Volume 58(10): 903–915, 2010
Journal of Histochemistry & Cytochemistry
http://www.jhc.org

ARTICLE

Fibrillins in Adult Human Ovary and Polycystic Ovary Syndrome: Is Fibrillin-3 Affected in PCOS?

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SUMMARY Polycystic ovary syndrome (PCOS) is a common endocrinopathy in women of reproductive age. Although genetic linkage analyses have demonstrated a susceptibility locus for PCOS mapping to the *fibrillin-3* gene, the presence of fibrillin proteins in normal and polycystic ovaries has not been characterized. This study compared and contrasted fibrillin-1, -2, and -3 localization in normal and polycystic ovaries. Immunohistochemical stainings of ovaries from 21 controls and 9 patients with PCOS were performed. Fibrillin-1 was ubiquitous in ovarian connective tissue. Fibrillin-2 localized around antral follicles and in areas of folliculolysis. Fibrillin-3 was present in a restricted distribution within the specialized perifollicular stroma of follicles in morphological transition from primordial to primary type [transitional follicles (TFs)]. Fibrillin-1 and -2 stainings of PCOS ovaries were similar to those of the controls. However, in eight of the nine PCOS ovaries, there was a decrease in the number of TFs associated with fibrillin-3, including no staining in five PCOS samples; decreased number of fibrillin-3-associated TFs/mm^2 was confirmed by quantitative analysis. Our findings support a role for fibrillin-3 in the pathogenesis of PCOS and suggest fibrillin-3 may function in primordial to primary follicle transition. We propose that loss of fibrillin-3 during folliculogenesis may be an important factor in PCOS pathogenesis. **(J Histochem Cytochem 58:903–915, 2010)**

KEY WORDS
fibrillin
fibrillin-3
folliculogenesis
polycystic ovary syndrome
ovary

POLYCYSTIC OVARY SYNDROME (PCOS) is a common endocrinopathy affecting an estimated 6–10% of women worldwide (Azziz et al. 2009). It represents the most common endocrinopathy of women of reproductive age. Although descriptions of polycystic ovaries date back to the early 1700s, variable degrees of virilization, menstrual abnormalities, and bilaterally enlarged polycystic ovaries form the basis for the definition of PCOS (Stein and Leventhal 1935). In addition to the clinical manifestations of infertility and virilization, metabolic consequences of PCOS include obesity, type II diabetes, hyperlipidemia, hypertension, and cardiovascular disease (Fleischman and Mansfield 2005).

To date, there is no overall consensus on the definition of PCOS or the criteria used for diagnosis. Current understanding for diagnosis encompasses the 1990 National Institutes of Health criteria (Zawadski and Dunaif 1992), Rotterdam 2003 criteria (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004), and most recently the Androgen Excess-PCOS (AE-PCOS) Society criteria (Azziz et al. 2009). According to the AE-PCOS Society's working definition, PCOS is a disorder of androgen excess or hyperandrogenism with the following clinical findings: hyperandrogenism (hirsuitism and/or hyperandrogenemia), ovarian dysfunction (oligo-anovulation and/or polycystic ovaries), and the exclusion of other androgen excess–related disorders (Azziz et al. 2009).

PCOS has a familial tendency and as such is considered to be a complex trait with a major genetic component (Amato and Simpson 2004). Numerous candidate genes have been proposed for involvement in PCOS

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Received for publication May 4, 2010; accepted June 28, 2010 [DOI: 10.1369/jhc.2010.956615].

(Urbanek et al. 1999; Amato and Simpson 2004; Unluturk et al. 2007; Dasgupta and Reddy 2008). In particular, a candidate gene region on chromosome 19p13.2 has been identified (Urbanek et al. 2005). This PCOS susceptibility locus maps closely to D19S884 by genetic linkage analysis of short-tandem repeat polymorphisms and PCOS family-based association in 367 families (Urbanek et al. 2005). Genes that map to within 100 kb of D19S884 are *FBN3* (encoding fibrillin-3), *ELAVL1* (encoding an mRNA-binding protein), and *CCL25* (encoding a thymus-expressed chemokine) (Urbanek et al. 2005). The dinucleotide repeat D19S884, which maps to intron 55 of the *FBN3*, was identified as the most likely PCOS susceptibility locus, and this polymorphic variant within *FBN3* confers an increased risk of PCOS (Urbanek et al. 2005; Stewart et al. 2006; Ewens et al. 2010). The PCOS susceptibility allele, A8, is also associated with metabolic consequences seen in both the women with PCOS and their brothers (Urbanek et al. 2007).

Association of PCOS with *FBN3* is controversial. A study using single-nucleotide polymorphisms (SNPs) in the vicinity of *FBN3* showed no significant associations of *FBN3* with PCOS (Prodoehl et al. 2009). However, a more recent SNP analysis reaffirmed the association between a region in *FBN3* and PCOS (Ewens et al. 2010). The differences between these two most recent studies are as follows: (1) Prodoehl et al.'s study was a case–control study, whereas Ewens et al.'s study was family based; (2) the sample size in the study by Ewens et al. was larger; and (3) the definition of PCOS in Ewens et al.'s study was much narrower. Because PCOS is known to be a heterogeneous disorder, family-based studies with a narrow clinical definition of PCOS were required to allow the identification of *FBN3* as a likely candidate gene.

Expression studies of ovarian tissues showed that levels of *FBN1* mRNA are greater than those of *FBN2* mRNA and much greater than those of *FBN3* mRNA (Prodoehl et al. 2009). These differences in mRNA expression levels do not provide important information about protein levels and locations. It is unknown if and where fibrillin-3 microfibrils are found in normal adult human ovary and whether the quantity or distribution of fibrillin-3 is abnormal in PCOS. This information is required in order to elucidate the role, if any, of fibrillin-3 in ovarian function and PCOS.

Fibrillins are multidomain extracellular matrix glycoproteins that are present in all connective tissues (Sakai et al. 1986; Maslen et al. 1991; Corson et al. 1993, 2004; Zhang et al. 1995). The expression and distribution of fibrillin-2 and fibrillin-3 are largely limited to fetal development (Zhang et al. 1995; Corson et al. 2004). Fibrillins form the backbone structure of small-diameter "microfibrils" (Sakai et al. 1986,1991; Reinhardt et al. 1996; Charbonneau et al. 2003; Corson et al. 2004). In addition to this architectural function, fibrillins perform regulatory functions by binding and sequestering growth factors (Charbonneau et al. 2004; Ramirez and Sakai 2010). Interactions between the large latent transforming growth factor-β (TGF-β) complex and fibrillin microfibrils are mediated through direct binding of latent TGF-β-binding protein (LTBP)-1 and LTBP-4 to fibrillin-1 (Isogai et al. 2003; Ono et al. 2009). These interactions have formed the basis for proposed pathogenetic mechanisms leading to activation of TGF-β signaling in the Marfan syndrome, an inherited disorder of connective tissue caused by mutations in fibrillin1 (Neptune et al. 2003). In addition, fibrillins interact with the prodomains of bone morphogenetic proteins (BMPs) and growth and differentiation factors (GDFs; Gregory et al. 2005; Sengle et al. 2008), raising the possibility that abnormalities in fibrillin microfibrils may alter BMP and GDF signaling and TGF-β signaling.

