

Corrigendum

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The authors apologise to readers for this mistake.

PTX3 plays a key role in the organization of the cumulus oophorus extracellular matrix and in in vivo fertilization

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Summary

PTX3 is a prototypic long pentraxin that plays a non-redundant role in innate immunity against selected pathogens and in female fertility. Here, we report that the infertility of *Ptx3*^{-/-} mice is associated with severe abnormalities of the cumulus oophorus and failure of in vivo, but not in vitro, oocyte fertilization. PTX3 is produced by mouse cumulus cells during cumulus expansion and localizes in the matrix. PTX3 is expressed in the human cumulus oophorus as well. Cumuli from *Ptx3*^{-/-} mice synthesize normal amounts of hyaluronan (HA), but are unable to organize it in a stable matrix. Exogenous PTX3 restores a normal cumulus phenotype. Incorporation in

the matrix of inter- α -trypsin inhibitor is normal in *Ptx3*^{-/-} cumuli. PTX3 does not interact directly with HA, but it binds the cumulus matrix hyaladherin tumor necrosis factor α -induced protein 6 (TNFAIP6, also known as TSG6) and thereby may form multimolecular complexes that can cross-link HA chains. Thus, PTX3 is a structural constituent of the cumulus oophorus extracellular matrix essential for female fertility.

Key words: Cumulus oophorus, Extracellular matrix, Fertility, Pentraxins, Mouse

Introduction

Fertilization of a mammalian oocyte requires a series of interactions between spermatozoa and the extracellular investments of the oocyte. In contrast to the zona pellucida, the cumulus oophorus had not received much attention until recently. However, findings reported in the past few years indicate that the cumulus matrix, though dispensable in vitro, plays a key role in the early events of in vivo fertilization (Hizaki et al., 1999; Zhuo et al., 2001; Fulop et al., 2003).

This matrix is synthesized by cumulus cells a few hours before ovulation and, in the mouse, the combined action of gonadotropins and soluble oocyte factors is required for triggering this process (Buccione et al., 1990; Salustri et al., 1990b). The major component is hyaluronan (HA), a large polyanionic glycosaminoglycan responsible for viscoelastic properties and expansion of the cumulus oophorus (Salustri et al., 1992). In vitro and in vivo studies have shown that serum-derived inter- α -trypsin inhibitor (α I or ITI) is essential for HA-matrix assembly and mouse female fertility (Chen et al., 1992; Chen et al., 1996; Zhuo et al., 2001; Fulop et al., 2003). α I consists of a small protein, named bikunin or light chain,

with a chondroitin sulfate moiety that connects two additional proteins, named heavy chains. The heavy chains of α I are covalently transferred to HA in the preovulatory follicle. It has been proposed that these polypeptides stabilize the HA-matrix by cross-linking HA molecules through covalent and ionic bonds. Another crucial component of the cumulus matrix is tumour necrosis factor α -induced protein 6 (TNFAIP6 or TSG6), a multifunctional protein usually associated with inflammation, which has the ability to specifically bind HA (Lee et al., 1992; Milner and Day, 2003). TNFAIP6 is synthesized by cumulus and granulosa cells in the preovulatory follicle (Fulop et al., 1997; Yoshioka et al., 2000), and *Tnfaip6*-deficient mice are unable to form stable cumulus matrix and are sterile (Fulop et al., 2003). Covalent transfer of heavy chains from α I to HA does not occur in *Tnfaip6*^{-/-} mice, indicating that TNFAIP6 is a key catalyst in this reaction. Oocytes, besides promoting cumulus matrix synthesis, inhibit hormone-induced proteolytic enzyme expression by mouse cumulus cells during matrix deposition, probably providing an additional mechanism for matrix stabilization (Canipari et al., 1995). Indeed, synthesis of cumulus matrix ceases at ovulation

and its degradation begins a few hours later, coinciding with an increase in protease production by the oocyte and the cumulus cells (D'Alessandris et al., 2001).

Recent findings have shown that the long pentraxin 3 (PTX3) is also involved in cumulus matrix stability (Varani et al., 2002). Pentraxins are a superfamily of conserved proteins, characterized by a cyclic multimeric structure (Emsley et al., 1994). The classical short pentraxins, C-reactive protein and serum amyloid P component, are acute phase proteins produced in the liver in response to inflammatory mediators (Steel and Whitehead, 1994; Szalai et al., 1997). Long pentraxins have an unrelated long N-terminal domain coupled to the C-terminal pentraxin domain, and differ in their gene organization, chromosomal localization, cellular source and inducing stimuli, as well as in the ligands they recognize (Mantovani et al., 2003). PTX3, the first long pentraxin identified (Breviario et al., 1992; Lee et al., 1993), is produced as a 10–20 subunit multimer protein by macrophages and other cell types or tissues upon stimulation with primary inflammatory mediators [lipopolysaccharide, interleukin 1 (IL1), tumor necrosis factor α (TNF α)] (Breviario et al., 1992; Lee et al., 1993; Lee et al., 1994; Introna et al., 1996; Bottazzi et al., 1997). PTX3 appears to have a protective effect in inflammatory sites limiting tissue damage, possibly by regulating apoptotic cell clearance (Mantovani et al., 2003; Ravizza et al., 2001). It also binds to selected microorganisms facilitating their recognition by macrophages (Garlanda et al., 2002). As a consequence, *Ptx3* deficiency renders mice susceptible to selected pathogens. Furthermore, *Ptx3*^{-/-} mice show a severe defect in female fertility (Garlanda et al., 2002; Varani et al., 2002). It has been reported that *Ptx3* is expressed by cumulus cells before ovulation, and that infertility of *Ptx3* deficient mice is due to defects in ovulation and oocyte fertilization, associated with loss of cumulus investment during extrusion from the ovary (Varani et al., 2002). The molecular basis for the loss of cumulus integrity in *Ptx3* deficient mice is currently unknown. It has been hypothesized that PTX3 might have antiproteolytic activity and functions to protect the oocyte and the extracellular matrix from proteases involved in rupture of the follicle wall.

We show that oocytes ovulated by *Ptx3*^{-/-} mice can be fertilized in vitro, indicating that the oocyte develops normally in the absence of PTX3, and that a defective cumulus expansion is the major cause for in vivo fertilization failure. PTX3 is produced by cumulus cells both in vivo and in vitro under stimuli inducing cumulus expansion, and localizes in the extracellular matrix. We also show that presence of PTX3 is essential for the expanding cumulus to retain HA molecules in the intercellular spaces, although it cannot prevent nor delay matrix degradation, which occurs at later times. The PTX3 role in retaining HA is independent of I α I incorporation into the matrix, but it is likely to be mediated by TNFAIP6. Finally, we show that *PTX3* is expressed by human cumulus cells as well, and that PTX3 protein is present in human cumulus matrix, suggesting that this molecule might have the same role in human female fertility.

Materials and methods

Ptx3 deficient mice

Ptx3 deficient (^{-/-}) mice were generated as described (Garlanda et al.,

2002). Phenotypic analysis was performed on two lines derived from independent clones, and results were confirmed in a 129/Sv-C57BL/6J-mixed and 129/Sv-inbred genetic background.

