Sphingosine-1-phosphate induces COX-2 expression and PGE2 production in human granulosa cells through a $S1P_{1/3}$-mediated YAP signaling

Jung-Chien Cheng[1], Hsun-Ming Chang[1], Pang-Pin Liu[1,2], Peter C.K. Leung[1]

[1]Department of Obstetrics and Gynaecology, Child & Family Research Institute, University of British Columbia, Vancouver, British Columbia, Canada V5Z 4H4

[2]Department of Obstetrics and Gynaecology, E-DA Hospital, Kaohsiung, Taiwan 82445

Short title: S1P induces COX-2 expression through YAP

Key words: S1P; COX-2; PGE2; YAP; Granulosa cells

Grant support: This work was supported by an operating grant from the Canadian Institutes of Health Research (#143317) to Peter C.K. Leung.

Disclosure summary: The authors have nothing to disclose.

Address all correspondence and requests for reprints to:

Peter C.K. Leung, PhD, FCAHS, FRSC

Department of Obstetrics and Gynaecology

Child & Family Research Institute

University of British Columbia

Room 317, 950 west 28th Ave,

Vancouver, British Columbia,

Canada, V5Z 4H4

Tel: 1-604-875-2718;

E-mail: peter.leung@ubc.ca

Abstract

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid that can regulate various physiological and pathological processes. The expression of S1P has been detected in human follicular fluid. In addition, two S1P receptors, $S1P_1$ and $S1P_3$, are expressed at a high level in human granulosa cells. Cyclooxygenase-2 (COX-2)-derived prostaglandin E2 (PGE2) production plays a critical role in the regulation of ovulation. However, thus far, the effect of S1P on COX-2 expression and PGE2 production in human granulosa cells remains unknown. In the present study, our results demonstrated that treatment with S1P significantly induced COX-2, but not COX-1, expression and increased PGE2 production in human granulosa cells. The stimulatory effects of S1P on COX-2 expression and PGE2 production were attenuated by treatment with specific antagonist of $S1P_1$ or $S1P_3$ and siRNA-mediated knockdown of $S1P_1$ or $S1P_3$. In addition, the COX-2 expression was induced by $S1P_1$ or $S1P_3$ agonist treatment. Interestingly, treatment with S1P activated YAP signaling via $S1P_1$ and $S1P_3$. Moreover, knockdown of YAP partially attenuated S1P-induced COX-2 expression and PGE2 production. These results provide evidence that S1P induces COX-2 expression and PGE2 production in human granulosa cells through a $S1P_{1/3}$-mediated YAP signaling pathway.

1. Introduction

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid metabolite that is formed by the phosphorylation of sphingosine, which is catalyzed by sphingosine kinase [1]. S1P exerts its functions by binding to and activating S1P receptors. S1P receptors are G protein-coupled receptors, and at present, 5 S1P receptors, $S1P_{1-5}$, have been identified. $S1P_{1-5}$ are coupled to different G proteins, which results in the activation or inhibition of different downstream signaling pathways [2, 3]. Many studies have demonstrated the important roles of S1P, which can regulate various physiological and pathological processes, in the reproductive system [4]. The S1P receptors, $S1P_1$ and $S1P_3$, are expressed at a high level in human granulosa cells [5, 6]. In addition, the expression of S1P can be detected in human follicular fluid [7]. However, thus far, only a handful of studies have examined the roles of S1P in human granulosa cells.

The cyclooxygenase (COX) enzyme mediates a key step of the prostaglandin synthesis [8]. Presently, two isoforms of the COX enzyme, COX-1 (*PTGS1*) and COX-2 (*PTGS2*), have been well characterized. Generally, COX-1 is expressed constitutively in most cells and is involved in maintaining homeostatic functions. In contrast, the expression levels of COX-2 are very low under normal physiological conditions, but can be rapidly induced by various external stimuli, such as different growth factors, hormones and cytokines [8]. Ovulation is a tightly regulated process, and the importance of COX-2 has been reported in the regulation of ovulation in different animal models [9-12]. In different cell types, S1P induces COX-2 expression and increases PGE2 production [13-21]. However, it is not yet clear whether the same is true for human granulosa cells.

The Hippo pathway was first identified in *Drosophila* and plays a critical role in controlling organ size [22]. In mammals, the YAP transcriptional coactivator is the major downstream effector of the Hippo pathway. Hippo pathway activation leads to the stimulation of the serine/threonine kinases Mst1/2, which phosphorylate the downstream kinases Lats1/2. Phosphorylated Lats1/2 subsequently phosphorylate YAP, which leads to its cytoplasmic localization and proteolytic degradation. Conversely, inactivation of the Hippo pathway results in the de-phosphorylation of YAP, which allows YAP to stay in the nucleus and

regulate the expression of its target genes [23]. S1P has been shown to rapidly activate YAP [24, 25]. Interestingly, a recent study has identified COX-2 as a novel target gene of YAP in pancreatic cells [26]. However, it is not known whether YAP contributes to S1P-induced COX-2 expression in other cell types.