Many members of the TGF-β superfamily are involved in the development and function of the ovary. Cell culture studies and studies in rodents have implicated various BMPs in functions ranging from specification of epiblast to become primordial germ cells to recruitment and selection of primordial follicles to enter folliculogenesis and to the maturation and subsequent atresia of follicles (Shimasaki et al. 1999; Kierszenbaum and Tres 2001; Nilsson and Skinner 2003; Drummond 2005). Genetic evidence, however, provides the most significant demonstration of the importance of growth factor signaling in ovarian follicle development. In sheep, mutations in BMP-15 and GDF-9 can either stimulate ovulation rate or result in ovarian failure after the earliest stages of follicle development (Galloway et al. 2000; Hanrahan et al. 2004; Bodin et al. 2007), and in humans, mutations in BMP-15 are associated with hypergonadotropic ovarian failure (DiPasquale et al. 2004).

The genetic linkage of *FBN3* to PCOS is intriguing, particularly because a significant role for fibrillins has recently emerged in the control of growth factor signaling. Whether fibrillins control growth factor signaling in the ovary is unknown. It is also unknown to what extent the functions of the fibrillins are overlapping or specific in the ovary. In order to consider potential roles for fibrillin-3 in PCOS, we first determined the presence and distribution of all three fibrillins in normal adult human ovary. In addition, we determined the distribution of fibrillins in ovaries from patients with a history of PCOS. In this report, we identify a significant difference in the quantity of fibrillin-3-associated follicles within the ovaries of women with a clinical history of PCOS as compared with our control population. Our demonstration of the restricted localization of fibrillin-3 to the perifollicular stroma of a subset of follicles suggests a role for fibrillin-3 in the regulation of TGF-β-related growth factors, particularly BMP-4, BMP-7, GDF-9, and/or TGF-β, in the perifollicular stroma. In this context, abnormalities in the structure or quantity of fibrillin-3

are hypothesized to alter the transition of primordial to primary follicles and subsequently influence the growth and maturation of follicles and competent oocytes. These data and hypothesis are consistent with the identification of a PCOS susceptibility locus in *FBN3* (Urbanek et al. 2007; Ewens et al. 2010).

Materials and Methods

Tissue Samples

Cases for study were identified by searching the University of Washington Medical Center Surgical Pathology archives in accordance with IRB#05-5943E/A01-03 and #05-9266E/A01-02. Search terms included "polycystic ovary syndrome/disease" and "Stein/Leventhal." All identified cases indicating PCOS or Stein/Leventhal syndrome in the clinical history section of the report or in the body of the pathology report were pulled from the archive for study. Where possible, the clinical history of PCOS was confirmed by accessing the patient's medical record. However, because of the archival nature of the study, the criteria for the diagnosis of PCOS were not standardized. Archived slides and blocks from non-neoplastic ovary samples were selected to serve as normal controls, based on non-neoplastic ovarian parenchyma with no histopathological abnormality. At the time of review of the slides, the nature of the sample (control tissue or tissue from patients with a history of PCOS) was withheld. Two surgical pathologists (CDJ and SDB) read the stained sections. In order to quantitate the staining, the slides were scanned by an Olympus Nanozoomer Digital Pathology (NDP) System (Center Valley, PA), and the NDP virtual slide viewer software (Hamamatsu Photonics; Hamamatsu City, Japan) was utilized to determine ovarian area on each of the slides studied.

Immunohistochemistry

Standard immunohistochemical studies were performed on slides from paraffin-embedded tissues using a modified avidin–biotin (ABC) immunoperoxidase method. Pretreatments consisted of 15-min microwave retrieval in 10 mM citrate buffer for fibrillin-1 monoclonal antibody (MAb15, 1 mg/ml, 1:5000 dilution) and 10-min pronase (Calbiochem; San Diego, CA) digestion and 10-min microwave retrieval in 10 mM citrate buffer for fibrillin-2 (MAb48, 1 mg/ml, 1:200 dilution) and fibrillin-3 (MAb129, 1.49 mg/ml, 1:200 dilution) antibodies. Primary antibodies for fibrillin-1 (Kuo et al. 2007), fibrillin-2 (Charbonneau et al. 2003), and fibrillin-3 (Corson et al. 2004) were all mouse MAbs that have been previously characterized. After incubation with primary antibody, sections were sequentially incubated with secondary antibody (biotinyl horse-anti-mouse) and ABC (Vectastain Elite, Vector Laboratories; Burlingame, CA). Slides were then developed in a solution of 0.5% hydrogen peroxide as substrate and DAB as chromogen for 7 min for each antibody. Positive control tissue was human fetal ovary and human fetal limb tissues that demonstrated positive staining with the studied antibodies. Negative controls consisted of each patient specimen incubated with normal mouse serum in place of the primary antibody.

Statistical Analyses

Ovarian area and number of follicles/mm^2 were calculated using NDP software (Hamamatsu Photonics). Welch's two-tailed *t*-test for data sets with unequal variances was used to calculate statistical significance. The average ovarian area of PCOS ovary sections was 508 mm^2 (SD $\pm$ 259.7), whereas that of the control set was 567 mm^2 (SD $\pm$ 368.8). This was not statistically significant ($p=0.714$). Data were considered statistically significant if $p<0.05$.

Results

Description of Study Samples

Thirty-one specimens were identified for study, of which nine specimens were from individuals with a clinical history of PCOS. Ages of control samples ranged from 17 to 51 years; for PCOS samples, ages ranged from 17 to 45 years. Non-neoplastic ovaries were reviewed from a variety of patients. Ovaries were resected as parts of staging procedures for neoplasms, endometriosis, or other benign disorders, or prophylactically in patients harboring BRCA gene mutations. Where available, additional clinical histories of PCOS samples included amenorrhea, oligomenorrhea, hirsuitism, and obesity. These and other characteristics of each specimen are recorded in Table 1.

Histology

Routine hematoxylin and eosin (H&E) staining was performed to evaluate each specimen and to compare control ovaries with PCOS ovaries (Figure 1). Descriptive characteristics are summarized individually in Table 1. In addition, after scanning the ovary sections and utilizing image analysis software, quantitative features of the samples, including area of ovary sections and enumeration of transitional follicles (TFs) in each of the samples, were recorded (Table 2).

