

Steroidal regulation of connective tissue growth factor (CCN2; CTGF) synthesis in the mouse uterus

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Abstract

Aims—To determine mechanisms regulating the production of connective tissue growth factor (CCN2; CTGF) and transforming growth factor β 1 (TGF- β 1) in the mouse uterus.

Methods—In situ hybridisation and immunohistochemistry were used to localise CCN2 (CTGF) and TGF- β 1 in uteri from sexually mature female mice that had either been (1) mated with sterile males to induce pseudopregnancy or (2) ovariectomised (OVX) and administered estradiol-17 β (E₂) or progesterone (P₄), either alone or in combination. Uteri collected on days 0.5, 1.5, 2.5, 3.5, 4.5, or 5.5 of pseudopregnancy or at one, three, six, 12, or 24 hours after steroid administration were fixed, sectioned, and incubated with specific riboprobes or antibodies to permit detection and localisation of mRNA or protein for CTGF and TGF- β 1.

Results—On days 0.5–2.5 of pseudopregnancy, CCN2 (CTGF) and TGF- β 1 were principally colocalised to uterine epithelial cells, with much smaller amounts in the stroma. On days 3.5–4.5, there was a reduction of CCN2 (CTGF) and TGF- β 1 in the epithelium but an increase in stromal and endothelial cells, corresponding to a period of extracellular matrix remodelling and neovascularisation within the endometrium. In OVX mice, epithelial cells were weakly positive for both CCN2 (CTGF) and TGF- β 1 in the absence of steroid hormones. Epithelial CTGF mRNA production were strongly but transiently stimulated in OVX mice cells by E₂. These effects were antagonised by P₄, which itself transiently stimulated epithelial CCN2 (CTGF) production, although less robustly than E₂. CTGF and TGF- β 1 protein amounts were high in epithelial cells throughout steroid treatment and were increased in the stroma, where they were relatively long lived. Stromal CCN2 (CTGF) and TGF- β 1 were lower after co-administration of E₂ and P₄ than in response to each hormone individually. Although *ccn2* (*ctgf*) is a TGF- β 1 inducible gene in other systems, and both growth factors were often co-localised in uterine tissues in these studies, several treatment regimens resulted in high amounts of TGF- β 1 protein in stromal cells without the concomitant production of *ccn2* (*ctgf*) mRNA.

Conclusions—Maternal factors are principal cues for CCN2 (CTGF) and TGF- β 1 production in the uterus because (1) their expression during pseudopregnancy is comparable to that seen in pregnancy and (2) they are regulated by ovarian steroids. TGF- β dependent and independent mechanisms of *ccn2* (*ctgf*) gene transcription exist in the uterus that are variably regulated by steroid hormones. Collectively, the data support a role for CCN2 (CTGF) in mediating the effects of steroid hormones and TGF- β on endometrial function.

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Connective tissue growth factor (CCN2; CTGF) is a multifunctional mosaic protein that is a member of the CCN2 (CTGF)/CCN1 (CYR61)/CCN3 (NOV) family.¹ CCN2 (CTGF) appears to be involved in diverse cellular functions such as proliferation, adhesion, migration, apoptosis, and extracellular matrix (ECM) production.^{1–3} These wide ranging biological activities, together with mRNA and protein expression studies, have resulted in proposed roles for CCN2 (CTGF) in processes such as embryonic development, tissue differentiation, angiogenesis, and wound healing, in addition to various pathologies such as fibrosis, inflammation, and tumour growth.¹ CCN2 (CTGF) is produced by and acts on a wide variety of cell types including fibroblasts, vascular smooth muscle cells, endothelial cells, epithelial cells, and cells of the nervous system.⁴ In certain cell types, *ccn2* (*ctgf*) is transcriptionally activated by transforming growth factor β (TGF- β).^{2–4,11} This mechanism of induction has become a central theme in accounting for certain biological actions that are shared both by CCN2 (CTGF) and TGF- β , most notably the stimulation of ECM production.^{2–12} Accordingly, CCN2 (CTGF) and TGF- β are frequently coexpressed in tissues that are undergoing ECM remodelling or synthesis, such as those that differentiate during development or exhibit pathological fibrotic transformation. Although most attention has been focused on the possible role of CCN2 (CTGF) as a downstream mediator of the activity of TGF- β in fibrotic disorders and wound repair, it is nonetheless clear that CCN2 (CTGF) also has important roles in regulating cellular homeostasis under normal circumstances. For example, it is present in

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diverse organ systems and tissues during embryogenesis,¹³⁻¹⁵ is involved in chondrogenesis and endochondral ossification,^{5, 16-20} and occurs in the uterus and ovary of cycling and pregnant mammals.^{14, 15, 21-26}

