Endocrinology

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Endocrinology 2005 146:2963-2975 originally published online Apr 7, 2005; , doi: 10.1210/en.2004-1440

0013-7227/05/$15.00/0
Printed in U.S.A.

Endocrinology 146(7):2963–2975

doi: 10.1210/en.2004-1440

Differential Expression of Procollagen Lysine 2-Oxoglutarate 5-Deoxygenase and Matrix Metalloproteinase Isoforms in Hypothyroid Rat Ovary and Disintegration of Extracellular Matrix

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Hypothyroid-induced reproductive malfunction in both the sexes is a common phenomenon of global concern. In an attempt to characterize the differentially expressed genes that might be responsible for these disorders, we have identified a number of clones in hypothyroid rat ovary by subtractive hybridization. One such clone is procollagen lysyl hydroxylase2 (Plod-2), the key enzyme for the first step of collagen biosynthetic pathway, which was down-regulated in hypothyroid condition. We have also demonstrated the reduced expression of other isoforms of Plods, namely Plod-1 and -3 in hypothyroid rat ovary. The current studies are the first of their kind to report that thyroid hormone regulates the Plod gene in rat ovary. Moreover, we have shown the up-regulation of matrix-degrading enzyme(s), matrix metalloproteinase(s) in the hypothyroid rat ovary, whereas the tissue-inhibitory metalloproteinase is down-regulated. Finally, the results of the present studies indicate that in hypothyroid condition, collagen biosynthesis in ovary seems to be disturbed with concomitant enhancement in collagen degradation, resulting in disintegration of overall ovarian structure. (*Endocrinology* 146: 2963–2975, 2005)

HYPOTHYROIDISM IN THE adult animal leads to a number of physiological disorders as total body metabolism depends on thyroid hormone. Thyroid hormone plays a vital role in reproduction in both the sexes (1, 2). The role of T_3 in the steroidogenesis is already reported (3, 4), but the regulatory mechanism is still not clearly known. The thyroid hormone receptor has been identified in porcine and human granulosa cells (5–7). Our earlier report shows the existence of thyroid hormone receptor in perch ovarian follicular cells (8), goat testicular Leydig cell (9), and human corpus luteal cell nuclei (10). Hypothyroidism impairs reproductive functions in human beings and experimental animals, although mechanism of this dysfunction is not known. These reproductive disorders include irregular estrous cycle (11, 12); ovarian atrophy (13); disturbed folliculogenesis; and absence of corpora lutea (14), delaying the onset of puberty (15), anovulation (16), amenorrhea or hypermenorrhea, menstrual irregularity, infertility, and increased frequency of continuous abortion (17). Numerous evidences exist in medical literature that link hypothyroidism to reproductive disorder, although the underlying molecular mechanism is poorly understood. The extracellular matrix proteins, especially collagens, play a very critical role in maintaining the normal function of ovary. Procollagen lysine 2-oxoglutarate 5-dioxygenase (Plod) is the key enzyme for the collagen biosynthesis. Three Plod isoforms have been characterized in humans, mice, and rats. The cells transfected with Plod gene have been reported to produce the functional protein (18–22).

The role of extracellular matrix (ECM) in the formation and maintenance of follicles and corpora lutea has been mentioned earlier (23–27). The cells interact with matrix through cell surface adhesion receptors including the integrins. These focal adhesions can transduce multiple intracellular signals as well as provide the cells with anchorage. Although there are many heterodimeric combinations of the integrins, only a few of them have been localized to granulosa cells (28–30). The role of thyroid hormone in regulating the ECM protein expression has already been elucidated (31), in which ECM protein has been shown to alter in hypothyroid condition. Matrix metalloproteinases (MMPs) are a family of extracellular proteases capable of degrading various proteinaceous components of the ECM. It has already been demonstrated that differential regulation of three thyroid hormone-responsive MMP genes implicates distinct functions during frog embryogenesis (32). Thyroid hormone stimulates the production of tissue inhibitor of metalloproteinase (TIMP)-1 in cultured granulosa cells (33).

The present study made an attempt to identify the differentially expressed genes from rat ovarian granulosa cells, which may affect the steroidogenesis or other reproductive function for their altered expression. Using PCR-select cDNA subtractive hybridization technique, along with a number of genes, we have already identified the Plod-2 gene from rat ovarian granulosa cells, which was down-regulated in the

First Published Online April 7, 2005

Abbreviations: ECM, Extracellular matrix, GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, matrix metalloproteinase, Plod, procollagen lysine 2-oxoglutarate 5-deoxygenase, PTU, propyl thio uracil; SDS, sodium dodecyl sulfate; SSC, saline sodium citrate; TBS, Tris-buffered saline; TIMP, tissue inhibitor of matrix metalloproteinase.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

hypothyroid condition. Our study also provides evidence of the differential expression of MMPs and TIMP-2 in hypothyroid ovary. Therefore, in hypothyroid rat ovary, the enzymes that enhance the collagen biosynthesis are down-regulated; concomitantly the enzymes degrade ECM are up-regulated.

Materials and Methods

Animals and treatment

Pregnant Sprague Dawley rats raised in our animal facilities were housed in a well ventilated and temperature-controlled room with a 12-h light and 12-h darkness schedule. They were fed with standard balanced rat pellet, and drinking water was made available *ad libitum*. Rats were divided into two groups: 1) euthyroid in which the rats were provided with normal drinking water and their pups were used as control; and 2) hypothyroid in which after birth (d 0) 0.02% 6-*N*-propyl-2-thiouracil (PTU, Sigma, St. Louis, MO) dissolved in water was administered as drinking water for mother rats until the end of experiment (34). Hormone treatment consisted of daily single ip injections of 15 ng T_3 (Sigma) per gram body weight. When the pups were 26 d old, 10 female pups from each hypothyroid and control groups were injected ip with pregnant Mayer's' serum gonadotropin (Sigma) at a concentration of 10 IU per rat. At 28 d of age, the ovarian granulosa cells were isolated and were pooled to isolate RNA. For ovarian RNA or protein isolation, ovaries from 10 pups of each group were pooled and homogenized followed by RNA or protein isolation. For T_3 and TSH measurement, sera from 10 individual rats were collected for each experiment and the measurement was performed in each sample separately for at least three different sets of experiments and the mean values were represented. The pups were killed after treatment with overdose of chloroform. All animal protocols that were followed during the experiments were approved by the institutional animal ethics committee.