Procedures involving animals and their care conformed with institutional guidelines in compliance with national (4D.L. N.116, G.U., Suppl. 40, 18-2-1992) and international law and policies (EEC Council Directive 86/609, OJ L 358,1,12-12-1987; NIH Guide for the Care and Use of Laboratory Animals, US National Research Council 1996). All efforts were made to minimize the number of animals used and their suffering.

In vivo and in vitro fertilization

Cumuli oophori, zygotes and embryos were recovered from the oviducts of untreated females after natural mating or after hormonally-induced superovulation (Hogan et al., 1994). Cumulus oophorus matrix was digested with *Streptomyces* hyaluronidase (Calbiochem), and cumulus cells and oocytes were separated as described (Hogan et al., 1994).

In vitro fertilization (IVF) was performed using intact oocytes (Hogan et al., 1994) or zona pellucida free eggs stained with 1 μ g/ml of Hoechst 33258 (Sigma-Aldrich) (Conover and Gwatkin, 1988). After insemination embryos were cultured in KSOM media (Cell & Molecular Technologies). Sperm-egg fusion was determined by counting eggs with fluorescent fertilizing sperm 4 hours after insemination of zona free eggs. Fertilization was assessed by counting two-cell stage embryos and blastocysts, 1 or 4 days after insemination of intact oocytes, respectively. Embryo transfer was performed as described (Hogan et al., 1994).

PTX3 mRNA and protein

Northern blot analysis, in situ hybridation, expression and purification of PTX3, and antibody assays were performed as described (Biffo and Tolosano, 1992; Introna et al., 1996; Bottazzi et al., 1997). Human PTX3 was expressed in CHO cells and purified under endotoxin-free conditions by immunoaffinity with a rat mAb (MNB4) (Muller et al., 2001). Purified PTX3 was checked for purity by SDS-PAGE, and for lipopolysaccharide (LPS) contamination by *Limulus ameobocyte* lysate assay (Bio-Whittaker).

Isolation and culture of cumuli

Ovaries were dissected from 8- to 12-week-old mice injected 48 hours earlier with 5 IU pregnant mares' serum gonadotropin (PMSG), and cumulus cell-oocyte complexes (COCs) were mechanically isolated. COCs were cultured in drop, under mineral oil, of MEM supplemented with 1% fetal bovine serum (FBS), 3 mM glutamine, 0.3 mM sodium pyruvate and 50 ng/ml gentamycin, in the presence of 100 ng/ml FSH (highly purified rat-FSH; kindly provided by the NIDDF and the National Hormone and Pituitary Program, NIH, MD, USA), or 1 mM 8-Bromo cyclic cAMP (8Br cAMP; Sigma), or 1 ng/ml epidermal growth factor (EGF; Sigma), or 200 ng/ml prostaglandin E₂ (PGE₂; Sigma), at 37°C, 5% CO₂, for the time indicated in the text. In certain cases, human recombinant PTX3 was added at the beginning of culture. Cultures of isolated cumulus cells were generated by mechanical dissociation of the COCs in the culture drop and removal of the oocytes, as described (Salustri et al., 1990b).

Ovulated COCs were collected from the oviducts 14 hours after the injection of 5 IU human chorionic gonadotropin (hCG) into PMSG-primed mice, and cultured in MEM, supplemented as reported above, at 37°C, 5% CO₂ for the time indicated in the text.

Human cumulus cells and cumulus matrix were obtained from patients undergoing IVF.

Western analysis

Mouse COCs and human cumulus fragments were directly solubilized in a reducing Laemmli loading buffer, or treated with 1 U *Streptomyces* hyaluronidase (Calbiochem) for 2 hours at 37°C in the presence of protease inhibitors (Boehringer Mannheim) before adding

loading buffer. In certain cases, the culture medium was collected at the end of culture and mixed with an equal volume of 2×loading buffer. For PTX3 localization analysis, after digestion of COCs with *Streptomyces* hyaluronidase, oocytes were collected under the microscope and the sample microcentrifuged. Oocytes, cell pellet and supernatant (matrix extract) were mixed with loading buffer.

Protein extracts were separated by SDS-PAGE (7% acrylamide). Western-blotting analysis of PTX3 was performed by using a polyclonal antibody against mouse PTX3 (1 µg/ml) and monoclonal antibody 16B5 against human PTX3 (1 µg/ml), for mouse and human cumulus extracts, respectively. For I α I immunoblotting, a polyclonal antibody against human I α I (1:2000; Dako) was used.

Quantitation of HA

Compact COCs were stimulated with 100 ng/ml FSH, 1% fetal bovine serum (FBS), in the presence of [³⁵S]-sulfate (60 µCi/ml) and [³H]-glucosamine (100 µCi/ml; NEN Life Science Products, Zaventem, Belgium), for the time indicated in the text, at 37°C in 5% CO₂. Medium and cell-matrix were collected separately, and the amount of HA in the two compartments determined as described elsewhere (Camaioni et al., 1993).

Immunofluorescence analysis of PTX3 and HA

For localization studies of PTX3 and HA, COCs were incubated with 10 µg/ml rabbit anti-mouse PTX3 polyclonal antibody and 5 µg/ml biotinylated HA binding protein (HABP; Seikagaku) in phosphate buffer with 3% BSA for 2 hours at room temperature. After washing, COCs were incubated with FITC-labelled anti-rabbit IgG and streptavidin AlexaFloor 568 (Molecular Probes) for 1 hour in phosphate buffer with 3% BSA at room temperature. Nuclei were stained with Hoechst 33258. Cumuli were visualized with a fluorescence microscope.

PTX3 binding to hyaluronan and TNFAIP6

The interaction of PTX3 with recombinant human TNFAIP6 (Nentwich et al., 2002), or with the Link module from human TNFAIP6 (termed Link_TNFAIP6) (Kohda et al., 1996), was investigated using colorimetric microtitre plate assays essentially as described before (Mahoney et al., 2001). Initial experiments (in PBS, 0.05% Tween-20, at room temperature) compared the binding of biotinylated-PTX3 (bPTX3; 1000 ng/well, which corresponds to 22.2 pmol/well assuming a molecular mass of 45 kDa for the PTX3 protomer) and biotinylated-HA (12.5 ng/well) to plates coated with 25 pmol/well full-length TNFAIP6. All other assays were carried out in 50 mM Na-acetate, 100 mM NaCl, 0.05% Tween-20 (pH 6.0) [conditions that are optimal for the interaction of HA with Link_TNFAIP6 (Parker et al., 1998)], and measured the binding of bPTX3 (5-1000 ng/well) to Link_TNFAIP6 coated wells (25 pmol/well). Competition experiments were performed using 200 ng/well bPTX3 binding (i.e. a saturating amount) in the absence and presence of HA (0.1-2500 ng/well), Link_TNFAIP6 (2-5000 ng/well) or unlabelled PTX3 (2-5000 ng/well). All absorbance measurements (405 nm) were corrected by subtracting values from uncoated control wells.

