Decorin is a part of the ovarian extracellular matrix in primates and may act as a signaling molecule

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STUDY QUESTION: Is decorin (DCN), a putative modulator of growth factor (GF) signaling, expressed in the primate ovary and does it play a role in ovarian biology?

SUMMARY ANSWER: DCN expression in the theca, the corpus luteum (CL), its presence in the follicular fluid (FF) and its actions revealed in human IVF-derived granulosa cells (GCs), suggest that it plays multiple roles in the ovary including folliculogenesis, ovulation and survival of the CL.

WHAT IS KNOWN ALREADY: DCN is a secreted proteoglycan, which has a structural role in the extracellular matrix (ECM) and also interferes with the signaling of multiple GF/GF receptors (GFRs). However, DCN expression and action in the primate ovary has yet to be determined.

STUDY DESIGN, SIZE, DURATION: Archival human and monkey ovarian samples were analyzed. Studies were conducted using FF and GC samples collected from IVF patients.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Immunohistochemistry, western blotting, RT–PCR, quantitative RT–PCR (qPCR) and enzyme-linked immunosorbent assay (ELISA) studies were complemented by cellular studies, including the measurements of intracellular Ca²⁺, reactive oxygen species (ROS), epidermal GF receptor (EGFR) phosphorylation by DCN and caspase activity.

MAIN RESULTS AND THE ROLE OF CHANCE: Immunohistochemistry revealed strong DCN staining in the connective tissue and follicular thecal compartments, but not in GCs of pre-antral and antral follicles. Pre-ovulatory follicles could not be studied, but DCN was associated with connective tissue of CL samples and the cytoplasm of luteal cells. DCN expression in monkey CL doubled (P < 0.05) towards the end of the luteal lifespan. DCN was found in human FF obtained from IVF patients (mean: 12.9 ng/ml; n = 20) as determined by ELISA. DCN mRNA and/or protein were detected in freshly isolated and cultured, luteinized human GCs. In the latter, exogenous human recombinant DCN increased intracellular Ca²⁺ levels and induced the production of ROS in a concentration-dependent manner. DCN, like epidermal GF, phosphorylated EGFR significantly (P < 0.05) and reduced the activity of caspase 3/7 in cultured GCs. The data indicate the expression of DCN in the theca of growing follicles, in FF of ovulatory follicles and in the CL. Therefore, DCN may exert paracrine actions via GF/GFR systems in multiple ovarian compartments.

LIMITATIONS, REASONS FOR CAUTION: Functional studies were performed in cultures of human luteinized GCs, which are an apt model but may not fully mirror the pre-ovulatory GC compartment or the CL. Other human ovarian cells, including the thecal cells, were not available.

WIDER IMPLICATIONS OF THE FINDINGS: In accordance with its evolving roles in other organs, ovarian DCN is an ECM-associated component, which acts as a multifunctional regulator of GF signaling in the primate ovary. DCN may thus be involved in folliculogenesis, ovulation and the regulation of the CL survival in primates.

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Introduction

The adult ovary is a dynamic organ, in which constant growth and regression of follicles, plus formation and regression of the corpus luteum (CL), require a constantly adapting extracellular matrix (ECM). The ECM of the ovary consists of basal laminae (BL), interstitial ECM and intra-epithelial matrix (focimatrix), i.e. plaques of aggregates between granulosa cells (GCs) of ovarian follicles (Irving-Rodgers et al., 2010).

While information about the ovarian ECM in rodent and bovine species is growing (Irving-Rodgers et al., 2010), little is known about ECM components in the human or non-human primate ovary (Irving-Rodgers et al., 2006; Ricciardelli and Rodgers, 2006). Collagen type IV, laminins and the proteoglycan versican were reported to be part of the ECM of the human CL (Irving-Rodgers et al., 2006). This study also described ECM in the form of sub-endothelial BL and aggregates of interstitial ECM between luteal cells that lack a continuous BL. Interestingly, laminins and collagen type IV are synthesized by luteal cells, implying an active role of endocrine cells in the formation of the ECM. Studies also indicated that other non-collagenous proteins, including the proteoglycan decorin (DCN) (Ricciardelli and Rodgers, 2006; Grisaru et al., 2007), are present in the ECM of normal and tumorous tissues in human ovaries. Its expression in normal ovarian tissue, namely in extracts of bovine follicles, that include the ovarian stroma and tunica albuginea (McArthur et al., 2000; Ricciardelli and Rodgers, 2006) further indicates that DCN is a component of the ovarian ECM. Whether DCN is expressed by follicular GCs or theca cells, secreted into the follicular fluid (FF) or is present in the CL is not known at present.

