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FSH stimulates the expression of the ADAMTS-16 protease in mature human ovarian follicles

Shuping Gao[1], Christian De Geyter[1,2,3], K.Kossowska[1] and Hong Zhang[1,2]

[1]Department of Research, Women's Hospital, University of Basel, Switzerland, [2]Department of Obstetrics and Gynecology, University Hospital of Basel, Spitalstrasse 21, CH-4031 Basel, Switzerland

[3]Correspondence address. Tel: +41 61 265 9315; Fax: +41 61 265 9194; E-mail: cdegeyter@uhbs.ch

We report the characterization of full-length human ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs)-16, a novel member of the disintegrin and metalloproteinase with thrombospondin motifs (hence ADAMTS) family. *ADAMTS-16* is highly expressed both in the kidney and in the ovary, where it is predominantly expressed in the parietal granulosa cells of pre-ovulatory follicles but only slightly expressed in cells of the cumulus oophorus. In fully differentiated luteinizing granulosa cells, follicle-stimulating hormone and forskolin induces expression of *ADAMTS-16*, suggesting that it is regulated via the cAMP pathway. Luteinizing hormone had a minor effect on the expression of *ADAMTS-16*. ADAMTS-16 is capable of cleaving α_2-macroglobulin (MG), a common substrate for proteases, which is present at high concentrations in the follicular fluid of ovarian follicles. These studies provide the first evidence that ADAMTS-16 is an active protease and suggest a physiological role of ADAMTS-16 in ovarian follicles, at least during the pre-ovulatory phase.

Keywords: ADAMTS-16/ovulation/FSH/ovarian follicle/α_2-macroglobulin

Introduction

The ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) are a family of 19 structurally related proteases, which are secreted in various organ systems and which can bind to specific proteins of the extracellular matrix (ECM). Since their first description (Kuno *et al.*, 1997) studies on ADAMTS have recently been rapidly expanding, because they have been implicated in a number of diseases including cancer (Coussens *et al.*, 2002). In addition to their involvement in ECM modelling, the ADAMTSs are involved in the functional regulation of a large number of cytokines, hormones, growth factors and proteases. Consequently, various members of the ADAMTS family are involved in the pathogenesis of apparently unrelated diseases such as osteoarthritis (Kevorkian *et al.*, 2004), bleeding disorders (Vazquez *et al.*, 1999), endothelial defects (Zheng *et al.*, 2001) and cancer (e.g. breast cancer, Porter *et al.*, 2004). Additionally, various members of the ADAMTS family play important roles in normal ovarian physiology. It has been demonstrated that *ADAMTS-1* mRNA and protein were induced in granulosa cells of periovulatory follicles by luteinizing hormone (LH) in wild type but not in progesterone receptor knockout mice (PRKO), indicating that *ADAMTS-1* is a transcriptional target of the progesterone receptor around the time of ovulation (Robker *et al.*, 2000; Doyle *et al.*, 2004). Studies in ADAMTS-1 (-/-) knockout mice demonstrated that the number of ovulated oocytes is significantly reduced as compared with heterozygous controls (Shindo *et al.*, 2000; Shozu *et al.*, 2005). The presence of ovulations, albeit significantly reduced, in *ADAMTS-1* (-/-) knockout mice and the involvement of other members of the ADAMTS family of proteases in the ovulatory process demonstrates a large degree of redundancy among the various members of this family (Richards *et al.*, 2005). Two proteases structurally and functionally closely related to ADAMTS-1, ADAMTS-4 and ADAMTS-5 are present in granulosa cells of small ovarian follicles, indicating that various members of the ADAMTS family also have functions beyond ovulation and the formation of the luteal body (Richards *et al.*, 2005).

Searching for novel markers of granulosa cell function, we identified *ADAMTS-16* during the analysis of our ovary-specific gene expression database based on its high expression level in the ovary. In this report, novel data concerning both the regulation and the function of *ADAMTS-16* in the ovary are presented.

Materials and Methods

Cloning of human ADAMTS-16 variants and chromosomal location of ADAMTS-16

OR0021 was identified based on its high level of expression in the ovary in our ovary- enriched gene expression database and OR0021 was found to be identical to *ADAMTS-16* (Cal *et al.*, 2002). Searching the GenBank database of human expressed sequence tags (ESTs) with the *ADAMTS-16* cDNA sequence, and another EST sequence (GenBank accession no. AK122980) was detected and sequenced; its gene was named *ADAMTS-16s.* The search of the GenBank database for STSs using *ADAMTS-16* as a template revealed that a human STS WI-6822 (accession number G06277) matches 100% to the nucleotides +941 to +1229 of the *ADAMTS-16* sequence. *ADAMTS-16* was found to be located on chromosome 5 by using the NCBI Unigen programme. The GenBank accession number for *ADAMTS-16*s is DQ266047.

Dragon Gene Start Finder was used to predict the transcriptional start site of *ADAMTS-16* upstream of the translational initiation codon (ATG). The programme PromotorInspector was used to analyse the genomic sequences at the 5′ end of the *ADAMTS-16* gene for putative promotor regions.

Tissue expression pattern analysis by RT–PCR

In order to determine the expression patterns of *ADAMTS-16* mRNA in different human tissues, RT–PCR was performed, using the primers 5′- AACT CAGCCTGCACGATTCAC -3′ and 5′- CAGTGCCCATTCAGGTAGTAC -3′ on a panel of first-strand cDNAs from various human tissues (Origene, MD, USA). The PCR conditions were as described in the manufacturer's protocol with 30 cycles performed on the β-actin control employing *Taq* polymerase.

Microarray data of mouse testis, generated by the GNF Mouse Atlas v2 project (Su *et al.*, 2004), were obtained from the hgFixed database of the UCSC Genome Browser (Karolchik *et al.*, 2003).

Hormonal treatment of ovarian cells in vitro

Human luteinizing granulosa cells were obtained by follicular aspiration from women undergoing oocyte retrieval for assisted reproduction. The study was approved by the local research ethics committee. The age range of the women was 24–41 years. For each experimental cell culture, the granulosa cells obtained from one to three patients collected during the same day were pooled, enzymatically dispersed with 0.1% hyaluronidase (Sigma Chemical Co., St Louis, MO, USA) and separated from red blood cells by centrifugation through Ficoll-Paque (Pharmacia Biotech, Sweden). The cells were plated at a density of $2–5 \times 10^5$ cells/well on 35 mm six-well dishes and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal calf serum (FCS; Gibco), 2 mmol/l L-glutamine and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) at 37ºC in a 95% air–5% CO_2 humidified environment. Cell culture media were changed every other day. On day 7, the cells were stimulated for 24 h with forskolin (10 μM) or with recombinant human follicle-stimulating hormone (rhFSH, 500 ng/ml, Gonal F, Serono, Zug, Switzerland) or with rhLH (100 IU/l, Luveris, Serono). Each experiment was repeated three times.