Granulosa cell isolation

The ovarian granulosa cells were isolated from 28-d-old female pups. The cells were obtained from the ovaries by puncturing the follicles with fine (26 gauge) needles gently allowing expulsion of cells into the 1× PBS (ice cold). Pooled cells were collected by brief centrifugation, washed, resuspended in RPMI 1640 medium, and kept in a humidified atmosphere containing 5% CO_2-95% air at 37 C. The cells were cultured for 4 h when the effect of T_3 was examined in *in vitro* system, 1 h without T_3, and another 3 h after addition of T_3 in the culture medium. The cell viability was more than 90% in all sets of experiments, as measured by the trypan blue dye exclusion test.

Histology and immunohistochemistry

The ovaries were dissected out and fixed by immersion in 10% paraformaldehyde diluted in 1× PBS, dehydrated in graded alcohol, and embedded in paraffin. Five-micrometer-thick sections were stained with hematoxylin/eosin. For each ovary, the total number of corpora lutea and Graafian follicles was counted under the light microscope (35).

Another set of section was processed for immunostaining. The sections were transferred to Tris-buffered saline (TBS) (pH 7.4), and the endogenous peroxidase activity was blocked by 1% H_2O_2 in TBS for 10 min. Anti Plod antibody (raised in rabbit in our laboratory, diluted 1:200) was added as primary antibody and incubated for 4 h, washed, and then incubated with secondary antibody (goat antirabbit AP, diluted 1:100) for 2 h. Immunoreactions were visualized under the Axiovert 25 microscope (Carl Zeiss, Gottinger, Germany).

RIA

For determination of plasma T_3 level, 100 μl blood from pups were collected and quickly mixed with 100 μl ice-cold 0.9% NaCl containing 0.24 mg EDTA. Plasma T_3 was determined by RIA using commercial T_3 RIA kit (RIAK-4, Board of Radiation and Isotope Technology, Bhaba Atomic Research Center, Mumbai, India). After incubation, the tubes were thoroughly decanted, and the bound radioactivity was determined by a γ-counter (Electronics Corp. of India Limited, Hyderabad, India). Standard curves were constructed by plotting the amount of total radioactivity bound against the hormone concentration (36). The sensitivity of T_3 was 0.24 ng/ml of the sample based on 90% B/B_0 intercept.

ELISA

ELISA was performed for serum TSH using Pathozyme TSH kit (Omega Diagnostics Ltd., Alva, UK) following manufacturer's instructions. The absorbance was noted immediately in a plate reader (Qualigens, Mumbai, India) using a 450-nm primary filter. The interassay and intraassay coefficient of variation was 6 and less than 5%, respectively. The minimum detectable concentration of TSH by Pathozyme TSH kit was estimated as 0.2 μIU/ml.

Western blot analysis

Total ovaries from 10 rats were isolated for each group (control, hypothyroid, and T_3-treated hypothyroid) for each experiment. The ovaries were homogenized in the buffer (150 mM NaCl, 500 mM Tris, and 10 mM EDTA) supplemented with protease inhibitors (1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml trypsin inhibitor) and 1% Triton X-100 (all from Sigma). The homogenate was then centrifuged at 8000 × *g* for 10 min at 4 C and the supernatant (an aliquot of it was used for protein concentration estimation) was subjected to 10% SDS-PAGE and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). The membrane was incubated with 5% blocking solution (TBS containing 0.1% Tween 20 and 5% nonfat dried milk) for 1 h, washed twice with TBS containing 0.1% Tween 20, and then incubated for 16 h with rabbit anticollagen I and III, respectively (Sigma), rabbit anti-MMP-2 (Sigma), goat anti-MMP-3 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-TIMP-2 antibody (Santa Cruz) and mouse anti-actin antibody (Santa Cruz). All primary antibodies were used in 1:1000 dilutions. Immunoreactive bands were visualized by reaction of horseradish peroxidase-labeled secondary goat antirabbit or antimouse antisera at 1:2000 dilutions (37) with horseradish peroxidase substrate.

RNA isolation and cDNA preparation

Total RNA was isolated from ovarian granulosa cells (in both control and hypothyroid groups) using TRIReagent solution (Sigma) following the manufacturer's instruction and the method described earlier (38), and cDNA was synthesized using Smart-PCR cDNA synthesis kit (Clontech, Palo Alto, CA) following the manufacturer's instruction.

Subtractive hybridization

Subtractive hybridization was performed using the PCR-select cDNA subtraction kit (Clontech), following the manufacturer's protocol with minor modification (39). In brief, 3 μg poly (A+) RNA isolated from the control and hypothyroid granulosa cells were used as driver and tester, respectively, to construct a forward subtracted library. Reverse subtracted library was also prepared, in which the driver was hypothyroid cDNA and the tester was control cDNA. The driver cDNA concentration was in excess, compared with the tester cDNAs, because during the second hybridization, only the driver cDNA, not tester cDNA, was added, which has been mentioned in the instruction manual supplied by the Clontech. The sequence of the cDNA synthesis primer used was 5′-TTTTGTACAA-GCTT$_{30}$N$_1$N-3′, which include *Rsa*I and *Hin*dIII restriction sites. The adaptor1 sequence used was 5′-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3′, the PCR primer1 was 5′-CTAATACGACTCACTATAGGGC-3′, the nested PCR primer-1 was 5′-TCGAGCGGCCGCCCGGGCAGGT-3′, the adapter-2 sequence was 5′-CTAATAC-GACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3′, and the nested PCR primer-2 sequence was 5′-AGCGTGGTCGCGGCCGAGGT-3′. Primary PCR condition was 94 C for 30 sec, 66 C for 30 sec, and 72 C for 90 sec for 30 cycles in 25 μl reaction volume. The secondary PCR condition was 94 C for 30 sec, 68 C for 30 sec, and 72 C for 90 sec for 16 cycles with 1 μl of one tenth diluted primary PCR product. For the PCR amplification, we used 50× PCR enzyme mix available with the Clontech Advantage cDNA polymerase mix. This 50× mix contains Klen-Taq-1 DNA polymerase (anexo-minus, N-terminal deletion of *Taq* DNA

polymerase), a proofreading polymerase, and TaqStart antibody for hot start. All PCR and hybridization were performed on a GeneAmp PCR system 9700 (PerkinElmer, Wellesley, MA).

Ligation, transformation, and preparation of subtracted plasmid

The subtracted cDNAs were cloned into T/A cloning vector (pGEM-T easy vector system 1, Promega, Madison, WI). Positive (white) recombinant plasmid DNAs were isolated and the insert cDNAs were released by digesting with *Eco*RI or *Not*I (New England Biolabs, Beverly, MA) and recovered from agarose gel by gel extraction kit (QIAGEN, Valencia, CA) (40).

