RESEARCH

Enhancement of cortisol-induced SAA1 transcription by SAA1 in the human amnion

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Abstract

Our previous studies have demonstrated that human fetal membranes are capable of *de novo* synthesis of serum amyloid A1 (SAA1), an acute phase protein of inflammation, wherein SAA1 may participate in parturition by inducing a number of inflammation mediators including interleukine-1β, interleukine-6 and prostaglandin E2. However, the regulation of *SAA1* expression in the fetal membranes remains largely unknown. In the current study, we examined the regulation of *SAA1* expression by cortisol, a crucial steroid produced locally in the fetal membranes at parturition, and the interaction between cortisol and SAA1 in the feed-forward induction of *SAA1* expression in human amnion fibroblasts. Results showed that cortisol-induced *SAA1* expression in a concentration-dependent manner, which was greatly enhanced by SAA1 despite modest induction of *SAA1* expression by itself. Mechanism studies revealed that the induction of *SAA1* expression by cortisol and SAA1 was blocked by either the transcription factor STAT3 antagonist AZD0530 or siRNA-mediated knockdown of STAT3. Furthermore, cortisol- and SAA1-induced STAT3 phosphorylation in a sequential order with the induction by SAA1 preceding the induction by cortisol. However, combination of cortisol and SAA1 failed to further intensify the phosphorylation of STAT3. Consistently, cortisol and SAA1 increased the enrichment of STAT3 at the *SAA1* promoter. Taking together, this study has demonstrated that cortisol and SAA1 can reinforce each other in the induction of *SAA1* expression through sequential phosphorylation of STAT3. The enhancement of cortisol-induced *SAA1* expression by SAA1 may lead to excessive SAA1 accumulation resulting in parturition-associated inflammation in the fetal membranes.

Key Words

- serum amyloid A1
- glucocorticoids
- STAT3
- inflammation
- preterm
- fetal membranes

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Introduction

Preterm birth is the leading cause of neonatal death (Romero *et al.* 2006, Blencowe *et al.* 2012). Premature neonates are at greater risks for a range of complications including respiratory distress syndrome, cerebral palsy and retinal problems and so on (Romero *et al.* 2006, MacLennan *et al.* 2015, Leung *et al.* 2018). Therefore, it is vitally important to understand the mechanisms underpinning preterm birth so that effective medical interventions can be developed.

The human fetal membranes, composed of the amnion and chorion, possess the largest capacity of cortisol regeneration through the reductase action of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) among fetal tissues (Murphy 1977, 1981).

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More intriguingly, the expression of 11β-HSD1 is under the feed-forward synergistic induction by glucocorticoids and pro-inflammatory cytokines in the fetal membranes (Sun *et al.* 2002, Sun & Myatt 2003, Li *et al.* 2006). This unique feature of cortisol regeneration in the fetal membranes is believed to be responsible for the accumulation of cortisol in the fetal membranes with the advance of gestational age (Wang *et al.* 2018).

Gestational tissue inflammation with consequently increased production of prostaglandin E2 (PGE2) and F2α (PGF2α) is known to be the final common mediators of parturition at both term and preterm births with actions including stimulation of myometrial contraction, cervical ripening and membrane rupture (Challis *et al.* 1997, Gibb 1998, Olson 2003). However, how inflammation can be initiated in the presence of accumulation of the well-described anti-inflammatory steroid cortisol (Barnes 2010) in the fetal membranes remains poorly understood. Of note, accumulating evidence indicates that cortisol is linked with the induction rather than inhibition of the inflammation mediators PGE2 and PGF2α in the fetal membranes (Zakar *et al.* 1995, Economopoulos *et al.* 1996, Sun *et al.* 2003, Li *et al.* 2017). It remains to be determined whether there are alternative pro-inflammatory mediators that can be induced by cortisol in the fetal membranes.

The acute phase protein serum amyloid A1 (SAA1) is produced in large quantities primarily by the liver in the acute phase of inflammation (Maury *et al.* 1985, Gabay & Kushner 1999, Sun & Ye 2016). Although the roles of SAA1 in the acute phase response are not fully understood, an existing body of literature indicates that SAA1 is engaged in a number of inflammatory reactions including induction of immune cell migration, stimulation of cytokine/chemokine production and mediation of neutrophil uptake of bacteria (Hershkoviz *et al.* 1997, Patel *et al.* 1998, Yan *et al.* 2014, Hansen *et al.* 2015, Connolly *et al.* 2016, De Buck *et al.* 2016). Recently, we found that the human fetal membranes were capable of *de novo* synthesis of SAA1 and its production was significantly increased at parturition (Li *et al.* 2017). We further demonstrated that SAA1 in the amnion was capable of stimulating the production of inflammation mediators including PGE2, interleukin 1β (IL-1β) and interleukin-6 (IL-6) (Li *et al.* 2017), suggesting that SAA1 may be an important mediator of inflammation in the fetal membranes at parturition. Moreover, we found that cortisol was able to stimulate the expression of *SAA1* in the fetal membranes (Li *et al.* 2017), which was further enhanced by the pro-inflammatory cytokine IL-1β, a phenomenon observed in the liver as well (Thorn & Whitehead 2002, Li *et al.* 2017).

Given the pro-inflammatory entity of SAA1, it is plausible to hypothesize that SAA1 may also synergize with glucocorticoids in the induction of its own expression in the fetal membranes. As such, a cascade of feed-forward reactions involving cortisol and SAA1 production may be set forth in the fetal membranes by the end of gestation resulting in the accumulation of both cortisol and SAA1 and the initiation of inflammation at parturition.