Fluorescence activated cell sorter and sorting of granulosa cells

Follicular fluid aspirates containing luteinizing granulosa cells were collected from infertile patients treated with exogenous gonadotrophins for assisted reproduction. The freshly collected clumps of granulosa cells were mechanically dissociated by nylon filters and stored frozen at −80ºC. After thawing, the isolated granulosa cells were identified by the simultaneous presence of two specific markers, FSH receptor (FSHR) and LH receptor (LHR), on their surface and subsequently sorted using a fluorescence activated cell sorter (FACS). In order to identify and sort granulosa cells, a dual labelling technique was introduced, where granulosa cells were identified as CD3-negative cells, distinguishing them from leukocytes, which are CD3-positive (De Neubourg *et al.*, 1998). The first polyclonal goat antibody rose against a peptide mapping either the FSHR or LHR of human origin (Santa-Cruz Biotechnology). The first antibody was added for 30 min and kept on ice in the dark. The second antibody was a donkey anti-goat IgG labelled with fluorescence isothiocyanate (FITC) (Santa-Cruz Biotechnology), incubated for 30 min on ice in the dark. In addition, granulosa cells without the primary antibody were used as a negative control. CD3 anti-leukocyte antibodies were used as a positive control (anti-CD3-APC monoclonal mouse antibodies—Becton Dickinson). The gating for the sorting of FSHR-positive cells was established by performing FACS with isotype control. Sorted cells were cultured in DMEM supplemented with either recombinant FSH (100 ng/ml or 3×10^{-4} IU/ml, Gonal F; Serono) or recombinant LH (100 ng/ml, Luveris, Serono) and with 1 : 50 dilution of 200 μg/ml of anti-FSHR (sc-7798, Santa Cruz Biotechnology) or anti-LHR antibody (sc-25828, Santa Cruz Biotechnology), respectively. As a negative control, granulosa cells were cultured in the presence of both anti-FSHR and anti-LHR antibodies (as above).

Quantitative real-time PCR

Total RNA was extracted from GCs using a commercially available RNeasy Total RNA kit from Qiagen (Hilden, Germany). The quantity of RNA was assessed by measuring the optical density at A260 nm. Total RNA (1 μg) from GCs was reversed transcribed into single strand cDNA using the cDNA synthesis kit (Boehringer Mannheim, Mannheim, Germany). Primers for real-time PCR were synthesized by Microsynth, Balgath, Switzerland: hA16 (forward) 5′ AAACTCCTCAGTGGCCCTTTC 3′ and R (reversed) 5′ CGTAT CATGCCTGACAAGCCT 3′. Power SYBR Green PCR Master Mix (AB Applied Biosystems) for real-time PCR and TaqMan GAPDH Control Reagent (PE Applied Biosystems) as internal control were used. cDNA was subjected to 40 cycles of real-time PCR amplification carried out using ABI PRISM 7000 Sequence Detector System (AB Applied Biosystems). The difference between the results was assessed statistically with the Mann–Whitney *U*-test.

Plasmid constructs

Pblue-script-k-Kiaa2029 (*ADAMTS-16*) was kindly provided by Kazusa DNA Research Institute (Kikuno *et al.*, 2004). Full-length ADAMTS-16 was cloned in-frame into pGEX-4T-2 Vector with Sal I /Not I sites. *ADAMTS-16s* was generated by PCR and cloned using the primers 5′-CGTGGATCCCATTTCTG-TGGAAGACGC-3′ and 5′-CGGGAATTCG CCACTTGCACT GTG TGT TT-3′. The PCR-product was digested with BamHI and EcoRI and cloned in-frame into the EcoRI/BamH I sites of pGEX-4T-1 or pcDNA3-HisC (Invitrogen, Groningen, The Netherlands). All constructs were confirmed by sequencing.

Purification of ADAMTS-16 GST-fusion and His-tagged proteins

In brief, pGEX-4T-2_ADAMTS-16 and pGEX-4T-1_ADAMTS-16 pep were transformed to DE3 bacteria (BL20). The positive clones were cultured in 250 ml LB (100 mg/ml ampicillin) for 3–4 h at 30ºC in a shaker until OD.600 nm reached 0.6–0.8. Then, IPTG was added to a final concentration of 0.1 mM (final concentration: 350 ng/ml), and the bacteria were cultured for an additional 4 h. Cells were collected by centrifugation and the pellets were stored at −70ºC. Subsequently, the pelleted cells were lysed in 3 ml cold lysis buffer (50 mM Na_2HPO_4, pH 8.0; 0.3 M NaCl; 1 mM PMSF with protease inhibitors) containing freshly added lysozyme (100 μg/ml) and sonicated 5 × 20 s until the sample cleared and was no longer viscous. Upon centrifugation, samples were boiled at 95ºC for 5 min and loaded on 10% SDS–PAGE. His-tagged proteins were produced by transfection of pcDNA3-Hisc-ADAMTS-16 in HEK293 cells. Protein concentration was determined by BioRad mini kit.

