

Transforming growth factor- β 1 increases lysyl oxidase expression by downregulating MIR29A in human granulosa lutein cells

Ying Fang^{1,2}, Hsun-Ming Chang², Jung-Chien Cheng², Christian Klausen², Peter C K Leung² and Xiaokui Yang¹

¹Department of Human Reproductive Medicine, Beijing Obstetrics and Gynecology Hospital, Capital Medical University, Beijing, China and ²Department of Obstetrics and Gynaecology, Child & Family Research Institute, University of British Columbia, Vancouver, British Columbia, Canada

Correspondence should be addressed to P C K Leung or X Yang; Email: peter.leung@ubc.ca or xiaokuiyang2012@163.com

Abstract

Lysyl oxidase (LOX), a key enzyme in the formation and stabilization of the extracellular matrix, is expressed in granulosa cells and plays a critical role in the regulation of granulosa cell differentiation, oocyte maturation and ovulation. To date, the regulation of LOX expression in human granulosa cells remains largely unknown. In this study, using primary and immortalized human granulosa lutein cells, we demonstrated that transforming growth factor (TGF)- β 1 (TGFB1) upregulated LOX expression and downregulated microRNA-29a (MIR29A) expression via a TGF- β type I receptor-mediated signaling pathway. Additionally, we showed that MIR29A downregulated the expression of LOX in both types of cells. Furthermore, the downregulation of MIR29A contributed to the TGFB1-induced increase in LOX expression because the inhibition of MIR29A with a MIR29A inhibitor not only reversed the MIR29A-induced downregulation of LOX but also enhanced the TGFB1-induced upregulation of LOX. Our findings suggest that TGFB1 and MIR29A may play essential roles in the regulation of extracellular matrix remodeling during the periovulatory phase.

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Introduction

Lysyl oxidase (LOX) is characterized by the ability to form and maintain the extracellular matrix (ECM) by initiating the covalent cross-linking of collagen and elastin during follicular development (Kagan & Trackman 1991, Smith-Mungo & Kagan 1998). Recent studies have shown that LOX is expressed in granulosa cells (Kendall *et al.* 2003), and this enzyme may play a critical role in the regulation of granulosa cell differentiation, oocyte maturation and ovulation (Slee *et al.* 2001, Jiang *et al.* 2010). In perch ovary, LOX mRNA levels were dramatically increased during ovulation (Langenau *et al.* 1999). In rabbit ovarian follicles, the activity of LOX reached its peak after human chorionic gonadotropin-induced follicle rupture (Himeno 1986). Indeed, the disruption of LOX activity has been shown to be related to copper-responsive subfertility due to abnormal follicular growth (Kendall *et al.* 2003). In rats, the expression of LOX in mural granulosa cells was strongly associated with the developmental competence of oocytes, indicating that LOX can be used as a potential marker for the quality of oocytes in assisted reproduction (Jiang *et al.* 2010). In view of the significant role of LOX

in folliculogenesis, a growing number of studies have focused on the regulation of *Lox* at both the endocrine and paracrine levels. In rat granulosa cells, *Lox* mRNA levels and enzyme activity were suppressed by FSH and 8-bromo-cAMP in a dose-dependent manner, whereas they were strongly enhanced by dihydrotestosterone (DHT). However, DHT enhanced the FSH-induced suppressive effect on *Lox* mRNA levels (Slee *et al.* 2001, Harlow *et al.* 2003). Many locally produced growth factors, such as transforming growth factor- β 1 (TGFB1), growth differentiation factor-9 and activin A, have been reported to stimulate LOX mRNA expression and activity (Harlow *et al.* 2003). Interestingly, *Lox* mRNA levels were increased more than threefold in a DHEA-induced rat polycystic ovary model (Henmi *et al.* 2001). In the DES-primed immature rat ovary, equine chorionic gonadotropin treatment significantly suppressed LOX transcripts, and this suppressive effect was further enhanced during gonadotropin-induced luteinization, suggesting that the expression of LOX is inversely related to the granulosa cell differentiation (Slee *et al.* 2001). To date, the regulation of LOX expression in human granulosa cells remains to be elucidated.