The signal transducer and activator of transcription 3 (STAT3) is a transcription factor, which can be activated upon phosphorylation at Tyr^{705} (Darnell 1997). *In silico* analysis of the *SAA1* gene reveals putative STAT3-binding sites in its promoter. Moreover, STAT3 has been shown to mediate *SAA1* transcription in non-gestational tissues (Hagihara *et al.* 2005, Tiwari *et al.* 2013). We demonstrated that the phosphorylation of STAT3 could be induced by glucocorticoids and the abundance of the phosphorylated STAT3 was significantly increased in the amnion at parturition (Wang *et al.* 2015). Given all these rationales, we anticipate that STAT3 may be involved in the regulation of *SAA1* expression by glucocorticoids and SAA1 in the amnion. Here we studied the interaction of cortisol and SAA1 in the regulation of *SAA1* expression and the involvement of STAT3 in the regulation in cultured human primary amnion fibroblasts, an important source of labor-associated mediators (Wang *et al.* 2018).

Materials and methods

Collection of human fetal membranes and preparation of amnion fibroblast cells

Human fetal membranes were collected from deliveries at elective cesarean sections at term without labor with written informed consents from patients under a protocol approved by the Ethics Committee of Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University. Pregnancies with complications such as preeclampsia, fetal growth restriction, gestational diabetes and chorioamnionitis were excluded from this study. After peeling the amnion off the fetal membranes, the amnion tissue was digested twice with 0.125% trypsin (Life Technologies Inc.) for 20 min at 37°C and then washed thoroughly with phosphate buffered saline (PBS) to remove epithelial cells. The remaining amniotic tissue was digested with 0.1% collagenase (Sigma) for 25 min at 37°C to release fibroblasts from the mesenchymal tissue. Fibroblast cells in the digestion medium were collected by centrifugation at 1200 ***g*** for 10 min and cultured at 37°C in 5% CO_2–95% air in Dulbecco's modified Eagle's medium

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(DMEM) containing 10% fetal bovine serum (FBS) plus 1% antibiotics (Life Technologies Inc.). The identity of cells was verified with staining for a mesenchymal cell marker vimentin and a relatively pure mesenchymal cells were prepared (Fig. 1A).

Treatments of amnion fibroblasts

Amnion fibroblasts were incubated for 3 days before treatments in phenol red/FBS-free culture medium. To study the concentration-dependent effect of cortisol or SAA1 on *SAA1* mRNA abundance, the cells were treated with cortisol (0.01, 0.1 and 1 μM) for 24 h or recombinant human apo-SAA1 (5, 10, 50 ng/mL; Peprotech Inc.) for 12 and 24 h. To study the time course of cortisol- or SAA1-induced *SAA1* mRNA expression, the cells were treated with cortisol (1 μM) or SAA1 (10 ng/mL) for 1, 3, 6, 12 and 24 h. To investigate the interaction between cortisol and SAA1 in the regulation of *SAA1* mRNA expression, the cells were treated with cortisol (1 μM), SAA1 (10 ng/mL) and combination of cortisol (1 μM) and SAA1 (10 ng/mL) for 12 and 24 h. To rule out the possibility that the observed effect was due to any trace amounts of endotoxin remained in the preparation of recombinant SAA1, the cells were treated with the combination of cortisol and SAA1 in the presence and absence of endotoxin inhibitor polymyxin B (50 μg/mL, Sigma). To examine the effect of cortisol or SAA1 on the phosphorylation of STAT3, the cells were treated with cortisol (1 μM) for 1, 2, 3, 6 and 12 h or SAA1 (10 ng/mL) for 0.5, 1, 2, 3 and 6 h. To test the individual and combined effects of cortisol and SAA1 on the phosphorylation of STAT3, the cells were treated with cortisol (1 μM), SAA1 (10 ng/mL) and cortisol (1 μM) plus SAA1 (10 ng/mL) for 1, 2, 3, 12 and 24 h. To study the role of STAT3 in the regulation of *SAA1* expression, the cells were treated with cortisol (1 μM), SAA1 (10 ng/mL), or combination of cortisol (1 μM) and SAA1 (10 ng/mL) in the presence and absence of a STAT3 antagonist S3I-201 (10 μM; Selleck) or with and without siRNA-mediated knockdown of STAT3. To examine the effect of cortisol or SAA1 on the phosphorylation of SRC, the cells were treated with cortisol (1 μM) for 1, 2, 3, 6 and 12 h or SAA1 (10 ng/mL) for 15, 30, 60, 120 and 180 min. To test whether SRC is involved in the phosphorylation of STAT3 and *SAA1* expression induced by cortisol or SAA1, the cells were treated with cortisol (1 μM, 6 h) or SAA1 (10 ng/mL, 1 h) in the presence and absence of the SRC inhibitor AZD0530 (20 μM, Selleck). All reagents were dissolved in DMSO, and the same amount of DMSO was used as the vehicle control. For antagonist study, antagonists were added 30 min before cortisol and SAA1 treatments. After treatments, total RNA and protein were extracted for analyses with real-time PCR (qRT-PCR) and Western blotting. Basal and cortisol-induced SAA1 secretions were determined by measuring SAA1 abundance in the culture medium with an ELISA kit (R&D Systems) according to a protocol provided by the manufacturer.

Transfection of siRNA in amnion fibroblasts with electroporation

Immediately after isolation, amnion fibroblasts were transfected with 50 nM siRNA (GenePharma Co.) against STAT3 (5′-CCACUUUGGUGUUUCAUAAtt-3′) or randomly scrambled siRNA (5′-UUCUCCGAACGUGUCACGUTT-3′) in Opti-MEM (Life Technologies Inc.) in 2 mm gap cuvettes. Fibroblasts were electroporated at 175 V for 5 ms using a NEPA21 electroporator (Nepa Gene). After dilution with DMEM containing 10% FBS, the cells were transferred to a six-well culture plate to incubate for 48 h and then treated with different reagent regimens as described earlier. After treatments, the cells were lysed for RNA extraction. The efficiency of knockdown was assessed in each experiment by measuring the target protein and mRNA abundance with Western blotting or qRT-PCR, which was 86% on the average.

