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Estrogens Reduce the Expression of YKL-40 in the Retina: Implications for Eye and Joint Diseases

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ABSTRACT

PURPOSE. To identify modifications in the gene expression profile of the ocular posterior segment in ovariectomized (OVX) mice with and without substitutive estradiol therapy and to select differentially expressed genes that could be relevant to the natural history of human age-related macular degeneration (AMD).

METHODS. Chorioretinal tissues from two groups of 25 treated and untreated OVX mice were analyzed by using cDNA array technology. The expression level of selected genes was confirmed in triplicate by RT-PCR and related to the estrogenic status of the animals. Expression of the YKL-40 gene was further investigated in intact or diseased human retinas and in a murine model of experimental choroidal neovascularization (CNV), using laser pressure catapulting.

RESULTS. Of the approximately 10,000 genes screened, only YKL-40 expression was significantly downregulated by 17-*β*-estradiol. YKL-40 was expressed in intact human neural retina and in the RPE. The expression of YKL-40 was upregulated in experimental CNV and in neovascular membranes extracted from patients affected by the exudative form of AMD.

CONCLUSIONS. These observations indicate that YKL-40 expression in the retina is modulated by serum levels of estradiol. This protein could be relevant to the development of AMD and is also a new mediator to take into account when evaluating the broad consequences of hormonal replacement therapy.

The presence of both types of estrogen receptors (ER) *α* and *β* has recently been demonstrated in the retina, suggesting a role for an intracellular receptor-mediated effect of estrogens in retinal biology.[1,2] The protective role of estradiol in the development of cataract has been convincingly established on experimental[3,4] and epidemiologic grounds.[5] However, the precise effects of estrogens on the retina remain largely unexplored.

Increasing experimental evidence suggests that estrogens are neuroprotective[6-8] and that estrogen replacement therapy may contribute to the prevention or delay the onset of Alzheimer's disease.[9] Menopause coincides also with the appearance or worsening of many common arthritic conditions[10] that are associated with increased levels of proinflammatory cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor-*α*.[11] Epidemiologic studies reported conflicting results on the protective effect of hormone replacement therapy (HRT) in the development of the neovascular form of AMD.[12,13] Others studies, however, failed to demonstrate a significant gender difference in the frequency of the disease.[14] A large clinical trial (Women's Health Initiative Sight Exam Study) investigating whether HRT may influence the development and the course of macular degeneration is under way.

The mechanisms by which 17-*β*-estradiol (E2) protects against AMD are not known. A protective effect has been demonstrated in the ischemia-reperfusion injury model in rat retina.[15] E2 enhances the expression of vascular endothelial growth factor (VEGF) and its receptor (VEGFR-2) in bovine retinal endothelial cells in vitro,[16] but this effect has recently been shown to depend on oxygen status in a murine model of retinopathy of prematurity.[17] Ovariectomy has been suggested to disturb Bruch's membrane turnover in mice fed with a high-fat diet (Alexandridou A, Elliot S, Espinosa D, Hernandez E, Csaky KG, Cousins SW, ARVO Abstract 1200, 2001).

In the present study, we used cDNA microarray technology to profile gene expression changes (~10,000 screened genes) in intact posterior segments of ovariectomized (OVX) mice treated or not with E2 pellets. In a second confirmation phase, the expression of selected genes was investigated by quantitative RT-PCR on individual mice retinas and correlated with the estrogen status. E2 supplementation significantly reduced the expression of one gene, YKL-40 (also known as human cartilage glycoprotein 39, HCgρ39). YKL-40 is a mammalian glycoprotein member of a family of 18 glysolyl hydrolases related in sequence to chitinases but

without chitinase activity.[18,19] Its function is unknown, but it is thought to be involved in tissue remodeling, acting as a growth factor for connective tissue cells[20] and as a potent migration factor for endothelial cells.[21] Most of the studies regarding YKL-40 have focused on rheumatoid arthritis (RA), because this protein is an articular autoan-tigen in mice[22] (with pathologic effects similar to those with immunization by collagen type II), and its serum levels are clinically correlated with the severity of the disease.[23.24] Because this glycoprotein has not been previously described in the retina, we studied its expression in human normal retina, in neovascular choroidal membranes extracted from patients with AMD, and in a murine model of experimental choroidal neovascularization (CNV).