In the current study, we first examined the effect of S1P on COX-2 expression and tested the hypothesis that YAP mediates S1P-induced COX-2 expression and PGE2 production in human granulosa cells. We found that S1P treatment induced COX-2 expression and PGE2 production. Interestingly, the expression of COX-1 was not affected by the treatment of S1P. In addition, treatment with S1P activated YAP, and knockdown of YAP attenuated S1P-induced COX-2 expression and PGE2 production. Using pharmacological antagonists and specific siRNA, our results further demonstrated that S1P-induced YAP activation, COX-2 expression, and PGE2 production were mediated by $S1P_1$ and $S1P_3$.

2. Materials and Methods

2.1 Cell culture

A non-tumorigenic SV40 large T antigen immortalized human granulosa cell line (SVOG) that was established previously by our group was used in the present study [27]. The cells were grown in DMEM/F12 medium (Sigma, Oakville, ON) and supplemented with 10% charcoal/dextran-treated fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT). The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.2 Preparation of primary human granulosa cells

Primary human granulosa cells were obtained with informed patient consent following approval from the University of British Columbia Research Ethics Board. The controlled ovarian stimulation protocol for *in vitro* fertilization patients consisted of either luteal-phase naferelin acetate (Synarel, Pfizer, Kirkland, Quebec, Canada) or follicular phase GnRH antagonist (Ganirelix; Merck Canada) down-regulation. Gonadotropin stimulation began on menstrual cycle day 2 with human menopausal gonadotropin (hMG; Menopur, Ferring, Canada) and recombinant FSH (Puregon, Merck, Canada) and was followed by human

chorionic gonadotropin administration 34-36 h before oocyte retrieval, based on follicle size. Granulosa cells were purified by density centrifugation from follicular aspirates collected from women undergoing oocyte retrieval, as previously described [28, 29]. Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C with DMEM/F-12 medium supplemented with 10% charcoal/dextran-treated FBS and 1X GlutaMAX for 5 days before treatment [30].

2.3 Antibodies and reagents

Polyclonal anti-COX-1 (#sc-1752) and monoclonal anti-α-tubulin (#sc-23948) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-COX-2 (#ab52237) and monoclonal anti-$S1P_1$ (#ab125074) and anti-$S1P_3$ (#ab108370) antibodies were obtained from Abcam (Cambridge, MA). Monoclonal anti-phospho-YAP^{ser127} (#13008) and anti-YAP (#12395) antibodies were obtained from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit IgG were obtained from Bio-Rad Laboratories (Hercules, CA). Horseradish peroxidase-conjugated donkey anti-goat IgG was obtained from Santa Cruz Biotechnology. Sphingosine-1-phosphate was obtained from Avanti Polar Lipids (Alabaster, AL) and prepared in PET solution (5% polyethylene glycol, 2.5% ethanol and 0.8% Tween-80) [31]. W146 and CAY10444 were obtained from Cayman Chemical (Ann Arbor, MI). $S1P_1$ agonist SEW 2871 and $S1P_3$ agonist CYM 5541 were obtained from Tocris (Minneapolis, MN).

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

Total RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies, Burlington, ON) according to the manufacturer's instructions. Reverse transcription was performed with 3 μg of RNA, random primers and M-MLV reverse transcriptase (Promega, Madison, WI). The primers were designed using Primer Express Software v2.0 (Applied Biosystems, Foster City, CA). All primers spanned at least one intron to detect specific mRNA sequences. The following primers were used for SYBR Green reverse transcription-qPCR (RT-qPCR): COX-1 (*PTGS1*), 5'-TGC CCA GCT CCT GGC CCG CCG CTT-3'

(sense) and 5'-GTG CAT CAA CAC AGG CGC CTC TTC-3' (antisense); COX-2 (*PTGS2*), 5'-CCC TTG GGT GTC AAA GGT AA-3' (sense) and 5'-GCC CTC GCT TAT GAT CTG TC-3' (antisense); YAP, 5'-CCC GAC TCC TTC TTC AAG C-3' (sense) and 5'-GAG AAA CAG CTC CCA ACT GC-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 using an Applied Biosystems 7300 Real-Time PCR System equipped with a 96-well optical reaction plate. The specificity of each assay was validated by a melting curve analysis and by agarose gel electrophoresis of the PCR products. All of the RT-qPCR experiments were performed in triplicate, and a mean value was used to determine the mRNA levels. Water and mRNA without RT were used as negative controls. Relative quantification of the mRNA levels was performed using the comparative Ct method with GAPDH as the reference gene and using the formula $2^{-\Delta\Delta Ct}$.

2.5 Western blot

Cells were lysed in cell lysis buffer (Cell Signaling). Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis and transferred onto PVDF membranes. After being blocked for 1 h with 5% non-fat dry milk in Tris-buffered saline (TBS), the membranes were incubated overnight at 4°C with primary antibodies that were diluted in 5% non-fat milk-TBS. Following primary antibody incubation, the membranes were incubated with the appropriate HRP-conjugated secondary antibody. Immunoreactive bands were detected using an enhanced chemiluminescent substrate and X-ray film. The intensities of the bands were quantified by densitometric analysis using Scion Image software (Scion Corp, Frederick, MD).

