

Contents lists available at ScienceDirect

Reproductive Biology

journal homepage: www.elsevier.com/locate/repbio

Original article

Clinical utility of decorin in follicular fluid as a biomarker of oocyte potential

Yuki Sawada, Takeshi Sato*, Chieko Saito, Fumiko Ozawa, Yasuhiko Ozaki, Mayumi Sugiura-Ogasawara

Department of Obstetrics and Gynecology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

ARTICLE INFO

Keywords:

Decorin
Fertilization
Follicular fluid
Granulosa cell
Intracytoplasmic sperm injection

ABSTRACT

This study investigated the concentration of decorin (DCN) in mature follicular fluid and the existence in the granulosa cells. It also investigated whether DCN is useful as a biomarker for outcomes of assisted reproductive technology (ART). A retrospective cohort study was performed involving 130 oocytes of 88 patients treated with ART because of unexplained infertility. The concentration of DCN in the follicular fluid (F-DCN) was 39.26 ng/ml (median value); it was higher than that in serum. F-DCN of the oocytes fertilized by intracytoplasmic sperm injection (ICSI) was significantly lower than that of oocytes that were not fertilized (33.24 ng/ml vs 40.18 ng/ml; $P = 0.043$). When a cut-off level of 34.5 ng/ml was set according to the receiver-operating characteristic curve, the fertilization rate of the oocytes from the follicles in which F-DCN was lower than the cut-off level tended to be good compared to that of the oocytes with F-DCN higher than the cut-off level ($P = 0.052$). DCN is less likely to be produced by the granulosa cells (GCs), because it was not detected in GCs by immunostaining and Western blot analysis. F-DCN has a possibility to be a biomarker indicating the quality of oocytes collected from the corresponding follicle.

1. Introduction

Various bioactive substances such as hormones and cytokines exist in the pre-ovulatory follicular fluid (FF). They are related to the development and maturity of oocytes. Oocyte development and maturity are important steps in the process of reproduction and infertility treatments. It is thought that treatment outcomes can be improved by applying clinical findings after analyzing the associated bioactive substances. Examples of these bioactive substances are granulocyte colony-stimulating factor (G-CSF) and macrophage inflammatory protein (MIP)-3 α . It has been reported that the concentration of G-CSF in FF is correlated with the number of the fertilized oocytes, and that it can be a useful biomarker of the quality of a corresponding oocyte [1,2]. On the other hand, it has been reported that MIP-3 α in FF is related to the maturity of an oocyte because its concentration in FF containing mature oocytes is significantly higher than that in FF containing immature oocytes [3].

Decorin (DCN), another bioactive substance, belongs to the small leucine-rich proteoglycan family and exists in some organs such as the bone, tendon, skin, aorta, and cornea. It has been reported that DCN constructs the extracellular matrix and has an effect on the formation of

collagen fiber by combining with collagen type I and type II [4]. In addition, it regulates cell proliferation by combining with epidermal growth factor receptor (EGFR) or insulin-like growth factor 1 receptor (IGF-1R). In normal cells such as endothelial and renal cells, it combines with EGFR, thereby inactivating EGFR and inhibiting cell proliferation [5,6]. However, by combining with IGF-1R, it promotes protein synthesis and controls apoptosis by activating the phosphatidylinositol 3-kinase/protein kinase B (PI3 K/Akt) pathway [5,7,8]. DCN has also been reported to halt tumor growth by antagonizing oncogenic tyrosine kinase receptors and restraining angiogenesis [9,10]. In addition, DCN actions at the fetal–maternal interface include restrained trophoblast migration, invasion, and uterine angiogenesis, and they might have a causal role in preeclampsia by compromising endovascular differentiation of the trophoblast and uterine angiogenesis, resulting in poor arterial remodeling [11,12]. However, it has also been reported that ectopic DCN overexpression in mouse cerebral endothelial cells upregulated vascular endothelial growth factor A to promote angiogenesis [13].

In the ovary, previous studies have shown that DCN presents in the connective tissue, follicular thecal compartments, FF of ovulatory follicles, and corpus luteum [14]. In addition, it has been reported that

* Corresponding author at: Takeshi Sato Department of Obstetrics and Gynecology, Nagoya City University Graduate School of Medical Sciences, 1, Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 4678601, Japan.

E-mail address: og.sato@med.nagoya-cu.ac.jp (T. Sato).

<https://doi.org/10.1016/j.repbio.2017.12.001>

Received 7 September 2017; Received in revised form 1 December 2017; Accepted 3 December 2017

1642-431X/© 2017 Society for Biology of Reproduction & the Institute of Animal Reproduction and Food Research of Polish Academy of Sciences in Olsztyn. Published by Elsevier Sp. z o.o. All rights reserved.

DCN does not exist in the granulosa cells (GCs) of pre-antral and antral follicles [14]. However, the origin of DCN is unclear and the expression of DCN in GCs of mature follicles was not analyzed.

Regarding the function of DCN, it has been reported that it plays roles in some important processes such as follicle growth, ovulation, and retaining the corpus luteum by regulating growth factors [14], but its detailed functions are unknown. The association between DCN and the outcomes of infertility treatments has not been examined.

In the present study, we examined the concentrations of DCN in FF (F-DCN) and in serum (S-DCN), the localization of DCN in GCs, and the relationships with outcomes of assisted reproductive technology (ART) and, as a result, whether DCN is useful as a biomarker for ART.