METHODS

Ovariectomized (OVX) Mice

OVX mice (ovariectomy was performed at 6 weeks of age) were purchased from Charles River France (Lyon, France). In the group (n = 25) receiving substitutive hormonal therapy, estrogens were administered through 3-mm subcutaneous pellets containing 1.7 mg E2 (Innovative Research of America, Sarasota, FL). In the control group, OVX mice (n = 25) received implants of a saline-containing pellet. E2 pellets provide continuous release, allowing for a constant level of circulating hormone. The selected pellet delivered 28 μg/d of E2, providing serum concentrations exceeding the usual physiological concentrations in female mice (~100 pg/mL) but lower than high-dose estrogen treatment (e.g., anti-tumor therapy).[25] Serum levels of E2 were monitored at the end of the study period by radioimmunoassay, according to the manufacturer's instructions (Immunotech; Westbrook, ME), as previously described.[2] After 30 days, mice were killed, and the ocular posterior segments (neural retina and RPE-choroid complex) were removed and frozen in liquid nitrogen. In the treated group, the efficiency of HRT was also controlled by means of uterine weight (approximate fivefold increase in the E2 treated group) and histologic evaluation of the endometrium (not shown). Three mice had to be removed from the study group because of defective pellet implantation. For experiments using RT-PCR, a second set of 20 OVX mice (10 in each group) was treated similarly (2 mice were not analyzed in the E2-treated group because of bad pellet implantation). Animal experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

***TABLE 1**. Oligonucleotide Sequences*

Gene and (Accession No.)	Position	Oligonucleotide Sequence (5 '-3')	Size of PCR Product (bp)	Cycles (n)
28S5 (U13369)	12403F 12614R	GTTCACCCACTAATAGGGAACGTGA GGATTCTGACTTAGAGGCGTTCAGT	212	19 (35 for LPC)
mYKL-40 (BC005611)	345F 486R	CTGTCTGTTGGAGGGTGGAAA CGAGATCCAGCCCATCAAA	142	32 (45 LPC)
mYKL-40 (BC005611)	1016F 1147R	GAAGGTTCCCTTCGCTACCAA TCATCCAAATCCAGTGCCCA	132	32
hYKL-40 (M80927)	271F 382R	ACACCTGGGAGTGGAATGATGT TGAGACCCAAAGTTCCATCCTC	112	35
mRBP (W83609)	174F 316R	GTCCCAAAAATGCCTGTGGAC TGCACGATCTCTTTGTCTGGC	143	30
mAngiogenin (NM_007447)	248F 377R	CGAATGGAAGCCCTTACAGAGAA ACATGTCTGAACCCTGCAGAGG	130	28
mTransthyretin (AA822116)	159F 300R	GTGGTTTTCACAGCCAACGAC TTTGGCAAGATCCTGGTCCTC	142	30
m,hVEGF-A (AH001553)	1208F 1687R	CCTGGTGGACATCTTCCAGGAGTA CTCACCGCCTCGGCTTGTCACA	407 347	33
mPEDF (NM_011340)	313F 569R	GAGCTGAACATCGAACAGAGTCTG AATCTCCTGAAGGTCTACTCGAGG	256	45

m and h, mouse and human, respectively.