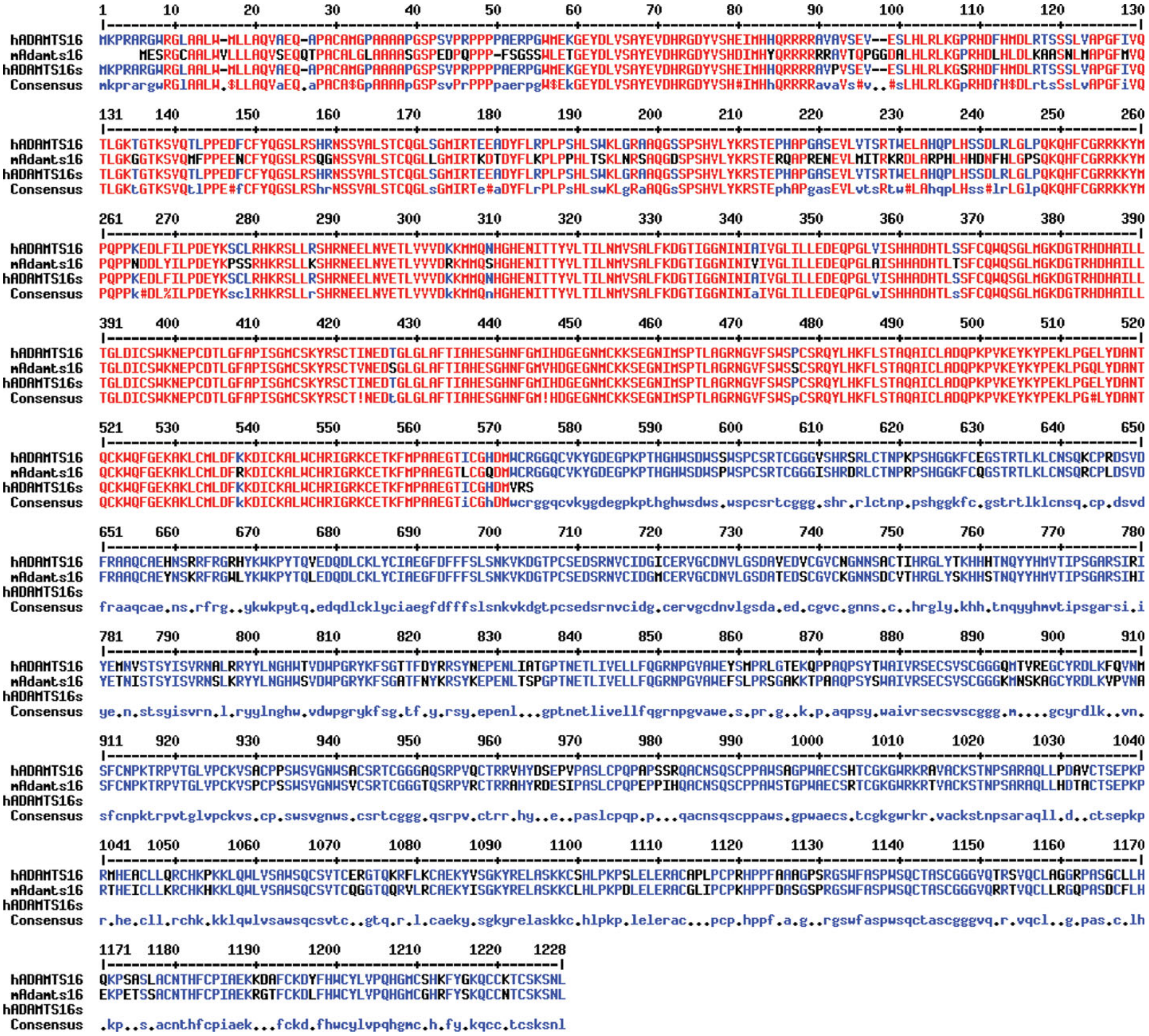
Cleavage of α_2-macroglobulin

About 45 μl of buffer (100 mM Tris–HCl, 20 mM $CaCl_2$ and 200 mM NaCl, pH 7.5) were added to 45 μl 50% GST beads (containing 5 mM Tris, 10 mM NaCl, pH 7.5, GE) and/or His Bead (Roche). ADAMTS-16 and ADAMTS-16s at a concentration of 20 ng/μl were incubated with 5 μl of 10 mM $ZnCl_2$ and 5 μl of 100 ng α_2-macroglobulin (MG) at 37ºC for 2 h in 1.5 ml Eppendorf tubes. Subsequently, the products were analysed for total protein by SDS–PAGE on 8–10% gels followed by staining with Coomassie Brilliant Blue R-250 to analyse for cleavage of MG.

Results

Molecular cloning of human ADAMTS-16 and its variant ADAMTS-16s

During the search of our ovarian-rich gene expression database, OR0021 was identified based on its high expression levels in the ovary (see below). This gene has been designated as *ADAMTS-16*, a member of the family of proteases, jointly denoted as ADAMTS. By screening the EST database, followed by PCR, we identified the full-length cDNA (Fig. 1), including the 3′ sequence, which was missing in an earlier report (Cal *et al.*, 2002). In addition, we identified a novel splicing variant (ABB70405) and termed it *ADAMTS-16*s for the short splicing form of the *ADAMTS-16* transcript (Fig. 1). Sequence analysis revealed that the full-length *ADAMTS-16* exhibits the typical ADAMTS modular structure (containing signal sequence, propeptide, metalloproteinase domain, disintegrin-like domain, central TS1 motif, cysteine-rich region and a C-terminal module with several TS1 submotifs) and that ADAMTS-16s encoded an open reading frame of 570 amino acids, containing only the peptidase domain (Fig. 1). Sequencing of AK031314 from NCBI provided an ORF encoding the full-length mouse *ADAMTS-16*. The human and



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           1       10        20        30        40        50        60        70        80        90        100       110       120       130
hADAMTS16  MKPRARGWRGLAALW-MLLAQVAEQ-APACAMGPAAAAPGSPSVPRPPPPAERPGWMEKGEYDLVSAYEVDHRGDYVSHEIMHHQRRRRAVAVSEV--ESLHLRLKGPRHDFHMDLRTSSSLVAPGFIVQ
mAdamts16         MESRGCAALWVLLLAQVSEQQTPACALGLAAAASGSPEDPQPPP-FSGSSWLETGEYDLVSAYEVDHRGDYVSHDIMHYQRRRRRAVTQPGGDALHLRLKGPRHDLHLDLKAASNLMAPGFMVQ
hADAMTS16s MKPRARGWRGLAALW-MLLAQVAEQ-APACAMGPAAAAPGSPSVPRPPPPAERPGWMEKGEYDLVSAYEVDHRGDYVSHEIMHHQRRRRAVPVSEV--ESLHLRLKGSRHDFHMDLRTSSSLVAPGFIVQ
Consensus  mkprargwRGlAALW.$LLAQVaEQ.aPACA$GpAAAApGSPsvPrPPPpaerpgW$EkGEYDLVSAYEVDHRGDYVSH#IMHhQRRRRavaVs#v..#sLHLRLKGpRHDfH$DLrtsSsLvAPGFiVQ