A subset of the control ovaries demonstrated very active folliculogenesis with follicles at several stages of development from primordial/primary (transitional) follicles (Figure 1A) to secondary and antral follicles (Figures 1B and 1C), including large antral follicles (Figure 1D) or Graafian follicles (Figure 1E). A few of the younger control ovaries mostly had TFs with only rare secondary follicles and occasional large antral follicles or follicular cysts. The term "transitional follicles" was chosen to represent the histological gray zone between primordial and primary follicles and to include the many observed follicles with intermediate features. On average, the control

Table 1 Summary of clinical history, H&E staining, and FBN3 staining in control and PCOS ovaries

Case number	Age	Clinical history of PCOS	History comments	H&E staining	FBN3 staining
1	43	No	Endometrial endometrioid CA, FIGO 1a	TF, AnF, GF, FC, FL, CAL	++
2	19	No	Salpingitis	TF to large AnF, AtF, foci of CF	+/−
3	17	No	Megacolon, rectovaginal fistula	Scant cortical tissue with cautery and surface fibrous adhesions. TF, AnF, GF clustered in focal area	+/−
4	29	No	Opposite ovary with mucinous cystadenoma	CL, AnF, TF, large FC	++
5	30	No	Placenta percreta, C/S hysterectomy	Many TF, AnF, FL, CAL	++
6	34	No	Endometriosis	Many TF, AnF, corpus hemorrhagicum	No
7	34	No	PSO; BRCA mutation detected	Wide range of follicle development	++
8	31	No	Corpus luteum—persistent ovarian cyst	Active folliculogenesis, TF to GF	++
9	27	No	Endometriosis	TF and multiple AnF	++
10	40	No	Irregular menses, DM, HTN, hypothyroid, endometrial endometrioid CA, FIGO I	TF, multiple FC and AnF, FL	++
11	38	No	Irregular menses, atypical hyperplasia	TF, AnF, GF, CL, FC, AtF, FL	++
12	38	No	IDM since 8 years old, hypothyroid, HTN, HCh, uterine CA	TF, AnF, FC, FL, AtF	+
13	27	No	IDM	TF, AnF, GF, FL	++
14	40	No	Endometrial CA, DM, HCh	TF, AnF, FC, FL, AtF, CF	+
15	34	No	Breast carcinoma	TF, AtF, CAL	++
16	45	No	Cervical dysplasia	TF, AtF, CAL	++
17	34	No	PSO; BRCA mutation detected	Range of follicles present—TF to GF, and FC	++
18	40	No	PSO; BRCA mutation detected	TF, FC, CF	++
19	45	No	Breast carcinoma	Senescent, AtF, CAL, rare TF	++[a]
20	46	No	PSO; BRCA mutation detected	Senescent, CAL, rare CEI, endometriosis	++[a]
21	51	No	Breast carcinoma. PSO; BRCA mutation detected	Senescent, focal CEI, AtF, CAL	++[a]
22	46	No	Strong family history breast and ovarian CA, no BRCA mutation detected	Senescent, AtF, FC	++[a]
23	38	Yes	Pulmonic stenosis, DM, enlarged ovary with cysts on ultrasound	Stromal hyperplasia, minimal HT, full follicle range, rare FC, SF	+
24	35	Yes	Oligomenorrhea, infertility, insulin resistance, obesity	Range of follicles present—TF to GF, AtF, and few FC	+/−
25	17	Yes	Amenorrhea, HTN, hirsuitism, obesity	Multiple FC, rare AnF, scattered TF, HT, SF	No
26	24	Yes	No additional history	Multiple FC, TF, rare GF	No
27	34	Yes	No additional history	TF, numerous large AnF, FL, SF	No
28	37	Yes	Infertile, hirsute, HTN	Range of follicles present—TF to GF, FC, SF, CF, FL. No HT	+
29	39	Yes	No additional history	FC, GF, TF, FL, SF. No HT	No
30	19	Yes	Oligomenorrhea, hirsuitism, obesity	TF, multiple AtF, FC, SF, CF	No
31	45	Yes	Irregular menses	Histologically normal ovary with active folliculogenesis	++

[a]FBN3 staining focal in cortical stroma without identifiable follicle.
H&E, hematoxylin and eosin; PCOS, polycystic ovary syndrome; CA, carcinoma; TF, transitional follicle (primordial to primary); AnF, antral follicle; GF, Graafian follicle; FC, follicle cyst; FL, folliculolysis; CAL, corpus albicans; AtF, atretic follicle; CF, cortical fibrosis; CL, corpus luteum; C/S, cesarean section; PSO, prophylactic salpingo-oophorectomy; DM, diabetes mellitus; HTN, arterial hypertension; IDM, insulin-dependent diabetes mellitus; HCh, hypercholesterolemia; CEI, cortical epithelial inclusion; HT, hyperthecosis; SF, subcapsular fibrosis.

ovaries displayed 0.334 TF/mm^2, and the average ovarian surface area observed on the control slides was 567 mm^2 (Table 2). Various stages of corpus luteum were also identified in different ovary sections (data not shown). Prominent large vessel hilar vascularity was most notable in a subset of ovaries, typically those that demonstrated senescent changes (data not shown). Small cortical vessels were readily identified in cortical stroma, and active angiogenesis was appreciated in regions of folliculolysis (Figure 1F) and of degeneration of corpora lutea (data not shown). Although many of the control ovaries demonstrated cortical stroma extending nearly subjacent to surface epithelium, subepithelial cortical fibrosis was common in the control set of ovaries. Hyperthecosis was inapparent in the control ovaries (data not shown).

The most consistent finding in the ovaries resected from patients with a history of PCOS was the presence

of variable numbers of TFs (Figure 1H) and the presence of numerous follicle cysts (Figure 1G) with degenerative linings (theca-lined cysts) or cysts with attenuated granulosa layers (Figures 1H and 1I). Quantitative analysis showed an average of 0.164 TF/mm^2 in PCOS ovaries. Only rare follicle cysts maintained significantly intact granulosa cell layers of late antral/Graafian follicles (data not shown). In addition, intermediary stages of follicle

