

DEVELOPMENT AND DISEASE

Impaired cumulus mucification and female sterility in tumor necrosis factor-induced protein-6 deficient mice

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SUMMARY

Mucification of the cumulus layer around the oocyte is an obligatory process for female fertility. Tumor necrosis factor-induced protein-6 (TNFIP6 or TSG6) has been shown to be specifically expressed during this process. We have generated TNFIP6-deficient mice and tested the ability of their cumulus cells to undergo mucification. Cumulus cell-oocyte complexes fail to expand in TNFIP6-deficient female mice because of the inability of the cumulus cells to assemble their hyaluronan-rich extracellular matrix. The impaired cumulus matrix formation is due to the lack of covalent complexes between hyaluronan and the heavy chains of the inter- α -trypsin inhibitor family. As a consequence, TNFIP6-deficient females are sterile. Cultured TNFIP6-deficient cumulus

cell-oocyte complexes also fail to expand when stimulated with dibutyryl cyclic AMP or epidermal growth factor. Recombinant TNFIP6 is able to catalyze the covalent transfer of heavy chains to hyaluronan in a cell-free system, restore the expansion of *Tnfip6*-null cumulus cell-oocyte complexes in vitro, and rescue the fertility in *Tnfip6*-null females. These results provide clear evidence that TNFIP6 is a key catalyst in the formation of the cumulus extracellular matrix and indispensable for female fertility.

Key words: Cumulus cell, Tumor necrosis factor-induced protein-6, *Tnfip6*, *Tsg6*, Inter- α -trypsin inhibitor, Hyaluronan, Extracellular matrix, Infertility

INTRODUCTION

Mammalian ovulation involves a variety of coordinated physiological and biochemical processes that eventually culminate in the expulsion of the cumulus cell-oocyte complex (COC) from the preovulatory follicle (Tsafriri and Reich, 1999). One of the pivotal processes in ovulation is the cumulus mucification, i.e. the synthesis and assembly of a highly viscoelastic extracellular matrix by the cumulus cells (Salustri et al., 1993). Several studies have shown that this extracellular matrix is crucial for female fertility (Chen et al., 1993; Hess et al., 1999; Zhuo et al., 2001). Although integral components of this matrix have been identified, the mechanism of matrix assembly is not completely understood.

Hyaluronan, a negatively charged glycosaminoglycan, is the major component of the COC matrix, and is responsible for the dramatic expansion of the COC after the midcycle luteinizing hormone (LH) surge (Salustri et al., 1992). The heavy chains

(HCs) of inter- α -trypsin inhibitor (α I) and pre- α -trypsin inhibitor (P α I) are also specifically incorporated into this matrix (Chen et al., 1992), and knockout studies have identified a crucial role for these molecules in COC matrix assembly and female fertility (Zhuo et al., 2001; Sato et al., 2001). A pivotal step of the matrix assembly seems to be the covalent transfer of these HCs to hyaluronan through a transesterification process (Chen et al., 1996). It has been suggested that these heavy chains could crosslink hyaluronan through covalent and ionic bonds and stabilize the mucified COC matrix (Chen et al., 1994; Chen et al., 1996).

We and others have recently demonstrated that the gene for tumor necrosis factor-induced protein-6 (*Tnfip6*; also known as tumor necrosis factor-stimulated gene-6, *Tsg6*) is specifically expressed in COCs undergoing mucification (Fulop et al., 1997; Yoshioka et al., 2000; Varani et al., 2002), and that TNFIP6 is incorporated into the COC matrix (Mukhopadhyay et al., 2001; Carrette et al., 2001). The LH surge also induces

Tnfr1 expression in granulosa cells, and the oocyte-derived growth differentiation factor-9 has been implicated in this process (Yoshioka et al., 2000; Varani et al., 2002). Thus, the synthesis of TNFIP6 appears to be under both autocrine and paracrine controls. The translated product of *Tnfr1* is an approx. 37 kDa glycoprotein (Lee et al., 1992) that is able to specifically bind hyaluronan (Kohda et al., 1996; Parkar and Day, 1997) and can form a complex with I α I (Wisniewski et al., 1994; Mukhopadhyay et al., 2001; Nentwich et al., 2002). TNFIP6 is usually expressed under inflammatory conditions (Wisniewski et al., 1993), and its anti-inflammatory and chondroprotective effects in arthritis are well documented (Wisniewski et al., 1996; Bardos et al., 2001; Glant et al., 2002). However, in the expanding COCs, it has been suggested that TNFIP6 plays the role as an extracellular matrix organizer by interacting with both hyaluronan and I α I (Fulop et al., 1997).

In this study we demonstrate that TNFIP6 is the catalyst that covalently transfers the heavy chains of the I α I-related proteins onto hyaluronan. TNFIP6-deficient mice are unable to complete this biochemical reaction, the females fail to assemble their cumulus extracellular matrix and consequently become sterile.

MATERIALS AND METHODS

Generation of *Tnfr1*-null (*Tnfr1*^{-/-}) mice

Tnfr1 is a 20.3 kb-long gene on mouse chromosome 2 (Fulop et al., 1997). A 10.7 kb genomic DNA fragment was isolated from a P1 clone (P-2199; Incyte Genomics) by *Hind*III digestion (Fig. 1A). This fragment contained the entire promoter region, exon 1 and a 5.2 kb fragment of intron 1. The 5.5 kb long 5' arm of the genomic fragment was inserted upstream of the PGK promoter-neo^r-poly(A) cassette of expression vector pPNT (Tybulewicz et al., 1991), into the *Nde*I cleavage site of exon 1, disrupting the reading frame and creating a stop codon 94 bp downstream of the translation start site. The 5.2 kb 3' arm genomic fragment was cloned downstream of the *Neo* cassette. Linearized vector was electroporated into embryonic stem (ES) cells of 129S6/SvEvTac origin (TC-1). Over 150 ES cell clones were identified after positive and negative selection. Homologous recombination was detected in three ES cell clones, which were karyotyped and then clone no. 135 (Fig. 1B) was injected into C57Bl/6 blastocysts. Two chimeric founder males were obtained and mated with wild-type BALB/c females to establish two independent colonies. Since female *Tnfr1*^{-/-} mice were infertile, the colonies were maintained by mating *Tnfr1*^{+/-} or *Tnfr1*^{-/-} males with BALB/c females. *Tnfr1*^{-/-} females were generated by intercrossing *Tnfr1*^{+/-} females and *Tnfr1*^{-/-} males. All mice were genotyped for the presence of the *Neo* gene and further tested for hetero- or homozygosity using gene-specific primers (Fig. 1C and Table 1). Embryonic fibroblast cells were isolated from wild-type, heterozygous and TNFIP6-deficient E18.5 embryos. *Tnfr1*-specific mRNA expression and protein secretion were confirmed in non-stimulated and TNF α -stimulated fibroblast cultures (Fig. 1D,E).

