

Syndecan 1 represses cell growth and FSH responsiveness in human granulosa cells

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Abstract

Albeit devoid of intrinsic catalytic activity, the transmembrane heparan sulphate proteoglycan syndecan 1 plays critical roles in cellular processes such as extracellular matrix crosstalk, cytoskeletal organization, cell spreading, proliferation and differentiation. During the ovarian cycle, the expression of syndecan 1 in granulosa cells shows cyclic variation suggesting that it might fulfil specific roles in follicle development. To investigate its physiological roles on granulosa cells, syndecan 1 was overexpressed in human granulosa cell line KGN which retains features of granulosa cells from small antral follicle such as estradiol (E2) synthesis and low expression of functional FSH receptor (FSHR). We demonstrated that overexpression of syndecan 1 in immature granulosa cells (KGN-SDC1) induces a profound alteration in their intrinsic characteristics including enhanced spreading and attachment, both associated with a reduced growth rate. Flow cytometry analysis revealed that syndecan 1 overexpression increases the percentage of KGN cells in quiescent phase. This partial cell cycle exit is concordant with downregulated levels of *CCND1* and *CDK4* and upregulated expression of CDK inhibitor *CDKN1A*. In parallel both unstimulated and FSH-induced E2 synthesis are reduced in KGN-SDC1 through both repression of *CYP19A1* and *FSHR* mRNA associated with decreased levels of potential regulators *NR5A1* and *ESR2*. Additionally, we provide evidence that transient cAMP accumulation reduction in cells overexpressing syndecan 1 is accompanied by an increase in cAMP-hydrolysing PDE activity. Our results demonstrated that syndecan 1 might regulate differentiation of granulosa cells and follicular development by means of various mechanisms involving morphological changes, control of signalling pathways and alterations in gene expressions.

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Introduction

Syndecan 1, a member of transmembrane heparan sulphate proteoglycan (HSPG) family composed of four members, is expressed in cell- and tissue-type specific patterns. Composed of three major domains – a short cytoplasmic domain, a membrane-spanning domain and a long extracellular domain bearing heparan sulphate chains – syndecan 1 acts as cell surface receptor, regulating adhesion-dependent signalling pathways during cell growth, migration and differentiation (Bernfield *et al.* 1999, Couchman 2010). Through binding with the extracellular matrix components (ECM) and/or soluble ligands, syndecan 1 could recruit or tether, near the inner plasma membrane, some scaffolding proteins, thus acting as a regulator of membrane signalling pathways (Couchman 2010). Syndecan 1 can potentiate or inhibit some growth factor/tyrosine kinase receptor interactions through its HS (Bernfield *et al.* 1999).

Additionally, syndecan 1 might be translocated into the nucleus to modulate transcription factors activities or induce histones post-translational modifications thus modulating the expression of genes (Brockstedt *et al.* 2002, Szatmari *et al.* 2012, Kovalszky *et al.* 2014). Hence, despite the lack of intrinsic catalytic activity, syndecan 1 plays significant roles in cell–cell and cell–matrix adhesion, in regulation of cell growth as well as in differentiation during the developmental processes.

During follicular development, granulosa cells are essential for normal cycle and oocyte development. From the time they enter the selectable stage during the follicular phase, human granulosa cells from pre-antral follicles express FSH receptors and become sensitive to cyclic changes of FSH in terms of granulosa cell proliferation (Oktay *et al.* 1997). A small decrease in FSH concentration in growing follicles is enough to induce apoptosis of granulosa cells (Chun *et al.* 1996).

Ovarian follicles co-ordinately respond to endocrine and paracrine signals in order to mediate maturation of oocytes and cyclic steroid production. Although soluble signalling molecules have been recognized as important factors in follicles growth, development and differentiation, extracellular matrix elements also play a critical role in these functions (Rodgers & Irving-Rodgers 2010, Woodruff & Shea 2011). The extracellular matrix has an influence in granulosa cells shape and functions as for instance proliferation, survival as well as expression of steroidogenic enzymes and gonadotrophin receptors (Huet *et al.* 2001, Berkholtz *et al.* 2006). General biosynthesis and cellular HSPG distribution in granulosa cells are FSH regulated (Yanagishita *et al.* 1981, Adashi *et al.* 1986, Hosseini *et al.* 1996) and syndecan-1 appeared to be strongly induced in response to the LH surge in cumulus oocyte complex (COC) but not in mural granulosa cells during oocyte maturation (Watson *et al.* 2012). In ovarian granulosa cells, syndecan 1 is thus expressed in a temporo-spatial manner (Ishiguro *et al.* 1999, Oksjoki *et al.* 1999, Princivalle *et al.* 2001) suggesting that syndecan 1 fulfils specific roles in the regulation of granulosa cell functions, follicular growth and oocyte maturation (Tsuiki *et al.* 1988, Sato *et al.* 1990). These assumptions were corroborated by *in vitro* experiments showing that deregulation of proteoglycan synthesis could affect FSH-mediated response in rabbit granulosa cells (Benhaim *et al.* 1995) as well as in rat Sertoli cells (Levallet *et al.* 2013).

Although syndecan 1 assumes crucial role during early development, SDC1 deficient mice are healthy and fertile (Alexander *et al.* 2000); this would imply that other syndecans or HSPGs can compensate for the loss of syndecan-1 (Teng *et al.* 2012). Thus, to investigate the biological functions of syndecan 1 on steroidogenesis and differentiated functions of granulosa cell, syndecan 1 has been overexpressed in a steroidogenic human granulosa-like tumour cell line KGN. The KGN cell line is a metastatic granulosa cell line established from a 73-year-old patient with recurrent, granulosa cell tumour (GCT) in the pelvic region (Nishi *et al.* 2001). Although KGN cell harbours C402G somatic mutation in a Forkhead transcription factor FOXL2, described in 97% of adult-type GCTs, these cells retain both proliferative and steroidogenic capacity of normal granulosa cells. The expression of a functional FSH signalling pathway that can stimulate aromatase activity and the absence of LH response (confirmed by absence of *LHCGR* expression (Supplementary data)) designated these cells as an immature granulosa cell model of interest to study early follicular development.

Materials and methods

Materials

Recombinant FSH was purchased from Merck-Serono (Gonal F-75, Merck-Serono, France). Dulbecco's Modified Eagle

Medium (DMEM), Ham F12 medium, trypsin, FCS (fetal calf serum), pcDNA3.1 Directional TOPO expression kit, Geneticin, Lipofectamine2000, ECL staining kit and Hybond-ECL nitrocellulose membrane were purchased from Life Technologies SAS. Δ 4-Androstenedione (Androst-4-ene-3,17-dione) [1β - 3 H(N)] (AS 26.3 Ci/mmol) was purchased from Perkin Elmer. [3 H] cAMP Assay Kit (TRK432) was purchased from GE Healthcare (Vélizy-Villacoublay, France). Anti-tyrosine-tubulin antibody, kanamycin, penicillin, peptidase A, streptomycin and mouse anti-tyrosine-tubulin (TUBA) were purchased from Sigma-Aldrich. FSHR (N-20) antibodies, HRP-, TR- and FITC-conjugated secondary antibodies were from Santa Cruz Biotechnology and monoclonal mouse anti-actin (Ab-1) from Millipore SAS. Cell cycle analysis was performed using Coulter DNA-Prep Reagents kit (Beckman Coulter, France). M-MLV-RT, random primers, dNTPs, RNasin, Taq DNA polymerase, GoTaq qPCR Master Mix were purchased from Promega.