2.6 Small interfering RNA (siRNA) transfection

To knock down endogenous YAP, $S1P_1$ or $S1P_3$, cells were transfected with 50 nM ON-TARGET*plus* SMART*pool* YAP siRNA, 75 nM ON-TARGETplus SMARTpool $S1P_1$ siRNA or 75 nM ON-TARGETplus SMARTpool $S1P_3$ siRNA (Dharmacon, Lafayette, CO) using Lipofectamine RNAiMAX

(Invitrogen, Life Technologies). siCONTROL NON-TARGETING *pool* siRNA (Dharmacon) was used as the transfection control.

2.7 Prostaglandin E2 ELISA

A human PGE2-specific ELISA was used in accordance with the manufacturer's protocol (Cayman Chemical). The culture media were collected and the PGE2 levels in the culture media were measured by ELISA. PGE2 levels were normalized to the protein concentrations from the cell lysates. Normalized PGE2 values from the treatments were represented as relative values by comparing to the control treatment.

2.8 Statistical analysis

The results are presented as the mean ± SEM of at least three independent experiments. Prism software was used for the statistical analysis. Multiple comparisons were analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. A significant difference was defined as $p<0.05$.

3. Results

3.1 S1P induces COX-2 but not COX-1 expression in human granulosa cells

To examine the effect of S1P on COX expression, we used SVOG cells established previously by our group using SV40 large T antigen transfection of human granulosa cells obtained from women undergoing *in vitro* fertilization (IVF) [27]. Treatment of SVOG cells with 0.3 μM S1P for different periods of time did not affect the mRNA levels of COX-1. However, treatment with 0.3 μM S1P significantly induced COX-2 mRNA levels, with the maximal effect observed 1 h after S1P treatment. The induced COX-2 mRNA levels declined after 3 h of S1P treatment (Figure 1A). The concentrations of S1P in the human follicular fluid and serum are ~0.17 μM and ~0.9 μM, respectively [4]. Therefore, we next examined the effect of different concentrations of S1P (0.1-3 μM) on expression of COX genes. As shown in Figure 1B, treatment with either low (0.1 μM) or high (3 μM) concentration of S1P did not

affect the mRNA levels of COX-1. COX-2 mRNA levels were induced by S1P treatment in a concentration-dependent manner. Treatment with 0.1 μM S1P slightly induced COX-2 mRNA levels but did not reach the statistical significance. Notably, treatment with 0.17 μM S1P could significantly induced COX-2 mRNA levels. In considering the physiological concentration of S1P in the human follicular fluid and to make more technically feasible, 0.3 μM S1P was used for the subsequent experiments. Western blot results showed a similar effect of S1P on COX-1 and COX-2 protein levels (Figures 1C and 1D). To further confirm the stimulatory effect of S1P on COX-2 expression in human granulosa cells, primary human granulosa cells obtained from patients undergoing an IVF procedure were treated with S1P. Consistent with our findings in SVOG cells, treatment with S1P induced COX-2 mRNA and protein levels in primary human granulosa cells (Figure 2).

3.2 S1P-induced COX-2 expression is mediated by $S1P_1$ and $S1P_3$

Two S1P receptors, $S1P_1$ and $S1P_3$ have been shown to be expressed at a high level in human granulosa cells [6]. To examine whether the stimulatory effect of S1P on COX-2 expression are mediated by $S1P_1$ and $S1P_3$, the $S1P_1$ antagonist W146 and the $S1P_3$ antagonist CAY10444 were used to block the binding of S1P to $S1P_1$ and $S1P_3$, respectively. As shown in Figure 3A, pretreatment with W146 attenuated S1P-induced COX-2 mRNA levels. Similarly, S1P-induced COX-2 mRNA levels were attenuated by pretreatment with CAY10444 (Figure 3B). Consistent with the RT-qPCR results, western blot results showed that inhibition of $S1P_1$ or $S1P_3$ activation attenuated S1P-induced COX-2 protein levels (Figures 3C and 3D). To further confirm the involvement of $S1P_1$ and $S1P_3$ in S1P-induced COX-2 expression and avoid off-target effects of the pharmacological inhibitor, the endogenous expression of $S1P_1$ or $S1P_3$ was knocked down by transfection with specific siRNA. As shown in Figure 4A, $S1P_1$ siRNA specifically down-regulated $S1P_1$ protein levels without affecting the expression levels of $S1P_3$. Similarly, the specific knockdown effect of $S1P_3$ siRNA was observed. In addition, western blot results showed that knockdown of $S1P_1$ or $S1P_3$ alone attenuated the S1P-induced up-regulation of COX-2 protein levels. Simultaneous knockdown of $S1P_1$ and $S1P_3$ abolished the S1P-induced up-regulation of COX-2 protein levels.

Moreover, treatment with S1P1 agonist SEW 2871 or S1P3 agonist CYM 5541 induced up-regulation of COX-2 protein levels (Figures 4B and 4C). These results clearly indicated that S1P-induced COX-2 expression was mediated by $S1P_1$ and $S1P_3$ in human granulosa cells.