Dot blot hybridization

The recombinant plasmids obtained by subtracted hybridization were amplified by PCR. Each product was spotted onto nitrocellulose membrane (Millipore) and denatured with 0.5 N NaOH, 0.5 M Tris-Cl, and 1.5 M NaCl. Hybridization was performed with α^{32}PdATP-labeled cDNA probe of control and experimental samples synthesized using Smart PCR cDNA synthesis kit (Clontech) keeping the same buffer and temperature as used for Northern hybridization. After hybridization, the membranes were washed twice with 0.5× saline sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) for 20 min each at 42 C and twice with 2× SSC, 0.1% SDS for 45 min each at 55 C, and exposed to x-ray film for autoradiography (Kodak, Rochester, NY) .

Northern hybridization

Northern hybridization was performed following the methods described earlier (37, 40). Briefly, 10 μg total RNA were loaded on each lane of 1% formaldehyde-agarose gel, electrophoresed, and transferred onto a nylon membrane (Nytran membrane, Millipore) by capillary suction method. Prehybridization was allowed for 2 h in 6× SSC with 50% formamide, 1× Denhardt's solution, 0.1 mg/ml salmon sperm DNA, and 0.5% SDS at 42 C. Hybridization was carried out for 18 h in the same buffer and temperature with the $^{\alpha 32}$PdATP-labeled cDNA fragments obtained from subtractive hybridization. The membrane was washed for 90 min (3 × 30 min each) at 65 C in 2× SSC containing 0.1% SDS with three subsequent changes of the buffer. The hybridized membrane was exposed to x-ray film (Kodak) followed by autoradiography. The RNA molecular size markers (0.2–6 kb) used in this experiments were purchased from MBI Fermentas (Hanover, MD).

Sequencing and analysis

Sequencing of the plasmids and the PCR products were performed by ABI Prism automatic DNA sequencer (PerkinElmer). Sequence alignment and data analysis were done through BLAST search from National Center for Biotechnology Information GenBank and using ClustalW software (41).

RT-PCR

First-strand cDNA synthesis was carried out with 2 μg total RNA using RevertAid M-MuLV reverse transcriptase (MBI Fermentas). To the tube oligo$(dT)_{18}$ primer, reverse transcription reaction buffer, Rnase inhibitor, deoxynucleotide triphosphates were mixed (final volume 20 μl) and incubated at 42 C for 1 h for first-strand cDNA synthesis. Two microliters from the cDNA prepared were used as template for RT-PCR with gene-specific primers, and relative expression was observed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer (37). A 50-μl PCR volume was made by adding 2.5 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) to a PCR mixture containing 1× reaction buffer [50 mM KCL, 10 mM Tris-HCl (pH 8.3), 0.1% Triton-X-100, and 2.5 mM $MgCl_2$], 200 μM of each deoxynucleotide triphosphates (MBI Fermentas), and 20 pmol of each primers. The PCR was performed for 25 cycles of denaturation at 94 C for 30 sec (5 min in the first cycle), annealing at specific temperature for each set of primers for 30 sec, and extension at 72 C for 30 sec (10 min in the last cycle; PerkinElmer 9700). The RT-PCR products were cloned, sequenced, and used for the expression purpose. The primers (used for RT-PCR) of the respective genes with the accession number and their amplified segments are listed in Table 1.

Real-time quantitative PCR

Relative quantitative RT-PCR was performed on iCycler (Bio-Rad Laboratories, Hercules, CA) real-time PCR machine using Quantitect SYBR green real-time RT-PCR kit (QIAGEN) following the instructions provided by the manufacturer to confirm the changes in gene expression observed during semiquantitative RT-PCR. In short, 1 μg of total RNA was reverse transcribed and PCR was performed with gene-specific primer in a total volume of 20 μl. Real-time RT-PCR conditions were as follows: reverse transcription step (50 C, 30 min), initial activation step (95 C, 15 min), and cycling step (denaturation 94 C, 15 sec, annealing 52–54 C, 30 sec, extension 72 C, 30 sec × 35 cycles) followed by melt curve analysis (42–45 C, 15 sec × 40). An internal control β-actin was amplified in separate tubes. The data were collected quantitatively and the cycle threshold value was corrected by cycle threshold reading of corresponding internal β-actin controls. Data from four determinations (mean ± SEM) are expressed in all experiments as fold changes, compared with normal rat (42). The oligonucleotide primers (used for real-time PCR) of the respective genes with their amplified segments are listed in Table 2.

Antibody raising

Because the Plod antibody is not commercially available, we raised the same in our laboratory. The RT-PCR fragment of Plod cDNA was cloned into *Eco*RI/*Sal*I site of pGEX 4T1 (Amersham, Uppsala, Sweden) expression vector. The clone was sequenced to check proper orientation followed by induction with isopropyl-1-thio-β-D-galactopyranoside. Polyclonal antibody was raised against overexpressed glutathione-S-transferase-Plod fusion protein in rabbit and checked by Western blotting. This polyclonal antiserum was used for immunolocalization study and in Western blotting (40).

TABLE 1. Primers used in semiquantitative RT-PCR

Gene products	Forward primer	Reverse primer	Region amplified
Plod-1 (NM053827)	5′-GGG GAT CCC GCT CAG CCC AGT TCT TCA AC-3′	5′-GGC TCG AGT CGG GGA CTG TGG GAT ACT TG-3′	nt. 79-527
Plod-2 (NM175869)	5′-GGG GAT CCA TGC TCG CCC TGC TCT CC-3′	5′-GGC TCG AGT TGT CTG CCA GCC GCT TAT C-3′	nt. 39-458
Plod-3 (NM178101)	5′-GGG GAT CCC TGC TCC TGC TGC TGC TG-3′	5′-GGC TCG AGT CGC CAG AAT CAC ATC GTA GC-3′	nt. 37-367
MMP-2 (NM_031054)	5′-ACC AGA ACA CCA TCG AGA CC-3′	5′-TGA ACA GGA AGG GGA ACT TG-3′	nt. 269-718
MMP-3 (NM_133523)	5′-GCT CAT CCT ACC CAT TGC AT-3′	5′-ATG AGC CAA GAC CAT TCC AG-3′	nt. 44-543
MMP-14 (NM031056)	5′-TGG CGG GTG AGG AAT AAC-3′	5′-CAG GAT GGG TGA GAA CAG C-3′	nt. 1186-1994
TIMP-2 (NM021989)	5′-AAA GCA GTG AGC GAG AAG GA-3′	5′-CCA GGA AGG GAT GTC AAA GC-3′	nt. 267-868
GADPH (BC059110)	5′-CAA ATG GGG TGA TGC TGG TG-3′	5′-CAT ACT TGG CAG GTT TCT CC-3′	nt. 326-837