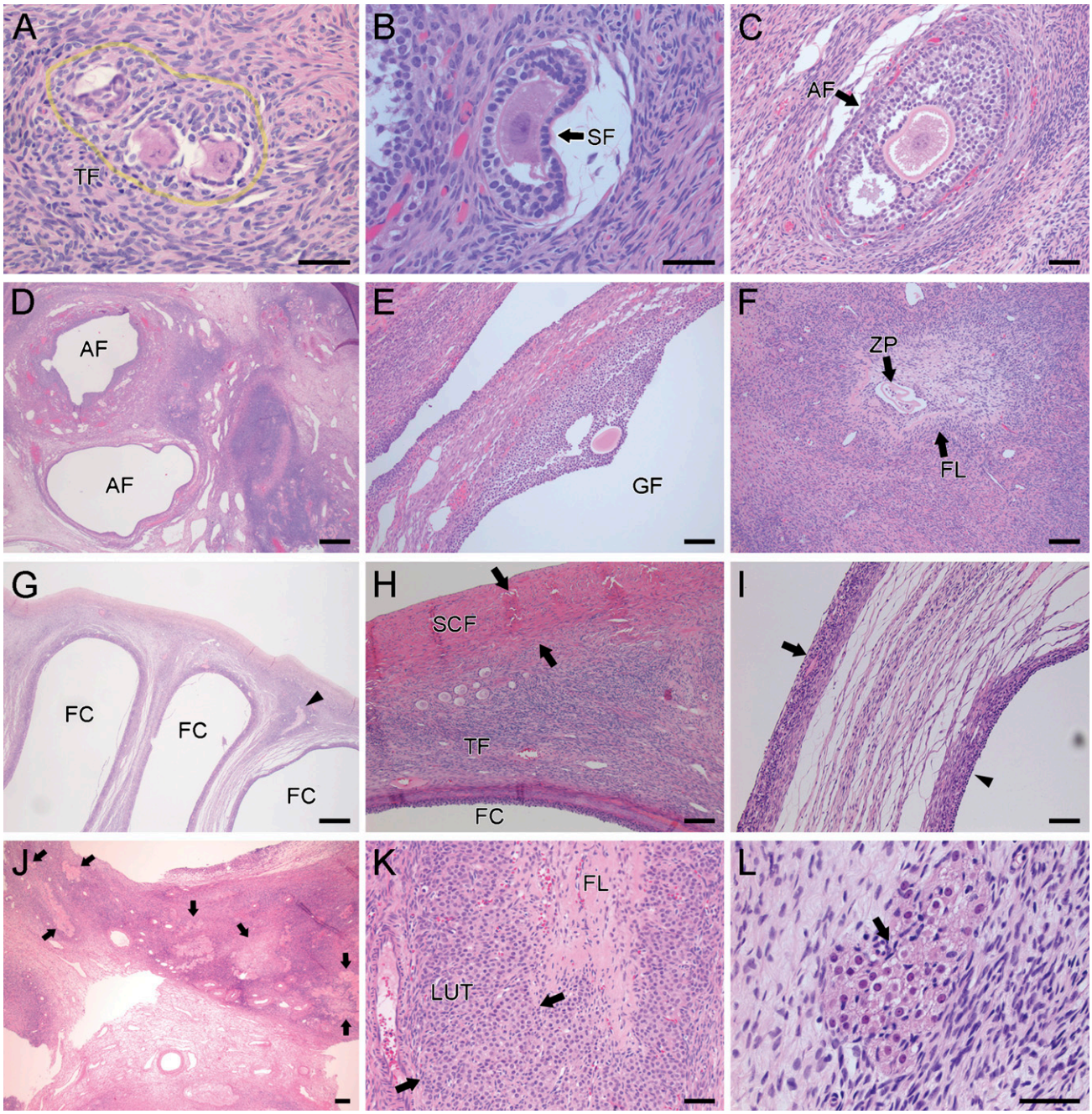

Figure 1 Hematoxylin and eosin (H&E) staining of control ovaries and polycystic ovary syndrome (PCOS) ovaries. Control ovaries: (**A**) transitional follicles (TF) with "specialized perifollicular stroma (SPS)" (inside yellow line); (**B**) secondary follicle (SF) or preantral follicle with solid granulosa cell layers; (**C**) early antral follicle (AF) with central oocyte, zona pellucida (ZP), thickened granulosa cell zone with small antrum, and discernable theca interna (TI) and theca externa (TE); (**D**) late AFs with enlarged follicle space and distinct theca; (**E**) Graafian follicle (GF) with cumulus oophorus; (**F**) folliculolysis (FL) showing destruction of a preovulatory follicle with vascular ingrowth and fibrosis; remnant of ZP noted. PCOS ovaries: (**G**) multiple follicle cysts (FCs), subepithelial fibrosis, and lytic follicle (arrow head); (**H**) thick subcapsular fibrosis (SCF between arrows), cluster of TFs, and FC; (**I**) theca-lined cyst (arrow) and FC (arrow head); (**J**) FL (arrows); (**K**) lytic follicle with luteinized theca (LUT between arrows); (**L**) cortical hyperthecosis (arrow). Bars: **A–C,K,L** = 50 μm; **D,G,J** = 500 μm; **E,F,H,I** = 100 μm.

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Table 2 Quantitation of ovarian section area, follicles, and fibrillin-3 staining in controls (case numbers 1–22) and PCOS samples (case numbers 23–31)

Case number	Age	Number of TF	Number of Fibr-3-positive TFs	Total number of all non-TF follicles	Ovary (mm^2/slide)	TF/mm^2	Fibr-3-positive TF/mm^2
1	43	354	39	63	1166	0.3	0.0334
2	19	307	4	19	656	0.47	0.0061
3	17	41	1	9	139	0.3	0.0072
4	29	83	3	7	296	0.28	0.0101
5	30	177	4	2	121	1.46	0.0331
6	34	410	11	18	515	0.8	0.0214
7	34	281	123	48	1146	0.25	0.1073
8	31	62	23	7	306	0.2	0.0752
9	27	103	5	8	279	0.37	0.0179
10	40	213	97	45	853	0.25	0.1137
11	38	116	22	19	719	0.16	0.0306
12	38	39	2	8	145	0.26	0.0138
13	27	272	8	15	924	0.29	0.0087
14	40	17	3	17	1221	0.01	0.0025
15	34	46	3	0	453	0.1	0.0066
16	45	40	21	0	442	0.09	0.0475
17	34	53	23	0	203	0.26	0.1133
18	40	104	54	13	614	0.17	0.0879
19	45	6	0	0	274	0.02	Stromal foci
20	46	0	Focal stroma	0	655	0	Stromal foci
21	51	0	Focal stroma	0	508	0	Stromal foci
22	46	0	Focal stroma	0	461	0	Stromal foci
23[a]	38	125	6	29	544	0.23	0.011
24[a]	35	20	1	6	287	0.07	0.0035
25[a]	17	128	0	17	430	0.3	0
26[a]	24	112	0	40	1084	0.01	0
27[a]	34	38	0	9	413	0.09	0
28[a]	37	29	4	26	573	0.05	0.007
29[a]	39	37	0	15	641	0.06	0
30[a]	19	76	0	5	171	0.44	0
31[a]	45	99	40	5	425	0.23	0.0941