3.3 YAP is activated by S1P treatment and partially involved in S1P-induced COX-2 expression

Phosphorylation of YAP at S127 results in the cytoplasmic sequestration of YAP. In contrast, YAP S127 de-phosphorylation activates YAP and allow it to stay in the nucleus [23]. Therefore, we examined the effect of S1P on YAP activation by measuring the phosphorylation levels of YAP at S127. As shown in Figure 5A, our western blot results revealed that S1P induced rapid YAP de-phosphorylation at S127 with a maximal effect observed 30 min after S1P treatment. In addition, the S1P-induced YAP de-phosphorylation at S127 was attenuated by the inhibition of $S1P_1$ or $S1P_3$ (Figures 5B and 5C). Similarly, western blot results showed that knockdown of $S1P_1$ or $S1P_3$ alone attenuated the S1P-induced YAP activation. Simultaneous knockdown of $S1P_1$ and $S1P_3$ abolished the S1P-induced activation of YAP (Figure 6A). Moreover, treatment with S1P1 agonist SEW 2871 or S1P3 agonist CYM 5541 activated YAP (Figures 6B and 6C). Next, to examine whether YAP is involved in S1P-induced COX-2 expression, siRNA-mediated knockdown was used to block the function of YAP. As shown in Figure 7A, transfection of cells with YAP siRNA significantly down-regulated YAP mRNA levels. In addition, S1P-induced COX-2 mRNA levels were attenuated by YAP knockdown. Similar to the RT-qPCR results, knockdown of YAP attenuated COX-2 protein levels that were induced by S1P treatment (Figure 7B).

3.4 $S1P_1$, $S1P_3$ and YAP are required for S1P-induced PGE2 production

PGE2 is a major COX product that regulates various reproductive functions in females [32]. Given the strong stimulatory effect of S1P on COX-2 expression, we examined the effect of S1P on PGE2 production in human granulosa cells. Our ELISA results showed that treatment with S1P for 3 and 6 h significantly induced PGE2 production (Figure 8A). Pretreatment with $S1P_1$ or $S1P_3$ antagonist and knockdown of $S1P_1$ or $S1P_3$ attenuated S1P-induced PGE2 production (Figures 8B and 8C). Moreover,

S1P-induced PGE2 production was attenuated by siRNA-mediated knockdown of YAP (Figure 8D). These results clearly indicated that S1P induced PGE2 production required $S1P_1$, $S1P_3$ and YAP activation in human granulosa cells.

4. Discussion

Our previous studies have demonstrated that different local factors within the ovary can induce COX-2 expression and PGE2 production in human granulosa cells [33-35]. Although the expression of S1P has been detected in human follicular fluid, to the best of our knowledge, only two studies have directly addressed the biological function of S1P in human granulosa cells. One study showed that treatment with S1P stimulates human granulosa cell migration, which may contribute to the development of the corpus luteum [5]. In addition, the stimulatory effect of S1P on granulosa cell migration is mediated by $S1P_3$, but not other S1P receptors [5]. Another study has shown that S1P is able to inhibit H_2O_2-induced granulosa cell apoptosis and that the anti-apoptotic effect of S1P is mainly mediated through the $S1P_1$ and $S1P_3$ pathways [6]. The expression levels of $S1P_2$ and $S1P_5$ are relatively low when compared with $S1P_1$ and $S1P_3$. In the present study, specific pharmacologic antagonist, agonist and siRNA of $S1P_1$ or $S1P_3$ were used to determine the involvement of S1P receptors in S1P-induced COX-2 expression and PGE2 production. Our results clearly demonstrated that S1P-induced COX-2 expression and PGE2 production were mediated by both $S1P_1$ and $S1P_3$. Taken together, these results indicate that S1P regulates distinct cellular functions in human granulosa cells through activating different receptors.

In the present study, we demonstrated that $S1P_1$ and $S1P_3$ were involved in S1P-induced COX-2 expression and PGE2 production in human granulosa cells. The expression levels of S1P receptors and the requirement of specific S1P receptors for S1P-induced COX-2 expression are varied in different types of cells. In human amnion-derived WISH cells, S1P induces COX-2 expression and PGE2 production by activating $S1P_1$ and $S1P_3$ [18]. In mouse embryonic fibroblast cells, $S1P_1$, $S1P_3$ and $S1P_5$-mediated NFκB signaling is necessary for S1P-induced COX-2 expression [19]. In rat vascular smooth muscle cells, S1P induces COX-2 expression via $S1P_3$ [21]. Interestingly, in human tracheal smooth muscle cells, both $S1P_1$

and $S1P_3$ are required for S1P-induced COX-2 expression and PGE2 production [13]. Moreover, S1P induces COX-2 expression and PGE2 production via $S1P_2$ in rat and human renal mesangial cells [17]. Taken together, these results indicate that the type of S1P receptor required for S1P-induced COX-2 expression is cell type-dependent.

To date, many studies have demonstrated the importance of Hippo signaling in the control of organ size, tissue regeneration, stem cell self-renewal, and cancer development in mammals [36, 37]. Recent studies have shown that YAP activation stimulates follicle growth and has been applied for the treatment of patients with primary ovarian insufficiency [38, 39]. In addition, increased YAP expression and nuclear localization have been detected in human granulosa cell tumors, and YAP stimulates tumor cell proliferation, migration and steroidogenesis [40]. These studies indicate that YAP may play an important role in the regulation of granulosa cell functions. In the present study, similar to studies in other cell types, we demonstrated that YAP was activated by S1P treatment in human granulosa cells. In addition, our results showed that YAP activation was involved in S1P-induced COX-2 expression and PGE2 production. These results are novel in granulosa cells and consistent with a recent study identifying COX-2 as a target gene of YAP [26]. YAP lacks a DNA binding domain and exerts its transcriptional activities mainly by interacting with the TEAD family transcription factors [23]. Therefore, additional studies will be needed to examine the mechanisms that mediate the transcriptional regulation of COX-2 by YAP.

