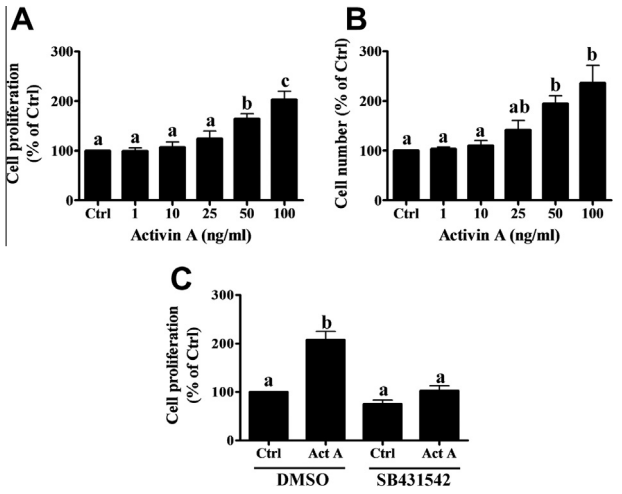

Fig. 1. Activin A stimulates KGN cell proliferation. (A) and (B) The cells were treated with increasing concentrations of activin A (1, 10, 25, 50 and 100 ng/ml) every 24 h for 72 h. Cell proliferation was evaluated by MTT assay (A) and trypan blue exclusion assay (B). (C) The cells were treated with 100 ng/ml activin A (Act A) every 24 h for 72 h in combination with vehicle control (DMSO) and SB431542 (10 μM). Cell proliferation was evaluated by MTT assay. The results are expressed as the mean ± SEM of at least three independent experiments. The values without a common letter are significantly different ($p < 0.05$).

3.2. Cyclin D2 is required for activin A-stimulated cell proliferation

In rat primary granulosa cells, treatment with activin A up-regulates cyclin D2 but does not affect the levels of cyclin D1 and cyclin D3 [22]. Therefore, we determined whether cyclin D2 was involved in activin A-stimulated KGN cell proliferation. Treatment with 100 ng/ml activin A for 24 and 48 h significantly up-regulated cyclin D2 protein levels in KGN cells (Fig. 2A). The activin A-up-regulated cyclin D2 protein levels were blocked by treating the cells with SB431542 (Fig. 2B). To further confirm whether cyclin D2 was required for activin A-stimulated KGN cell proliferation, an siRNA-mediated depletion approach was used to knock-down the endogenous cyclin D2. As shown in Fig. 2C, cyclin D2 siRNA not only down-regulated the basal levels of cyclin D2 but also abolished cyclin D2 expression induced by activin A treatment. The MTT assay results showed that activin A-stimulated KGN cell proliferation was attenuated by knockdown of cyclin D2 (Fig. 2D). These results indicate that cyclin D2 is involved in activin A-stimulated KGN cell proliferation.

3.3. Smad activation signaling is required for activin A-induced cyclin D2 expression

It is well known that both TGF-β and activins activate Smad2 and Smad3 [23]. To determine whether activin A activates Smad2