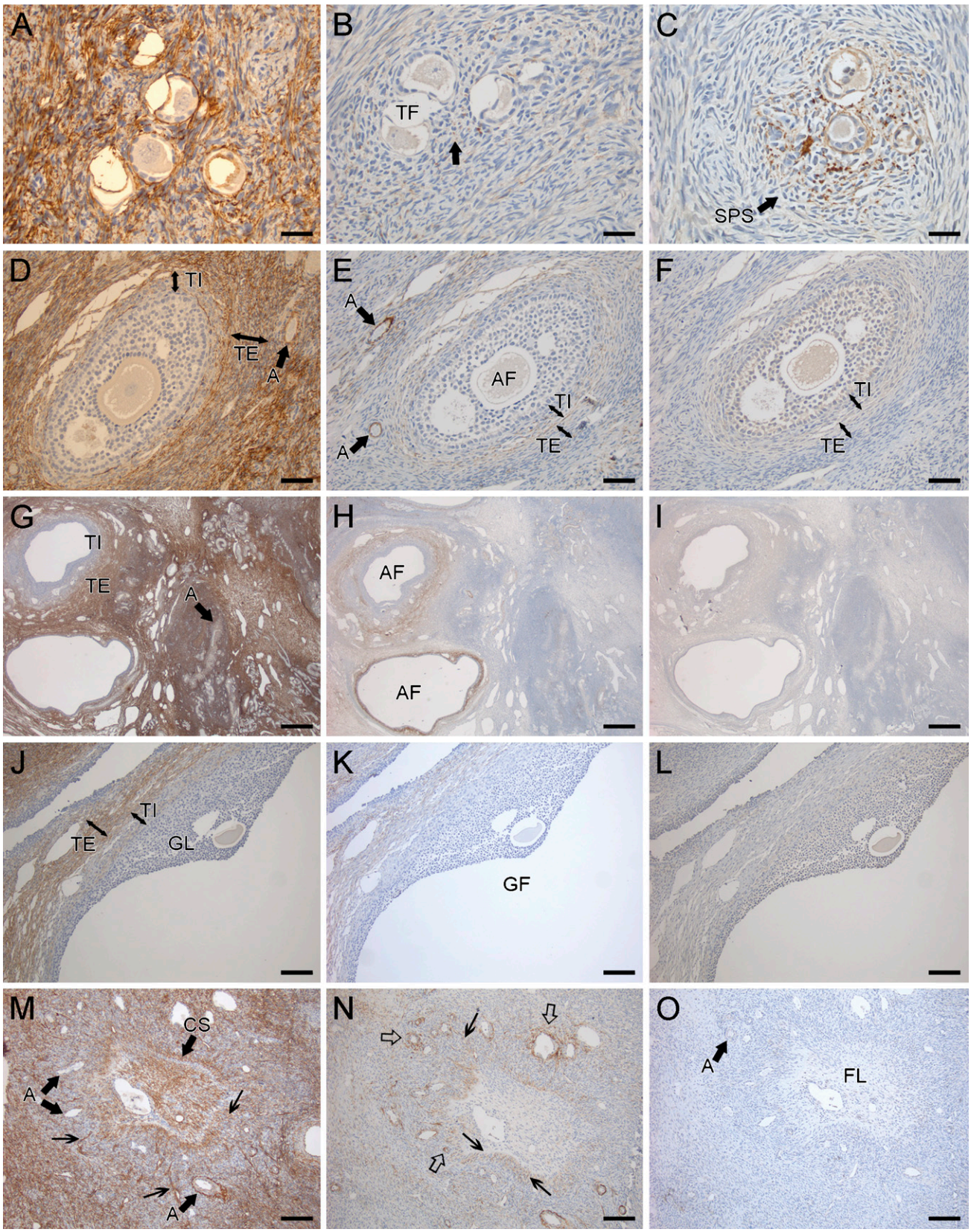
[a]PCOS ovaries.
Control samples (cases 1–18) and PCOS ovaries (cases 23–31) are included in the statistical analysis. Senescent ovaries (cases 19–22) are excluded from statistical analysis. Fibr-3, fibrillin-3.

development were quite rare or absent in PCOS ovaries as were corpus luteum (data not shown). Extensive evidence of folliculolysis with multiple scarred follicles was observed (Figure 1J). In addition, luteinization of residual theca was readily identified in association with lytic follicles in a subset of the PCOS ovaries (Figure 1K), a finding not apparent in the control set of ovaries. These areas of luteinization were seen in addition to the small clusters of luteinized theca cells (Figure 1L) (interpreted as hyperthecosis) in most of the PCOS ovaries. Subcapsular fibrosis was common in the PCOS samples (Figure 1H), and in a few samples, more extensive cortical fibrosis was identified (data not shown). One of the ovary samples from a patient with a reported history of PCOS (case number 31) was histologically normal with a range of follicle maturation.

Fibrillins in Control Ovaries

All three fibrillins were identified in the control ovaries after immunohistochemical staining (Figure 2). Previous studies have demonstrated that fibrillin-2 and fibrillin-3 are restricted to fetal development (Zhang et al. 1995;

Figure 2 Fibrillin staining of control ovaries. (**A**) Fibrillin-1 densely stained ovarian cortex and extended into SPS of TFs. (**B**) Minimal fibrillin-2 staining was identified in SPS (arrow) of TFs. (**C**) Fibrillin-3 staining was present in SPS (arrow) of TFs. Fibrillin-1 (**D,G,J**) and fibrillin-2 (**E,H,K**) were identified within the TE (double-headed arrow) of early and late AFs and GFs with very few fibrils extending into TI (double-headed arrow). No staining was seen in the granulosa layer (GL). (**D,G**) Fibrillin-1 in the ovarian cortex and associated with vessels (A, thick arrow). (**E,H**) Fibrillin-2 in theca layers (TI and TE, double-headed arrows) and in association with small cortical vessels (A, thick arrow). (**M**) Perpendicularly oriented fibrillin-1 fibrils extend from the exterior of follicle (thin arrows) and form a dense central scar (CS, thick arrow). Fibrillin-1 staining associated with vessels (A, thick arrow). (**N**) Fibrillin-2 in FL (thin arrows) and in neoangiogenesis with associated vascular staining (open arrows). (**O**) No fibrillin-3 staining was identified during FL or around vessels (A, thick arrow). (**F,I,L,O**) No staining of theca, ovarian stroma outside the "specialized perifollicular stroma," or vasculature was observed on staining with antibodies to fibrillin-3. Bars: **A–C** = 50 μm; **D–F** = 100 μm; **G–I** = 500 μm; **J–O** = 200 μm.



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Charbonneau et al. 2003; Corson et al. 2004). Therefore, these are the first findings to document the presence of fibrillin-2 and fibrillin-3 in adult tissue.

Fibrillin-1

Strong fibrillar staining with antibodies to fibrillin-1 was found diffusely in the ovarian cortex surrounding individual stromal cells and also in association with cortical vessels (Figures 2A, 2D, and 2G). Variable degrees of decreased staining or a lack of staining subjacent to the surface epithelium were observed (data not shown). This typically correlated with the thickness of subcortical fibrosis. The concentration of fibrillin-1 fibrils was decreased within the region of the theca externa, and only rare fibrillin-1 fibrils were identified within the theca interna (Figures 2G and 2J). Fibrillin-1 was observed in association with a subset of TFs, usually the same follicle sets that were also stained with fibrillin-2 antibody or fibrillin-3 antibody or both (see below and Figure 2A). Increasingly dense fibrillar fibrillin-1 staining was observed in progressive stages of folliculolysis and typically overshadowed the notable staining for fibrillin-2 (Figures 2M and 2N). The orientation of the fibrillin-1 fibrils changed during the process of folliculolysis. During the growing follicle stages, fibrillin-1 was predominantly oriented in a concentric fashion following the contour of the theca externa at the perimeter of the follicle (Figures 2D, 2G, and 2J). As follicle atresia progressed, fibrillin-1 fibrils assumed a perpendicular orientation to the margin of the pre-existing follicle (Figure 2M), and by the end of folliculolysis, fibrillin-1 fibrils filled the previous luminal space of the follicle and were a significant component of the subsequent scar (Figure 2M). A similar progression of fibrillin-1 staining was observed in association with corpora lutea undergoing regression (data not shown). Fibrillin-1 staining was ubiquitous in the hilar loose stromal connective tissue and vessels and was often accentuated around the coiled hilar arteries producing a very dense or globular perivascular staining pattern (data not shown).

