

Oestrogen formation and connective tissue growth factor expression in rat granulosa cells

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Abstract

Ovarian follicular development involves continual remodelling of the extracellular matrix (ECM) forming the basement membrane and intercellular framework that support granulosa cell (GC) growth and differentiation. Insight into the molecular regulation of ovarian ECM remodelling is potentially translatable to tissue remodelling elsewhere in the body. We therefore studied the link between a gene marker of ECM remodelling (connective tissue growth factor (CTGF)) and oestrogen biosynthesis (cytochrome P450_{aromatase} (P450_{arom})) in rat granulosa cells. To determine if a cause–effect interaction exists, we used semi-quantitative *in situ* hybridisation to analyse patterns of CTGF and P450_{arom} mRNA expression and immunohistochemistry to detect CTGF protein localisation throughout follicular development, and tested the actions of CTGF on oestrogen biosynthesis and oestradiol on CTGF mRNA expression in isolated GC *in vitro*. CTGF mRNA levels in GC rose gradually through small preantral (SP) and small antral (SA) stages of development to a maximum (fivefold higher) in large antral (LA) follicles. In preovulatory (PO) follicles, the CTGF mRNA level fell to 30% of that in SP follicles. P450_{arom} mRNA also increased (threefold in LA relative to SP) throughout antral development follicles, but in contrast to CTGF continued to increase (12-fold) in PO follicles. In the cumulus oophorus of PO follicles, the residual GC CTGF mRNA expression increased with proximity to the oocyte, being inversely related to P450_{arom}. Elsewhere in the follicle

wall, there was a mural-to-antral gradient of CTGF mRNA expression, again inversely related to P450_{arom}. Immunohistochemistry showed CTGF protein localisation broadly followed mRNA expression during follicular development, although the protein was also present in the theca interna and ovarian surface epithelium. Gradients in CTGF expression across the cumulus oophorus and follicle wall were similar to those observed for mRNA with CTGF protein expression being greatest in proximity to the oocyte. Treatment of isolated GC from preantral and SA follicles with recombinant human CTGF (1–100 ng/ml) did not affect basal or FSH-stimulated GC aromatase activity. However, in the absence of FSH, oestradiol (10^{-7} – 10^{-5} M) stimulated CTGF mRNA expression up to twofold. Conversely, FSH (10 ng/ml) alone reduced CTGF mRNA expression by 40% and combined treatment with FSH and oestradiol further suppressed CTGF to 10% of the control value. The oestrogen receptor (ER) antagonist, ICI 182 780 blocked the stimulatory and inhibitory effects of oestradiol, suggesting a specific ER-mediated mode of action on CTGF. Therefore, CTGF gene expression in GC is under local control by oestrogen whose effect (positive or negative) is modulated by FSH. This helps explain why gene expression of CTGF and P450_{arom} diverge in FSH-induced PO follicles and has implications for oestrogenic regulation of CTGF formation elsewhere in the body.

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Introduction

Ovarian follicular development requires sustained formation and breakdown of the extracellular matrix (ECM) with collateral development and disruption of local vasculature. A paracrine factor newly implicated in this process in the ovary is connective tissue growth factor (CTGF, CCN2; *Slee et al.* 2001). CTGF is a member of the multi-domain gene family known as CCN (CTGF/Cysteine-rich 61/Nephroblastoma) and has been identified in numerous epithelial-derived cells with multiple roles connected with tissue remodelling (*Brigstock* 1999). Through mediation or modulation of

TGF β action, CTGF regulates connective tissue synthesis in fibroblasts (*Duncan et al.* 1999), osteoblasts (*Pereira et al.* 2000), chondrocytes (*Eguchi et al.* 2001), vascular smooth muscle cells (*Fan et al.* 2000) and renal mesangial cells (*Goppelt-Strube et al.* 2001).

We previously showed that rat granulosa cells in preantral and early antral follicles abundantly express CTGF (*Slee et al.* 2001), which is upregulated by TGF β superfamily members and androgens but downregulated by follicle stimulating hormone (FSH) *in vitro* (*Harlow et al.* 2002). In addition, we observed an apparent inverse relationship, detected by *in situ* hybridisation, between CTGF and cytochrome P450_{aromatase}

(P450_{arom}) mRNA expression (i.e. oestrogen synthesis) in the developing rat follicle (Slee *et al.* 2001). Ovarian CTGF immunoreactivity has previously only been observed in the pig (Wandji *et al.* 2000), where it paralleled mRNA expression in granulosa, theca and vascular endothelial cells of the theca and corpus luteum. Several ECM components have been characterised during mouse follicular development (Berkholtz *et al.* 2006). For example, fibronectin and type IV collagen are detectable in theca, stroma and basement membrane of small preantral (SP) follicles and increase as the follicle size increases (Berkholtz *et al.* 2006). Thus, based on the pattern of CTGF expression during folliculogenesis in rat (Slee *et al.* 2001, Harlow *et al.* 2002), luteal development in pig (Wandji *et al.* 2000) and ECM component expression during mouse follicle development (Berkholtz *et al.* 2006), granulosa cell-derived CTGF likely plays a critical role in theca cell recruitment, follicle growth and corpus luteum vascularisation.

The intrafollicular role of oestrogens in rodents remains unclear, with conflicting reports on whether oestrogens directly affect the development of follicles *in vivo* (for reviews, see Hillier *et al.* 1980, Rosenfeld *et al.* 2001). *In vitro* studies suggest that oestrogens can stimulate P450_{arom} activity and induce gonadotrophin receptors (Hsueh *et al.* 1984). In the light of our previous observation that androgens modulate CTGF mRNA expression (Harlow *et al.* 2002), here we ask if oestrogen locally affects CTGF gene expression and vice versa in rat ovarian follicles. Results reveal evidence for a direct action of oestrogen on CTGF gene and protein expression in the developing preovulatory (PO) follicle.

Materials and Methods

Hormones and tissue culture reagents

Diethylstilbestrol (DES), oestradiol, testosterone and BSA (fraction V) were obtained from Sigma. Oestrogen receptor (ER) antagonist ICI 182 780 was obtained from Tocris Crookson Ltd (Bristol, UK). Recombinant human FSH (rhFSH; 3860 IU/mg) was donated by Dr C Howles (Serono Laboratories, Inc. Welwyn Garden City, UK). rhCTGF was provided by Fibrogen, Inc. (South San Francisco, CA, USA). Culture medium was medium 199 with 25 mM HEPES, without phenol red, supplemented with 2 mM L-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin (all from Invitrogen Ltd) and 0.1% (w/v) BSA. Donor calf serum and Dulbecco's PBS were also obtained from Invitrogen Ltd.

Animals

Twenty-one-day-old female Wistar rats (Charles River Laboratories, Inc., Margate, Kent, UK) were housed under temperature-controlled conditions on a 12 h light:12 h darkness cycle and fed rat chow available *ad libitum*. Handling and treatment of animals were according to the Animals (Scientific Procedures) Act, 1986. For *in situ* hybridisation

and immunohistochemistry studies (see below), rats were either left untreated or injected with equine chorionic gonadotropin (eCG; 10 IU) and ovaries collected 48 h later. Six animals were used for each experiment. For *in vitro* studies, proliferating but essentially undifferentiated, granulosa cells were induced by giving twice daily s.c. injections of DES (2 mg/day) in ethanol:propylene glycol (5:95) to stimulate preantral/early antral follicular development. Thirty animals were used in each experiment.

