REPRODUCTION
RESEARCH

Regulation of ovulatory genes in bovine granulosa cells: lessons from siRNA silencing of *PTGS2*

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Abstract

Prostaglandin endoperoxide synthase-2 (*PTGS2*), tumour necrosis factor-alpha-induced protein-6 (*TNFAIP6*), pentraxin-3 (*PTX3*), epidermal growth factor-like factors: amphiregulin (*AREG*) and epiregulin (*EREG*) are essential for successful ovulation. In this study, we compared the induction of these ovulatory genes in bovine granulosa cells (GCs) *in vivo* (after LH surge) and *in vitro* (forskolin (FRS) treatment). These genes were markedly stimulated in GCs isolated from cows 21 h after LH-surge. In isolated GCs, FRS induced a distinct temporal profile for each gene. Generally, there was a good agreement between the *in vivo* and *in vitro* inductions of these genes except for *PTX3*. Lack of *PTX3* induction in isolated GCs culture suggests that other follicular compartments may mediate its induction by LH. Next, to study the role of *PTGS2* and prostaglandins (PGs) in the cascade of ovulatory genes, *PTGS2* was silenced with siRNA. *PTGS2* siRNA caused a marked and specific knockdown of *PTGS2* mRNA and PGE2 production (70% compared with scrambled siRNA) in bovine GCs. Importantly, *PTGS2* silencing also reduced *AREG*, *EREG* and *TNFAIP6* mRNA levels but not *PTX3*. Exogenous PGE2 increased *AREG*, *EREG* and *TNFAIP6* mRNA levels, further confirming that these genes are prostanoid dependent. A successful and specific knockdown of *PTGS2* was also achieved in endometrial cells (EndoCs) expressing *PTGS2*. Then, cholesterol-conjugated *PTGS2* (chol-*PTGS2*) siRNA that facilitates cells' entry was investigated. In EndoCs, but not in GCs, chol-*PTGS2* siRNA succeeded to reduce *PTGS2* and *PGE2* levels even without transfection reagent. *PTGS2* knockdown is a promising tool to critically examine the functions of *PTGS2* in the reproductive tract.
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Introduction

Ovulation is triggered by LH surge. The LH receptor activates several families of heterotrimeric G-proteins, but the activation of Gs and cAMP/PKA cascade is widely accepted as an important downstream signalling of LH action (Fan *et al*. 2011, Breen *et al*. 2013). It brings about series of changes such as resumption of meiosis in the oocyte, reprogramming of the granulosa cells (GCs) and theca cells (TCs) layer of the follicular wall. These events are accompanied by the expression of new mRNAs and proteins and result in the release of a fertilisable oocyte and the luteinisation of follicular cells (Richards 2001, 2005). In preparation for oocyte expulsion, the extracellular matrix (ECM) of cumulus–oocyte complex (COC) undergoes remodeling (Richards 1994, Salustri *et al*. 1996). A number of genes mediating these processes have been identified, amongst them are prostaglandin endoperoxide synthase 2 (*PTGS2*), tumour necrosis factor-alpha-induced protein 6 (*TNFAIP6*), pentraxin 3 (*PTX3*), epidermal growth factor (EGF)-like factors: amphiregulin (*AREG*) and epiregulin (*EREG*) (Mukhopadhyay *et al*. 2001, Salustri *et al*. 2004, Conti *et al*. 2006, Bridges & Fortune 2007).

LH-induced *PTGS2* in the GCs layer increases prostaglandins (PGs), especially PG endoperoxide 2 (PGE2) in the follicular fluid (Sirois & Richards 1992, Sirois 1994, Tsai *et al*. 1996, Liu *et al*. 1997). The essential role of *PTGS2* is demonstrated in gene-knockout experiments and PTGS inhibitors studies which resulted in failed ovulation with normal luteinisation and corpus luteum formation (Dinchuk *et al*. 1995, Davis *et al*. 1999, Peters *et al*. 2004). Later, it was demonstrated that LH/hCG upregulate EGF-like factors in murine GCs; these peptides mediated LH action in COC expansion and oocyte maturation (Park *et al*. 2004, Conti *et al*. 2006, Shimada *et al*. 2006). During COC expansion, LH surge also induces TNFAIP6, which is a hyaluronan (HA)-binding protein (Lee *et al*. 1992) critical for the stability of glycosaminoglycan HA-rich ECM (Ochsner *et al*. 2003). A study of *Tnfaip6*-knockout mice showed defective COC expansion and infertility (Fulop *et al*. 2003).

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Likewise, *PTX3*, belonging to the long pentraxin family of inflammatory proteins, was expressed in COC (Varani *et al*. 2002, Salustri *et al*. 2004). It plays a protective role during the formation of HA-rich matrix of the COC by cross-linking HA polymers through interactions with heavy chains of inter-α trypsin inhibitor (IαI) and/or TNFAIP6 (Ievoli *et al*. 2011). An abnormal COC expansion characterised by unstable extracellular matrix was reported in *Ptx3*-deficient mice (Salustri *et al*. 2004). Thus, *PTGS2*, EGF-like factors (*AREG* and *EREG*) and HA-binding proteins (TNFAIP6 and PTX3) are all the obligatory factors for ECM stability, COC expansion and follicular rupture.