Fibrillin-2

Staining of control ovaries with antibodies to fibrillin-2 revealed a much more restricted distribution than that observed for fibrillin-1 (Figures 2B, 2E, 2H, 2K, and 2N). Fibrillin-2 was found within and around subsets of smaller cortical arteries (Figure 2N). Fibrillin-2 was seen as concentric layers of fibrils surrounding the periphery of the theca externa and within the outer aspect of the theca externa (Figures 2E, 2H, and 2K). Most TFs did not have associated fibrillin-2 staining (data not shown). However, occasionally, slight fibrillin-2 staining was seen in the perifollicular stroma of TF clusters that also demonstrated staining with antibodies to fibrillin-3 and/or fibrillin-1 (Figure 2B). Staining for fibrillin-2 typically intensified in midstages of folliculogenesis of secondary or later stage follicles (Figures 2E, 2H, and 2K). In addition, the orientation of the fibrillin-2 fibrils changed from concentric around the follicle perimeter to perpendicular with subsequent extension of fibrillin-2 fibrils from the periphery to the center of the follicle during folliculolysis (Figure 2N); angiogenesis emerged alongside these changes of fibrillin-2. In the late stages of folliculolysis, fibrillin-1 staining was more abundant than that of fibrillin-2 as the scarring process ensued (Figures 2M and 2N). Fibrillin-2 staining of vessels at the periphery of the scar was noted (Figure 2N).

Fibrillin-3

Fibrillin-3 was identified in a very restricted distribution within adult human ovary (Figures 2C, 2F, 2I, 2L, and 2O). Fibrillin-3 was localized in the stroma immediately adjacent to a minor subset of TFs (Figures 2C and 3K–3N). Often, only rare or a few TFs in a given ovary section demonstrated this perifollicular staining (Table 2). On average, 0.0409 TF/mm^2 had adjacent fibrillin-3 staining in the control ovaries (Figure 4) (only 12% of the TFs identified by histology were fibrillin-3 positive). Ovaries with more active folliculogenesis typically had readily identifiable staining for fibrillin-3 (Tables 1 and 2). On the H&E stains of these latter ovaries, clusters of TF have associated plump or slightly epithelioid stromal cells, thus termed "specialized perifollicular stroma" (Figure 1A). Fibrillin-3 staining was most dense in such areas of specialized perifollicular stroma (Figures 2C, 3K, 3L, and 3N). Staining with fibrillin-2 or accentuation of cortical staining with fibrillin-1 was also noted in a subset of clustered TFs with specialized perifollicular stroma (Figures 2A and 2B). A few of the TFs that have associated staining for fibrillin-3 do not contain observable oocytes possibly representing barren follicles (Figures 3K and 3M). Outside the regions of specialized perifollicular stroma, no staining for fibrillin-3 was identified, except in senescent ovaries where small foci of cortical stroma with the appearance of specialized perifollicular stroma demonstrate staining for fibrillin-3

Figure 3 Fibrillin-3 staining of PCOS patient ovaries (**A–I**) and control ovaries (**K–O**). Figure labels corresponding to case numbers are as follows: (**A**) 25, (**B**) 26, (**C**) 27, (**D**) 29, (**E**) 24, (**F**) 30, (**G**) 28, (**H**) 23, (**I**) 31, (**J**) 28, (**K**) 16, (**L**) 21, (**M**) 4, (**N**) 8, and (**O**) 15. No staining in PCOS ovaries was found in some samples (**A–D,F**). Equivocal staining was identified in case number 24 (**E**). Focal detectable staining was identified in case numbers 28 (**G**, arrow) and 23 (**H**, arrow), and readily identifiable staining was observed in case number 31 (**I**, arrows). Staining was observed within perifollicular stroma of control ovaries (**K–N**, arrows) and focally in the cortical stroma of a senescent ovary (**O**, arrows). Case number 28 showed staining for fibrillin-3 (**G**, arrow) but no staining with the negative control, normal mouse serum (NMS; **J**). Bar = 50 μm.

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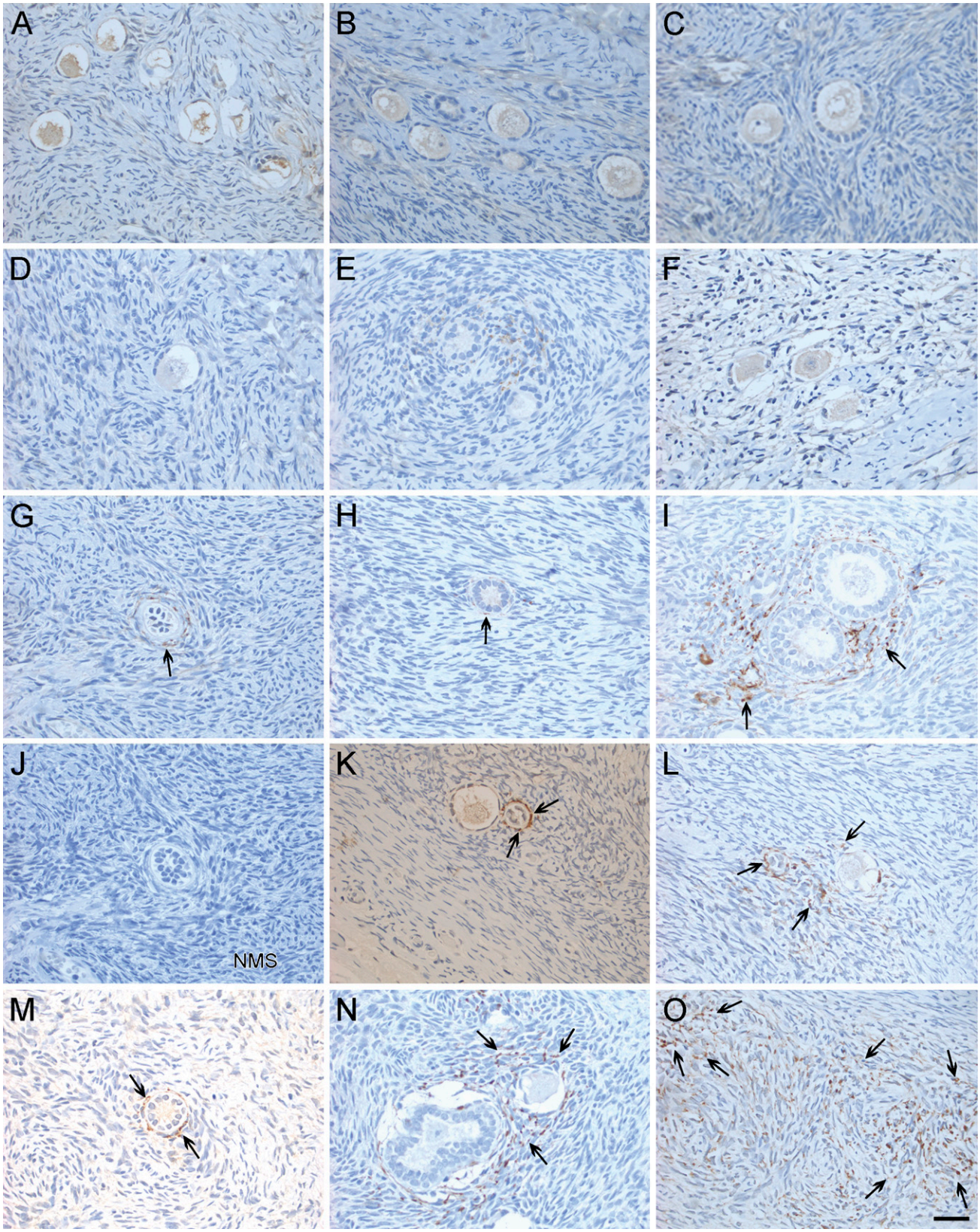