Recombinant TNFIP6 proteins

Human recombinant TNFIP6 [recTNFIP6, the Arg-144 allele (Nentwich et al., 2002) which is 94% identical to the murine protein] was expressed in Schneider 2 cells [derived from *Drosophila melanogaster* (Schneider, 1972)], purified using ion exchange and reverse-phase high performance liquid chromatography, and the protein concentration was determined by amino acid analysis as described previously (Nentwich et al., 2002). Mouse recTNFIP6 was

Table 1. Primer sequences used for genotyping and RT-PCR

Primer name	Location	Sequence (5'→3')	Product size (bp)*
Neo-13Fw	Neo	GGATCGGCCATTGAACAAG	600
Neo-14Rev	Neo	CACCATGATATTCGGCAAGC	
Tnfr1-Fw1	Neo	GGGTGGGATTAGATAAATGC	441
Tnfr1-Rev1	Intron-1	TCACTCCTTCCATTCAGTCC	
Tnfr1-Fw2	Exon-1	TGCCAATGCAAATGAGTCAGAGG	356
Tnfr1-Rev2	Intron-1	GGATCGATATTCCTCAACATGAAGC	2,156
Tnfr1-Fw3	Exon-1	CGTCCTCCTTTGCTTATGC	490
Tnfr1-Rev3	Exon-4	AATGTGCCAGTAGCAGACC	

*For the corresponding amplification results see Fig. 1C and D.

purified on a Hyaluronan-Sepharose column from supernatants of Chinese hamster ovary cells (CHO-K1; American Type Culture Collection) stable-transfected with Lonza pEE14.1 vector (Lonza Biologics plc) containing a full-length (1654 bp) mouse *Tnfr1* cDNA clone as described previously (Bardos et al., 2001). Purified recTNFIP6 was tested by western blot using a rabbit polyclonal antibody (TSG-6-CR21) raised against a synthetic peptide (¹³⁵CGGVFTDPKRFKSPG) located at the N-terminal end of the CUB domain and quantitated with sandwich enzyme-linked immunosorbent assay as described previously (Bardos et al., 2001).

Induction of ovulation

Superovulation was induced by consecutive injections of gonadotropins. First, 21-day old female mice were injected intraperitoneally with 5 U pregnant mares' serum gonadotropin (PMSG, Sigma) in 100 μ l phosphate-buffered saline (PBS) to induce follicle maturation. Ovulation was induced by intraperitoneal injection of 5 U human chorionic gonadotropin (hCG, Sigma) in 100 μ l PBS 48 hours after the PMSG injection.

In vivo fertilization

Superovulation was induced as described above, and the females were paired with wild-type males 2 hours after the hCG injection (at midnight). Females were checked for vaginal plugs the next morning, and sacrificed 36 hours after the hCG injection. Oocytes and two-cell embryos were collected from the oviducts to assess in vivo fertilizability.

Isolation and culture of COCs

Ovaries and fallopian tubes were dissected in Minimum Essential Medium (MEM) containing 25 mM Hepes, 0.1% bovine serum albumin and 50 ng/ml gentamycin. Ovulated COCs were collected from the fallopian tubes 13-13.5 hours after the hCG injection. For cultures, ovaries were dissected from the mice 48 hours after PMSG treatment, and COCs were isolated by puncturing the larger follicles with a sterile needle. The isolated compact COCs were washed once with standard COC culture medium (MEM supplemented with 5% fetal bovine serum (FBS), 3 mM glutamine, 0.3 mM sodium pyruvate and 50 ng/ml gentamycin), then cultured in standard COC culture medium in the absence or the presence of either epidermal growth factor (EGF, 3 ng/ml; Sigma) or dibutyryl cyclic AMP (dbcAMP, 1 mM; Sigma) at 37°C for 18 hours. In certain cases, COCs from *Tnfr1*^{-/-} females were cultured in the presence of 1 μ g/ml human recTNFIP6 protein to restore cumulus mucification. Matrix expansion was monitored morphologically using an Olympus inverted microscope.

Immunohistochemistry

Ovaries were harvested, fixed in 4% paraformaldehyde, and embedded in paraffin. Deparaffinized sections were blocked in Hank's

balanced salt solution (HBSS) containing 2% FBS at room temperature for 1 hour and stained with biotinylated hyaluronan binding protein (HABP, 5 $\mu\text{g}/\text{ml}$; Seikagaku) in HBSS, 2% FBS at 4°C for 16 hours. Fluorescein isothiocyanate-conjugated streptavidin (2 ng/ml; Vector Laboratories) was used as a secondary conjugate in HBSS, 2% FBS at 25°C for 1 hour. The slides were washed in HBSS and mounted with Vectashield containing DAPI (Vector Laboratories). Sections were visualized with a confocal microscope (Leica TCS-SP). Hematoxylin and Eosin staining was performed using standard procedures.

Protein extraction from ovaries

The ovulatory process was induced as described above, and mice were sacrificed 10 hours after the hCG injection. Dissected ovaries were minced and digested with 200 mU *Streptomyces hyaluronidase* in 100 μl PBS at 37°C for 24 hours in the presence of protease inhibitors (5 mM EDTA, 1 mM iodoacetamide, 5 $\mu\text{g}/\text{ml}$ pepstatin A, 5 $\mu\text{g}/\text{ml}$ aprotinin, 5 $\mu\text{g}/\text{ml}$ leupeptin and 10 mM benzamide). Each digestion mixture, including the tissue debris, was dried in a Speed-Vac concentrator and resuspended in reducing Laemmli-buffer (Laemmli, 1970). Samples were analyzed by SDS-PAGE and western blotting using a polyclonal antibody against $\text{I}\alpha\text{I}$ (Dako). Mouse sera with or without chondroitinase ABC digestion were used as a reference for the size of $\text{I}\alpha\text{I}$, $\text{P}\alpha\text{I}$ and their HCs.