In summary, the present study demonstrates the stimulatory effect of S1P on COX-2 expression and PGE2 production in human granulosa cells. In addition, our results showed that YAP was activated by S1P and partially mediated the S1P-induced COX-2 expression and PGE2 production. Moreover, the effect of S1P on YAP activation, COX-2 expression, and PGE2 production are mediated by both $S1P_1$ and $S1P_3$. These results increase understanding of the physiological roles of S1P in granulosa cells and provide important insight into the molecular mechanisms that mediate S1P-induced COX-2 expression.

5. Acknowledgements

This work was supported by an operating grant from the Canadian Institutes of Health Research

(#143317) to P.C.K.L. P.C.K.L. is a Scientist Level 3 of the Child and Family Research Institute.

6. Conflict of Interest

The authors declare no conflict of interest.

7. References

[1] S. Spiegel, S. Milstien, Sphingosine 1-phosphate, a key cell signaling molecule, The Journal of biological chemistry, 277 (2002) 25851-25854.

[2] S. Spiegel, S. Milstien, Sphingosine-1-phosphate: an enigmatic signalling lipid, Nature reviews. Molecular cell biology, 4 (2003) 397-407.

[3] K. Mendelson, T. Evans, T. Hla, Sphingosine 1-phosphate signalling, Development, 141 (2014) 5-9.

[4] X. Ye, Lysophospholipid signaling in the function and pathology of the reproductive system, Human reproduction update, 14 (2008) 519-536.

[5] S. Becker, S. von Otte, H. Robenek, K. Diedrich, J.R. Nofer, Follicular fluid high-density lipoprotein-associated sphingosine 1-phosphate (S1P) promotes human granulosa lutein cell migration via S1P receptor type 3 and small G-protein RAC1, Biology of reproduction, 84 (2011) 604-612.

[6] T. Nakahara, A. Iwase, T. Nakamura, M. Kondo, Bayasula, H. Kobayashi, S. Takikawa, S. Manabe, M. Goto, T. Kotani, F. Kikkawa, Sphingosine-1-phosphate inhibits H2O2-induced granulosa cell apoptosis via the PI3K/Akt signaling pathway, Fertility and sterility, 98 (2012) 1001-1008 e1001.

[7] S. von Otte, J.R. Paletta, S. Becker, S. Konig, M. Fobker, R.R. Greb, L. Kiesel, G. Assmann, K. Diedrich, J.R. Nofer, Follicular fluid high density lipoprotein-associated sphingosine 1-phosphate is a novel mediator of ovarian angiogenesis, The Journal of biological chemistry, 281 (2006) 5398-5405.

[8] W.L. Smith, D.L. DeWitt, R.M. Garavito, Cyclooxygenases: structural, cellular, and molecular biology, Annu Rev Biochem, 69 (2000) 145-182.

[9] D.M. Duffy, R.L. Stouffer, Follicular administration of a cyclooxygenase inhibitor can prevent oocyte release without alteration of normal luteal function in rhesus monkeys, Hum Reprod, 17 (2002) 2825-

2831.

[10] K.E. Hester, M.J. Harper, D.M. Duffy, Oral administration of the cyclooxygenase-2 (COX-2) inhibitor meloxicam blocks ovulation in non-human primates when administered to simulate emergency contraception, Hum Reprod, 25 (2010) 360-367.

[11] M. Mikuni, M. Pall, C.M. Peterson, C.A. Peterson, P. Hellberg, M. Brannstrom, J.S. Richards, L. Hedin, The selective prostaglandin endoperoxide synthase-2 inhibitor, NS-398, reduces prostaglandin production and ovulation in vivo and in vitro in the rat, Biol Reprod, 59 (1998) 1077-1083.

[12] H. Lim, B.C. Paria, S.K. Das, J.E. Dinchuk, R. Langenbach, J.M. Trzaskos, S.K. Dey, Multiple female reproductive failures in cyclooxygenase 2-deficient mice, Cell, 91 (1997) 197-208.

[13] C.K. Hsu, I.T. Lee, C.C. Lin, L.D. Hsiao, C.M. Yang, Sphingosine-1-phosphate mediates COX-2 expression and PGE2 /IL-6 secretion via c-Src-dependent AP-1 activation, Journal of cellular physiology, 230 (2015) 702-715.

[14] H.L. Hsieh, C.B. Wu, C.C. Sun, C.H. Liao, Y.T. Lau, C.M. Yang, Sphingosine-1-phosphate induces COX-2 expression via PI3K/Akt and p42/p44 MAPK pathways in rat vascular smooth muscle cells, Journal of cellular physiology, 207 (2006) 757-766.