Granulosa cell isolation and culture

Animals were killed by asphyxiation with CO₂ and the ovaries removed. Granulosa cells were isolated by puncturing follicles with a 25 gauge hypodermic needle and gently expelling the cells into medium. Pooled cells were centrifuged, resuspended in fresh medium and their viability assessed by counting a Trypan blue-stained preparation in a haemocytometer. Cell viability was 25–30%. Tissue culture grade 24-well plastic dishes (Corning, Inc., Corning, NY, USA) were precoated with 0.25 ml donor calf serum and washed twice with Dulbecco's PBS (0.5 ml) before inoculating with 0.25 ml culture medium containing 1 × 10⁵ viable cells. Following overnight preincubation at 37 °C in a humidified atmosphere containing 5% CO₂ in air, 0.25 ml prewarmed medium containing hormone treatments was added (24–36 wells per treatment) and the incubation continued for 48 h. Aromatase activity was measured in a separate group of four culture wells after the incubation by washing the cell monolayers twice with prewarmed Dulbecco's PBS and adding fresh medium containing 1 µM testosterone as substrate. This medium was collected after a further 3-h incubation and assayed for oestradiol by RIA (Hillier & de Zwart 1982).

In situ hybridisation

Ovaries were fixed in 4% paraformaldehyde in PBS and embedded in paraffin blocks. Following digestion with proteinase K and acetylation, the sections (10 µm) were hybridised (overnight incubation at 55 °C) with cRNA probes generated from cDNA templates labelled with [³⁵S]UTP (Amersham International) using an RNA transcription kit (Promega). Slides were washed in buffers of decreasing salt concentrations, dehydrated through ethanol gradients and processed for liquid emulsion autoradiography (Kodak NTB-2). After exposure for 3 weeks at 4 °C, slides were developed, counterstained with haematoxylin and taken for photomicrography. Sense cRNA probes were used as a control for non-specific binding.

CTGF immunohistochemistry

Five-micrometre sections of ovaries of eCG-treated rats were dewaxed and antigen retrieval was achieved by pressure cooking in 10 mM citrate buffer. Serum blocking was with normal goat serum in PBS-BSA followed by avidin-biotin

block. Primary antibody (rabbit anti-CTGF raised against full-length 38 kDa glycosylated human CTGF, kindly donated by Dr David Brigstock, Center for Cell and Vascular Biology, Children's Research Institute, Columbus, OH, USA) was applied at 40 µg/ml overnight followed by secondary antibody (biotinylated goat anti-rabbit 1:500 in NGS/PBS/BSA, Molecular Probes; Invitrogen) for 30 min. Antigen binding was localised using Streptavidin Alexa 546 (Molecular Probes; Invitrogen) diluted in the ratio of 1:200 in PBS for 1 h followed by nuclear counterstaining with Sytox Green (Molecular Probes; Invitrogen) 1:1000 in PBS for 5 min. Slides were mounted in Permafluor and visualised using a LSM510 confocal microscope with argon laser (505–550 nm filter) and helium–neon laser with long-pass 560 nm filter. For negative control slides, the primary antibody was replaced with the same concentration of antibody that had previously been incubated overnight with a fivefold excess of recombinant human CTGF (Fibrogen) to block active binding sites.

RNA isolation and northern blot analysis

Total RNA was isolated from rat granulosa cell monolayers using RNeasy (Tel-Test, Friendswood, TX, USA), with the following modification of the manufacturer's instructions. Cell monolayers were drained of excess medium and 0.1 ml RNeasy added to each well. The lysates from 12 wells were pooled and mixed with 0.2 ml chloroform. This mixture was added to a Phase Lock Gel tube (Eppendorf AG, Hamburg, Germany) and the aqueous phase separated by centrifugation according to the manufacturer's instructions.

Total RNA (5 µg) was size-fractionated by electrophoresis on a 1% agarose–formaldehyde denaturing gel for 3 h at 80 V and visualised with ethidium bromide. The RNA was transferred overnight to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech) which was then baked for 2 h at 80 °C. Rat CTGF transcripts were detected by probing with a cDNA clone corresponding to nucleotides 934–1340 of the full-length rat CTGF cDNA (Xu *et al.* 2000; Genbank accession number: AF120275), as described previously (Slee *et al.* 2001). Northern blot hybridisation was performed using standard methods. Hybridisation was in Ultrahyb buffer (Ambion (Europe) Ltd, Huntingdon, Cambs, UK) at 42 °C using ³²P-labelled probes (Redivue, Amersham Pharmacia Biotech). Posthybridisation washes were two 5-min washes in 2× SSC and two 15-min washes in 0.1× SSC each with 0.1% SDS at 42 °C. Hybridisation was quantified by electronic autoradiography using an Instant Imager (Packard, Downers Grove, IL, USA), and exposed to autoradiographic film (XAR-5, Eastman Kodak Co.) for 2–24 h at –70 °C. After stripping, blots were reprobed with a rat 18S rRNA cDNA probe (donated by Dr G Scobie, MRC Human Reproductive Sciences Unit, Edinburgh, UK) to allow correction for gel loading and transfer. Northern blots were quantified by electronic autoradiography (Instant Imager, Packard, Downers Grove, IL, USA) with normalisation of mRNA abundance to 18S rRNA. Experimental results are presented relative to unstimulated, control values.

RNA extraction and TaqMan real-time PCR analysis

RNA was extracted from granulosa cells using RNeasy minispinn columns (Qiagen Ltd) as per the manufacturer's protocol, including DNase treatment (1 U) on the column. Portions (1 µl) of purified RNA were removed for quantification and quality assessment, using the Agilent 2100 Bioanalyser system for total RNA in combination with RNA6000 nano chips (Agilent Technologies, Cheshire, UK). Only RNA that displayed intact 18S and 28S peaks was reverse transcribed to cDNA for real-time PCR analysis. This quality control step was included for each experimental run to avoid generation of false negative results due to RNA degradation prior to and during extraction steps, and also as a quantification method to ensure equal amounts of RNA were transcribed in each RT reaction.

Real-time PCR analysis

Total RNA (200 ng) was reverse transcribed (random hexamer kit; Applied Biosystems), and 2 µl RT mix was analysed. The final reaction volume was 25 µl containing 300 nmol/l primers and 200 nmol/l TaqMan hybridisation probe (Biosource UK Ltd). Primers and probes were designed using Primer-Express software (Perkin-Elmer, Beaconsfield, Bucks, UK). Target mRNA was quantified in relation to 18S rRNA abundance in each sample, with suitable positive-control RNA (rat liver total RNA (Ambion (Europe) Ltd) and in-house prepared rat placental mRNA), and negative controls consisting of RT-negative (RNA template with no reverse transcriptase enzyme), and RT-H₂O (water in place of RNA template) samples generated at the time of reverse transcription of samples, plus a TaqMan reaction negative control where cDNA was replaced with water. The sequences of primers and probe were as follows: forward primer: CGCCAACCGCAA-GATTG; reverse primer: ACACGGACCCACCGAAGAC; probe: TGCCCTGCCAAAGATGGTGCAC. The primer/probe set was validated prior to use.