2. Materials and methods

2.1. Patients

A retrospective cohort study involving 88 patients treated with ART because of unexplained infertility was performed at Nagoya City University Hospital between April 2010 and March 2016.

FF, serum, and GCs were collected from the patients. F-DCN, S-DCN, and the concentration of IGF-1 in FF were examined. We investigated the relationships between F-DCN and patient age, pulsatility index (PI) of the blood flow around the follicle at ovum pick-up (OPU), S-DCN, the concentration of IGF-1 in FF, fertilization, and quality of the embryo. We tried to identify the existence and localization of DCN in GCs.

The study was conducted with the approval of the Research Ethics Committee of Nagoya City University. Written informed consent was obtained from all patients.

2.2. Ovarian stimulation

Controlled ovarian stimulation was performed with a long protocol of gonadotropin-releasing hormone (GnRH) agonist, a short protocol of GnRH agonist, a GnRH antagonist protocol, or a clomiphene citrate (CC) protocol. The protocol was selected according to patient age and the ovarian reserve predicted by serum anti-Müllerian hormone. The details of each protocol are described here.

2.2.1. Long protocol of GnRH agonist

Downregulation of the pituitary gland was performed by administering buserelin acetate in a nasal spray (0.9 mg/day) (Suprecur; Mochida Pharmaceutical, Tokyo, Japan) starting from the mid-luteal phase of the previous cycle. When the serum estradiol levels were reduced to less than 30 pg/ml during the early phase of the menstrual cycle, intramuscular injection of gonadotropin (Gonpure; ASKA Pharmaceutical, Tokyo, Japan) was started (150–450 IU/day).

2.2.2. Short protocol of GnRH agonist

Administration of buserelin acetate in a nasal spray was started during the early phase of the menstrual cycle. Then, intramuscular injection of gonadotropin was started 2 days later.

2.2.3. GnRH antagonist protocol

Intramuscular injection of gonadotropin was started on day 3 to day 5 of the menstrual cycle. Since the leading follicle reached 16–18 mm in diameter when measured by transvaginal ultrasonography, daily subcutaneous injections of 0.25 mg Cetrotide (Merck Serono Co., Ltd., Tokyo, Japan) were administered until stimulation was completed.

2.2.4. CC protocol

On day 5 of the menstrual cycle, 100–150 mg/day of CC (Fuji Pharmaceutical, Toyama, Japan) was started orally. When the leading follicle reached 10–12 mm, intramuscular injection of gonadotropin was started.

2.3. OPU, fertilization, and embryo evaluation

During all protocols, ovulation was induced by intramuscular injection of 10,000 IU human chorionic gonadotropin (Gonotropin; ASKA Pharmaceutical, Tokyo, Japan) when the leading follicle reached a diameter of more than 20 mm as measured by transvaginal ultrasonography. Thirty-six hours later, transvaginal ultrasonography-assisted OPU was performed after PI of the blood flow around the follicle was calculated by transvaginal ultrasonography. The oocytes collected from the first punctured follicles that were selected according to accessibility and size at least 18 mm in diameter were cultured individually in separate dishes. In vitro fertilization (IVF) was performed; however, intracytoplasmic sperm injection (ICSI) was performed for patients with low fertilization rates during previous IVF. In addition, when more than six oocytes were collected, split ICSI was performed if requested by the patient. Embryo quality was evaluated 2 days after OPU according to the Veeck classification. Grade 1 or grade 2 embryos were considered good, whereas the others were determined to be poor embryos.

2.4. Samples (serum, FF, and GC)

On the day of OPU, serum and FF samples were collected. Blood was sampled just before OPU. Blood samples were centrifuged at $250 \times g$ for 5 min to separate and collect serum. At OPU, FF was aspirated and collected from the first punctured follicle of each ovary to avoid contamination with blood. These samples were centrifuged at $250 \times g$ for 5 min to separate the supernatant as FF and the precipitate as the cellular components. The serum and FF samples were cryopreserved at -20°C until analysis.

Some FF samples were layered over a Ficoll-Paque gradient at $250 \times g$ for 30 min at 4°C . The cell samples in the mononuclear cell layer were cryopreserved as GCs at 4°C for use during immunocytochemistry and SDS-PAGE and Western blot analyses.

2.5. Enzyme-linked immunosorbent assay

Commercial enzyme immunoassay kits were used to examine the concentration of DCN (RayBiotech, Norcross, GA, USA) in serum and FF and the concentration of IGF-1 (R&D Systems, Minneapolis, MN, USA) in FF. Because the kits were validated for serum samples but not for FF, before using FF samples, the recovery rate of at least three FF samples was checked. All samples were measured in duplicate according to the manufacturer's instructions. In cases with very high or low concentrations, the measurements were repeated with the appropriate dilution.

2.6. Immunostaining

2.6.1. Immunocytochemistry

Immunocytochemistry was performed for three GC samples to examine the localization of DCN in GCs.