[15] T. Ohama, M. Okada, T. Murata, D.L. Brautigan, M. Hori, H. Ozaki, Sphingosine-1-phosphate enhances IL-1{beta}-induced COX-2 expression in mouse intestinal subepithelial myofibroblasts, American journal of physiology. Gastrointestinal and liver physiology, 295 (2008) G766-775.

[16] S. Mahajan-Thakur, B.D. Sostmann, A.C. Fender, D. Behrendt, S.B. Felix, K. Schror, B.H. Rauch, Sphingosine-1-phosphate induces thrombin receptor PAR-4 expression to enhance cell migration and COX-2 formation in human monocytes, Journal of leukocyte biology, 96 (2014) 611-618.

[17] A. Volzke, A. Koch, D. Meyer Zu Heringdorf, A. Huwiler, J. Pfeilschifter, Sphingosine 1-phosphate (S1P) induces COX-2 expression and PGE2 formation via S1P receptor 2 in renal mesangial cells, Biochimica et biophysica acta, 1841 (2014) 11-21.

[18] J.I. Kim, E.J. Jo, H.Y. Lee, M.S. Cha, J.K. Min, C.H. Choi, Y.M. Lee, Y.A. Choi, S.H. Baek, S.H. Ryu, K.S. Lee, J.Y. Kwak, Y.S. Bae, Sphingosine 1-phosphate in amniotic fluid modulates

cyclooxygenase-2 expression in human amnion-derived WISH cells, The Journal of biological chemistry, 278 (2003) 31731-31736.

[19] S.H. Ki, M.J. Choi, C.H. Lee, S.G. Kim, Galpha12 specifically regulates COX-2 induction by sphingosine 1-phosphate. Role for JNK-dependent ubiquitination and degradation of IkappaBalpha, The Journal of biological chemistry, 282 (2007) 1938-1947.

[20] N.N. Rumzhum, M.M. Rahman, B.G. Oliver, A.J. Ammit, Sphingosine 1-phosphate Increases COX-2 Expression and PGE Secretion: Effects on beta-adrenergic Receptor Desensitization, American journal of respiratory cell and molecular biology, DOI 10.1165/rcmb.2014-0443OC(2015).

[21] A. Nodai, T. Machida, S. Izumi, Y. Hamaya, T. Kohno, Y. Igarashi, K. Iizuka, M. Minami, M. Hirafuji, Sphingosine 1-phosphate induces cyclooxygenase-2 via Ca2+-dependent, but MAPK-independent mechanism in rat vascular smooth muscle cells, Life sciences, 80 (2007) 1768-1776.

[22] D. Pan, Hippo signaling in organ size control, Genes & development, 21 (2007) 886-897.

[23] S. Piccolo, S. Dupont, M. Cordenonsi, The biology of YAP/TAZ: hippo signaling and beyond, Physiological reviews, 94 (2014) 1287-1312.

[24] E. Miller, J. Yang, M. DeRan, C. Wu, A.I. Su, G.M. Bonamy, J. Liu, E.C. Peters, X. Wu, Identification of serum-derived sphingosine-1-phosphate as a small molecule regulator of YAP, Chemistry & biology, 19 (2012) 955-962.

[25] F.X. Yu, B. Zhao, N. Panupinthu, J.L. Jewell, I. Lian, L.H. Wang, J. Zhao, H. Yuan, K. Tumaneng, H. Li, X.D. Fu, G.B. Mills, K.L. Guan, Regulation of the Hippo-YAP pathway by G-protein-coupled receptor signaling, Cell, 150 (2012) 780-791.

[26] W. Zhang, N. Nandakumar, Y. Shi, M. Manzano, A. Smith, G. Graham, S. Gupta, E.E. Vietsch, S.Z. Laughlin, M. Wadhwa, M. Chetram, M. Joshi, F. Wang, B. Kallakury, J. Toretsky, A. Wellstein, C. Yi, Downstream of mutant KRAS, the transcription regulator YAP is essential for neoplastic progression to pancreatic ductal adenocarcinoma, Science signaling, 7 (2014) ra42.

[27] B.L. Lie, E. Leung, P.C. Leung, N. Auersperg, Long-term growth and steroidogenic potential of human granulosa-lutein cells immortalized with SV40 large T antigen, Molecular and cellular

endocrinology, 120 (1996) 169-176.

[28] H.M. Chang, C. Klausen, P.C. Leung, Antimullerian hormone inhibits follicle-stimulating hormone-induced adenylyl cyclase activation, aromatase expression, and estradiol production in human granulosa-lutein cells, Fertility and sterility, 100 (2013) 585-592 e581.

[29] M.C. Quinn, S.B. McGregor, J.L. Stanton, P.A. Hessian, W.R. Gillett, D.P. Green, Purification of granulosa cells from human ovarian follicular fluid using granulosa cell aggregates, Reproduction, fertility, and development, 18 (2006) 501-508.

[30] L. Ophir, Y. Yung, E. Maman, N. Rubinstein, G.M. Yerushalmi, J. Haas, E. Barzilay, A. Hourvitz, Establishment and validation of a model for non-luteinized human mural granulosa cell culture, Molecular and cellular endocrinology, 384 (2014) 165-174.

