TGFB1 represses the expression of SF1 and LRH1 to inhibit E₂ production in rat LCs

Qianqian Yang^{1,2,*}, Binfang Ma^{1,*}, Huilian Qiao¹, He Ma¹, Yuhang Dong¹, Liang Cao², Jing Ma² and Zhen Li¹

¹Department of Histology and Embryology, The Fourth Military Medical University, Xi'an, Shaanxi, People's Republic of China and ²Department of Traditional Chinese Medicine, Xijing Hospital, The Fourth Military Medical University, Xi'an, Shaanxi, People's Republic of China

Correspondence should be addressed to J Ma or Z Li; Email: jingma@fmmu.edu.cn or lizhenhe@fmmu.edu.cn

*(Q Yang and B Ma contributed equally to this work)

Abstract

Leydig cells (LCs) in the adult testis have been identified as the major sites of oestrogen production, which is crucial for mammalian germ cell differentiation. Our previous work showed that transforming growth factor beta 1 (TGFB1) inhibits estradiol (E₂) secretion via down-regulating *Cyp19* gene expression in mature rat LCs. However, the mechanism remains unclear. In the present study, the effects of TGFB1 on the expression levels of steroidogenic factor 1 (SF1), liver receptor homolog 1 (LRH1), cAMP response element-binding protein (CREB) and cAMP responsive element modulator (CREM) were evaluated both in primary cultured LCs and in rat testis. The involvement of TGFB1 signalling in the regulation of SF1 and LRH1 expression was then validated by applying the inhibitor of the TGFB type 1 receptor (TGFBRI) SB431542. Moreover, the expression of CYP19 in testicular LCs was investigated and the production of E₂ in testicular interstitial fluid (TIF) was measured. The results showed that TGFB1 especially down-regulated the expression levels of SF1 and LRH1 both in primary cultured LCs and in rat testis. The down-regulations of TGFB1 in the production of E₂ in TIF and the expression of CYP19 in testicular LCs were also observed *in vivo*. These inhibitory effects could be reversed by TGFBRI inhibitor SB431542. Our findings suggest that TGFB1 may act through the canonical signalling pathway involving ALK5 to restrain SF1 and LRH1 accumulation and eventually attenuate *Cyp19* transcription and oestrogen production in LCs.

Reproduction (2017) **153** 621–629

Introduction

It has become increasingly clear that oestrogens are closely related to the maintenance of spermatogenesis and normal testicular development (Carreau *et al.* 1999). The demonstration that oestrogen receptor alpha knockout mouse results in a disruption of sperm morphology (Hess 2003), inactive sperm and testicular atrophy (Hamilton *et al.* 2014), together with the fact that aromatase knockout mouse displays testicular dysfunction (Hess 2003), has reinforced the idea that oestrogens play a key role in the male reproductive system.

Leydig cells (LCs) in the adult testis have long been considered as the main site of conversion of androgens into oestrogens, although Sertoli cells (SCs) of prepuberty and germ cells can generate oestrogens as well (Lambard *et al.* 2005). Estradiol (E₂) is converted from testosterone (T) in LCs, during which the catalysis is involved by microsomal enzymatic complex termed cytochrome P450 aromatase (P450arom). The P450arom

is encoded by a unique gene called aromatase cytochrome P450 gene (*Cyp19*), a single-copy gene, which belongs to cytochrome P450 gene superfamily (O'Donnell *et al.* 2001).

The regulation of aromatase in testis is influenced by many autocrine/paracrine factors such as tumour necrosis factor alpha (TNFα), insulin-like growth factor (IGF) and transforming growth factor beta 1 (TGFB1) (Carreau *et al.* 2004, Bjelic *et al.* 2014). Particularly, TGFB1 has an essential impact on testicular functions and spermatogenesis (Ozguden-Akkoc & Ozer 2012). For instance, TGFB1 has been demonstrated to be a potent inhibitor of LCs differentiating function (Le Roy *et al.* 1999) and to inhibit LH/HCG stimulated steroidogenesis in rat LCs (Lin *et al.* 1987). These studies are in accordance with our previous work, which documented that TGFB1 inhibits E₂ secretion via down-regulating *Cyp19* gene expression in mature rat LCs (Liu *et al.* 2013). Despite the extensive studies on TGFB1

regulation of LC function, little is known regarding the mechanism by which TGFB1 exerts its inhibitory effect on *Cyp19* gene expression in LCs.