TGFB1, a canonical member of the TGF- β superfamily, is expressed and secreted by growing follicles, including oocytes, theca cells and granulosa cells (Knight & Glister 2006). In mammals, this growth factor plays a critical role in the regulation of follicular development and oocyte maturation (Ingman et al. 2006, Pangas 2007). Higher serum levels and increased ovarian expression of TGFB1 have been found in patients with polycystic ovary syndrome (PCOS), indicating that the dysregulation of TGFB1 may be involved in the pathogenesis of PCOS (Raja-Khan et al. 2010, Hatzirodos et al. 2011, Tal et al. 2013). MicroRNAs (miRNAs) are endogenous small noncoding RNAs that negatively regulate gene expression by binding to specific mRNAs and promoting their degradation and/or translational repression (Bartel 2004). MicroRNA-29a (MIR29A), one of the earliest identified miRNA families, is strongly expressed in the ovaries of mammals, including humans (Hossain et al. 2009, da Silveira et al. 2012, Sang et al. 2013). MIR29A is expressed in bovine oocytes and follicular cells (cumulus/granulosa cells and theca cells) and can be detected in the follicular fluid at different developmental phases. Notably, MIR29A is implicated in the regulation of cell–cell communication (oocytes and granulosa cells), progesterone production and luteinization (Hossain et al. 2009). Clinical data have shown that MIR29A levels are reduced in the serum of PCOS patients (Ding et al. 2015). Interestingly, emerging results from *in vitro* studies have demonstrated that the expression of MIR29A is downregulated by TGFB1 in many cell types, including fibroblasts and tubular epithelial cells (Maurer et al. 2010, Qin et al. 2011, Wang et al. 2012, Yang et al. 2013). Given that PCOS patients have higher expression levels of TGFB1 and LOX as well as lower expression levels of MIR29A, we hypothesized that TGFB1 increases the expression of LOX by downregulating MIR29A in human granulosa cells. In this study, using primary and immortalized human granulosa lutein cells, we sought to investigate the biological role of TGFB1 in the regulation of LOX expression and the underlying molecular mechanisms.

Materials and methods

Preparation of primary human granulosa lutein cells

Primary human granulosa lutein (hGL) cells were collected after obtaining informed consent from patients and approval from the University of British Columbia Research Ethics Board. The controlled ovarian stimulation protocol for *in vitro* fertilization patients consisted of downregulation of either luteal-phase nafarelin acetate (Synarel; Pfizer) or follicular-phase GnRH antagonist (Ganirelix; Merck, Frosst, Montreal, Canada). Gonadotropin stimulation began on menstrual cycle day 2 with human menopausal gonadotropin (hMG; Menopur, Ferring) and recombinant FSH (Puregon, Merck, Montreal,

Canada) and was followed by human chorionic gonadotropin (Pregnyl, Merck, Montreal, Canada) administration 34–36 h before oocyte retrieval, based on the follicle size. Follicular aspirates from women undergoing oocyte retrieval were density centrifuged as described previously and hGL cells were then purified (Quinn et al. 2006, Chang et al. 2013). Individual primary cultures comprised cells from one patient. Then, 2×10^5 viable cells were seeded 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) supplemented with 10% charcoal/dextran-treated fetal bovine serum (HyClone, Logan, UT, USA), 100 U/mL of penicillin (Life Technologies, Inc/BRL), 100 μ g/mL of streptomycin sulfate (Life Technologies), and 1X GlutaMAX (Life Technologies). The culture medium was changed every other day in all of the experiments.

Human granulosa cell immortalized with Simian virus 40 large T antigen

A non-tumorigenic immortalized human granulosa lutein cell line (SVOG), which was previously established by transfecting human granulosa lutein cells with the SV40 large T antigen (Lie et al. 1996), was used in the present study. SVOG cells retain the physiological characteristics of hGL cells, such as the steroidogenic function, and also respond to many different treatments in a similar manner as hGL cells (Chang et al. 2014, 2015a,b, Chen et al. 2015). SVOG cells were counted with a hemocytometer, and cell viability was assessed using Trypan blue (0.04%) exclusion. The cells were seeded ($4\text{--}8 \times 10^5$ 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 of penicillin (Life Technologies), 100 μ g/mL of streptomycin sulfate (Life Technologies) and 1X 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 treatment with growth factor.

miRNA and miRNA inhibitor transfection

SVOG cells were seeded in 6 well plates until 50–60% confluence was reached on the day of transfection. Cells were transfected with 25 nM MIR29A mimic, 25 nM miR negative control, 25 nM MIR29A inhibitor or 25 nM inhibitor negative control (Ambion) using Lipofectamine RNAiMAX (Life Technologies) as a transfection reagent, according to the manufacturer's instructions. The MIR29A mimic is a small, double-stranded RNA molecule that mimics mature endogenous MIR29A, while the MIR29A inhibitor is a single-stranded RNA oligonucleotide that is antisense to the endogenous MIR29A. The inhibitor negative control is a scrambled nonspecific sequence with no identifiable effects on any reported miRNA function. The medium was changed to DMEM-F12 containing 10% FBS the day after transfection. The transfected cells were harvested at the indicated time points following specific treatments.