TNFIP6-mediated transfer of the heavy chains of $\text{I}\alpha\text{I}$ family members to hyaluronan

High molecular mass hyaluronan (12 μg , mol. mass: 5000 kDa; Healon GV, Pharmacia & Upjohn) and mouse serum (10 μl , Rockland Immunochemicals) were incubated in the absence or presence of human recTNFIP6 (500 ng) in 100 μl PBS at 37°C for 24 hours. A 10 μl aliquot of each reaction mixture was further digested with 200 mU *Streptomyces hyaluronidase* at 37°C for 1 hour. The transfer of the heavy chains was monitored by Western blot analysis as described above.

Rescue of female fertility in vivo

The rescue of fertility in *Tnfrp6*^{-/-} females was attempted by two different approaches. First, *Tnfrp6*^{-/-} female mice were superovulated as described above and also received 100 μg of purified mouse recTNFIP6 protein intraperitoneally together with the hCG injection (total volume of 100 μl). Stud males were transferred into the female cages 6 hours later. Vaginal plugs were identified next morning. A second rescue approach was performed using in vivo genetic manipulations by generating *Tnfrp6*^{-/-} females that also carried the *Tnfrp6* transgene. Initially, transgenic females (*Tnfrp6-Tg*^{+/+}), constitutively expressing *Tnfrp6* (Glant et al., 2002), were mated with *Tnfrp6*^{-/-} males, first to generate heterozygous *Tnfrp6*^{+/-}*Tnfrp6-Tg*^{+/-} offsprings and then to select females lacking the wild-type *Tnfrp6* gene but having the transgene. Although the *Tnfrp6-Tg* expression vector (pSP/44-3) was designed to drive cartilage-specific expression (Glant et al., 2002), recently we found that the construct is 'leaking' and all tissues and organs, including ovaries, constitutively express some levels of transgene-derived *Tnfrp6* (data not shown). *Tnfrp6*^{-/-}*Tnfrp6-Tg*^{+/-} or *Tnfrp6*^{-/-}*Tnfrp6-Tg*^{+/+} females without superovulation were mated with wild-type stud males.

RESULTS

Tnfrp6-null females demonstrate severe subfertility

We have obtained two chimeric males and established two independent *Tnfrp6*-null (*Tnfrp6*^{-/-}) mouse colonies by backcrossing these chimeric males with BALB/c females (Fig. 1). To confirm the expression or the lack of *Tnfrp6* and the corresponding protein, embryonic fibroblasts were isolated from

Table 2. Average litter sizes of matings among different *Tnfrp6* genotypes

Mating pairs (<i>Tnfrp6</i> genotype)		Number of pregnant females/number of matings	Average size of litter per pregnancy
Female*	Male		
+/+	+/+	11/12	7.20
+/+	+/-	14/14	6.05
+/+	-/-	6/7	6.29
+/-	+/+	6/6	6.50
+/-	+/-	56/58	6.87
+/-	-/-	9/10	7.00
-/- [†]	+/+	0/48 (16)	0
-/- [†]	+/-	0/31 (9)	0
-/- [†]	-/-	0/14 (9)	0
-/- [‡]	+/+	8/9	1.75
-/- [§]	+/+	3/15	1.0
-/- [¶] (<i>Tnfrp6-Tg</i> ⁺)	+/+	5/9	4.0

*All females were offspring of *Tnfrp6*^{+/-} parents or wild-type mothers and *Tnfrp6*^{-/-} fathers. In all cases, one female mouse was mated with a male in a 3-day rotation cycle until the female became pregnant.

[†]For *Tnfrp6*^{-/-} females, the numbers in parentheses indicate the number of females. These 34 females were mated repeatedly with stud males in the 'standard' 3-day rotation cycle. Some of these stud males were selected from other wild-type colonies.

[‡]*Tnfrp6*^{-/-} females were superovulated and the hCG co-injected with 100 μg of mouse recTNFIP6.

[§]*Tnfrp6*^{-/-} females were superovulated without adding exogenous TNFIP6; only 3 females became pregnant and delivered one baby of each.

[¶]*Tnfrp6*^{-/-} females without superovulation, but carrying the *Tnfrp6* transgene, were mated with wild-type stud males.

all genotypes and tested for expression of *Tnfrp6*-specific mRNA (Fig. 1D) and for protein in both culture media and cell lysates with or without TNF α stimulation (Fig. 1E). These results clearly showed the absence of TNFIP6 mRNA and protein in *Tnfrp6*^{-/-} fibroblasts (Fig. 1D,E). *Tnfrp6*^{-/-} mice were normal at birth and showed normal growth. The only apparent phenotypic abnormality was the sterility of the *Tnfrp6*^{-/-} females (Table 2). *Tnfrp6*^{+/-} females and *Tnfrp6*^{-/-} males were fertile, and the average litter sizes in all combinations were highly comparable (Table 2). In order to determine if the sterility of *Tnfrp6*^{-/-} females resulted from their inability to ovulate, females of the three *Tnfrp6* genotypes were induced to superovulate by consecutive injections of PMSG and hCG. Oocytes were recovered from the fallopian tubes 13.5 hours after hCG injection and counted. Although *Tnfrp6*^{-/-} females were able to ovulate, the average number of oocytes recovered from their oviducts after superovulation (14 \pm 2, n =8) was significantly lower (P <0.001) than the average recovered from their wild-type (29 \pm 10, n =8) or heterozygous littermates (32 \pm 12, n =12). In addition, sexually mature (12-week old) *Tnfrp6*^{-/-} females also appeared to be able to ovulate under normal endocrine conditions as evidenced by the presence of corpus lutea in their ovaries (Fig. 2A-C). In order to investigate if oocytes from *Tnfrp6*^{-/-} females can be fertilized in vivo, superovulated females were mated with wild-type males, and the ability of the oocytes to reach the two-cell stage was assessed. While the vast majority (>90%) of oocytes from wild-type and *Tnfrp6*^{+/-} females successfully reached the two-cell stage in these assays, we were unable to detect any two-cell embryos in *Tnfrp6*^{-/-} females (Fig. 3). Thus, the sterility of the *Tnfrp6*^{-/-} females was the result of the inability of their oocytes to be fertilized in vivo.