The signalling of TGFB1 occurs at the cell surface via a heterogenic complex of specific type 1 and 2 serine/threonine kinase receptors. In general, upon TGFB1 binding, the type 2 receptor (TGFB2) phosphorylates and activates TGFB1, also termed ALK5. ALK5 then propagates the signal inside the cell through the phosphorylation of receptor-regulated Smads proteins (Smad2/Smad3). Activated Smad2/Smad3 form a heterotrimeric complex with Smad4, which then translocates to the nucleus, where they activate transcription of target genes (Shi & Massague 2003). The transcription of aromatase occurs through divergent areas of nine distinct tissue-specific promoters that locate in the first exon of the *Cyp19* gene (Prosperi & Robertson 2006). Of note, a promoter proximal to the translation start site, termed promoter II (PII), is the specific one in rat LCs (Lanzino *et al.* 2001) and acts as the main promoter that drives the aromatase gene expression (Carreau *et al.* 2004). Two response elements of PII along with their transcription factors have been verified to induce aromatase transcription in testis (Haverfield *et al.* 2011). The first is steroidogenic factor response element (SFRE) (Pezzi *et al.* 2004), which requires the binding of steroidogenic factor 1 (SF1; also called NR5A1) and liver receptor homolog 1 (LRH1; also named NR5A2) to induce aromatase expression under basal condition (Kato *et al.* 1992, Sierens *et al.* 2010). SF1 and LRH1 are closely related members of orphan nuclear receptor family, which share common DNA binding domain and transactivation properties. Both SF1 and LRH1 have been shown to exist in LCs, indicating a potential to regulate aromatase expression in testis (Sierens *et al.* 2010). The second is cyclic AMP responsive element (CRE) (Young & McPhaul 1998), which binds to CRE-binding protein (CREB) and cyclic AMP (cAMP) responsive element modulator (CREM) following the activation of PKA under FSH and LH stimulated condition (Don & Stelzer 2002, Itman *et al.* 2006). CREB is known as the major mediator of cAMP-induced changes in gene expression and is required for normal testicular function (Chrivia *et al.* 1993). Similarly, the expression of the closely related protein, CREM, also plays an essential role in spermiogenesis (Martianov *et al.* 2010).

To explore the mechanism by which TGFB1 regulated E_2 secretion in mature rat LCs, in the present study the effects of TGFB1 on the expression of the related transcription factors of *Cyp19* gene, namely SF1, LRH1, CREB and CREM, were evaluated in primary LCs and rat testis. The involvement of TGFB1 signalling in SF1 and LRH1 accumulation, CYP19 expression and E_2 production was also investigated.

Materials and methods

Animals

Three-month-old adult male Sprague Dawley (SD) rats (weighing 300 ± 10 g) were purchased from the Laboratory Animal Center of the Fourth Military Medical University. They were maintained under controlled temperature ($25 \pm 2^\circ\text{C}$) and constant photoperiodic conditions (12 h light–12 h darkness) with food and water *ad libitum*. Experimental procedures were approved by the local ethical committee. All surgery was performed under sodium pentobarbital anaesthesia (0.04 – 0.05 mg/g body weight, intraperitoneally (i.p.)).

Isolation and culture of rat LCs

Male SD rats were killed at 3 months of age and the testes were decapsulated under aseptic conditions. LCs were isolated by enzymatic digestion and purified on continuous Percoll gradients (Amersham Pharmacia Biotech) as described previously (Liu *et al.* 2013). Briefly, the testes were incubated for 10–15 min in culture medium containing 0.25 mg/mL collagenase (Type II, Sigma-Aldrich) in a shaking water bath at 34°C . Separated cells were filtered through two layers of nylon mesh (100 mm pore), centrifuged at $800g$ for 10 min and re-suspended in 55% isotonic Percoll. Density gradient centrifugation was carried out at $18,000g$ for 60 min at 4°C . LCs fractions with densities between 1.070 and 1.088 g/mL were collected according to the Percoll density marker beads (Cospheric, Santa Barbara, CA, USA). The cells were washed twice with D-Hank's buffered saline solution and then cultured in a 34°C , 5% CO_2 humid incubator in DMEM-F12 medium with 10% fetal bovine serum (FBS) supplemented with penicillin/streptomycin, and L-glutamate for further studies. The purity of LCs was assessed by histochemical staining of 3β -HSD and the cell viability was determined by trypan blue exclusion. The purity of LCs was 90–95% and viability was above 90%. The cells were cultured with the addition of 10 ng/mL LH (Merck Millipore) according to a previous experiment (Liu *et al.* 2015). LCs were then incubated either with 5 ng/mL TGFB1 (PeproTech Inc., Rocky Hill, NJ, USA) for 20 h according to our previous work (Liu *et al.* 2013) or with 5 ng/mL TGFB1 plus 0.1 μM ALK5 inhibitor SB431542 (Sigma-Aldrich) for 20 h. The inhibitor was added 1 h before TGFB1 treatment.

Western blotting

Western blotting was performed by following a routine protocol. Briefly, LCs were washed with phosphate buffered saline (PBS) and scraped into 200 μL sodium dodecyl sulphate (SDS) electrophoresis sample buffer. Cell lysates were then sonicated (60 Hz, 10 s for 3 times) and heated for 10 min at 95°C . Protein samples were separated by 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and were then transferred to a nitrocellulose membrane (NC membrane). The blotting membranes were blocked with 5% w/v non-fat milk and probed with rabbit anti-SF1 (1:800 dilution, Cell Signaling Technology, D1Z2A), rabbit anti-LRH1 (1:800 dilution, Abcam,

ab189876), rabbit anti-CREB (1:1000 dilution, Cell Signaling Technology, 48H2), rabbit anti-CREM (1:1000 dilution, Abcam, ab64832) and rabbit anti-CYP19 (1:400 dilution, Santa Cruz Biotechnology, sc-30086) respectively. After being washed, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:1000 dilution, Sigma-Aldrich, A0545) for 1 h at room temperature. The bound antibodies were visualized by a chemiluminescence system (BIO-RAD Biosciences). The optical densities of the bands were quantified by using Image J software (NIH). GAPDH served as loading control.