DCN is a small leucine-rich proteoglycan associated with the ECM, especially with collagen type I, and has a widespread distribution in the human body (Iozzo and Schaefer, 2010). It was found in human and monkey testes, while interestingly only trace amounts of DCN were detectable in the mouse testis (Ungefroren et al., 1995; Adam et al., 2011, 2012). DCN is remarkable in that it can interfere with the signaling of growth factors (GFs; Santra et al., 2002). Two types of interaction can occur, including binding and storage of soluble GFs, thereby reducing the levels available for receptor binding and activation. In addition, DCN can bind to many GF receptors (GFRs), for example the epidermal GFR (EGFR; Iozzo et al., 1999; Iozzo and Schaefer, 2010). The binding causes phosphorylation and activation of EGFR and can be followed by internalization and down-regulation of EGFR signaling, resulting in a chronic blockage of EGFR/ERBB actions (Csordas et al., 2000; Zhu et al., 2005; Adam et al., 2011). This may be relevant to the regulation of the immature primate testis and in states of human male infertility. In both of these situations, DCN is massively increased and may interfere with paracrine signaling of GFs (Adam et al., 2011, 2012).

In the ovary, especially of non-primate species, paracrine signaling via the EGFR and EGF-like molecules is known to be of importance. Recently studies in primate species, including the human, suggest a similar role (Inoue et al., 2009; Peluffo et al., 2012; Yu et al., 2012). LH surge causes local production of EGF-like molecules, especially ampiregulin and epiregulin, which are crucial in the orchestration of the events leading to ovulation and subsequent development and survival of the CL (Park et al., 2004; Ben-Ami et al., 2006). In support for the latter action, anti-apoptotic actions of EGF-like molecules, via the activation of EGFR, were described. These studies used IVF-derived human GCs (Ben-Ami et al., 2006) and the authors concluded that EGFR activation is crucial for the survival of the human CL. Interestingly, EGF is a factor, which also targets human oocytes and positively affects human oocyte maturation (Yu et al., 2012). The activation of the EGFR is linked to a complex signaling cascade, which includes among others the generation of reactive oxygen species (ROS; Finkel, 2011). Whether this occurs also in ovarian cells is however not known. As recently described, ROS evolved as physiological signaling molecules and as such may also be involved in ovulatory events (Shkolnik et al., 2011; Saller et al., 2012).

Whether DCN, via its ability to interfere with EGFR signaling, is a potential regulator of ovarian functions is unknown. To explore this point, we studied DCN expression in rhesus monkey and human ovary. We also determined DCN levels in the FF from IVF patients and used luteinized human GCs to explore its potential to act as a signaling molecule and to affect apoptosis.

Materials and Methods

Human GC culture

FF, containing human GCs, was obtained from IVF patients stimulated according to routine protocols. The local ethics committee of the University of Munich (LMU) approved the study (project number 323/05) and patients granted written informed consent. Aspirates with cells from two to four patients were pooled for each experiment. For ELISA studies the aspirates from individual patients were pooled. Cells were separated by centrifugation at 560g for 3 min and washed in serum-free Dulbecco’s modified eagle’s medium (DMEM)/Ham’s F-12 (PAA GmbH, Co¨lbe, Germany). The supernatant, i.e. FF, was frozen at −80°C until used in DCN ELISA studies. Cells were resuspended in culture medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal calf serum (FCS) (all from PAA GmbH), as previously described (Mayerhofer et al., 1992; Mayerhofer et al., 1993; Bulling et al., 2000; Mayerhofer et al., 2006; Saller et al., 2012). Fresh, uncultured cells (Day 0) and cultured cells were used. The latter were harvested on Days 1–4 for RT–PCR and western blot studies. Other experiments were performed on Day 2 of culture, unless otherwise indicated. The numbers of replicates are provided for each experiment.
Ovarian samples and immunohistochemistry