Extraction of RNA and analysis with quantitative real-time PCR

Total RNA was extracted from the cells using a commercial kit (Omega Bio-Tek) following a protocol provided by the manufacturer. RNA concentration and quality were determined by measuring OD260 (optical density at 260 nm) and the ratio of OD260/OD280 with a Nanodrop ND-2000. mRNA from the total RNA was reverse-transcribed to the cDNA using a PrimeScript RT Master Mix Perfect Real Time kit (TaKaRa). The amounts of *SAA1* mRNA were determined with qRT-PCR using the above transcribed cDNA and SYBR Premix Ex Taq (TaKaRa). The annealing temperature was set at 61°C. Data were obtained as Ct values which were used to calculate ΔCt values. Fold-change in expression was determined by subtracting ΔCt values for treated cells from their controls. The resulting ΔΔCt values were used to calculate fold-change using the equation $2^{\Delta\Delta Ct}$. The housekeeping gene *GAPDH* was amplified in parallel for internal loading control. The primer sequences used for amplifying *SAA1* and *GAPDH* were *SAA1*, 5′-TTTCTGCTCCTTGGTCCTGG-3′ (forward) and 5′-CTCTGGCATCGCTGATCACT-3′ (reverse);

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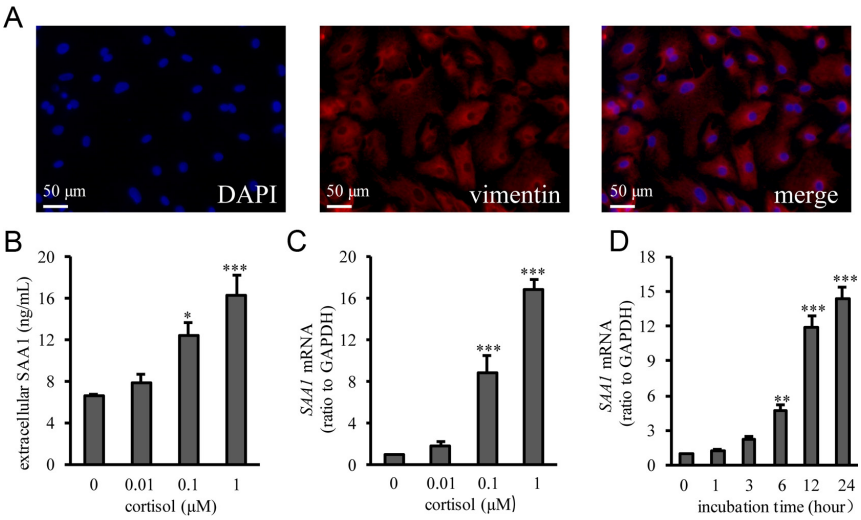

Figure 1
Induction of *SAA1* expression by cortisol in human amnion fibroblasts. (A) Immunofluorescence staining of vimentin (red), a marker of mesenchymal cells, in human amnion fibroblasts. Nuclei were counterstained blue with DAPI. Scale bar, 50 μm. (B and C) Concentration-dependent effects of cortisol (0.01, 0.1 and 1 μM, 24 h) on secreted SAA1 protein in the culture medium (B, $n = 4$) and on *SAA1* mRNA in the cells (C, $n = 5$). (D) Time course study of the effect of cortisol (1 μM; 1, 3, 6, 12, 24 h) on the abundance of *SAA1* mRNA. $n = 4$. Data are the mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs respective control.

GAPDH, 5′-GTCAAGGCTGAGAACGGGAA-3′ (forward) and 5′-GCAGGAGGCATTGCTGATGA-3′ (reverse). The ratio of the target gene over *GAPDH* in each sample was obtained as an indication of the target gene expression.

Extraction of protein and analysis with Western blotting

Total cellular protein was extracted from the above treated cells using an ice-cold radio immunoprecipitation assay (RIPA) lysis buffer (Active Motif) containing a protease inhibitor cocktail (Roche) and a phosphatase inhibitor (Roche). The abundance of total STAT3, phosphorylated STAT3 at Tyr^{705}, total SRC, phosphorylated SRC at Tyr^{416}, phosphorylated SRC at Ser^{17}, was determined following a standard Western blotting protocol. Briefly, after determination of protein concentration with Bradford assay, 25 μg of protein from each sample was electrophoresed in 10% SDS-polyacrylamide gel and transferred to the nitrocellulose membrane. After blocking with 5% nonfat milk, the membrane was incubated with antibodies against STAT3 (1:1000; Cell Signaling), phosphorylated STAT3 at Tyr^{705} (1:1000; Cell Signaling), total SRC antibody (1:1000; Cell Signaling), phosphorylated SRC at Tyr^{416} (1:500; Cell Signaling) and phosphorylated SRC at Ser^{17} (1:1000; Cell Signaling) respectively overnight at 4°C. After washing with 1× Tween/Tris-buffered saline solution, the membrane was incubated with appropriate secondary antibodies conjugated with horseradish peroxidase (1:5000; Proteintech) for 1 h. The enhanced chemiluminescent detection system (Millipore) was used to detect the bands with peroxidase activity. To control sampling error, the same blot was probed for α-tubulin (1:1000; Proteintech) for internal loading control. The bands were visualized using a G-Box iChemi Chemiluminescence Image Capture system (Syngene). The abundance of phosphorylated STAT3 and SRC was expressed as the ratio of their band densities over total STAT3 or SRC respectively.