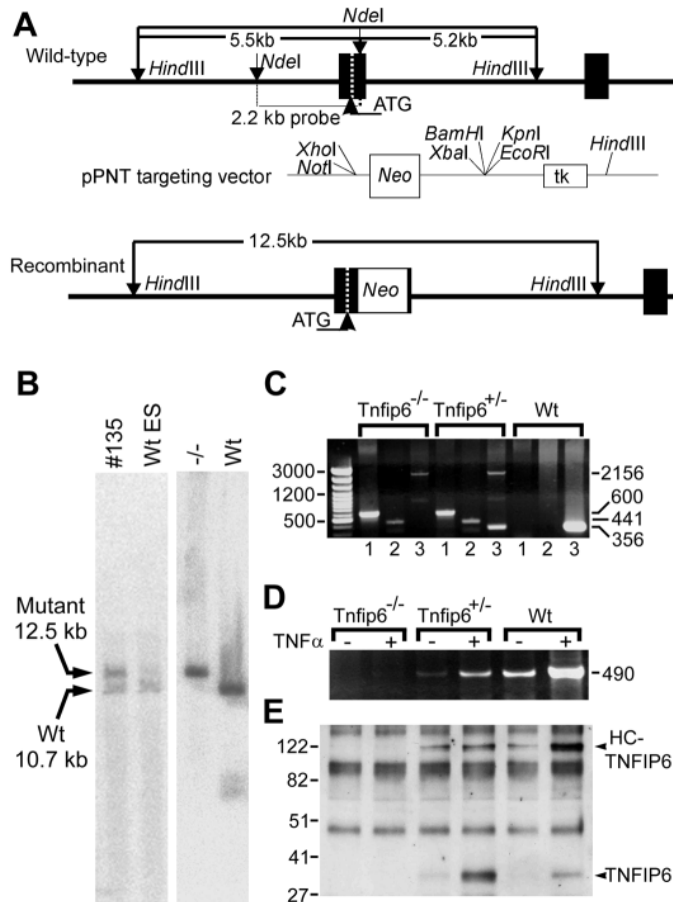


Fig. 1. Generation of *Tnfrp6*^{-/-} mice. (A) Genomic organization of wild-type and recombinant alleles, and the targeting vector pPNT. The location of the probe used for Southern blot hybridization is shown underneath the wild-type allele. Black boxes represent exons. (B) Southern blot analysis of the genomic DNA samples (10 µg) derived from ES cell clones (wild type and clone no.135 with selected disruption of *Tnfrp6*) or from mouse tails were digested with *Hind*III, separated in a 0.6% agarose gel, transferred to Nytran membrane and probed with a 2.2 kb ³²P-labeled genomic DNA probe shown in A. (C) Genotyping results of *Tnfrp6*^{-/-}, *Tnfrp6*^{+/-} and wild-type (wt) littermates. PCR products of genomic DNAs using neomycin-specific primers (600 bp; lane 1); neomycin and intron 1-specific primers (441 bp; lane 2); exon 1 and intron 1-specific primers (365 bp without *Neo* gene and 2156 with *Neo*; lane 3). (D) RT-PCR results using total RNA samples from fibroblasts of *Tnfrp6*^{-/-}, *Tnfrp6*^{+/-} and wild-type mice without and with TNFα stimulation (20 ng/ml; 24 hours) as indicated. (E) Media from the same fibroblast cultures used for RNA isolation were collected and TNFIP6 protein detected by Western blot analysis using affinity purified and biotinylated rabbit TSG-6-RC21 antibody (Bardos et al., 2001). The locations of TNFIP6 (36 kDa) and the previously described HC-TNFIP6 complex (~125 kDa) (Mukhopadhyay et al., 2001) are marked.

Abnormal cumulus mucification in *Tnfrp6*^{-/-} females in vivo

Previous studies have demonstrated that the mucified COC extracellular matrix plays an important role in the fertilizability of the oocyte (Chen et al., 1993; Hess et al., 1999; Zhuo et al., 2001), and that TNFIP6 specifically incorporates into this

matrix during the ovulatory process (Mukhopadhyay et al., 2001; Carrette et al., 2001). Thus, one possible explanation for the sterility of the *Tnfrp6*^{-/-} females is that the COCs do not mucify correctly, which in turn could lead to the infertilizability of the oocyte. To explore this possibility, female mice were superovulated, and cumulus matrix expansion was evaluated using histochemistry. Preovulatory follicles formed 48 hours after the PMSG injection, contained compact COCs in all three *Tnfrp6* genotypes and were indistinguishable (Fig. 4A-C). However, when cumulus mucification was induced by hCG injection, the morphology of the homozygous preovulatory follicles differed dramatically from their wild-type and heterozygous counterparts. While wild-type and *Tnfrp6*^{+/-} females demonstrated normal cumulus expansion 10 hours after hCG injection, *Tnfrp6*^{-/-} females failed to do so (Fig. 4D-F). Instead, oocytes in the homozygotes shed a large number of cumulus cells at this stage. The few cumulus cells retained around the oocyte did not show any signs of expansion (Fig. 4F), suggesting defects in cumulus matrix organization and stability. These observations were further supported when ovarian sections were stained for hyaluronan, the major structural component of the mucified cumulus extracellular matrix. Wild type and heterozygous females showed intense hyaluronan staining in their cumulus matrix 10 hours after hCG injection (Fig. 4G,H). In contrast, homozygous littermates had only faint hyaluronan staining at this stage, indicating the lack of the normal cumulus matrix in this genotype (Fig. 4I). High power magnification of sections revealed that the detectable hyaluronan staining in *Tnfrp6*^{-/-} mice was associated with the cell surface of the cumulus cells and with the follicular fluid (Fig. 4L). These observations suggested that the cumulus cells of the *Tnfrp6*^{-/-} mice still synthesized hyaluronan but were unable to organize it into an extracellular matrix.

The differences in the structure of the COCs were even more dramatic after ovulation (13.5 hours after hCG injection). Ovulated COCs from wild-type or heterozygous females had a characteristic shape with a fully expanded cumulus layer around the oocyte (Fig. 5A,B,D,E). However, most of the oocytes collected from the fallopian tubes of the homozygotes were nude (Fig. 5C), lacking the cumulus layers. A few oocytes were associated with a loose network of granulosa/cumulus cells, but without showing the normal structure (Fig. 5F). In fact, this association was very weak, because even gentle pipetting released the oocytes from the somatic cells (not shown).

Impaired expansion of *Tnfrp6*^{-/-} COCs in vitro

Cumulus matrix expansion can be induced by a variety of factors in vitro, as described by previous studies (Dekel and Kraicer, 1978; Eppig, 1981; Downs, 1989). In order to study the ability of TNFIP6-deficient COCs to mucify in vitro, COCs were isolated from PMSG-primed female mice and cultured for 18 hours in the absence or presence of either EGF or dbcAMP. In the untreated cultures, the COCs from all three *Tnfrp6* genotypes remained compact and morphologically indistinguishable (Fig. 6A-C). While both EGF and dbcAMP were able to induce expansion of COCs from wild-type and *Tnfrp6*^{+/-} females (Fig. 6D,E,G,H), COCs from *Tnfrp6*^{-/-} females shed most of their cumulus cells (Fig. 6F,I), which closely resembled the phenotype observed in vivo (Fig. 4F).