Antibodies and reagents

Recombinant human TGFB1 was obtained from R&D Systems as a Chinese hamster ovary cell-derived protein of more than 97% purity based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Lyophilized TGFB1 was reconstituted in a solution of 4mM HCl with 0.1% BSA as a carrier protein. The TGF-β type I receptor inhibitor SB431542 (S4317) was purchased from Sigma-Aldrich. Monoclonal mouse anti-α-tubulin antibody (sc-23948; diluted 1:2000) was obtained from Santa Cruz Biotechnology. The polyclonal rabbit anti-LOX (ab31238) antibody (diluted 1:1000) was obtained from Abcam. The horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse immunoglobulin G antibodies were obtained from Bio-Rad Laboratories.

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

Total RNA was extracted with TRIzol Reagent (Life Technologies) in accordance with the manufacturer’s instructions. Briefly, for the mRNA assay, RNA (2 μg) was reverse transcribed into first-strand cDNA with random primers and Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega). RT-qPCR was performed on Applied Biosystems 7300 Real-Time PCR System in 96 well optical reaction plates. Each 20 μL RT-qPCR reaction contained 1X SYBR Green PCR Master Mix (Applied Biosystems), 20 ng cDNA and 250 nM of each specific primer. The primers used were *LOX*, 5'-GCCTCAGGCTGCAC AATTC-3' (sense) and 5'-TCAGAACACCAGGCACTGATTT-3' (antisense); and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5'-GAGTCAACGGATTTGGTCTG-3' (sense) and 5'-GACAAGCTTCCCGTTCTCAG-3' (antisense). The specificity of each assay was validated using dissociation curve analysis and agarose gel electrophoresis of the PCR products. The assay performance was validated by evaluating amplification efficiencies using calibration curves and ensuring that the plot of the log input amount vs ΔCq (also known as ΔCt) had a slope <|0.1|.

For the miRNA assay, 1 μg RNA was reverse transcribed into cDNA using MultiScribe Reverse Transcriptase from the TaqMan MicroRNA RT Kit (Applied Biosystems) and specific stem-loop RT primers of MIR29A and U6 small nuclear RNA from TaqMan microRNA assays (Applied Biosystems). RT-qPCR was performed on the Applied Biosystems 7300 Real-Time PCR System in 96 well optical reaction plates. The TaqMan microRNA assays were used to detect the expression level of MIR29A according to the manufacturer’s instructions. Each 20 μL RT-qPCR reaction contained 1X TaqMan Universal PCR Master Mix II, TaqMan Small RNA Assay for MIR29A and U6 (Applied Biosystems), and 20 ng cDNA. Three independent experiments were performed on different cultures, and each sample was assayed in triplicate. Mean value was used for the determination of mRNA levels by the comparative Cq method with *GAPDH* or *U6* as the reference gene and using the formula $2^{-\Delta\Delta Cq}$. *GAPDH* and *U6* levels were unaffected by the various treatments in our study.

Western blotting analysis

Cells were washed with cold PBS and lysed in lysis buffer (Cell Signaling Technology) containing a protease inhibitor cocktail (Sigma-Aldrich). Extracts were centrifuged at 20,000g for 10 min at 4°C, and protein concentrations were determined using the DC Protein Assay (Bio-Rad Laboratories). Equal amounts of protein (50 μg) were separated on 10% SDS-PAGE gels and 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 washing, the membranes were incubated with a peroxidase-conjugated secondary antibody (Bio-Rad Laboratories) for 1 h. Immunoreactive bands were detected using enhanced chemiluminescence reagents or SuperSignal West Femto chemiluminescence substrate (Pierce), followed by exposure to CL-XPosure film (Thermo Fisher). Membranes were stripped with stripping buffer (50mM Tris-HCl