Paraffin-embedded ovaries from six adult rhesus monkeys (age 5–6 years) were the same as used in previous studies (Mayerhofer et al., 2006). All samples displayed pre-antral and antral follicles (maximum diameter of 3 mm) and three of these contained functional CL. All tissue collections were approved by the Oregon National Primate Research Center Institutional Animal Care and Use Committee. Human ovarian sections were from the tissue archive of the Institute of Anatomy and Cell Biology, Munich. Ovaries (n = 8) contained pre-antral and antral follicles with a maximum diameter of 10 mm. Three functional CL were present in the samples. For immunohistochemistry, 5-μm-thick sections were cut and deparaffinized. Sections were subjected to antigen retrieval by heating in citrate buffer (1.8 mM citric acid, 8.2 mM sodium citrate, pH 6.0) at 90°C for 30 min and endogenous peroxidase activity was blocked. Sections were incubated overnight at 4°C with a goat anti-human DCN antiserum (1:100; R&D Systems, Wiesbaden-Nordenstadt, Germany), which also recognizes monkey DCN (Adam et al., 2012) followed by a secondary antibody (biotinylated anti-goat; 1:500; Vector Laboratories, Inc. Burlingame, CA, USA), and avidin–biotin complex peroxidase (ABC, Vector Laboratories). Incubation with normal non-immune serum instead of the specific antiserum, or omission of the primary antiserum, served as controls.

Isolation of RNA and RT–PCR

Total RNA from fresh (Day 0; n = 6) and cultured human GCs at several time points (1, 2, 3 and 4 days after collection; n = 3/time point) was isolated using RNasy minikit (Qiagen GmbH, Hilden, Germany). Four hundred nanograms of total RNA was subjected to reverse transcription using random hexamer primers and Superscript-RT II (Life Technologies, Karlsruhe, Germany). RT–PCR was performed using oligonucleotide primers for DCN, GFRs and cyclophilin as detailed in a previous study (Adam et al., 2011). Controls with RNA input instead of cDNA input or without cDNA were performed. Amplified PCR products were separated and visualized by ethidium bromide stained agarose gels. The identities of all PCR products were verified by sequencing (Adam et al., 2011; Saller et al., 2012).

Gene array of monkey ovarian samples and quantitative real-time RT–PCR

The level of DCN mRNA in macaque CL obtained throughout the luteal phase of the menstrual cycle was determined from a published DNA microarray database (Bogan et al., 2008). Briefly, RNA from individual CL was isolated, labeled and hybridized to Affymetrix™ Rhesus Macaque Total Genome Arrays (Affymetrix™, Santa Clara, CA, USA). CL stages collected included Days 3–5 (early stage, developing CL), Days 7–8 (mid-stage, fully functional CL), Days 10–12 (mid-late stage, functional CL on the verge of regression), Days 14–16 (late stage, functionally regressing CL) or Days 18–19 (very-late stage, menses) after the LH surge. RNA isolation, array hybridization and data normalization parameters were previously described (Bogan et al., 2008). The sequence from the corresponding Affymetrix™ DCN probe set was used to BLAST the human and rhesus macaque genome sequence to ensure its proper annotation.