Most of our knowledge on ovulatory genes stems from rodents, especially mice; however reproductive strategies differ in rodents and large mono-ovulatory animals. Therefore, data from rodents cannot always be extrapolated in mono-ovulatory mammals such as bovine (Bahr & Wolf 2012). In addition, these animals are not amendable for gene deletion studies. In such species, siRNA can be used as a means for gene knockdown (Fang *et al*. 2013). Therefore, we aimed herein to study the induction of ovulatory genes in bovine GCs under *in vivo* and *in vitro* conditions. siRNA silencing of *PTGS2* was used to critically examine the role of *PGE2* in the cascade of events in GCs that leads to ovulation. As a proof of concept we also employed endometrial cells (EndoCs), these cells, similar to GCs, express *PTGS2* and their synthesised PGs play significant physiological roles.

Materials and methods

Animals and sample collection

Follicles were collected from Holstein–Friesian cows as described (Vanselow *et al*. 2010, Christenson *et al*. 2013). Large dominant follicles before LH surge were collected after carefully, monitoring a growing cohort of follicles in normally cycling cows by transrectal ultrasonography (Aloka SSD-500, Aloka GmbH, Meerbusch, Germany). The animals were slaughtered at days 7 ($n=1$) and 8 ($n=2$) of the estrous cycle during the first follicular wave. Only the largest growing, but not stagnating or regressing, follicle of each animal was collected. To collect dominant follicles after the LH surge, normally cycling cows were treated with 500 μg PGF2a (PGF Veyx forte, Veyx Pharma GmbH, Schwarzenborn, Germany) at day 8 of the cycle, to induce luteolysis. Forty-eight hours later, the animals were injected with 100 μg of a gonadotrophin-releasing hormone (GNRH) analogue (GonavetVeyx, Depherelin, Veyx Pharma GmbH) to induce the LH surge. The animals were slaughtered 23 h later and the largest growing follicle of each animal was isolated ($n=3$). The GCs were collected by aspiration of the follicular fluid with an 18G needle. The fluid was centrifuged (2 min, 400 ***g***) and the sediment cells were frozen in liquid nitrogen and stored at −80 °C for RNA preparation. RNA isolation, RT and qPCR were carried out as described previously (Christenson *et al*. 2013).

All procedures involving living animals (injections and ultrasonography examinations in cattle) were performed according to the German law for animal protection (8a TierSchG i.V.m. 29 Tierschutz-Versuchstierverordnung). The named procedures are not subjected to specific permit by the governmental authority Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) Rostock.

Isolation and culture of GCs

The ovaries were collected at a local abattoir as described previously (Meidan *et al*. 1990). The GCs were enzymatically dispersed by using a combination of collagenase I (125 units/ml), hyaluronidase III (36 units/ml) and deoxyribonuclease I (11 units/ml) in DMEM/F-12 containing 2 mM L-glutamine and 100 μg/ml of penicillin/streptomycin. Only large follicles (>10 mm in diameter) containing ≥4 million viable cells were used. The GCs were cultured overnight in a medium containing 3% FCS. The cells were then incubated with DMEM/F-12 containing 1% FCS (control) and with PGE2 (1 μM; Cayman Chemical Co., Ann Arbor, MI, USA) or with forskolin (FRS; 10 μM) for various time points as indicated. Unless otherwise stated all biochemicals were from Sigma–Aldrich and cell culture materials were from Biological Industries, Kibbutz Beit Haemek, Israel.

Isolation and culture of EndoCs

Uterine horns from cows at late luteal phase were collected from the abattoir (Arosh *et al*. 2002). Endometrial strips were dissected and incubated in an enzyme solution (18 units/ml DNAse and 315 units/ml collagenase I) at 38 °C for 25 min in a shaking water bath. The cell suspension was filtered with a cell strainer, centrifuged and cultured in DMEM/F-12 containing 10% FCS. Confluent plates were trypsinised with trypsin EDTA solution (0.25% trypsin and 0.02% EDTA). The cells of passages 2–3 were utilised in this study. Based on morphology and growth in culture, the cells were identified as endometrial stromal cells (Cherny & Findlay 1990).

Transfection of cells

For transfection experiments, GCs were trypsinised with trypsin EDTA solution (0.05% trypsin and 0.02% EDTA) immediately after isolation from follicles. The trypsinised cells were seeded (3×10^5 GCs; 0.8×10^5 EndoCs) in six-well plates and cultured for up to 24 h in 3% FCS. Then cells were transfected using Lipofectamine 2000 reagent (Invitrogen) in 1% FCS, according to the manufacturer's protocol. The cells were transfected with siRNA sequence, targeting (50 nmol/l) naked *PTGS2* siRNA or cholesterol-conjugated *PTGS2* (chol-*PTGS2*) siRNA or scrambled siRNA. The sequence of naked *PTGS2* siRNA was sense (S), GUGAAAGGCUGUCCCUUUA[dT][dT], antisense (AS), UAAAGGGACAGCCUUUCAC[dT][dT] corresponding to bases 1781–1799 of the bovine *PTGS2* mRNA sequence (NM_174445). The sequence for chol-*PTGS2* siRNA was same as for naked *PTGS2* siRNA with cholesterol conjugation in 3′ end of sense strand: S, GUGAAAGGCUGUCCCUUUA [dA][dT][CholTEG], AS, UAAAGGGACAGCCUUUCAC [dA][dT]. Scrambled siRNA sequence-negative control was S,

UUCUCCGAACGUGUCACGU[dT][dT], and AS, ACGUGACACGUUCGGAGAA[dT][dT]. A day after transfection, PTGS2 was induced in GCs by FRS (10 μM) and in EndoCs by phorbol 12-myristate 13-acetate (PMA; 100 ng/ml), then the cells were harvested and total RNA was extracted from cells 48 h after transfection.