Relatively little is known about the role of CCN2 (CTGF) in the uterus or the mechanisms by which uterine *ccn2* (*ctgf*) gene transcription is regulated. Studies in the pig have revealed that a variety of CCN2 (CTGF) isoforms, ranging from 10 kDa to 38 kDa, are present in uterine fluids that exhibit cyclic and pregnancy associated changes in their concentrations.²⁴⁻²⁶ Low mass CCN2 (CTGF) molecules correspond principally to one or two structural modules located at the C-terminus of the primary translational product and arise through limited proteolysis of full length 38 kDa CCN2 (CTGF).^{24, 26} A variety of CCN2 (CTGF) isoforms have also been identified in mouse uterine fluids.¹⁴ In the cycling mouse, CCN2 (CTGF) was localised mainly in luminal and glandular epithelial cells in which the signal is somewhat attenuated at the time of oestrus.¹⁴ CCN2 (CTGF) localisation in pregnant animals mimicked that in cycling animals until day 4.5, when the blastocyst was first detected in the uterine lumen. At this time, epithelial expression of CCN2 (CTGF) was substantially reduced and followed by high expression in decidual cells after invasion and implantation by the embryo.¹⁴ Collectively, these data suggest that in the mouse uterus, CCN2 (CTGF) is physiologically important in epithelial cells during the oestrous cycle and in differentiating stromal cells during early pregnancy. Similar observations have been made in women, where it was shown that epithelial cells and decidua expressed relatively high amounts of CCN2 (CTGF) compared with other cell types.²¹ Although the pig does not exhibit a classic decidual cell reaction, epithelial cells are a major site of CCN2 (CTGF) synthesis, but have transiently reduced CTGF during the peri-attachment period, coincident with stromal ECM remodelling and neovascularisation.⁴ In all three species (pig, mouse, human), CTGF expression has been documented in other cell types such as endothelial cells, vascular smooth muscle cells and, to a much lesser extent, stromal cells.^{4, 14, 21} These observations suggest that uterine CCN2 (CTGF) may control a variety of diverse biological processes that are delicately integrated in time and space by steroid dependent and independent mechanisms. Thus, the purpose of these studies was to determine whether the expression of CCN2 (CTGF) is embryo or steroid dependent and to define the extent to which CCN2 (CTGF) expression mimics that of TGF- β .

Materials and methods

MATERIALS

Histochoice was obtained from Amresco (Solon, Ohio, USA). Superfrost Plus slides, Crystalmount, and Permount were from Fisher Scientific (Pittsburgh, Pennsylvania, USA). Phosphate buffered saline (PBS) was obtained from Gibco BRL (Gaithersburg, Maryland, USA). Plasmid pCRII was from Invitrogen

Corp (Carlsbad, California, USA). Normal rabbit IgG was from Vector Laboratories (Burlingame, California, USA). Power BlockTM and StrAvidGen Multilink kit were purchased from BioGenex (San Ramon, California, USA). Haematoxylin was obtained from Newcomer Supply (Middleton, Wisconsin, USA). Digoxigenin RNA labelling kit and sheep antidigoxigenin-alkaline phosphatase were from Roche Molecular Diagnostics (Indianapolis, Indiana, USA). NBT/BCIP chromogenic substrates were from Promega Corp (Madison Wisconsin, USA). Other chemicals were from Sigma Chemical Co (St Louis, Missouri, USA).

CCN2 (CTGF) antiserum raised in rabbits against residues 80-93 of mouse CCN2 (CTGF) (a peptide region that is not shared with other CCN proteins) was produced and affinity purified as described previously.^{14, 15, 27} Affinity purified rabbit anti-TGF- β 1 antiserum was obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA). A cDNA corresponding to nucleotides -1 to 1046 of the published mouse *ccn2* (*fisp-12*) sequence²⁸ was generated from mRNA from TGF- β 1 stimulated mouse 3T3 cells via the reverse transcription polymerase chain reaction (RT-PCR) using the primers 5'-gcaggatccatgctgcctc cgtcgca-3' and 5'-ccagcgccgcgaattcttacgcat gtctcc-3'. The same RNA was also used to amplify a 460 bp murine TGF- β 1 cDNA using the primers 5'-ccggctcctgtccaaactaaggctcgc-3' and 5'-cctctagaccagtgcgtcaaaagacagcc-3'. The amplified products from each reaction were cloned into pCRII and the resulting pCRII/*ccn2* (*ctgf*) or pCRII/TGF- β 1 plasmids were cut with diagnostic restriction enzymes to verify insert directionality. Digoxigenin-UTP labelled RNA sense and antisense probes were made using a digoxigenin RNA labelling kit, according to the manufacturer's instructions. pCRII/*ccn2* (*ctgf*) and pCRII/TGF- β 1 were linearised with EcoRV for SP6 generation of the sense probe and with Kpn I for T7 generation of the antisense probe.

ANIMALS

Swiss Webster mice (Harlan Sprague Dawley, Indianapolis, Indiana, USA) were used for all experiments, which were approved by the institutional animal use and care committee of the Children's Research Institute.

Pseudopregnancy

Adult male mice were vasectomised and kept for 35 days to clear all viable sperm from the genital tract. Mating was induced between these sterile males and fertile female mice in the evening and mating was verified at 8.00 am on the morning of the next day by the appearance of the vaginal plug. This time point was defined as day 0.5 of pseudopregnancy. The uteri of pseudopregnant females were harvested daily at 8.00 am between days 0.5 and 5.5 ($n = 3/\text{time point}$), the last time point being the probable day of return of oestrus.

Steroid hormone treatment

Adult virgin female mice were ovariectomised (OVX) and kept for two weeks to deplete them

of circulating ovarian steroids. On day 15 post-ovariectomy, animals were given a single subcutaneous injection of either estradiol-17 β (E₂) (250 ng), progesterone (P₄) (2 mg), or a combination of both hormones in 200 μ l sesame oil (n = 3/treatment group). OVX animals that received 200 μ l sesame oil only were used as negative controls. The uteri of steroid injected animals or oil injected controls were collected one, three, six, 12, and 24 hours after injection.

TISSUE PROCESSING

Mice were humanely sacrificed at the designated times and the uteri were removed by dissection. Uteri were fixed in Histochoice for 24 hours and kept in 70% ethanol at 4°C until use. Paraffin wax embedded sections (5 μ m thick) were cut using a microtome and placed on Superfrost Plus slides, which were then heated at 60°C for one to two hours and dewaxed in xylene for five minutes. Sections were then hydrated through a series of graded ethanol washes (95%, 90%, 70%, and 50% ethanol) and then placed in distilled water for a few minutes.