Fig. 2. Hematoxylin and eosin stained ovaries from sexually mature (12-week old) mice. Note the presence of corpus luteum (CL) in all three *Tnfip6* genotypes. Scale bar: 500 μ m.

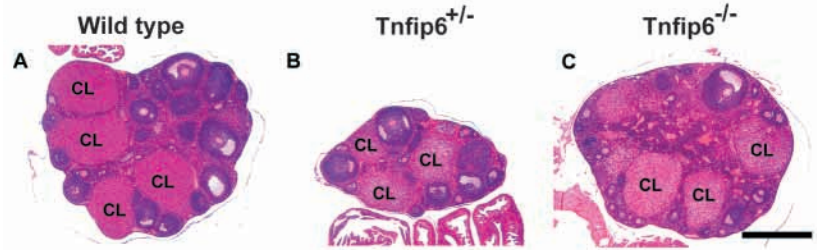
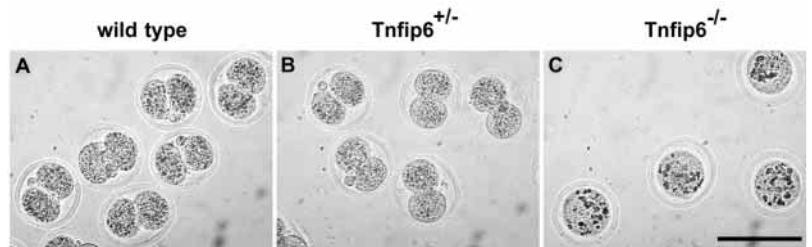


Fig. 3. Lack of fertilizability of oocytes from *Tnfip6*^{-/-} females. Fertilizability was assessed by the ability of the oocytes to reach the two-cell stage using in vivo fertilization. Scale bar: 100 μ m.



Culturing TNFIP6-deficient COCs in the presence of recTNFIP6 successfully restored their ability to expand (Fig. 6J,K), further supporting the crucial role of this protein in cumulus matrix organization.

TNFIP6 is necessary for the transfer of heavy chains (HCs) from α 1 family members onto hyaluronan

Previous studies demonstrated that after the LH surge, α 1-related molecules diffuse from the blood into the preovulatory follicle (Powers et al., 1995), and that the covalent transfer of the HCs of these molecules onto hyaluronan was crucial for the formation of the COC extracellular matrix (Zhuo et al., 2001). In order to investigate the presence of hyaluronan-HC covalent complexes in the ovaries of the three *Tnfip6* genotypes, we digested the ovaries (10 hours after hCG injection) with Streptomyces hyaluronidase, an enzyme highly specific for hyaluronan. Western blot analysis revealed that ovaries from all three genotypes contained α 1 and α 2. However, hyaluronan-linked HCs could only be detected in wild-type and heterozygous mice (Fig. 7A). Hyaluronidase extracts of homozygous ovaries lacked the ~85 kDa band (representing single heavy chains) and additional high molecular mass bands (Fig. 7A, open arrowheads). These latter bands most likely represent dimers and clusters of

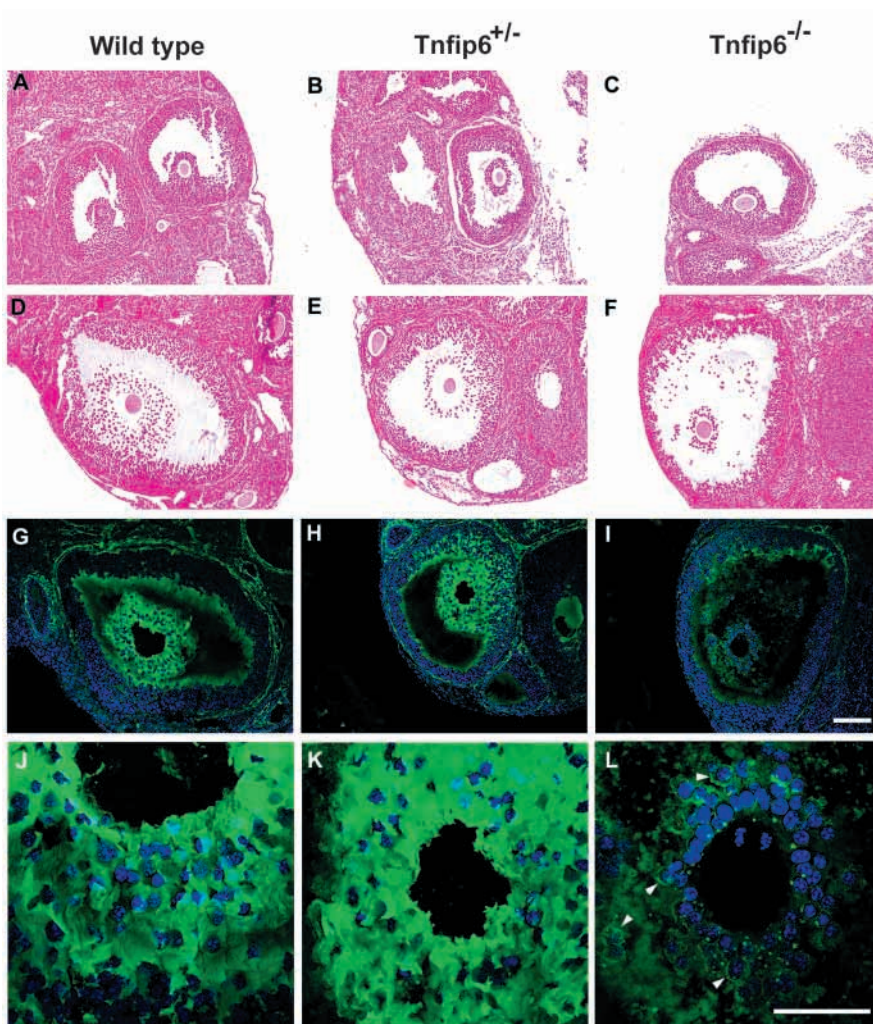
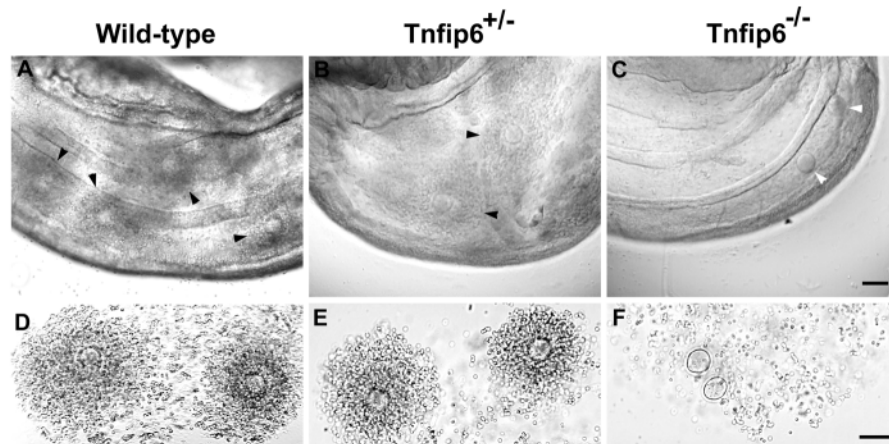