For treatment of GCs and EndoCs in the absence of transfection reagent, the cells were incubated with chol-*PTGS2* siRNA for 24 h and total RNA was extracted from cells after 48 h.

RNA extraction and real-time PCR

Total RNA was isolated from the cells using TriFast reagent (Peqlab Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's instructions: 1 μg of total RNA was reverse transcribed using M-MuLV Reverse Transcriptase (200 units/μl), M-MuLV RT Buffer (New England Biolabs, Ipswich, MA, USA), random primer (100 nM), oligo-dT (100 μM) and dNTPs mix (100 mM) (Bioline Reagents Limited, London, UK). Real-time PCRs were carried out using the Mx3000P system (Stratagene, Garden Grove, CA, USA), using Platinum SYBR Green (SuperMix, Invitrogen), as previously described (Klipper *et al.* 2009). Gene expression (*PTGS2*, *TNFAIP6*, *PTX3*, *AREG* and *EREG*) was analysed by quantitative real-time PCR. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as the housekeeping gene. The threshold cycle number (*C*t) was used to quantify the relative abundance of the gene; arbitrary units were calculated as $2^{-\Delta Ct} = 2^{-(Ct\ \text{target gene} - Ct\ \text{housekeeping gene})}$. The primer sequences used were as follows: *GAPDH* (NM_001034034) forward: 5′-GTCTTCACTACCATGGAGAAGG-3′, reverse: 5′-TCATGGATGACCTTGGCCAG-3′; *PTGS2* (NM_174445) forward: 5′-CAGCGGTGCAGCAAATCCTTG-3′, reverse: 5′-CTGTGTTGGGAGTGGGTTTCA-3′; *AREG* (NM_001099092) forward: 5′-CTATAGCTGCTTTCGTCTCTGC-3′, reverse: 5′-CGTTCTTCAGCGACACCTTCA-3′; *EREG* (XM_002688367) forward: 5′-CTGCTGCTCGTCCTGGTTTTC-3′, reverse: 5′-GCTGTGCAGTTATCTCCCGAC-3′; *TNFAIP6* (NM_001007813) forward: 5′-ATGGCTTGAACAAGCAGCAGG-3′, reverse: 5′-GCCATCCACCCAGCAGCACA-3′; *PTX3* (NM_001076259) forward: 5′-AGCCTCTTGCCTCGTCCCCTC-3′, reverse: 5′-TCTGAGTTCTCCGCCGACACT-3′; *VEGF* (NM_174216) forward: 5′-CCATGAACTTTCTGCTCTCTTGG-3′, reverse: 5′-TCCATGAACTCCACCACTTCG-3′; *SLC2A1* (NM_174602) forward: 5′-CGCTTCCTGCTCATTAACCG-3′, reverse: 5′-CCTTCTTCTCCCGCATCAT-3′; *TNFR1* (NM_174674) forward: 5′-GGCGAGACACGGACTGCA-3′, reverse: 5′-TCCCGGTCCACTACACAAGG-3′.

PGE2 analysis

Media from the cell cultures were collected on the day of RNA isolation. The levels of PGE2 were measured by PGE2 enzyme Immunoassay Kit – monoclonal (Cayman Chemical Co.) according to manufacturer's instruction. The standard curve ranged from 6.4 to 1000 pg/ml. Cross reactivity of the assay with PGE2 was 100 and 0.01% for PGF2a.

Statistical analyses

Data are presented as means ± S.E.M.; experiments were repeated at least three times. The expression of specific mRNA transcripts was normalised relative to the abundance of *GAPDH* mRNA. Data were analysed by Student's *t*-test. Differences were considered significant at $P<0.05$. Asterisks represent significant differences from their respective controls. *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.

Results

In vitro *and* in vivo *induction of ovulatory genes*

hCG (Fig. 1A inset) and LH (data not shown) induced *PTGS2*; however, LH/hCG response was variable therefore FRS was used instead as an activator of adenylyl cyclase. The primary bovine GCs were cultured in the absence or presence of FRS (10 μM) for 6–24 h. There was a distinct temporal profile of induction for each gene (Fig. 1). A significant induction of *PTGS2* (Fig. 1A) was