Following the isolation of RNA from individual CL, cDNA was synthesized and qPCR was performed as previously described (Bogan et al., 2008). The rhesus macaque DCN and mitochondrial ribosomal protein S10 (MRPS10) DNA sequences served as templates for the design of qPCR primers and TaqMan probes via PrimerExpress software (Applied Biosystems, Foster City, CA, USA). The DCN primer sequences are 5′-TGGGCTGGCCAGAGCATAAGT-3′ (forward) and 5′-CCAGGTTGGCAGAAGTCACT-3′ (reverse), whereas the MRPS10 primer sequences are 5′-AATGTTGCCCCAATCTGTC-3′ (forward) and 5′-TCCAGG CAAACGTGTCTCCA-3′ (reverse). The DCN probe sequence is 6FAM-5′-CATCCAGTTGCTTACCTC-3′-MGBNFQ and the MRPS10 probe sequence is VIC-5′-TGAAGGCATGAGCTCTCAATGACC C-3′-MGBNFQ. Primers and probes were purchased from Invitrogen (Carlsbad, CA) and Applied Biosystems, respectively. Target gene concentrations were normalized to MRPS10 levels. Results are given as the mean ± SEM of four to eight samples/stage of the luteal phase.

Enzyme-linked immunosorbent assay

To examine DCN in FF, a commercial immunoassay (R&D Systems, GmbH) was used. The assay was performed according to the manufacturer’s protocol, as described previously (Adam et al., 2011). Pooled follicular aspirates of 20 randomly selected IVF patients were examined in duplicates.

Measurements of intracellular Ca²⁺ levels

Ca²⁺ measurements were performed as described previously (Mayerhofer et al., 1992, 1993; Rey-Ares et al., 2007). In brief, GCs from three different pools of patients (Days 2 and 3) were grown on glass cover slips for 24 h in DMEM/Ham’s F-12 supplemented with 10% FCS and 1% penicillin/streptomycin, loaded with 5 μM fluo-4, acetoxy-methylester (fluor-4 AM, Molecular Probes, Eugene, OR, USA) for 30 min at 37°C and 5% CO₂. Thereafter, cells were washed with FCS-free medium and the cover slip was transferred into a recording chamber mounted on a TCS SP2 confocal microscope (Leica Microsystems, Bensheim, Germany). Fluorescence was monitored at 500–540 nm (λex = 488 nm) every 2 s and the intensity was quantified over single cells. To assess whether human GCs respond to DCN, changes of intracellular Ca²⁺ were recorded on-line. In pilot experiments, 5 μg/ml of human recombinant (Immundagnostik AG, Bensheim, Germany) or bovine articular cartilage DCN (Sigma-Aldrich, Munich, Germany) were evaluated. No differences in the ability of the two DCN preparations to mobilize intracellular Ca²⁺ were observed, as was described in a previous study (Adam et al., 2011). Measurements of Ca²⁺ levels after adding 5 μg/ml of bovine serum albumin (BSA; pH 7.8; PAA GmbH) were conducted to exclude non-specific protein effects.

Western blot analyses

Western blotting was performed as previously described (Mayerhofer et al., 1999). In brief, human GCs (n = 3 pools) were cultured on 60-mm dishes (Nunc GmbH & Co. KG, Wiesbaden, Germany) in DMEM/Ham’s F-12 medium containing 10% FCS and 1% penicillin/streptomycin. We performed immunoblots using the same DCN antisera as for immunohistochemistry. The use of a β-actin antibody (1:5,000, Sigma-Aldrich) allowed us to detect possible differences in loading. Western blot bands were detected with chemiluminescent reagents (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce, Thermo Scientific, Rockford, IL, USA).

The ability of DCN and EGF to phosphorylate EGFR was studied by western blotting (Adam et al., 2011). Human GCs were incubated with 5 μg/ml of recombinant bovine DCN and 50 ng/ml of human recombinant EGF (New England Biolabs, Frankfurt, Germany) for 10 min in DMEM/Ham’s F-12 without FCS. To exclude non-specific protein effects, cells were treated with 5 μg/ml of BSA (pH 7.8). In additional controls PBS was added. Immunoblotting was performed using 0.4 μg/ml of rabbit anti-phospho-EGFR antibody (1:250; R&D Systems). For control purposes, a monoclonal rabbit anti-human EGFR antibody (1:100; Thermo Fisher Scientific Inc., Fremont, CA, USA) was used. Bands were analyzed densitometrically with ImageJ Software (National Institutes of Health, Bethesda, MD, USA; version 1.40) and the results normalized to those obtained for
EGFR. Four independent experiments using cells from different patients were evaluated.