GC samples were smeared on MAS-coated glass slides (Matsunami Glass Ind. Ltd., Osaka, Japan) after fixation using Mildholm (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After washing in phosphate-buffered saline (PBS) (Dulbecco's phosphate-buffered saline; Invitrogen, Inc., Carlsbad, CA, USA) for 5 min three times, treatment with 0.1% TritonX (Sigma-Aldrich Co., St. Louis, MO, USA) in PBS was performed for 5 min. Endogenous peroxidase activity was blocked by incubating GC samples with 0.3% H_2O_2 in methanol (Sigma-Aldrich Co.) for 30 min. GC samples were blocked with Histofine SAB-PO (M) (Nichirei Bioscience Inc., Tokyo, Japan) for 1 h at room temperature. Then, GC samples were incubated with primary antibodies diluted in PBS overnight at 4°C . Anti-decorin antibody sc-73896 (mouse, 1/100 and 1/500 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was used. Negative controls were created by omitting the primary antibodies and incubating with PBS only. GC samples were incubated with

Histofine SAB-PO (M) (Nichirei Biosciences Inc.) for 30 min at room temperature. Color development was performed using DAB (Nichirei Biosciences Inc.). GC samples were washed in water, and nuclei were counterstained with 1% methylgreen (Muto Pure Chemicals Co., Ltd, Tokyo, Japan) for 10 min. Permanent specimens were created by dehydration using ethanol and xylene and sealing with ENTELLAN (Merck KGaA, Darmstadt, Germany). To examine the localization of DCN, hematoxylin and eosin staining was performed using GCs.

2.6.2. Immunohistochemistry

Immunohistochemistry was performed for normal human ovarian tissue sections (US Biomax, Inc., Rockville, MD, USA).

After deparaffinization and rehydration through a series of xylene and ethanol, endogenous peroxidase activity was blocked by incubating the section with 3% H₂O₂ in distilled water (Sigma-Aldrich Co.) for 30 min.

2.7. SDS-PAGE and western blot analysis

Western blot analysis was performed for two clinical GC samples and recombinant human DCN (recDCN) (ATGen Co., Ltd, Seongnam-si, Gyeonggi-do, Korea) as positive control using anti-decorin antibody to examine the presence of DCN in GCs.

The samples were diluted with Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) and subjected to SDS-PAGE according to the Laemmli method [15–17]. An equal amount of protein (2.5 µg) for each sample was applied in each lane of 5–20% Criterion ready gradient gels J (Bio-Rad) with a mid-range molecular weight standard marker (Bio-Rad). Electrophoresis was performed using SDS-PAGE cassettes (Bio-Rad), a power supply (Bio-Rad), and electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3; Bio-Rad). Trans-Blot Turbo (Bio-Rad) was performed to transfer the proteins. The membranes were blocked with 5% skim milk (Meg Milk Snow Brand Co., Ltd, Tokyo, Japan) in Tris-buffered saline (TBS) and 0.1% Tween for 1 h at room temperature on a shaker (Taitec, Tokyo, Japan) prior to primary antibodies. Anti-decorin antibody (1/200 dilution; Santa Cruz Biotechnology, Inc.) was used. The primary antibodies were diluted with primary ab dilution buffer (3% bovine serum albumin, 9 mM Na₂CO₃, TBS-T). As loading control, α-tubulin (Cell Signaling Technology Inc., Danvers, MA, USA) and β-actin (Cell Signaling Technology Inc.) bolts were used. An alkaline phosphatase-conjugated goat anti-mouse IgG-HRP secondary antibody (1/1000 dilution; Bio-Rad) was used. Color development was achieved with the Super Signal West Dura Extended Duration Substrate kit (Bio-Rad). The immunoreactive bands were semi-quantified using Science Lab 2005 Multi Gauge version 3.0 software (Fuji Photo Film Co., Tokyo, Japan).

2.8. Statistical analysis

Each FF and serum samples was analyzed as a single event. All statistical analyses were performed using R version 3.3.2. Comparisons between groups were performed using the Mann-Whitney *U* test or Fisher's exact probability test. Correlations between measured parameters were evaluated using the Spearman *R* test. *P* < 0.05 was considered statistically significant.

3. Results

A total of 130 oocytes and the corresponding FF and serum samples from 88 patients with unexplained infertility aged 27 to 50 years were included in the study. The median (interquartile range [IQR]) age at the time of OPU and total dose of gonadotropin administered were 40 (38–43) years and 1800 (1275–3056) IU (Table 1). The fertilization rate and good embryo rates for the 130 oocytes were 82% (107/130) and 44% (61/107), respectively (Table 1). The medians (IQR) of F-DCN and S-DCN from 130 samples were 39.26 (28.41–44.44) ng/ml and 4.88

Table 1
Characteristics of the study subjects.

Age	40 (38–43)
Controlled ovarian stimulation protocol, n	
Long	54
Short	35
GnRH antagonist	24
CC	17
Total dose of gonadotropin administered [IU]	1800 (1275–3056)
Pulsatility index	0.80 (0.68–0.94)
Fertilization rate [%]	82 (107/130)
Good embryo rate [%]	44 (61/107)

Values of age, total dose of gonadotropin administered, pulsatility index of the blood flow around the follicle are presented as median (interquartile range). The fertilization rate is presented as the percentage of the total number (fertilized oocytes/all oocytes). The good embryo rate is presented as the percentage of the total number (good embryos/fertilized oocytes).

(3.95–5.75) ng/ml, respectively (Table 2). F-DCN was significantly higher than S-DCN (95% confidence interval: 35.39–39.51 vs 4.79–5.28). Each F-DCN and S-DCN showed no significant difference among controlled ovarian stimulation protocols (Table 2).

Correlations were not observed between F-DCN and patient age, the total dose of gonadotropin administered, IGF-1 concentration in FF, and PI of the blood flow around the follicle (Table 3). However, F-DCN showed a weak negative correlation with S-DCN (*R* = −0.189; *P* = 0.031) (Fig. 1).