TABLE 2. Primers used in real-time RT-PCR

Gene product	Forward primer	Reverse primer	Region amplified
Plod-1 (NM053827)	5′-CCA ACG CAG ACG CCA GGA AC-3′	5′-CCA GAC GCC AAC ACG ACG G-3′	nt. 1216-1484
Plod-2 (NM175869)	5′-GGC TGT GCT CTT TGT GAC TTT G-3′	5′-CTG GCT TCC GCT TGA CTT AGG-3′	nt. 1091-1374
Plod-3 (NM178101)	5′-TCT CCA GCA GCG ACA CAG AC-3′	5′-TTC TCA TAC CCT CCA GCC AAC C-3′	nt. 1478-1850
β-actin (BC014861)	5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3′	5′-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3′	nt. 542-1202

Statistical analysis

All data are expressed as the mean ± SD, and statistical analysis was performed by Sigmaplot 2000 for Windows (version 6, SPSS Inc., Chicago, IL) using Student's *t* test. $P < 0.05$ was considered to be significant. Experiments were repeated at least three times in duplicate unless otherwise stated. To make the variance independent of the mean, statistical analyses of real-time PCR data were performed after logarithmic transformation.

Results

Change in ovarian morphology in hypothyroid condition

The level of serum T_3 was decreased and the TSH was increased by several folds in PTU-treated hypothyroid rats, compared with that in normal rats (Fig. 1A). The T_3 level was increased when it was injected into the hypothyroid rats and after withdrawal of PTU. TSH was decreased when T_3 was injected into the hypothyroid rats and also after withdrawal of PTU from the drinking water. However, T_3-injected control rats showed significantly higher level of serum T_3 and lower level of TSH, respectively, compared with control. Ovaries from the control, hypothyroid, and T_3-injected hypothyroid rats were collected, fixed, and histological slides prepared. These slides were stained with eosin and hematoxylin. The stained sections of hypothyroid ovary showed a markedly reduced number and the size of mature antrum filled follicles, compared with that observed in the control rat (Fig. 1B). Moreover, follicles of different stages of maturation were not clearly visible in hypothyroid rat ovary in contrast to that observed in the control rats. The ECM was also found disintegrated in the hypothyroid rat ovary, compared with the control set, whereas, in the T_3-injected hypothyroid rat ovaries, a clear indication of the ECM recovery was observed, although the follicular structure was not completely recovered up to 15 d after T_3 injection.

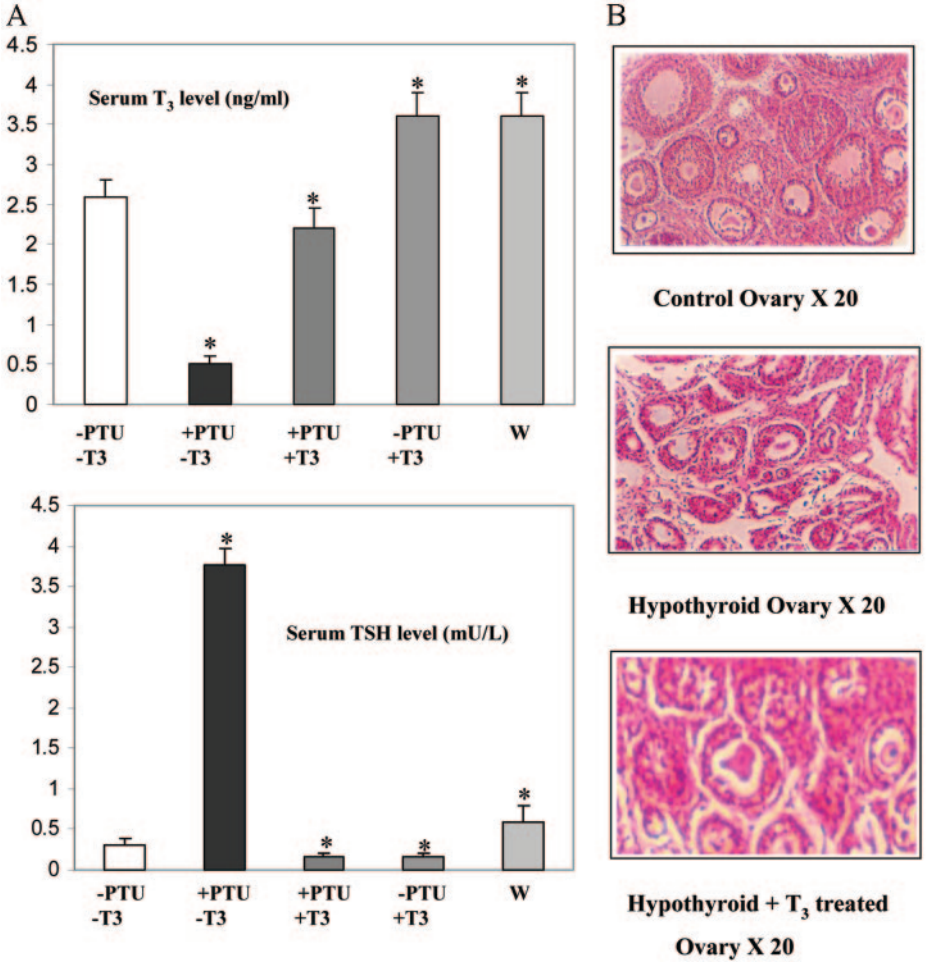


FIG. 1. Effect of PTU on serum T_3 and TSH level and histological study of the hypothyroid rat ovary. T_3 and TSH were measured in blood serum collected from control (−PTU-T_3) and hypothyroid (+PTU-T_3) animals. The rats mentioned as +PTU +T_3 were ip injected daily with T_3 (15 ng/g body weight) in hypothyroid animals, and rats mentioned as −PTU +T_3 were injected with T_3 (15 ng/g body weight) daily. In both sets, the rats were injected from d 15 of their birth for 15 d. PTU was withdrawn from the drinking water after 2 wk of PTU treatment and continued for another 15 d (W). Serum samples were prepared from the blood of 10 individual rats of each set. Three experiments were performed in duplicate, the data represented as mean ± SD. *, $P < 0.05$ (A). B, Histological staining of control and hypothyroid rat ovary. The histological sections of rat ovary were processed as mentioned in *Materials and Methods* and stained with hematoxylin and eosin. It shows that both number and size of the ovarian follicles in hypothyroid condition were drastically reduced, compared with control. The hypothyroid +T_3-treated ovarian section shows indication of recovery of number and size of the follicles.