Immunofluorescence microscopy analysis of cultured LCs

LCs were fixed with 4% paraformaldehyde for 20 min at room temperature, rinsed with PBS and permeabilized with 0.1% v/v Triton for 15 min. Cells were then incubated with rabbit anti-SF1 (diluted 1:50) and rabbit anti-LRH1 (diluted 1:50), in PBS containing 1% w/v bovine serum albumin (BSA) overnight at 4°C respectively. After three rinses, cells were incubated at room temperature for 1 h with CY3-conjugated donkey anti-rabbit IgG (diluted 1:500, Jackson ImmunoResearch, 126883). Nucleus staining was performed with 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific). Immunofluorescence images were captured using a Nikon E800 microscope with a Spot-2 camera (Nikon).

Double immunofluorescence labelling of testis tissue

Rat testes were fixed by intracardial perfusion with 4% buffered paraformaldehyde for 100 min, embedded in paraffin and processed into 5 µm sections. Antigen retrieval was performed by microwaving at 95°C for 10 min in sodium citrate buffer (pH 6.0). The sections were then incubated with mouse anti-AMHR2 (1:500 dilution, Abcam, ab64762) and rabbit anti-SF1 or rabbit anti-LRH1, followed by the mixed secondary antibodies, Alexa Fluor 488-conjugated donkey anti-mouse IgG (1:500 dilution, Jackson ImmunoResearch, 123589) and CY3-conjugated donkey anti-rabbit IgG (1:500 dilution). Control sections were incubated with isotype-matched mouse IgG1 (Abcam, ab91353), rabbit mAb IgG (Cell Signaling Technology, DA1E) or rabbit polyclonal IgG (Abcam, ab171870) in the same working dilution as the corresponding primary antibodies.

Injection of testis with TGFB1 or TGFB1 plus SB431542

The rats were randomly divided into three groups ($n=3$ for each group). Injection of PBS, TGFB1 or TGFB1 plus SB431542 into rat testes was performed on the basis of the previous depicted method with some modifications (Ogawa *et al.* 1997, Sato *et al.* 2002). Briefly, 200 µL of PBS solution was slowly injected into the rat testicular interstitium with a sharp pipette in a diameter of 50–60 µm. In TGFB1 plus SB431542 group, 100 µL PBS containing 1 µM SB431542 was injected 1 h before TGFB1 (1 µM in 100 µL PBS) supplement. A small amount of trypan blue (0.4%) was added to the solution as the indicator. The testis interstitium filling time is about 2 min. 24 h after

testes injection, the animals were killed for LCs isolation as well as testicular interstitial fluid (TIF) collection.

Estradiol assays

TIF collection was performed as described elsewhere (Stojkov *et al.* 2013). Briefly, testes from control and stimulated groups were quickly removed, decapsulated, and placed on Falcon nylon mesh with 100-micron pores (Corning) in 50 mL plastic tube (one testis per tube). After being centrifuged at 100g for 7 min at room temperature, TIF was collected. The volume of TIF was recorded and stored at –80°C until further analysis of estradiol (Haakensen *et al.* 2011). The estradiol contents were measured using electrochemiluminescence immunoassays (ECLIA) on a Roche cobas e 601 instrument (Roche Diagnostics) using a commercially available Elecsys Estradiol II kit (Roche) by local clinical laboratory, Xijing Hospital, China. The estradiol values are given as picograms per millilitre (pg/mL). The functional sensitivity of the estradiol assay was 12 pg/mL (44 pmol/L) with the intra-assay and inter-assay coefficients of variation of <3.3% and <4.9% respectively. All measurements were repeated at least three times independently, and data were presented as mean ± the standard error of mean (S.E.M.).

Statistical analysis

Results are presented as mean ± S.E.M. from at least three independent experiments. Statistical analysis was performed by using the one-way ANOVA followed by Bonferroni's multiple comparison test and $P < 0.05$ was considered to be statistically significant. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software).

Results

TGFB1 specifically repressed the expression of SF1 and LRH1 in cultured LCs

Western blot analysis was performed to investigate the potential regulation of the *Cyp19* transcription factors by TGFB1 in primary cultured LCs. Treatment with 5 ng/mL TGFB1 for 20 h significantly reduced the protein content of SF1 and LRH1. In contrast, no significant alteration was observed in the expression levels of the other two transcription factors CREB and CREM (Fig. 1A and B). Consistently, the SF1 and LRH1 positive staining in the nuclei of LCs was notably attenuated in the presence of TGFB1 (Fig. 1C). These data together suggest a specific suppressive effect of TGFB1 on the expression of SF1 and LRH1 in rat LCs.