Cell culture

The KGN cell line originated from a Stage III granulosa cell carcinoma removed from a 63-year-old Japanese women in 1984 (Nishi *et al.* 2001) was obtained from RIKEN Bioresource Center (Tsukuba, Japan). Cells were cultured at 37°C under 5% CO₂ atmosphere in Ham F12-Dulbecco's Modified Eagle Medium (HamF12/DMEM, 1/1: v/v (H/D)) containing antibiotics (50,000 IU/L penicillin, 50 mg/L streptomycin and 50 mg/L kanamycin), fungizone (0.25 mg/L) and 2.2 g/L sodium bicarbonate. The culture medium was supplemented with 10% (v/v) fetal calf serum (FCS) as described by Nishi and coworkers. Two or three times a week, when confluence reached 70–80%, the cells were harvested by trypsinization. They were then centrifuged for 5 min at 400g and subjected to experiments or were cultured at 1×10^4 cells/cm² in 25 cm² culture flask. Cells were cultured for at most 10 successive passages during which the properties of the KGN granulosa cells did not undergo significant changes. Human luteinized granulosa cells (LGC) were collected from patients with normal ovulatory function undergoing IVF at the ART unit of the reproductive medicine department of the University Hospital of Caen. All patients gave informed consent before their inclusion in the study. Cells were collected by centrifugation for 10 min at 300g, overlaid on Ficoll (PAN Biotech, France) and centrifuged at 600g for 5 min as described in Khalaf and coworkers (Khalaf *et al.* 2010). Lutein granulosa cells were aspirated from the interface, washed twice and submitted to RNA extraction.

Generation of stably engineered cell lines

The full length of human syndecan 1 cDNA (933 bp length) was prepared from luteinized human granulosa cells and subcloned into a pcDNA 3.1(+) Directional TOPO Expression vector. The PCR primers were designed based on the full length of mRNA (NM_001006946) with the following sequences: forward primer, 5'-CACCATGAGGCGCGCGGCGCTC-3'; reverse primer and 5'-TCAGGCATAGAATTCCTCCTG-3'. The control plasmid was made by inserting into pcDNA 3.1(+) Directional TOPO Expression vector, a 750 bp PCR product provided by supplier that does not contain Kozak sequence

and ATG codon. Stable transfection of KGN cells was done using Lipofectamine2000 and selected with Geneticin at 400 µg/mL and subsequently grown in HamF12/DMEM-10% FCS in the presence of Geneticin at 50 µg/mL.

Cell growth assays and cell cycle analysis

Cellular growth was assayed using the crystal violet (CV) staining method with a 24-h interval, over a period of 5 days and after a 24-h adhesion period (starting density of 1×10^4 cells/well in 96-well plates). Cells were washed with PBS and stained with 0.1% CV. Dye was eluted with 100 µL of 10% acetic acid, and absorbance at 600 nm was measured using a Metertech Σ 960 Microplate reader. For flow cytometry analysis, cells were cultured in HamF12/DMEM-10% FCS until 70–80% confluence and harvested with PBS-EDTA containing 0.25% Trypsin. One million cells were fixed in ice-cold 75% ethanol, and stained with propidium iodide (DNA-Prep Reagents kit). The cell cycle analysis was carried out using the Gallios Flow Cytometer System and analysed with Kaluza Analysis Software (Beckman Coulter).

Immunofluorescence and microscopy

The cells were seeded onto a cover glass (3000 cells/12 mm diameter) in HamF12/DMEM-10% FCS for 24 h. Afterward, cells were fixed for 15 min in 3% paraformaldehyde and permeabilized with ice-cold 100% methanol for 10 min at -20°C . Non-specific antigen sites were blocked with PBS-0.3% Triton X-100 supplemented with 5% BSA for 1 h at room temperature and cells were then incubated overnight in a humidified dark chamber at $+4^\circ\text{C}$ with polyclonal Syndecan 1 (1:50) or monoclonal anti-tyrosine-tubulin (1:100) antibodies diluted in PBS containing 0.3% Triton X-100 and 1% BSA. After primary antibody incubation and washing, the cells were incubated with fluorescent conjugated secondary antibody (1:200) for 1 h at room temperature. The coverslips were mounted with UltraCruz mounting medium containing 1.5 µg/mL of 4',6-diamidino-2-phenylindole (DAPI) for DNA counterstaining and observed by confocal laser scanning microscopy using a FluoView-FV1000, Olympus. Fluorescence signal collection, cell spreading and scaling were performed through the control software (FV10-ASW-1.7, Olympus).

Quantitative and semi-quantitative RT-PCR

Total RNA was extracted from subconfluent cell cultures using an RNeasy kit. The synthesis of cDNA was performed through reverse transcription of 250 ng RNA using 200 IU M-MLVRT, 20 IU RNasin, 0.5 µg oligo dT and 100 µmol/L dNTP in a total volume of 20 µL.

Semi-quantitative RT-PCR was performed with 1.5 IU Taq DNA polymerase in PCR buffer containing 200 µmol/L dNTP, 1.5 mmol/L MgCl_2 and 25 pmol of each primer in a total volume of 25 µL. The PCR primers used and the size of the resulting PCR products are listed in [Supplementary Table 1](#) (see section on [supplementary data](#) given at the end of this article). For semi-quantitative real-time RT-PCR, reverse transcription was made with 250 ng of total RNA in 20 µL

containing 20 nmol dNTP, 100 ng random primers, 12 IU RNasin and 200 IU M-MLVRT for 1 h 30 min at 37°C . QPCR was performed in duplicate using an Agilent Technologies Mx3005P real-time PCR machine (Agilent Technologies) and the detection was done by the fluorescent dye SYBRGreen with GoTaq qPCR Master Mix (Promega). Expression levels were calculated according to the $2^{-\Delta\text{Ct}}$ method normalized to the Actin mRNA expression level.

Hormonal assays

Production of estradiol (E2) and progesterone (P4) were determined in 25–100 µL incubation mediums by radioimmunoassay. Cells were seeded at 5×10^4 cells/well in 96-well plates in triplicate and cultured for 48 h in HamF12/DMEM-10% FCS followed by 24 h in 1% FCS medium. Cells were incubated for 48 h with HamF12/DMEM-1% FCS with or without 100 ng/mL rFSH. For E2 assay, medium was supplemented with 200 ng/mL testosterone. Supernatants were collected and stored at -20°C until RIA with specific antibodies (Bioss, Paris antibody, France). Cell number per well was estimated using the crystal violet (CV) method and steroid production expressed as ng of steroids per million cells.