Identification of differentially expressed genes by subtractive hybridization

By PCR-select cDNA subtractive hybridization, as described in *Materials and Methods*, we identified approximately 500 clones from the rat ovarian granulosa cells (Fig. 2A), which were either up- or down-regulated in hypothyroid condition. The clones were then screened again by dot-blot technique and the positive clones were then subjected to Northern hybridization to confirm their differential expression in the ovarian granulosa cells of the hypothyroid rat. Figure 2B shows some of the selected differentially expressed clones as demonstrated by Northern hybridization. The clones that showed differential expression in tertiary screening (Northern hybridization) were chosen for nucleotide sequencing and further characterization. The nucleotide sequences obtained were subjected to BLAST search (41) to identify any existing homology with other known genes at the nucleotide level. Among all the clones obtained so far, one represented the Plod or procollagen lysyl hydroxylase or lysyl hydroxylase gene. Table 3 represents the differentially expressed clones that were sequenced after tertiary screening and the BLAST search performed to identify the clones. In the right column, percent decrease or increase of their expression has been mentioned.

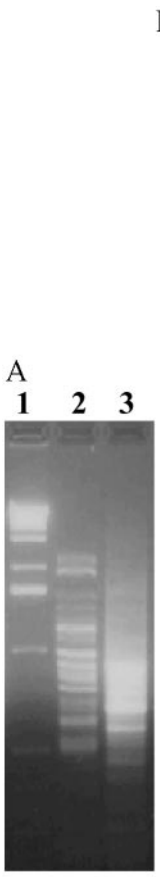
Plod isoforms are down-regulated in ovary of hypothyroid animals

After identification of the Plod-2 gene, it was subjected to Northern hybridization (Fig. 3A), and its expression level was significantly reduced in the hypothyroid rat ovarian granulosa cells. Figure 3B shows the 28S rRNA band indicating equal loading on each lane.

Eventually we found that there are three isoforms of Plod in human, mouse, and rat; these are Plod-1, -2, and -3. We wanted to check whether all the Plod isoforms were down-regulated in hypothyroid rat ovary. The oligonucleotide primers directed for each Plod isoforms were synthesized and used for semiquantitative RT-PCR using total ovarian RNA from control, hypothyroid, and T_3-injected hypothyroid rats. The RT-PCR products were cloned and subsequently sequenced. The nucleotide sequences showed complete homology with rat Plod-1, -2, and -3 cDNAs that have already been published (21). The RT-PCR products were electrophoresed in agarose gel, and the ethidium bromide-stained DNA bands were scanned (Fig. 3C). The scanning data showed that each Plod isoform was down-regulated in the hypothyroid condition, whereas their expression was increased on T_3 add-back.

Plod isoforms are down-regulated in hypothyroid ovarian granulosa cells and recovers on T_3 treatment

Granulosa cells were isolated from the ovaries of control rat and hypothyroid rats and cultured as described in *Materials and Methods*. The cells were divided into four groups, *e.g.* 1) control group (C), 2) hypothyroid group (H), 3) Hypo-T_3 group (HT) (where T_3 has been added in the culture medium of granulosa cells isolated from hypothyroid rat



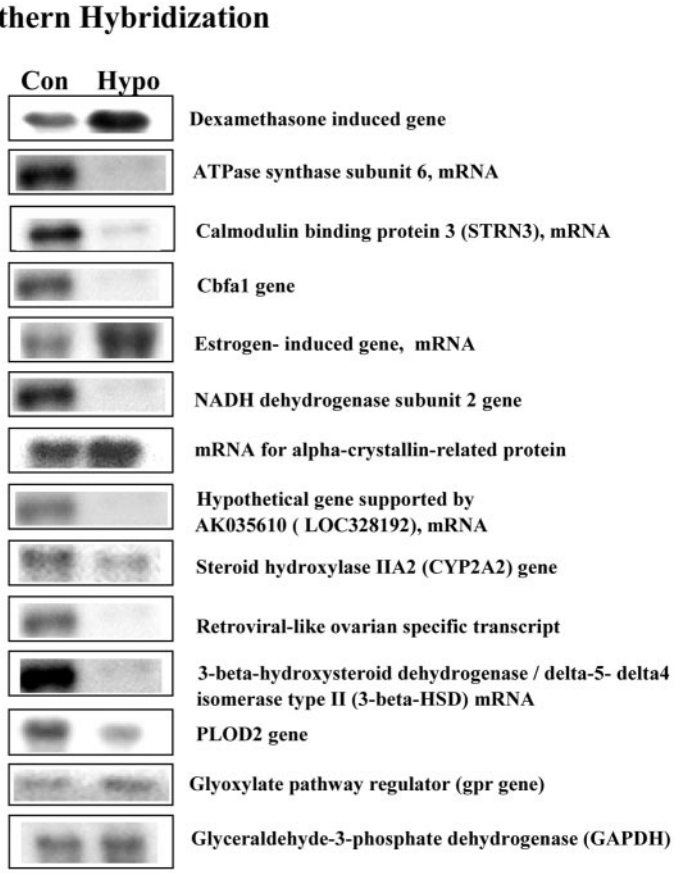


FIG. 2. Subtractive hybridization and Northern hybridizations of selective clones. Three micrograms poly (A+) RNA each from control (Con) or hypothyroid (Hypo) granulosa cells used as tester and driver and vice versa. Two rounds of PCRs were performed with these cDNAs and then subjected to agarose gel electrophoresis and loading on each lane as follows: DNA marker, *i.e.* 1 kb DNA ladder (Invitrogen) (lane 1); forward-subtracted clones, *i.e.* the clones specifically expressed in control set (lane 2); reverse-subtracted clones, *i.e.* the clones specifically expressed in hypothyroid set (lane 3) (A). After secondary screening by dot-blot hybridization, the selective clones were again screened (tertiary screening) by Northern hybridization. B, Northern hybridization results of some of the differentially expressed genes resulting from subtractive hybridization. NADH, Nicotinamide adenine dinucleotide (reduced).