Chromatin immunoprecipitation assay

Binding of STAT3 to the *SAA1* promoter was measured with chromatin immunoprecipitation (ChIP) assay. After treatments with and without cortisol (1 μM) or SAA1 (10 ng/mL) for 12 h, amnion fibroblasts were fixed with 1% formaldehyde to cross-link the proteins to chromatin DNA, which was terminated with 0.125 M glycine. The cells were then lysed with a 1% SDS lysis buffer supplemented with a protease inhibitor cocktail on ice. The lysed cells were sonicated to shear the chromatin DNA to an optimal size around 500 bp. After precleaning with Protein A Agarose/Salmon Sperm DNA (Millipore), sheared chromatin DNA was immunoprecipitated with the antibody against STAT3 (Cell Signaling). An equal amount of preimmune IgG served as the negative control. The immunoprecipitate was then incubated with the Magna ChIP Protein A Agarose Magnetic Beads (Millipore) and the complex was pulled down on a magnetic stand. After washing, reverse cross-linking was performed in 5 M NaCl at 65°C overnight. After cleaning contaminating RNA with ribonuclease A and digestion of protein with proteinase K, the sheared DNA recovered from reverse cross-linking was extracted using a DNA extraction kit for further analysis with qRT-PCR. The sequences of the primers used for qRT-PCR are 5′-CTTCCAGCAGCCCAGGTG-3′ (forward) and 5′-GATGTGGTCCCTGGGGAAAG-3′ (reverse), which amplify the region between −258 bp and −35 bp spanning

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the putative STAT3-binding sites. The same amount of sheared DNA without antibody precipitation after reverse cross-linking served as input control. Data were obtained as Ct values and used to determine ΔCt values. Fold-change in expression was determined by subtracting ΔCt values for treated cells from their controls. The resulting ΔΔCt values were used to calculate fold-change using the equation $2^{-\Delta\Delta Ct}$. The ratio of DNA precipitated by STAT3 over input control was obtained to indicate the amount of STAT3 bound to the *SAA1* promoter.

Statistical analysis

All data are reported as the mean ± S.E.M. The number for each study indicates repeated experiments using fetal membranes from different patients. After examination of normal distribution using the Kolmogorov–Smirnov test, paired Student's *t* test or one-way ANOVA followed by the Newman–Keuls multiple comparison test where appropriate was performed. Significance was set at $P<0.05$.

Results

Induction of *SAA1* expression by cortisol and SAA1 in human amnion fibroblasts

Cortisol (0.01, 0.1 and 1 μM, 24 h) treatment of amnion fibroblasts increased SAA1 protein abundance in the culture medium in a concentration-dependent manner, with significant increases observed at ≥0.1 μM (Fig. 1B). Consistently, cortisol (0.01, 0.1 and 1 μM, 24 h) also increased the abundance of *SAA1* mRNA in a concentration-dependent manner with significant increases observed at ≥0.1 μM (Fig. 1C). Time course (1, 3, 6, 12 and 24 h) studies showed a time-dependent induction of *SAA1* mRNA by cortisol (1 μM) with significant induction observed from 6 to 24 h and the maximal induction was observed at 24 h (Fig. 1D). These results suggest that cortisol can induce SAA1 production through upregulation of *SAA1* mRNA expression in human amnion fibroblasts.

Time course studies with SAA1 (10 ng/mL; 1, 3, 6, 12 and 24 h) also revealed a time-dependent induction of *SAA1* mRNA expression in amnion fibroblasts with significant increases observed from 3 h to 12 h and the maximal effects was observed at 12 h (Fig. 2A). Notably, the induction was no longer present at 24 h (Fig. 2A). To further verify the effects of SAA1 at 12 and 24 h, concentration-dependent effects were examined. Treatment of the cells with SAA1 (5, 10 and 50 ng/mL) for 12 h increased the abundance of *SAA1* mRNA in a concentration-dependent manner with significant increases observed at ≥10 ng/mL (Fig. 2B). However, SAA1 at the same concentration range of failed to induce *SAA1* mRNA expression at 24 h (Fig. 2B).

Combination of cortisol (1 μM) and SAA1 (10 ng/mL) at either 12 or 24 h further increased *SAA1* mRNA expression as compared with either SAA1 or cortisol alone (Fig. 2C and D). Of interest, despite ineffectiveness of SAA1 alone at 24 h, more pronounced enhancement was observed in terms of *SAA1* expression with combination of cortisol and SAA1 at this time point (Fig. 2D). The average fold increases by cortisol, SAA1 and combination of cortisol and SAA1 were 13.2-, 1.1- and 24.3-fold respectively. The combined effects were much greater than the sum of the individual effects, suggesting that cortisol may synergize with SAA1 to induce *SAA1* expression. The endotoxin inhibitor polymyxin B (50 μg/mL) failed to block the