PTX3 binding to sperm

Spermatozoa were isolated from the cauda epididymis and vas deferens of male mice of proven fertility and capacitated for 1 hour at 37°C (Hogan et al., 1994). Binding of soluble PTX3 to spermatozoa was characterized by cytofluorimeter analysis, and by immunofluorescence using 100 µg/ml FITC-labelled PTX3 and 10 µg/ml biotin-labelled PTX3, respectively.

For the adhesion assay, 35 mm cell culture plates were first coated with polylysine and, second, one half of the surface was layered with purified PTX3 (20 µg/ml, overnight at 4°C). PTX3 solution was then removed taking care not to spill into the other half of the surface. Plates were washed with PBS, filled with 50 mg/ml BSA in PBS and

incubated at 37°C for 1 hour. Plates were washed and, subsequently, 10⁶ sperm, suspended in 2 ml Whittingham's medium, were added. After 4 hours of incubation at 37°C, non-adherent cells were gently washed two times with PBS and the number of cells adhering to the two different coatings were blindly counted in eight random fields at 20× magnification.

Results

Role of Ptx3 in female fertility

Ptx3^{-/-} mice generated by homologous recombination display a severe defect in female fertility (Garlanda et al., 2002; Varani et al., 2002). Whereas heterozygous (+/-) females and males, and homozygous (-/-) males are normal and fertile, *Ptx3*^{-/-} females show compromised fertility, independently of the male genotype. *Ptx3*^{-/-} females on the 129/Sv background were sterile, and those on the 129/Sv×C57BL/6J background displayed severe subfertility as we observed occasional pregnancies (4 out of 10 females during a 60 day period) with one to two pups/mother versus a mean of one pregnancy every month with a mean of nine pups/mother in wild-type (+/+) females. All experiments reported in the present paper were carried out on 129/Sv females. Histological analysis of ovaries collected at various times from hormonally stimulated or naturally cycling females did not show morphological alteration of the follicles at any stage of development, except for at a few hours before ovulation, when cumulus cells failed to acquire a polarized and elongated shape during deposition of the viscoelastic extracellular matrix (cumulus expansion) and cumulus cell layers became disorganized (Fig. 1A-D). Oocytes collected from the oviduct of *Ptx3*^{-/-} mice at 14 hours after hCG injection were still associated with cumulus cells, but the cumuli showed several abnormalities. In wild-type and *Ptx3*^{+/-} mice, cumuli were well structured, with cumulus cells radiating out from a central oocyte (Fig. 1E). By contrast, cumuli from *Ptx3*^{-/-} mice were disorganized, with cumulus cells uniformly dispersed in the cumulus mass, and the oocytes randomly located (Fig. 1F). In addition, the viscoelastic matrix, in which cumulus cells and oocytes were embedded, spontaneously dissolved in a short time in vitro (15-60 minutes in *Ptx3*^{-/-} versus several hours in *Ptx3*^{+/-} cumuli), quickly leading to oocyte denudation (Fig. 1G,H). Instability of the matrix was observed in vivo as well: a few hours after ovulation (20 hours after hCG), *Ptx3*^{+/-} cumuli were still arranged around the oocytes, whereas *Ptx3*^{-/-} cumuli were dissociated, and cumulus cells and oocytes were dispersed in the oviduct. The number of oocytes collected at day 0.5 after natural mating, or at 14 hours after hCG injection, was comparable in *Ptx3*^{+/-} and *Ptx3*^{-/-} mice (Table 1). These data indicate that ovulation rate is normal in *Ptx3*-deficient mice.

In agreement with previous observations (Varani et al., 2002), fertilization of oocytes was impaired in *Ptx3*^{-/-} mice. We never found oocytes developing to the two-cell stage (day 1.5) (Table 1), nor oocytes with two pronuclei (day 0.5; data not shown), in spontaneously ovulating or superovulated *Ptx3*^{-/-} females after mating. Fertilization failure could not be ascribed to impaired oocyte meiotic maturation, as we did not find any difference in the percentage of oocytes showing the first polar body (Table 1). To evaluate whether lack of in vivo fertilization might be secondary to abnormalities of the oocytes preventing sperm penetration, in vitro fertilization (IVF)

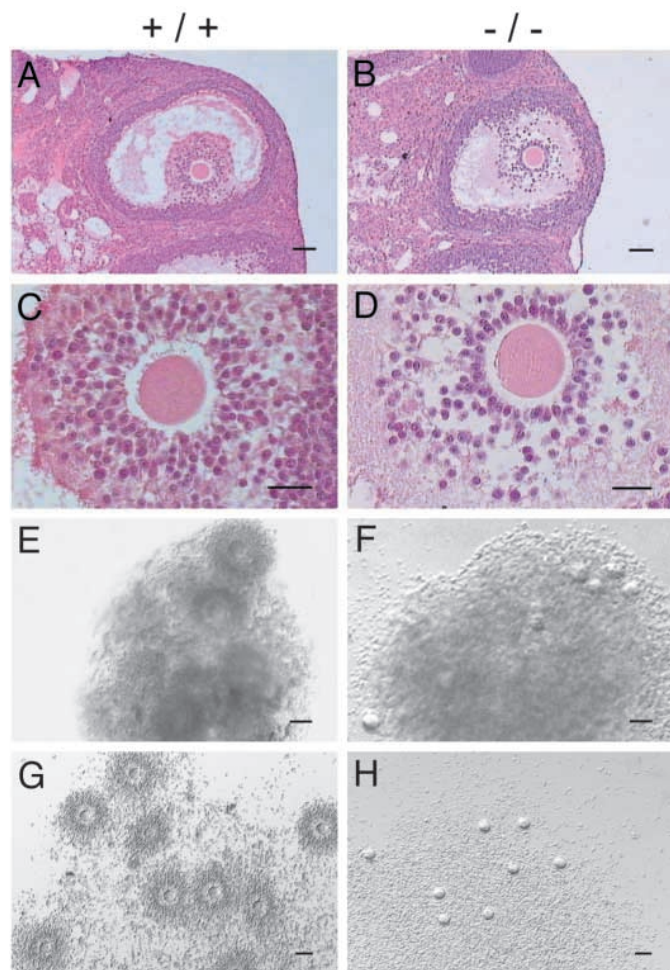


Fig. 1. Defective cumulus expansion in *Ptx3*^{-/-} mice. (A-D) Morphology of cumuli oophori during matrix deposition. Micrographs show individual preovulatory follicles 10 hours after hCG (A,B) and an enlargement of the enclosed cumuli (C,D). (E-H) Morphology of *Ptx3*^{+/+} and *Ptx3*^{-/-} ovulated cumuli immediately after isolation (E,F), or after 1 hour of in vitro culture (G,H). Scale Bars: 100 μ m in A,B,E-H; 50 μ m in C,D.

experiments were performed using wild-type sperm from adult males to inseminate *Ptx3*^{+/+} or *Ptx3*^{-/-} oocytes (Table 1). IVF was first conducted with oocytes freed of the zona pellucida and stained with the DNA-specific fluorochrome Hoechst 33258 to observe fusion. Under these conditions we observed normal sperm binding to the *Ptx3*^{-/-} oocyte plasma membrane, and comparable fusion of *Ptx3*^{+/+} and *Ptx3*^{-/-} oocytes with sperm (Table 1). Zona pellucida-intact *Ptx3*^{-/-} oocytes collected 13-15 hours after hCG were also fertilized and progressed to the two-cell stage (Table 1), or to the blastocyst stage (not shown), with percentages comparable to *Ptx3*^{+/+} oocytes. In addition, zygotes obtained from in vitro fertilized *Ptx3*^{-/-} oocytes were able to develop and implant in vivo when transferred to the uterus of wild-type pseudopregnant females (data not shown). Reciprocally, when *Ptx3*^{+/+} blastocysts were transferred to the uterus of *Ptx3*^{-/-} pseudopregnant females, normal pregnancy and delivery was observed (data not shown).