Aromatase assay

The aromatase activity was determined by measuring the amount of $[3\text{H}] \text{H}_2\text{O}$ released upon the conversion of $[1\beta\text{-}3\text{H}]$ androstenedione to estrone. Cells were seeded at 15×10^4 cells/well on a 6-well plate for 48 h in HamF12/DMEM-10% FCS then for an additional 24-h period in 1% FCS containing medium. Cells were further incubated with 42.5 nM $[1\beta\text{-}3\text{H}]$ androstenedione (SA, 26.5 Ci/mmol) for 3 h at 37°C . After incubation, the reaction was stopped by the addition of 3 mL ice-cold chloroform, and the supernatant was centrifuged for 10 min at 4000 g to remove precipitated proteins. Aqueous phase was removed (800 µL) and combined to 800 µL of activated-charcoal (7%) suspension containing dextran (1.5%). After 10 min the charcoal was separated from the aqueous phase by centrifugation (2700 g, 15 min, 4°C), and the radioactivity of an aliquot of supernatant was counted by liquid scintillation. The cell protein content was determined after the cell layers were dissolved in 0.1 mol/L NaOH and the aromatase activity was expressed as picomoles per mg of cell protein per hour of incubation.

Cyclic AMP extraction and measurement

KGN cells were seeded at 12×10^4 cells/well on a 6-well plate for 48 h in HamF12/DMEM-10% FCS and then again for 24 more hours in 1% FCS containing medium prior to the addition of FSH (100 ng/mL) or Forskolin (1 µM) for varying times up to 3 h. At the end of incubation, the cultured medium (1 mL) was collected and cells were scraped with 2 mL 100% ethanol. Then both the medium and cell lysate were pooled and centrifuged (2000 g, 15 min, 4°C). Supernatant was collected and evaporated before being resuspended in 200 µL Tris (5 mM, pH 7.5)-EDTA (4 mM). Aliquots of 50 µL were assayed for cAMP content using cAMP assay kit following

manufacturer's instructions ([³H] cAMP Assay Kit (TRK432), GE Healthcare). Total cAMP contents were firstly calculated as pmol/well and further corrected by cell number counted from parallels plates to give final values in pmol/cells.

Cell fractions' preparation

Cultured KGN granulosa cells were washed with cold PBS and were recovered by gentle scraping in lysis buffer (20mM Tris-HCl pH 7.2, 1mM EDTA, 250mM sucrose) supplemented with 0.1mM phenylmethanesulfonyl fluoride, 2mM benzamidin and a mixture of antiproteases (antipain, aprotinin, leupeptin, pepstatin A) at a final concentration of 1 µg/mL and were centrifuged at 100,000g for 1h at 4°C as previously described (Levallet *et al.* 2007). The calculation of protein concentration of the samples was done using Bradford method and the supernatant (soluble fraction) and the pellet (particulate fraction) were then stored at -20°C.

PDE assay

Phosphodiesterase activities were assayed according to the Thompson and Appleman two-step procedure (Thompson & Appleman 1971). Protein mixture (5–20 µg) was incubated at 34°C for 15 min in 200 µL of reaction buffer (40mM Tris-HCl pH 8, 1mM MgCl₂, 1.25mM β-mercapto-ethanol, 0.14mg BSA) in the presence of 1 µM cAMP ([2,8-³H]-cAMP (3.7 kBq/tube)). The reaction was then stopped by the addition of 200 µL of stop solution (40mM Tris-HCl pH 7.5, 10mM EDTA) followed by heat denaturation at 100°C for 1 min. *Crotalus atrox* snake venom (50 µg) was added to each sample and incubated at 34°C for 20 min. The reaction products were separated by anion exchange chromatography using a freshly prepared solution of Dowex (Dowex/water/ethanol: 1/1/1: w/v/v) and an aliquot of supernatant was then counted by liquid scintillation.

Western blot

Cell fractions were obtained by ultracentrifugation as previously described, the supernatant contained soluble cytosolic proteins and the pellet contained membrane-associated proteins (Levallet *et al.* 2013). The proteins (25 µg) of each subcellular fraction were separated by SDS-PAGE and electroblotted onto a Hybond-ECL nitrocellulose membrane. The membranes were probed with syndecan 1 (C-20) or FSHR (N-20) primary antibodies diluted (1:500) in PBS-Tween, 0.1% BSA. Membranes were washed and incubated for 1h at 4°C with HRP-conjugated secondary antibody and revealed with ECL reagents. Subsequently, densitometric analyses of the bands were semi-quantitatively conducted using TotalLab Image Analysis software (Nonlinear Dynamics Ltd., Newcastle, UK). For loading control, the membranes were stripped and re-probed with mouse anti-actin (Ab-1).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism software. All experiments were performed at least three times.

Statistical comparisons were performed with Student's *t*-test when two variables were involved or by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test when appropriate, using the Prism 5 software package (GraphPad Software). A *P* value of <0.05 was considered significant.

Results

Syndecan 1 overexpression enhances attachment and spreading of KGN cells

Plasmids containing full length human syndecan 1 (SDC1) cDNA or control (Ctr) sequence were transfected into granulosa cell line KGN to generate through not clonal selection KGN-SDC1 and KGN-Ctr cell lines respectively. The SDC1 overexpression in KGN-SDC1, compared with KGN-Ctr and wild type (KGN-WT) cell lines, was confirmed both at mRNA level by semi-quantitative and real-time RT-qPCR and at protein level by western blot and immunofluorescence staining. Interestingly, syndecan 1 expression level obtained in KGN-SDC1 cell line is comparable to those measured in luteinized granulosa cells. Furthermore, it is of note that Syndecan-4 expression level, another member of syndecan family, was not affected by SDC1 overexpression; this suggests the absence of compensatory mechanism between these two syndecans (Supplementary Fig. 1). As shown in Fig. 1A, differences in cell attachment were observed after 30, 60 and 90s of trypsin-EDTA treatment between KGN-SDC1 and KGN-Ctr with lower numbers of rounded cells observed in KGN-SDC1 line (*P*<0.001 at respective time). Additionally, using tyrosine-tubulin staining, we observed that cell morphology and cytoskeletal system were affected by syndecan 1 overexpression (Fig. 1B). Unlike KGN control cell lines, displaying a spindle shape at low seeding density with diffuse microtubules structure, KGN-SDC1 cells exhibited increasing spreading with microtubule network, stretching radially from the perinuclear region to the plasma membrane. The morphometric analysis reveals that KGN-SDC1 cells display 3-fold enlarged cytoplasm than KGN control cells (Fig. 1C).