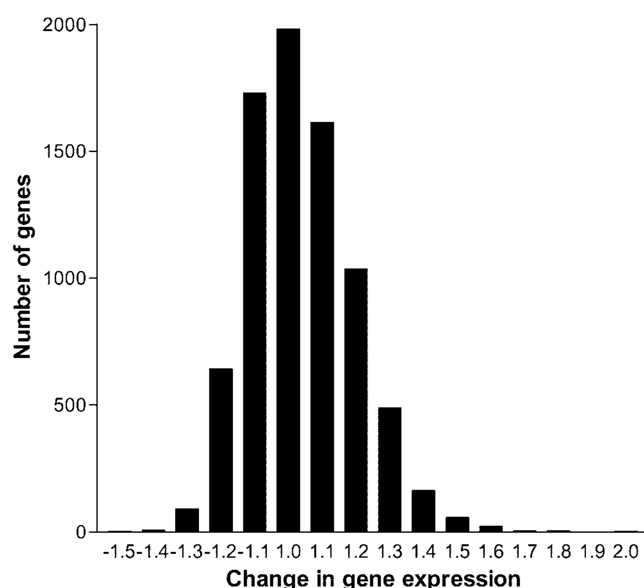
cDNA Array

Total RNA was isolated from posterior segments (retina and RPE-choroid complex) from OVX control and OVX-E2-treated mice by using an RNA isolation protocol (TRIzol; Sigma, Belgium), as recommended by the manufacturer. The frozen tissues were first pulverized with a dismembrator (B. Braun Biotech International, GmBH, Melsungen, Germany) and homogenized in the extraction reagent. RNA quality was controlled on 1% agarose gel. RNA samples from each group were pooled and then processed by Incyte Genomics (St. Louis, MO) and hybridized to a cDNA array (Mouse UniGeneI; Incyte Genomics, Inc.). This array is designed to provide a broad view of the mouse genome and includes targets for 9596 elements with 8985 unique annotated genes and expressed sequence tag (EST) clusters (list available at http://www.incyte.com/expression/easy_to_spot/catalog/txt_ files/mouse_unigenel_gene_list.txt).
Gene expression levels were measured and displayed using the manufacturer's software (GEMTools 2.5; Incyte Genomics).

Human Intact and Pathologic Retinas

Eight consecutive submacular CNV specimens were surgically removed (performed by Carel Claes, MD, A.Z Middelheim, Antwerpen, Belgium) during 360° macular translocations performed on patients with exudative AMD (three men, five women; mean age, 77 years; range, 72-83). These patients were not eligible for conventional laser photodynamic therapy because of the presence of occult new vessels or submacular bleeding, or, in the case of one patient, because of a recurrence of the disease. The methods conformed to the Declaration of Helsinki for research involving human subjects. The specimens were snap frozen in liquid nitrogen and stored at -80°C. Ten human male and female donor eyes were also obtained from the Cornea Bank, University of Liège, Belgium, as a source of intact neural retina and RPE-choroid. After removal of the anterior segment structures, 5-mm diameter punches were made in the macular region and in the peripheral retina (to estimate differential expression between the two regions), frozen in liquid nitrogen, and stored at -80°C.

***FIGURE 1.** Frequency of genes exhibiting different levels of expression (in multiples of change) in OVX mice supplemented or not with E2 pellets (increased expressions are shown as negative values).*



Laser Pressure Catapulting

For laser pressure catapulting (LPC), experimental CNV was induced by laser in C57BL mice (n = 12), as previously described,[26] to evaluate the spatial and temporal pattern of YKL-40 expression. The eyes were enucleated at selected intervals after induction (days 3, 6, 14, and 40), embedded in mounting compound (Tissue Tek; Miles Laboratories, Naperville, IL), and frozen in liquid nitrogen. Serial frozen sections (n = 8-10) were mounted directly on 1.35-mm thin polyethylene foil (Palm, Wolfratshausen, Germany), which had been mounted on the glass slide using a robotic technique (Microbeam-Moment; Palm).[27] The robot microbeam (Palm) focused the laser (337 nm) on the specimen, catapulting the selected area into the microfuge cap. The entire subretinal CNV area (as shown in Fig. 4C) and an adjacent intact chorioretinal zone (control) were microdissected

separately on frozen sections (10 μm thick). The specimens were covered with 100 μL lysis buffer, and total RNA isolation was performed with an RNA isolation kit (PureScript RNA; BioZym, Landgraaf, The Netherlands) according to the manufacturer's protocol. Total RNA was dissolved in a 10 μL RNA hydration solution supplied by the manufacturer, and RT-PCR was performed.

***TABLE 2.** Gene Expression in the Absence of E2 Treatment*

Gene Description	Accession No.	Change (×)
Glutathione *S*-transferase like	W16059	2
YKL-40	W10705	2
EST	AI447967	2
EST	AI549666	1.9
CDC-like kinase (cell cycle)	AA684191	1.8
Lactate dehydrogenase 1, A chain	A1325819	1.7
Transthyretin	AA822116	1.7
Ribosomal protein S16	AA624897	1.7
Ribosomal protein L41	AI120332	1.7
Ribosomal protein L7	W82240	1.7
Ribosomal protein S5	AA444231	1.7
Insulin-like growth factor-binding protein 4	AA145454	1.7
Cathepsin S	AA146437	1.7
Small inducible cytokine A6	AA821932	1.6
Prothymosin-β4	W09641	1.6
Matrix γ-carboxyglutamate (gla) protein	W88093	1.6
Fas apoptotic inhibitory molecule	AA623998	1.6
Retinol binding protein, cellular	W83609	1.5
Cystatin C	AA770768	1.5
Crystallin-α	AI325730	1.5
Vitronectin	AA980366	1.5
Decorin	AA755007	1.5
Hypoxia inducible factor-1α (HIF-α)	AA422602	-1.4

cDNA microarray expression data of selected genes analyzed on RNA samples pooled from E2-treated (n = 22) or E2-untreated (n = 25) OVX mice. All expression changes are positive except for HIF-α.