IMMUNOHISTOCHEMISTRY FOR CCN2 (CTGF) AND TGF- β 1

Endogenous peroxidase activity was blocked by placing slides in 3% H₂O₂ for 15 minutes, after which they were rinsed in distilled water followed by PBS. Slides were then incubated in Power Block for up to 10 minutes to block non-specific binding sites, and then rinsed with PBS for 10 minutes. Details for CCN2 (CTGF) immunohistochemistry were as described previously,^{14,15} with some modifications. Sections were incubated for one hour at room temperature with PBS/2% bovine serum albumin (BSA) containing either 10 μ g/ml anti-CTGF IgG, 2 μ g/ml anti-TGF- β 1 IgG, or equivalent concentrations of preimmune rabbit IgG (obtained before immunisation with the CCN2 (CTGF) (81–94) peptide) or non-immune rabbit IgG. Slides were rinsed in PBS for 10 minutes, incubated with a 1/50 dilution of Multilink biotinylated secondary antibody in PBS/2% BSA for 20 minutes, washed in PBS for 10 minutes, and then incubated with a 1/50 dilution of concentrated streptavidin conjugated peroxidase for 20 minutes. Slides were washed for 10 minutes and developed with diaminobenzidine for three minutes, after which the chromogenic colour reaction was stopped using distilled water. Slides were counterstained using haematoxylin, washed in distilled water, dehydrated, and mounted.

IN SITU HYBRIDISATION FOR CCN2 (CTGF) OR TGF- β 1

Prehybridisation was carried out by successive incubations in water, PBS, PBS/100mM glycine, PBS/0.3% Triton X-100, PBS, and Tris/EDTA (TE) buffer containing 20 μ g/ml proteinase K. All solutions were treated with diethylpyrocarbonate. Sections were postfixed in PBS/4% paraformaldehyde at 4°C, washed with PBS, incubated with 0.1M triethanolamine buffer (pH 8.0) containing 0.25%

(vol/vol) acetic acid, followed by prehybridisation buffer (4 \times saline sodium citrate (SSC) containing 50% vol/vol deionised formamide) at 37°C. Slides were then incubated with 300 ng/ml digoxigenin labelled sense or anti-sense *ccn2* (*ctgf*) or TGF- β 1 RNA probes in 300 μ l hybridisation buffer (4 \times SSC containing 10% dextran sulphate, 40% deionised formamide, 1 \times Denhardt's, 10mM dithiothreitol (DTT), 1 mg/ml yeast tRNA, and 1 mg/ml denatured and sheared salmon sperm DNA). Sections were incubated at 42°C overnight in a humid chamber. Posthybridisation steps were 2 \times SSC for 10 minutes at room temperature, two 15 minute washes with 2 \times SSC at 37°C, two 15 minute washes with 1 \times SSC at 37°C, two 30 minute washes with 0.1 \times SSC at 37°C, two 10 minute washes with buffer 1 (100mM Tris HCl/150mM NaCl, pH 7.5), and a 30 minute incubation with blocking solution (buffer 1 containing 0.1% Triton X-100/2% goat serum). Sections were then incubated for two hours with PBS/0.1% Triton X-100/1% goat serum containing a 1/750 dilution of sheep antidigoxigenin-alkaline phosphatase. Sections were washed with buffer 1 and buffer 2 (100mM Tris HCl/100mM NaCl/50mM MgCl₂, pH 9.5) and developed with NBT/BCIP chromogenic substrate. The reaction was stopped using 10mM Tris HCl (pH 8.1) containing 1mM EDTA. Slides were washed in water, counterstained with nuclear fast red, and mounted with crystal mount.

Results

In all experiments, verification that the immunohistochemical signal was specific for each protein was achieved through the use of non-immune controls (data not shown). Similarly, sense mRNA in situ hybridisation performed on all control and treatment groups was used to confirm the specificity of staining (data not shown).

PSEUDOPREGNANCY

Both *ccn2* (*ctgf*) and TGF- β 1 were transcribed and translated in the uteri of pseudopregnant mice (fig 1). On days 0.5–2.5 of pseudopregnancy, strong immunohistochemical staining for CCN2 (CTGF) and TGF- β 1 was seen in the cytoplasm of the columnar luminal and glandular cells, whereas moderate to weak staining was present in the condensed aggregates of the stromal fibroblasts (fig 1). In addition, the smooth muscle fibres of the myometrium exhibited moderate CCN2 (CTGF) protein expression (data not shown). Over the same period, large amounts of *ccn2* (*ctgf*) and TGF- β 1 mRNA were also colocalised in luminal and glandular epithelial cells, and smaller amounts were found in the underlying stroma. On days 3.5–4.5, *ccn2* (*ctgf*) and TGF- β 1 mRNA and protein values were decreased in epithelial cells but increased in stromal fibroblasts. This corresponded to stromal ECM remodelling and massive invasion of blood capillaries, which were also positive for *ccn2* (*ctgf*) expression. By the next day (day 5.5; corresponding to the probable return of oestrus), both *ccn2* (*ctgf*) and TGF- β 1 protein and mRNA expression increased in the luminal and