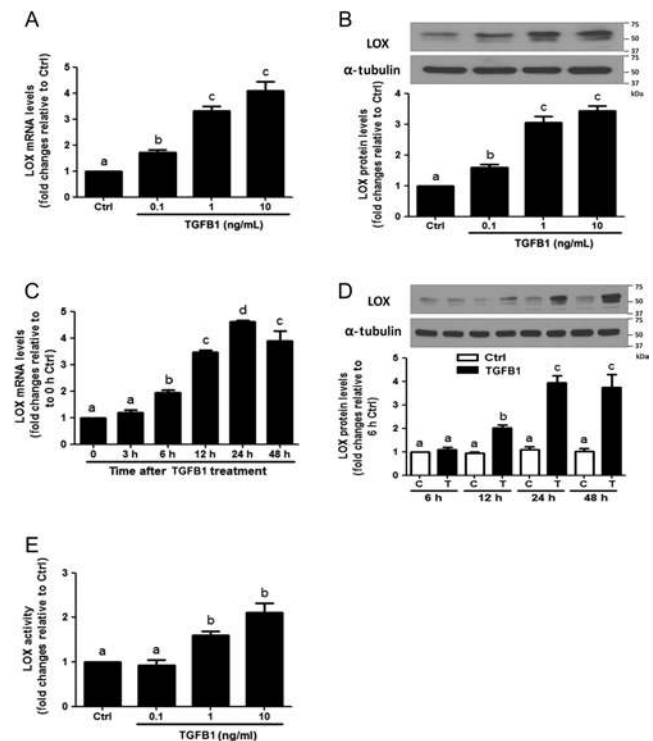


Figure 1 TGFB1 induces the upregulation of LOX expression and activity in SVOG cells. (A and B) SVOG cells were treated with vehicle control or increasing concentrations (0.1, 1 or 10 ng/mL) of TGFB1 for 12 h or 24 h, and LOX mRNA (A) or protein levels (B) were examined using RT-qPCR or Western blotting. (C and D) SVOG cells were treated with 5 ng/mL of TGFB1 for 0, 3, 6, 12, 24 and 48 h, and LOX mRNA (C) or protein (for 6, 12, 24 and 48 h) (D) levels were examined using RT-qPCR or Western blotting. (E) SVOG cells were treated for 24 h with increasing concentrations of TGFB1 (0.1, 1 or 10 ng/mL) and LOX activity in conditioned medium was determined using a fluorescent enzyme assay. The results are presented as the mean ± S.E.M. from at least 3 independent experiments, and values without a common letter are significantly different ($P < 0.05$). Ctrl, control.

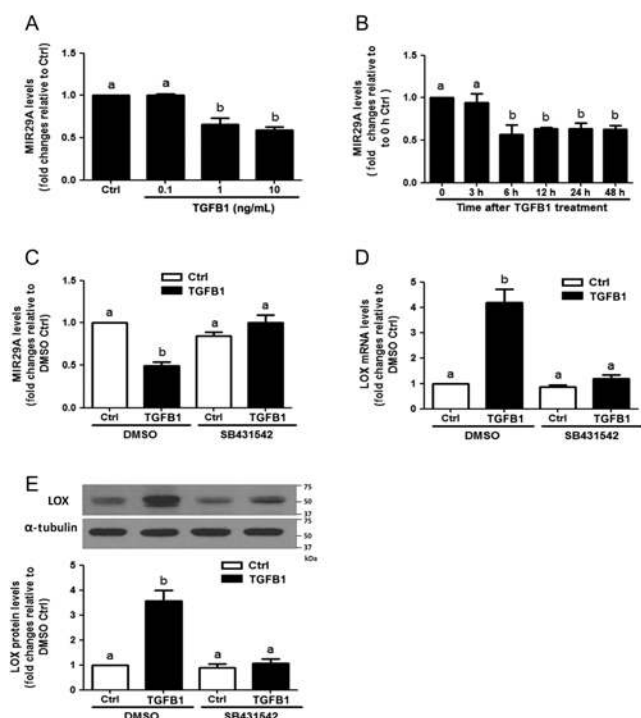


Figure 2 SB431542 reverses the TGFβ1-induced downregulation of MIR29A, and abolishes the TGFβ1-induced upregulation of LOX (A) SVOG cells were treated with vehicle control or increasing concentrations (0.1, 1 or 10 ng/mL) of TGFβ1 for 12 h, and the expression level of MIR29A was examined using RT-qPCR. (B) SVOG cells were treated with 5 ng/mL of TGFβ1 for 0, 3, 6, 12, 24 and 48 h and the expression level of MIR29A was examined by RT-qPCR. (C, D and E) Cells were treated with 5 ng/mL of TGFβ1 for 12 h (C), 12 h (D) or 24 h (E) in the presence of vehicle control (DMSO) or 10 μM SB431542, and the expression level of MIR29A (C) and LOX (D) mRNA were examined using RT-qPCR, and LOX protein (E) levels were examined using Western blotting. The results are presented as the mean ± s.e.m. from at least 3 independent experiments, and values without a common letter are significantly different ($P < 0.05$).

pH 7.6, 10 mmol/L β-mercaptoethanol and 1% SDS) at 50°C for 30 min and then reprobed with mouse anti-α tubulin antibody as a loading control. Immunoreactive band intensities were quantified by densitometry (Scion Image software, Scion Corporation, Frederick, MD, USA), target levels were normalized to those of α-tubulin, and the results are expressed as fold change relative to the respective control.

Measurement of LOX activity

Following the specified treatment, culture medium was assayed immediately or stored at -80°C until it was assayed. Relative quantification of LOX activity was performed according to the manufacturer's instructions using a fluorescent enzyme assay (Abcam, ab112139). The coefficient of variation for this assay was 7.94% and the reported detection limit of LOX in solution is 40 ng. Each sample was measured in triplicate and LOX activity was normalized to total cellular protein content for each sample, and the results are expressed as fold change relative to the respective control.

Statistical analysis

PRISM software (GraphPad Software) was used to perform Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparison tests. The results are presented as the mean ± s.e.m. of at least three independent experiments performed on different cultures and were considered significantly different if $P < 0.05$.