RT-PCR Analysis

The frozen tissues were pulverized in a dismembrator (B. Braun Biotech International), and total RNA was extracted with a kit (RNeasy; Qiagen, Paris, France), according to the manufacturer's protocol. 28S rRNA was amplified with an aliquot of 10 ng total RNA (for tissues) or 1 μL total RNA (LPC samples), by using an RNA PCR kit (GeneAmp with Thermostable rTth reverse transcriptase; Applied Biosystems, Foster City, CA) and two pairs of primers (Eurogentec, Liège, Belgium). Oligonucleotide sequences are shown in Table 1. Reverse transcription was performed at 70°C for 15 minutes followed by 2 minutes incubation at 95°C for denaturation of RNA-DNA heteroduplexes. Amplification was performed in cycles of 15 seconds at 94°C, 20 seconds at 60°C, and 10 seconds at 72°C. RT-PCR products were resolved on 2% agarose gels and analyzed by fluorescence imager (Fluor-S Multilmager; BioRad, Richmond, CA), after staining with ethidium bromide (FMC BioProducts, Philadelphia, PA).

Statistics

Gene expression levels in both groups were compared on computer by *t*-test (Prism 3.0; GraphPad, San Diego, CA).

RESULTS

Effects of E2 on Chorioretinal mRNA Profile

The mean E2 serum level was 15.08 ± 4.2 pg/mL in OVX-untreated mice and 537 ± 79.7 pg/mL in E2-treated mice, indicating that ovariectomy reduced endogenous circulating E2 concentrations, whereas supplementation with 1.7-mg estradiol pellets restored circulating concentrations of E2 moderately over the physiological range (100 pg/mL).[25] Only a minority of the genes tested on the cDNA array showed a noticeable change of their expression after 30 days of substitutive E2 therapy. Less than 1% of tested genes displayed a differential expression ratio of 1.5 or more, and most of them appeared to be upregulated by E2 deficiency (Fig. 1). The cDNA microarray expression data of selected genes are shown in Table 2. The largest difference in expression (ratio = 2) was seen for the YKL-40 gene with lower expression in OVX E2-treated mice. The influence of E2 supplementation on YKL-40 expression was confirmed by semiquantitative RT-PCR (in triplicate and with two different couples of oligonucleotides normalized to 28S signal) performed on a second set of animals and related to the presence or absence of E2 supplementation (Fig. 2). Several genes showing similar expression levels in the E2-treated and untreated groups were assessed in parallel by RT-PCR and used as the negative control (Fig. 3).

Figure 2. *Influence of E2 treatment on YKL-40 gene expression in OVX mice. (**A**) Representative example of YKL-40 mRNA expression in the eye of individual mice. (**B**) Relative levels of YKL-40 mRNA (den-sitometric quantification and normalization to the 28S signal) in the E2-treated (+E2) and untreated (-E2) group.*