ICSI was performed for 70 of the 130 oocytes. The fertilization rate of the IVF group was significantly higher than that of ICSI group, but patient age, PI of the blood flow around the follicle, F-DCN, S-DCN, IGF-1 concentration in FF, and the good embryo rate did not show significant differences between the two groups (Table 4).

In all samples, there were no significant differences in F-DCN and S-DCN of the fertilized oocytes and the oocytes that were not fertilized (Table 5). However, F-DCN of the fertilized oocytes was significantly lower than that of the oocytes that were not fertilized only for those who underwent ICSI (*P* = 0.043) (Fig. 2). F-DCN of the ICSI group had the ability to predict successful fertilization, with the receiver-operating characteristic (ROC) area under the curve for elevated F-DCN being 0.662 (Fig. 3). When a cut-off level of 34.5 ng/ml was set according to the ROC curve, the fertilization rate of the oocytes from the follicles in which F-DCN was lower than the cut-off level tended to be good compared to that of the oocytes with F-DCN higher than the cut-off level (*P* = 0.052). Sensitivity, specificity, positive predictive value and negative predictive value of F-DCN for fertilization outcomes were 0.72, 0.58, 0.86, and 0.37, respectively.

Regarding the quality of embryos, there was no significant difference between F-DCN of good and poor embryos or between the S-DCN of the two groups. However, S-DCN of the good embryos was significantly higher than that of the poor embryos (Table 5) only for those oocytes fertilized by ICSI.

Immunocytochemistry was performed for three GC samples. Staining with antibodies against DCN was not observed in all samples (Fig. 4). The localization of DCN was not found in GCs. In addition, immunohistochemistry was performed for normal human ovarian tissue sections. Staining with antibodies against DCN was observed in the stromal cells and the degeneration form of the fibroblasts (Fig. 5). The follicles were not found in these sections.

Western blot analysis of two GC samples using anti-decorin antibody did not show a band, but recDCN showed a band with molecular weights of 43 kDa. On the other hands, using anti-α-tubulin antibody and anti-β-actin antibody as loading controls showed single bands with molecular weights of 52 kDa and 45 kDa, respectively (Fig. 6). The presence of DCN was not ascertained in the present study.

Table 2
F-DCN and S-DCN classified by controlled ovarian stimulation protocols.

	Total, n = 130	Controlled ovarian stimulation protocol			P	
		Long, n = 54	Short, n = 34	GnRH antagonist, n = 24		CC, n = 17
F-DCN [ng/ml]	39.26 (28.41–44.44)	35.56 (24.06–43.81)	41.34 (31.66–44.55)	41.50 (33.88–47.61)	35.37 (27.23–41.72)	0.129
S-DCN [ng/ml]	4.88 (3.95–5.75)	4.92 (3.97–5.93)	4.90 (4.22–5.48)	4.61 (3.68–5.60)	4.87 (3.93–5.70)	0.831

F-DCN and S-DCN are presented as median (interquartile range). The *P*-values for those parameters were evaluated using the Mann-Whitney *U*-test.

Table 3
Correlations between F-DCN and each parameter.

Correlation between F-DCN and the following:	Spearman <i>R</i>	<i>P</i>
Age	−0.031	0.731
Total dose of gonadotropin administered	0.108	0.222
S-DCN	−0.189	0.031
Follicular fluid IGF-1	−0.097	0.557
PI	−0.027	0.781

Correlations between F-DCN and other parameters were evaluated using the Spearman *R*-test (n = 130).

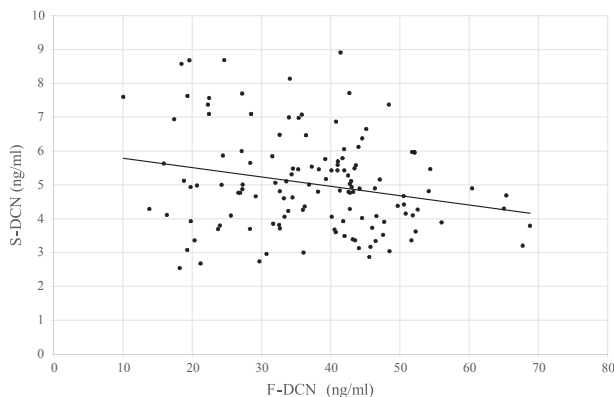


Fig. 1. Correlation between F-DCN and S-DCN. The vertical axis indicates S-DCN and the horizontal axis indicates F-DCN. Correlation between S-DCN and F-DCN was evaluated using the Spearman *R*-test. A weak negative correlation was observed between two parameters ($R = -0.189$; $P = 0.031$).

Table 4
Comparison of parameters of the IVF group and ICSI group.

	IVF group, n = 60	ICSI group, n = 70	<i>P</i>
Age	41 (37–43)	40 (38–42)	0.225
Total dose of gonadotropin administered [IU]	1800 (1463–3113)	1837 (1275–3000)	0.689
F-DCN [ng/ml]	41.87 (33.1–47.22)	35.59 (27.24–42.91)	0.068
S-DCN [ng/ml]	4.67 (3.91–5.46)	5.01 (4.07–6.48)	0.062
Follicular fluid IGF-1 [ng/ml]	92.98 (71–101.06)	73.29 (68.57–83.93)	0.151
PI	0.79 (0.66–0.91)	0.8 (0.68–0.94)	0.906
Fertilization rate [%]	92 (55/60)	74 (52/70)	0.011

Values of age, total dose of gonadotropin administered, F-DCN, S-DCN, IGF-1 concentration in FF, and PI of the blood flow around the follicle are presented as median (interquartile range). The *P*-values for those parameters were evaluated using the Mann-Whitney *U*-test. The fertilization rate is presented as the percentage of the total number (fertilized oocytes/all oocytes). The *P*-value was calculated using Fisher's exact probability test.