           131     140       150       160       170       180       190       200       210       220       230       240       250       260
hADAMTS16  TLGKTGTKSVQTLPPEDFCFYQGSLRSHRNSSVALSTCQGLSGMIRTEEADYFLRPLPSHLSWKLGRAAQGSSPSHVLYKRSTEPHAPGASEVLVTSRTWELAHQPLHSSDLRLGLPQKQHFCGRRKKYM
mAdamts16  TLGKGGTKSVQMFPPEENCFYQGSLRSQGNSSVALSTCQGLLGMIRTKDTDYFLKPLPPHLTSKLNRSAQGDSPSHVLYKRSTERQAPRENEVLMITRKRDLARPHLHHDNFHLGPSQKQHFCGRRKKYM
hADAMTS16s TLGKTGTKSVQTLPPEDFCFYQGSLRSHRNSSVALSTCQGLSGMIRTEEADYFLRPLPSHLSWKLGRAAQGSSPSHVLYKRSTEPHAPGASEVLVTSRTWELAHQPLHSSDLRLGLPQKQHFCGRRKKYM
Consensus  TLGKtGTKSVQtlPPE#fCFYQGSLRShrNSSVALSTCQGLsGMIRTe#aDYFLrPLPsHLswKLgRaAQGsSPSHVLYKRSTEphAPgasEVLvtsRtw#LAhqpLHss#lrLGlpQKQHFCGRRKKYM

           261     270       280       290       300       310       320       330       340       350       360       370       380       390
hADAMTS16  PQPPKEDLFILPDEYKSCLRHKRSLLRSHRNEELNVETLVVVDKKMMQNHGHENITTYVLTILNMVSALFKDGTIGGNINIAIVGLILLEDEQPGLVISHHADHTLSSFCQWQSGLMGKDGTRHDHAILL
mAdamts16  PQPPNDDLYILPDEYKPSSRHKRSLLKSHRNEELNVETLVVVDRKMMQSHGHENITTYVLTILNMVSALFKDGTIGGNINIVIVGLILLEDEQPGLAISHHADHTLTSFCQWQSGLMGKDGTRHDHAILL
hADAMTS16s PQPPKEDLFILPDEYKSCLRHKRSLLRSHRNEELNVETLVVVDKKMMQNHGHENITTYVLTILNMVSALFKDGTIGGNINIAIVGLILLEDEQPGLVISHHADHTLSSFCQWQSGLMGKDGTRHDHAILL
Consensus  PQPPk#DL%ILPDEYKsclRHKRSLLrSHRNEELNVETLVVVDkKMMQnHGHENITTYVLTILNMVSALFKDGTIGGNINIaIVGLILLEDEQPGLvISHHADHTLsSFCQWQSGLMGKDGTRHDHAILL

           391     400       410       420       430       440       450       460       470       480       490       500       510       520
hADAMTS16  TGLDICSWKNEPCDTLGFAPISGMCSKYRSCTINEDTGLGLAFTIAHESGHNFGMIHDGEGNMCKKSEGNIMSPTLAGRNGVFSWSPCSRQYLHKFLSTAQAICLADQPKPVKEYKYPEKLPGELYDANT
mAdamts16  TGLDICSWKNEPCDTLGFAPISGMCSKYRSCTVNEDSGLGLAFTIAHESGHNFGMVHDGEGNMCKKSEGNIMSPTLAGRNGVFSWSSCSRQYLHKFLSTAQAICLADQPKPVKEYKYPEKLPGQLYDANT
hADAMTS16s TGLDICSWKNEPCDTLGFAPISGMCSKYRSCTINEDTGLGLAFTIAHESGHNFGMIHDGEGNMCKKSEGNIMSPTLAGRNGVFSWSPCSRQYLHKFLSTAQAICLADQPKPVKEYKYPEKLPGELYDANT
Consensus  TGLDICSWKNEPCDTLGFAPISGMCSKYRSCT!NEDtGLGLAFTIAHESGHNFGM!HDGEGNMCKKSEGNIMSPTLAGRNGVFSWSpCSRQYLHKFLSTAQAICLADQPKPVKEYKYPEKLPG#LYDANT

           521     530       540       550       560       570       580       590       600       610       620       630       640       650
hADAMTS16  QCKWQFGEKAKLCMLDFKKDICKALWCHRIGRKCETKFMPAAEGTICGHDMWCRGGQCVKYGDEGPKPTHGHWSDWSSWSPCSRTCGGGVSHRSRLCTNPKPSHGGKFCEGSTRTLKLCNSQKCPRDSVD
mAdamts16  QCKWQFGEKAKLCMLDFRKDICKALWCHRIGRKCETKFMPAAEGTLCGQDMWCRGGQCVKYGDEGPKPTHGHWSDWSPWSPCSRTCGGGISHRDRLCTNPRPSHGGKFCQGSTRTLKLCNSQRCPLDSVD
hADAMTS16s QCKWQFGEKAKLCMLDFKKDICKALWCHRIGRKCETKFMPAAEGTICGHDMVRS
Consensus  QCKWQFGEKAKLCMLDFkKDICKALWCHRIGRKCETKFMPAAEGTiCGhDMwcrggqcvkygdegpkpthghwsdws.wspcsrtcggg.shr.rlctnp.pshggkfc.gstrtlklcnsq.cp.dsvd

           651     660       670       680       690       700       710       720       730       740       750       760       770       780
hADAMTS16  FRAAQCAEHNSRRFRGRHYKWKPYTQVEDQDLCKLYCIAEGFDFFFSLSNKVKDGTPCSEDSRNVCIDGICERVGCDNVLGSDAVEDVCGVCNGNNSACTIHRGLYTKHHHTNQYYHMVTIPSGARSIRI
mAdamts16  FRAAQCAEYNSKRFRGWLYKWKPYTQLEDQDLCKLYCIAEGFDFFFSLSNKVKDGTPCSEDSRNVCIDGMCERVGCDNVLGSDATEDSCGVCKGNNSDCVTHRGLYSKHHSTNQYYHMVTIPSGARSIHI
hADAMTS16s
Consensus  fraaqcae.ns.rfrg..ykwkpytq.edqdlcklyciaegfdfffslsnkvkdgtpcsedsrnvcidg.cervgcdnvlgsda.ed.cgvc.gnns.c..hrgly.khh.tnqyyhmvtipsgarsi.i

           781     790       800       810       820       830       840       850       860       870       880       890       900       910
hADAMTS16  YEMNVSTSYISVRNALRRYYLNGHWTVDWPGRYKFSGTTFDYRRSYNEPENLIATGPTNETLIVELLFQGRNPGVAWEYSMPRLGTEKQPPAQPSYTWAIVRSECSVSCGGGQMTVREGCYRDLKFQVNM
mAdamts16  YETNISTSYISVRNSLKRYYLNGHWSVDWPGRYKFSGATFNYKRSYKEPENLTSPGPTNETLIVELLFQGRNPGVAWEFSLPRSGAKKTPAAQPSYSWAIVRSECSVSCGGGKMNSKAGCYRDLKVPVNA
hADAMTS16s
Consensus  ye.n.stsyisvrn.l.ryylnghw.vdwpgrykfsg.tf.y.rsy.epenl...gptnetlivellfqgrnpgvawe.s.pr.g..k.p.aqpsy.waivrsecsvscggg.m....gcyrdlk..vn.