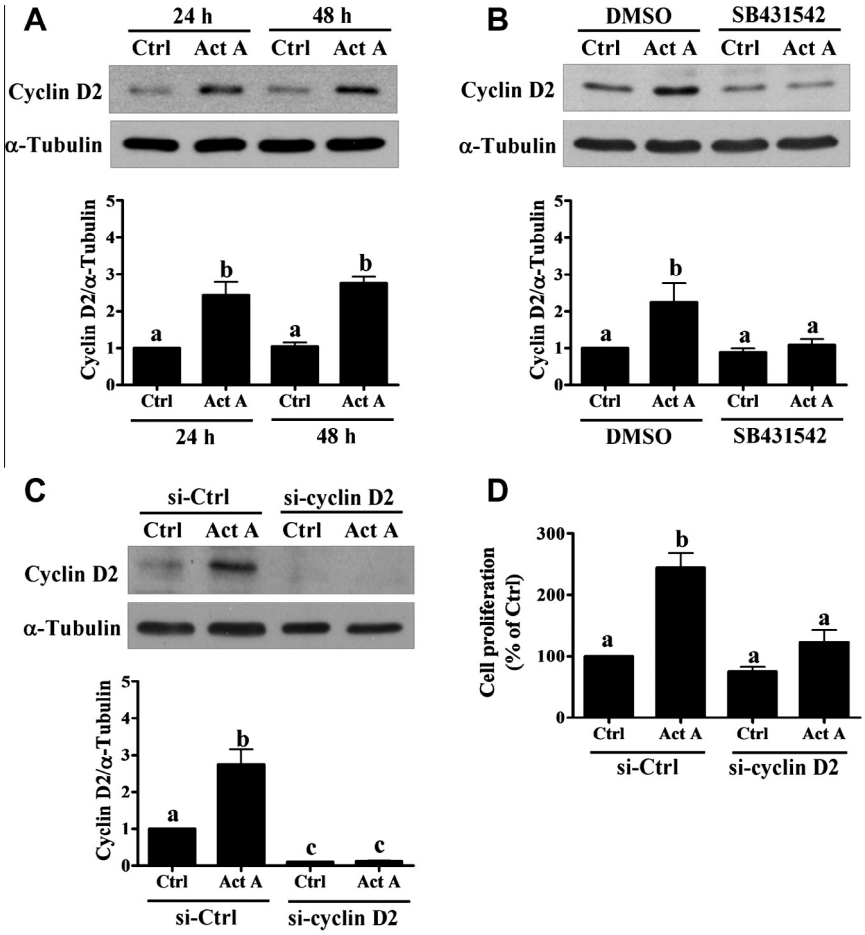

Fig. 2. Cyclin D2 is required for activin A-stimulated KGN cell proliferation. (A) The cells were treated with 100 ng/ml activin A (Act A) for 24 and 48 h. The protein levels of cyclin D2 were evaluated by Western blot. (B) The cells were treated with 100 ng/ml activin A (Act A) every 24 h for 48 h in combination with vehicle control (DMSO) and SB431542 (10 M). The protein levels of cyclin D2 were evaluated by Western blot. (C) The cells were transfected with 50 nM control siRNA (si-Ctrl) or cyclin D2 siRNA (si-cyclin D2) for 48 h and then treated with 100 ng/ml activin A (Act A) every 24 h for 48 h. The protein levels of cyclin D2 were evaluated by Western blot. (D) The cells were transfected with 50 nM control siRNA (si-Ctrl) or cyclin D2 siRNA (si-cyclin D2) for 48 h and then treated with 100 ng/ml activin A (Act A) every 24 h for 72 h. Cell proliferation was determined by MTT assay. The results are expressed as the mean ± SEM of at least three independent experiments. The values without a common letter are significantly different ($p < 0.05$).

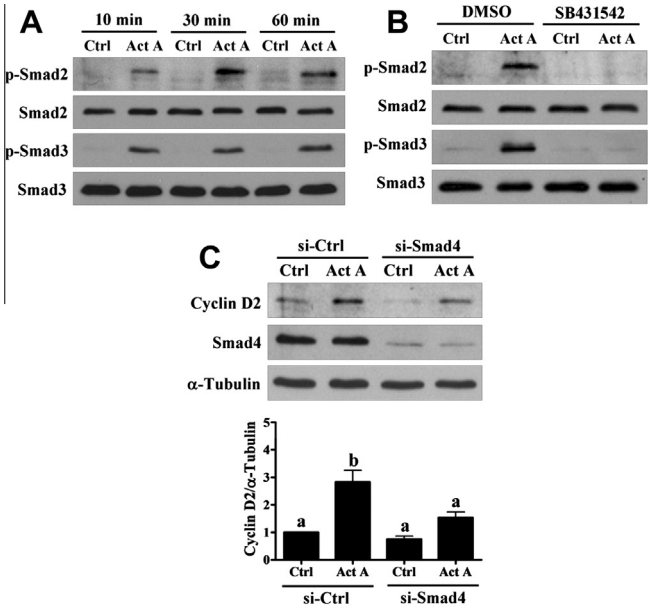

Fig. 3. Activation of Smad signaling is required for activin A-induced cyclin D2 expression in KGN cells. (A) The cells were treated with 100 ng/ml activin A (Act A) for 10, 30 and 60 min. The phosphorylation levels of Smad2 and Smad3 were evaluated by Western blot. (B) The cells were treated with 100 ng/ml activin A (Act A) in combination with vehicle control (DMSO) and SB431542 (10 μM) for 60 min. The phosphorylation levels of Smad2 and Smad3 were evaluated by Western blot. (C) The cells were transfected with 50 nM control siRNA (si-Ctrl) or Smad4 siRNA (si-Smad4) for 48 h and then treated with 100 ng/ml activin A (Act A) every 24 h for 48 h. The protein levels of cyclin D2 were evaluated by Western blot. The results are expressed as the mean ± SEM of at least three independent experiments. The values without a common letter were significantly different ($p < 0.05$).

and Smad3 in human GCTs, KGN cells were treated with 100 ng/ml activin A for different periods of time. Western blot analysis showed that treatment with activin A induced the phosphorylation of Smad2 and Smad3 (Fig. 3A). Moreover, the activin A-induced Samd2 and Smad3 activations were blocked by treating the cells with SB431542 (Fig. 3B). To determine whether Smad signaling was involved in activin A-up-regulated cyclin D2 expression, an siRNA-mediated depletion approach was used to knockdown the endogenous common Smad, Smad4. Western blot analysis showed that Smad4 siRNA significantly knocked down the endogenous Smad4 protein levels. Moreover, activin A-up-regulated cyclin D2 protein levels were attenuated by knockdown of Smad4 (Fig. 3C).