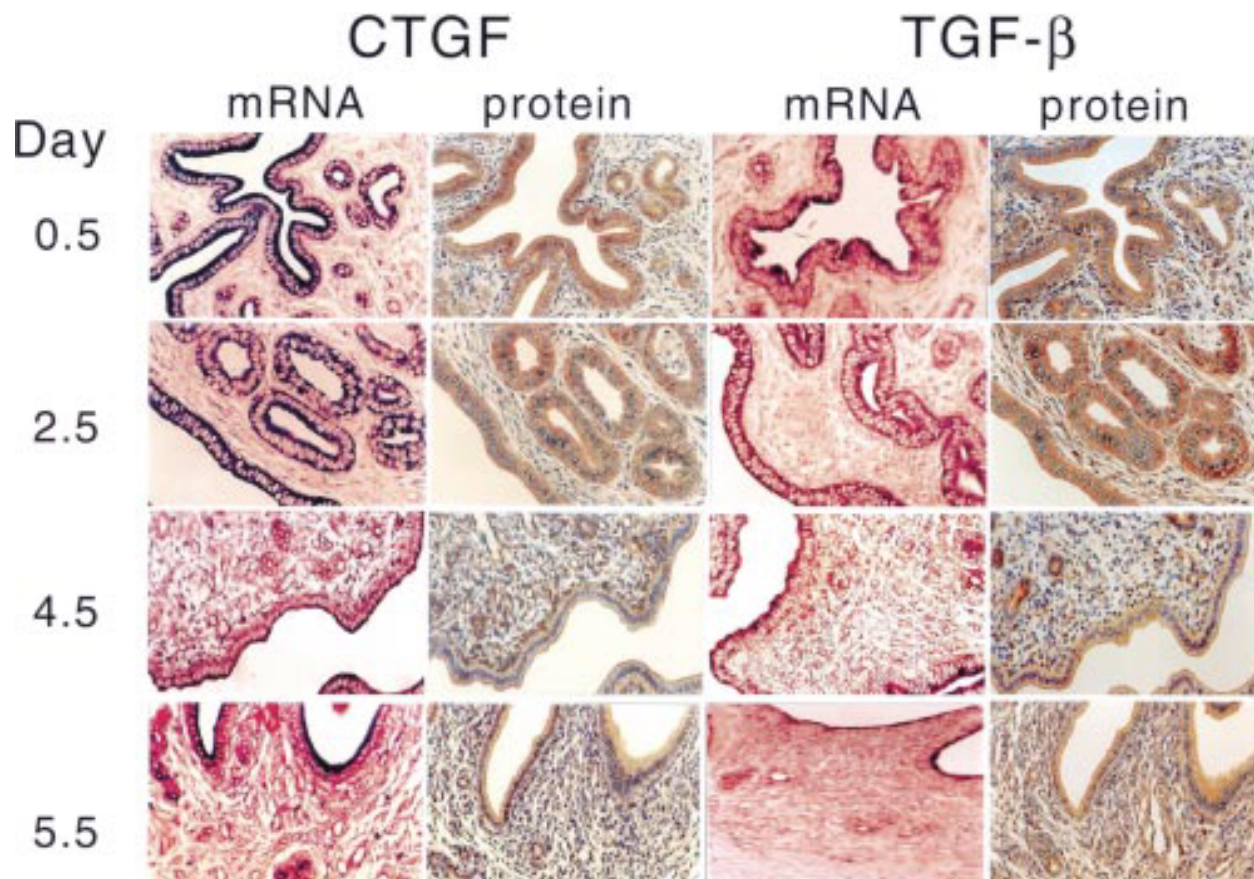


Figure 1 Uterine *ccn2* (*ctgf*) and transforming growth factor β (*TGF- β*) in pseudopregnant mice. Female Swiss Webster mice were mated with vasectomised males and uterine *ccn2* (*ctgf*) and *TGF- β* mRNA and protein were assessed on the indicated days of pseudopregnancy by *in situ* hybridisation or immunohistochemistry, respectively.

glandular epithelial cells and was apically localised within the cells. Stromal staining for *ccn2* (*ctgf*) or *TGF- β 1* mRNA was quite low, whereas their respective protein values were relatively high.

Based on our previously published data,¹⁴ the cellular distribution of *ccn2* (*ctgf*) in uterine tissues of pseudopregnant mice was generally comparable to that found in preimplantation pregnancy. These data suggest that during the preimplantation period, *ccn2* (*ctgf*) expression is predominantly regulated by maternal factors. Although embryonic signals may influence *ccn2* (*ctgf*) expression during the peri-implantation or postimplantation period, these and previously published data suggested that maternal factors, such as steroid hormones, drive uterine *ccn2* (*ctgf*) expression in cycling or early pregnant mice. To investigate this question, we next evaluated *ccn2* (*ctgf*) and *TGF- β 1* expression in the uteri of OVX mice that had been treated with acute administration of E_2 or P_4 , either alone or in combination.

STEROID HORMONE EFFECTS

Because the steroidal regulation of mRNA and protein for *TGF- β 1*, *TGF- β 2*, and *TGF- β 3* in the mouse uterus has been well established from previous work,^{29,30} we focused our investigation on the effects of E_2 and P_4 on the *TGF- β 1* protein and performed this in conjunction with a comprehensive analysis of *ccn2* (*ctgf*) mRNA and protein.