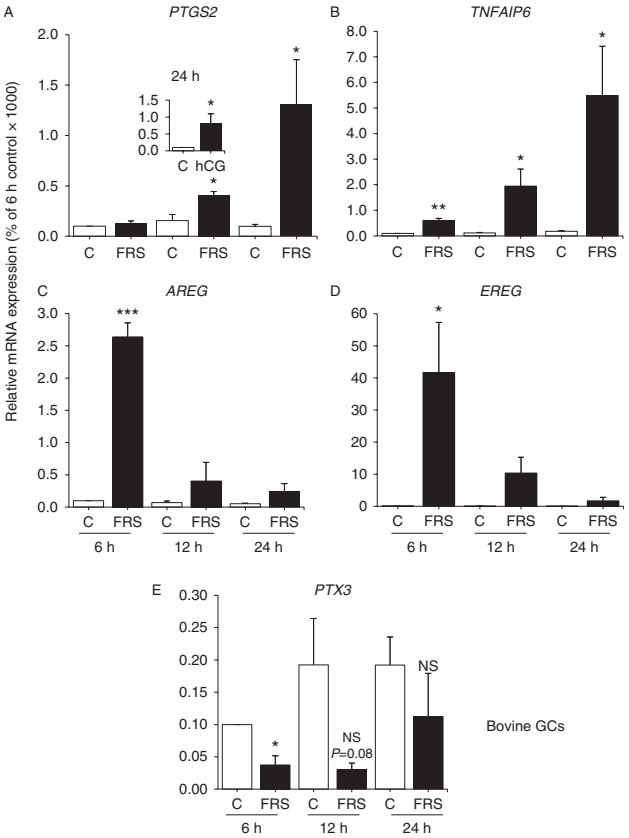

Figure 1 *In vitro* expression of (A) *PTGS2*, (B)*TNFAIP6*, (C) *AREG*, (D) *EREG* and (E) *PTX3* in bovine GCs. GCs were incubated with or without (control: C) forskolin (FRS; 10 μM) for 6, 12 and 24 h, when cells were collected for RNA extraction. mRNA expression was measured by quantitative real-time PCR. Control levels at 6 h were designated 100%. The results are mean ± S.E.M. from three independent experiments. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ indicate significant differences from their respective controls. Inset: GCs were incubated with hCG (10 IU) for 24 h.

observed at 12 h (threefold), which was further elevated (13-fold compared with its control) at 24 h. Induction of *TNFAIP6* followed a similar pattern to that of *PTGS2*, but a maximal fold stimulation was much higher (30-fold; Fig. 1B). Unlike to these two genes, *AREG* and *EREG* were maximally induced already at 6 h and then FRS effect decreased gradually until 24 h (Fig. 1C and D). Amongst the genes studied, *EREG* was highly expressed. In contrast to other genes, *PTX3* was decreased in the presence of FRS at all time point examined (Fig. 1E).

In vivo as well, comparing the mRNA expression in GCs collected before and 21 h after LH administration to cows reveals a marked stimulation of these genes by LH. *PTGS2*, *TNFAIP6* and *PTX3* were strongly upregulated (Fig. 2A, B and E). *AREG* and *EREG* on the other hand were only moderately induced at this time point (Fig. 2C and D).

Effect of PGE2 on ovulatory genes

To examine the role of endogenous PGs we employed siRNA targeting *PTGS2*. The mRNA levels of *PTGS2* were significantly inhibited. *PTGS2* levels were only 30% of those present in GCs treated with scrambled siRNA (Fig. 3A). In accordance, the concentration of PGE2 in the cell culture media was also strongly reduced to <1/3 as compared with scrambled siRNA (Fig. 3B). Silencing was specific to *PTGS2* because non-related genes such as vascular endothelial growth factor (*VEGF*) and solute carrier family 2 (facilitated glucose transporter), member 1 (*SLC2A1*) were not affected (Fig. 3C). *PTGS2* knockdown affected some of the ovulatory genes studied. Along with 70% inhibition of *PTGS2*, the mRNA

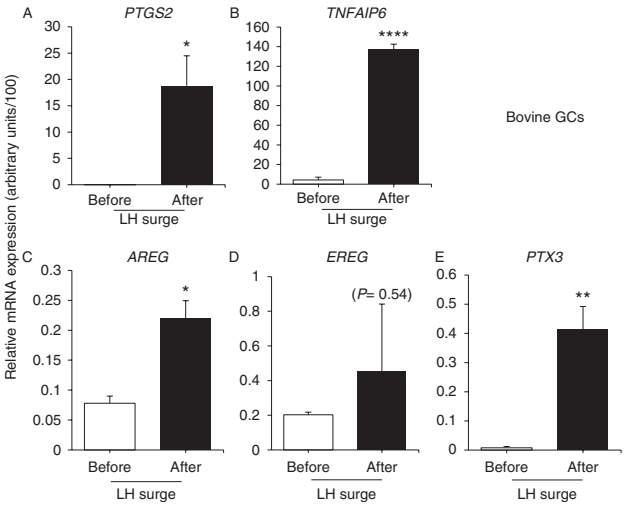

Figure 2 *In vivo* expression of (A) *PTGS2*, (B) *TNFAIP6*, (C) *AREG*, (D) *EREG* and (E) *PTX3* in bovine GCs. GCs were aspirated from cows before ($n=3$) and 21 h after GNRH-induced LH surge ($n=3$). mRNA expression was measured by quantitative real-time PCR. The results are mean±S.E.M. $*P<0.05$, $**P<0.01$ and $****P<0.0001$ indicate significant differences from the follicles collected before LH surge.