Image analysis

In situ hybridisation slides were visualised using bright- and dark-field microscopy and images captured using a charge-coupled device (CCD) camera. Openlab v2.08 (Improvision Ltd, Coventry, UK) was used as a platform to create an automated image analysis system for the measurement of follicle diameter and hybridisation signal intensity. Diameter was determined by drawing around the outside surface of the granulosa compartment. To ensure that maximum follicle diameter was measured, the analysis was restricted to sections passing through the oocyte. The programme calculated the mean diameter from the area measured. Follicles were divided into five class sizes based on their mean diameter, identified as follows: small preantral (SP), 50–117 µm; large preantral (LP), 118–165 µm; small antral (SA), 166–273 µm; large antral (LA), 274–449 µm; PO, 450–600 µm. Silver grain intensity was assigned a value on an arbitrary scale from 1 (black) to 255 (white). For cumulus oophorus readings, signal intensity was

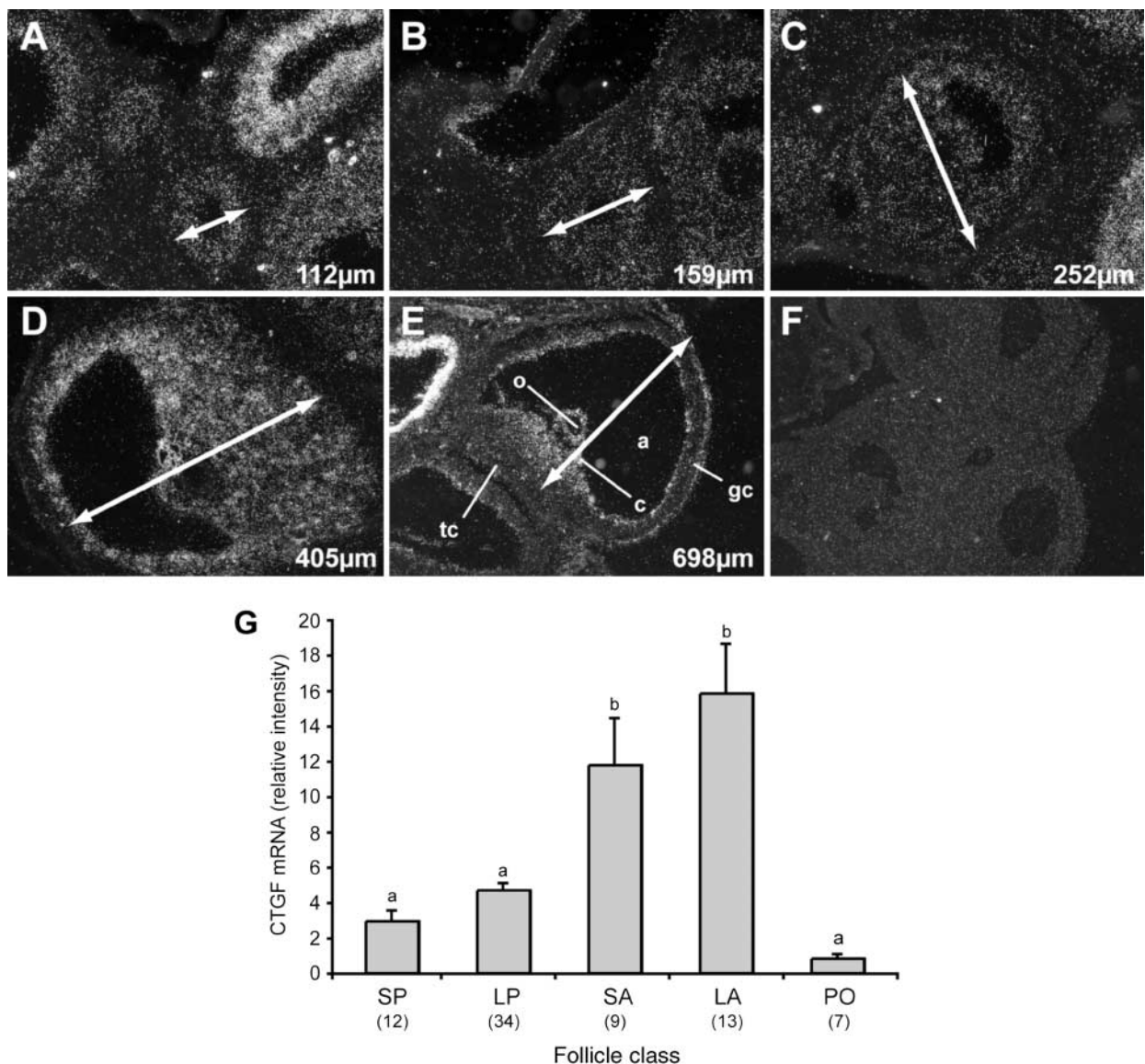


Figure 1 Granulosa cell CTGF mRNA expression in relation to follicular maturity in rat ovary. *In situ* hybridisation analysis using a S^{35} -labelled CTGF cRNA probe was carried out on ovarian sections from untreated and eCG-treated immature rats containing follicles at varying stages of follicular maturation, as described in Materials and Methods. (A–F) Digital photomicrographs of CTGF *in situ* hybridisation images. (A) Small preantral follicle, (B) large preantral follicle, (C) small antral follicle, (D) large antral follicle, (E) preovulatory follicle, and (F) sense strand control depicting follicles ranging from small preantral to large antral. Most intense signal was observed in the large antral follicles. Diameter of the granulosa cell (gc) compartment (arrow) is displayed in bottom right. a, antral cavity; c, cumulus; o, oocyte; tc, theca cell compartment. (G) Quantitative analysis of CTGF *in situ* hybridisation data from granulosa cell compartment of ovarian follicles: SP, small preantral; LP, large preantral; SA, small antral; LA, large antral; PO, preovulatory. Mean \pm S.E.M. Number of follicles analysed is given at the base of each bar. One-way ANOVA showed a significant effect of follicle size ($F=17.24$; d.f. = 4,70; $P<0.0001$). Bars with different superscript letters are significantly different ($P<0.001$, Fisher's PLSD).

read sequentially at 15 equidistant locations across the cumulus granulosa cell layer, progressing from the mural granulosa cell layer (position 1) to cells adjacent to the oocyte (position 15) in five PO follicles. Measurements from elsewhere comprised nine successive readings from the mural (position 1) to antral (position 9) granulosa cell layers of the same follicles.