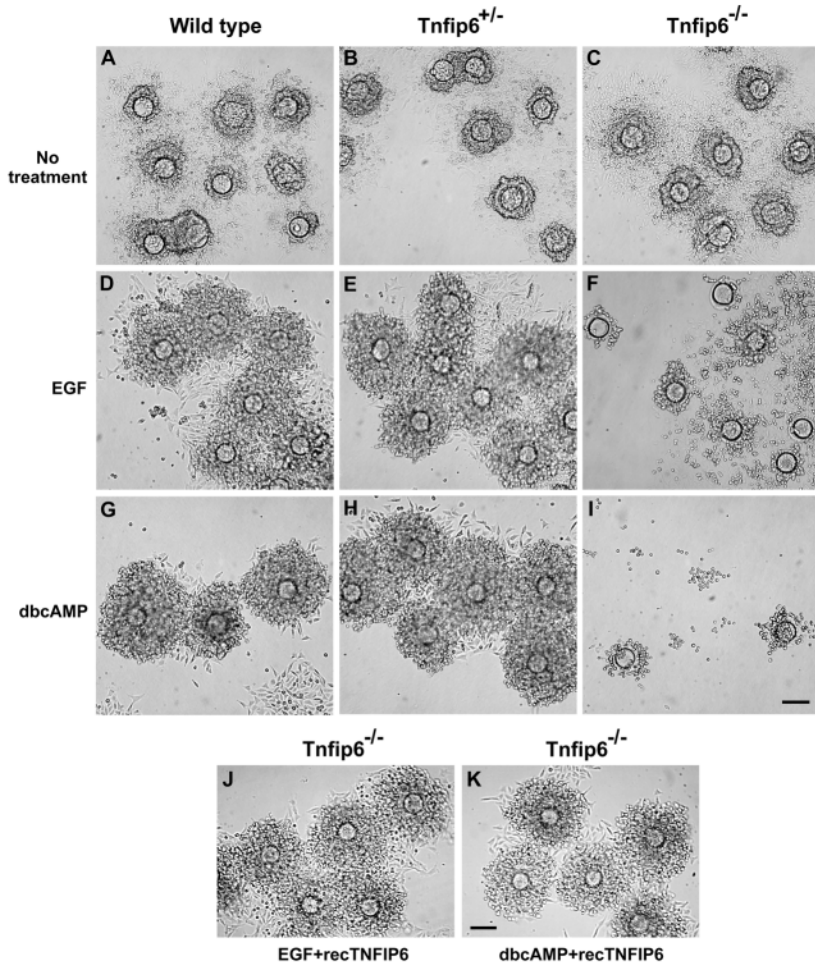
Fig. 4. Deficient cumulus mucification in *Tnfip6*^{-/-} females. (A-C) Hematoxylin stained preovulatory follicles in the three *Tnfip6* genotypes 48 hours after the PMSG treatment (before hCG injection). (D-F) Hematoxylin stained preovulatory follicles 10 hours after induction of ovulation by hCG injection. (G-I) Localization of hyaluronan (green) in preovulatory follicles 10 hours after hCG injection. Low (G-I) and high (J-L) power magnifications are shown. White arrowheads in L point to cell surface hyaluronan. Nuclei are stained blue by DAPI. Scale bars: 100 μ m (A-I) and 50 μ m (J-L).

Fig. 5. Morphology of ovulated COCs in mice with different *Tnfrp6* genotypes. Females of all three *Tnfrp6* genotypes are able to ovulate as indicated by the presence of oocytes within the oviduct (A-C). The dense cloud-like material (black arrowheads) around the oocytes in the oviducts of wild-type and *Tnfrp6*^{+/-} females are the expanded cumulus layer. Oocytes in the oviduct of the *Tnfrp6*^{-/-} females lack the same dense material (C, white arrowheads). This difference is clearly seen when isolated COCs are compared (D-F). COCs from wild-type and heterozygous females show normal morphology, while oocytes from the homozygotes do not have a cumulus layer. These latter oocytes are either completely nude (not shown) or they are associated with a dispersed network of granulosa/cumulus cells (F). Scale bars: 100 µm.



HCs located close to each other on a single hyaluronan chain. The close clustering of these chains would render resistance of these structures to hyaluronidase digestion as suggested by Chen et al. (Chen et al., 1996). In order to investigate whether TNFIP6 was necessary for the transfer of the HCs onto hyaluronan, we incubated hyaluronan with mouse serum (the physiological source of IαI and PαI) in the absence or presence

of recTNFIP6. Covalent transfer of the HCs to hyaluronan did not occur in the absence of recTNFIP6 (Fig. 7B, lanes 1 and 2). However, when recTNFIP6 was added to the reaction mixture, the IαI and PαI bands disappeared, and a new band immunoreactive with anti-IαI antibody was detected at the top of the gel indicating the formation of the high molecular mass hyaluronan-HC complexes (Fig. 7B, lane 3). These complexes could not be dissociated in reducing Laemmli buffer, but could be readily broken down by *Streptomyces hyaluronidase* (Fig. 7B, lane 4), implying the covalent nature of the hyaluronan-HC complex.