Results

TGFβ1 induces the upregulation of LOX expression and activity in SVOG cells

It has been shown that TGFβ1 can stimulate LOX mRNA and enzyme activity in rat granulosa cells (Harlow et al. 2003). However, neither the regulation nor the potential mechanism has been defined in human granulosa cells. To examine whether TGFβ1 can regulate the expression of LOX in human granulosa cells, SVOG cells were treated with a vehicle control or increasing concentrations (0.1, 1 or 10 ng/mL) of human recombinant TGFβ1. As shown in Fig. 1A, treatment with TGFβ1 for 12 h increased the LOX mRNA levels in a concentration-dependent manner. Consistent with the results from mRNA, treatment with TGFβ1 increased LOX protein levels (Fig. 1B). As shown in Fig. 1C, the time course experiments following TGFβ1 treatment demonstrated that LOX mRNA levels began to increase 6 h after treatment, reaching a peak level at 24 h, and the effect was sustained until 48 h after treatment. Western blotting analysis revealed that LOX protein levels increased starting at 12 h, and persisted until 48 h after treatment (Fig. 1D). To investigate whether TGFβ1-induced upregulation of LOX expression correlates with an increase in LOX activity, we used a fluorescent enzyme assay to measure LOX activity in conditioned medium following treatment of SVOG cells with increasing concentrations of TGFβ1. The results showed that TGFβ1 could significantly increase the LOX activity in a concentration-dependent manner (Fig. 1E).

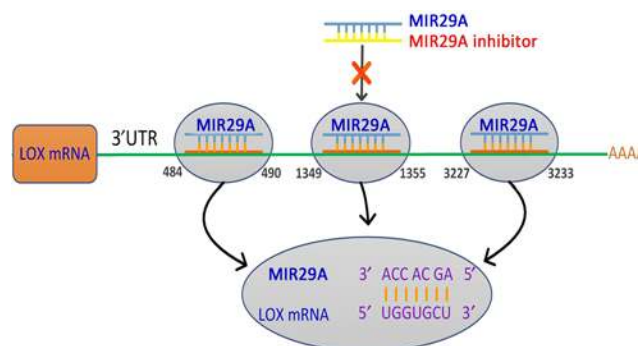


Figure 3 Schematic diagram representing the consequential pairing of the LOX region and MIR29A using the TargetScan program.

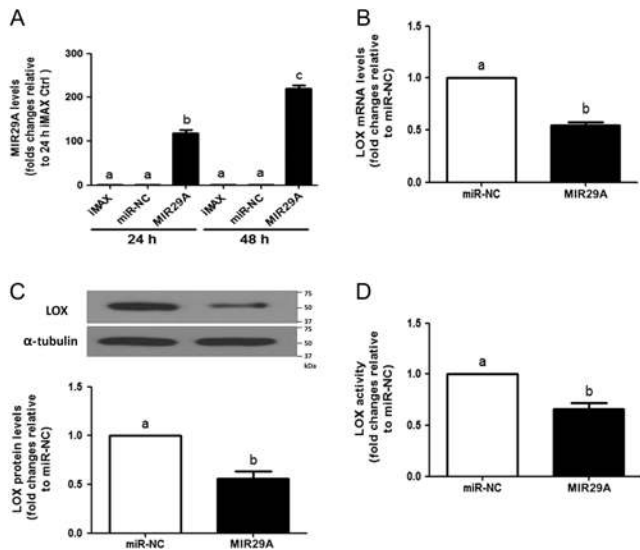


Figure 4 MIR29A inhibits the expression and activity of LOX in human granulosa cells. (A) SVOG cells were transfected with 25 nM Lipofectamine RNAiMAX, miR-NC or MIR29A mimic for 24 h or 48 h, and the transfection efficiency was examined using RT-qPCR. (B, C and D) SVOG cells were transfected for 24 h with 25 nM miR-NC and MIR29A mimic for 24 h and LOX mRNA (B), protein (C) and activity (D) were measured by RT-qPCR, Western blotting and fluorescent enzyme assay, respectively. The results are presented as the mean \pm S.E.M. from at least 3 independent experiments, and values without a common letter are significantly different ($P < 0.05$). IMAX, lipofectamine RNAiMAX; miR-NC, miRNA mimic negative control; MIR29A, MIR29A mimic.