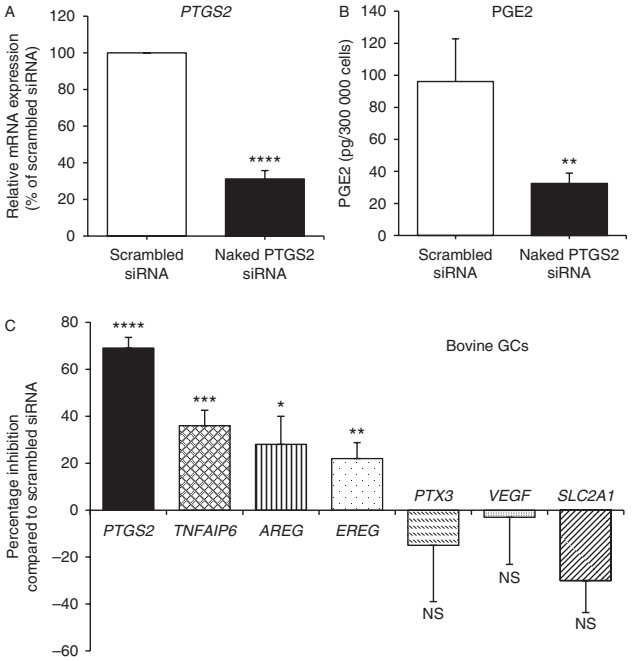

Figure 3 Effects of *PTGS2* gene silencing on PGE2 concentration and ovulatory gene expression in bovine GCs. Bovine GCs were transfected with 50 nmol/l of scrambled siRNA; designated 100% or siRNA targeting *PTGS2* (naked *PTGS2* siRNA). 24 h post-transfection forskolin (10 μM) was added. RNA was extracted 48 h post transfection and mRNA levels were measured using quantitative real-time PCR. Scrambled siRNA was designated as 100%. (A) *PTGS2* expression (B) PGE2 concentrations in culture media of cells transfected with scrambled siRNA or naked *PTGS2* siRNA. (C) Percent inhibition of *PTGS2*, *TNFAIP6*, *AREG*, *EREG*, *PTX3*, *VEGF* and *SLC2A1* compared with scrambled siRNA (0%). The data were obtained from five independent experiments. $*P<0.05$, $**P<0.01$, $***P<0.001$ and $****P<0.0001$ indicate significant differences from scrambled siRNA.

levels of *TNFAIP6*, *AREG* and *EREG* were significantly inhibited by 40, 30 and 20% respectively (as compared with 0% inhibition by scrambled siRNA; Fig. 3C). Notably, however, *PTX3* was not affected by *PTGS2* silencing (Fig. 3C).

To further strengthen these findings, we examined next the effect of exogenous PGE2 on *AREG*, *EREG*, *TNFAIP6* and *PTX3*. Bovine GCs were cultured in presence and absence of PGE2 (1 μM) for 3–24 h. The effect of PGE2 subsided with time. At 6 h, as given in Table 1, maximal effect of PGE2 was observed and expression of *AREG*, *EREG* and *TNFAIP6* was significantly upregulated. As with *PTGS2* silencing, the expression of *PTX3* was not affected by PGE2. PGE2 did not modulate *PTGS2* expression (data not shown).

PTGS2 *silencing with naked siRNA and cholesterol-conjugated siRNA in GCs and EndoCs*

We then compared naked siRNA with cholesterol-conjugated siRNA molecule that does not necessitate transfection reagent. First, in bovine GCs, we examined

Table 1 Induction of ovulatory genes in bovine granulosa cells by 6 h treatment with PGE2 (1 μM).

Gene	Fold over control	*P* value
AREG	3.4±1.08	0.0016
EREG	11.8±3.42	0.019
TNFAIP6	2.2±0.49	0.04
PTX3	1.0±0.12	NS

Expression of each gene is as compared with its control designated as 1. $n=4$.

the efficiency of chol-*PTGS2* siRNA using a transfection reagent. Similar to the naked siRNA molecule, the mRNA levels of *PTGS2* were declined to 65% as compared with scrambled siRNA (Fig. 4A). As expected PGE2 level were also reduced (data not shown). Likewise, *TNFAIP6* expression was inhibited by 43% (Fig. 4B). However, when bovine GCs were treated with chol-*PTGS2* siRNA without transfection reagent, no inhibition of *PTGS2* or *TNFAIP6* was observed.

Since bovine EndoCs also express the *PTGS2*, we next examined the effects of these siRNA molecules in bovine EndoCs. To induce *PTGS2* in these cells, PMA (100 ng/ml) (Parent & Fortier 2005) was utilised (Fig. 5A). When transfected with naked si*PTGS2*, a significant reduction was observed in the mRNA levels and PGE2 concentration by 65 and 91% respectively (Fig. 5B and C). Tumour necrosis factor receptor (*TNFR1*), another gene expressed in same levels as that of *PTGS2* in bovine EndoCs, was unaffected by the siRNA (Fig. 5D), indicating specificity of the silencing process. In a similar manner to naked siPTGS2, transfection of chol-*PTGS2* siRNA in EndoCs showed 68% reduction in *PTGS2* mRNA levels (Fig. 6A). But in this cell type, chol-*PTGS2* siRNA treatment without transfection reagent significantly reduced *PTGS2* mRNA levels and PGE2 concentration (25 and 50% respectively) (Fig. 6B and C).