Epithelial cells

Two weeks postovariectomy there was a massive reduction in the overall uterine size, a decrease in the height of the luminal and glandular epithelial cells, and invasion of blood capillaries perforating the stroma (fig 2A). This phenotype was associated with greatly reduced expression of *ccn2* (*ctgf*) mRNA, especially in epithelial cells, which normally show a very strong *ccn2* (*ctgf*) mRNA signal in cycling animals (fig 1; and data not shown¹⁴). In control OVX animals, the low basal expression of *ccn2* (*ctgf*) mRNA remained unaltered for up to 24 hours after oil administration, and was confined to the apical region of the cells (fig 2A). However, despite their reduced *ccn2* (*ctgf*) mRNA expression, the amount of CCN2 (CTGF) protein detected immunohistochemically in these cells was moderately high, as was *TGF- β 1*. Both CCN2 (CTGF) and *TGF- β 1* were distributed throughout the cell and their values were not altered in response to oil treatment at any of the time points examined (fig 2A). Thus, whereas steroid deprivation for two weeks after OVX resulted in a significant reduction of *ccn2* (*ctgf*) mRNA expression in epithelial cells, compared with cycling or pseudopregnant animals, both CCN2 (CTGF) and *TGF- β 1* proteins were quite stable and readily detectable.

Treatment of OVX animals with E_2 (fig 2B) resulted in a dramatic but transient increase in the *ccn2* (*ctgf*) mRNA and protein in uterine epithelial cells, which was detectable within one

hour, had peaked by six hours, and had subsided by 24 hours (fig 2B). During the induction phase (one to six hours), *ccn2* (*ctgf*) mRNA was localised throughout the epithelial cells, whereas during the attenuation phase (24 hours), it was confined to the apical region of the cells and resembled the localisation seen in control uteri. Greatly increased epithelial CCN2 (CTGF) and

TGF- β 1 protein expression was seen at one to 24 hours after E₂ treatment (fig 2B). P₄ treatment of OVX animals resulted in stimulation of *ccn2* (*ctgf*) mRNA within one hour in epithelial cells, which was distributed throughout the entire cytoplasm (fig 3A). However, this induction was less robust than that stimulated by E₂, and by six to 24 hours of P₄ treatment,

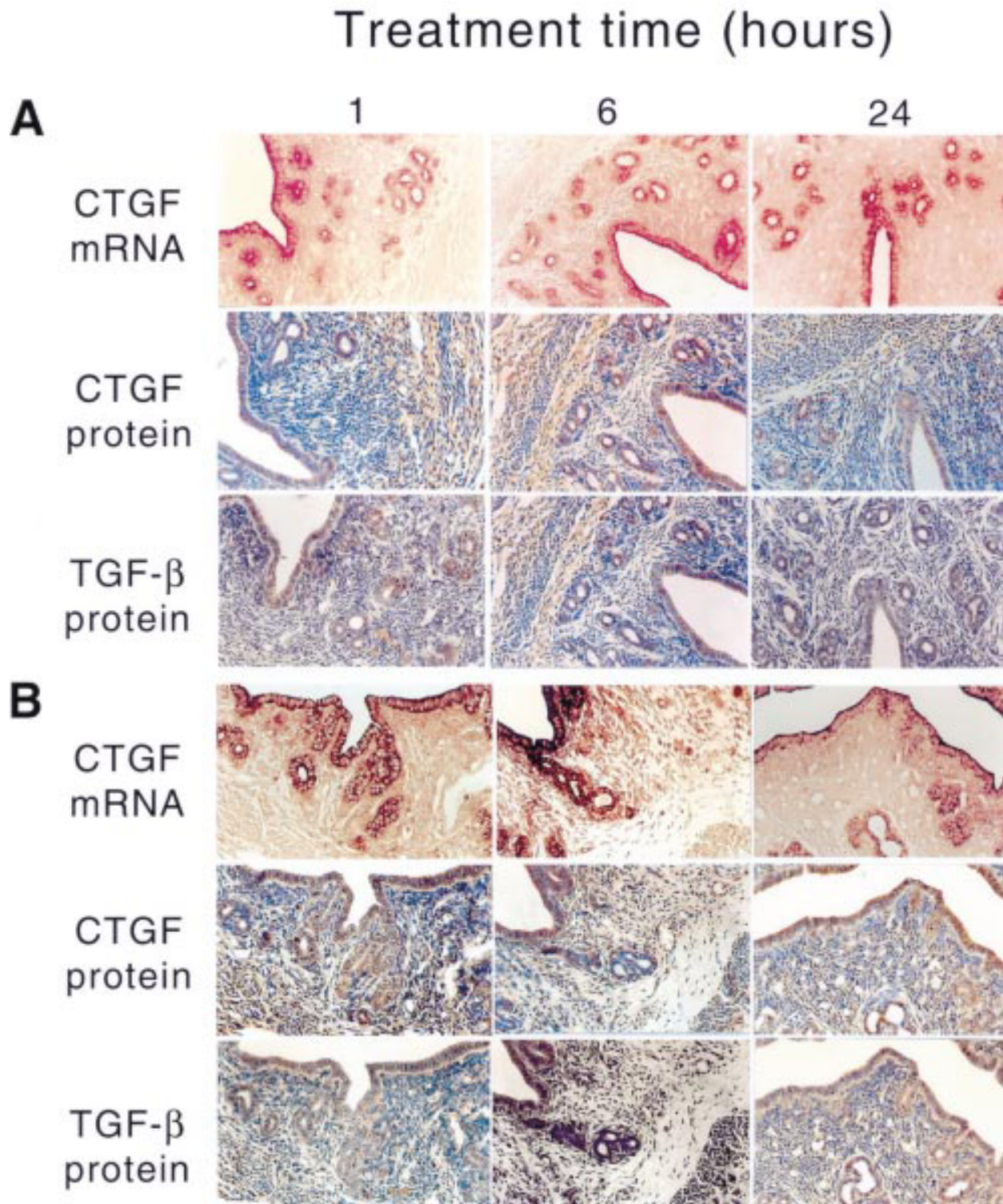


Figure 2 Steroidal regulation of uterine *ccn2* (*ctgf*) and transforming growth factor β . Ovariectomised mice were treated with (A) sesame oil control or (B) estradiol-17 β for the indicated amounts of time (1-24 hours). The presence of uterine *ccn2* (*ctgf*) and TGF- β was determined by the situ hybridisation or immunohistochemistry.