Data analysis

Data were analysed using one-way ANOVA with Fisher's protected least-square differences (PLSD) to determine the effects of individual treatments. Image analysis data were compared by one-way ANOVA and general linear model

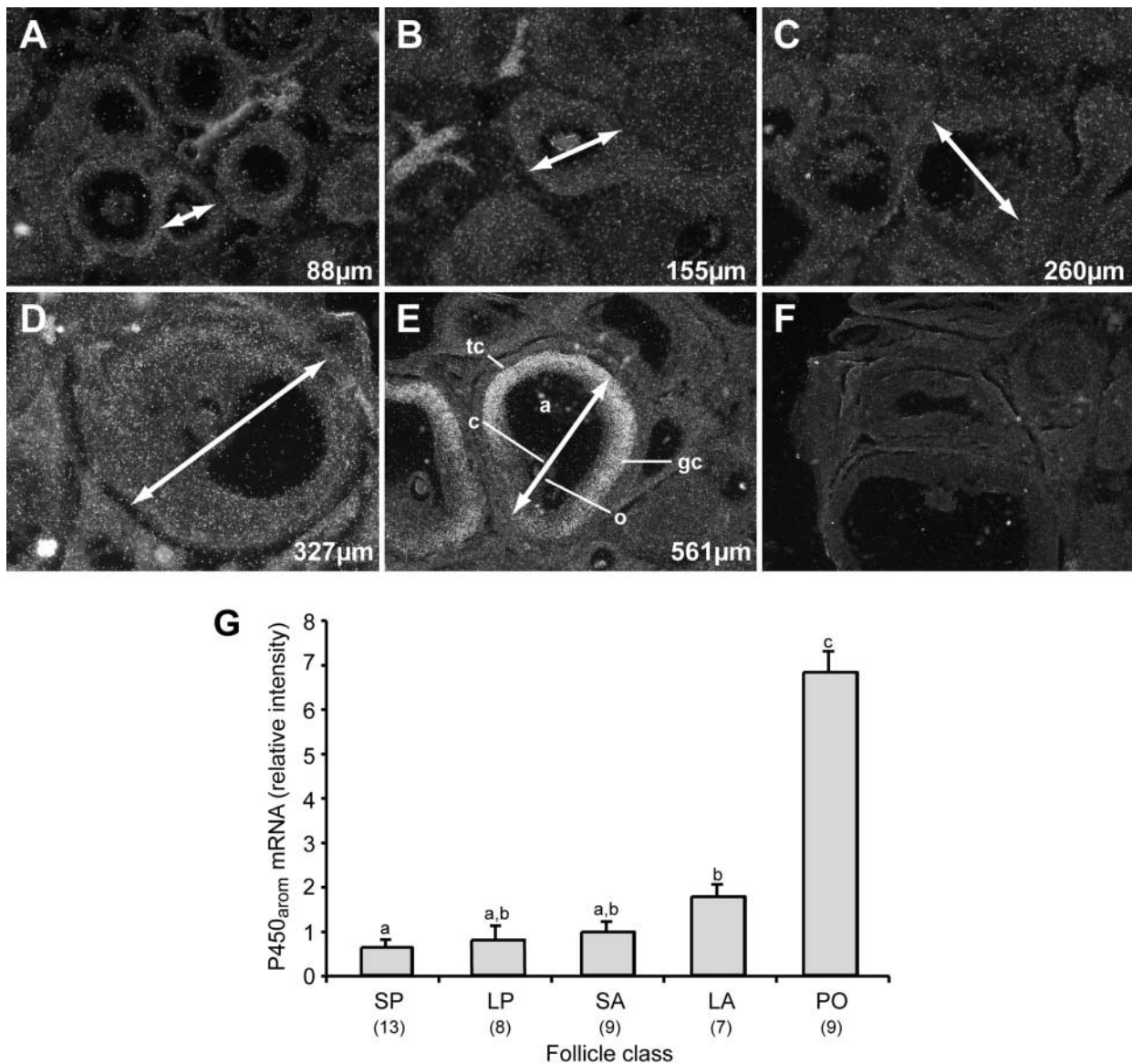


Figure 2 Granulosa cell P450_{arom} mRNA expression in relation to follicular maturity in rat ovary. *In situ* hybridisation analysis using a S^{35} -labelled P450_{arom} cRNA probe was carried out on ovarian sections from untreated and eCG-treated immature rats containing follicles at varying stages of follicular maturation, as described in Materials and Methods. (A–F) Digital photomicrographs of P450_{arom} *in situ* hybridisation images from (A) small preantral follicle, (B) large preantral follicle, (C) small antral follicle, (D) large antral follicle, (E) preovulatory follicle, and (F) sense strand control depicting follicles from large preantral to preovulatory stages. Most intense signal was observed in the preovulatory follicles. Diameter of the granulosa cell (gc) compartment (arrow) is displayed in bottom right. a, antral cavity; c, cumulus; o, oocyte; tc, theca cell compartment. (G) Quantitative analysis of P450_{arom} *in situ* hybridisation data from granulosa cell compartment of ovarian follicles: SP, small preantral; LP, large preantral; SA, small antral; LA, large antral; PO, preovulatory. Mean \pm S.E.M. Number of follicles analysed is given at the base of each bar. One-way ANOVA showed a significant effect of follicle size ($F=62.65$, d.f. = 4,41, $P<0.0001$). Bars with different superscript letters are significantly different ($P<0.05$, Fisher's PLSD).

to dissect the main effects and interactions. Differences with $P<0.05$ were regarded as significant. TaqMan results from different experiments were normalised relative to control (untreated) cells, and the statistical analysis was performed on the raw data using one-way ANOVA with Fisher's PLSD to determine the effects of individual treatments.

Results

Spatiotemporal relationships between CTGF and P450_{arom} mRNA expression

CTGF mRNA abundance increased gradually from low levels in SP follicles to maximum levels that were fivefold

higher in LA follicles (Fig. 1). In PO follicles, CTGF mRNA decreased to 30% of that observed in SP follicles (Fig. 1). By contrast, P450_{arom} did not increase in LP and SA follicles, increased threefold in LA follicles, but increased a further fourfold in PO follicles (Fig. 2).

Measurement of signal intensity across the cumulus oophorus of PO follicles showed a gradual linear increase in CTGF mRNA from cells on the basement membrane to those in the vicinity of the oocyte, with an inverse relationship to P450_{arom} mRNA (Fig. 3). Elsewhere in the follicle wall, the rise in CTGF mRNA

from mural to antral granulosa cells was exponential in nature and again inversely related to P450_{arom} (Fig. 4).

Spatiotemporal changes in localisation of CTGF protein

Granulosa cell CTGF protein localisation was detected immunohistochemically at all stages of follicular development (Fig. 5) but was greatest in SA follicles (Fig. 5D). Specific CTGF immunostaining was also noted in theca cells of some follicles (Fig. 5B and D) and in the ovarian surface epithelium