           911     920       930       940       950       960       970       980       990       1000      1010      1020      1030      1040
hADAMTS16  SFCNPKTRPVTGLVPCKVSACPPSWSVGNWSACSRTCGGGAQSRPVQCTRRVHYDSEPVPASLCPQPAPSSRQACNSQSCPPAWSAGPWAECSHTCGKGWRKRAVACKSTNPSARAQLLPDAVCTSEPKP
mAdamts16  SFCNPKTRPVTGLVPCKVSPCPSSWSVGNWSVCSRTCGGGTQSRPVRCTRRAHYRDESIPASLCPQPEPPIHQACNSQSCPPAWSTGPWAECSRTCGKGWRKRTVACKSTNPSARAQLLHDTACTSEPKP
hADAMTS16s
Consensus  sfcnpktrpvtglvpckvs.cp.swsvgnws.csrtcggg.qsrpv.ctrr.hy..e..paslcpqp.p...qacnsqscppaws.gpwaecs.tcgkgwrkr.vackstnpsaraqll.d..ctsepkp

           1041 1050     1060      1070      1080      1090      1100      1110      1120      1130      1140      1150      1160      1170
hADAMTS16  RMHEACLLQRCHKPKKLQWLVSAWSQCSVTCERGTQKRFLKCAEKYVSGKYRELASKKCSHLPKPSLELERACAPLPCPRHPPFAAAGPSRGSWFASPWSQCTASCGGGVQTRSVQCLAGGRPASGCLLH
mAdamts16  RTHEICLLKRCHKHKKLQWLVSAWSQCSVTCQGGTQQRVLRCAEKYISGKYRELASKKCLHLPKPDLELERACGLIPCPKHPPFDASGSPRGSWFASPWSQCTASCGGGVQRRTVQCLLRGQPASDCFLH
hADAMTS16s
Consensus  r.he.cll.rchk.kklqwlvsawsqcsvtc..gtq.r.l.caeky.sgkyrelaskkc.hlpkp.lelerac...pcp.hppf.a.g..rgswfaspwsqctascgggvq.r.vqcl..g.pas.c.lh

           1171 1180     1190      1200      1210      1220    1228
hADAMTS16  QKPSASLACNTHFCPIAEKKDAFCKDYFHWCYLVPQHGMCSHKFYGKQCCKTCSKSNL
mAdamts16  EKPETSSACNTHFCPIAEKRGTFCKDLFHWCYLVPQHGMCGHRFYSKQCCNTCSKSNL
hADAMTS16s
Consensus  .kp..s.acnthfcpiaek...fckd.fhwcylvpqhgmc.h.fy.kqcc.tcsksnl
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Figure 1: Isolation of cDNAs encoding the human gene *ADAMTS*-16 and its variant *ADAMTS-16s*
Alignment of the amino acid sequence of *ADAMTS-16* and its splice variant ADAMTS-16s from human and mouse using the multiple alignment programme (Multalin). Letters in red indicate 100% indentity within 3 sequences, letters in blue are those identical between two sequences, letters in black indicate low consensus value

mouse nucleotide and predicted amino acid sequences have an overall identity of 86% and 91%, respectively (Fig. 1).

The full-length cDNA sequence of human *ADAMTS-16* was mapped by a BLAST comparison to the BAC clones CTD-2297D10 (GenBank accession no. AC022424) and CTC-485I21 (AC010269) on human chromosome 5p15. *ADAMTS-16* appears to consist of 23 exons spanning 180 kb of genomic DNA (Table 1). Analysis of the genomic region at the 5′ end of *ADAMTS-16* indicated that it has a CpG island (AC022424, nt 28 391–27 251; %GC = 66.6; O/E = 0.848; number of CpG2 = 112), suggesting that it is expressed in a tissue-specific manner (Fig. 2B). A single transcriptional start site was predicted at the upstream position of 560 bp relative to the translational codon (ATG). The programme PromotorInspector was used to analyse the genomic sequences at the 5′ end of the *ADAMTS-16* gene for putative promotor regions. The presence of one strong promotor region of *ADAMTS-16* was predicted at nt 27 728–27 478 of AC0022424. Two sp1 sites and one egr-1 site are present in the promotor (Fig. 2C), suggesting that they mediate the expression of *ADAMTS-16* during development.

Expression of ADAMTS-16 mRNA in the ovary

To determine the tissue-specific expression of *ADAMTS-16* in the human, semi-quantitative RT–PCR analysis (semi-Q-RT–PCR) was performed by using a pair of primers corresponding to sequences flanking the first intron. As shown in Fig. 2A, *ADAMTS-16* was expressed mainly in adult kidney, pancreas and ovary. We subsequently analysed the expression of the *ADAMTS-16* transcript in the UniGene (Build #184). Expression of *ADAMTS-16* was also present in ESTs from brain medulloblastoma, brain amygdala, lung focal fibrosis, lung large cell carcinoma, endometrial adenocarcinoma and retinoblastoma (Wheeler *et al.*, 2003). These results obtained from