TGFB1 specifically repressed the expression of SF1 and LRH1 in LCs in vivo

To determine whether the specific suppressive effect of TGFB1 on the expression of SF1 and LRH1 could

Figure 1 Effects of TGFβ1 on the expression of *Cyp19* transcription factors in cultured rat LCs. (A) Representative Western blots of SF1, LRH1, CREB and CREM immunoreactivity in LCs treated with or without TGFβ1 at the concentration of 5 ng/mL for 20 h. The GAPDH expression served as the loading control. (B) Densitometric quantification of Western blots expressed as ratios of the control value. Histograms represent the mean $\pm$ S.E.M. of three independent experiments (*** P < 0.001). Significant differences between groups were analyzed with one-way ANOVA. (C) Immunofluorescent staining of SF1 and LRH1 in LCs in the absence or presence of TGFβ1 at the concentration of 5 ng/mL for 20 h. Cell nuclei were counterstained with DAPI. A distinct decline of SF1 and LRH1 immunoreactivity in the nuclei was observed after TGFβ1 treatment. Scale bars = 100 μ m.

take place *in vivo*, 1 μ M TGFβ1 was injected into the interstitium of rat testis under stereoscopic microscope according to the protocol described above. After 24 h, LCs were isolated and the expression of the *Cyp19* transcription factors was investigated by Western blot analysis. In line with the *in vitro* data, administration of TGFβ1 significantly reduced the expression levels of SF1 and LRH1, while no inhibitory effect on the expression of CREB or CREM was detected (Fig. 2A and B). By using anti-AMHR2 antibody, the specific marker of LCs (Mendis-Handagama *et al.* 2006), we next found that SF1 was expressed in the nuclei of both LCs and Sertoli cells, whereas LRH1 was predominantly expressed in LCs nuclei of testicular interstitium (Fig. 2C, left panel). Clear decreases in the immunoreactivity of SF1 and LRH1 in testicular LCs were observed under the treatment of TGFβ1 (Fig. 2C, right panel). The specificity of the primary antibodies was checked by replacing the primary antibodies with isotype IgG (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

Inhibition of ALK5 recovered the expression of SF1 and LRH1 in cultured LCs

To further validate the involvement of TGFβ1 signalling in the negative regulation of SF1 and LRH1 expression, LCs were subjected to TGFβ1 in the absence or presence of SB431542, a potent inhibitor of ALK5. As expected, TGFβ1 treatment resulted in a significant reduction in the expression levels of SF1 and LRH1 protein, which could be remarkably reversed by SB431542 treatment (Fig. 3A and B).

Inhibition of ALK5 recovered the expression of SF1, LRH1 and CYP19 in LCs and the production of E_2 in TIF *in vivo*

Consistent with the above *in vitro* data, the reduction of SF1 and LRH1 induced by TGFβ1 were prevented obviously when the rats were treated with SB431542 before the addition of TGFβ1 compared to the TGFβ1 group (Fig. 4A). Then the involvement of TGFβ1 signalling in the expression of *Cyp19*, the gene

Figure 2 Effects of TGFB1 on the expression of *Cyp19* transcription factors in LCs of rat testis. (A) Representative Western blots of SF1, LRH1, CREB and CREM immunoreactivity in LCs isolated from rats treated with or without TGFB1 at the concentration of 1 μ M for 24 h. The GAPDH expression served as the loading control. (B) Densitometric quantification of Western blots expressed as ratios of the control value. Histograms represent the mean $\pm$ S.E.M. of three independent experiments (*** P < 0.001). Significant differences between groups were analyzed with one-way ANOVA. (C) Double immunofluorescent staining for AMHR2 and SF1 or LRH1 in the interstitium of adult rat testis. The red signal of SF1 and LRH1 (CY3 labelled) was predominantly seen in the nuclei of LCs (green, Alexa 488 labelled). A distinct decrease of SF1 and LRH1 immunoreactivity was observed after TGFB1 treatment. Scale bars = 20 μ m.

Figure 3 Effects of SB431542 on TGFB1-inhibited expression of SF1 and LRH1 in cultured rat LCs. (A) Representative Western blots of SF1 and LRH1 immunoreactivity in LCs treated with or without SB431542 at the concentration of 0.1 μ M 1 h before incubation with TGFB1 for 20 h. The GAPDH expression served as the loading control. (B) Densitometric quantification of Western blots expressed as ratios of the control value. Histograms represent the mean $\pm$ S.E.M. of three independent experiments (** P < 0.01, *** P < 0.001 compared to control group; # P < 0.05, compared to TGFB1 group). Significant differences between groups were analyzed with one-way ANOVA.