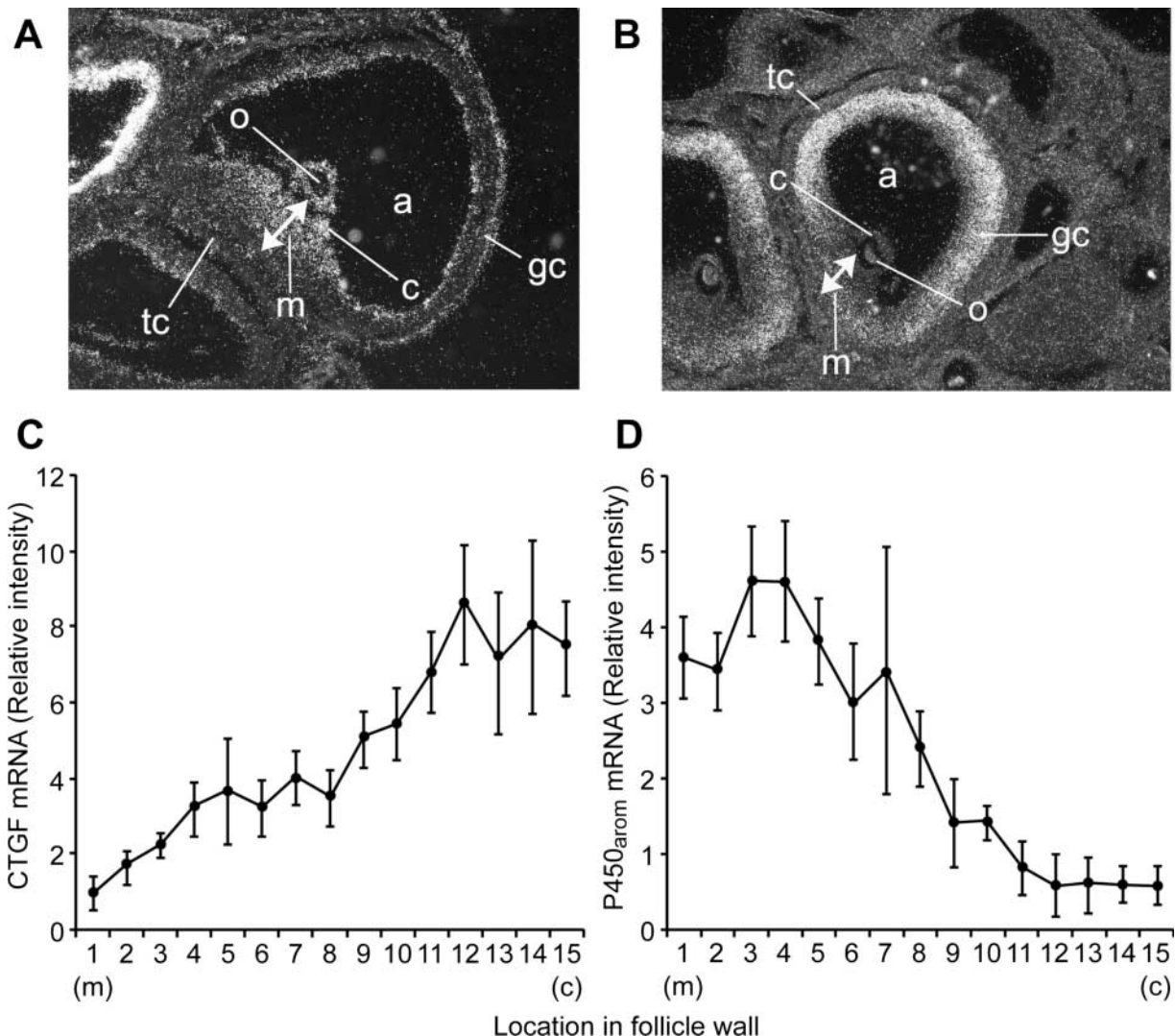


Figure 3 Reciprocal relationship between CTGF and P450_{arom} mRNA expression in cumulus oophorus granulosa cells of preovulatory follicles. *In situ* hybridisation analysis using a ³⁵S-labelled CTGF and P450_{arom} cRNA probes was carried out on ovarian sections from eCG-treated immature rats, as described in Materials and Methods. Digital photomicrographs of (A) CTGF and (B) P450_{arom} *in situ* hybridisation showing the gradient of CTGF mRNA intensity across the cumulus oophorus from mural (m) to cumulus (c) granulosa cells. a, antrum; gc, granulosa cells; o, oocyte; tc, theca cells. The arrows show representative sampling regions. (C) CTGF and (D) P450_{arom} signal intensities read sequentially from 15 equidistant locations across the cumulus granulosa cell layer, progressing from the mural granulosa cell layer (position 1) to the corona radiata (position 15; see Materials and Methods) Mean ± s.e.m., n=5. One-way ANOVA showed a significant effect of distance (CTGF: $F=4.33$, d.f.=4,41, $P<0.0001$; P450_{arom}: $F=8.12$, d.f.=14,60, $P<0.0001$).