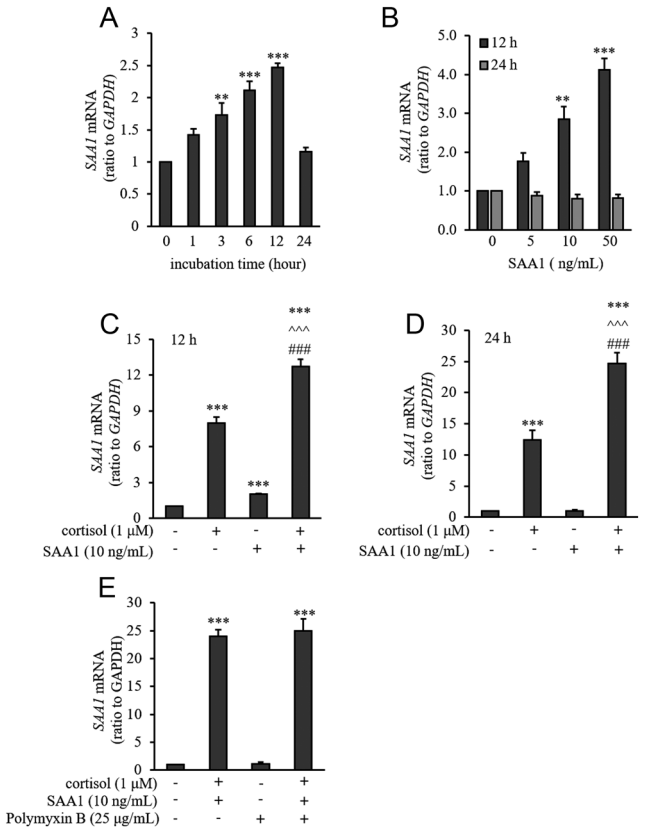

Figure 2
Induction of *SAA1* expression by cortisol and SAA1 in human amnion fibroblasts. (A) Time course study of the effect of SAA1 (10 ng/mL; 1, 3, 6, 12, 24 h) on *SAA1* mRNA expression. $n = 4$. (B) Concentration-dependent effects of SAA1 (5, 10, and 50 ng/mL, 12 and 24 h) on the abundance of *SAA1* mRNA. $n = 4$. (C and D) Effect of combination of cortisol (1 μM) and SAA1 (10 ng/mL) on *SAA1* mRNA abundance. (C, 12 h and D, 24 h, $n = 4$). (E) Effect of combination of cortisol (1 μM) and SAA1 (10 ng/mL) on *SAA1* mRNA abundance in the presence of an endotoxin inhibitor polymyxin B (50 μg/mL) (24 h, $n = 3$). Data are the mean ± S.E.M. $^{**}P < 0.01$, $^{***}P < 0.001$ vs respective control; $^{\wedge\wedge\wedge}P < 0.001$ vs cortisol; $^{\#\#\#}P < 0.001$ vs SAA1.

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synergistic induction of *SAA1* expression by cortisol and SAA1 (Fig. 2E), suggesting that the observed synergistic effect was not due to any trace amounts of endotoxin remained in the preparation of recombinant SAA1.

Involvement of STAT3 in the induction of *SAA1* expression by cortisol and SAA1 in human amnion fibroblasts

Either a STAT3 antagonist S3I-201 (10 μM) or siRNA-mediated knockdown of STAT3 completely blocked the induction of *SAA1* mRNA expression by SAA1 (10 ng/mL, 12 h) (Fig. 3A and B). The same dose of S3I-201 or siRNA-mediated knockdown of STAT3 partially blocked the induction of *SAA1* mRNA expression by cortisol (1 μM, 24 h) (Fig. 3C and D) or by combination of cortisol (1 μM) and SAA1 (10 ng/mL) at 24 h (Fig. 3C and D). These results suggest that STAT3 is involved in the induction of *SAA1* expression by either SAA1 or cortisol alone or combination of cortisol and SAA1. However, there may be additional transcription factors involved in the induction by cortisol alone or by the combination of cortisol and SAA1 since incomplete blockade was observed with inhibition of STAT3.

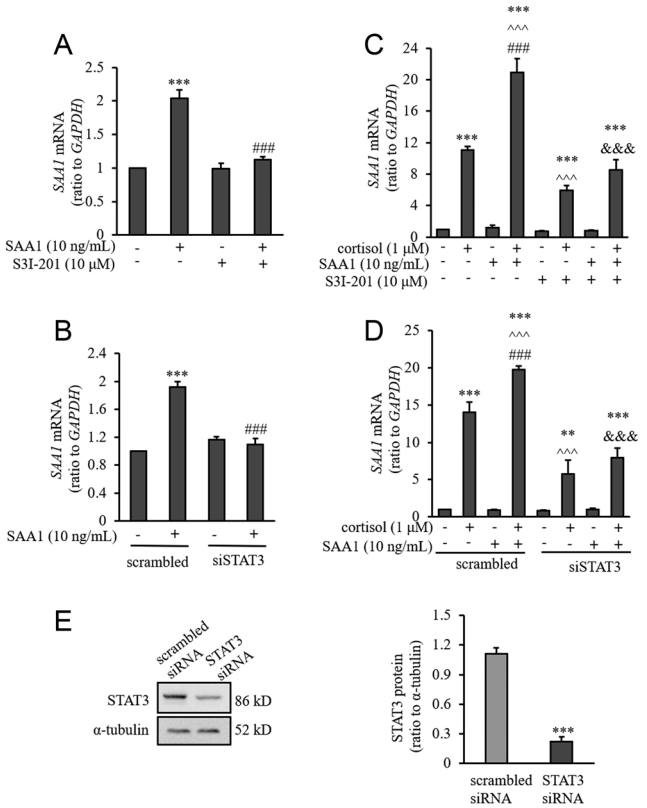

Figure 3
Role of STAT3 in the induction of *SAA1* mRNA expression by cortisol and SAA1 in human amnion fibroblasts. (A and B) Blockade of the induction of *SAA1* mRNA expression by SAA1 (10 ng/mL, 12 h) with a STAT3 antagonist S3I-201 (10 μM) and siRNA-mediated knock-down of STAT3. $n = 3$. (C and D) Attenuation of the induction of *SAA1* mRNA expression by cortisol (1 μM, 24 h) or combination of cortisol (1 μM, 24 h) and SAA1 (10 ng/mL, 24 h) with the STAT3 antagonist S3I-201 (10 μM) or siRNA-mediated knock-down of STAT3. (E) Efficiency of siRNA-mediated knock-down STAT3. Left panel is the representative Western blots and right panel is the average data. $n = 4$. Data are the mean ± S.E.M. $^{**}P < 0.01$, $^{***}P < 0.001$ vs respective control; $^{\wedge\wedge\wedge}P < 0.001$ vs cortisol, $^{\#\#\#}P < 0.001$ vs SAA1; $^{\&\&\&}P < 0.001$ vs cortisol plus SAA1.