Discussion

The present data demonstrated that ovulatory genes, namely *PTGS2*, *TNFAIP6*, *AREG*, *EREG* and *PTX3* were upregulated in bovine GCs 21 h after an induced LH surge. Except *PTX3*, the other genes studied were also induced by FRS treatment in isolated GCs culture *in vitro*. *AREG* and *EREG* were induced abruptly (at 6 h) during culture and progressively declined until 24 h. *PTGS2* and *TNFAIP6* showed a different pattern with gradual induction of FRS, reaching a maximum at 24 h. Using siRNA, we achieved a marked and specific silencing of *PTGS2* in bovine GCs and another cell type expressing *PTGS2*, EndoCs. Reduction in *PTGS2* mRNA levels in turn results in declined PGE2 concentrations in the culture media of both cell types. *PTGS2* knockdown in GCs also inhibited the expression of ovulatory genes *TNFAIP6*, *AREG* and *EREG*, but not *PTX3*. In agreement, PGE2 treatment of GCs elevated the expression of these genes, except *PTX3*. Together these studies suggest that PGs, most likely PGE2, which are induced in GCs by LH/cAMP promote the expression of *TNFAIP6*, *AREG* and *EREG* (Fig. 7).

Interesting observations emerged from the comparison of *in vivo* and *in vitro* data. Although *AREG* and *EREG* were induced by LH surge, their fold inductions were low, in comparison with *PTGS2* and *TNFAIP6*. The *in vitro* temporal pattern suggests that this may be due to the fact that *AREG* and *EREG* expression had already resided at 24 h after being highly induced at earlier time points. Another important observation is related with *PTX3*, which is considered as a bona fide ovulatory protein necessary for ECM stability during cumulus expansion (Varani *et al*. 2002, Salustri *et al*. 2004). *PTX3*, as shown before and also in this study, is induced in GCs by the LH surge in mice and bovine (Varani *et al*. 2002, Christenson *et al*. 2013). However, our study demonstrates that although GCs do express *PTX3*, it was not induced in isolated cell culture but rather inhibited by elevated cAMP levels. In fact, the mode of *PTX3* induction is not well established yet and warrants

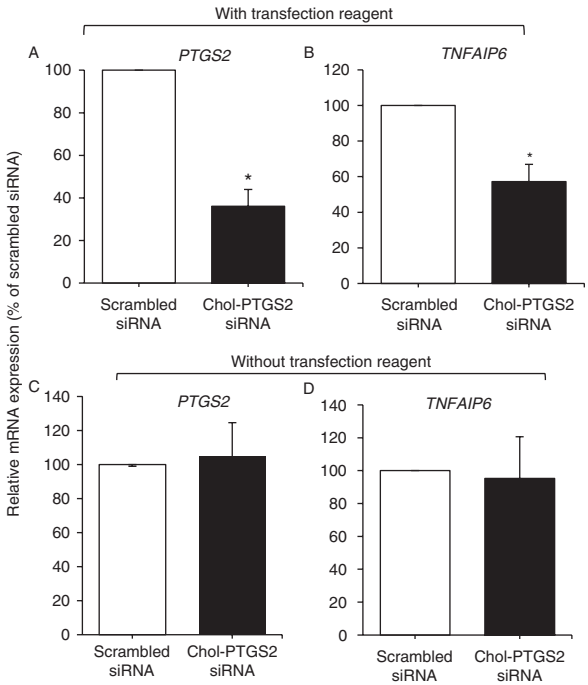

Figure 4 Cholesterol-conjugated *PTGS2* siRNA treatment in bovine GCs with and without transfection reagent. Bovine GCs were treated with 50 nmol/l of scrambled siRNA (negative control; designated 100%) or cholesterol-conjugated *PTGS2* siRNA (chol-*PTGS2* siRNA) with transfection reagent (A and B) or without transfection reagent (C and D). 24 h post transfection/treatment forskolin (10 μM) was added and RNA was extracted 48 h post transfection/treatment the cells. mRNA levels were measured using quantitative real-time PCR. Scrambled siRNA was designated as 100%. The data were obtained from three independent experiments. *$P<0.05$ indicates significant difference from scrambled siRNA.