Table 1: Human ADAMTS-16 gene structure

Exon number	Exon size (bp)	Position in cDNA	Intron size (bp)	Splice acceptor	Splice donor	Intron phase	Ensembl exon report number
1		−72	124	5′ UTR	AGCAG**gt**gag	0	ENSE00001323050
2	103	73–175	5.363	cgc**ag**GCACC	GGGCG**gt**aag	1	ENSE00001228221
3	326	176–501	35.588	ttc**ag**AATAT	GCTTG**gt**gag	0	ENSE00001228213
4	262	502–763	3.746	tcc**ag**TCAGG	GAAAT**gt**atg	0	ENSE00001228207
5	200	764–963	1.473	cat**ag**ACATG	ACATG**gt**agg	0	ENSE00000996102
6	84	964–1047	2.162	ttc**ag** GTATC	AACAG**gt**agt	0	ENSE00000970889
7	160	1048–1207	1.554	cac**ag**CCAGG	TTTGG**gt**gag	1	ENSE00000970890
8	106	1208–1313	8.341	cac**ag**GATTT	CACAA**gt**aag	2	ENSE00000970891
9	138	1314–1451	8.823	cat**ag**CTTTG	CTAAG**gt**agg	2	ENSE00000970892
10	154	1452–1605	13.542	ttc**ag**CACCG	AAAAG**gc**aag	0	ENSE00000996101
11	96	1606–1701	9.483	ttt**ag**GACAT	ACATG**gt**aag	0	ENSE00001228162
12	149	1702–1850	2.497	ttt**ag**TGGTG	CCCAA**gt**aag	2	ENSE00001228154
13	173	1851–2023	1.782	tcc**ag**GCCAT	AGAAG**gt**aaa	1	ENSE00001228147
14	131	2024–2154	2.051	tta**ag**ATCAG	GTGAG**gt**aat	0	ENSE00001228138
15	124	2155–2278	406	tcc**ag**AGAGT	CAACC**gt**gag	1	ENSE00001228133
16	245	2279–2523	2.127	tct**ag**AGTAT	TGGAG**gt**aaa	0	ENSE00001228127
17	139	2524–2662	20.465	tgt**ag**CTGCT	AGGGG**gt**agg	1	ENSE00001228120
18	127	2663–2789	40.484	tgc**ag**GACAG	CCCAG**gt**aag	2	ENSE00001228114
19	202	2790–2991	102	ccc**ag**CTGGT	CAGAG**gt**aac	0	ENSE00001318371
20	195	2992–3186	2.737	tgc**ag**TGCTC	CCCAG**gt**agg	0	ENSE00001318062
21	225	3187–3411	11.405	ttt**ag**TGCTC	CTCAG**gt**agg	0	ENSE00001312646
22	148	3412–3559	741	cac**ag**TGCAC	GAAAG**gt**gag	1	ENSE00001294092
23	116	3560–3676		ttc**ag**ATGCC	3′ UTR		ENSE00001312496

RT–PCR analyses were also supported by microarray expression analyses collected from GNF (Genomics Institute of the Novartis Research Foundation) Expression Atlas Chips U133A, GNF1H and Affy U95 (data not shown, http://symatlas.gnf.org/SymAtlas), confirming that *ADAMTS-16* is highly expressed in the ovary, but that it is also expressed to some extent in other tissues.

Hormonal regulation of ADAMTS-16 expression

We subsequently examined the effects of both FSH and LH on the induction of *ADAMTS-16* mRNA expression in both luteinizing granulosa cells and cumulus cells *in vitro*, collected from patients treated with exogenous gonadotrophins for assisted reproduction. As shown in Fig. 3A, *ADAMTS-16* mRNA was predominantly detected in luteinizing granulosa cells with little expression in cumulus cells. A weak but distinct expression pattern was also found in cells of an immortalized granulosa cell line developed from a granulosa cell tumour (KGN, Nishi *et al.*, 2001). By semi-quantitative RT–PCR analysis, the increase in the *ADAMTS-16* expression level was detected in the presence of FSH and forskolin (Fig. 3B, lane 3–5), whereas LH (at a concentration of 100 IU/l) induced minor changes in the expression level of *ADAMTS-16* as compared with FSH (Fig. 3B, lane 1).

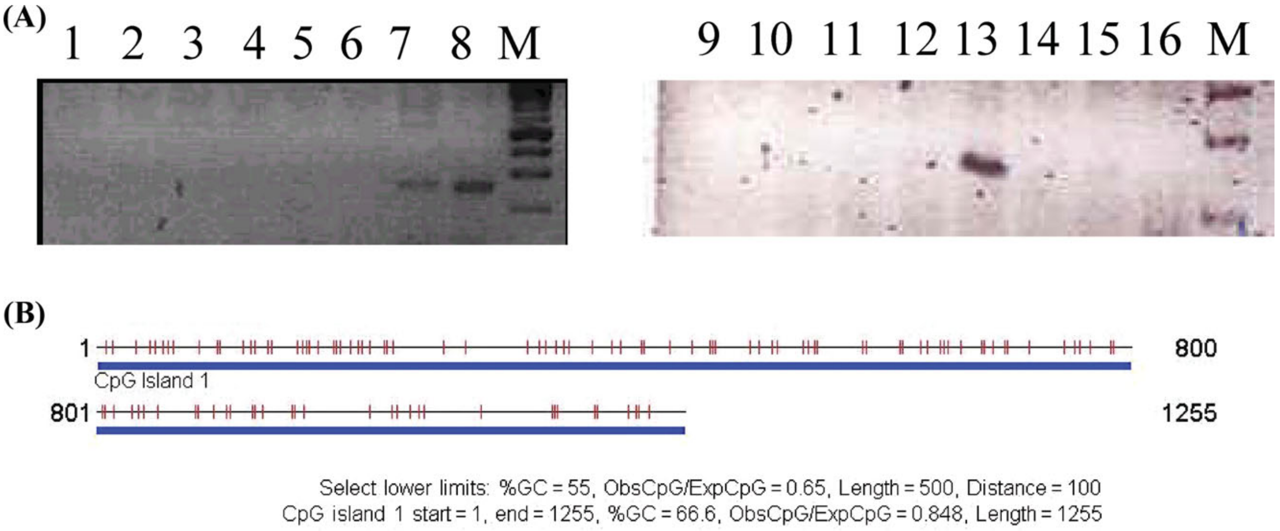
In order to further dissect the effects of FSH and LH on the expression of ADAMTS-16 in granulosa, luteinizing granulosa cells were collected from infertile patients treated with exogenous gonadotrophins for assisted reproduction and sorted with FACS based on the concomitant presence of both the FSHR and the LHR. These sorted granulosa cells were then cultured in the presence of recombinant FSH together with anti-LHR-antibodies or in the presence of recombinant LH together with anti-FSHR-antibodies, respectively. As shown in Fig. 3C, under these conditions, the expression level of *ADAMTS-16* in granulosa cells cultured in the presence of recombinant FSH and anti-LHR antibody was significantly higher as compared with that of granulosa cells cultured in the presence of recombinant LH and anti-FSHR-antibodies ($P = 0.02$, Mann–Whitney *U*-test).