Syndecan 1 overexpression promotes cell cycle exit through cell cycle transcriptional regulation

Because increased spreading is often associated with alterations in cell growth, we have analysed the growth of KGN cell lines. We demonstrated that cell line expressing high level of syndecan 1 exhibits a significant reduction in growth rate obtained after 72 h and 96 h of culture in H/D medium supplemented with 10% FCS (Fig. 2A) or only after 96 h in H/D-1% FCS (not shown). The calculated doubling times revealed that KGN-SDC1, cultured in H/D-10% FCS, needs nearly twice as much time for doubling than control (52.4 ± 2.3 h vs 32.1 ± 6.5 h respectively) (Fig. 2B). In line with these

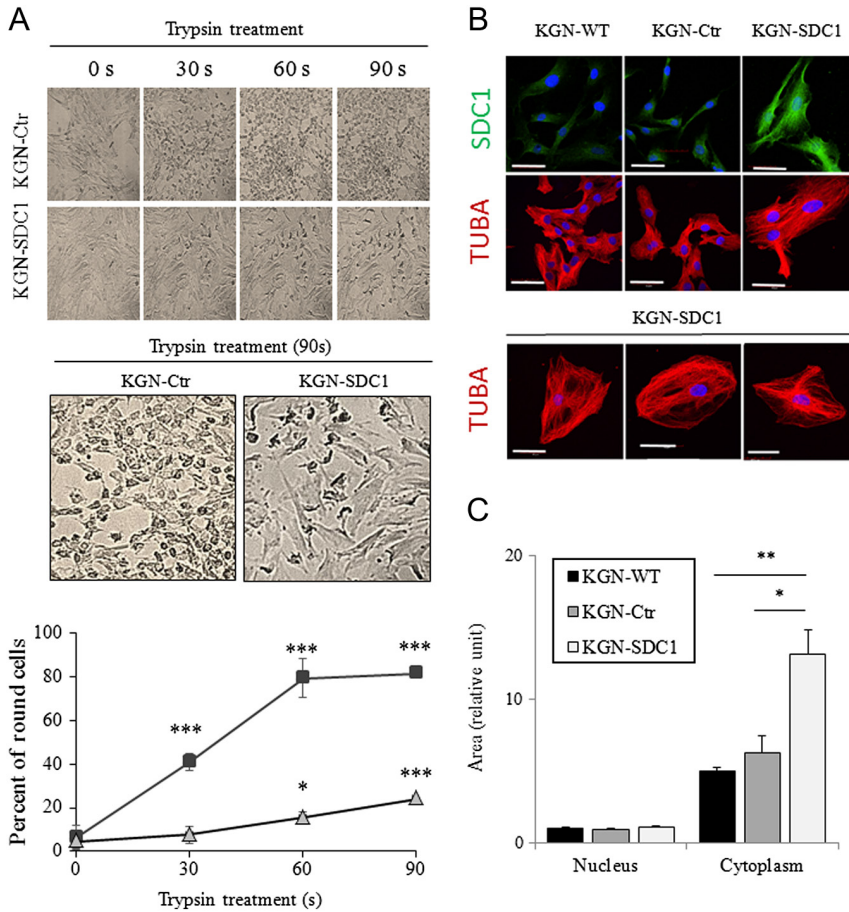


Figure 1 Syndecan 1 overexpression increases the attachment and spreading of granulosa cells. (A) KGN-Ctr and KGN-SDC1 cells were cultured in 10% FCS culture media until 70–80% confluence; cell attachment was monitored with phase-contrast light microscope after 30, 60 and 90 s of trypsin-treatment with 0.25% (w/v) trypsin in PBS containing 1 mM EDTA. (Magnification $\times 20$ or $\times 40$ for upper and lower panel respectively.) Rounded cells were counted and express as mean \pm s.e.m. of three independent experiments (each performed in duplicate wells with 2–3 counting/well). $*P < 0.05$ and $***P < 0.001$ vs 0 s for respective cell line. (B) Morphology of growing KGN cell lines observed by immunocytochemistry using anti-SDC1 (SDC1) or anti-tyrosine-tubulin (TUBA) antibodies respectively stain with FITC-labelled and TR-labelled secondary antibodies and counterstained with nuclear dye DAPI. (Scale bar = 50 μ m.) (C) Cell and nucleus areas were measured using FV10-ASW-1.7, Olympus software and express as relative unit compared to KGN-WT. Data were means \pm s.e.m. from three independent immunocytochemistry preparation (around 40 individualized cells were analysed). $*P < 0.05$; $**P < 0.01$ for cytoplasm area.

results, significant accumulation of KGN-SDC1 cells in G0/G1 phases, with a concomitant reduction in G2/M phases, was observed in comparison with the distribution of both KGN-Ctr cells (Fig. 2C and D) or wild type cells (data not shown). Among KGN-SDC1 cells, a significant increase of cells with deficit in DNA content, defined as ‘sub-G1’ subpopulation, has been observed, but proportion of these cells remains very low (less than 2%). The mRNA expression of the cell cycle’s main regulators was then estimated by RT-qPCR. In the KGN control cell line (as in KGN-WT (not shown)), a specific expression panel of cyclins (Fig. 3A), cyclin-dependent kinases (Fig. 3B) and CDK inhibitors (Fig. 3C) was observed with *CCND1* and *CDKN1A* having the highest expression levels while the *CDKs* display comparable levels. Overexpression of SDC1 in KGN cell line was accompanied by a significant reduction of *CCND1*, *CCNE1*, *CDK4* and *CDKN1B* gene expression while only *CDKN1A* expression was found significantly increased.

Syndecan 1 overexpression alters oestrogen synthesis through transcriptional inhibition of CYP19A1

To investigate involvement of syndecan 1 as a modulator of steroidogenesis in granulosa cells, KGN cells were

cultured in H/D medium supplemented with only 1% FCS to minimize cross-reaction with serum components; progesterone (P4) and estradiol (E2) levels were measured in medium after 48 h of culture. Secreted progesterone levels were similar (around 3 ng/10⁶ cells) into culture medium of wild type KGN or in both transfected cell lines (Fig. 4A). However, estradiol production by KGN-SDC1 cells was significantly reduced (3.4 \pm 1.6 ng/10⁶ cells) as compared to levels measured in parental or control KGN cells medium (11.7 \pm 3.5 and 10.1 \pm 2.3 ng/10⁶ cells respectively) (Fig. 4B). In agreement, both cytochrome P450 aromatase activity (Fig. 4C) and *CYP19A1* mRNA expression (Fig. 4D) were reduced in KGN-SDC1 cells respectively by 5- and 20-fold as compared to the control cell lines. The expression of other key enzymes involved in P4 and E2 synthesis were also analysed. As shown in Fig. 4E, the cholesterol side-chain cleavage enzyme, encoded by the *CYP11A1* gene, which initiates steroidogenesis by converting cholesterol to pregnenolone, was also found downregulated by syndecan 1 while steroidogenic acute regulatory protein (*STAR*) and 3beta-hydroxysteroid dehydrogenase/delta5-delta4 isomerase (*HSD3B2*) remained unaffected. Additionally, the expression of transcriptional regulators of genes encoding steroidogenic enzymes including *NR5A1* (–50%) or *ESR2* (–83%) was significantly