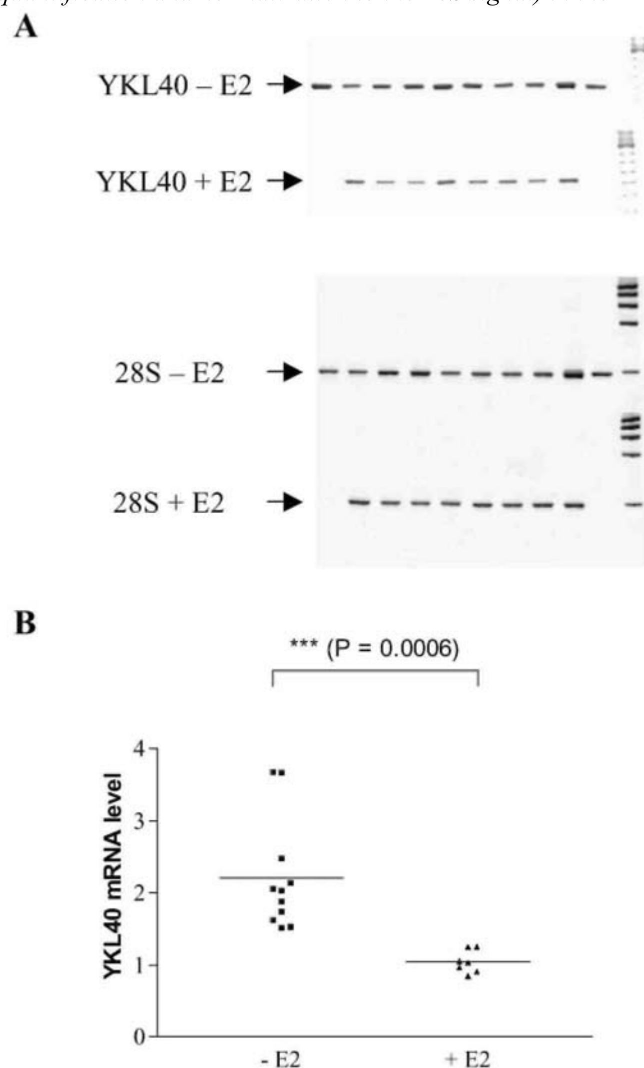


***FIGURE 3.** Confirmation by RT-PCR of the cDNA array results for selected genes (used as negative control). Transthyretin, retinol binding protein (RBP), angiogenin, and VEGF-A mRNA expression levels (densito-metric quantification and normalization to 28S signal) in ocular posterior segments of individual mice. There was no statistically significant difference between mean mRNA expression in the E2-treated or untreated OVX mice (P > 0.05, t-test).*

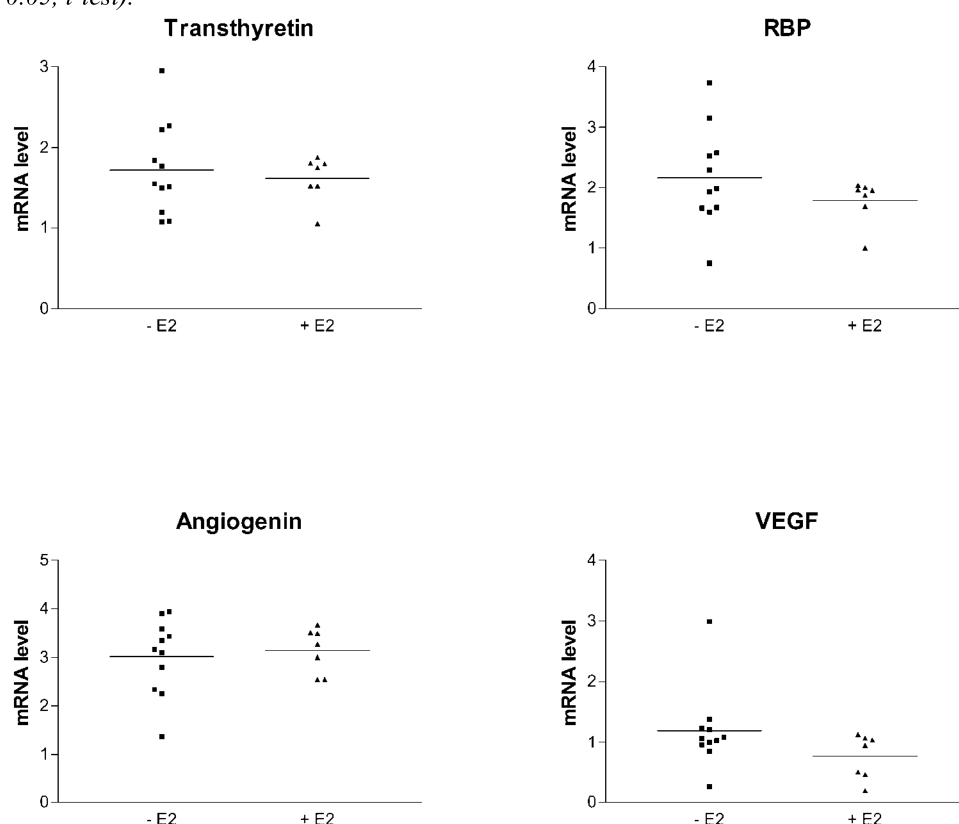


Table 3 shows the relative expression (signal intensity) of several groups of genes potentially involved in chorioretinal diseases in the untreated and E2-treated group.