TGFB1 downregulates the expression of MIR29A

Recent studies have shown that TGFB1 could suppress the expression of MIR29A in the fibroblasts, tubular epithelial cells and primary chondrocytes (Maurer *et al.* 2010, Qin *et al.* 2011, Wang *et al.* 2012, Yang *et al.* 2013). To date, no study has investigated the regulatory effect of TGFB1 on MIR29A expression in human granulosa cells. To address this issue, we treated SVOG cells with a vehicle control or different concentrations (0.1, 1 or 10 ng/mL) of TGFB1, and the results showed that TGFB1 decreased MIR29A levels in a concentration-dependent manner (Fig. 2A). In addition, time course studies revealed that MIR29A level began to decrease as early as 6 h after TGFB1 treatment, and the suppressive effect was sustained until 48 h after treatment (Fig. 2B).

TGF- β type I receptor is required for the TGFB1-induced downregulation of MIR29A and upregulation of LOX

A potent and specific TGFB1 type I receptor inhibitor, SB431542, was used to verify the involvement of the receptor in the effects of TGFB1 (Inman *et al.* 2002). As shown in Fig. 2C, pretreatment with SB431542 reversed the downregulation of MIR29A caused by TGFB1. Similarly, the stimulatory effects of TGFB1 on the expression of LOX (mRNA and protein) were

completely abolished by pretreatment with SB431542 (Fig. 2D and E).

MIR29A inhibits the expression and activity of LOX

We used the TargetScan database to predict the potential binding sites of MIR29A in the LOX mRNA 3'-UTR region (Fig. 3). A previous study identified a direct interaction between the upregulated LOX mRNA and the downregulated MIR29A in anaplastic thyroid carcinomas, which was experimentally validated by a luciferase assay in HEK cells (Hebrant *et al.* 2014). To examine whether MIR29A can inhibit the expression of LOX in human granulosa cells, we first transfected SVOG cells with a MIR29A mimic or a miRNA mimic negative control at a final concentration of 25 nM for 24 or 48 h. The transfection efficiency was examined using RT-qPCR. As shown in Fig. 4A, the intracellular MIR29A levels were increased by more than 100-fold at 24 h and 200-fold at 48 h after transfection. Next, we investigated the regulatory role of MIR29A on the expression of LOX in human granulosa cells. As shown in Fig. 4B and C,

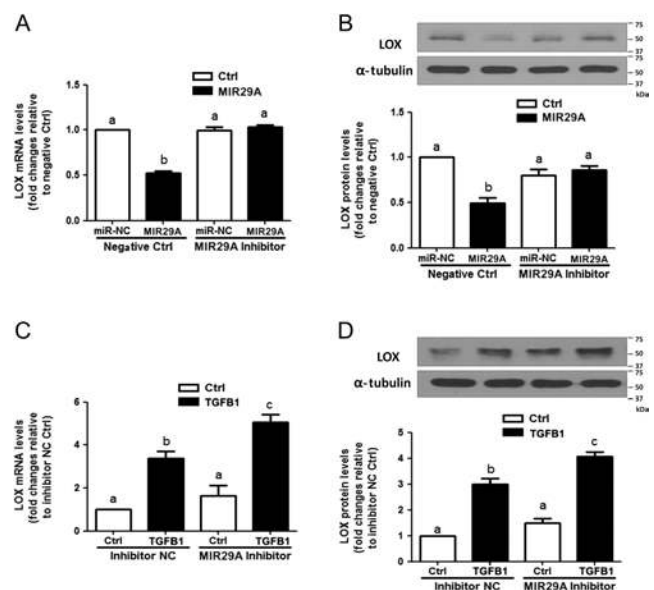


Figure 5 The downregulation of MIR29A contributes to the TGFB1-induced increase in LOX expression. (A and B) SVOG cells were transfected for 24 h with 25 nM of miRNA inhibitor negative control or 25 nM of MIR29A inhibitor before the transfection with miR-NC or MIR29A mimic for additional 24 h. The LOX mRNA (A) or protein levels (B) were examined using RT-qPCR or Western blotting. (C and D) SVOG cells were transfected with 25 nM of miRNA inhibitor negative control or 25 nM of MIR29A inhibitor for 24 h, and then treated with vehicle control or 5 ng/mL of TGFB1 for additional 24 h. The LOX mRNA (C) and protein levels (D) were examined using RT-qPCR or Western blotting. The results are presented as the mean \pm S.E.M. from at least 3 independent experiments, and values without a common letter are significantly different ($P < 0.05$). Ctrl, control; inhibitor NC, miRNA inhibitor negative control; miR-NC, miRNA mimic negative control; MIR29A, MIR29A mimic.