4. Discussion

The present study indicated that DCN existed in FF of mature follicles and could be measured. In addition, F-DCN was higher than S-DCN. F-DCN of oocytes fertilized by ICSI was significantly lower than that of the oocytes that were not fertilized. Regarding the origin of DCN in FF of mature follicles, it was speculated that DCN was less likely to be produced by GCs because DCN was not detected by both immunocytochemistry and Western blotting in GCs.

DCN existed in FF of mature follicles, and their concentration in FF was 39.26 ng/ml (median, n = 130). Adam et al. reported that the concentration of DCN in FF collected from IVF patients was 12.9 ng/ml (mean, n = 20) [14], which was considerably lower than the concentration shown in this study. The discrepancy between the two studies may have been caused by differences in methods of collecting FF and the enzyme-linked immunosorbent assay (ELISA) kit used for the measurement of F-DCN. In the present study, F-DCN was approximately eight-times higher than S-DCN. The results suggest that DCN is necessary and has a role in the mature follicles.

F-DCN of the oocytes fertilized by ICSI was significantly lower than that of the oocytes that were not fertilized (33.24 ng/ml vs 40.18 ng/ml; $P = 0.043$), although correlations were not shown between F-DCN and patient age, the total dose of gonadotropin administered, IGF-1 concentration in FF, and PI of the blood flow around the follicle. When a cut-off level of 34.5 ng/ml was set according to the ROC curve, the fertilization rate of the oocytes from the follicles in which F-DCN was lower than the cut-off level tended to be good compared to that of the oocytes with F-DCN higher than the cut-off level ($P = 0.052$). In addition, positive predictive value was higher than the fertilization rate of ICSI group (0.86 vs 0.74). Therefore, F-DCN has a possibility to be a predictor of successful fertilization by ICSI. In the case of ICSI, because it could be postulated that the fertilization outcome was more dependent on the quality of the oocytes rather than the fertilization ability of sperm, it was suggested that DCN affected the development and maturity of oocytes. Adam et al. reported that DCN phosphorylated EGFR of GCs [14]. In that report, it was suspected that the action of DCN in FF was related to epidermal growth factor (EGF). Therefore, we also measured EGF in FF, but we could not investigate the relation between F-DCN and EGF because the concentration of EGF was below the lower limit of the measurement value of the ELISA kit used in the present study. Although the concentration of IGF-1 in FF could be measured, a correlation was not shown between IGF-1 and F-DCN. Therefore, DCN in mature follicles was less likely to regulate growth factors through IGF-1, despite the report that indicated that DCN activated PI3k-Akt by combining with IGF-1R in endothelium [4,6–8].

DCN was not detected in GCs of mature follicles. In addition, considering the results of the previous study demonstrating that DCN did not exist in GCs of pre-antral and antral follicles [14], it was thought that DCN was less likely to be produced by GCs. We discussed about some hypotheses about the origin of F-DCN. First, DCN may be produced by the stromal cells and secreted into the follicles. In the present study, the expression of DCN in the stromal cells of ovary was detected by immunohistochemistry as Adam et al. showed in their study [14].

Table 5
Comparison of the roles of F-DCN and S-DCN in fertilization outcomes and embryo quality.

F-DCN [ng/ml]	Fertilization, n = 130		P	Embryo quality, n = 107		
	Success	Failure		Good	Poor	P
Total	38.30 (27.80–44.36) {107}	41.04 (32.16–44.91) {23}	0.435	40.71 (28.49–44.02) {61}	36.43 (28.33–44.18) {46}	0.811
IVF	41.80 (33.72–47.34) {55}	42.76 (31.68–42.79) {5}	0.873	41.93 (35.78–47.65) {31}	38.70 (30.35–45.95) {25}	0.687
ICSI	33.24 (26.67–41.43) {52}	40.18 (33.11–45.64) {18}	0.043	33.61 (27.21–42.56) {30}	33.32 (26.97–41.98) {21}	0.992
S-DCN [ng/ml]						
Total	4.87 (3.98–5.83) {107}	4.90 (4.29–5.49) {23}	0.949	5.00 (4.08–6.12) {61}	4.77 (3.93–5.47) {46}	0.115
IVF	4.66 (3.92–5.47) {55}	4.77 (3.89–4.94) {5}	0.659	4.38 (3.85–5.55) {31}	4.65 (4.13–5.26) {25}	0.942
ICSI	5.01 (4.05–6.71) {52}	4.96 (4.71–5.65) {18}	0.804	5.63 (4.65–7.30) {30}	4.98 (3.87–5.59) {21}	0.036

F-DCN and S-DCN are presented as median (interquartile range) and {number of samples}. The *P*-values for those parameters were evaluated using the Mann-Whitney *U*-test.