Collectively, these data provide evidence that abnormalities

Table 1. Defective in vivo fertilization in *Ptx3*^{-/-} mice

	<i>Ptx3</i> ^{+/+}	<i>Ptx3</i> ^{-/-}	<i>P</i> value
Ovulated oocytes			
Spontaneous ovulation	7/mouse (<i>n</i> =4)	7.8/mouse (<i>n</i> =8)	NS [¶]
After superovulation	35/mouse (<i>n</i> =9)	27/mouse (<i>n</i> =18)	NS
Oocytes with first polar body	77%	70%	NS
In vivo fertilization ^{***}			
Spontaneous ovulation	17/28 (60%)	0/39 (0%)	<0.0001 [§]
After superovulation	81/162 (50%)	0/192 (0%)	<0.0001
In vitro fertilization ^{**}			
Zona-free oocytes [†]	21/27 (77%)	21/31 (68%)	NS
Intact oocytes [‡]	79/189 (42%)	68/169 (40%)	NS

*Embryos were collected at 1.5 days post coitum, at the two-cell stage.
[†]Fusion was assessed by the dye transfer technique 4 hours after insemination.
[‡]Two-cells embryos were counted the day after insemination.
[§]Fischer's exact test. *P*<0.05 indicates significance.
[¶]NS, not significantly different from control *Ptx3*^{+/+} or *Ptx3*^{+/-} mice.
^{**}Numbers represent fertilized eggs over total.

in the cumulus underlie in vivo fertilization failure and infertility of *Ptx3*^{-/-} females, and strongly suggest that additional developmental defects are unlikely.

We also examined whether *Ptx3* deficiency could affect the implantation process as well. We observed normal pregnancy and delivery (data not shown) when *Ptx3*^{+/+} blastocysts were transferred to the uterus of *Ptx3*^{-/-} pseudopregnant females, suggesting that implantation and subsequent processes are not altered in *Ptx3*^{-/-} mice.

PTX3 is produced by cumulus cells and localizes in the matrix

Ptx3 mRNA expression in naturally cycling mice was confined to cumulus cells and to a few granulosa cells lining the follicle antrum of preovulatory follicles, with no evidence of *Ptx3* transcripts in oocytes, peripheral granulosa cells, theca cells or interstitial ovarian tissue (Fig. 2A). Northern blot analysis of whole ovary revealed that *Ptx3* mRNA expression starts 2 hours after injection of an ovulatory dose of hCG in PMSG-primed mice, peaks at 6 hours and declines thereafter (Fig. 2B), showing close temporal correlation to matrix deposition by cumulus cells and cumulus expansion (Salustri et al., 1989; Salustri et al., 1992).

We then analyzed PTX3 localization in ovulated cumuli. Western blot analysis indicated that PTX3 was associated with the extracellular matrix, as cumulus cells and oocytes isolated by hyaluronidase digestion did not show immune reactivity (Fig. 2C). Immunofluorescence analysis of *Ptx3*^{+/+} cumuli confirmed the localization of PTX3 in the extracellular matrix (Fig. 2D).

Previous studies have shown that cumuli oophori are induced to expand in vitro by the combined action of a soluble factor produced by the oocytes and follicle-stimulating hormone (FSH), or cyclic AMP, EGF or PGE₂ (Salustri et al., 1990a; Salustri et al., 1990b; Buccione et al., 1990; Tirone et al., 1997). Results reported in Fig. 3 show that such synergistic action is also required to induce PTX3 expression by in vitro cultured cumulus cells. FSH, 8Br-cAMP, EGF and PGE₂ stimulated PTX3 synthesis by intact cumuli (Fig. 3A), but

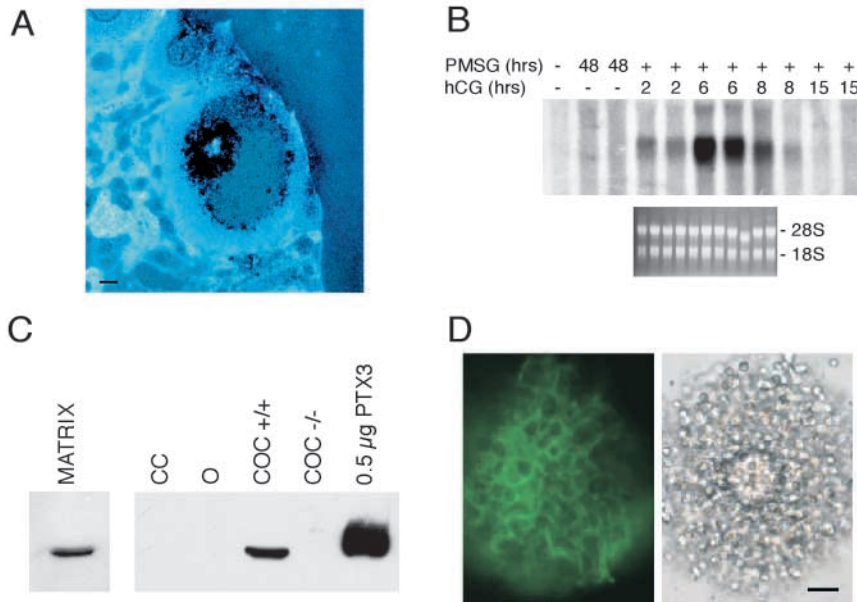


Fig. 2. PTX3 is expressed by cumulus cells in the preovulatory follicle and localizes in the matrix. (A) In situ hybridization of an ovary isolated from a naturally cycling mouse. (B) Kinetics of *Ptx3* mRNA expression in ovaries during hormonally induced ovulation. Each lane was loaded with 10 μ g of total RNA. Ethidium bromide staining of the gel is shown in the lower panel. (C) Detection of PTX3 protein in ovulated intact cumuli oophori (COC), isolated cumulus cells (CC), denuded oocytes (O) and cumulus matrix (matrix) assessed by western blotting. (D) Immunofluorescence analysis (left panel) and phase contrast (right panel) of a cumulus oophorus. Scale bars: 100 μ m in A; 50 μ m in D.

FSH, and all of the above-mentioned factors, failed to do so when cumulus cells were dissected from the cumuli and cultured in the absence of oocytes (Fig. 3B, and data not shown). Interestingly, most PTX3 synthesized during in vitro expansion was retained in the matrix, mimicking the in vivo preovulatory condition.