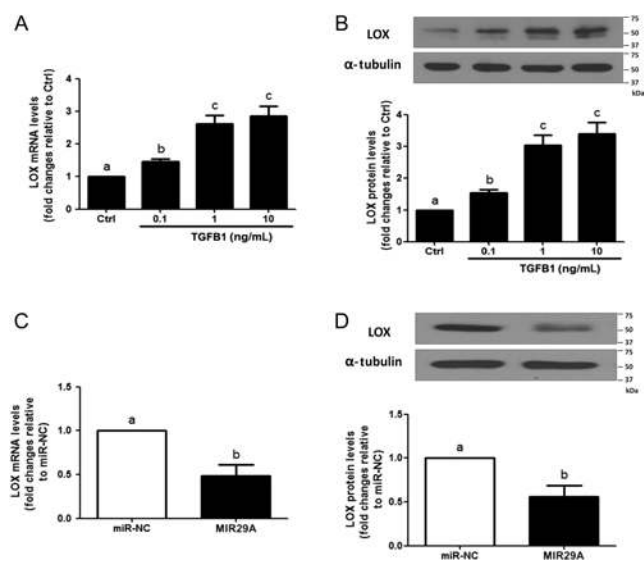


Figure 6 Effects of TGF β 1 and MIR29A on the regulation of LOX in primary hGL cells. (A and B) Primary hGL cells were treated with vehicle control or increasing concentrations (0.1, 1 or 10 ng/mL) of TGF β 1 for 12 h or 24 h, and LOX mRNA (A) or protein levels (B) were examined using RT-qPCR or Western blotting. (C and D) Primary hGL cells were transfected with miR-NC or MIR29A mimic for 24 h, and the levels of LOX mRNA (C) and protein (D) were examined using RT-qPCR or Western blotting. The results are presented as the mean \pm s.e.m. from at least 3 independent experiments, and values without a common letter are significantly different ($P < 0.05$). Ctrl, control; hGL, human granulosa-lutein.

transfection with the MIR29A mimic for 24 h resulted in significant decreases in the levels of LOX mRNA and protein. Furthermore, compared with the miRNA mimic negative control, transfection of SVOG cells with the MIR29A mimic for 24 h significantly decreased LOX activity in conditioned medium (Fig. 4D).

Downregulation of MIR29A contributes to TGF β 1-induced increase in LOX expression

To further confirm the role of MIR29A in the suppression of LOX expression, we treated SVOG cells with a miRNA inhibitor negative control or the MIR29A inhibitor for 24 h before transfection with the miRNA mimic negative control or the MIR29A mimic. Our results show that the decreases in LOX mRNA (Fig. 5A) and LOX protein (Fig. 5B) levels induced by the MIR29A mimic were reversed by transfection with a MIR29A inhibitor. Next, we investigated whether MIR29A plays a role in the TGF β 1-induced upregulation of LOX expression. The SVOG cells were transfected with the miRNA inhibitor negative control or the MIR29A inhibitor for 24 h before treatment with 5 ng/mL of TGF β 1 (additional 24 h). The results showed that the stimulatory effects of TGF β 1 (5 ng/mL) on LOX mRNA (Fig. 5C) and protein (Fig. 5D) levels were further enhanced by the MIR29A inhibitor, which suppressed the effects from the endogenous MIR29A.

Effects of TGF β 1 and MIR29A on the regulation of LOX in primary hGL cells

Non-immortalized primary hGL cells were used to further confirm the regulatory effects of TGF β 1 and MIR29A on the expression of LOX. As shown in Fig. 6A and B, treatment of primary hGL cells with TGF β 1 (0.1, 1 or 10 ng/mL) increased LOX mRNA (12 h) and protein levels (24 h) in a concentration-dependent manner. Consistent with the results from SVOG cells, transfection with MIR29A mimic for 24 h significantly decreased the levels of LOX mRNA and protein in primary hGL cells (Fig. 6C and D).

Discussion

The ECM in the ovary plays an essential role in follicular growth and subsequent ovulation (Woodruff & Shea 2007). LOX, a key enzyme involved in the remodeling and stabilization of the ECM, has been shown to be closely associated with granulosa cell differentiation, oocyte maturation and ovulation. TGF β 1 has been identified as a key regulator of LOX expression and activity in many cell types, including lung fibroblasts (Boak et al. 1994), osteoblasts (Feres-Filho et al. 1995) and aortic smooth muscle cells (Gacheru et al. 1997). In gingival fibroblastic cells, TGF β 1 strongly induced the expression of connective tissue growth factor (CTGF) and LOX, and CTGF itself increased the LOX enzyme activity up to 1.5-fold (Hong et al. 1999). Additionally, TGF β 1 inhibited cell proliferation by increasing LOX mRNA and protein levels in neonatal rat aortic smooth muscle cells (Gacheru et al. 1997). Furthermore, signaling studies have found that both SMAD-dependent and -independent (e.g. JNK/AP-1, PI3K/Akt, and MAPK) signaling pathways were involved in the regulation of LOX induced by TGF β 1 in cardiac fibroblasts and human trabecular meshwork cells (Sethi et al. 2011, Voloshenyuk et al. 2011). A recent study revealed that LOX mRNA and enzyme activities were induced by TGF β 1 in a dose-dependent manner in rat granulosa cells (Harlow et al. 2003). However, the underlying mechanism by which TGF β 1 regulates LOX expression was not investigated in these studies. In the present study, we demonstrated that TGF β 1 upregulated the expression of LOX (both mRNA and protein) and increased LOX activity in human granulosa lutein cells. Furthermore, we showed that downregulation of MIR29A contributed to the TGF β 1-induced upregulation of LOX. These conclusions are based on the fact that the downregulation of MIR29A induced by TGF β 1 occurred at 6 h after treatment, while the upregulation of LOX started later, at 12 h (mRNA level) and at 24 h (protein level). In addition, the inhibition of MIR29A using a specific inhibitor not only reversed the MIR29A-induced downregulation of LOX but also enhanced the TGF β 1-induced