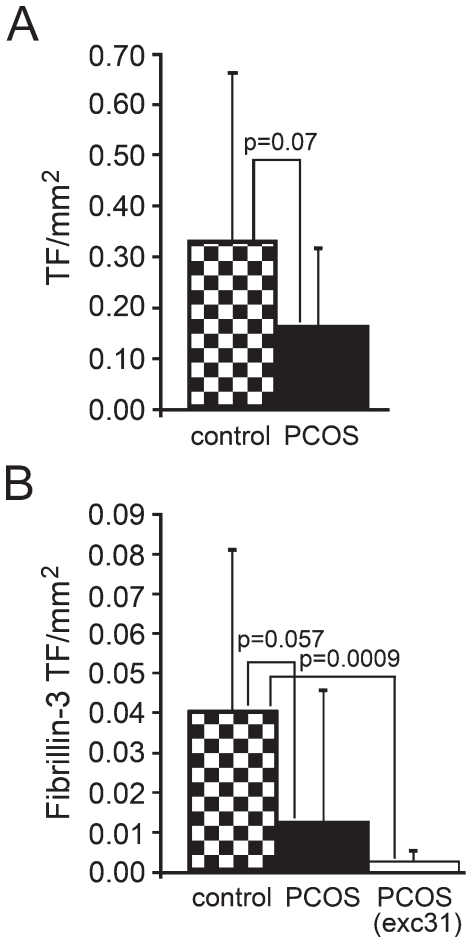

Figure 4 Quantitation of total TF/mm^2 and fibrillin-3-associated TF/mm^2 in control and PCOS ovaries. **(A)** PCOS ovaries had fewer average TF/mm^2 (0.164 TF/mm^2) than control ovaries (0.334 TF/mm^2; $p=0.07$). **(B)** Control ovaries had an average of 0.0409 fibrillin-3-associated TF/mm^2. The entire set of PCOS ovaries had a decreased average of fibrillin-3-associated TF/mm^2 (0.0128 fibrillin-3-associated TF/mm^2; $p=0.057$). After exclusion of PCOS case number 31, the average number of fibrillin-3-associated TF/mm^2 in PCOS was 0.0027 ($p=0.0009$). Standard deviations and p values are shown. Raw data are shown in Table 2.

(Figure 2O). No fibrillin-3 staining was identified in association with maturing antral follicles (Figures 2F, 2I, and 2L), folliculolysis (Figure 2O), or corpora lutea (data not shown). In addition, there was no staining of cortical or hilar vasculature with antibodies to fibrillin-3 (data not shown).

Fibrillins in PCOS Ovaries

Staining of PCOS ovaries was similar to that of control ovaries for both fibrillin-1 and fibrillin-2. However, staining for fibrillin-3 was decreased in PCOS ovaries compared with control ovaries.

Fibrillin-1 in PCOS

The staining pattern of fibrillin-1 was very similar in the PCOS and control ovaries. Of note, however, was the appearance of fibrillin-1 in areas of cortical fibrosis. In these areas, fibrillin-1 appeared as larger bundles of fibrils between collagen fibers rather than the usual fine reticulated meshwork surrounding individual cortical stromal cells (data not shown).

Fibrillin-2 in PCOS

The distribution of fibrillin-2 staining within PCOS ovaries was very similar to that observed within the control ovaries. However, in the PCOS ovaries, staining for fibrillin-2 was more prominent, probably because of increased folliculolysis in the PCOS ovaries compared with the controls (data not shown).

Fibrillin-3 in PCOS

Fibrillin-3 staining in PCOS ovaries varied between samples. However, fibrillin-3 staining appeared to be mostly decreased or completely absent in the sections examined (Figures 3A–3H; Table 2). The average number of follicles with associated fibrillin-3 staining in the PCOS samples was 0.0128 TF/mm^2 (Figure 4). When identified, fibrillin-3 staining was usually limited to faint perifollicular staining around individual TFs, including some without discernable oocytes, which were included in the counted TFs (Figures 3G and 3H). One patient with a clinical history of PCOS (case number 31) had histologically normal ovaries with active folliculogenesis and demonstrated fibrillin-3 staining similar to the control ovaries in both quantity and distribution (Figure 3I). Similar to the control ovaries, no fibrillin-3 staining was identified in association with maturing antral/Graafian follicles, folliculolysis, corpora lutea, or vasculature (data not shown).

Quantitation of fibrillin-3 staining in the nine PCOS samples revealed an average of 0.0128 fibrillin-3-associated TF/mm^2. Follicle-associated fibrillin-3 staining in the control set of ovaries (excluding the senescent ovaries, all of which showed stromal foci of fibrillin-3 staining) was 0.0409 fibrillin-3-associated TF/mm^2. This approximates a significant difference using Welch's two-tailed t-test with a p value of 0.0572. If the one PCOS ovary that was histologically normal (case number 31) is excluded from the analysis, the difference in staining for fibrillin-3 in control and PCOS ovaries is statistically significant with a p value of 0.0009 (Figure 4).

Discussion

PCOS is primarily a disorder of androgen excess or hyperandrogenism. In addition to phenotypic virilization and reproductive abnormalities, associated metabolic disturbances including diabetes mellitus and increased cardiovascular risk lead to significant morbidity in these patients (Azziz et al. 2009). PCOS is considered to be a multifactorial polygenic disorder. The strongest susceptibility locus associated with PCOS implicates

fibrillin-3 in the pathogenesis of PCOS (Urbanek et al. 2007; Ewens et al. 2010). Our results support a role for fibrillin-3 in PCOS.

Contradictory conclusions by the two most recent SNP analyses of PCOS (Prodoehl et al. 2009; Ewens et al. 2010) may be due to differences in the methodologies used (case–control vs family-based approaches, and the different clinical definitions of PCOS used to select the samples). Although significant differences between PCOS and non-PCOS controls in *FBN* mRNA expression have not been found (Prodoehl et al. 2009), our study of protein localization in ovarian tissue did find differences in fibrillin-3 in our PCOS and non-PCOS samples. Therefore, even with a small randomly selected sample, our study supports a role for fibrillin-3 in PCOS.

Immunolocalization studies in control ovaries demonstrated that the presence of fibrillin-3, in contrast to fibrillin-1 and fibrillin-2, is very limited. Although sparse in quantity, fibrillin-3 consistently localized to the stroma adjacent to a minor subset of primordial or primary follicles (designated here as TFs). It is possible that fibrillin-3 may serve as a biomarker for a specific type of TFs because, of the total 0.334 TF/mm^2, only 0.0409 TF/mm^2 was associated with fibrillin-3 staining. Because fibrillin-1 and fibrillin-2 were identified around TFs that also displayed fibrillin-3, fibrillin-3 may be expressed before the other fibrillins, marking an earlier subset of follicles than those marked by all three fibrillins. In general, we found the most abundant fibrillin-3 staining in a subset of those ovaries with active folliculogenesis, displaying follicles at various stages of maturation (Table 1; case numbers 1, 6–8, 10, 11, 18). Although fibrillin-3 was also identified in the stroma adjacent to only rare or a few isolated TFs within a given ovary, dense fibrillin-3 staining was notable in the stroma associated with clusters of some TFs. On the basis of these observations, we propose that fibrillin-3 forms a specialized stroma around transient TFs as they differentiate from primordial to primary follicles.