Figure 4 Effects of SB431542 on TGFB1-inhibited expression of SF1, LRH1 and CYP19 in LCs and E_2 production in TIF. (A) Representative Western blots of SF1 and LRH1 immunoreactivity in LCs isolated from rats treated with or without SB431542 at the concentration of 1 μ M 1 h before administration with TGFB1 for 24 h. The GAPDH expression served as the loading control (upper panel). Densitometric quantification of Western blots expressed as ratios of the control value. Histograms represent the mean $\pm$ S.E.M. of three independent experiments (** P < 0.01, *** P < 0.001, compared to PBS group; # P < 0.05, ## P < 0.01 compared to TGFB1 group). Significant differences between groups were analyzed with one-way ANOVA (lower panel). (B) Representative Western blot of CYP19 immunoreactivity in LCs isolated from rats. The GAPDH expression served as the loading control (left panel). Densitometric quantification of Western blots expressed as ratios of the control value. Histograms represent the mean $\pm$ S.E.M. from three independent experiments (*** P < 0.001 compared to PBS group; ## P < 0.01 compared to TGFB1 group). Significant differences between groups were analyzed with one-way ANOVA (right panel). (C) The concentrations of E_2 in TIF measured by ECLIA. The results are expressed as mean $\pm$ S.E.M. of three independent experiments (* P < 0.05, compared to PBS group; # P < 0.05, compared to TGFB1 group).

Figure 5 Schematic presentation of the postulated mechanisms of TGFB1 action in aromatase expression in LCs. TGFB1 binds to its receptor complex (TGFB2 and ALK5) to activate Smads. The complex is formed by Smad2 and Smad3 with Smad4, and translocate to nucleus, where the complex, together with other repressors, could down-regulate SF1 and LRH1 expression to indirectly inhibit aromatase gene transcription (dashed line: negative regulation).

encoding P450arom, was evaluated. TGFB1 treatment resulted in a notable decrease in the expression level of CYP19, and this inhibitory effect could be effectively reversed by the supplement with SB431542 (Fig. 4B). ECLIA showed that the concentration of E_2 in TIF of TGFB1-injected group was significantly decreased from 37.86 pg/mL to 26.37 pg/mL, whereas SB431542 treatment significantly attenuated the change of E_2 concentration (Fig. 4C).

Discussion

Our recent study demonstrated that TGFB1 significantly down-regulated the level of oestrogen secretion, the activity of P450arom, and the expression of Cyp19 in rat LCs (Liu *et al.* 2013). The results from the present study showed that TGFB1 specifically inhibited the expression of two Cyp19 transcription factors, SF1 and LRH1, in the rat LCs both *in vitro* and *in vivo*, and that the inhibitory effects of TGFB1 could be significantly reversed by the supplement with SB431542. Hence, TGFB1 may exert its inhibitory effect on oestrogen production in LCs through TGFB1 signalling pathway to decrease endogenous SF1 and LRH1 levels.

In the current study, we demonstrated that TGFB1 specifically down-regulated the expression of SF1 and LRH1, the two transcription factors binding to SFRE of PII, in adult rat LCs, indicating that SF1 and LRH1 may

serve as downstream signal mediators in the TGFB1 signalling pathway to regulate CYP19 expression during steroidogenesis. These findings concur with those obtained in Y-1 adrenocortical cells, where TGFB1 inhibits the expression of *Sf1* gene at a transcriptional level (Lehmann *et al.* 2005). The importance of SF1 in LCs function has been demonstrated by cell-specific knockout (Jeyasuria *et al.* 2004) and oestrogen-producing testicular Leydig cell tumour with elevated levels of SF1 and over-expression of CYP19 (Straume *et al.* 2012). Over-expression of LRH1 in primary rat LCs has been shown to strongly increase endogenous aromatase mRNA level (Sierens *et al.* 2010). Given that the binding domains of SF1 and LRH1 DNA are highly conserved, and both proteins bind to the same DNA regions in steroidogenesis tissues (Yazawa *et al.* 2015), whether SF1 and LRH1 act alone or synergistically in the regulation of aromatase expression in LCs remains to be further clarified. In this study, the inhibitory effect of TGFB1 on the expression of these transcription factors was mostly restricted to the nuclei in LCs. However, the expression of CREB and CREM remains unchanged under TGFB1 treatment. Other studies also showed TGFB1 could not decrease the abundance of CREB but decrease the phosphorylation of CREB in mink lung epithelial cells (Yoshizumi *et al.* 1997). To our knowledge, the regulation of CREM by TGFB1 has not been reported so far.