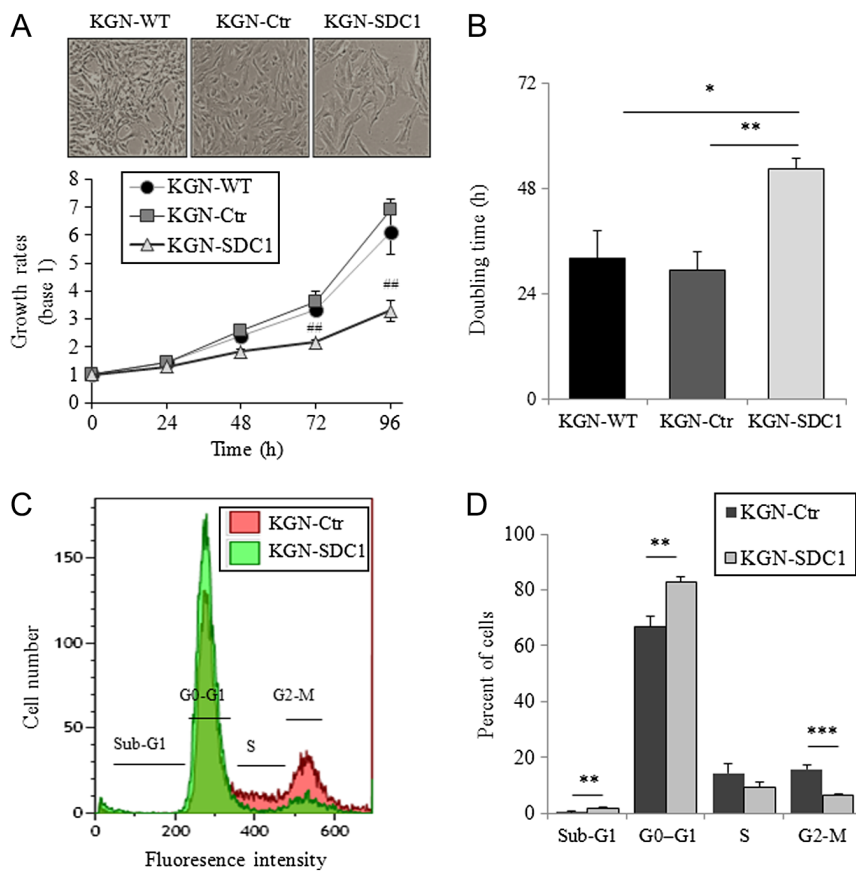


Figure 2 Overexpression of syndecan 1 reduces growth rate and promotes cell cycle exit of KGN granulosa cells. (A) KGN cells were cultured for up to 96 h in 10% FCS culture media and cell densities observed by phase-contrast microscopy (upper panel) and were quantified with crystal violet as described in the 'Materials and methods' section. Values were means \pm S.E.M. of absorbance at 600 nm expressed as fold increased related to each cell lines ($n=7$) and compared by Student's t -test at each culture time (** $P<0.01$). (B) Doubling time was calculated from the log phase (Roth V. 2006 <http://www.doubling-time.com/compute.php>). * $P<0.05$; ** $P<0.01$. (C) Cell cycle progression was determined by flow cytometry using Coulter DNA-Prep Reagents kit (representative experiment). (D) Percentages of cells in sub-G1; G0-G1, S and G2-M phases were means \pm S.E.M. from 3 independent experiment and compared by Student's t -test. ** $P<0.01$ and *** $P<0.001$.

reduced in KGN-SDC1. No significant differences were found for oestrogen receptors alpha (*ESR1*) but a decrease in *PGR* expression has been noticed. In our experimental conditions, and in agreement with Nishi and coworkers observations, luteinizing hormone/choriogonadotrophin receptor (*LHCGR*) expression could be evidenced neither in wild type KGN nor in transfected KGN cell lines.

Syndecan 1 suppresses FSH-mediated estradiol synthesis and forskolin-dependent cAMP accumulation

During follicular development, FSH enhances estradiol biosynthesis through the classical signalling cascade activated by binding FSH to its receptor (FSHR) and adenylyl cyclase (AC)/cAMP/protein kinase A (PKA) pathway. To go further into the consequences of syndecan 1 overexpression, the FSH signalling pathway was directly investigated by the addition of FSH or by bypassing FSHR step using the adenylate cyclase activator forskolin (10 μ M) or the dbcAMP (1 mM), a non-hydrolysable analogue of cAMP. To preserve their sensitivity to hormone stimulation, the cells were cultured in a medium containing only 1% FCS. As shown in Fig. 5A, FSH-induced estradiol secretion was totally abrogated in KGN-SDC1 cells although relatively low in KGN-WT and KGN-Ctr. Consistently, a drastic repression of *FSHR* mRNA expression was observed

(Fig. 5B) in these cells which is also evidenced at protein level, by a 4-fold reduction of the FSHR band intensity in membrane-associated fraction of KGN-SDC1 cells (Fig. 5C). In contrast, dbcAMP which increases oestrogen production in both control cell lines (Fig. 5A) totally restores this ability in the syndecan-1 overexpressing KGN cells. Unlike dbcAMP, amplitude of forskolin-induced E2 production was found reduced in KGN-SDC1 cell line, from 10.2- or 9.1-fold respectively in KGN-WT and KGN-Ctr to 3.9-fold in KGN-SDC1. The difference between forskolin and dbcAMP responses in KGN-SDC1 cells was also observed in term of CYP19A1 mRNA expression (Fig. 5D) as illustrated by a significant reduction of Forskolin inducing factor, as compared to dbcAMP which has not been observed in control cell line.

Overexpression of syndecan-1 reduces forskolin-induced cAMP accumulation and increases the phosphodiesterase activity

The production of cAMP was measured in KGN cells in response to FSH and forskolin treatment. As shown in Fig. 6A, a slight but significant 2-fold increase in cAMP production was measured after 3 h of FSH treatment in KGN-WT, while in agreement with reduced FSHR expression; FSH is unable to increase cAMP production in granulosa cells expressing high levels of syndecan 1.

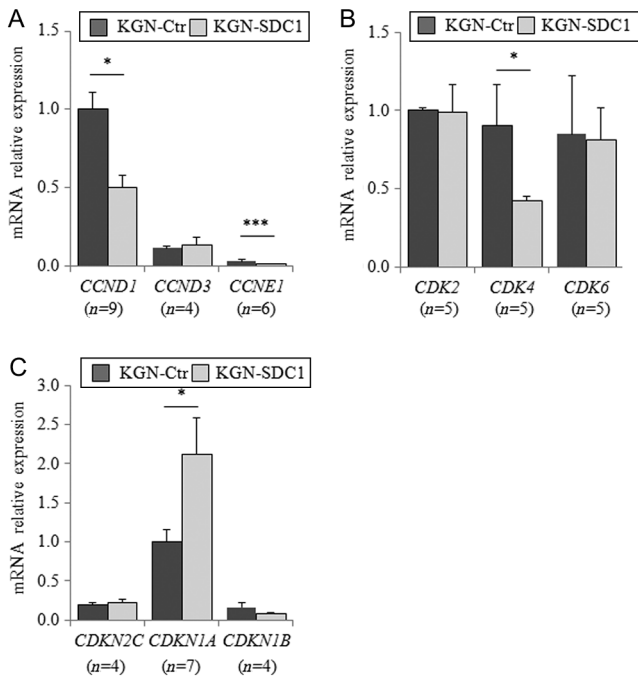


Figure 3 Syndecan 1 overexpression alters expression of cell cycle regulators. Expressions of cyclin (A), cyclin-dependent kinase (B) and their inhibitors (C) were measured by real-time PCR after RNA extraction and reverse transcription from cells cultured in H/D-10% FCS until 70–80% confluence. The results were normalized using actin as an internal control, calculated according to $2^{-\Delta Ct}$ method, and then expressed as relative expression compared to KGN-Ctr *CCND1*, *CDK2* and *CDKN1A*. Values are means \pm S.E.M. of qRT-PCR from 4 to 9 separate cell cultures. Significantly different at * $P < 0.05$; and *** $P < 0.001$ vs KGN-Ctr using Student's *t*-test.