[31] Y. Morita, G.I. Perez, F. Paris, S.R. Miranda, D. Ehleiter, A. Haimovitz-Friedman, Z. Fuks, Z. Xie, J.C. Reed, E.H. Schuchman, R.N. Kolesnick, J.L. Tilly, Oocyte apoptosis is suppressed by disruption of the acid sphingomyelinase gene or by sphingosine-1-phosphate therapy, Nature medicine, 6 (2000) 1109-1114.

[32] Y. Sugimoto, T. Inazumi, S. Tsuchiya, Roles of prostaglandin receptors in female reproduction, Journal of biochemistry, 157 (2015) 73-80.

[33] L. Fang, J.C. Cheng, H.M. Chang, Y.P. Sun, P.C. Leung, EGF-like growth factors induce COX-2-derived PGE2 production through ERK1/2 in human granulosa cells, The Journal of clinical endocrinology and metabolism, 98 (2013) 4932-4941.

[34] L. Fang, H.M. Chang, J.C. Cheng, P.C. Leung, Y.P. Sun, Nitric oxide and cGMP induce COX-2 expression and PGE2 production in human granulosa cells through CREB signaling pathway, The Journal of clinical endocrinology and metabolism, 100 (2015) E262-269.

[35] L. Fang, H.M. Chang, J.C. Cheng, P.C. Leung, Y.P. Sun, TGF-beta1 induces COX-2 expression and PGE2 production in human granulosa cells through Smad signaling pathways, The Journal of clinical endocrinology and metabolism, 99 (2014) E1217-1226.

[36] J. Dong, G. Feldmann, J. Huang, S. Wu, N. Zhang, S.A. Comerford, M.F. Gayyed, R.A. Anders, A.

Maitra, D. Pan, Elucidation of a universal size-control mechanism in Drosophila and mammals, Cell, 130 (2007) 1120-1133.

[37] B. Zhao, K. Tumaneng, K.L. Guan, The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal, Nature cell biology, 13 (2011) 877-883.

[38] K. Kawamura, Y. Cheng, N. Suzuki, M. Deguchi, Y. Sato, S. Takae, C.H. Ho, N. Kawamura, M. Tamura, S. Hashimoto, Y. Sugishita, Y. Morimoto, Y. Hosoi, N. Yoshioka, B. Ishizuka, A.J. Hsueh, Hippo signaling disruption and Akt stimulation of ovarian follicles for infertility treatment, Proceedings of the National Academy of Sciences of the United States of America, 110 (2013) 17474-17479.

[39] A.J. Hsueh, K. Kawamura, Y. Cheng, B.C. Fauser, Intraovarian control of early folliculogenesis, Endocrine reviews, 36 (2015) 1-24.

[40] D. Fu, X. Lv, G. Hua, C. He, J. Dong, S.M. Lele, D.W. Li, Q. Zhai, J.S. Davis, C. Wang, YAP regulates cell proliferation, migration, and steroidogenesis in adult granulosa cell tumors, Endocr Relat Cancer, 21 (2014) 297-310.

Figure legends

Figure 1. S1P induces COX-2 expression in SVOG cells. A, Cells were treated with vehicle control (PET) or 0.3 μM S1P for different periods of time, and the mRNA levels of COX-1 and COX-2 were examined by RT-qPCR. The level of COX-1 or COX-2 mRNA at each time point was normalized to the GAPDH mRNA level at the same time point. B, Cells were treated with vehicle control (PET) or different concentrations of S1P (0.1-3 μM) for 1 h. The mRNA levels of COX-1 and COX-2 were examined by RT-qPCR. C and D, Cells were treated with vehicle control (PET) or 0.3 μM S1P for 1 and 3 h. The protein levels of COX-1 (C) and COX-2 (D) were examined by western blot. The results are expressed as the mean ± SEM of at least three independent experiments. Values without a common letter were significantly different ($p<0.05$).

Figure 2. S1P induces COX-2 expression in primary culture of human granulosa cells. A, Cells were treated with vehicle control (PET) or 0.3 μM S1P for 0.5, 1 and 3 h and the mRNA levels of COX-2 were examined by RT-qPCR. The level of COX-2 mRNA at each time point was normalized to the GAPDH mRNA level at the same time point. B, Cells were treated with vehicle control (PET) or 0.3 μM S1P for 1 and 3 h. The protein levels of COX-2 were examined by western blot. The results are expressed as the mean ± SEM of four independent experiments. Values without a common letter were significantly different (p<0.05).

Figure 3. $S1P_1$ and $S1P_3$ mediate S1P-induced COX-2 expression. A and B, SVOG cells were pre-treated with vehicle control (DMSO), 10 μM $S1P_1$ antagonist, W146 (A) or 10 μM $S1P_3$ antagonist CAY10444 (B) for 1 h and then treated with vehicle control (PET) or 0.3 μM S1P for 1 h. The mRNA levels of COX-2 were examined by RT-qPCR. C and D, SVOG cells were pre-treated with vehicle control (DMSO), 10 μM $S1P_1$ antagonist W146 (C) or 10 μM $S1P_3$ antagonist CAY10444 (D) for 1 h and then treated with vehicle control (PET) or 0.3 μM S1P for 3 h. The protein levels of COX-2 were examined by western blot. The results are expressed as the mean ± SEM of at least three independent experiments. Values without a common letter were significantly different (p<0.05).