Cortisol (1 μM) (Fig. 4A) or SAA1 (10 ng/mL) treatment (Fig. 4B) induced STAT3 phosphorylation at Tyr^{705} in a time-dependent manner. Like the sequential induction of *SAA1* mRNA expression by SAA1 and cortisol, SAA1 induced STAT3 phosphorylation from 0.5 to 2 h while cortisol caused STAT3 phosphorylation from 2 to 12 h. However, there were no further increases in STAT3 phosphorylation by combination of cortisol (1 μM) and SAA1 (10 ng/mL) at all the time points examined (1, 2, 3 12 and 24 h) (Fig. 4C). These data suggest that the phosphorylation of STAT3 can be maintained for a longer time when cortisol and SAA1 were combined. Chromatin immunoprecipitation assays showed that both cortisol (1 μM, 12 h) and SAA1 (10 ng/mL, 12 h) significantly increased the binding of STAT3 to the *SAA1* promoter region that contains the putative-binding sites for STAT3 (Fig. 5).

Role of SRC in the phosphorylation of STAT3 and synergistic induction of *SAA1* expression by cortisol and SAA1 in human amnion fibroblasts

SRC inhibitor AZD0530 (20 μM) completely blocked the induction of *SAA1* mRNA expression by SAA1 (10 ng/mL, 12 h) (Fig. 6A). Similarly, the same dose of AZD0530 partially inhibited the induction of *SAA1* expression by cortisol (1 μM, 24 h) (Fig. 6B) or by combination of cortisol (1 μM) and SAA1 (10 ng/mL) at 24 h (Fig. 6B). Consistently, the phosphorylation of STAT3 at Tyr^{705} induced by SAA1 (10 ng/mL, 1 h) (Fig. 6C) or by cortisol (1 μM, 6 h) (Fig. 6D) was completely blocked by AZD0530 (20 μM). Time course studies showed sequential phosphorylation of SRC at Tyr^{416} and Ser^{17} by SAA1 (Fig. 7A) and cortisol (Fig. 7B). As in the case of STAT3 phosphorylation, SAA1 stimulated a relatively early onset of SRC phosphorylation, which started at 15 min and ended at 60 min (Fig. 7A), while cortisol caused a relatively late onset of SRC phosphorylation, which started at 1 h and ended at 6 h (Fig. 7B). Moreover, the phosphorylation of SRC occurred ahead of STAT3 phosphorylation following either cortisol or SAA1 treatment. These results suggest that phosphorylated SRC is upstream to the phosphorylation

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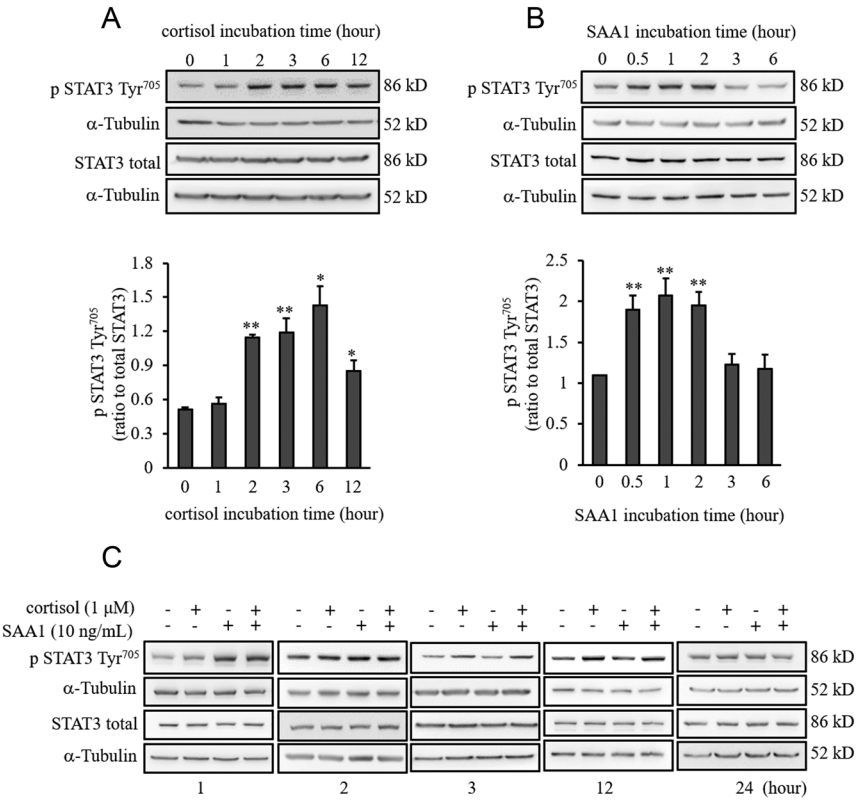

Figure 4
Effects of cortisol and SAA1 on the phosphorylation of STAT3 in human amnion fibroblasts. (A and B) Time-dependent effects of cortisol (1 μM; 1, 2, 3, 6, 12 h) and SAA1 (10 ng/mL; 0.5, 1, 2, 3, 6 h) on the phosphorylation of STAT3 at Tyr[705]. $n = 4$. (C) The interaction of cortisol (1 μM) and SAA1 (10 ng/mL) in the phosphorylation of STAT3 at Tyr[705] at different time points (1, 2, 3, 12, 24 h). Data are the mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, vs respective control (0 h).

of STAT3 in the induction of *SAA1* mRNA expression following cortisol and SAA1 treatments.