Several lines of evidence suggest a crucial role of TGFB1 in the regulation of LCs function. For instance, TGFB1 has been shown to inhibit oestrogen secretion (Liu *et al.* 2013), to suppress proliferation of LCs, and to be involved in the morphological differentiation of immature LCs into the adult form (Khan *et al.* 1999, Dickson *et al.* 2002) and the shift between the active and inactive state in LCs (Gonzalez *et al.* 2012). However, the mechanism by which TGFB1 exerts its effects on LCs remains to be established. The presence of TGFB1, TGFB1R1 and TGFB1R2 has long been described in adult LCs by us (Zhang *et al.* 2004). Moreover, we and other authors also proved that Smad 2, 3, 4 and the intracellular signalling molecules of TGFB1 are all expressed in rat LCs (Hu *et al.* 2003, Xu *et al.* 2003). These results remind us that LCs are the major targets of TGFB1 in the testis, and that TGFB1 may bind to the receptor on LCs to activate a series of biological effects through paracrine/autocrine action. It has been reported that TGFB1 inhibits cAMP-induced testosterone production via activating ALK5 in mouse primary cultured LCs (Park *et al.* 2014), and that TGFB1 signalling through ALK5 signalling is also involved in the proliferation of mouse LCs (Gonzalez *et al.* 2010). ALK5 has been identified here as a receptor that mediates the down-regulation of SF1 and LRH1 expression induced by TGFB1 at both *in vitro* and *in vivo* levels. However, whether other repressors or corepressors are involved in this transcriptional regulation is required to be further

clarified. Furthermore, TGFB1 suppresses the expression of the CYP19 as well as the E₂ secretion in TIF *in vivo*, which is consistent with our previous *in vitro* study showing that TGFB1 inhibits oestrogen production via down-regulating the expression of the CYP19 in adult LCs. Interestingly, the reduction of CYP19 protein content is more significant than that of E₂ synthesis. This observation suggests that the production of E₂ may well be due to both the testosterone and reduced CYP19 expression, for the reason that the main source of oestrogen in the testis is the conversion of androgen catalyzed by P450arom. In the experiments detailed here, the SB431542 concentrations of 0.1 µM *in vitro* and 1 µM *in vivo* have been found to be sufficient to intervene in TGFB1 signalling. Our above data collectively raise the possibility that ALK5 may be involved in the down-regulation of SF1 and LRH1 expression induced by TGFB1 to indirectly inhibit aromatase transcription and E₂ secretion in LCs (Fig. 5).

In summary, we have provided evidence that TGFB1 may act through the canonical signalling pathway involving ALK5 to restrain SF1 and LRH1 accumulation and eventually attenuate *Cyp19* transcription and oestrogen production in LCs. The present study helps us to understand the signalling pathway of TGFB1/ALK5 regulating oestrogen secretion in LCs.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0044>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by Natural Science Foundation of China (NSFC) (grant numbers 31271606, 81471447 and 81573851).

Acknowledgements

The authors thank Ce Jing, Pang Chen, Jinhua Wei and Wei Li for their expert technical assistance.

References

- Bjelic MM, Stojkov NJ, Baburski AZ, Sokanovic SJ, Mihajlovic AI, Janjic MM, Kostic TS & Andric SA 2014 Molecular adaptations of testosterone-producing leydig cells during systemic *in vivo* blockade of the androgen receptor. *Molecular and Cellular Endocrinology* **396** 10–25. (doi:10.1016/j.mce.2014.08.007)