Role of PTX3 in the formation of the cumulus matrix

The role of PTX3 in cumulus matrix synthesis and stability was investigated by in vitro expansion of cumuli. Cumuli were isolated from PMSG-primed *Ptx3*^{+/-} and *Ptx3*^{-/-} mice, and cultured in the presence of FSH and FBS. Radiolabeled precursors of glycosaminoglycans were added to the culture to detect production and organization of HA at different culture times. At 16 hours of culture, most of the HA synthesized by *Ptx3*^{+/-} cumuli was retained in the matrix, and cumulus cells remained embedded within the expanded viscoelastic matrix surrounding the oocytes (Fig. 4Aa). *Ptx3*^{-/-} cumuli synthesized normal amounts of HA but were unable to form a stable matrix, as shown by both the release of HA in the medium and the dispersion of cumulus cells (Fig. 4Ab). Note, if *Ptx3*^{-/-} cumuli were stimulated in the presence of recombinant PTX3, a normal cumulus phenotype was restored (Fig. 4Ac). A dose-response curve (data not shown) revealed that 50% rescue was achieved with 0.3 μ g/ml PTX3 and 100% rescue was achieved with 1 μ g/ml. The matrix formed by *Ptx3*^{-/-} cumuli cultured in the presence of recombinant PTX3 showed the same stability as that formed in vitro or in vivo by *Ptx3*^{+/-} cumuli, undergoing spontaneous degradation during the following 8-12 hours of culture (24-28 hours total culture; Fig. 4B). Accordingly, supplementation of the culture medium with recombinant PTX3 did not prevent or delay degradation of the HA-matrix assembled by *Ptx3*^{+/-} cumuli (Fig. 4B). Altogether, these data clearly indicate a role for PTX3 in assembling the HA-enriched matrix of the cumulus rather than in preventing its degradation, as previously hypothesized (Varani et al., 2002).

A crucial step of cumulus matrix assembly seems to be the transfer and covalent linkage of the heavy chains of serum I α I

protein to HA (Chen et al., 1996; Zhuo et al., 2001a; Fulop et al., 2003). Therefore, we assessed whether the formation or the integrity of the heavy chain-HA complexes in the cumulus matrix was impaired in *Ptx3*^{-/-} mice. Western blot analysis with anti-I α I antibody showed that the bands obtained after hyaluronidase treatment of *Ptx3*^{+/-} and *Ptx3*^{-/-} ovulated cumuli were identical in both molecular weight and intensity (Fig. 5A). The formation of a covalent complex between a heavy chain of I α I and a TNFAIP6 molecule has been previously reported and hypothesized to contribute to the cumulus matrix (Carrette et al., 2001; Mukhopadhyay et al., 2001; Fulop et al., 2003; Ochsner et al., 2003). This complex is detectable as an ~120 kDa band by the I α I antibody in hyaluronidase-digested and undigested extracts of *Ptx3*^{+/-}, as well as of *Ptx3*^{-/-} cumuli (Fig. 5A). These results indicate that PTX3 is not involved in the incorporation of I α I-heavy chains in the matrix or their protection from proteolytic enzymes, and that its stabilizing activity is exerted through a mechanism independent of I α I.

PTX3 does not appear to form covalent or tight linkages with the HA matrix as digestion of cumuli with hyaluronidase did

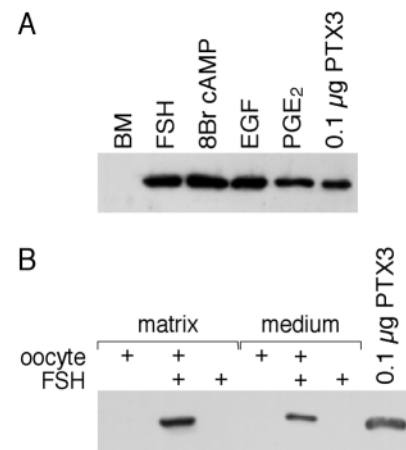
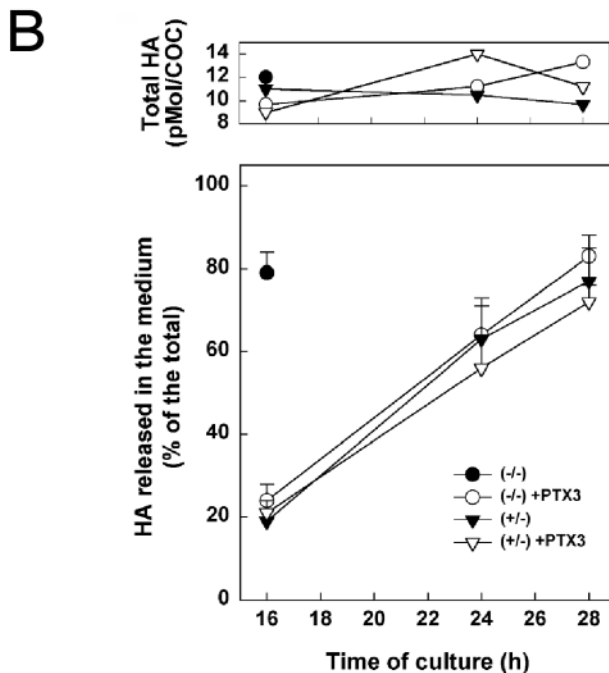
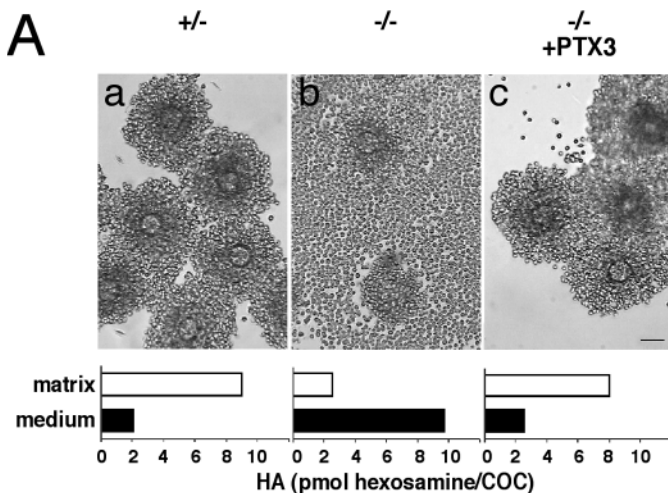


Fig. 3. PTX3 synthesis by in vitro cultured cumulus cells. (A) COCs (30/20 μ l) were cultured for 16 hours in the absence (BM, basal medium) or presence of the indicated stimuli. (B) Cumulus cells isolated from 30 COCs were cultured for 16 hours in the presence of 60 oocytes or FSH, or both. PTX3 was assessed in the matrix and medium by western blotting.



not increase the intensity of the band recognized by the anti-PTX3 antibody in western blot analysis of matrix extracts (Fig. 5B).