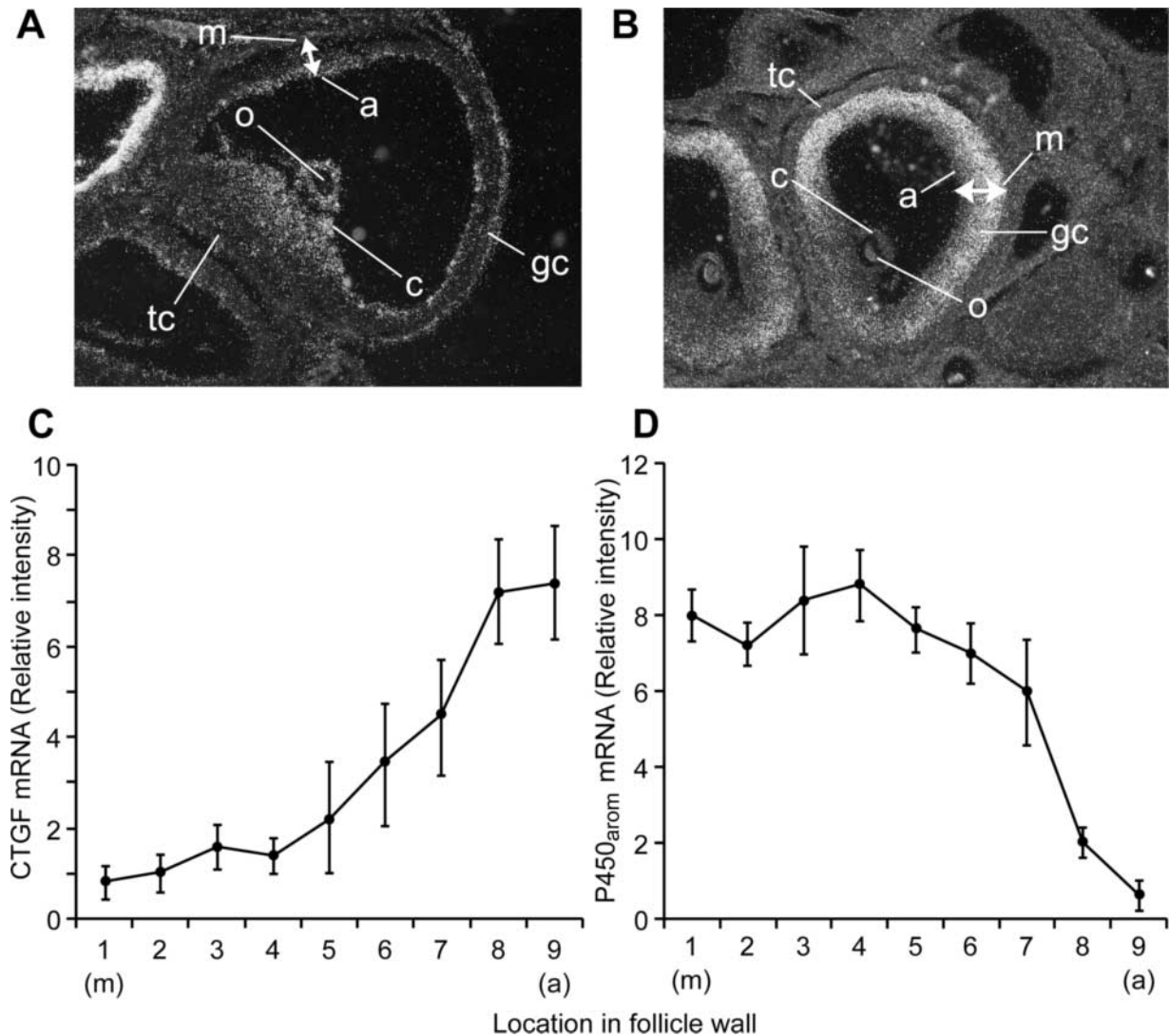


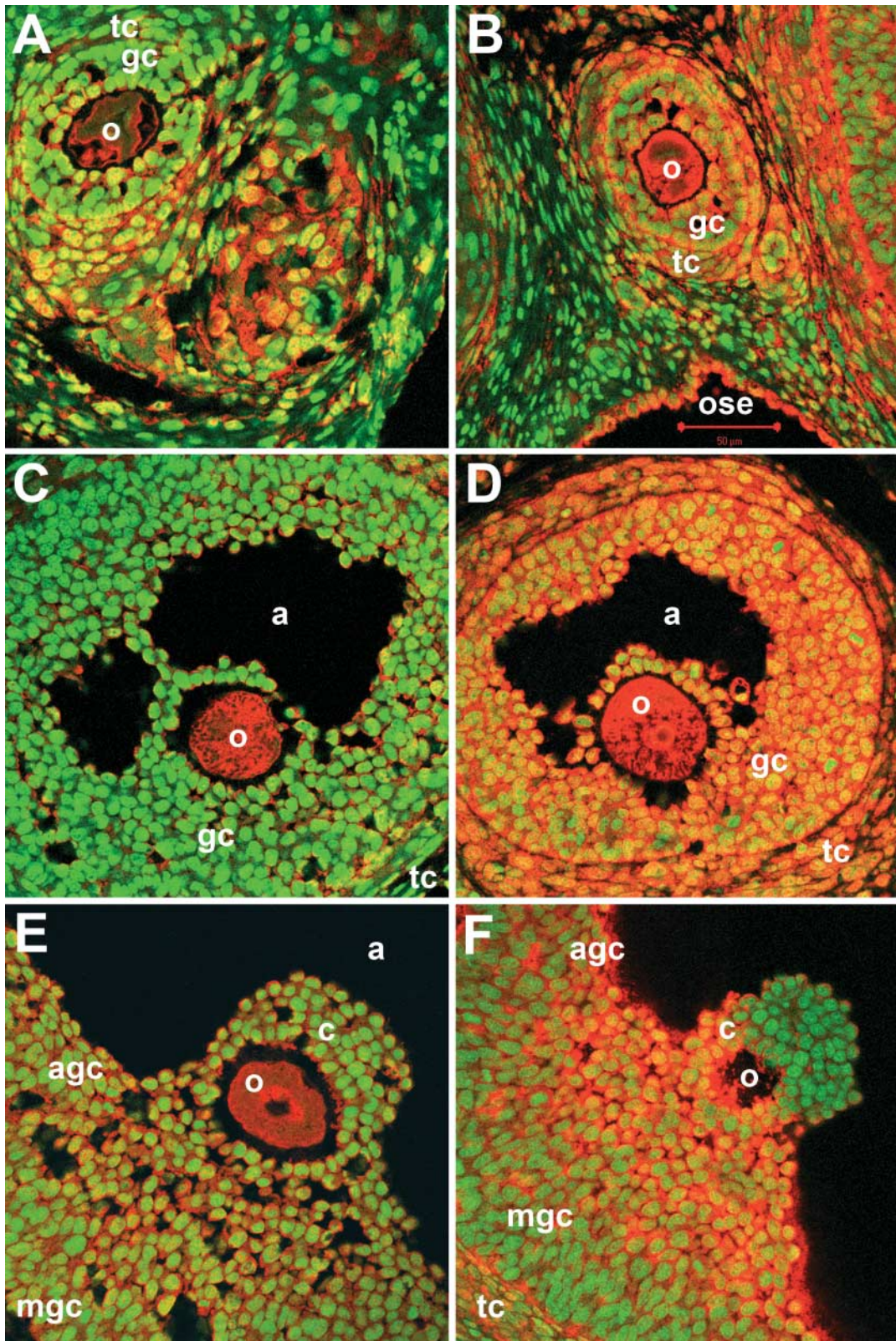
Figure 4 Reciprocal relationship between CTGF and P450_{arom} mRNA expression across the walls of preovulatory follicles. *In situ* hybridisation analysis using a ³⁵S-labelled CTGF and P450_{arom} cRNA probes was carried out on ovarian sections from eCG-treated immature rats, as described in Materials and Methods. Digital photomicrographs of (A) CTGF and (B) P450_{arom} *in situ* hybridisation showing the gradient of CTGF mRNA intensity across the preovulatory follicle wall from mural (m) to antral (a) granulosa cells. gc, granulosa cells; o, oocyte; tc, theca cells. The arrows show representative sampling regions. (C) CTGF and (D) P450_{arom} signal intensities read sequentially from nine equidistant locations progressing from the mural (position 1) layer to the antral (position 9) granulosa cell layer (see Materials and Methods). Mean \pm S.E.M., $n=5$. One-way ANOVA showed a significant effect of distance (CTGF: $F=6.85$, d.f. = 8,36, $P<0.0001$; P450_{arom}: $F=10.60$, d.f. = 8,36, $P<0.0001$).

(Fig. 5B). Apparent intense staining of oocytes was non-specific (Fig. 5C and D). In PO follicles, there was intense specific staining of cumulus cells and a clear gradient of expression from coronal/antral to mural granulosa cells (Fig. 5F). Overall expression was less in PO than in SA follicles (Fig. 5D and F).

In vitro studies

The above *in situ* hybridisation and immunohistochemistry results show that in PO follicles that have been exposed to

maturing levels of FSH *in vivo*, and responded by increased expression of P450_{arom}, there is a dramatic reduction in expression of CTGF mRNA and protein. To investigate the interaction of these genes further, and to assess whether CTGF has a direct effect on aromatase activity, we cultured granulosa cells with rhCTGF in the absence or presence of FSH, mimicking the environment in preantral/early antral and PO follicles respectively. To examine the effects of oestradiol on CTGF mRNA expression, we also tested the effects of oestradiol in the presence or absence of FSH to mimic the conditions that occur after PMSG treatment, and



in the presence or absence of an ER antagonist to assess whether the effect of oestradiol was ER mediated.

Effects of CTGF on granulosa cell P450_{arom} enzymic activity

To test for direct effects of CTGF on granulosa cell function, granulosa cells from preantral/SA follicles were cultured for 48 h with CTGF (1–100 ng/ml) in the presence or absence of a fixed dose of FSH (10 ng/ml) before determining aromatase enzyme activity. In four out of four experiments, the presence of CTGF had no effect on basal- or FSH-induced activity (data not shown).

Effects of oestradiol on CTGF mRNA expression

To test the direct effects of oestrogen on CTGF mRNA expression, preantral/SA granulosa cell cultures were treated with oestradiol (10^{-7} – 10^{-5} M) with and without FSH (10 ng/ml) before quantifying CTGF mRNA expression by northern analysis. Oestradiol alone caused a dose-dependent increase in CTGF mRNA expression which was significant at 10^{-5} M (Fig. 6). However, in the presence of FSH (which alone significantly reduced CTGF mRNA expression by 40%), oestradiol caused dose-dependent inhibition of CTGF mRNA expression, which was also significant at a dose of 10^{-5} M (Fig. 6).