MG is a substrate of ADAMTS-16

Several sequence features of the human ADAMTS-16 are very similar to those of other members of the ADAMTSs family of proteases including the number of cysteine residues (Porter *et al.*, 2005). Thus, this 'trapping' mechanism induces a delayed migration of MG in SDS–PAGE upon incubation with a protease and is considered an experimental evidence of its proteolytic activity, which is different from the cleavage pattern induced by trypsin (Kuno *et al.*, 1999). As shown in Fig. 4A and B, in the presence of ADAMTS-16 produced either by *Escherichia coli* (Fig. 4A, lane 1 and 2) or by HEK293 (Fig. 4B), migration of MG on SDS–polyacrylamide gel was altered. Conversion of intact MG to the 90 kDa species was also observed, indicating the formation of MG-ADAMTS-16 complexes, together with proteolysis of MG by ADAMTS-16. MG-ADAMTS-16 complexes were mainly detected in HEK293 cells (Fig. 4B, lane 1). In contrast, high levels of MG could not form high order complexes in the absence of ADAMTS-16 (Fig. 4B, lane 2). In the control experiments, trypsin attack sites were different to those affected by ADAMTS-16 (Fig. 4C). These studies demonstrate that ADAMTS-16 was able to cleave MG.

Discussion

Various members of the ADAMTS protease family have been implicated in the process of ovulation (Espey *et al.*, 2000; Robker *et al.*, 2000; Madan *et al.*, 2003; Doyle *et al.*, 2004; Mittaz *et al.*, 2004; Shozu *et al.*, 2005; Richards *et al.*, 2005). The liberation of an oocyte from its mature follicle during ovulation is the climax of complex remodelling processes occurring both in the follicular wall and in the cumulus–oocyte complex. This involves remodelling of various components of the ECM through the action of a multitude of proteases. The efficacy of each of these processes results from the equilibrium between the specific components of the ECM and each of the proteases. Both the amount of various components of the ECM and the expression of specific proteases seem to be hormonally regulated during the entire process of follicular development and



(C)

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ggaccctccggtggcccctagcccctcggagcgctcctggATGAAGCCCCGCGCGCGCGG
ATGGCGGGGCTTGGCGGCGCTGTGGATGCTGTTGGCGCAGGTGGCCGAGCAG
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Figure 2: Both the expression level and the distribution of *ADAMTS-16* mRNA species vary in different tissues
(**A**) RT–PCR was performed on a panel of first-strand cDNAs prepared from different human tissues: (1) brain, (2) heart, (3) placenta, (4) lung, (5) liver, (6) smooth muscle, (7) kidney, (8) panceas, (9) spleen, (10) thymus, (11) prostate, (12) testis, (13) ovary, (14) small intestine, (15) colon and (16) peripheral blood leukocytes. (**B**) Analysis of the genomic region at the 5′ end of *ADAMTS-16* using CpGplot. (**C**) The programme PromoterInspector was used to analyse the genomic sequences at the 5′ end of the *ADAMTS-16* gene for putative promotor regions

ovulation. Due to the dependency of the expression and secretion of each component on a particular endocrine signal, which may be conveyed to the tissue both locally and during particular time intervals, the whole process becomes tightly regulated. The stringent control of these processes and the specificity of the various substances involved carries the risk of making ovulation—and with this also the ultimate success of reproduction—crucially dependent on the exact functioning of each individual process. Therefore, the involvement of a large number of substrates and proteases adds an aspect of redundancy to this system (Richards *et al.*, 2005), permitting the ongoing of successful ovulations even in the absence of one of the players (e.g. ADAMTS-1, Mittaz *et al.*, 2004; Shozu *et al.*, 2005).

One of the components of the ECM of the cumulus oophorus, versican, is induced in the granulosa cells under the influence of LH (Russell *et al.*, 2003a) and is a substrate for various members of the ADAMTS protease family, mainly ADAMTS-1 (Russell *et al.*, 2003b). In the PRKO mouse, which fails to ovulate in response to LH, the expression of *ADAMTS-1* is significantly reduced (Robker *et al.*, 2000), suggesting an important role of the ADAMTS protease family in the downstream pathway leading to ovulation.

ADAMTS-16 is another member of this family and has not yet been studied in detail. Phylogenetically, it is most related to ADAMTS-18 (Cal *et al.*, 2002) and has been implicated in the pathogenesis of osteoarthritis (Kevorkian *et al.*, 2004). We identified high-expression levels of *ADAMTS-16* in the ovary during our search of an ovary-specific gene expression database. The *ADAMTS-16* gene was shown to consist of 23 exons spanning 180 kb of genomic DNA and is located on the human chromosome 5p15. *ADAMTS-16* was expressed only in the parietal granulosa cells of pre-ovulatory follicles, not in the cumulus oophorus, both previously collected from patients treated with exogenous gonadotrophins for assisted reproduction. The increase in *ADAMTS-16* expression in luteinizing granulosa cells was only detected in the presence of FSH and forskolin. The change in the expression levels of *ADAMTS-16* in sorted granulosa cells provoked by recombinant LH was significantly lower than those induced by recombinant FSH ($P = 0.02$, Fig. 3C). LH considerably reduced *ADAMTS-1* expression in human granulosa cells and was modestly repressed after treatment with FSH (Freimann *et al.*, 2005). Most dramatically, forskolin highly suppressed *ADAMTS-1* expression as compared with non-treated controls (Freimann *et al.*, 2005). These results