- Carreau S, Genissel C, Bilinska B & Levallet J 1999 Sources of oestrogen in the testis and reproductive tract of the male. *International Journal of Andrology* **22** 211–223. (doi:10.1046/j.1365-2605.1999.00172.x)
- Carreau S, Bourguiba S, Lambard S, Silandre D & Delalande C 2004 The promoter(s) of the aromatase gene in male testicular cells. *Reproductive Biology* **4** 23–34.
- Chrivia JC, Kwok RP, Lamb N, Hagiwara M, Montminy MR & Goodman RH 1993 Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* **365** 855–859. (doi:10.1038/365855a0)
- Dickson C, Webster DR, Johnson H, Cecilia MA & Khan SA 2002 Transforming growth factor-beta effects on morphology of immature rat leydig cells. *Molecular and Cellular Endocrinology* **195** 65–77. (doi:10.1016/S0303-7207(02)00216-2)
- Don J & Stelzel G 2002 The expanding family of CREB/CREM transcription factors that are involved with spermatogenesis. *Molecular and Cellular Endocrinology* **187** 115–124. (doi:10.1016/S0303-7207(01)00696-7)
- Gonzalez CR, Gonzalez B, Rulli SB, Huhtaniemi I, Calandra RS & Gonzalez-Calvar SI 2010 TGF-beta1 system in leydig cells. Part I: effect of HCG and progesterone. *Journal of Reproduction and Development* **56** 389–395. (doi:10.1262/jrd.09-166N)
- Gonzalez CR, Calandra RS & Gonzalez-Calvar SI 2012 Influence of the photoperiod on TGF-beta1 and p15 expression in hamster leydig cells. *Reproductive Biology* **12** 201–218. (doi:10.1016/S1642-431X(12)60086-2)
- Haakensen VD, Bjoro T, Luders T, Riis M, Bukholm IK, Kristensen VN, Troester MA, Homen MM, Ursin G & Borresen-Dale AL 2011 Serum estradiol levels associated with specific gene expression patterns in normal breast tissue and in breast carcinomas. *BMC Cancer* **11** 332. (doi:10.1186/1471-2407-11-332)
- Hamilton KJ, Arao Y & Korach KS 2014 Estrogen hormone physiology: reproductive findings from estrogen receptor mutant mice. *Reproductive Biology* **14** 3–8. (doi:10.1016/j.repbio.2013.12.002)
- Haverfield JT, Ham S, Brown KA, Simpson ER & Meachem SJ 2011 Teasing out the role of aromatase in the healthy and diseased testis. *Spermatogenesis* **1** 240–249. (doi:10.4161/spmg.1.3.18037)
- Hess RA 2003 Estrogen in the adult male reproductive tract: a review. *Reproductive Biology and Endocrinology* **1** 52. (doi:10.1186/1477-7827-1-52)
- Hu J, Zhang YQ, Liu XP, Wang RA, Jin Y & Xu RJ 2003 Expression and localization of Smad1, Smad2 and Smad4 proteins in rat testis during postnatal development. *Asian Journal of Andrology* **5** 51–55.
- Itman C, Mendis S, Barakat B & Loveland KL 2006 All in the family: TGF-beta family action in testis development. *Reproduction* **132** 233–246. (doi:10.1530/rep.1.01075)
- Jeyasuria P, Ikeda Y, Jamin SP, Zhao L, De Rooij DG, Themmen AP, Behringer RR & Parker RL 2004 Cell-specific knockout of steroidogenic factor 1 reveals its essential roles in gonadal function. *Molecular Endocrinology* **18** 1610–1619. (doi:10.1210/me.2003-0404)
- Kato S, Tora L, Yamauchi J, Masushige S, Bellard M & Chambon P 1992 A far upstream estrogen response element of the ovalbumin gene contains several half-palindromic 5'-TGACC-3' motifs acting synergistically. *Cell* **68** 731–742. (doi:10.1016/0092-8674(92)90148-6)
- Khan SA, Mirsafian M, Howdeshell K & Dorrington JH 1999 Transforming growth factor-beta inhibits DNA synthesis in immature rat leydig cells in vitro. *Molecular and Cellular Endocrinology* **148** 21–28. (doi:10.1016/S0303-7207(98)00244-5)
- Lambard S, Silandre D, Delalande C, Denis-Galeraud I, Bourguiba S & Carreau S 2005 Aromatase in testis: expression and role in male reproduction. *Journal of Steroid Biochemistry and Molecular Biology* **95** 63–69. (doi:10.1016/j.jsbmb.2005.04.020)
- Lanzino M, Catalano S, Genissel C, Ando S, Carreau S, Hamra K & Mcphaul MJ 2001 Aromatase messenger RNA is derived from the proximal promoter of the aromatase gene in leydig, sertoli, and germ cells of the rat testis. *Biology of Reproduction* **64** 1439–1443. (doi:10.1095/biolreprod64.5.1439)
- Le Roy C, Lejeune H, Chuzel F, Saez JM & Langlois D 1999 Autocrine regulation of leydig cell differentiated functions by insulin-like growth factor 1 and transforming growth factor beta. *Journal of Steroid Biochemistry and Molecular Biology* **69** 379–384. (doi:10.1016/S0960-0760(99)00075-8)
- Lehmann TP, Biernacka-Lukanty JM, Trzeciak WH & Li JY 2005 Steroidogenic factor 1 gene transcription is inhibited by transforming growth factor beta. *Endocrine Research* **31** 71–79. (doi:10.1080/07435800500229110)
- Lin T, Blaisdell J & Haskell JF 1987 Transforming growth factor-beta inhibits leydig cell steroidogenesis in primary culture. *Biochemical and Biophysical Research Communications* **146** 387–394. (doi:10.1016/0006-291X(87)90541-9)
- Liu ML, Wang H, Wang ZR, Zhang YF, Chen YQ, Zhu FH, Zhang YQ, Ma J & Li Z 2013 TGF-beta1 regulation of estrogen production in mature rat leydig cells. *PLoS ONE* **8** e60197. (doi:10.1371/journal.pone.0060197)
- Liu HC, Zhu D, Wang C, Guan H, Li S, Hu C, Chen Z, Hu Y, Lin H & Lian QQ 2015 Effects of etomidate on the steroidogenesis of rat immature leydig cells. *PLoS ONE* **10** e0139311. (doi:10.1371/journal.pone.0139311)
- Martianov I, Choukrallah MA, Krebs A, Ye T, Legras S, Rijkers E, Van Ijcken W, Jost B, Sassone-Corsi P & Davidson I 2010 Cell-specific occupancy of an extended repertoire of CREM and CREB binding loci in male germ cells. *BMC Genomics* **11** 530. (doi:10.1186/1471-2164-11-530)
- Mendis-Handagama SM, Di Clementi N, Ariyaratne HB & Mrkonjich L 2006 Detection of anti-mullerian hormone receptor II protein in the postnatal rat testis from birth to sexual maturity. *Histology and Histopathology* **21** 125–130. (doi:10.14670/HH-21.125)
- O'Donnell L, Robertson KM, Jones ME & Simpson ER 2001 Estrogen and spermatogenesis. *Endocrine Reviews* **22** 289–318. (doi:10.1016/b0-12-341103-3/00087-5)
- Ogawa T, Arechaga JM, Avarbock MR & Brinster RL 1997 Transplantation of testis germinal cells into mouse seminiferous tubules. *International Journal of Developmental Biology* **41** 111–122.
- Ozguden-Akkoc CG & Ozer A 2012 Immunohistochemical localization of transforming growth factor beta1 and beta2 in mouse testes during postnatal development. *Biotechnic and Histochemistry* **87** 154–159. (doi:10.3109/10520295.2011.592150)
- Park E, Song CH, Park JI, Ahn RS, Choi HS, Ko C & Lee K 2014 Transforming growth factor-beta1 signaling represses testicular steroidogenesis through cross-talk with orphan nuclear receptor nur77. *PLoS ONE* **9** e104812. (doi:10.1371/journal.pone.0104812)
- Pezzi V, Sirianni R, Chimento A, Maggiolini M, Bourguiba S, Delalande C, Carreau S, Ando S, Simpson ER & Clyne CD 2004 Differential expression of steroidogenic factor-1/adrenal 4 binding protein and liver receptor homolog 1 (LRH1)/fetoprotein transcription factor in the rat testis: LRH1 as a potential regulator of testicular aromatase expression. *Endocrinology* **145** 2186–2196. (doi:10.1210/en.2003-1366)
- Prosperi JR & Robertson FM 2006 Cyclooxygenase-2 directly regulates gene expression of p450 cyp19 aromatase promoter regions pII, p1.3 and p1.7 and estradiol production in human breast tumor cells. *Prostaglandins Other Lipid Mediators* **81** 55–70. (doi:10.1016/j.prostaglandins.2006.07.003)
- Sato M, Ishikawa A & Kimura M 2002 Direct injection of foreign DNA into mouse testis as a possible in vivo gene transfer system via epididymal spermatozoa. *Molecular Reproduction and Development* **61** 49–56. (doi:10.1002/mrd.1130)
- Shi Y & Massague J 2003 Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113** 685–700. (doi:10.1016/S0092-8674(03)00432-X)
- Sierens J, Jakody I, Poobalan Y, Meachem SJ, Knower K, Young MJ, Sirianni R, Pezzi V & Clyne CD 2010 Localization and regulation of aromatase liver receptor homologue-1 in the developing rat testis. *Molecular and Cellular Endocrinology* **323** 307–313. (doi:10.1016/j.mce.2010.03.001)
- Stojkov NJ, Janjic MM, Kostic TS & Andric SA 2013 Orally applied doxazosin disturbed testosterone homeostasis and changed the transcriptional profile of steroidogenic machinery, cAMP/cGMP signalling and adrenergic receptors in leydig cells of adult rats. *Andrology* **1** 332–347. (doi:10.1111/j.2047-2927.2012.00035.x)
- Straume AH, Lovas K, Miletic H, Gravdal K, Lonning PE & Knappskog S 2012 Elevated levels of the steroidogenic factor 1 are associated with over-expression of cyp19 in an oestrogen-producing testicular leydig cell tumour. *European Journal of Endocrinology* **166** 941–949. (doi:10.1530/EJE-11-0849)