ER-mediated oestradiol action on CTGF mRNA expression

To establish an ER-mediated mode of oestradiol action on granulosa cell CTGF mRNA expression, preantral/SA granulosa cells were treated *in vitro* with oestradiol and/or FSH in the presence and in the absence of the selective ER antagonist ICI 182 780, using real-time RT-PCR analysis of CTGF mRNA as readout. As shown in Fig. 7, the increase in CTGF mRNA induced by 10^{-5} M oestradiol was blocked by an equimolar concentration of ICI 182 780 (10^{-5} M). The inhibitory effect of FSH on CTGF mRNA levels in the presence and in the absence of oestradiol was also partially ameliorated by the presence of ICI 182 780 (Fig. 7).

Discussion

These results are novel in three ways. First, we quantify a clear reciprocal relationship between CTGF and P450_{arom} gene expression in ovarian granulosa cells. Secondly, we establish a spatio-temporal pattern of CTGF mRNA expression and protein localisation that strongly implicates CTGF in peri/postovulatory follicular physiology. Thirdly, we

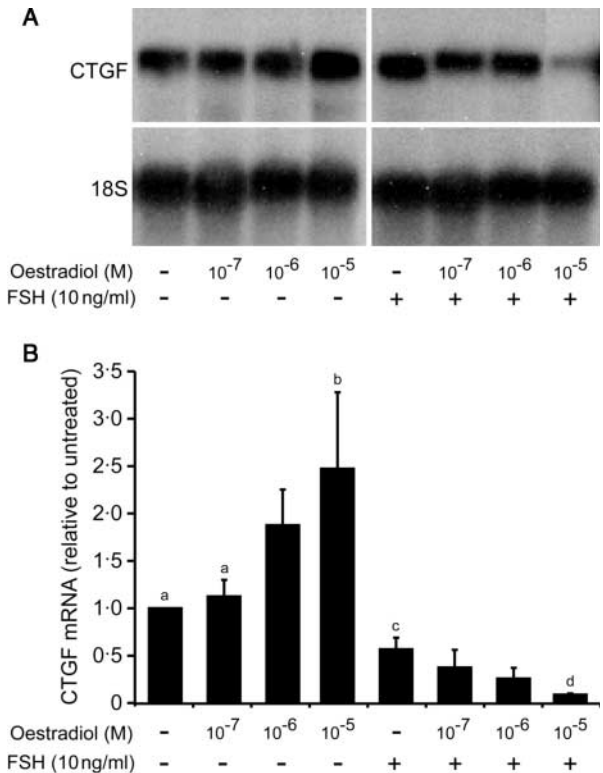


Figure 6 Oestrogenic regulation of granulosa cell CTGF mRNA expression. Granulosa cells from PA/SA follicles in immature rat ovaries were cultured for 48 h with oestradiol and/or FSH, as indicated, then total RNA was extracted for northern analysis of CTGF mRNA. (A) Autoradiogram of a typical northern blot showing CTGF mRNA compared with the signal for 18S rRNA (18S). Due to the large variation in the intensity of the CTGF mRNA signal between treatments with and without FSH, different exposure times were required for the two arms of the experiment (without FSH, 8 h; with FSH, 48 h). (B) Composite quantitative data from three separate experiments. Bars indicate the mean \pm S.E.M. CTGF mRNA intensity relative to the signal for 18S measured by electronic autoradiography. All data were normalised to the control value. ANOVA showed a significant effect of treatment ($F=6.41$, d.f. = 7,16, $P=0.001$). Significant effects comparing individual treatments are indicated by different superscript letters ($P<0.05$, Fisher's PLSD).

demonstrate a direct ER-mediated mode of oestradiol action on granulosa cell CTGF mRNA expression.

Concerning the relationship between CTGF and P450_{arom}, our quantitative *in situ* hybridisation results quell any doubt that these genes might be reciprocally related in PO follicles. Not only are they temporally linked in the sense that as follicles become PO, their granulosa cells show overall massively reduced expression of CTGF in

Figure 5 Immunohistochemical analysis of CTGF protein localisation in rat ovary. Fluorescent double-antibody immunohistochemistry, using rabbit anti-human CTGF primary antiserum, as described in Materials and Methods. Digital photomicrographs showing the distribution of immunoreactive CTGF in A, B small preantral, C, D small antral and E, F preovulatory follicles. A, C and E were from sections in which the primary antibody was preabsorbed with a fivefold excess of CTGF protein. a, antrum; agc, antral granulosa cells; c, cumulus cells; gc, granulosa cells; mgc, mural granulosa cells; o, oocyte; ose, ovarian surface epithelium; tc, theca cells. Scale bar = 50 μ m.

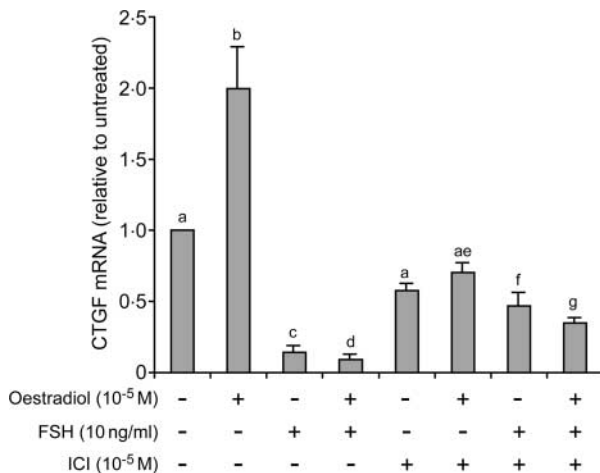


Figure 7 Blockade of oestradiol effects on granulosa cell CTGF mRNA expression by the ER antagonist ICI 182,780 (ICI). Granulosa cells from PA/SA follicles in immature rat ovaries were cultured for 48 h with the treatments indicated. Total RNA was extracted and analysed for CTGF mRNA by quantitative real-time RT-PCR as described in Materials and Methods. Results are expressed as the mean (\pm S.E.M.) of five experiments. ANOVA showed a significant effect of treatment ($F=2.824$, d.f. = 7,31, $P=0.02$). Significant effects comparing individual treatments are indicated by different superscript letters ($P<0.05$, Fisher's PLSD).

the face of increased expression of P450_{arom}, but also a reciprocal pattern of residual expression persists within the follicle wall. Most notably, CTGF mRNA expression and protein localisation persist in antrally located granulosa cells and those closest to the oocyte, implying a role for CTGF in the remodelling of the follicle wall associated with extrusion of the oocyte and formation of the corpus luteum.

The role of CTGF in follicular and luteal physiology seems unlikely to include direct effects on P450_{arom} expression. Instead, we favour stimulation of CTGF expression by oestrogen. In the uterus, there is evidence that oestradiol can upregulate CTGF mRNA in glandular epithelium (Rageh *et al.* 2001), and the related CCN family member, Cyr61 (CCN1) is upregulated in human myometrial explants by oestradiol (Sampath *et al.* 2001). We found no effect of CTGF on oestradiol production *in vitro* despite being able to show that the CTGF added to our culture system was active in stimulating matrix metalloproteinase-2 activity by granulosa cells (AC Bradshaw unpublished observations). Therefore, oestradiol biosynthesis appears not to be affected by biologically active levels of CTGF *in vitro*. Conversely, we found a modest effect of oestradiol on CTGF mRNA levels, consistent with the studies in the uterus. The level of oestradiol required to elicit an effect is within the range measured in rat follicular fluid (Fujii *et al.* 1983). Furthermore, the inhibition by ICI 182 780 of oestradiol-stimulated CTGF mRNA, strongly points to an ER-mediated effect.