The addition of forskolin, used as adenylylase activator, provokes a time-dependent cAMP accumulation in KGN-WT cells (Fig. 6B). However, in KGN-SDC1 cell line, in which basal level of cAMP was reduced, forskolin induced only a 3.4-fold accumulation cAMP after 3 h of treatment corresponding to 70% reduction of the response intensity as compared to KGN-WT (Fig. 6C). According to the of Thompson and Appleman two-step modified procedure (Thompson & Appleman 1971), we evaluated cAMP specific phosphodiesterase activity associated either to cytosolic or to membrane compartment of KGN cells (Fig. 6D). In both cytosol and particulate fraction of KGN-SDC1, cAMP-PDE activity was found to be significantly increased as compared to the level measured in KGN-WT cells (2.3- and 2.8-fold respectively) thus showing that syndecan 1 might regulate activities of enzymes which catalyse cAMP hydrolysis known as keys regulators of intracellular concentration of this second messenger.

Discussion

In the present study, we demonstrated that syndecan 1 overexpression in human immature granulosa cells

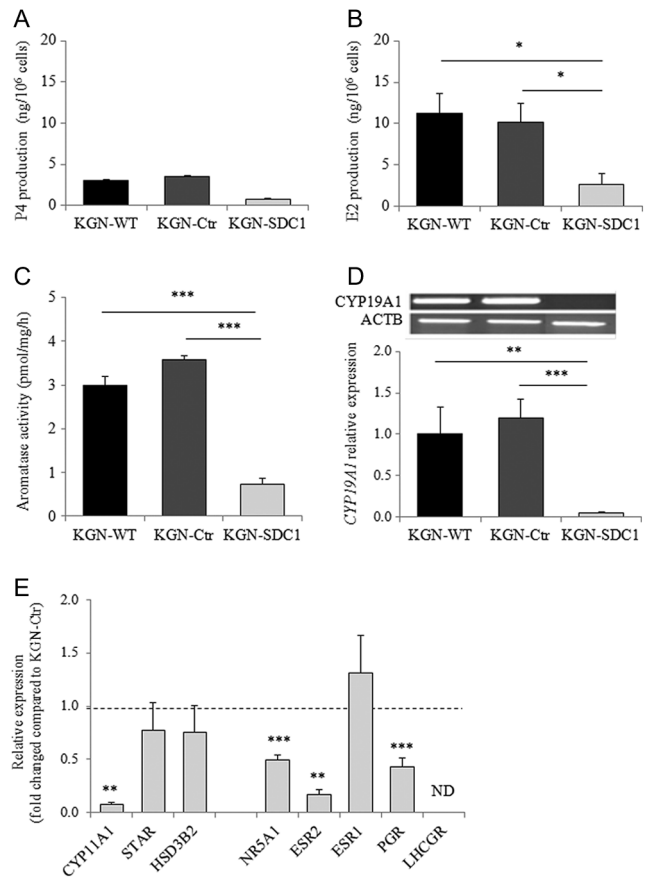


Figure 4 Syndecan 1 overexpression inhibits estradiol synthesis and aromatase expression in KGN cells. (A) Progesterone and (B) estradiol secretion by KGN cells cultured in H/D-1% FCS supplemented with 200 ng/mL of testosterone for E2 measurements. Steroids were measured in medium by RIA after 48 h of culture. The results were the mean \pm S.E.M. of 8 separate triplicate experiments. (C) Aromatase activity was evaluated by measuring tritiated water released by 42.5 nM (1 β -3H) androstenedione for 3 h at 37°C as described in the 'Materials and methods' sections. Values were the mean \pm S.E.M. of three triplicated measurements. (D) Expression of CYP19A1 mRNA was estimated by semi-quantitative (upper panel) and real-time RT-PCR (lower panel). The results were normalized using actin as an internal control and were the mean \pm S.E.M. of qRT-PCR from 10 separated culture. The obtained data were compared to the expression in KGN-Ctr using Student's *t*-test. ** $P < 0.01$ and *** $P < 0.001$.

KGN induces a profound alteration of their intrinsic characteristics such as changes in cell morphology and the expression of genes related to differentiated function of granulosa cell that leads to a reduced growth rate, alteration of FSH signalling and steroidogenesis. Granulosa cells expressing high level of syndecan 1 (KGN-SDC1) exhibited an enlargement of the cytoskeletal network, associated with an increased cell spreading and adhesion. Consistently, through its ability to transmit signals from outer to inner membrane (Hozumi *et al.* 2010, Ishikawa & Kramer 2010), syndecan 1-mediated morphological changes were

also accompanied by a reduced growth rate of KGN-SDC1 cells. Accumulation of KGN cells overexpressing SDC 1 in quiescent phase G0/G1 and the concomitant reduction of cells progressing throughout S and G2/M phases is in line with a downregulation of cyclin D1 (*CCND1*) and *CDK4* expression and an increased level of the cyclin-dependent kinase inhibitor 1A (*CDKN1A*) observed in KGN-SDC1. Involvement of syndecan 1 in cell cycle control and proliferation had already been described (Zong *et al.* 2010, Ibrahim *et al.* 2012) as in human granulosa cell tumour, in which a decreased expression of syndecan 1 was observed (Owens *et al.* 2002). Interestingly, in a human malignant mesothelioma

cell line, genes altered by syndecan 1 overexpression belong to cell adhesion and cell cycle regulation, with the majority being downregulated especially those driving the G1 phase and G1/S transition (Szatmari *et al.* 2012). The cytoplasmic tail of syndecan 1 core protein possesses a nuclear localization signal sequence and a tubulin-dependent translocation of syndecan 1 into the nucleus have been observed (Zong *et al.* 2011). Nuclear syndecan 1 can interact with transcription factors or histones to regulates the transcriptional machinery and transcription level of target genes (Brockstedt *et al.* 2002, Chen & Sanderson 2009, Szatmari & Dobra 2013, Stewart & Sanderson 2014).