It is interesting to note the relatively high level of expression of CD59a (an inhibitor of complement activation[28]), in view of the recent reports of complement involvement as a drusen component[29] and as a mediator of CNV (Bora PS, Sohn JH, Kang SG, Bora NS, Kaplan HJ, ARVO Abstract 1291, 2002). Elevated expression of β-crystallin in the retina has been reported previously, without explanation of its potential role.[30] Membrane-type matrix metaËoproteinases (MT1-MMP) and tissue inhibitor of matrix metalloproteinase (TIMP)-3 also had a relatively high level of expression. Angiopoietin-like 2 had the highest level of expression in the angiogenesis regulator group,[31] whereas some members of the VEGF family were weakly expressed (placental growth factor) or absent (VEGF-B and -C).

YKL-40 Expression Profile in Human and Mouse Neovascular Membranes

YKL-40 expression was detected in normal human posterior segments from different donors (Fig. 4A). YKL-40 was detected in ocular tissues from patients of different age and gender, regardless of the region of the sample (inside or outside the macula, retina or RPE-choroid complex). YKL-40 mRNA was also detected in all CNV specimens obtained during surgery (Fig. 4B). Although sharing in common the unfeasibility of conventional treatment, these neovascular membranes had a documented different natural history (size of the lesion, duration of symptoms, previous treatment). To evaluate more precisely the spatial (lesion versus intact adjacent regions) and temporal pattern of YKL-40 expression, RT-PCR analysis was applied to laser-induced murine neovascular choroidal membranes microdissected (Fig. 4C, left) by LPC at different time end points (days 3-40 after laser-induced CNV). When compared with intact adjacent regions, upregulation of YKL-40 mRNA expression was evident in CNV lesions at all end points (Fig. 4C).

DISCUSSION

DNA microarrays are powerful tools that allow for genome-wide gene expression profiling of cells or tissues.[32] In this study, we used a cDNA array testing for approximately 10,000 genes to identify modification of the gene expression profile of pooled ocular posterior segments in ovariectomized mice rescued or not by substitutive E2 therapy. In this experimental setting, less than 1% of the genes tested showed noticeable expression changes. Among them, the YKL-40 gene showed the largest expression change, with lower values in OVX E2-treated mice. This observation was confirmed by semiquantitative RT-PCR in a second set of experiments performed on individual mice tissues. YKL-40 gene expression was further studied in normal and pathologic human retinas.

YKL-40 expression has been reported in human RPE, with serial analysis of gene expression technology (SAGE).[33] In the current study, YKL-40 was consistently expressed in normal human eyes both in neural retina and in the RPE-choroid complex. High concentrations of YKL-40 in serum correlates with morbidity in different human diseases such as active rheumatoid arthritis and hepatic fibrosis, as well as with death of recurrent colorectal cancer.[34-36] YKL-40 is produced by monocytes in the media of arteritic vessels, in inflamed synovial membranes, and in atherosclerotic plaques, suggesting a role for YKL-40 in tissue remodeling.[37,38] In vitro, YKL-40 is one of the most abundant proteins secreted by cultured chondrocytes.[39] The regulation of YKL-40 expression, however, is largely unknown. In this study YKL-40 was significantly down-regulated by estrogen supplementation in the ocular posterior segment of OVX mice. Furthermore, YKL-40 expression was upregulated both in pathologic human and experimental CNV, although we cannot exclude bias caused by our selecting specimens from patients for whom standard laser therapy could not be used. Taken together with the migratory properties of YKL-40 in endothelial cells,[21] these data suggest a proangiogenic role of YKL-40 in the development of exudative AMD and could at least partly verify a protective role for estrogen replacement therapy.[12,13] A direct effect of YKL-40 on angiogenesis in other in vitro and in vivo models, such as corneal pellets or aortic rings, has yet to be evaluated. If a similar regulation were to be demonstrated in the synovium, our results could also provide a putative explanation for the protective effect of estrogens in rheumatoid arthritis, because YKL-40 has been proposed as a candidate autoantigen in this disease,[23] and its serum levels are related to disease activity.[25] It is of interest that, in our model of OVX mice, only a limited number of genes were affected by estrogen therapy in the retina. This could be explained by the pooling of samples for cDNA array, which selected only for strong and constant differences of gene expression and minimized individual variations. In particular, the expression of cathepsin D, an aspartic protease highly expressed in human retinal pigment epithelial lysosomes[40] with transcription classically regulated by estrogens in breast cancer,[41] was not influenced in vivo in the murine retina. Experimental impairment of cathepsin D results in accumulation of rod outer segment debris in the RPE and as been suggested as a murine model of dry AMD.[42] A similar discrepancy has been reported for endometrial cells and attributed to differential tissue-dependent regulation.[43] Individual variations in the level of mRNA expression for a specific gene are evident from the confirmation phase of our study, especially in the OVX untreated group. There was up to a 15-fold modulation of VEGF-A expression between mice of the same group. This could be explained by variations in very low level E2 concentrations (picomolar range), which have been demonstrated to enhance gene expression in different models.[44] It is also known that for some genes, the mechanism of regulation by estrogens is posttranscriptional rather than at the transcriptional level. This was indeed demonstrated for hepatic apolipoprotein E.[45] Finally, because the levels of ER-α and -β mRNA were weak in murine posterior segment, we cannot exclude the possibility that HRT could modify other genes in the human retina, in which these receptors are expressed at a higher level.[2]