The stimulatory effect of oestradiol on CTGF mRNA expression by immature granulosa cells could explain the

associated rise in P450_{arom} and CTGF in follicles that occurs up to the LA stage but not the abrupt decline in CTGF observed in PO follicles at the time of maximum P450_{arom} expression. The latter, and the inverse relationship between CTGF and P450_{arom} across the granulosa cell compartment, suggest an interaction between FSH and oestradiol to downregulate CTGF, which is confirmed in the present study *in vitro*. Since these culture experiments were carried out on granulosa cells from preantral/early antral follicles, they should have been a relatively homogeneous population of immature cells strongly expressing CTGF. However, the inhibition of CTGF expression by oestradiol *in vitro* may not reflect a parallel situation *in vivo*, where multiple positive regulators, including theca-derived androgen (Harlow *et al.* 2002) would be expected positively to impact CTGF expression.

CTGF protein was detected at all stages of follicular development and, unlike mRNA, was present in both granulosa and theca cells of follicles up to SA stage, as well as in the ovarian surface epithelium. There was evidence for an increase in distribution of protein from SP to SA follicles with a decline in PO follicles. This pattern is very similar to that observed during porcine follicular development (Wandji *et al.* 2000). Since CTGF mRNA is generally expressed only at low abundance in thecal cells relative to granulosa cells, we interpret the presence of immunoreactive CTGF protein due to formation in granulosa cells. Based on the established roles of CTGF in collagen biosynthesis, cell motility and angiogenesis, it can be predicted that the theca is a paracrine target of granulosa-derived CTGF (Harlow *et al.* 2002). We previously proposed that granulosa-derived CTGF is one of the main signals for formation of the thecal compartment when follicles form and begin to grow. A continuing role for CTGF in the extensive thecal remodelling required during antral stages of follicular development seems equally likely, particularly in light of the recent finding that key ECM markers, such as collagen IV and fibronectin appear to be developmentally regulated in the mouse follicle (Berkholtz *et al.* 2006). In this connection, CTGF is implicated in the deposition of collagen, through its role in modulating lysyl oxidase (Hong *et al.* 1999), a crucial enzyme required for cross-linking of collagen monomers (Smith-Mungo & Kagan 1998). We also found lysyl oxidase to be developmentally regulated in the rat follicle (Harlow *et al.* 2003).

Previous studies have shown a gradient of P450_{arom} protein expression across the rat PO follicle wall, with intensity declining centripetally (Turner *et al.* 2002). Here, this pattern was confirmed at the mRNA level, inversely related to CTGF mRNA and protein. The gradient in CTGF expression is intriguing, and highlights the probable compartmentalisation within the granulosa layer. However, the biological significance of these findings is unclear. CTGF is implicated in collagen synthesis: for example, CTGF stimulates lysyl oxidase enzyme activity and collagen deposition in human gingival fibroblasts (Hong *et al.* 1999). Granulosa cells are

capable of synthesising many of the components of the basal lamina (Rodgers *et al.* 2000). The presence of increasing CTGF mRNA and protein during follicle growth to the PO stage could provide a driving force to promote the collagen biosynthesis necessary for expansion of the basal lamina. By the time, the CTGF expression declines, follicles have reached close to their maximum size so that further ECM synthesis should be unnecessary. If we assume that the decline in CTGF expression is regulated by rising sensitivity to FSH in the granulosa, it is possible that the gradient in CTGF expression across the granulosa compartment may reflect local differences in FSH receptors. These results may reflect the dominant role of FSH and oestradiol as regulators of granulosa cell differentiation, and demonstrate a divergence between P450_{arom} and CTGF mRNA expression in PO follicles.

We also show that the residual CTGF mRNA and protein in the PO follicle is strongest in the cumulus cells, the same cells that exhibit the lowest P450_{arom} mRNA expression, and protein levels (Turner *et al.* 2002). Higher CTGF in these cells may result from the action of GDF-9 secreted by the oocyte, which we previously showed to enhance CTGF mRNA *in vitro* (Harlow *et al.* 2002). Indeed, a concentration gradient of GDF-9 as distance from the oocyte increases has been proposed (Vitt & Hsueh 2001). However, since GDF-9 synergises with FSH to downregulate CTGF mRNA, this stimulatory effect of GDF-9 could only occur in the absence of appreciable FSH or FSH receptors. Evidence for the expression of FSH receptors in rat cumulus cells is scant, but Magnusson *et al.* (1982) showed responsiveness of rat cumulus cells to FSH in terms of progesterone stimulation, and bovine oocyte cumulus complexes responded to FSH by expanding in size (Van Tol *et al.* 1996). However, there are oocyte-secreted factors, such as BMP-15 that downregulates FSH receptors (Otkay *et al.* 1997) and BMP-6 that downregulates FSH-induced cAMP by suppressing adenylate cyclase (Otsuka *et al.* 2001). Further studies on the expression of FSH receptor, FSH availability and ER isotypes in cumulus cells together with the effects of other oocyte-derived factors are required to confirm cause and consequence of elevated CTGF in the cumulus oophorus. Studies of the effect of ER antagonists on cumulus expansion and CTGF expression *in vivo* might also provide further mechanistic insight. Given the importance of the ECM in the structure and function of the cumulus, and the role of FSH in regulating paracrine interactions via transzonal processes between the oocyte and cumulus (Albertini *et al.* 2001), it is likely that CTGF may play a unique role in cell–cell communication within this highly specialised follicular compartment.

An additional intraovarian role of CTGF may relate to postovulatory angiogenesis (Brigstock 2002). Wandji *et al.* (2000) noted increased CTGF mRNA and protein around vascular endothelial cells in pig ovarian follicles and in association with migrating endothelial cells in the corpus luteum. We have also observed residual presence of CTGF protein in the most antrally located granulosa cells of periovulatory rat follicles which mirrors the pattern of

mRNA expression (Slee *et al.* 2001). This, with sustained expression by adluminal luteal cells noted in the newly formed rat (Slee *et al.* 2001) and human corpus luteum (Duncan *et al.* 2005), further emphasises a likely angiogenic role for CTGF in corpus luteum formation.

In conclusion, we show both *in vivo* and *in vitro* that CTGF mRNA and protein, and P450_{arom}/oestradiol rise in concert during early follicular development up to the LA stage of follicular development. The evidence suggests that oestradiol, at least in part, drives the increase in CTGF. During PO development, the role of oestradiol, under the influence of FSH, switches to suppression of CTGF. The negative spatiotemporal relationship between P450_{arom} and CTGF in the PO follicle extends across the follicle wall, such that antrally located and cumulus granulosa cells show low P450_{arom} but high CTGF gene and protein expression. The particular relevance of CTGF to cumulus cell biology remains to be determined but it could be involved in cumulus expansion or paracrine communication between cumulus cells and oocyte.

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