ccn2 (*ctgf*) mRNA expression had declined and was confined to the apical regions of the cells. However, these changes were not paralleled by *ccn2* (*ctgf*) or TGF- β 1 proteins, which remained at a constant moderate value (comparable to epithelial cells of control (oil treated)

OVX uteri) over the entire time course (fig 3A). The high amounts of *ccn2* (*ctgf*) mRNA induced by E₂ alone at one to six hours or P₄ alone at one hour were absent in animals receiving both hormones at the same time (fig 3B). Nonetheless, at one to six hours, *ccn2* (*ctgf*)

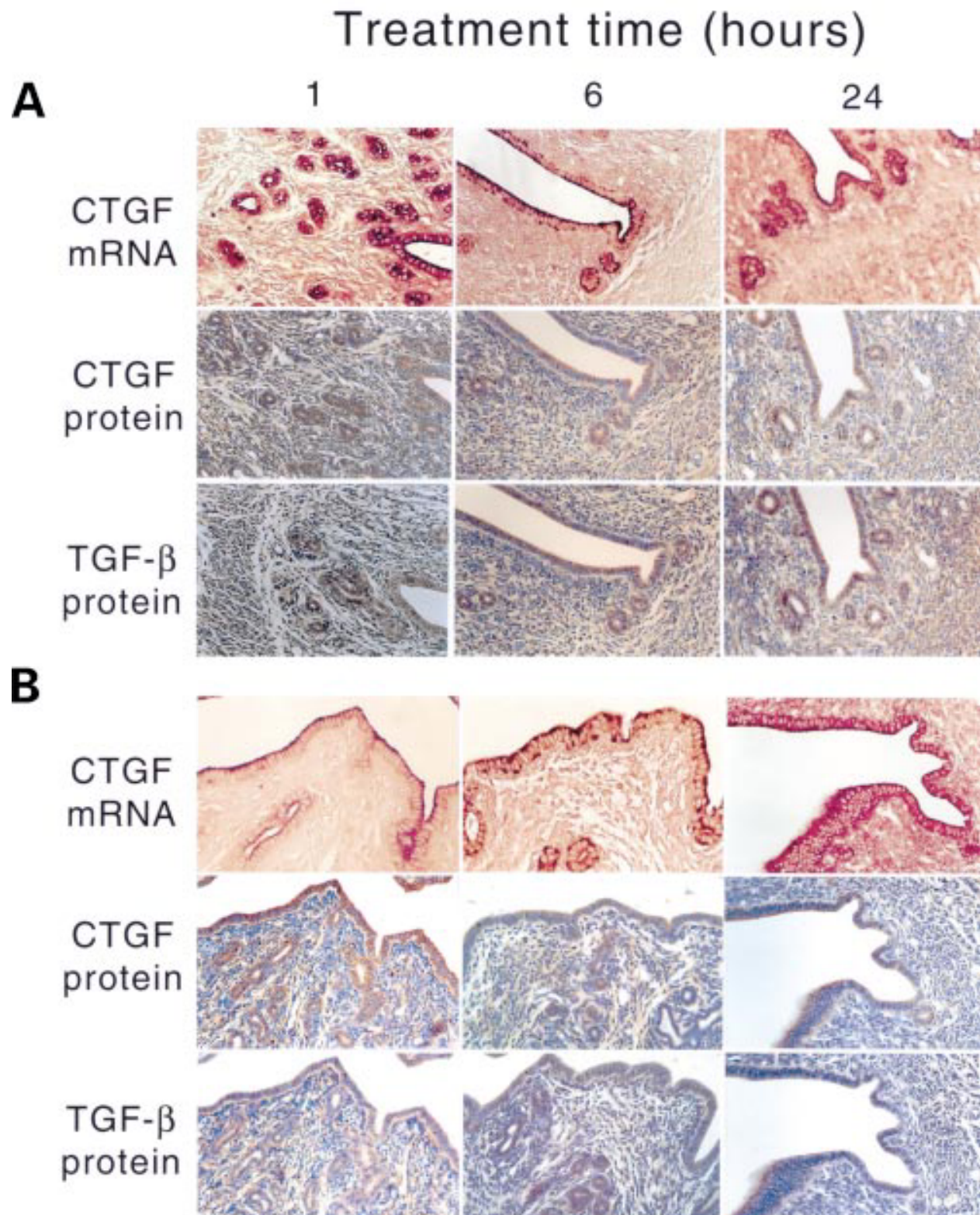


Figure 3 Steroidal regulation of uterine *ccn2* (*ctgf*) and transforming growth factor β (TGF- β). Ovariectomised mice were treated with (A) progesterone or (B) estradiol-17 β and progesterone for the indicated amounts of time (1–24 hours). The presence of uterine *ccn2* (*ctgf*) and TGF- β were determined by *in situ* hybridisation or immunohistochemistry.

mRNA was slightly higher in OVX animals receiving E_2 and P_4 than in OVX animals receiving the oil control. This signal was mainly apical and its intensity declined to that seen in the oil treated controls by 24 hours.