Discussion

The present study documented a novel phenomenon that there was self-induction of *SAA1* expression not only on its own but also in synergy with cortisol in human amnion fibroblasts. Fibroblasts in the human amnion play an important role in human parturition. They are not only a major source of PGE2, a crucial stimulator of myometrium contraction and cervical ripening (Mitchell *et al.* 1995, Challis *et al.* 1997, Olson 2003), but also possess a unique feature of feedforward cortisol regeneration which signals a number of crucial events in human parturition including prostaglandin synthesis and extracellular matrix remodeling (Economopoulos *et al.* 1996, Zhu *et al.* 2009, Wang *et al.* 2015, 2016, 2018, Liu *et al.* 2016, Li *et al.* 2017, Mi *et al.* 2017, 2018). Notably, human amnion fibroblasts are also capable of producing other pro-inflammatory mediators such as IL-1β, TNFα as well as SAA1 (Li *et al.* 2017, Zhang *et al.* 2017). Hence, human amnion fibroblasts are important as both endocrine and immune cells in human parturition. Data derived from human aortic smooth muscle cells, hepatocytes as well as amnion fibroblasts indicate that the endocrine signal cortisol and the inflammatory mediator IL-1β can interact to induce the expression of SAA1 synergistically (Kumon *et al.* 2001, Thorn & Whitehead 2002, Li *et al.* 2017). Here, we presented evidence for the first time that the synergism can be extended to the interaction between cortisol and the target gene product SAA1 itself. This self-induction in synergy with cortisol may be particularly important in terms of local feed-forward accumulation of SAA1 in the fetal membranes given the feed-forward cortisol regeneration in human amnion fibroblasts (Sun & Myatt 2003, Li *et al.* 2006, Yang *et al.* 2007). These feedforward loops may reinforce each other resulting in overwhelming accumulation of cortisol and SAA1 in the fetal membranes toward the end of gestation (Wang *et al.* 2015, Liu *et al.* 2016, Li *et al.* 2017). However, it should be kept in mind that in addition to the autocrine signals from amnion fibroblasts per se, paracrine signals from amnion epithelial cells and chorion trophoblasts in the fetal membranes as well as endocrine signals from the placenta may also influence the endocrine and immune functions of amnion fibroblasts *in vivo*.

As a transcription factor, STAT3 is activated upon phosphorylation at Tyr[705] (Darnell 1997). The current study revealed that STAT3 was involved in the induction of *SAA1* mRNA transcription by cortisol or SAA1 alone or by combination of cortisol and SAA1 in human amnion

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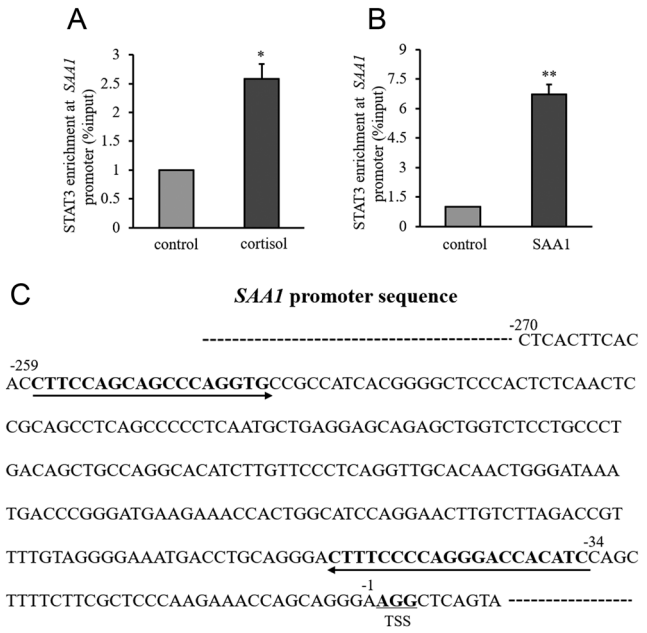

Figure 5
Chromatin immunoprecipitation assay showing the enrichment of STAT3 at the *SAA1* promoter in responses to cortisol and SAA1 in human amnion fibroblasts. (A and B) Increased enrichment of STAT3 at the *SAA1* promoter in response to cortisol (1 μM, 12 h) and SAA1 (10 ng/mL, 12 h). (C) Sequence of the *SAA1* promoter spanning −270 to +10 base pairs (bp). Arrows indicate the primer aligning positions in the ChIP assay. TSS, transcription start site. **P* < 0.05, ***P* < 0.01 vs respective control. Data are the mean ± S.E.M. of three experiments.