Double staining of *Pttx3*^{+/-} ovulated cumuli for HA and PTX3 revealed that PTX3 co-localizes with HA in the extracellular matrix (Fig. 6). Indeed, PTX3 was organized in a fine fibril network, extending from the outer region of the cumulus to the zona pellucida, which appears to be intimately associated to HA.

The HA-binding glycoprotein TNFAIP6 is synthesized by cumulus cells in parallel with PTX3 (Varani et al., 2002), and plays an essential role in cumulus matrix formation (Fulop et al., 2003). Therefore, we assessed whether PTX3 could interact with TNFAIP6. Microtitre plate-binding experiments revealed an interaction between immobilized full-length human TNFAIP6 and soluble bPTX3 (Fig. 7A); however, there was no binding of bPTX3 to HA-coated wells (data not shown). As

Fig. 4. Effects of PTX3 on matrix organization and cumulus expansion. (A) PTX3 is necessary for matrix assembly and cumulus expansion. COCs (20/20 μ l) were cultured for 16 hours in the presence of FSH and FBS, and with 1 μ g/ml recombinant PTX3 where indicated. HA evaluation was performed as indicated in Materials and methods. Cumulus expansion and HA distribution between matrix and medium were assessed for (a) *Pttx3*^{+/-} cumuli, (b) *Pttx3*^{-/-} cumuli and (c) *Pttx3*^{-/-} cumuli cultured with recombinant PTX3. Reported data refer to one representative experiment of three performed. Scale bar: 100 μ m. (B) Degradation of expanded cumulus matrix is not affected by recombinant PTX3. Degradation of the HA-rich matrix was assessed at 24 and 28 hours of culture by calculating the proportion of the total HA released in the culture medium, as described previously (D'Alessandris et al., 2001). In the upper panel the total amount of HA (pmol hexosamine)/COC at each time culture is reported. Error lines indicate the standard deviation of duplicate cultures.

can be seen from Fig. 7B, biotinylated-PTX3 also bound in a dose-dependent manner to wells coated with Link_TNFAIP6, the isolated Link module from human TNFAIP6 [i.e. its HA-binding domain (Kohda et al., 1996)]. This interaction could be competed out with unlabelled PTX3 and Link_TNFAIP6, but not with HA (Fig. 7C); in molar terms the estimated IC₅₀ values were essentially equivalent for the TNFAIP6 Link module (11 kDa) and the PTX3 protomer (45 kDa). Under the same experimental conditions, HA interacted with immobilized Link_TNFAIP6 [as described previously (Mahoney et al., 2001) (Fig. 7A)], but no binding of HA to immobilized PTX3 was detectable (data not shown). Single site mutants of Link_TNFAIP6 (K11Q, Y12F, Y59F, F70V and Y78F), which have significantly impaired HA-binding activities (Mahoney et al., 2001; Getting et al., 2002), interact with PTX3 with the same apparent affinity as the wild-type protein (data not shown). These data indicate that PTX3 interacts with the TNFAIP6 Link-module domain at a site distinct from its HA-binding surface and that this interaction does not interfere with its ability to bind HA. In addition, the

Fig. 5. Incorporation of I α I and PTX3 in the matrix.

(A) Covalent linkage of I α I heavy chains to HA normally occurs in the cumulus matrix of *Pttx3*^{-/-} mice. Western blot analysis, with anti-I α I, of *Pttx3*^{+/-} and *Pttx3*^{-/-} ovulated cumuli undigested and digested with hyaluronidase (ha'ase). A ~85 kDa band (representing single I α I heavy chains) and additional high molecular weight bands (most likely representing dimers and clusters of I α I heavy chains) were detected after

hyaluronidase digestion in

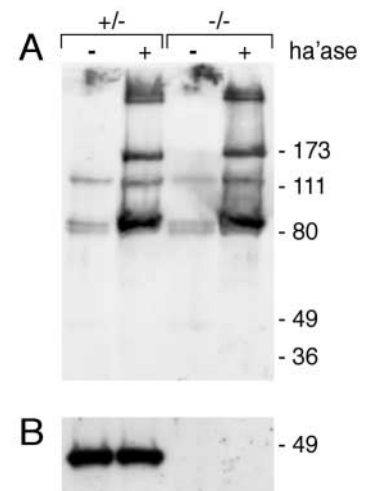
both *Pttx3*^{+/-} and *Pttx3*^{-/-}

cumulus matrix. (B) PTX3 is not covalently bound to HA in the

cumulus matrix. The band recognized by anti-PTX3 shows the same

intensity in undigested and digested *Pttx3*^{+/-} cumuli. As expected, no

PTX3 positivity was found in *Pttx3*^{-/-} cumuli.



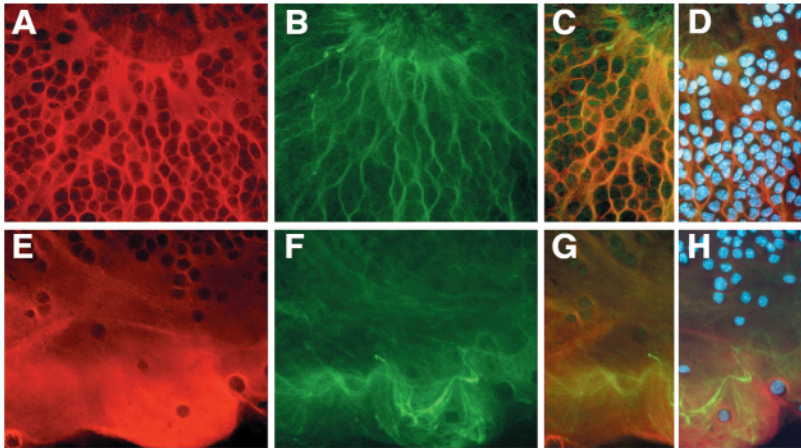


Fig. 6. Co-localization of PTX3 and HA in ovulated cumuli. *Ptx3*^{+/-} cumuli were stained for HA (red), PTX3 (green) and chromatin (blue), as reported in Materials and methods, and the innermost (A-D) and outermost (E-H) layers of a cumulus were photographed using a fluorescence microscope. HA histochemical staining (A,E); PTX3 immunostaining (B,F); merging of the two signals (C,G); and staining of cumulus cell nuclei (D,H). Cumuli incubated with secondary probes only showed no specific staining (data not shown).

competition studies (Fig. 7C) suggest that each protomer in a PTX3 10/20-mer (Bottazzi et al., 1997) can bind an individual TNFAIP6 molecule and, therefore, may form a multi-