**Qualitative measurement of ROS and quantification of ROS**

The 2,7-dichlorodihydrofluoresceindiacetate (DCFH$_2$-DA) method (Kim et al., 2008) was used, as described previously (Saller et al., 2012). The DCFH$_2$-DA dye (Invitrogen, Life Technologies GmbH Karlsruhe, Germany), added at the beginning of the measurements (at 10 µM), is taken up by cells and in the presence of intracellular ROS converted to the highly fluorescent compound DCF (dichlorofluorescein). For qualitative assessment this was monitored using a fluorescence microscope (Zeiss Axioskop FSZ+; Zeiss, Jena, Germany). For quantification purposes of ROS, human GCs were placed in 96-well plates with buffer containing 140 mM NaCl, 3 mM KCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 10 mM HEPES and 10 mM glucose (pH 7.4). Cells were treated with human recombinant (10, 100 ng/ml and 1, 10 µg/ml) or bovine articular cartilage (1, 5, 10 µM) DCN alone or together with 1 mM N-acetylcystein (NAC; Sigma-Aldrich). For the measurements with DCN, this substance was added 30 min before starting ROS measurements and incubated at 37°C. For each treatment, eight replicate measurements/treatment group were performed. Fluorescence was measured at 485 nm excitation/520 nm emission monitored by a fluorimeter at RT (FluoStar, BMG Labtech GmbH, Offenburg, Germany). The differences between the values determined after 2 h, when the experiments were terminated, were statistically analyzed. Fluorescence intensities shown were normalized to untreated controls. For all ROS experiments, GCs on Day 2 of culture were used and experiments were repeated two to three times with cells from different patients.

**Caspase assay**

The activities of the effector caspases 3/7 were determined following exposure of human GCs to DCN or EGF (Saller et al., 2010). Cells were cultured for 2 or 5 days (three experiments using cells from different patients/time point) and subsequently treated with DCN (5 µg/ml) and EGF (50 ng/ml) for 24 h. Staurosporine (1 µM; Sigma-Aldrich) was used as a positive control. Hundred microlitres of CaspaseGlo® 3/7 reagent (Promega, Mannheim, Germany) were added to each well of a white-walled 96-well microplate containing 100 µl of untreated or stimulated cells. Contents of the plate were mixed on a plate shaker at 500 cycles per minute for 30 s and incubated for 1 h afterwards. The luminescence of each sample was measured in a plate-reading luminometer (FluoStar).

**Data analysis and statistics**

Data were analyzed using PRISM 4.0 (GraphPad Software, Inc., San Diego, CA). T-test or one-way ANOVA tests were performed (gene expression, qPCR, caspase assay; ROS). Differences between the groups were evaluated with a post hoc test (Newman–Keuls).

**Results**

**DCN expression in the non-human primate and human ovary**

Immunohistochemistry revealed an almost identical DCN staining pattern in all sections of adult rhesus monkey and human ovaries (Fig. 1). In both species, DCN staining was associated with the stromal connective tissue (Fig. 1A and D) and the theca layers of follicles (Fig. 1B and C). In the human ovary, DCN immunostaining is associated with the connective tissue and luteal cells (Fig. 1E and F). In the monkey CL, DCN immunostaining is associated with the connective tissue and luteal cells (Fig. 1C and F). In the human CL, DCN immunostaining is associated with the connective tissue and luteal cells (Fig. 1E and F). In the human CL, DCN immunostaining is associated with the connective tissue and luteal cells (Fig. 1E and F). In the human CL, DCN immunostaining is associated with the connective tissue and luteal cells (Fig. 1E and F). In the human CL, DCN immunostaining is associated with the connective tissue and luteal cells (Fig. 1E and F).
follicles (Fig. 1D). GCs within all classes of pre-antral and antral follicles were immuno-negative for DCN (Fig. 1A, B and D), including large antral follicles within human ovarian follicles (diameters up to 10 mm) and the equivalent stage in monkey sections (diameters up to 3 mm). DCN staining was readily found in luteal cells and connective tissue cells of all CL present in the sections examined (Fig. 1B, C, E and F). In human CL, DCN was localized to small areas between luteal cells as well as in the connective tissue (Fig. 1F). DCN was furthermore detected in the cytoplasm of some monkey luteal cells (Fig. 1C). Controls with non-immune serum (data not shown) and omission of primary antiserum were negative (Fig. 1A and D inserts).