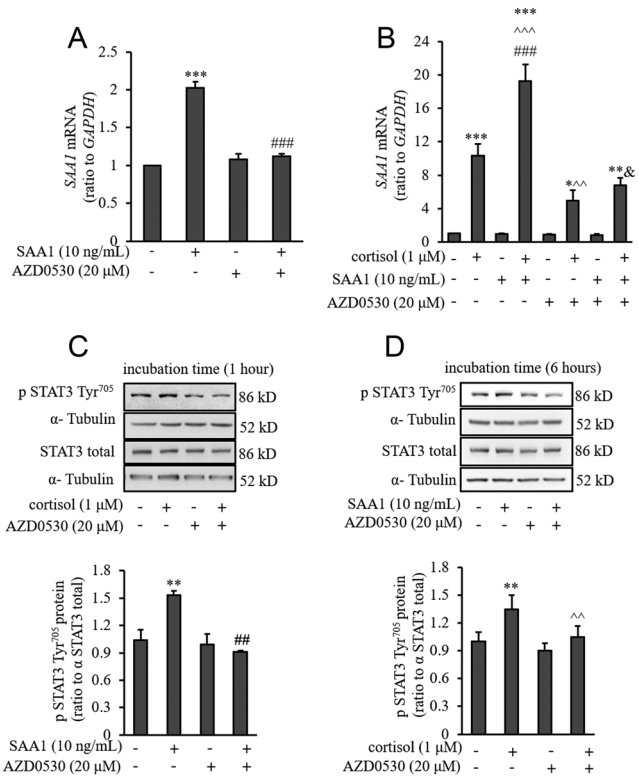

Figure 6
Role of SRC in the induction of *SAA1* mRNA expression and the phosphorylation of STAT3 by cortisol and SAA1 in human amnion fibroblasts. (A) Blockade of the induction of *SAA1* mRNA expression by SAA1 (10 ng/mL, 12 h) with a SRC antagonist AZD0530 (20 μM). *n* = 4. (B) Attenuation of the induction of *SAA1* mRNA expression by cortisol (1 μM, 24 h) or combination of cortisol (1 μM, 24 h) and SAA1 (10 ng/mL, 24 h) by AZD0530 (20 μM). *n* = 4. (C and D) Blockade of the phosphorylation of STAT3 by cortisol (1 μM, 6 h) and SAA1 (10 ng/mL, 1 h) with AZD0530 (20 μM). *n* = 3. Data are the mean ± S.E.M. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs respective control; ##*P* < 0.01, ###*P* < 0.001 vs SAA1; ^^*P* < 0.01, ^^^*P* < 0.001 vs cortisol; &*P* < 0.05 vs cortisol plus SAA1.

fibroblasts, which is supported by our previous studies showing concurrent increases in the abundance of phosphorylated STAT3, cortisol and SAA1 in the human amnion tissue at parturition (Wang *et al.* 2015, Li *et al.* 2017). Here we found that cortisol and SAA1 induced the phosphorylation of STAT3 in a sequential manner rather than in a mutual enhancement way. The sequential phosphorylation of STAT3 by SAA1 and cortisol may compensate each other so that extended phosphorylation of STAT3 can be maintained resulting in sustained expression of SAA1. In keeping with the sequential phosphorylation of STAT3, the phosphorylation of SRC, the kinase responsible for the phosphorylation of STAT3, also manifested a similar sequential manner in responses to cortisol and SAA1 treatments. Our previous study has demonstrated that cortisol induces the phosphorylation of SRC indirectly via stimulation of PGE_2 production in human amnion fibroblasts (Wang *et al.* 2015), then PGE2 induces the phosphorylation of SRC by activating the cAMP-PKA pathway through its EP2/EP4 receptors (Wang *et al.* 2015). This indirect mechanism may account for the late onset of both SRC and STAT3 phosphorylation in response to cortisol treatment in this study. Since the phosphorylation of SRC occurred more rapidly in response to SAA1 treatment, it appears that a more direct mechanism is involved, which is to be revealed in the future.

Of interest, the synergistic induction of *SAA1* expression by cortisol and SAA1 was more dramatic at 24 h than 12 h despite the ineffectiveness of SAA1 on its own expression at 24 h, suggesting that a complex synergistic mechanism is involved, which requires longer acting time. The incomplete blockade of the induction of SAA1 by cortisol or combination of cortisol and SAA1 by inhibition of STAT3 further suggests that unidentified transcriptional factors may be involved in the induction by cortisol alone or combination of cortisol and SAA1 in addition to STAT3. Identification of this transcriptional factor may help to explain how the synergistic induction is maintained when the phosphorylation of STAT3

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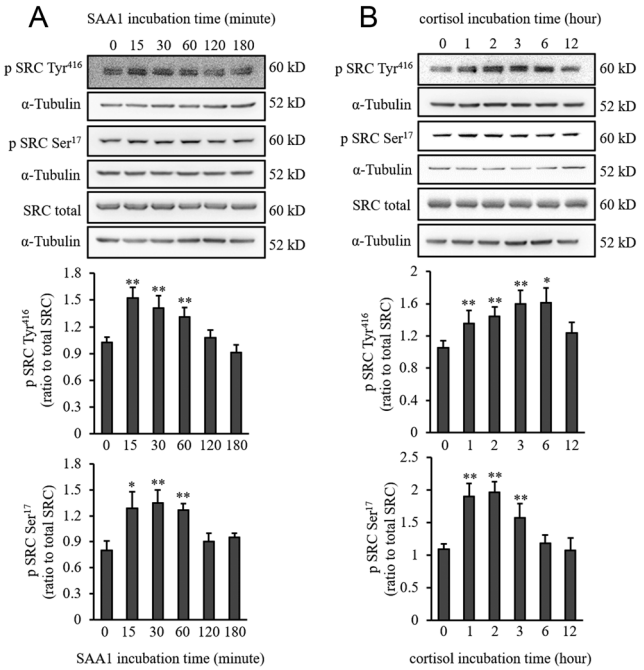

Figure 7
Phosphorylation of SRC by cortisol and SAA1 in human amnion fibroblasts. (A) Phosphorylation of SRC at Tyr^{416} and Ser^{17} in responses to cortisol (1 μM, 1, 2, 3, 6, 12 h). (B) Phosphorylation of SRC at Tyr^{416} and Ser^{17} in responses to SAA1 (10 ng/mL, 15, 30, 60, 120, 180 min). $n = 4$. Data are the mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ vs respective control.

disappears and the induction of *SAA1* expression by itself no longer exists at 24 h.

In conclusion, we have documented a novel feedforward self-induction of *SAA1* expression either on its own or in synergy with cortisol in human amnion fibroblasts and phosphorylated STAT3 appears to be involved in the induction of *SAA1* transcription by either cortisol or SAA1 or combination of cortisol and SAA1 in human amnion fibroblasts. However, additional unidentified transcription factors may also participate in the synergistic induction of SAA1 by cortisol and SAA1. These feedforward loops of SAA1 induction may result in overwhelming accumulation of SAA1 in the fetal membranes toward the end of gestation. Given the demonstrated pro-laboring inflammatory effects of SAA1 (Li *et al.* 2017), we believe that accumulation of SAA1 under the drive of cortisol and SAA1 in the fetal membranes may be a crucial event in the initiation of local inflammatory reactions, which comprises an important component of a series of feed-forward reactions at the onset of human parturition.

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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