TABLE 3. The differentially expressed clones obtained from subtractive hybridization

No.	GenBank accession no.	Name of the genes obtained by S.H.	Decrease/ increase, %
1	U48828	Retroviral-like ovarian specific transcript	−89.74
2	XM_286485	Hypothetical gene supported by AK035610 (LOC328192), mRNA	−83.69
3	AB013129	Cbfal gene	−79.50
4	AF504920	ATPase synthase subunit 6 mRNA	−78.75
5	AJ428514	NADH dehydrogenase subunit 2 gene	−75.89
6	M38179	3-β-hydroxysteroid dehydrogenase/δ-5-δ-4 isomerase type II (3-β-HSD) mRNA	−69.81
7	NM_014574	Calmodulin binding protein 3 (STRN3), mRNA	−67.54
8	AJ430861	Procollagen 2-oxogluterate 5-deoxygenase gene2 (PLOD2)	−51.84
9	M33313	Steroid hydroxylase IIA2 (CYP2A2) gene	−48.52
10	V00665	Genes coding for three transfer RNAs (specific for Phe, Val, and Leu)	−48.47
11	XM_193959	Casein kinase II, α 1 polypeptide (Csnk2a1), mRNA	−46.75
12	M27315	tRNA-Gly and tRNA-Arg genes	−46.34
13	gi313871	Mss 4	−38.24
14	gi3551053	Class 1 β-tubulin	−33.75
15	AK088955	Neonate thymus thymic cells cDNA	−32.45
16	AF448862	Dendrobium chrysotoxum trnK gene, intron	−24.77
17	AF115770	BHE/Cdb tRNA-Lys gene	−20.89
18	D44443	Dexamethasone-induced gene	+77.92
19	S74324	Estrogen-induced gene	+55.52
20	AJ534345	Glyoxylate pathway regulator (gpr gene)	+36.54
21	AB047592	mRNA for α-crystallin-related protein	+28.32
22	HSU17989	Nuclear autoantigen GS2NA mRNA	+23.12
23	AF151726	Putative MtN3-like protein mRNA	+24.98
24	XM_213508	RNA-dependent helicase p68 (DEAD-box protein p68) (DEAD-box protein 5)	+23.96

Negative and positive signs indicate the reduction and increment of expression of the clones in hypothyroid condition with respect to control value. NADH, Nicotinamide adenine dinucleotide; S.H., subtractive hybridization; STRN3, striatin 3; DEAD, aspartic acid (D), glutamic acid (E), alanine (A), aspartic acid (D).

ovaries), and 4) control-T_3 group (CT) (where T_3 has been added in the culture medium of granulosa cells isolated from control rat ovaries). The granulosa cells of all the groups were cultured for 4 h, the first two groups without adding T_3 in the medium, whereas in the third and fourth groups, the cells were incubated for 1 h without T_3 and then for 3 h with T_3. After incubation, total RNAs were isolated from each group and RT-PCRs were performed with gene-specific primers of Plod-1, -2, and -3. The products were electrophoresed on agarose gel and the ethidium bromide-stained bands were scanned. Figure 4A shows all the isoforms were down-regulated in hypothyroid ovarian granulosa cells; their expression increased in both T_3-added hypothyroid and T_3-added control granulosa cells, compared with that in hypothyroid condition. The expression of each isoforms was higher in T_3-added control granulosa cells than that in T_3-added hypothyroid granulosa cells.

To know whether the Plod expression and their up- or down-regulation are restricted to the granulosa cells, we did experiments with the residual ovaries of rat. We prepared residual ovaries by isolating and removing granulosa cells from the ovaries and then vigorously washing the tissue with PBS. Total RNAs were isolated from the residual ovaries, and subsequently RT-PCR was performed with the gene-specific primers of Plod isoforms. Figure 4B shows that all the isoforms were down-regulated in hypothyroid residual ovaries and showed little increase in their expression when T_3 was injected into hypothyroid animals. In RT-PCR experiments, the expression level of GAPDH was used as loading control.

We further confirmed the reduction of expression of different Plod isoforms in hypothyroid rat ovary by using real-time quantitative PCR. The results reveal a significant decrease in the expression of Plod-1, -2, and -3 in hypothyroid rat ovary when compared with control (Fig. 4C).

Western immunoblotting with Plod antibody shows that the expression of Plod protein was significantly decreased in hypothyroid ovary, whereas its expression recovers upon T_3 add-back (Fig. 4D). By immunohistochemistry using Plod antibody, it has been shown that Plod protein is expressed both in theca and granulosa cells. Figure 4E shows the expression of Plod in the rat ovarian section, which is not found in the absence of primary antibody (negative control).

Collagens are reduced in hypothyroid rat ovary

The protein level of collagen types I and III decreased by more than 60% in the ovary of hypothyroid rats, compared with control, as demonstrated by Western immunoblot (Fig. 5, A and B). The level of these proteins, however, increased in the ovary when the hypothyroid rats were injected with T_3. Actin antibody was used as a loading control (Fig. 5C) in this experiment.

MMP expression is increased in hypothyroid condition

MMPs are a family of Zn^{2+}-dependent extracellular proteases capable of degrading various proteinaceous components such as different types of collagens present in the ECM. There are many types of MMPs, which are specific for the degradation of particular type of collagens. The Western immunoblot data showed that the active MMP-2 protein level was increased by more than 5-fold in hypothyroid rat ovary (Fig. 6A). Another MMP, *i.e.* MMP-3, protein level was also significantly increased in hypothyroid condition, whereas its expression was much reduced in T_3 add-back (Fig. 6B). The TIMP-2 protein was reduced in hypothyroid