DCN was readily detected in the FF from 20 patients undergoing IVF. ELISA measurements showed that the DCN levels ranged from 6.7 to 23.6 ng/ml with a mean of 12.9 ± 4.5 (standard deviation; Fig. 2A). Freshly isolated luteinizing GCs expressed DCN, as shown by RT–PCR in a total of six different pools of GCs (Fig. 3A).

The analysis of gene expression data revealed overall high levels of DCN mRNA in monkey CL samples obtained from different stages of the natural luteal phase (data not shown). Moreover, significant but constitutive levels of DCN mRNA are present in the naturally selected rhesus macaque follicle (whole follicles including granulosa and theca compartments) prior to and after an ovulatory stimulus, as determined by a separate DNA microarray study (Xu et al., 2011; data not shown).

DCN expression was noted in human ovary (data not shown), as well as freshly isolated, luteinizing GCs (Day 0) and luteinized GCs cultured for 1–4 days (Fig. 3A and B). Western blot analyses of several batches (3–6 each) of cultured human GCs (Fig. 3C) also showed DCN protein expression.

DCN actions in human luteinized GCs

The addition of DCN (5 and 10 μg/ml) to human GCs after 2 or 3 days of culture induced, within seconds, transient elevations of intracellular Ca^{2+}, which is suggestive of interaction with and activation of GFRs (Fig. 1A and B). Both human recombinant and bovine DCN were equally effective. BSA (5 μg/ml), which served as a negative control to exclude non-specific protein effects, did not alter intracellular Ca^{2+} levels (Fig. 4B). The experiment was repeated four times using different batches of pooled human GCs, consisting of at least 100 cells. The effect of DCN was found in all experiments, yet the magnitude and onset of individual responses of cultured GCs varied somewhat as seen in Fig. 4A.

The addition of 1 and 10 μg/ml of DCN to cultured GCs generated ROS (Fig. 4C–E), an effect that was observed within minutes and was shown).
statistically significant ($P < 0.05$) after 2 h when the experiments were terminated. The addition of the antioxidant NAC inhibited the DCN-dependent increase in ROS levels. The magnitude of ROS generation increased with higher concentrations of DCN. Lower doses of DCN (10–100 ng/ml) did not significantly increase ROS within 2 h (not shown).

To examine whether DCN could act via GFRs expressed by GCs, we screened GCs for GFRs by RT–PCR, with the DNA product identity being verified by sequencing (Fig. 5A and data not shown). Cultured human GCs (Days 1–4 in the culture) express several GFRs, which are known to be partners of DCN in other tissues. They include receptors of the EGFR family, namely EGFR, ErbB2...
DCN acts in part via EGFR expressed by human GCs. (A) RT–PCR products showing the expression of individual epidermal GFR (EGFR) family members (EGFR, ErbB2, ErbB3, ErbB4). Results were obtained from GCs on Days 1–4 of culture; (–): RNA instead of cDNA input. Cyclophilin was co-amplified as an internal standard. (B) Representative western blot experiment (top panel) showing that DCN causes phosphorylation of EGFR in human GCs: DCN and EGF robustly increase phosphorylation of EGFR within 10 min, addition of PBS (–) and BSA do not. Results using an antibody recognizing non-phosphorylated EGFR in the same samples are shown in the middle panel and were used to normalize the results. In the densitometric evaluation of four western blot experiments (bottom panel) EGF and DCN robustly phosphorylate EGFR to the same extent (mean ± SD). (C) The result of a representative caspase 3/7 activity assay. Both EGF and DCN significantly reduced the levels after 24 h (P < 0.05; mean ± SEM; 6 wells/group). Results are expressed in relative luminescence units (RLU). Two additional experiments yielded similar results.