STROMAL CELLS

ccn2 (*ctgf*) mRNA was not detectable in the stromal fibroblasts of OVX animals for one to 24 hours after the administration of the oil control. Accordingly, low amounts of CCN2 (CTGF) and TGF- β 1 proteins were found in the stroma—lower than that seen in the myometrium (fig 2A). After E_2 treatment, there was a slight increase in *ccn2* (*ctgf*) mRNA in a few scattered stromal cells by six hours after treatment, but this increase was far less than that seen in the epithelium. Nonetheless, stromal staining for CCN2 (CTGF) and TGF- β 1 proteins was higher than in oil treated controls, and appeared to increase modestly with time. P_4 did not appear to stimulate *ccn2* (*ctgf*) mRNA production in the stroma, although relatively high amounts of CCN2 (CTGF) and TGF- β 1 protein were present at all time points (fig 3A). No stromal staining for *ccn2* (*ctgf*) mRNA was present in animals receiving both E_2 and P_4 , and the CCN2 (CTGF) and TGF- β 1 protein values were much reduced compared with the increases seen in the stroma in response to each hormone individually (fig 3B).

Discussion

The uterus is an intriguing and unique example of an organ system in which tissue turnover is cyclical and regulated primarily by steroid hormones. It is now evident that the cyclic changes in uterine cell organisation are attributable, at least in part, to the regulation by E_2 and/or P_4 of the synthesis of uterine polypeptide growth factors and their signal transducing receptors.³¹ Through the integrated action of numerous steroidal regulated molecules, including growth factors and receptors, endometrial cells undergo proliferation, differentiation, ECM remodelling, and regression.³² In addition, during pregnancy, endometrial stromal cells are transformed to decidual cells under the influence of E_2 and P_4 , a process that also involves changes in growth factor expression. Apart from decidualisation, uterine growth factors are also implicated in other processes during pregnancy, such as embryo–uterine signalling, placental development, and angiogenesis.^{32–34} Synchrony between the hormonal status of the uterus and its receptiveness for an appropriately developed blastocyst is central to successful implantation in many mammalian species.^{35–38} This highlights the pivotal role that steroidal regulated growth factors are likely to play in the embryo–maternal interactions that enable successful establishment and maintenance of pregnancy.^{32–34} 39 Our data establish for the first time that maternal sex steroids, including E_2 and P_4 , are among the maternal factors that can regulate the expression of *ccn2* (*ctgf*) in the

uterus, suggesting that CCN2 (CTGF) probably mediates some of the growth and differentiation processes in uterine tissues that are steroid dependent. Although steroidal regulation of *ccn2* (*ctgf*) has not been reported previously, our data are consistent with an earlier subtractive cDNA library strategy, which showed that the CCN2 (CTGF) related molecule CCN1 (CYR61) was induced in ovariectomised rat uteri after four hours of treatment with 17- α -ethynyl estradiol.⁴⁰

Our experiments revealed several features regarding the regulation of *ccn2* (*ctgf*) in the mouse uterus. Interestingly, the only scenarios in which uterine stromal or connective tissue cells produce large amounts of CCN2 (CTGF) are during the P_4 dominated decidualisation phase of pregnancy, after P_4 administration, or during periods of neovascularisation and ECM remodelling during pseudopregnancy.¹⁴ In contrast, one of the most striking features of these and previous¹⁴ data is that epithelial cells in cycling, early pregnant, or pseudopregnant animals are the principal site of uterine CCN2 (CTGF) synthesis. These data suggest that CCN2 (CTGF) may influence uterine function through its action on epithelial cells, directly or by paracrine effects on the underlying stroma. CCN2 (CTGF) is produced by a variety of epithelial cell types and regulates functional properties including adhesion,¹³ differentiation,^{41 42} and apoptosis.⁴³ Although direct effects of CCN2 (CTGF) on uterine epithelial cells have yet to be examined, our results also support the possibility that CCN2 (CTGF) is translocated from the epithelium to the stroma, as has been suggested previously for TGF- β .⁴⁴ Evidence for diffusion of CCN2 (CTGF) from its site of synthesis is shown by the presence of large amounts of CCN2 (CTGF) protein in both epithelial and stromal cells under conditions in which only the epithelial cells contain appreciable amounts of *ccn2* (*ctgf*) mRNA (for example, on days 0.5–2.5 of pseudopregnancy or in control or hormone treated OVX animals). Thus, CCN2 (CTGF) may be one of the mediators of epithelial–mesenchymal cell interactions that drive uterine differentiation and support the establishment and maintenance of pregnancy.

The pseudopregnancy experiments allowed us to investigate which of the pregnancy associated changes in CCN2 (CTGF) production are independent of the presence of an activated blastocyst. The data clearly show that most of the preimplantation changes in CCN2 (CTGF) production that we reported previously¹⁴ are embryo independent and, thus, maternally regulated. Similarly, previous studies have shown that the uterine expression of TGF- β 1, TGF- β 2, and TGF- β 3 appears to be independent of blastocyst regulation.^{29 44} Moreover, the pattern of CCN2 (CTGF) production that we found was comparable to that of TGF- β 1 and was principally epithelial over the first three days of pseudopregnancy. In view of previous studies supporting TGF- β dependent *ccn2* (*ctgf*) gene induction in various cell types, including epithelial cells, a functional relation between TGF- β and CCN2 (CTGF)

in the uterine epithelium is plausible. In addition, a striking decrease in epithelial CCN2 (CTGF) values was observed on day 4.5 in both pregnant¹⁴ and pseudopregnant (our present data) mice. This time is the normal day of implantation and is marked by extensive stromal remodelling and angiogenesis. Suppression of epithelial CCN2 (CTGF) production may allow for a net degradation of stromal ECM as an essential prerequisite to ECM remodelling and neovascularisation. In addition, the presence of CCN2 (CTGF) in the endothelial cells of the new vessels is consistent with the proposed role for CCN2 (CTGF) in regulating endothelial cell proliferation, adhesion, and survival.