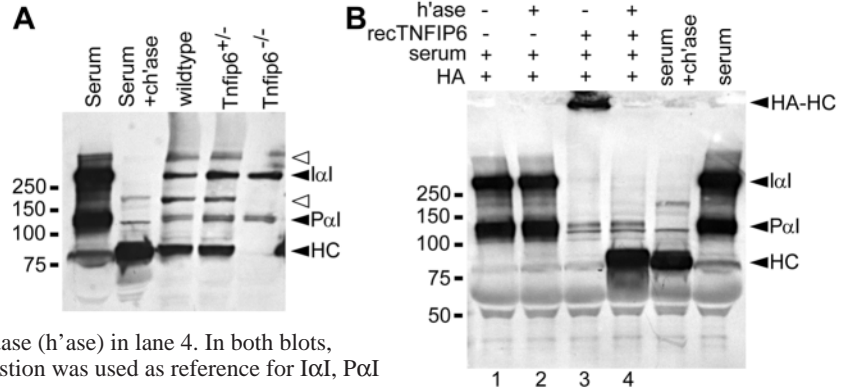
Rescue of fertility in *Tnfrp6*^{-/-} female mice

Infertile *Tnfrp6*^{-/-} females were superovulated and also injected intraperitoneally with mouse recTNFIP6 at the time of hCG administration. After mating with wild-type males, eight of nine superovulated and recTNFIP6-treated *Tnfrp6*^{-/-} female mice with a vaginal plug became pregnant, although the average litter size was very low (1-2 newborns per mother, Table 2). All pups were healthy, showed normal growth and were fertile when they reached maturity. The low number of newborns from TNFIP6-rescued females, however, was in contrast to the litter size of *Tnfrp6*^{+/-} or wild-type females, which usually delivered 6-8 newborns (Table 2). Although the TNFIP6-rescued pregnancy yielded a low number of offspring, this did not seem to be a direct consequence of superovulation, as only 3 of 15 *Tnfrp6*^{-/-} superovulated females became pregnant

Fig. 6. Impaired expansion of *Tnfrp6*^{-/-} COCs in vitro. (A-I) Compact COCs (48 hours after PMSG injection) from the three *Tnfrp6* genotypes were cultured in the absence (A-C) or in the presence of either 3 ng/ml EGF (D-F) or 1 mM dbcAMP (G-I). (J-K) Expansion of *Tnfrp6*^{-/-} COCs can be restored by supplementing recombinant TNFIP6 in the culture medium. Scale bars: 100 µm.

Fig. 7. TNFIP6 is necessary for the transfer of heavy chains (HCs) from I α I and P α I to hyaluronan.

(A) *Tnfrp6*^{-/-} mice lack HCs covalently transferred to hyaluronan in their ovaries. Western blot of *Streptomyces* hyaluronidase-digested ovarian extracts are shown. Open arrowheads indicate dimers or clusters of HCs located close to each other on a single hyaluronan chain and resistant to hyaluronidase digestion. (B) TNFIP6 facilitates the covalent transfer of HCs from I α I and P α I to hyaluronan in vitro. Note the formation of a high molecular mass hyaluronan-HC complex (HA-HC) in lane 3 in the presence of recTNFIP6, and the sensitivity of this band to hyaluronidase (h'ase) in lane 4. In both blots, serum with or without chondroitinase ABC (ch'ase) digestion was used as reference for I α I, P α I and their HCs.



and delivered only one baby without recTNFIP6 treatment (Table 2). In addition, when *Tnfrp6* was constitutively expressed by a transgene (Glant et al., 2002) in otherwise *Tnfrp6*^{-/-} females, we found significantly higher litter sizes (Table 2). These results indicate that the introduction of TNFIP6, either as a recombinant protein or as the product of the *Tnfrp6* transgene, can rescue fertility in *Tnfrp6*^{-/-} females.

DISCUSSION

In this study, we have demonstrated that TNFIP6 is indispensable for the formation of the COC extracellular matrix during ovulation and consequently for female fertility. *Tnfrp6*^{-/-} female mice appear to develop morphologically normal preovulatory follicles (Fig. 4A-C), concurring with previous findings that TNFIP6 is expressed and accumulated in the preovulatory follicles only after the midcycle LH surge (Fulop et al., 1997; Yoshioka et al., 2000; Mukhopadhyay et al., 2001; Carrette et al., 2001). Thus, TNFIP6 does not seem to have a crucial role in follicular development up to the preovulatory stage. However, TNFIP6 has a pivotal importance in the mucification of the COC during the preovulatory period. The COC extracellular matrix fails to assemble in TNFIP6-deficient mice in vivo, as visualized by the lack of hyaluronan incorporation and ensuing COC expansion. Most of the cumulus cells detach from the COCs just prior to the follicular rupture and disperse into the follicular space. This observation suggests that the extracellular matrix is absolutely necessary to maintain the cumulus mass in the oocyte's vicinity once the contacts between cumulus cells are severed. Similar conclusions can be drawn from the lack of cumulus matrix expansion after stimuli by either EGF or dbcAMP (Fig. 6), potent initiators of COC expansion in vitro (Downs, 1989; Salustri et al., 1990).

Tnfrp6^{-/-} female mice have markedly lower number of oocytes in their oviducts after superovulation than their wild-type or heterozygous littermates. This difference could be the result of (i) lower number of preovulatory follicles, (ii) lower rate of ovulation or (iii) inefficient pickup of the ovulated oocytes by the fimbria. Although we did not compare the actual number of preovulatory follicles, the morphology of these follicles was very similar among the three genotypes. In addition, *Tnfrp6* is expressed only after the LH surge (i.e., after preovulatory follicle formation) (Fulop et al., 1997) and is not

expected to influence the number of preovulatory follicles. Lower numbers of COCs (or nude oocytes) in the oviducts have also been demonstrated in other experimental animal models in which COC expansion was inhibited, such as during 6-diazo-5-oxo-1-norleucine treatment (Chen et al., 1993), hyaluronan oligosaccharide treatment (Hess et al., 1999), and in the bikunin knockout mice (Zhuo et al., 2001; Sato et al., 2001). While these studies have resulted in the speculation that the reduced number of oocytes in the oviducts is due to the lower ovulation rate, inefficient fimbrial pickup cannot be excluded (Mahi-Brown and Yanagimachi, 1983). In fact, polycationic macromolecules, such as poly-L-lysine have been shown to inhibit COC transport along the fallopian tube (Norwood et al., 1978), suggesting that the negatively charged hyaluronan-rich COC matrix may play an important role in this process. The lack of the hyaluronan-rich COC matrix (such as in *Tnfrp6*^{-/-} mice) could result in less efficient pickup of the nude oocytes by the ciliary structures of the fimbria.