To test whether EGFR is among the receptors targeted by DCN in the ovary, DCN (5 μg/ml) was added to human GCs for 10 min and the phosphorylation of the EGFR was determined by western blotting (Fig. 5B). The results of four independent experiments, using cells from different patients, indicate that EGFR phosphorylation occurred within 10 min upon addition of DCN. The normalization of phosphorylated EGFR to total EGFR levels revealed an increase of 1.4–4.6-fold following DCN treatment versus untreated controls. Similar responses were observed upon addition of 50 ng/ml of EGF (1.2–4.1-fold). The addition of BSA (5 μg/ml) did not cause EGFR phosphorylation and therefore, a non-specific protein effect can be excluded.

Finally, we tested whether exogenous DCN or EGF can affect apoptotic events in cultured GCs (Fig. 5C). The treatment of human GCs (Day 2 of culture; 3 experiments) with EGF (50 ng/ml) or DCN (5 μg/ml) for 24 h led to variable results but did not robustly affect caspase 3/7 activity levels, which are a measure of apoptosis. However, when GCs were tested on Day 5 following a 24 h treatment period, DCN and EGF significantly reduced caspase 3/7 activity levels in three independent experiments (Fig. 5C). Staurosporine (1 μM), used as positive control, strongly induced caspase 3/7 activity (data not shown).

Discussion

The results of this study revealed that DCN is a member of the ECM within the primate ovary, where it is found in stromal, follicular thecal and luteal tissue. DCN is absent from follicular (non-luteinized) GCs. In the CL it is localized to small aggregates between granulosa lutein cells and to the cytoplasm of some luteal cells, and is found in the connective tissue. Lastly, DCN is present in human FF and freshly isolated, luteinizing human GCs obtained from IVF patients. Hence, DCN is induced in GCs around ovulation and its levels furthermore increase in the late CL at the end of the menstrual cycle.

The study also provides insight into a possible functional role of DCN in the ovary. Using cultured luteinized human GCs as a test system, DCN behaved as a paracrine factor. DCN increased intracellular Ca²⁺ levels, caused the generation of ROS and reduced apoptosis. These actions are most likely due to the ability of DCN to target GFRs, including EGFR and others. Given the widespread distribution of DCN in the primate ovary and distinct changes of its levels during the ovarian cycle, the results suggest that DCN is a hitherto unknown factor involved in the regulation of important ovarian functions.

The composition of the ECM in the human or non-human primate ovary, and especially the CL, is not well defined. Aggregates of interstitial ECM between luteal cells in the human CL were reported in a previous study (Irving-Rodgers et al., 2006) with the ECM components laminin and collagen type IV being synthesized by human luteal cells. The synthesis of DCN by primate luteal cells was clearly shown by immunohistochemistry and by cellular assays in the present study. Some differences between monkey and human CL staining became apparent. In macaques, DCN stained the cytoplasm of luteal cells and outlined their circumference, indicative of synthesis and secretion. Cytoplasmic DCN in human luteal cells was not evident, possibly due to differences in fixation; but its synthesis by freshly isolated luteinizing human GCs and cultured luteinized GCs was unequivocally shown. Thus, periovulatory events led to an induction of DCN expression by GCs. Furthermore, FF from IVF patients contained DCN. It is
possible that luteinizing GCs are responsible for this production, but a contribution from the theca cells, which strongly stain for DCN, is possible as well.

In general, the ECM actively participates in the regulation of important processes, including cell migration, proliferation and differentiation. The capacity of ECM to affect cellular function stems from its ability to serve as a storage compartment for GFs and to affect GF activities. DCN, known to stabilize collagen fibrils of collagen type I (Reed and Iozzo, 2002), can also store GFs and furthermore activate/interfere with multiple GF signaling systems. A number of GF partners for DCN are now known and include EGFR (Schafer and Iozzo, 2008), as well as insulin-like GFR-1 (IGF-1R; Schaefer and Iozzo, 2008), vascular endothelial GFR (Iacob et al., 2008), PDGFR-α/β (Nili et al., 2003) and HGFR (Goldoni et al., 2009).