Figure 4. Knockdown of $S1P_1$ or $S1P_3$ attenuates S1P-induced COX-2 expression. A, SVOG cells were transfected with 75 nM control siRNA (si-Ctrl), $S1P_1$ siRNA (si-$S1P_1$), $S1P_3$ siRNA (si-$S1P_3$) or $S1P_1$ siRNA in combination with $S1P_3$ siRNA (si-$S1P_{1+3}$) for 48 h and then treated with vehicle control (PET) or 0.3 μM S1P for 3 h. The protein levels of COX-2, $S1P_1$ and $S1P_3$ were examined by western blot. B, SVOG cells were treated with vehicle control (DMSO) or different concentrations of $S1P_1$ agonist SEW 2871 (0.1, 0.5 and 1 μM) for 3 h. The protein levels of COX-2 were examined by western blot. C, SVOG cells were treated with vehicle control (DMSO) or different concentrations of $S1P_3$ agonist CYM 5541 (0.1, 0.5 and 1 μM) for 3 h. The protein levels of COX-2 were examined by western blot. The results are

expressed as the mean ± SEM of at least three independent experiments. Values without a common letter were significantly different (p<0.05).

Figure 5. $S1P_1$ and $S1P_3$ mediate S1P-induced YAP activation. A, SVOG cells were treated with vehicle control (PET) or 0.3 μM S1P for the indicated durations. The phosphorylation levels of YAP at S127 (p-YAP^{S127}) were examined by western blot. B and C, SVOG cells were pre-treated with vehicle control (DMSO), 10 μM $S1P_1$ antagonist W146 (C) or 10 μM $S1P_3$ antagonist CAY10444 (D) for 1 h and then treated with vehicle control (PET) or 0.3 μM S1P for 30 min. The phosphorylation levels of YAP at S127 (p-YAP^{S127}) were examined by western blot. The results are expressed as the mean ± SEM of at least three independent experiments. Values without a common letter were significantly different (p<0.05).

Figure 6. Knockdown of $S1P_1$ or $S1P_3$ attenuates S1P-induced YAP activation. A, SVOG cells were transfected with 75 nM control siRNA (si-Ctrl), $S1P_1$ siRNA (si-$S1P_1$), $S1P_3$ siRNA (si-$S1P_3$) or $S1P_1$ siRNA in combination with $S1P_3$ siRNA (si-$S1P_{1+3}$) for 48 h and then treated with vehicle control (PET) or 0.3 μM S1P for 30 min. The phosphorylation levels of YAP at S127 (p-YAP^{S127}) and protein levels of $S1P_1$ and $S1P_3$ were examined by western blot. B, SVOG cells were treated with vehicle control (DMSO) or different concentrations of $S1P_1$ agonist SEW 2871 (0.1, 0.5 and 1 μM) for 30 min. The phosphorylation levels of YAP at S127 (p-YAP^{S127}) were examined by western blot. C, SVOG cells were treated with vehicle control (DMSO) or different concentrations of $S1P_3$ agonist CYM 5541 (0.1, 0.5 and 1 μM) for 30 min. The phosphorylation levels of YAP at S127 (p-YAP^{S127}) were examined by western blot. The results are expressed as the mean ± SEM of at least three independent experiments. Values without a common letter were significantly different (p<0.05).

Figure 7. YAP is involved in S1P-induced COX-2 expression. A and B, SVOG cells were transfected with 50 nM control siRNA (si-Ctrl) or YAP siRNA (si-YAP) for 48 h and then treated with vehicle

control (PET) or 0.3 μM S1P. The mRNA (A) and protein (B) levels of COX-2 and YAP were examined by RT-qPCR and western blot, respectively. The results are expressed as the mean ± SEM of at least three independent experiments. Values without a common letter were significantly different (p<0.05).

Figure 8. S1P induces PGE2 production through $S1P_{1/3}$-mediated YAP activation. A, SVOG cells were treated with vehicle control (PET) or 0.3 μM S1P for 3 and 6 h. The levels of PGE2 in culture media were examined using ELISA. B, SVOG cells were pre-treated with vehicle control (DMSO), 10 μM $S1P_1$ antagonist W146 or 10 μM $S1P_3$ antagonist CAY10444 for 1 h and then treated with vehicle control (PET) or 0.3 μM S1P for 3 h. The levels of PGE2 in culture media were examined by ELISA. C, SVOG cells were transfected with 50 nM control siRNA (si-Ctrl), $S1P_1$ siRNA (si-$S1P_1$) or $S1P_3$ siRNA (si-$S1P_3$) for 48 h and then treated with vehicle control (PET) or 0.3 μM S1P for 3 h. The levels of PGE2 in culture media were examined using ELISA. D, SVOG cells were transfected with 50 nM control siRNA (si-Ctrl) or YAP siRNA (si-YAP) for 48 h and then treated with vehicle control (PET) or 0.3 μM S1P for 3 h. The levels of PGE2 in culture media were examined using ELISA. The results are expressed as the mean ± SEM of at least three independent experiments. Values without a common letter were significantly different (p<0.05).