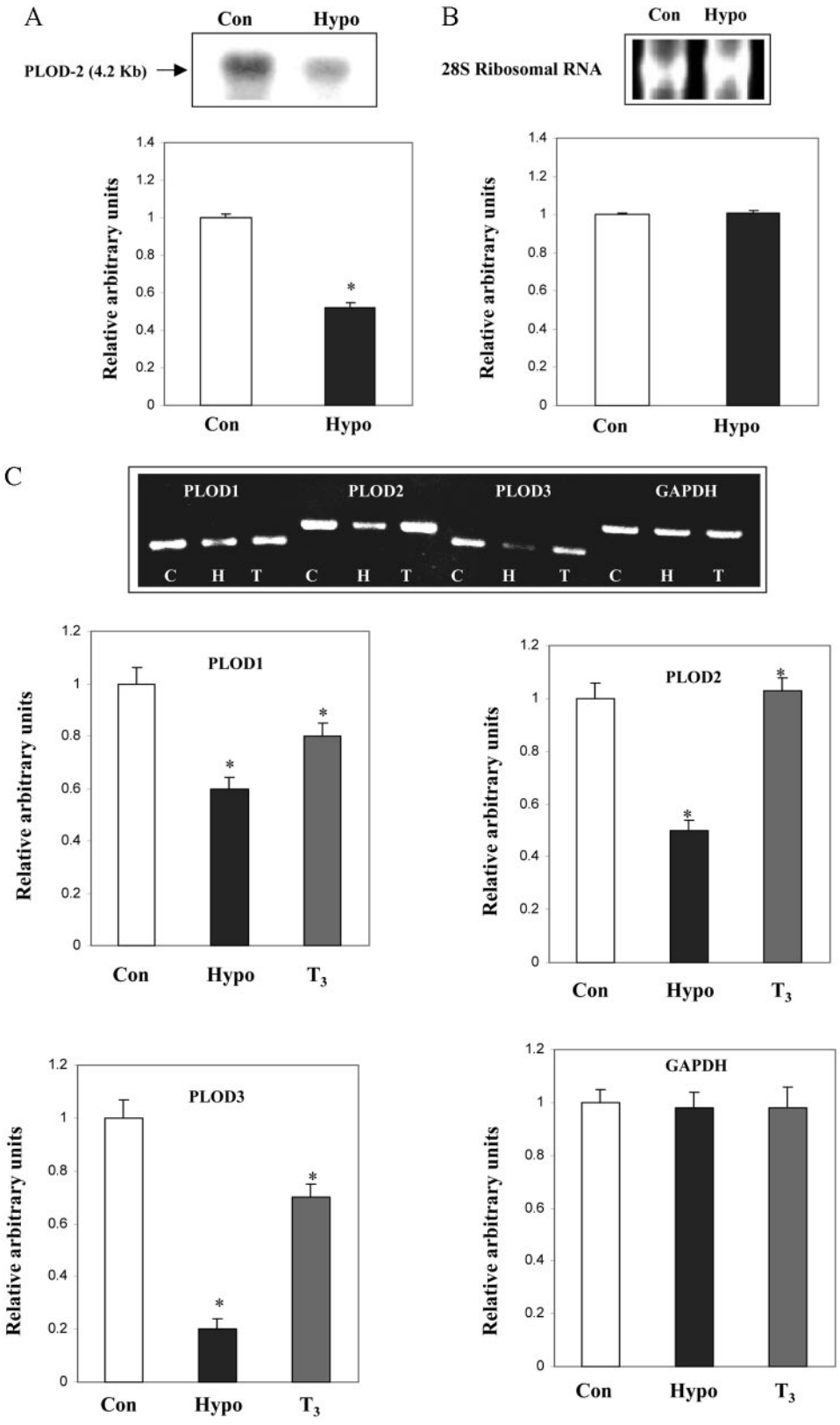
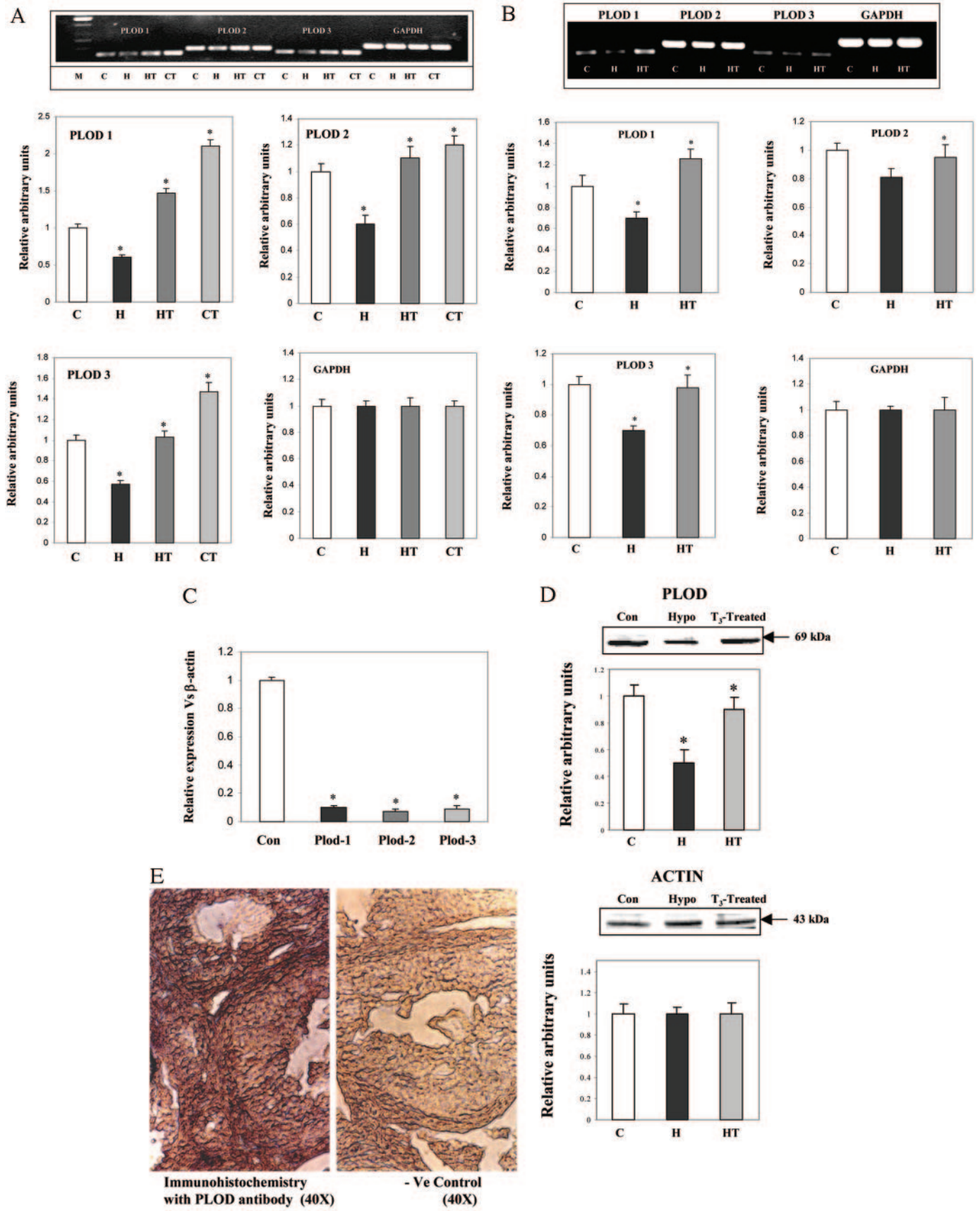


FIG. 3. Plod isoforms are down-regulated in hypothyroid condition and the effect of T_3 in their expression in ovary. A, Northern hybridization of Plod-2 cDNA. Fifteen micrograms of each control (Con) and hypothyroid (Hypo) rat granulosa cell RNAs were subjected to 1% formaldehyde agarose gel electrophoresis and then transferred onto Nytran membrane. This Northern blot was hybridized with radiolabeled Plod-2 cDNA obtained from subtractive hybridization. B, 28S rRNA from the same gel to show equal loading of RNAs in each lanes. C, Expression of Plod-1, -2, and -3 in the ovary of control, hypothyroid, and T_3 injected-hypothyroid rats by semiquantitative RT-PCR. The gene-specific oligonucleotide primers of Plod-1, -2, and -3 and GAPDH primers (for loading control) were used for RT-PCR and the amplified products were loaded in agarose gel as control (C), hypothyroid (H), and T_3 injected-hypothyroid animals (T). The pixel densities of the bands were quantified with ImageJ software [National Institutes of Health (NIH)] and have been represented in the *lower panel* (C) as relative arbitrary units considering the control value as 1. All the experiments were performed three times in duplicate, and the mean ± SD values have been shown. *, $P < 0.05$.

ovary, whereas its expression increased in T_3 add-back experiment (Fig. 6C). In these Western blotting experiments, actin antibody was used as loading control (Fig. 6D).