We explored the possibility that DCN may activate ovarian GFR signaling systems. Most human ovarian cells (theca and stromal cells, oocytes) are not available for such experiments. GCs, which correspond to periovulatory GCs and granulosa-derived luteal cells of the CL are available and were studied. GCs are a relevant model because they express several GFRs (Kwintkiewicz and Giudice, 2009; Karakida et al., 2011; results of the present study). Furthermore, DCN in FF and DCN expression by GCs undergoing luteinization, show that the potential ligand for all these GFR is present as well.

The observed actions of DCN, namely transient increases in Ca2+ and ROS generation, are indicative of GFR activation by DCN (Iozzo and Schafer, 2010; Finkel 2011). However, it is an intrinsic property of DCN to bind to several GFs albeit with different binding affinities (Iozzo and Schafer, 2010). Thus, the expression pattern of GFRs, their abundance and the local concentration of DCN will determine whether and how DCN acts. With regard to the ovary, many details thus remain to be studied but the actions of DCN observed in GCs demonstrate that DCN is able to act as a signaling molecule.

Our results furthermore indicate that the EGFR is one of the GFRs directly targeted by DCN in human GCs, as shown directly by rapid phosphorylation of EGFR upon addition of DCN. Phosphorylation is an early step in EGFR signaling and this action of DCN is similar to that described in previous studies with EGFR-expressing cells (Iozzo et al., 1999; Csordas et al., 2000; Zhu et al., 2005; Adam et al., 2011). We studied EGFR activation, because in the context of ovarian physiology (Saller et al., 2012), it is unlikely that this rise is related to structural properties of DCN in the ECM. Given the ability of DCN to store and thus lower available GF levels, the increase in DCN in the CL could be interpreted as a timed event, which may be involved in the demise of the CL by limiting important paracrine signaling events. Down-regulation of GFRs, namely EGFRs, is another mode of DCN action that was described to occur after an initial activation (Csordas et al., 2000; Zhu et al., 2005). Whether this occurs as a response to high levels of DCN in the CL remains to be shown.

How DCN is regulated in the ovary, e.g. how it is induced in GCs during ovulation and increased in the regressing CL is unknown. Hormonal signals might be involved, but in other organs there is only evidence for DCN regulation by local factors, including products of immune cells, e.g. mast cell-derived tryptase and tumor necrosis factor alpha (Adam et al., 2011, 2012), thrombin (Ivey and Little, 2008), interleukin-1 (IL-1) and IL-4 and others (Pulkkinen et al., 1992; Mauviel et al., 1995; Wegrowski et al., 1995).

In summary, the results identify DCN as part of the ovarian ECM in the ovum-stroma, follicular theca cells and luteal cells. Moreover, DCN is present in the FF of ovulatory follicles. Distinct changes of DCN expression are associated with ovulation and the demise of the CL. In human GCs, DCN can act as a paracrine signaling factor due to its ability to interfere with GF/GFR systems. Our study thus raises the possibility that DCN is a local regulator of folliculogenesis, ovulation and the functional lifespan of the CL in primates. The identification of the GF/GFR partners of ovarian DCN and elucidation of the regulation of DCN may open new strategies to target follicular growth, ovulation and the function of the CL.

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**Authors’ roles**

M.A. performed the majority of the experiments and analyzed the results. S.S. contributed to the work with ROS measurements and Ss by performing DCN ELISA and caspase assays. J.H. performed research on monkey tissues, including qPCR and analyzed the results.
D.B. and U.B. contributed essential human GCs, GAD, RLS and SRO provided monkey tissue and provided important conceptual input. A.M. conceived of the study and designed the research. Together with M.A. he drafted the manuscript. All authors contributed to the final version of the paper and approved it.

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**Conflict of interest**

None declared.

**References**