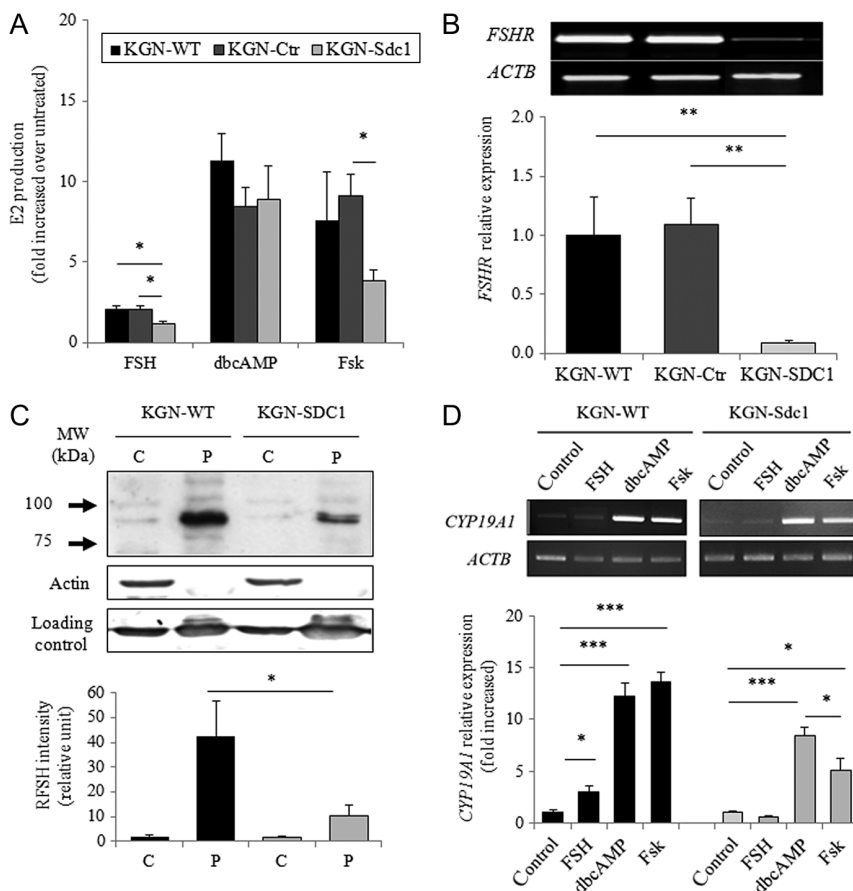


Figure 5 Syndecan 1 overexpression decrease FSH responsiveness through downregulation of FSHR expression. (A) Estradiol production by KGN cells incubated during 48 h in HamF12/DMEM-1% FCS medium supplemented with 200 ng/mL testosterone with or without 100 ng/mL rFSH, 1 mM dbcAMP or 10 μ M Forskolin was measured by RIA. Estradiol contents are expressed as fold induction compared to untreated cells. Each value indicates the mean \pm s.e.m. of 3 experiments. * P < 0.05 by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test for each treatment. (B) Expression of FSHR mRNA in KGN cells by semi-quantitative (upper panel) and real-time RT-PCR. The results were normalized using actin as an internal control and were the mean \pm s.e.m. of 4 separate experiments performed in duplicate. ** P < 0.01. (C) Expression of FSHR in cytosolic (C) and particulate (P) fractions estimated by Western blot with anti-FSHR antibody and re-probed with mouse anti-actin (Ab-1) to estimated purity of subcellular fraction. FSHR levels were normalized with loading control and densitometry quantification was performed with ImageJ software. The results were the mean \pm s.e.m. of 3 separate experiments. * P < 0.05 vs KGN-Ctr. (D) Expression of CYP19A1 mRNA in KGN-WT and KGN-Sdc1 cells stimulated with FSH, dbcAMP or Forskolin. Semi-quantitative RT-PCR was performed after 24 h of treatment (upper panel). Optimal numbers of PCR cycles were adjusted for CYP19A1 in order to quantify the difference in transcripts levels according to cell line. Signals were quantified using ImageJ software, normalized using actin as an internal control and expressed as fold increase over respective control for each cell line. Values are means \pm s.e.m. of 4 separate experiments (lower panel). * P < 0.05; *** P < 0.001 estimated by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test for each cell line.

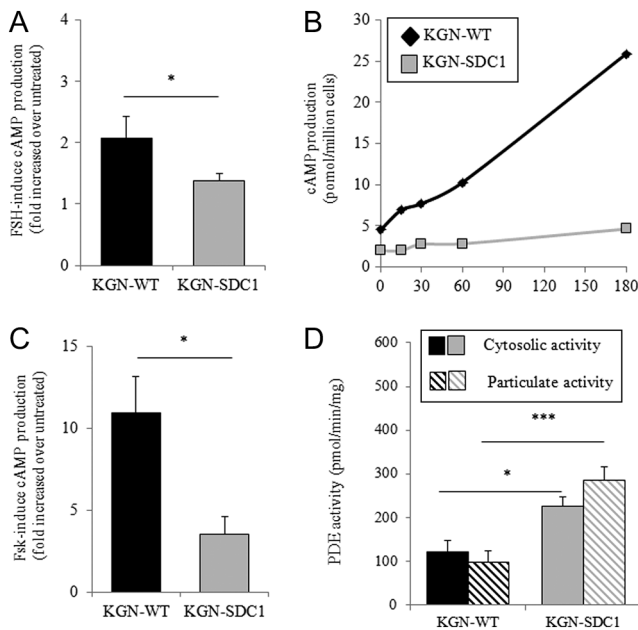


Figure 6 Reduction of Forskolin-dependent cAMP accumulation is associated with increased phosphodiesterase activity in KGN-SDC1 cells. KGN Cells were kept in culture for 24 h in 1% FCS containing medium before addition of FSH (100 ng/mL) or Forskolin (1 μ M) for increasing period of time. (A) cAMP contents were measured after 3 h of FSH treatment and expressed as fold-increase vs control from four independent experiments. * $P < 0.05$ using *t*-test. (B) Time-dependent accumulation of cAMP following Forskolin stimulation measured by $[3H]cAMP$ assay and expressed as pmol/million cells (representative experiment). (C) cAMP contents were measured after 3 h of Forskolin treatment and expressed as fold increase vs control from four independent experiments. * $P < 0.05$ using *t*-test. (D) cAMP phosphodiesterase activities in particulate (filled bar) and cytosolic (hatched bar) proteins fractions were measured as described in the material and methods section by incubating proteins with $[2,8-3H]-cAMP$. The determined PDE activity was expressed as pmoles of cAMP hydrolyzed per min and per mg protein. Values were means \pm S.E.M. of four independent experiments performed in triplicate. ** $P < 0.01$; *** $P < 0.001$ estimated by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test.