3.4. Wild-type FOXL2-induced follistatin inhibits activin A-stimulated KGN cell proliferation

Follistatin binds to activin to inhibit its functions; however, whether FOXL2 regulates follistatin in human GCT-derived KGN cells remains unclear. Therefore, KGN cells were transiently transfected with FOXL2 to determine the effects of FOXL2 on follistatin expression. The overexpression of wild-type and C134W mutant FOXL2 was confirmed by RT-qPCR (Fig. 4A). The overexpression of wild-type FOXL2 up-regulated follistatin mRNA levels; however, the overexpression of C134W mutant FOXL2 did not significantly affect the follistatin mRNA levels (Fig. 4B). Similarly, the ELISA results showed that the overexpression of wild-type but not the C134W mutant FOXL2 increased follistatin production in KGN cells (Fig. 4C). In addition, treatment with exogenous recombinant human follistatin attenuated activin A-stimulated KGN cell proliferation (Fig. 4D). Moreover, the overexpression of wild-type FOXL2 or C134W FOXL2 did not significantly affect the basal cell proliferation, which was consistent with our recent previous study (Fig. 4E) [20]. Interestingly, only the overexpression of wild-type FOXL2, but not C134W FOXL2, attenuated activin A-stimulated KGN cell proliferation (Fig. 4E). Overall, these results indicate that the overexpression of wild-type FOXL2 induced follistatin production, which in turn blocked the pro-proliferative effect of activin A on KGN cells.

4. Discussion

To date, the pathogenesis of human GCTs and the cellular pathways that regulate cell proliferation and survival in GCTs remain poorly understood [24]. It is well known that activin regulates many physiological functions, particularly in the reproductive system [25]. In addition, activin has been shown to be involved in the regulation of the progression of numerous types of human cancers [26]. Interestingly, activin A is pleiotropic; therefore, it is either tumorigenic or anti-tumorigenic, depending on the cell type [27]. In normal human granulosa cells, treatment with activin A induces cell proliferation [3]. In human epithelial ovarian cancer cells, activin A increases cell proliferation and Matrigel invasion [28]. Moreover, epithelial ovarian cancer cells that respond to activin A are more aggressive in xenograft models [28]. It has been shown that inhibin A subunit-deficient mice develop GCTs [8]. The serum levels of activin A are more than 10-fold higher in homozygous inhibin-deficient mice than wild-type or heterozygous mice [29]. Interestingly, the overexpression of follistatin or inhibition of activin signaling reduces tumor progression in inhibin-deficient mice [30–32]. These animal studies indicate that activin may be involved in the progression of GCTs. However, whether activin A is directly involved in the tumorigenesis of GCTs in humans remains unclear. In the present study, our results demonstrated that treatment with activin A induced human GCT-derived KGN cell proliferation. These results suggest a tumorigenic role of activin A in human GCTs, which is similar to the results obtained from animal model studies.

In mouse alphaT3-1 pituitary gonadotroph cells and the mouse GCT-derived cell line KK1, FOXL2 has been shown to increase follistatin promoter activity and up-regulate follistatin mRNA levels, respectively [16,33]. The FOXL2 C134W mutation is detected in KGN cells, whereas FOXL2 wild-type mRNA and protein expression are not detected in another human GCT-derived cell line, COV434 [17,34]. Therefore, it has been postulated that KGN cells are derived from adult GCTs and COV434 cells are derived from juvenile GCTs [24]. A recent study showed that overexpression of the wild-type FOXL2 in COV434 cells does not affect follistatin promoter activity, whereas overexpression of the C134W FOXL2 mutant increases follistatin promoter activity [35]. In the present study, our results showed that overexpression of wild-type FOXL2, but not the C134W mutant, increased follistatin mRNA levels and protein production in KGN cells. In addition to the promoter luciferase assay, our results demonstrated, for the first time, that wild-type FOXL2 regulated follistatin in human GCTs. Moreover, our results suggest that FOXL2 may have different roles in the regulation of follistatin in human adult and juvenile GCTs.