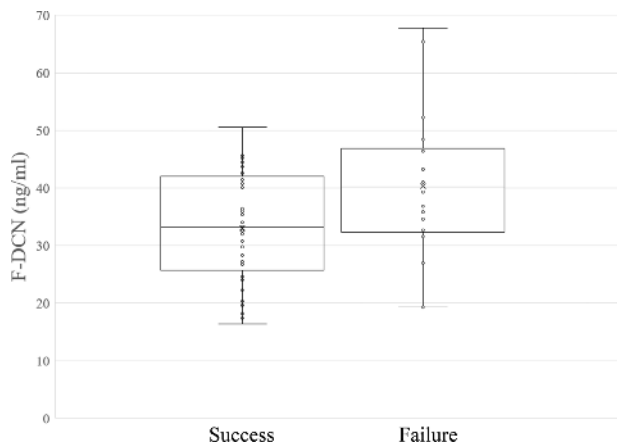


Fig. 2. F-DCN in the case of successful and failure fertilization using ICSI. The medians (IQR) of F-DCN of the oocytes fertilized by ICSI were 33.24 (26.67–41.43) ng/ml. On the other hands, that of the oocytes that were not fertilized were 40.18 (33.11–45.64) ng/ml. F-DCN of the oocytes fertilized by ICSI was significantly lower than that of the oocytes that were not fertilized ($P = 0.043$).

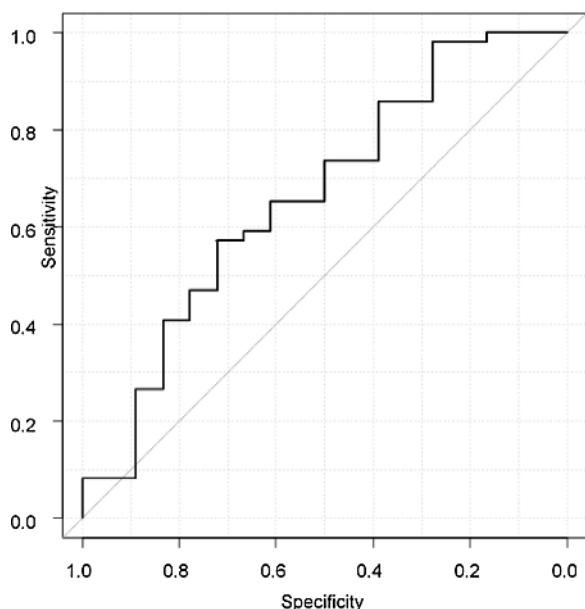


Fig. 3. Receiver-operating characteristic curve for F-DCN for the prediction of fertilization using ICSI. Receiver-operating characteristic (ROC) curve for decorin concentration in follicle fluid for the prediction of fertilization using intracytoplasmic sperm injection. ROC area under the curve was 0.662.

However, we could not examine existence of DCN in the stromal cells of mature follicles, because we collected the samples at OPU in which only GCs but not stromal cells were obtained. Second, there is a possibility that DCN flow into FF from the blood through the blood–follicle barrier. The blood–follicle barrier was found to be permeable by protein with a molecular weight less than 500 kDa [18]. That indicates, DCN can pass the blood–follicle barrier because its molecular weight is 43 kDa. In addition, McArthur et al. showed that large molecules such as proteoglycans including DCN do not traverse the basal lamina of follicle rapidly [19]; therefore, it is thought that DCN gradually flows into FF from the blood during follicle growth. Because F-DCN was higher than S-DCN in this study, it may be possible that DCN is actively taken into FF from the blood. So, DCN probably positively functions in the mature follicles even though it might affect the development and maturity of oocytes in this study. It has been reported that DCN was related to cell proliferation and restraint in the endothelial cells [4–8] and to promotion and restraint in angiogenesis [11–13]. Similarly, DCN in mature follicles may promote and restrain oocyte maturity and follicle growth.

In the present study, S-DCN was measured and analyzed in relation to other factors. However, it is not thought that S-DCN, unlike other bioactive substances mainly secreted by GCs, directly reflects GCs and oocytes because DCN is secreted and exists in various organs and flows into blood. Therefore, we cannot definitely determine whether the S-DCN of the good embryos was significantly higher than that of the poor embryos, or whether S-DCN was negatively correlated with F-DCN.

We acknowledge that there were several limitations to this study. First, the FF and serum samples were cryopreserved until analysis. It is possible that the freezing and thawing steps affected the ELISA results. Second, the sample size in the present study was relatively small, which made it difficult to analyze the association between DCN and pregnancy or birth rate. As the reason for that, our study was limited to patients treated with ART only because of unexplained infertility and FF samples were collected from the first punctured follicles. To analyze the relationship between DCN and treatment outcomes, it will be necessary to increase analysis samples and to perform further investigations in the future.

In conclusion, the present study found that DCN existed in FF of mature follicles with high concentrations and was related to fertilization after ICSI, probably through its involvement with the development and maturity of oocytes. The fertilization rate of oocytes from follicles in which F-DCN was less than 34.5 ng/ml was high, indicating that it is possible to predict the potential of oocytes to be fertilized by examining F-DCN of the corresponding follicle during ICSI treatment cycles. Therefore, it is useful for planning embryo transfer and cryopreservation. F-DCN in the mature follicles has a possibility to be a biomarker indicating the quality of the oocyte collected from the corresponding follicle. Regarding the origin of DCN, it is less likely to be produced by GCs. Identifying the detailed functions of DCN in reproduction requires further investigations of the correlation between F-DCN and other bioactive substances that are related to follicle growth, pregnancy establishment, and chromosomal abnormalities in oocytes and embryos.