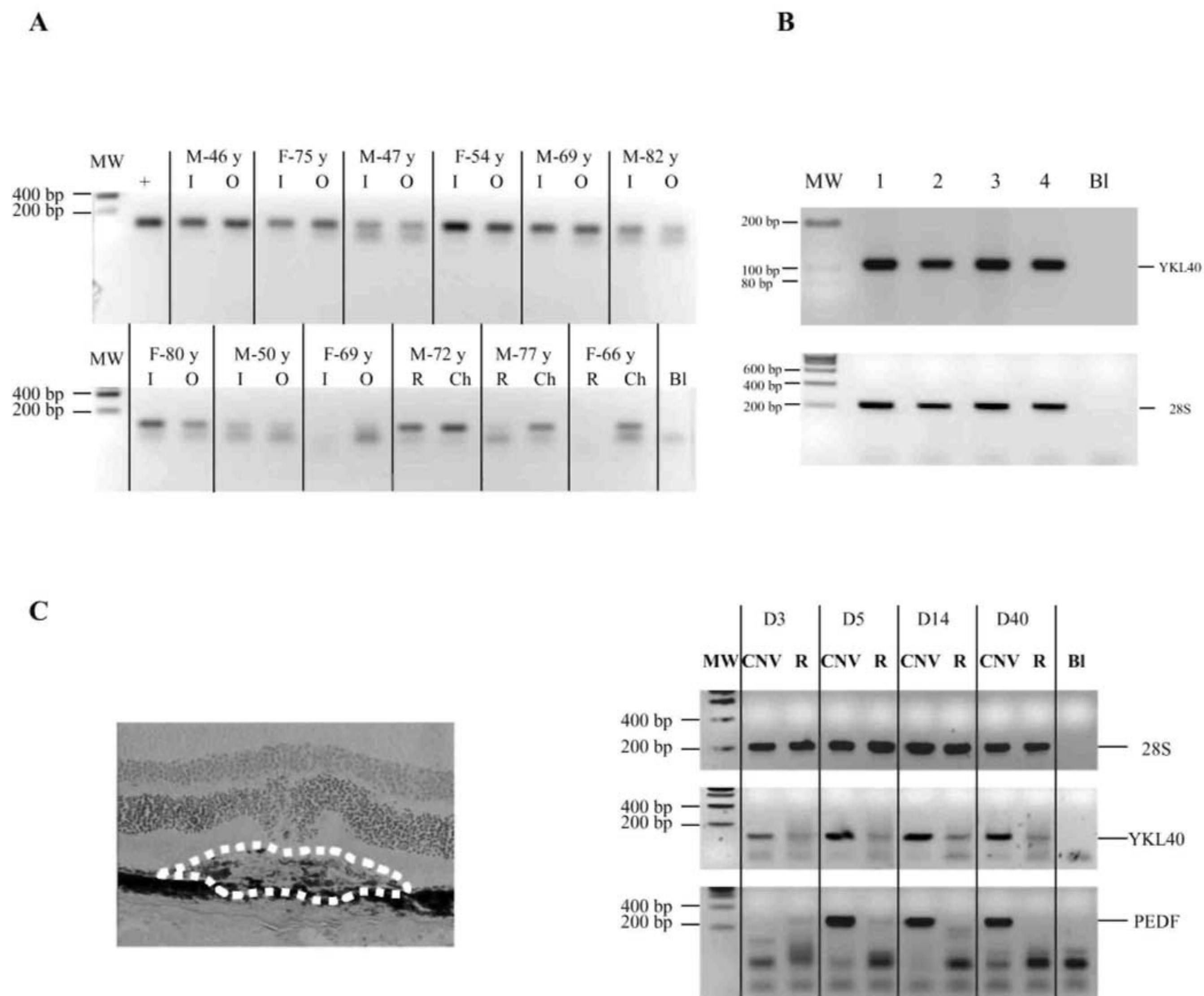
This study is obviously a preliminary phase in the understanding of the potential influences of HRT on AMD, and it would be of great interest to evaluate by cDNA microarray HRT-treated and untreated patients, as well as to compare intact and AMD tissue specimens. Our data nevertheless identify for the first time the YKL-40 gene as a potential mediator of estrogens effects both in the normal eye and during the development of CNV.