In order to test whether fibrillin-3 may contribute to the pathogenesis of PCOS, we quantitated the number of TFs/mm^2 and the number of fibrillin-3-positive TFs/mm^2 in archived samples diagnosed as PCOS compared with control samples. Criteria for diagnosis of PCOS were not standardized, and only nine samples were available. However, using this approach, we were nevertheless able to determine that the number of TFs/mm^2 and the number of fibrillin-3-positive TFs/mm^2 were reduced in the PCOS samples. The reduction in the number of TFs/mm^2 did not reach statistical significance ($p=0.070$). However, the reduction in the number of fibrillin-3-positive TFs/mm^2 approached statistical significance ($p=0.057$). Of the nine PCOS samples, one (case number 31) was histologically normal in appearance and in fibrillin-3 staining. Although this patient had a clinical history of PCOS, the morphological findings within this ovary sample including active folliculogenesis were atypical for a patient with this disorder, which is commonly associated with abnormal folliculogenesis and infertility. If this sample is omitted from the calculations, then the reduction in the number of fibrillin-3-positive TFs/mm^2 was statistically significant ($p=0.0009$). On the basis of these results, we conclude that PCOS is a heterogeneous disorder at the molecular level, and we hypothesize that fibrillin-3 may play a primary role in most cases of PCOS.

The importance of TGF-β superfamily members in the control of folliculogenesis, together with evolving concepts of how the fibrillin microfibril network regulates TGF-β and BMP signaling (Charbonneau et al. 2004; Ramirez and Sakai 2010), suggests that fibrillins may perform significant roles in ovarian function, including folliculogenesis. Some members of the TGF-β superfamily interact with fibrillin microfibrils, either directly (Gregory et al. 2005; Sengle et al. 2008) or through an intermediary molecule, LTBP (Isogai et al. 2003; Ono et al. 2009). Fibrillins have been shown to be required for specific developmental sequences and cellular functions involving growth factor signaling. *FBN2*-null mice demonstrated a limb-patterning defect associated with loss of BMP signaling (Arteaga-Solis et al. 2004). *FBN1*-deficient mice displayed defects in distal alveolar septation caused by abnormal activation of TGF-β signaling (Neptune et al. 2003).

Whether fibrillin-3 binds to and regulates BMPs and/or TGF-β is unknown. However, because both fibrillin-1 and fibrillin-2 share the same binding repertoires (Isogai et al. 2003; Sengle et al. 2008), it seems likely that fibrillin-3 also performs similar molecular functions. However, distinct organ-specific functions appear to be determined by temporal and tissue-specific differences in gene expression rather than by distinct molecular interactions. For example, the early and specific expression of fibrillin-2 in the interdigital space (Charbonneau et al. 2010) may explain the defect in limb patterning in *FBN2*-null mice, whereas the ubiquitous postnatal expression of *FBN1*, in contrast to *FBN2*, may explain defects in homeostasis found in *FBN1*-deficient mice. From the restricted localization and apparent transient temporal expression of fibrillin-3 in the TF-specialized perifollicular stroma, we predict that fibrillin-3 may be responsible for the regulation of BMPs and/or TGF-β in these TFs. Evidence exists in rodents that BMP-4 and BMP-7 produced by prethecal stromal cells are involved in the transition of a primordial follicle to a primary follicle and in the subsequent survival of follicles (Lee et al. 2001; Nilsson and Skinner 2003). The production of these growth factors by prethecal stroma (specialized perifollicular stroma) in conjunction with the demonstrated restricted localization of fibrillin-3 to this specialized stroma suggests an important role for fibrillin-3 in the regulation of growth

factors required for the transition of primordial to primary follicles.

Dysregulation of multiple TGF-β-related proteins including BMP-4, BMP-7, anti-Müllerian hormone, activins, inhibins, GDF-9, and BMP-15 has been implicated in the impaired follicular growth that characterizes PCOS (Jonard and Dewailly 2004; Maciel et al. 2004; Pangas and Matzuk 2004; Dumesic et al. 2007a,b). A balancing act between these growth factors influences androgen production by the follicle and subsequent follicle growth and maturation. Intrafollicular androgen excess is thought to be a primary factor in altered and arrested follicle development leading to the morphology of polycystic ovaries and secondary endocrine abnormalities (Jonard and Dewailly 2004). A fibrillin-3 microfibrillar scaffold may be required to appropriately balance growth factor signaling and androgen production during folliculogenesis.

The tissue-specific localization of fibrillins in adult ovaries has not been previously investigated. In the early embryo, all three fibrillins are expressed ubiquitously in the connective tissue space. The major differences between fibrillins are accounted for by differences in temporal expression rather than tissue-specific expression. On the basis of these studies (Zhang et al. 1995; Corson et al. 2004), immunolocalization of fibrillin-2 and fibrillin-3 was not expected in adult tissues. We report here that fibrillin-2 and fibrillin-3, as well as fibrillin-1, are present in adult ovarian tissues.

We showed that fibrillin-1, the most ubiquitous fibrillin in postnatal connective tissues, is the dominant fibrillin in the adult human ovary. This correlates with recent data that *FBN1* mRNA expression levels in human ovary are 50–100 times greater than those of *FBN2* and 200–1000 times greater than those of *FBN3* (Prodoehl et al. 2009). In ovarian parenchyma, we identified fibrillin-1 throughout the cortical stroma, hilar connective tissues, and vessels. Fibrillin-1 accumulates secondarily in regions of neoangiogenesis, vascular regression, and scarring associated with folliculolysis and regression of corpora lutea. It is found only rarely in stroma around TF clusters.

Fibrillin-2 is localized to what may be imagined as activity zones in the ovary. Fibrillin-2 microfibrils are present at the periphery and external layers of the theca externa. They appear to be active participants in the evolution of folliculolysis, often aligning densely in areas of neoangiogenesis. Fibrillin-2 microfibrils are present in a subset of cortical vessels and are occasionally localized to the specialized stroma of TF clusters.

The spatial and temporal expression patterns of BMPs are active throughout folliculogenesis in mammals (Erikson and Shimasaki 2003; Shimasaki et al. 2004). We demonstrate here that like the BMPs and potentially in concert with BMPs, all three fibrillins are expressed in specific spatial and temporal patterns in adult human ovary.

As such, the fibrillin microfibril network within human ovary is the first example of an adult organ in which there is a dynamic interplay between all three fibrillins. We propose that each of the fibrillins is spatially and temporally located in the same adult organ and that coordination of the specific spatial and temporal expression of fibrillins may be required for normal folliculogenesis. Loss of fibrillin-3 function, because fibrillin-3 is the specific fibrillin associated with the transition from primordial to primary follicle, may be the most disruptive to folliculogenesis. In contrast to recently published results (Prodoehl et al. 2009), our data strongly indicate that additional studies aimed at determining the role of fibrillin-3 in the pathogenesis of PCOS merit investigation.

Acknowledgments

Funding was provided by the University of Washington and by the Shriners Hospitals for Children.

We thank Dr. Paul Swanson for his review of the manuscript and suggestions and the members of the University of Washington immunohistochemistry laboratory for their expert technical assistance.

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