We also demonstrate, in this study, that syndecan 1 overexpression deeply affects granulosa cell steroidogenesis. The relationships between cell shape and function (Gutierrez *et al.* 1997) or cell polarization and steroid production/secretion have been demonstrated *in vitro* in granulosa cell (Lawrence *et al.* 1979) and the involvement of glycosaminoglycans was suggested (Huet *et al.* 2001). We provided evidence that the expression of high level of syndecan 1 in granulosa cells drastically reduces basal and FSH-induced estradiol production mainly through transcriptional repression of *CYP19A1* and *FSHR* genes. Besides, the expression of the steroidogenic factor 1 (*NR5A1*), oestrogen receptor beta (*ESR2*) and progesterone receptor (*PGR*), which are all crucial to promote the differentiation of mesenchymal stem cells into steroidogenic cells or to maintain

follicular growth and steroidogenesis (Conneely *et al.* 2002, Hegele-Hartung *et al.* 2004, Ferraz-de-Souza *et al.* 2011, Mizutani *et al.* 2014), were also reduced in KGN-SDC1 cells. The presence of a binding site for NR5A1 and ESR2 in the *CYP19A1* and *FSHR* genes promotor (Levallet *et al.* 2001, Luo & Wiltbank 2006, Stocco 2008), in addition to downregulation of these transcription factors in KGN-SDC1, further confirms syndecan 1 involvement in oestrogen synthesis regulation and FSH signalling. In contrast to estradiol production, synthesis and secretion of progesterone were not affected by syndecan 1 overexpression, and among the three enzymes whose expression level reflects progesterone production i.e. *STAR*, *CYP11A1* and *HSD3B2* (Mizutani *et al.* 2015) only *CYP11A1* expression was downregulated. These observations suggest that SDC1 regulates a panel of gene activated for definite granulosa cell function at a specific stage of follicle development. Interestingly, in mice, during *in vitro* maturation, treatment of cultured COCs with heparin, which inhibits HSPG-dependent growth factor signalling, significantly altered the expression of more than 600 genes, including *LHCGR* and *CYP11A1* (Watson *et al.* 2012).

The results presented in this work brought to light another mechanism by which syndecan 1 may affect granulosa cell function and FSH/cAMP/PKA-mediated response. Whether, overexpression of syndecan 1 abrogates FSH response, mainly through transcriptional repression of *FSHR*, estradiol synthesis, after addition of non-hydrolysable cAMP (dbcAMP) was not affected and measured in comparable range reported previously in KGN cells (Tsutsumi *et al.* 2008, Nishi *et al.* 2001). Nevertheless, overexpression of syndecan 1 reduces forskolin-mediated response in terms of cAMP accumulation, *CYP19A1* expression and estradiol synthesis. Downstream to membrane signalling complex (*FSHR/pG/Adenylate cyclase*), the regulation of phosphodiesterases (PDEs) is crucial for the cAMP signal termination (Conti *et al.* 2002). We previously described, in rat Sertoli cells, that the alterations of proteoglycans synthesis impaired *FSHR/G* protein coupling, decreased PDE activity and finally FSH-stimulated estradiol synthesis (Phamantu *et al.* 1999, Levallet *et al.* 2007, 2008, 2013). Through their cytoplasmic domain, proteoglycans can associate with a large number of adaptor and signalling molecules including PKA (Hayashida *et al.* 2006) or phosphodiesterases (Levallet *et al.* 2007), both involved in intracellular cAMP steady state either by direct interaction with enzyme activity or by promoting scaffolding signalling complexes (Tkachenko *et al.* 2005, Lambaerts *et al.* 2009). Thus, the discrepancy between forskolin- and dbcAMP-response as observed in KGN-SDC1 cells could be related either to adenylate cyclase activity decreases or cAMP hydrolysis activity increases, both suggesting that syndecan 1 may also affect the intensity, duration and spatial propagation of cAMP signal in granulosa cells. Such regulation is critical

to activate a specific pattern of gene expression and to assume particular granulosa cell functions such as proliferation or steroidogenesis, and thus differentiation between follicular cumulus and mural granulosa cell (Conti 2002, Park *et al.* 2003, Hunzicker-Dunn & Maizels 2006). Interestingly, in granulosa cells, functional activity of PDE (Sasseville *et al.* 2009) and syndecan 1 expression (Watson *et al.* 2012) exhibit a change during follicular growth and both drastically increase in cumulus cells. During this transition, FSH enhances, via increasing production of adenosine monophosphate, estradiol biosynthesis together with the expression of proliferation and differentiation genes, including *CYP19A1* (Hunzicker-Dunn & Maizels 2006) and becomes essential to prevent granulosa cell apoptosis and follicular atresia (Chun *et al.* 1996). The transition from pre-antral to antral follicle was associated with the differentiation of GC into the steroidogenic mural GCs, which lines the follicular wall, critical for steroidogenesis and the cumulus cells that nurse the oocyte by promoting its growth and developmental competence through paracrine action with oocyte-produced factors (Oktay *et al.* 1997, Edson *et al.* 2009). Heparan sulphate proteoglycans (HSPGs) are critical modulators of growth factor activities (Huet *et al.* 2001, Tkachenko *et al.* 2005) including growth factors which play important roles within the follicular growth and oocytes' maturation such as the insulin-like growth factor, transforming growth factor beta families or fibroblast growth factors (FGFs) (Knight & Glister 2006, Gilchrist *et al.* 2008, Price 2016). Thus the variation of SDC1 expression or of subcellular location (Hosseini *et al.* 1996), by modulating the signalling gradients of growth factors, might regulate follicular development. Additional experiments have to be done to delineate mechanism by which syndecan-1 modulates granulosa cells' functions, but we demonstrated, in the present paper, that overexpression of syndecan 1, reaching a level comparable to those present in luteinized granulosa cells (Supplementary Fig. 1), promotes cell cycle exit, loss of FSH responsiveness and repression of oestrogen synthesis in human granulosa cells. Thus, a low level of syndecan 1 is probably required for the onset of oestrogen production and for the initiation of FSH-mediated follicular growth. Such hypothesis is consistent with the weak level of syndecan 1 expressed by immature granulosa cells (Adashi *et al.* 1986, Ishiguro *et al.* 1999, Princiville *et al.* 2001, Watson *et al.* 2012). Consistently, after the LH surge, granulosa cells exit the cell cycle (Quirk *et al.* 2004) in a timing matching with the peak of syndecan-1 expression (Ishiguro *et al.* 1999, Oksjoki *et al.* 1999, Watson *et al.* 2012). Concomitantly, luteinized granulosa cells lose their proliferative activity, the expression of *FSHR* and *CYP19A1* mRNA as well as estradiol synthesis declines (Sugino 2014).

Extracellular matrix within the follicle plays a critical role in various ovarian cell functions, such as adhesion,

migration, survival, differentiation and proliferation, via mechanical and chemical signals (Rodgers & Irving-Rodgers 2010, Woodruff & Shea 2011). Through multiple mechanisms involving a dynamic linkage between the ECM and the cytoskeleton, the control of signalling pathways and the alteration of gene expression, syndecan 1 might regulate granulosa cell proliferation and differentiated functions including steroidogenesis and gonadotrophin responsiveness. Thus, the data presented herein demonstrated for the first time that, despite lack of catalytic activity, the ECM receptor syndecan 1 might participate in early ovarian follicular development. In this respect, KGN-SDC1 cell line is a useful cellular model to further investigate the physiological and pathological consequences of syndecan 1 expression in granulosa cells, and the molecular mechanisms involved.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-17-0074>.

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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