- Xu J, Beyer AR, Walker WH & McGee EA 2003 Developmental and stage-specific expression of Smad2 and Smad3 in rat testis. *Journal of Andrology* **24** 192–200. (doi:10.1002/j.1939-4640.2003.tb02662.x)
- Yazawa T, Imamichi Y, Miyamoto K, Khan MR, Uwada J, Umezawa A & Taniguchi T 2015 Regulation of steroidogenesis, development, and cell differentiation by steroidogenic factor-1 and liver receptor homolog-1. *Zoological Science* **32** 323–330. (doi:10.2108/zs140237)
- Young M & McPhaul MJ 1998 A steroidogenic factor-1-binding site and cyclic adenosine 3',5'-monophosphate response element-like elements are required for the activity of the rat aromatase promoter in rat leydig tumor cell lines. *Endocrinology* **139** 5082–5093. (doi:10.1210/en.139.12.5082)
- Yoshizumi M, Wang H, Hsieh CM, Sibinga NE, Perrella MA & Lee ME 1997 Down-regulation of the cyclin a promoter by transforming growth factor-beta1 is associated with a reduction in phosphorylated activating transcription factor-1 and cyclic AMP-responsive element-binding protein. *Journal of Biological Chemistry* **272** 22259–22264. (doi:10.1074/jbc.272.35.22259)
- Zhang YQ, He XZ, Zhang JS, Wang RA, Zhou J & Xu RJ 2004 Stage-specific localization of transforming growth factor beta1 and beta3 and their receptors during spermatogenesis in men. *Asian Journal of Andrology* **6** 105–109.

Received 24 January 2016

First decision 3 March 2016

Revised manuscript received 10 February 2017

Accepted 28 February 2017