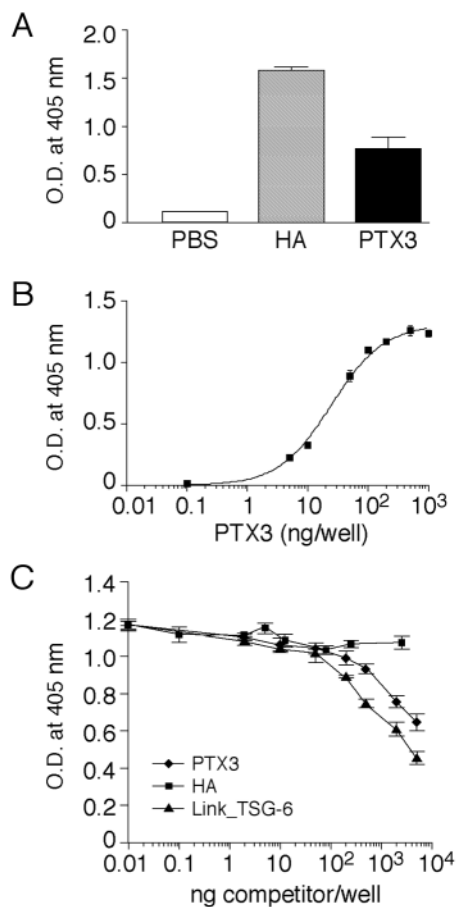


Fig. 7. Binding of PTX3 to TNFAIP6. (A) Binding of bHA (12.5 ng/well) or bPTX3 (22.2 pmol/well), or PBS as a control, to immobilized TNFAIP6 (25 pmol/well; $n=3$). (B) Dose-dependent binding of different amounts of soluble bPTX3 to immobilized Link_TNFAIP6 (25 pmol/well; $n=4$). (C) Effect of different amounts of competitors (PTX3, HA, Link_TNFAIP6) on the interaction of bPTX3 (200 ng/well) with Link_TNFAIP6-coated plates ($n=4$).

molecular complex that acts as a 'node' for cross-linking HA chains in the cumulus matrix.

PTX3 binding to sperm

Having found that PTX3 is an essential component of the extracellular matrix of the cumulus oophorus, it was important to assess whether spermatozoa could interact with this matrix component. As indicated by cytofluorimetric and immunofluorescence analysis (Fig. 8A,B), spermatozoa bound soluble PTX3 and the binding site was localized to the subacrosomal region. To test whether spermatozoa could also interact with plastic-immobilized PTX3, mimicking a matrix substrate, capacitated live sperm cells were incubated in culture dishes coated with either PTX3 or BSA. Consistent with a possible role of PTX3 in sperm-cumulus matrix interaction, the number of sperm adhering to the PTX3-coated surface was significantly higher than the number adhering to a BSA-coated control surface (704 ± 69 versus 298 ± 38 sperm/mm², $P=0.013$, as assessed by Student's *t*-test) (Fig. 8C).

Expression of PTX3 in human ovarian tissues

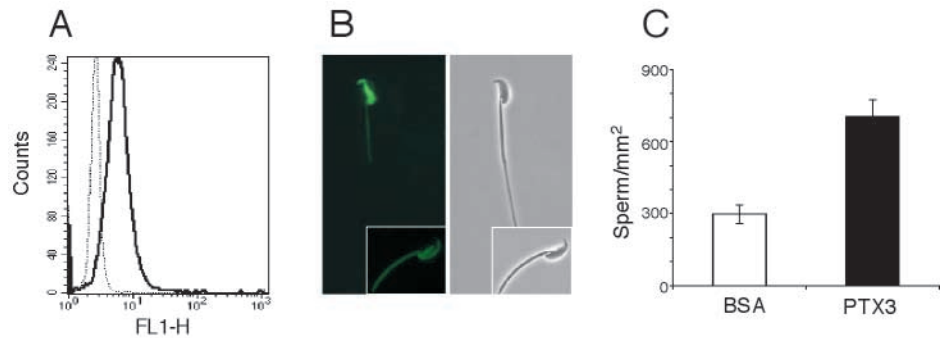
Given the conserved primary structure and regulation of PTX3 between mouse and man (Introna et al., 1996), it was important to study the expression of PTX3 by human cumuli oophori. Northern blot analysis revealed that cumulus cells obtained from patients undergoing IVF express *PTX3* mRNA (Fig. 9A). Furthermore, PTX3 protein could be detected by western blotting in the cumulus matrix (Fig. 9B), and by ELISA in the follicular fluid (mean 11.4 ng/ml, range 3.2-27.9 ng/ml; Fig. 9C).

Discussion

Cross-linking of HA molecules by proteins is essential for cumulus matrix formation (Zhuo et al., 2001a; Fulop et al., 2003). The results reported here provide a new insight into the molecular structure of this oocyte investment, showing that the long pentraxin PTX3 plays a crucial role in the assembly of the HA-rich matrix of the cumulus oophorus.

Expression of PTX3 is induced by primary inflammatory signals in various cell types in vitro and in vivo (Breviaro et al., 1992; Lee et al., 1993; Introna et al., 1996). Several lines of evidence, including the phenotype of COX2- and prostaglandin receptor E receptor subtype EP(2)-deficient mice (Lim et al., 1997; Davis et al., 1999; Hizaki et al., 1999; Tilley et al., 1999), point to analogies between the process of ovulation and inflammation (Espsey, 1994; Richards et al., 2002). In addition to a hormonal ovulatory stimulus, oocyte

Fig. 8. Binding of soluble PTX3 (2.2×10^{-6} M) to spermatozoa as assessed by FACS (A) and immunofluorescence (B). (C) Differential localization and binding of spermatozoa cultured on PTX3- or BSA-coated plastic ($P=0.013$; Student's *t*-test).



soluble factors are required for eliciting HA synthesis and cumulus expansion (Salustri et al., 1990b; Buccione et al., 1990). Likewise, we show here that expression of *Ptx3* is induced in cumulus cells by an ovulatory stimulus, and that the oocyte influences this response. *Ptx3* expression was also detected in granulosa cells lining the antral cavity of the preovulatory follicle. This finding is consistent with previous observations showing that such granulosa cell subpopulations synthesize HA-rich matrix and become included in the expanded cumulus. Experimental evidence suggests that a gradient of oocyte factor(s) is established in the preovulatory follicle that influences cumulus cells as well as antral granulosa cells (Salustri et al., 1992). Growth differentiation factor 9 (GDF9), is probably the oocyte factor involved in the control of such processes (Elvin et al., 1999; Varani et al., 2002). Therefore, interplay between different signals is likely to be required for temporally and anatomically restricted PTX3 expression during the periovulatory period. The synthesis of PTX3 during both ovulation and inflammation adds a further element linking these processes.