The observed phenotype of *Tnfrp6*^{-/-} females is similar to that previously described in bikunin-null females (Zhuo et al., 2001). Bikunin is the light chain component of P α I and I α I (Salier et al., 1996). These complex serum proteins also contain one or two evolutionarily-related heavy chains (HC1 and HC2 in I α I, and HC3 in P α I). The polypeptide chains are covalently linked together by a single chondroitin sulfate chain through ester and glycosidic bonds (for schematic representation see Fig. 8). Bikunin null mice are unable to assemble I α I and P α I (Zhuo et al., 2001). Although unprocessed HCs are present in the sera of these animals, these HCs cannot be covalently transferred to hyaluronan in the expanding COCs. The absence of this biochemical reaction leads to impaired cumulus mucification, ovulation of nude oocytes, and female sterility (Zhuo et al., 2001).

The comparable phenotypes of the *Tnfrp6*^{-/-} and bikunin-null (i.e., I α I-null and P α I-null) female mice suggest a cooperative role of TNFIP6 and the HCs of the I α I family members in the formation of the COC extracellular matrix (Fig. 8). After the midcycle LH surge, cumulus cells start to synthesize hyaluronan, while both cumulus and granulosa cells synthesize TNFIP6 (Yoshioka et al., 2000; Mukhopadhyay et al., 2001; Carrette et al., 2001; Varani et al., 2002). These processes are accompanied by the diffusion of the I α I family members into the preovulatory follicle (Powers et al., 1995). Our present data provide the first evidence that TNFIP6 facilitates the covalent transfer of HCs from I α I and P α I to

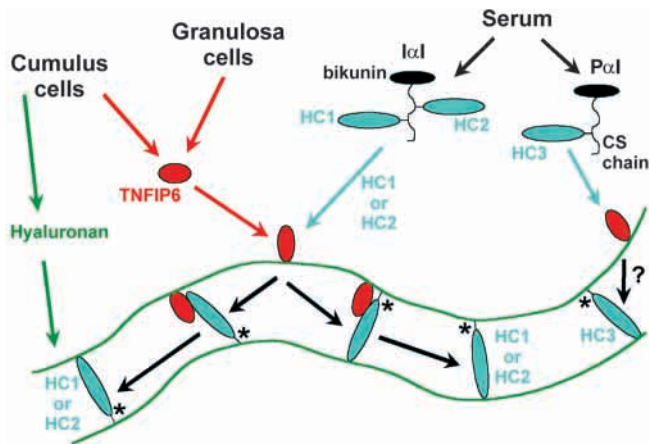


Fig. 8. Schematic model for the proposed role of TNFIP6 in the stabilization of the cumulus extracellular matrix. For simplicity, only two hyaluronan chains are shown, but the proposed interactions can crosslink many of these chains creating a very stable hyaluronan meshwork. Asterisks indicate covalent interactions (ester bonds) between the heavy chains (HCs) and hyaluronan. All other protein-hyaluronan interactions are non-covalent. The presence of HC(1 or 2)-TNFIP6 complexes in the cumulus matrix was demonstrated in our previous study (Mukhopadhyay et al., 2001). Note that the exact structure of the HC-TNFIP6 has not yet been elucidated, therefore two hypothetical complex structures are shown. '?' indicates that no HC3-TNFIP6 complex has been detected yet. CS, chondroitin sulfate.

hyaluronan. These HCs can stabilize the COC extracellular matrix by crosslinking separate hyaluronan chains through covalent and ionic interactions as hypothesized before (Fig. 8) (Chen et al., 1994; Chen et al., 1996). According to our model, deficiencies in either TNFIP6 or I α I (and P α I) result in the same phenotype, i.e. lack of cumulus matrix formation. A previous study by Chen et al. (Chen et al., 1996) suggested that the factor that facilitates HC transfer to hyaluronan is produced by granulosa cells. While granulosa cells are potent sites of TNFIP6 synthesis (Yoshioka et al., 2000; Mukhopadhyay et al., 2001; Carrette et al., 2001; Varani et al., 2002), TNFIP6 synthesized by cumulus cells alone is clearly sufficient to form the extracellular matrix as demonstrated by COC cultures in the absence of granulosa cells (Fig. 6). However, granulosa cell-derived TNFIP6 can substantially strengthen the stability of the COC matrix, since the preovulatory follicle contains ~50 times more granulosa cells than cumulus cells (Pedersen, 1970; Salustri et al., 1992). Indeed, COCs expanded *in vivo* have been shown to be ~6 times more resistant to mechanical forces than those expanded *in vitro* (Chen et al., 1996).

We have shown recently that Tnfp6 forms covalent complexes with HC1 and HC2 of I α I (but not with HC3 of P α I) in the COC matrix, and that these complexes, similarly to the single HCs, are very tightly associated with hyaluronan in the COC matrix (Mukhopadhyay et al., 2001). We have hypothesized that these HC-TNFIP6 complexes play a role in the stabilization of the COC matrix by crosslinking separate hyaluronan chains. Our current findings now further suggest that HC-TNFIP6 complexes could be intermediates or by-products during the TNFIP6-facilitated HC transfer to hyaluronan (Fig. 8). This hypothesis is favored by our previous observation that only a small portion of the HCs are present in

the form of HC-TNFIP6 complexes in the COC matrix (Mukhopadhyay et al., 2001). The small amount of HC-TNFIP6 complexes, however, could still significantly contribute to matrix stabilization as a result of the very strong hyaluronan-binding affinity of TNFIP6 (Kohda et al., 1996; Parkar and Day, 1997). Therefore, the cumulus matrix is more likely stabilized by multiple crosslinks involving individual HCs and HC-TNFIP6 complexes (Fig. 8).

Our studies present clear evidence that correct mucification of the cumulus mass is an obligatory step for female fertility. Successful *in vitro* fertilization has been previously correlated with the degree of cumulus matrix formation (Chen et al., 1993), and complete failure of cumulus expansion results in the lack of fertilizability of the oocytes in bikunin-null (Zhuo et al., 2001) and pentraxin-3-null (Varani et al., 2002) female mice. Similar to these animal models, TNFIP6 deficiency also causes impaired cumulus matrix formation, the ovulation of nude oocytes and the lack of fertilizability of these oocytes.

Our findings may lead to new approaches for the treatment of certain women with unexplained infertility. TNFIP6 deficiency could be a cause in a group of these women, and genetic screening should be applied to identify this possibility. TNFIP6-deficient women are not expected to respond to gonadotropin therapy (as shown in our animal model); rather a treatment with recombinant TNFIP6 should be developed. Our *in vitro* and *in vivo* rescue experiments lend feasibility to this approach.

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