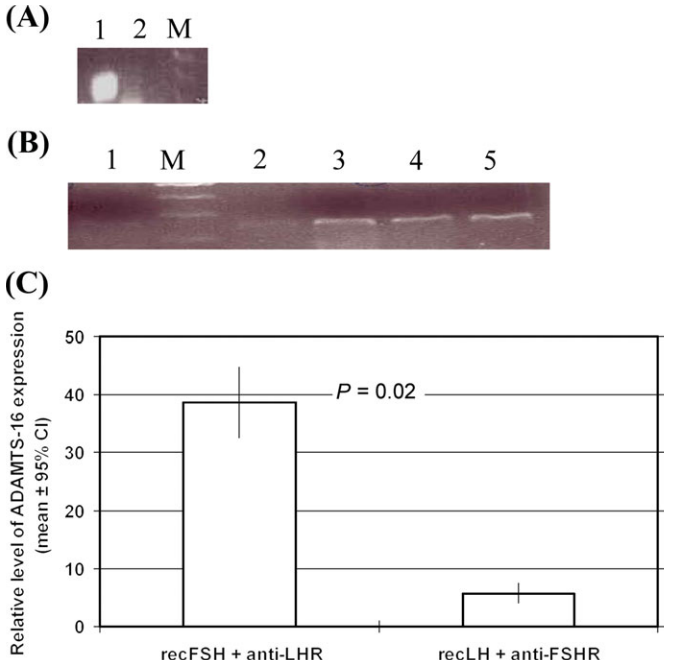

Figure 3: Induction of *ADAMTS-16* expression
Representative PCR analysis of *ADAMTS-16* expression. (**A**) PCR was performed on first-strand cDNAs prepared from luteinizing granulosa cells (lane 1) or cells of the cumulus oophorus (lane 2). Semiquantitive-based PCRs were performed. The PCR products obtained from each reaction following 30 cycles were separated on a 1.2% agarose gel and visualized by serber green I. (**B**) Human granulosa cells were isolated from patients treated with IVF or ICSI. PCR was performed on first-strand cDNAs prepared from the cells, which after 7 days of culture in DMEM were treated with (1) LH, (3) forskolin, (4) FSH and (5) FSH + Forskolin, (2) served as a control. PCRs (30 cycles) of the housekeeping gene β-actin were performed in parallel. The amounts of the β-actin PCR products are equivalent in the different tissues (not shown). (**C**) Expression of ADAMTS16 mRNA prepared from luteinizing granulosa cells sorted with FACS based upon both FSHR and LHR. The expression of GAPDH mRNA was used as an internal control. The relative level of mRNA expression was determined by real-time PCR and indicated as fold changes over GAPDH expression (ADAMTS16/GAPDH ratio). Data are compiled from four experiments and presented by their mean values ± 95% confidence intervals

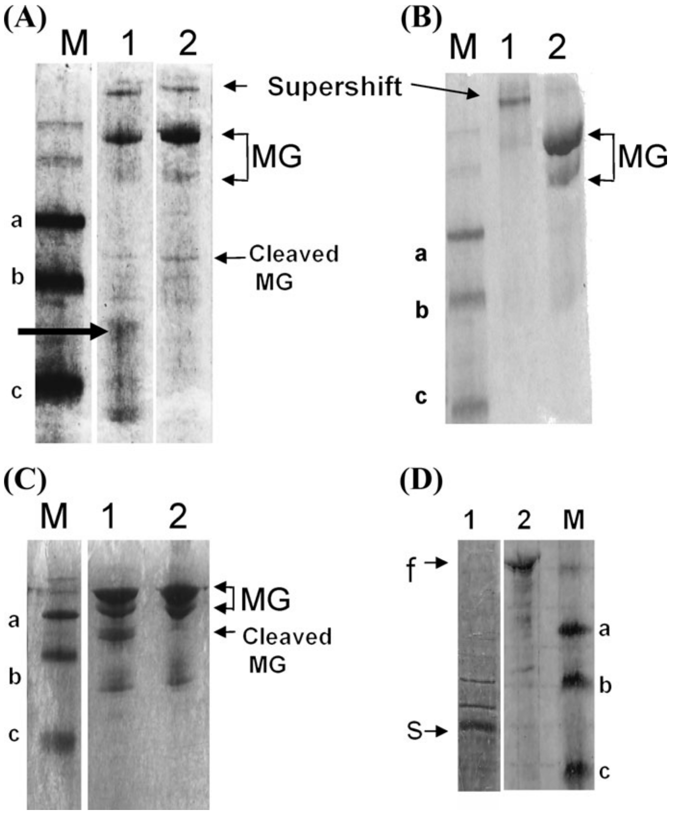

Figure 4: Cleavage of MG by ADAMTS-16
(**A**) Trapping and cleavage of MG by incubation of ADAMTS-16s (1) and ADAMTS-16 (2) together with MG. In all experiments, equivalent amounts of GST-ADAMTS-16 coupled to GSH beads were used. M indicates a protein marker with a (103 kD), b (77 kD) and c (50 kD). (**B**) Trapping of MG by incubating with His-ADAMTS-16 (1). (**C**) Cleavage of MG by trypsin (0.5 μg). (**D**) GST-ADAMTS-16s (1) and GST-ADAMTS-16 (2) produced in *E. Coli*. f indicates full-length GST-ADAMTS-16 and s indicates GST-ADAMTS-16s

suggest a distinct role of ADAMTS-16 in ovarian function as compared with that of other members of the ADAMTS protease family such as ADAMTS-1. Since the present studies were carried out with parietal granulosa cells aspirated from follicles primed with exogenous gonadotrophins and collected during oocyte pick-up for IVF or ICSI, further studies may be needed to address regulation of this protease in the follicles at earlier stages of development.

MG is a highly conserved proteinase inhibitor present at high concentration levels both in human serum and in ovarian follicular fluid (Gaddy-Kurten *et al.*, 1989; Ireland *et al.*, 2004). For the first time, our results clearly demonstrate that ADAMTS-16 and its variant are able to cleave MG, thereby proving unequivocally that ADAMTS-16 is an active protease. Previously, it was demonstrated that MG is a substrate of ADAMTS-4 and ADAMTS-5 as well (Tortorella *et al.*, 2004). Although the entire spectrum of its cognate substrates *in vivo* is still unknown, the interaction between ADAMTS-16 and MG may well be physiologically significant, because both are present in high concentrations in the follicular fluid of mature ovarian follicles. MG has been demonstrated to be involved in the regulation of estradiol production by granulosa cells (Ireland *et al.*, 2004). MG interacts with the vascular endothelial growth factor leading to the inactivation of the latter (Bhattarjargee *et al.*, 2000) and is thought to restrict the development of small capillary blood vessels to the thecal layer until final follicular maturation (Gruemmer *et al.*, 2005). *ADAMTS-16*, which has now been shown to be expressed in the granulosa cell layer of the follicular wall under the influence of FSH, may thus be involved in regulating the local concentration of MG, thereby regulating the development of perifollicular capillaries. These have been demonstrated to be important for follicular development and for the provision of competent oocytes (Van Blerkom *et al.*, 1997). Other members of the ADAMTS family have also been implicated in modulating the formation of intraovarian capillaries, such as ADAMTS-1 (Shozu *et al.*, 2005).

Our studies provide the first evidence that *ADAMTS-16* is expressed in parietal granulosa cells of ovarian follicles under the influence of FSH and that it is an active protease of MG, which is present at high concentrations in follicular fluid. We therefore suggest a physiological role of ADAMTS-16 in ovarian follicles, at least during the pre-ovulatory phase.

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