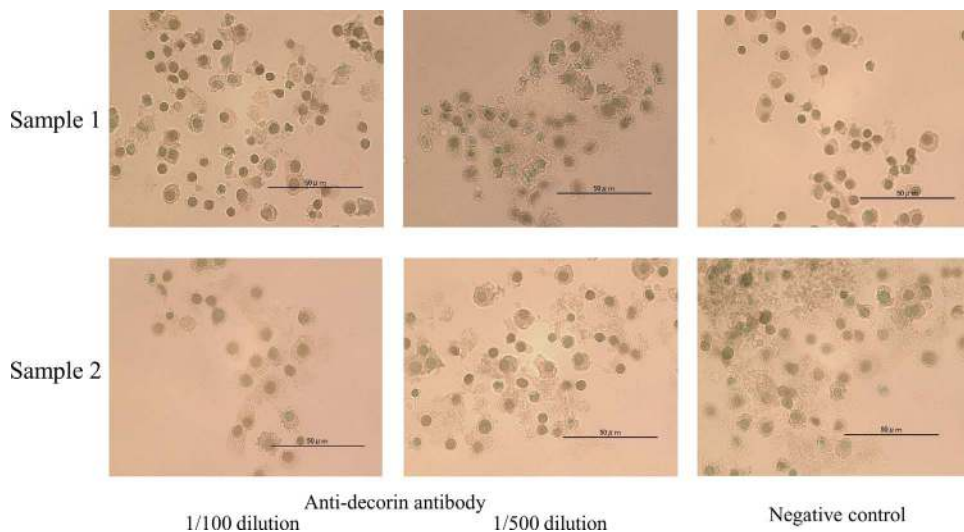


Fig. 4. Immunocytochemistry of GCs. Immunocytochemistry was performed for granulosa cells from patients treated with assisted reproduction technology. The pictures of two samples of three were presented. Brown color represents positive staining of the anti-decorin antibody. Immunoreactivity of decorin was not observed in all samples and no remarkable difference in the negative control was observed. Original magnification: $\times 400$, Scale bars: $50 \mu\text{m}$.

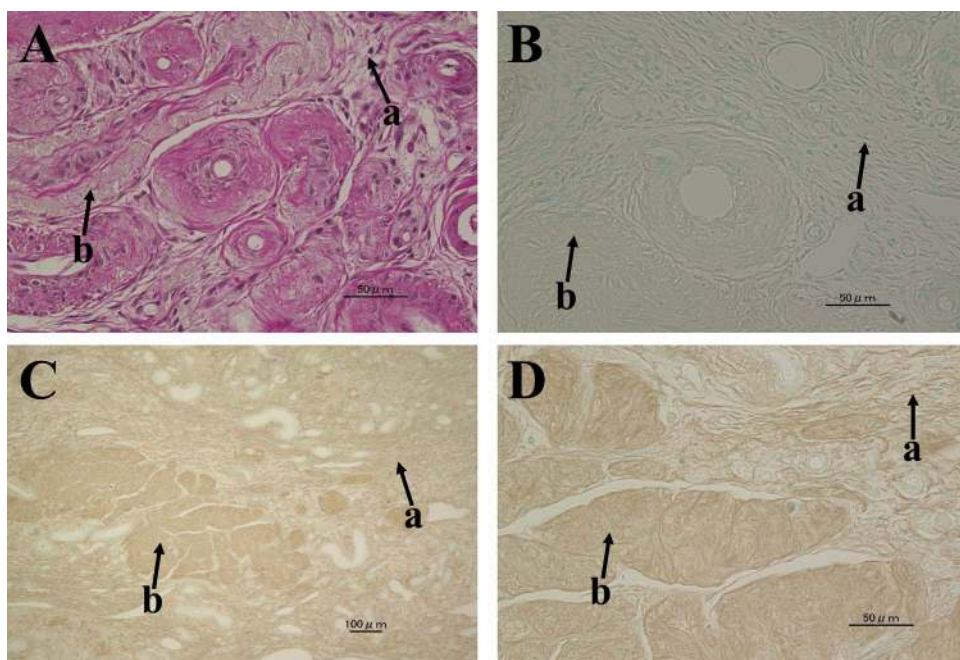


Fig. 5. Immunohistochemistry of normal human ovarian tissue. Immunohistochemistry was performed for normal human ovarian tissue sections. The follicles were not found in these sections. Brown color represents positive staining of the anti-decorin antibody. (A) Hematoxylin and eosin staining was performed. (B) Negative controls were created by omitting the primary antibodies. (C, D) Immunoreactivity of decorin was observed in the stromal cells (a) and the degeneration form of the fibroblasts (b). Original magnification: $\times 400$, scale bars: $50 \mu\text{m}$ (A, B, D). Original magnification $\times 100$, scale bar: $100 \mu\text{m}$ (C).