The presence of *ccn2* (*ctgf*) mRNA or protein or TGF- β 1 protein was not completely abolished within the two week period after ovariectomy. Similarly, the mRNA encoding TGF- β 1–3 was expressed at high basal values in the uterus of immature mice³⁰ and TGF- β 2 or TGF- β 3 mRNA persisted in the uteri of adult ovariectomised mice.²⁹ These data show that transcriptional activation of the uterine *ccn2* (*ctgf*) and TGF- β genes are, at least partly, steroid independent. Nonetheless, our data show that both CCN2 (CTGF) and TGF- β 1 are individually induced by E_2 and P_4 , although the effects of each hormone are quite different. In response to a single dose of E_2 , *ccn2* (*ctgf*) mRNA was induced in epithelial cells within one hour, peaked at six hours, and declined by 24 hours. Previous studies of the mouse uterus showed that diethylstilbestrol transiently upregulated TGF- β 1 and TGF- β 2 mRNA by three hours and TGF- β 3 mRNA within 30 minutes, with subsequent decreases to basal values by six hours.³⁰ In another study, E_2 caused a rapid (six hour) but transient induction of TGF- β 2 mRNA, whereas there was no significant effect of the hormone on TGF- β 3 mRNA in mouse uteri.²⁹ In situ hybridisation localised the mRNAs for all three TGF- β isoforms primarily to the uterine epithelium.^{29, 30} Unlike the transient nature of TGF- β mRNA induction elicited by E_2 , the TGF- β proteins persisted in the epithelium for a relatively long time.³⁰ Following E_2 stimulation, we found that CCN2 (CTGF) and TGF- β 1 proteins were long lived in both the epithelium and stroma, and were thus more broadly distributed than would be predicted from their mRNA, which was localised primarily to epithelial cells. These data suggest that epithelial CCN2 (CTGF) or TGF- β act in a paracrine fashion to regulate stromal cell function. Similar conclusions were drawn from studies of TGF- β protein localisation during the preimplantation period, which showed that ECM of the stroma actively accumulated TGF- β 1 that was synthesised in and secreted from the epithelium.⁴⁴

P_4 is an essential hormone in the establishment and maintenance of pregnancy, at least partly because it is required to sensitise stromal cells to differentiate in response to an embryonic or artificial stimulus and to maintain their differentiated state. Nonetheless, direct induction of gene expression by P_4 is not widely

documented, although some growth factors (such as heparin binding epidermal growth factor (HB-EGF), insulin-like growth factor type 1 (IGF-1), vascular endothelial growth factor (VEGF), tumour necrosis factor α (TNF- α), and amphiregulin) and growth factor receptors or binding proteins (such as the EGF receptor, IGF binding protein 1, and the TNF- α receptor type 1) appear to be P_4 dependent.³² Although detailed studies of the effects of P_4 on TGF- β are lacking in the literature, neither TGF- β 2 nor TGF- β 3 were reported to be significantly affected by P_4 treatment.²⁹ However, in our studies, a single injection of P_4 was effective in stimulating the expression of *ccn2* (*ctgf*) and TGF- β 1 over control mice, but this response was less robust than that seen with E_2 . The P_4 responsiveness of the *ccn2* (*ctgf*) and TGF- β genes may account, at least in part, for their high expression in the P_4 dominated decidualisation phase following implantation.¹⁴ While epithelial rather than stromal *ccn2* (*ctgf*) mRNA was induced by P_4 , a similar pattern of regulation has been reported for other uterine genes including amphiregulin, a member of the EGF family, which is produced by epithelial cells in response to P_4 .⁴⁵ Although co-injection of P_4 with E_2 did not antagonise the transient E_2 stimulated accumulation of TGF- β 2 mRNA in an earlier study,²⁹ P_4 appeared to antagonise the E_2 induced increase in TGF- β 1 and *ccn2* (*ctgf*) expression by epithelial cells in our studies. Similar opposing effects between E_2 and P_4 on their effector molecules are not uncommon and have been documented previously for several uterine growth factors including HB-EGF,⁴⁶ amphiregulin,⁴⁵ and basic fibroblast growth factor.³²

The similarity between CCN2 (CTGF) and TGF- β in their patterns of expression and localisation are consistent with TGF- β dependent mechanisms of *ccn2* (*ctgf*) gene transcription, which could be both steroid dependent and independent. However, in several situations we found high amounts of TGF- β protein in stromal cells but no detectable *ccn2* (*ctgf*) mRNA. These findings may suggest that: (1) TGF- β was present in its inactive latent form, (2) stromal cells were TGF- β responsive but the *ccn2* (*ctgf*) gene was refractory to the effect of TGF- β , and/or (3) functional TGF- β receptors were not present on the stromal cells. However, this last possibility seems unlikely because of the previously reported presence of the type I and II TGF- β receptors in stromal cells on days 1–3 of pregnancy and of the type I, II, and III TGF- β receptors in stromal cells on day 4 and the decidua thereafter.⁴⁷

In conclusion, these and previous data demonstrate that maternal factors are principal cues for CCN2 (CTGF) production in the uterus because it is produced in cycling and pseudopregnant animals, its expression during pseudopregnancy is comparable to that seen in the preimplantation period, and its expression is regulated by ovarian steroids. Our studies further suggest that *ccn2* (*ctgf*) gene transcription in the uterus is not only regulated by female sex steroids but also by TGF- β 1

dependent and independent mechanisms. Thus, CCN2 (CTGF) is likely to mediate at least some of the effects of steroid hormones and TGF- β on endometrial function in cycling and pregnant animals.

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