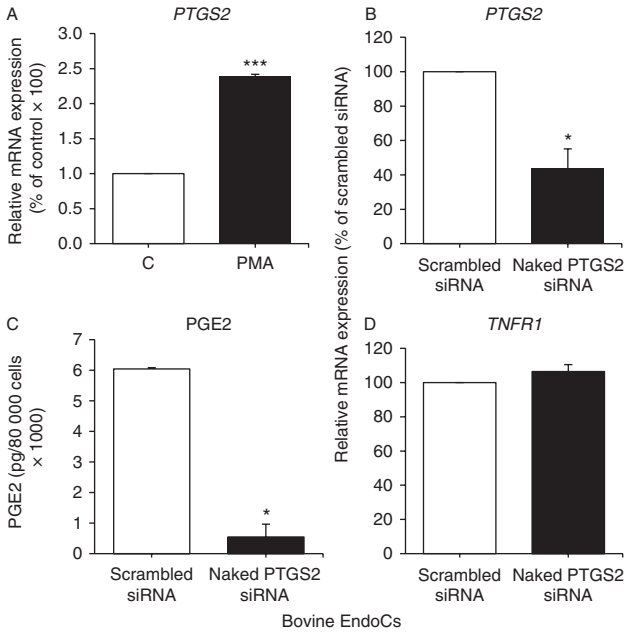

Figure 5 Expression of PTGS2 and PGE2 concentrations in bovine endometrial cells. (A) *PTGS2* expression in bovine EndoCs incubated with or without PMA (100 ng/ml) for 24 h. (B) Silencing of *PTGS2* in bovine EndoCs. The cells were transfected with 50 nmol/l of scrambled siRNA; designated 100% or siRNA-targeting *PTGS2* (naked PTGS2 siRNA). 24 h post transfection PMA (100 g/ml) was added and RNA was extracted 48 h post transfection. mRNA levels were measured using quantitative real-time PCR. (C) PGE2 concentrations in culture media of cells transfected with scrambled siRNA or naked PTGS2 siRNA. (D) *TNFR1* expression in scrambled siRNA and naked *PTGS2* siRNA transfected cells. The data were obtained from four independent experiments. $*P<0.05$ and $***P<0.001$ indicate significant differences from scrambled siRNA.

further investigation. Yet, lack of induction in isolated GCs culture strongly suggests that other follicular compartments may mediate its induction by LH. Indeed *in vitro* induction of *PTX3* (by cAMP and PGE2) was demonstrated in the past only in murine COC, where the presence of oocyte appears to be essential (Salustri *et al*. 2004).

The temporal and functional relationship between PGs and EGF-like factors has been inconclusive. *Ptgs2*-knockout mice have reduced level of *Areg* and *Ereg* (Shimada *et al*. 2006). In agreement, PGE2 induces *AREG* and *EREG* in a dose-dependent fashion in human GCs (Ben-Ami *et al*. 2006). Moreover, injecting intra-follicular indomethacin (INDO) in cows showed reduced *AREG* expression in GCs (Li *et al*. 2009). These reports tend to suggest that PTGS2 induces EGF-like factors. Other studies favour the concept of *PTGS2* being induced by EGF-like factors. For instance, explants of preovulatory mice follicles treated with LH and AG14780 (EGFR kinase inhibitor) showed no expression of *Ptgs2* (Ashkenazi *et al*. 2005). Similarly, reduced *PTGS2* was noted when bovine GCs that were treated with the FRS, an adenylyl cyclase activator and an inhibitor of EGFR tyrosine kinase activity (Sayasith *et al*. 2013). In a recent study, *EGFR* knock-down has resulted in reduced expression of *PTGS2* and decreased *PGE2* (Fang *et al*. 2013).

We found that *PTGS2* knockdown resulted in decreased expression of *AREG* and *EREG*. Also, exogenous PGE2 upregulate the expression of both *AREG* and *EREG*. Expression of *TNFAIP6* was similarly reduced in *PTGS2*-silenced GCs and induction of *TNFAIP6* by exogenous PGE2. Our data therefore support the notion that *AREG* and *EREG* are regulated by *PTGS2* as observed in *Ptgs2*-knockout mice. Our data also point out that *TNFAIP6* is yet another downstream target of PTGS2. Considering the major effect induced by LH/cAMP on these genes, shown here and in many other studies, a likely scenario of post-LH events would suggest that PGs provide a secondary, autocrine pathway to regulate the expression of EGF-like peptide in GCs. However, it is also plausible that *AREG* and *EREG* can in turn provide an additional signal to further induce *PTGS2* and PGs. These multiple-positive autocrine loops would ensure a successful ovulatory process.

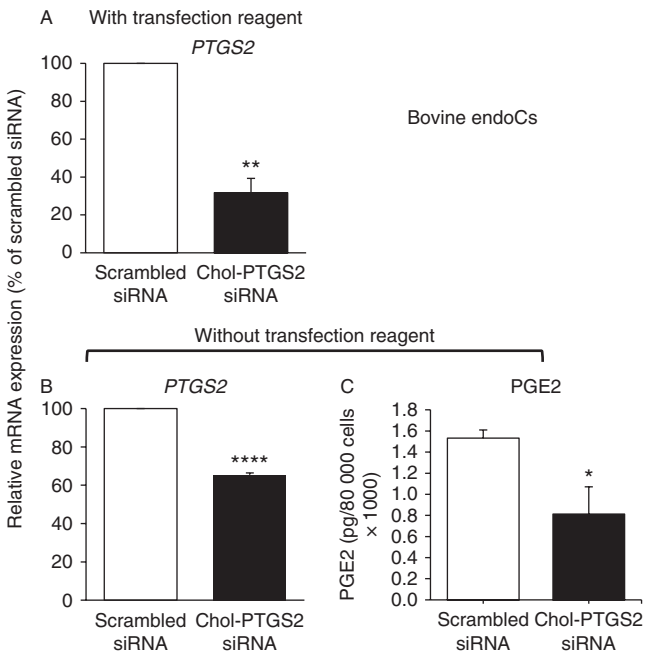

Figure 6 Cholesterol-conjugated *PTGS2* siRNA treatment in bovine endometrial cells (bovine EndoCs) with and without transfection reagent. Endometrial cells were transfected or treated with 50 nmol/l of scrambled siRNA; designated 100% or cholesterol-conjugated *PTGS2* siRNA (chol-*PTGS2* siRNA). 24 h post transfection/treatment PMA (100 g/ml) was added and RNA was extracted 48 h post transfection/treatment for determination of *PTGS2* mRNA using quantitative real-time PCR. (A) *PTGS2* expression in transfected cells. *PTGS2* expression (B) and PGE2 concentrations in culture media (C) of siRNA treated non-transfected cells. The data were obtained from three independent experiments. $*P<0.05$, $**P<0.01$ and $****P<0.0001$ indicate significant differences from scrambled siRNA.