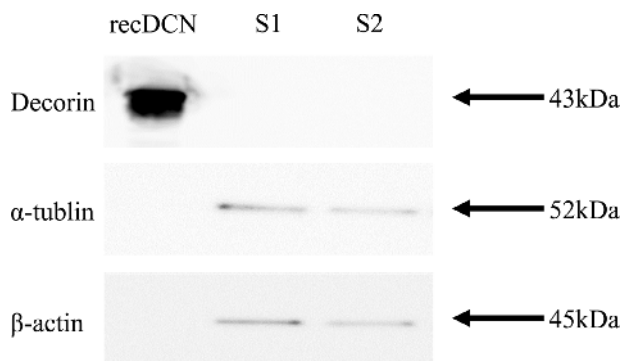


Fig. 6. Western blot analysis of GCs and recDCN. Anti- α -tubulin antibody and anti- β -actin antibody are loading controls. The two samples (S1, S2) are granulosa cells from patients treated with assisted reproduction technology. RecDCN is positive control. No band was observed in GC samples using anti-decorin antibody.

Sources of funding

This study was supported by a grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Declaration

The authors report no financial or commercial conflicts of interest.

References

- [1] Lédée N, Petitbarat M, Rahmati M, Dubanchet S, Chaouat G, Sandra O, et al. New pre-conception immune biomarkers for clinical practice: interleukin-18, interleukin-15 and TWEAK on the endometrial side, G-CSF on the follicular side. *J Reprod Immunol* 2011;88(2):118–23.
- [2] Gaafar TM, Hanna MO, Hammady MR, Amr HM, Osman OM, Nasef A, et al. Evaluation of cytokines in follicular fluid and their effect on fertilization and pregnancy outcome. *Immunol Invest* 2014;43(6):572–84.
- [3] Kawano Y, Fukuda J, Nasu K, Nishida M, Narahara H, Miyakawa I. Production of macrophage inflammatory protein-3 α in human follicular fluid and cultured granulosa cells. *Fertil Steril* 2004;82(Suppl. 3):1206–11.

- [4] Schaefer L, Iozzo RV. Biological functions of the small leucine-rich proteoglycans: from genetics to signal transduction. *J Biol Chem* 2008;283(31):21305–9.
- [5] Schönherr E, Levkau B, Schaefer L, Kresse H, Walsh K. Decorin-mediated signal transduction in endothelial cells. Involvement of Akt/protein kinase B in up-regulation of p21(WAF1/CIP1) but not p27(KIP1). *J Biol Chem* 2001;276(44):40687–92.
- [6] Zhu JX, Goldoni S, Bix G, Owens RT, McQuillan DJ, Reed CC, et al. Decorin evokes protracted internalization and degradation of the epidermal growth factor receptor via caveolar endocytosis. *J Biol Chem* 2005;280(37):32468–79.
- [7] Schönherr E, Sunderkötter C, Iozzo RV, Schaefer L. Decorin a novel player in the insulin-like growth factor system. *J Biol Chem* 2005;280(16):15767–72.
- [8] Schaefer L, Tsalastra W, Babelova A, Baliova M, Minnerup J, Sorokin L, et al. Decorin-mediated regulation of fibrillin-1 in the kidney involves the insulin-like growth factor-I receptor and Mammalian target of rapamycin. *Am J Pathol* 2007;170(1):301–15.
- [9] Grant DS, Yenisey C, Rose RW, Tootell M, Santra M, Iozzo RV. Decorin suppresses tumor cell-mediated angiogenesis. *Oncogene* 2002;21(31):4765–77.
- [10] Iozzo RV, Schaefer L. Proteoglycans in health and disease: novel regulatory signaling mechanisms evoked by the small leucine-rich proteoglycans. *FEBS J* 2010;277(19):3864–75.
- [11] Järveläinen H, Sainio A, Wight TN. Pivotal role for decorin in angiogenesis. *Matrix Biol* 2015;43:15–26.
- [12] Lala PK, Nandi P. Mechanisms of trophoblast migration, endometrial angiogenesis in preeclampsia: the role of decorin. *Cell Adh Migr* 2016;10(1-2):111–25.
- [13] Santra M, Santra S, Zhang J, Chopp M. Ectopic decorin expression up-regulates VEGF expression in mouse cerebral endothelial cells via activation of the transcription factors Sp1, HIF1alpha, and Stat3. *J Neurochem* 2008;105(2):324–37.
- [14] Adam M, Saller S, Ströbl S, Hennebold JD, Dissen GA, Ojeda SR, et al. Decorin is a part of the ovarian extracellular matrix in primates and may act as a signaling molecule. *Hum Reprod* 2012;27(11):3249–58.
- [15] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227(5259):680–5.
- [16] Goto S, Ozaki Y, Suzumori N, Yasukochi A, Kawakubo T, Furuno T, et al. Role of cathepsin E in decidual macrophage of patients with recurrent miscarriage. *Mol Hum Reprod* 2014;20(5):454–62.
- [17] Obayashi Y, Ozaki Y, Goto S, Obayashi S, Suzumori N, Ohyama F, et al. Role of indoleamine 2,3-Dioxygenase and tryptophan 2,3-Dioxygenase in patients with recurrent miscarriage. *Am J Reprod Immunol* 2016;75(1):69–77.
- [18] Schweigert FJ, Gericke B, Wolfram W, Kaisers U, Dudenhausen JW. Peptide and protein profiles in serum and follicular fluid of women undergoing IVF. *Hum Reprod* 2006;21(11):2960–8.
- [19] McArthur ME, Irving-Rodgers HF, Byers S, Rodgers RJ. Identification and immunolocalization of decorin, versican, perlecan, nidogen, and chondroitin sulfate proteoglycans in bovine small-antral ovarian follicles. *Biol Reprod* 2000;63(3):913–24.