PTX3 localizes in the cumulus matrix and plays a crucial role in cumulus expansion. In *Ptx3*-deficient mice, corona radiata cells fail to polarize and randomly surround the oocyte in the preovulatory follicle. After ovulation, single COCs are no longer identifiable: the oocytes appear scattered in an unstable, uniform mass that quickly disaggregates into single cells. Experiments performed *in vitro* clearly show that *Ptx3*^{-/-} cumuli are unable to retain HA within the matrix. Presently, two additional molecules have been identified that are involved in HA organization in the cumulus matrix, TNFAIP6 and I α I. TNFAIP6 is a protein able to tightly bind HA through a link module (Kohda et al., 1996; Milner and Day, 2003). It is produced by cumulus cells during the expansion process with a temporal pattern identical to that of PTX3 and HA. I α I is a serum protein complex, formed by two heavy chains and a light chain (bikunin) covalently linked to a chondroitin sulfate moiety, which diffuses into the follicle after

the luteinizing hormone (LH) surge, when cumulus expansion is triggered (Powers et al., 1995). Bikunin^{-/-} mice (which cannot assemble I α I) (Zhuo et al., 2001a) and *Tnfaip6*^{-/-} mice (Fulop et al., 2003) are infertile because of instability of the cumulus matrix and lack of oocyte fertilization, like *Ptx3*^{-/-} mice. Co-operative interaction between TNFAIP6 and I α I has been demonstrated. In the expanding cumulus the heavy chains of I α I are covalently transferred from the chondroitin sulfate to HA (Chen et al., 1996), and TNFAIP6 is clearly essential for completing this coupling reaction, as heavy chain-HA complexes do not form in *Tnfaip6*-deficient mice (Fulop et al., 2003). In addition, a covalent complex between one TNFAIP6 molecule and one of the heavy chains is also formed, and accumulates in the cumulus matrix (Mukhopadhyay et al., 2001). It has been proposed that HA-linked heavy chains and TNFAIP6-heavy chain complexes stabilize the cumulus matrix by cross-linking separate HA molecules (Chen et al., 1992; Chen et al., 1996; Zhuo et al., 2001a; Fulop et al., 2003). Here, we demonstrate that both types of complexes are present in the *Ptx3*^{-/-} cumuli. This indicates that, although necessary, these complexes are not sufficient to confer stability to the cumulus matrix and that the PTX3 matrix-stabilizing activity is exerted through an independent mechanism. In this regard, PTX3 binds TNFAIP6 (through a site that is distinct from its HA-binding surface) and could therefore serve as an additional way of cross-linking the matrix via the association of TNFAIP6 with HA. PTX3, as TNFAIP6 (Carrette et al., 2001), co-localizes with HA throughout the matrix, from the periphery of the cumulus to the zona pellucida. As PTX3 is unable to bind to or form covalent bonds with HA, TNFAIP6 is the likely matrix component involved in mediating such interaction. PTX3 is predominantly assembled as a large multimer complex consisting of two decamers (Bottazzi et al., 1997), and competition-binding studies suggest that each protomer can bind an individual TNFAIP6 molecule. As illustrated in Fig. 10, PTX3/TNFAIP6 complexes might thus serve as an anchoring site for multiple HA molecules, thereby

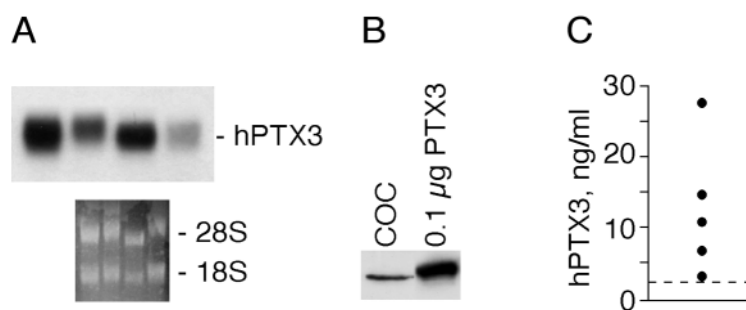


Fig. 9. Expression of PTX3 in human ovarian tissues.

(A) *PTX3* mRNA expression by human cumulus cells in four different patients undergoing IVF. Each lane was loaded with 10 μ g of total RNA. Ethidium bromide staining of the gel is shown in the lower panel. (B) Detection of PTX3 in human cumulus matrix by western blot analysis. Lane 1, human cumuli; lane 2, recombinant PTX3. (C) PTX3 protein levels in the follicular fluid of five different patients undergoing IVF. The dotted line indicates PTX3 plasma levels in healthy donors.

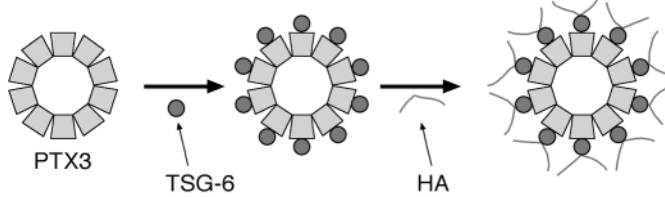


Fig. 10. Schematic model of complex formed between PTX3 and TNFAIP6 in the cumulus matrix that could act as a node to cross-link multiple HA chains.

substantially strengthening and stabilizing the HA network. HA-protein interaction is crucial for the formation and stability of extracellular matrix in several tissues in both physiological and pathological conditions (Day and Prestwich, 2002; Tammi et al., 2002). The finding that the long pentraxin PTX3 is a component of the extracellular matrix of the cumulus oophorus, essential for HA organization, raises the likely possibility of a similar localization and function of this molecule in certain HA-enriched inflammatory tissues, such as occur in rheumatoid arthritis, where TNFAIP6 is also expressed (Milner and Day, 2003).

Oocytes, albeit loosely associated to the cumulus masses, were ovulated in normal numbers and transferred to the oviduct in *Ptx3*^{-/-} mice. This result is in apparent conflict with data reported by Varani et al. (Varani et al., 2002), who observed a drastic (50–70%) reduction in the number of oocytes in the ampullae of superovulated *Ptx3*^{-/-} female mice. A possible explanation for this is the rapid dispersion of the cumulus mass in these mice, as compared with in wild-type mice, and the difficulty of recovering the oocytes at 20 hours after hCG, the time at which Varani et al. performed their evaluation. Oocytes ovulated by *Ptx3*-null female mice were not fertilized in vivo, but could be fertilized in vitro. These results are consistent with the crucial role assigned to normal cumulus expansion for sperm recruitment in vivo, a role possibly connected with the presence of a very low number of spermatozoa at the site of in vivo fertilization, and with the necessity for sperm to be guided to the oocyte (Eisenbach and Tur-Kaspa, 1999). Defects in cumulus matrix assembly and the absence of a corona radiata in *Ptx3*^{-/-} mice most likely alter the mechanisms facilitating access of sperm to the oocyte. The finding that spermatozoa can bind soluble and immobilized PTX3 are intriguing, and raise the additional possibility that the absence of PTX3 might perturb interactions of the sperm with the matrix during in vivo fertilization. However, the significance of these observations is at present only speculative, and further studies are needed to validate this hypothesis. Besides the in vivo fertilization process, PTX3 deficiency did not affect any additional step of mouse reproduction, including ovulation, implantation, embryo development and delivery.

Finally, the primary structure of PTX3 is conserved between mouse and humans (Introna et al., 1996), and we report that PTX3 is also expressed in the human periovulatory cumulus oophorus, and that it is present in the cumulus matrix as well. Therefore, it is likely that PTX3 may play the same role in human, as it does in mouse female fertility. This implies that PTX3 deficiency might be a cause of unexplained infertility in women. Based on the *Ptx3*^{-/-} mouse model, PTX3-deficient women would be infertile despite a normal ovulation. Hormonal therapy

and/or artificial insemination would be ineffective in overcoming pregnancy failure in these women, and in vitro fertilization should be considered the first-choice treatment for them.

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