***TABLE 3**. Relative Expression of Selected Groups of Genes*

Gene Description	**Accession No.**	**Relative Level of Expression**	
		Untreated	**E2-Treated**
Apolipoproteins			
Apolipoprotein E	AI325603	3500	2436
Apolipoprotein D	AI120641	2294	2211
Apolipoprotein CII	AA617232	1938	1880
Apolopoprotein AII	AA051684	1056	902
Apolipoprotein AI	AA822098	393	408
Cathepsins			
Cathepsin F	AA726152	3590	3464
Cathepsin D	W16244	3103	2096
Cathepsin J	AA013726	2585	2535
Cathepsin E	AA839435	1421	1144
Cathepsin Z	AI892501	1301	1089
Cathepsin M	AA024360	921	1047
MMPs/ΓIMPS			
TIMP-3	AI197159	6979	6220
MMP-14 (MT1-MMP)	AA727488	2486	2565
MMP-24(MT5-MMP)	AA726203	935	753
MMP-2	AA756126	419	347
MMP-7	AA689037	173	182
Serine proteases			
uPA receptor (uPAR)	W82324	3557	3547
Plasminogen activator inhibitor l(PAI-l)	AA600496	511	354
Plasminogen	AA106793	318	255
Urokinase plasminogen activator (uPA)	AA510298	190	142
Tissue plasminogen activator (tPA)	AA426892	155	125
Angiogenesis			
Angiopoietin-like 2	AA755981	2630	2672
Prothymosin beta 4	W09641	2067	1277
PEDF	AA727967	2031	1509
VEGF-A	AA793036	2030	2275
Angiogenin	AI121741	1076	1232
Placenta growth factor (PIGF)	AA982549	329	299
Complement			
CD59a antigen	AA162378	8903	8824
Complement receptor 2	AA254235	1548	1639
C1q-α	AA145122	743	731
C1q-β	AI182838	340	306
C9	AA237324	592	673
Others			
Hyaluronidase 1	AA688635	13474	14120
Rod outer segment membrane protein 1	AA444932	8774	6569
ATP-binding cassette D, member 4	AA105879	8065	8493
Crystallin α-1	AI323082	7555	5914
Integrin-α4 (CD49d)	AA152636	5800	6426
Clusterin	AA210481	2413	1814
YKL-40	W10705	774	388

cDNA microarray relative expression data normalized to microarray, internal control signals, and analyzed on RNA samples pooled from E2-treated (n = 22) or E2-untreated (n = 25) OVX mice. PEDF. (pigmented epithelium-derived growth factor).

FIGURE 4. *Expression of YKL-40 mRNA in human retina and in CNV. (**A**) YKL-40 mRNA was present in human normal neural retina (R) and in the RPE-choroid complex (Ch), both in the macular region (I) and in the periphery (O). Age of the donors is given in years with gender. (**B**) Representative example of YKL-40 mRNA expression in surgically extracted choroidal neovascular membranes of four patients with exudative AMD. Total RNA (10 ng) was subjected to RT-PCR. (**C**) LPC followed by RT-PCR analysis of YKL-40 expression (with pigmented epithelium-derived growth factor [PEDF] used as an additional control gene on microdissected new choroidal vessels growing in the subretinal space (CNV, delineated by dotted line) and on adjacent (control) intact chorioretinal tissue (R) from days 3 to 40. Multiple bands under YKL-40 or PEDF signals represent multimers of primers. Molecular weights are shown at left (expected size for 28S product is 212 bp; for PEDF, 256 bp; for human YKL-40, 112 bp; and for murine YKL-40 142 bp).*



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