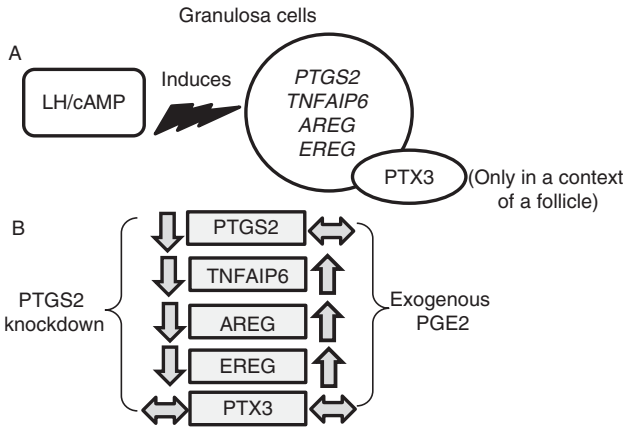

Figure 7 Schematic summary demonstrating induction of ovulatory genes by LH/cAMP and PGE2 in bovine GCs. (A) Role of LH/cAMP on the induction of ovulatory genes. GCs collected from cows 21 h after GNRH-induced LH surge upregulated the expression of following ovulatory genes: *PTGS2*, *TNFAIP6*, *AREG*, *EREG* and *PTX3*. Except *PTX3*, the other genes were also induced *in vitro* by forskolin, directly activating adenynyl cyclase in isolated GCs culture. (B) Role of PGE2 on the expression of ovulatory gene. The mRNA levels of *PTGS2* were significantly inhibited by 70% compared with cells treated with scrambled siRNA. Concentrations of PGE2 in the cell culture media were also markedly reduced to $<1/3$ as compared with control. Along with the silencing of *PTGS2*, the expression of *TNFAIP6*, *AREG* and *EREG* was significantly downregulated. *PTX3* was not affected by *PTGS2* silencing. In agreement with *PTGS2* silencing, incubation with PGE2 (1 μM) for 6 h elevated the mRNA levels of *TNFAIP6*, *AREG* and *EREG*, but not *PTX3*.

Previous studies employed *PTGS2* inhibitors to examine the role of PTGS2 and PGE2 on ovulatory genes. The inhibitors used such as INDO are non-specific as they also affect PTGS1, but even PTGS2 inhibitors can have non-specific effects if not calibrated precisely. In this study, we employed siRNA silencing of *PTGS2* instead, which provides a specific and effective tool to critically examine the role of PGs in GCs *in vitro* but may also allow manipulation of ovulatory process. Furthermore, by inhibiting the mRNA rather than the protein, a more profound and longer inhibition can be achieved. Introducing the inhibitory sequence into small hairpin RNA (shRNA) plasmid can be used for stable knockdown. Furthermore, siRNA provides a means for a post-transcriptional gene regulation *in vitro*, but also *in vivo* in species where gene knockout is not feasible. Under *in vivo* conditions transfection reagents may exhibit immunostimulatory effects and toxicity (Dass 2004). Conjugation of cholesterol, a lipophilic molecule, with siRNA can facilitate the entry of siRNA without the need of transfection reagent (Yuan *et al*. 2008, Medvedeva *et al*. 2009, Wu *et al*. 2009). Chol-*PTGS2* siRNA can be therefore used in various cell types expressing *PTGS2*. In the reproductive tract, EndoCs are most relevant where *PTGS2*-derived PGs plays a central role in the regulation of the estrous cycle, pregnancy recognition, pregnancy maintenance and parturition (Charpigny, Reinaud *et al*. 1997, Asselin, Lacroix *et al*. 1997, Fuchs, Rust *et al*. 1999, Liu, Antaya *et al*. 2001). In this study, we found chol-*PTGS2* siRNA without transfection reagent to be effective in EndoCs, suggesting that this molecule can be administered locally in the uterine horn of farm animals to decisively examine the role of *PTGS2* during the estrous cycle or pregnancy. In contrast, chol-*PTGS2* siRNA was not effective in GCs without transfection reagent, the possible reasons are not clear, but it might be related with differences in membrane composition and/or the fact that EndoCs are proliferating at higher rates as compared with GCs.

In conclusion, our data confirm that the induction of ovulatory genes in bovine GCs, such as *PTGS2*, *AREG*, *EREG* and *TNFAIP6*, is dependent on LH/cAMP *in vivo* and *in vitro*. We had achieved a successful and specific knockdown of *PTGS2* in GCs. *PTGS2* silencing also caused significant reduction in mRNA levels of *AREG*, *EREG* and *TNFAIP6*. Exogenous PGE2 increased these genes, further confirming that these genes are prostanoid dependent. In isolated GCs culture, neither cAMP nor PGE2 elevated *PTX3*. *PTGS2* knockdown in GCs and EndoCs can be utilised to critically determine the functions of *PTGS2 in vitro*. siRNA ablation of PTGS2 in the reproductive tract as well as of other genes through local delivery may provide a novel approach for studying gene functions in large animals *in vivo*.

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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