In summary, our studies demonstrate that activin A exerts pro-proliferative effect on human GCTs-derived cells, KGN. The activin A-stimulated cell proliferation is inhibited by follistatin. Moreover, overexpression of wild-type FOXL2 up-regulates follistatin, whereas the mutant C134W FOXL2 does not up-regulate follistatin. These results suggest that FOXL2 may act as a tumor suppressor in human adult-type GCTs by inducing follistatin expression, which in turn inhibits activin-stimulated cell proliferation.

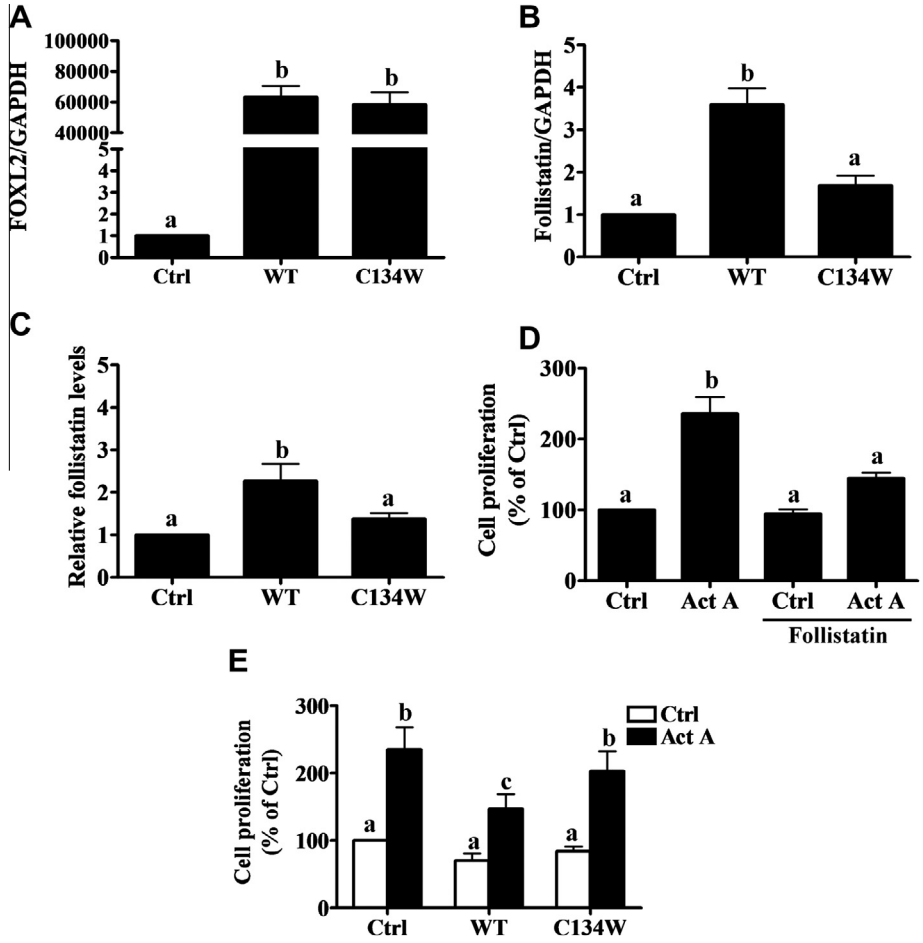

Fig. 4. Overexpression of wild-type but not the C134W mutant FOXL2 induces follistatin expression in KGN cells. (A) and (B), The cells were transfected with empty pcDNA3 plasmid (Ctrl), wild-type FOXL2 (WT) or C134W mutant FOXL2 (C134W) for 24 h. The mRNA levels of FOXL2 (A) and follistatin (B) were evaluated by RT-qPCR. (C) The cells were transfected with empty pcDNA3 plasmid (Ctrl), wild-type FOXL2 (WT) or C134W mutant FOXL2 (C134W) for 48 h. The protein levels of follistatin in the culture medium were determined by ELISA. (D) The cells were treated with 100 ng/ml activin A (Act A) in combination with 500 ng/ml follistatin every 24 h for 72 h. Cell proliferation was examined by MTT assay. (E) Cells were transfected with empty pcDNA3 plasmid (Ctrl), wild-type FOXL2 (WT) or C134W mutant FOXL2 (C134W) for 24 h and then treated with treated with 100 ng/ml activin A (Act A) every 24 h for 72 h. Cell proliferation was examined by MTT assay. The results are expressed as the mean ± SEM of at least three independent experiments. The values without a common letter were significantly different ($p < 0.05$).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We thank Dr. Toshihiko Yanase for providing the KGN cells and Dr. Aaron Hsueh for providing the wild-type human FOXL2 plasmid. This work was supported by Grants from the Canadian Institutes of Health Research to P.C.K.L.

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