upregulation of LOX. Our results indicate that MIR29A may act as a downstream negative regulator for the intracellular function of TGFB1 in human granulosa cells.

MIR29A is expressed in granulosa cells at various follicular stages and plays an essential role in folliculogenesis. Studies have shown that the gonadotropins (FSH and LH) regulate MIR29A in a biphasic manner. Notably, treatment with FSH for 12 h decreased the expression of MIR29A, while treatment with FSH for 48 h caused a twofold increase in MIR29A expression (Yao *et al.* 2010). In addition to gonadotropins, many growth factors have been shown to regulate the expression of MIR29A. In particular, TGFB1 (10 ng/mL) suppressed the expression of MIR29A up to 60% in human orbital fibroblasts in a time-dependent manner (Tan *et al.* 2014). Another study has revealed the involvement of TGFB1/Smad3 signaling in the downregulation of miR-29 in cultured fibroblasts and tubular epithelial cells. Notably, the TGFB1-induced downregulation of miR-29 was mediated by the binding of Smad3 to the promoter of miR-29 (Qin *et al.* 2011). In this study, we demonstrated for the first time that TGFB1 negatively regulates the expression of MIR29A in human granulosa cells. Future research will focus on how TGFB1 downregulates the expression of MIR29A in human granulosa cells.

In a rat model of premature ovarian failure (POF), the expression of MIR29A was substantially lower in POF ovaries than in normal ovaries, suggesting a role for the deregulation of MIR29A in POF development (Kuang *et al.* 2014). In addition, serum levels of MIR29A were lower in PCOS patients (Ding *et al.* 2015). These results indicate that deregulation of MIR29A may lead to several ovarian disorders. Interestingly, compelling evidence has shown that the deregulation of TGFB1 is also involved in the pathogenesis of PCOS (Raja-Khan *et al.* 2010, Hatzirodos *et al.* 2011, Tal *et al.* 2013). Furthermore, the levels of LOX mRNA and protein were upregulated in DHT-treated rat granulosa cells (Harlow *et al.* 2003) and DHEA-treated PCOS rat ovaries (Henmi *et al.* 2001). Using *in vitro* cell models, we have provided a potential molecular mechanism by which TGFB1 positively regulates LOX expression by downregulating the expression of MIR29A in human granulosa cells. Our findings may help explain the molecular changes that often occur in PCOS patients and may lead to the development of novel therapeutic strategies.

It is well known that miRNAs function post-transcriptionally as negative regulators of target gene expression. Using the TargetScan database, we found three potential binding sites for MIR29A in the 3'-UTR of LOX mRNA. Importantly, our *in vitro* studies demonstrated that LOX mRNA and protein levels were suppressed by MIR29A mimic treatment, and the effect was reversed by the MIR29A inhibitor, suggesting a

functional role for MIR29A in the regulation of LOX expression. Indeed, previous studies using 3'-UTR luciferase assays demonstrated a direct interaction of LOX mRNA and MIR29A in HEK293 cells (Hebrant *et al.* 2014). Taken together, previous studies and our results suggest that MIR29A may downregulate LOX mRNA through a direct interaction with its 3'-UTR site in human granulosa cells. However, MIR29A is not the sole regulator of basal LOX expression because transfection with MIR29A mimic yielded only a 2-fold reduction in LOX despite 100–200-fold increases in MIR29A levels. Furthermore, the addition of a MIR29A inhibitor in the presence of TGFB1 induced a further increase in LOX expression. These findings suggest that there are likely other mechanisms by which TGFB1 upregulates LOX expression, which will be an interesting area for future investigation.

In summary, we demonstrated that TGFB1 not only upregulated the expression and activity of LOX but also downregulated the expression of MIR29A in human granulosa cells. In addition, our results indicate that the TGF- β type I receptor is required for these actions induced by TGFB1. Furthermore, the downregulation of MIR29A contributed to the TGFB1-induced upregulation of LOX. Our *in vitro* findings suggest that TGFB1 and MIR29A may play crucial roles in the control of ECM remodeling during the periovulatory stage.

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.

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