When RT-PCR was performed using the gene-specific primers, it was found that the expression of MMP-2, MMP-3, and MMP-14 significantly increased in hypothyroid ovary, and their expression


A
PLOD 1
PLOD 2
PLOD 3
GAPDH
M C H HT CT C H HT CT C H HT CT C H HT CT
B
PLOD 1
PLOD 2
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C H HT C H HT C H HT C H HT
Relative arbitrary units
C H HT CT
C
Relative expression Vs β-actin
Con Plod-1 Plod-2 Plod-3
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69 kDa
ACTIN
43 kDa
E
Immunohistochemistry with PLOD antibody (40X)
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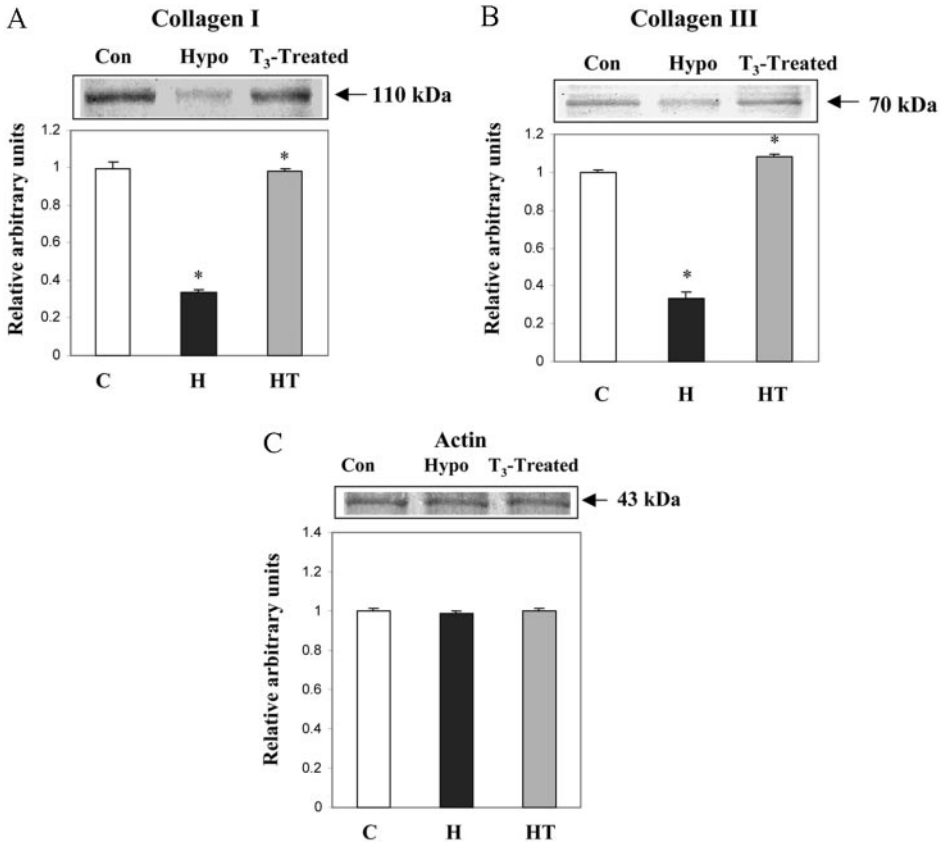

FIG. 5. Western immunoblot shows the collagen status in hypothyroid condition and the effect of T_3 in their expression in ovary. Thirty micrograms of ovarian proteins from each set were fractionated on 10% SDS-polyacrylamide gel, transferred onto polyvinyl difluoride membrane, and subjected to immunodetection with either rabbit anticollagen I (A), rabbit anticollagen III (B), or mouse anti-β-actin antibody as an internal control (C). The lanes indicated by Con, Hypo, and T_3-treated represent the protein loaded were isolated from control, hypothyroid, and T_3-injected hypothyroid animals, respectively. Collagen I and III and β-actin protein bands were quantified with the help of ImageJ software (NIH) and represented in the *lower panel*, in which C represents control (1 RAU), H represents hypothyroid, and HT represents T_3-injected hypothyroid ovarian tissue. All experiments were performed three times in duplicate and the mean ± SD values have been shown. *, $P <$ 0.05.

was normalized after T_3 add-back (Fig. 6, E–G). However, the TIMP-2 gene expression was decreased in hypothyroid condition and increased when hypothyroid animals were injected with T_3 (Fig. 6H). In all the experiments, GAPDH expression, however, remained unchanged (Fig. 6I). The increased expression of MMP-2, -3, and -14 and the down-regulation of TIMP-2 in hypothyroid rat ovary indicate degradation of ECM in this condition.

Expression of Plod in different tissues of control and hypothyroid rats

Total RNAs were isolated from different tissues, *i.e.* brain, heart, lung, kidney, liver, and ovary of control and hypothyroid rats, and RT-PCR was performed using Plod-2 gene-specific primers. Figure 7A shows a significant increase of Plod2 gene expression in heart, lung, and kidney of hypothyroid rats, whereas no significant change of expression was observed in brain tissue. The expression of Plod-2 was down-regulated in hypothyroid ovary. The expression of GAPDH was used as a loading control (Fig. 7B).

Discussion

Hypothyroidism-induced reproductive disorders are very common in both the sexes in most mammals including humans. Although the disorders are of various types in males

FIG. 4. Plod isoforms are down-regulated in both granulosa cell and residual ovary and role of T_3 in their expression. A, Differential expression of Plod-1, -2, and -3 genes in hypothyroid condition as shown by semiquantitative RT-PCR. Double-stranded cDNAs were prepared from 2 μg of total RNA of ovarian granulosa cells from control (C), hypothyroid (H), T_3-treated hypothyroid (HT), and T_3-treated control rats (CT). PCR was performed with these cDNA samples using gene-specific primers of Plod-1, -2, and -3 and GAPDH (for loading control). B, Expression of Plod isoforms in the residual ovarian tissue. RT-PCR was performed with the gene-specific primers of Plod-1, -2, and -3 and GAPDH with the residual ovary RNA samples isolated from the control (C), hypothyroid (H), and T_3-injected hypothyroid animals (HT). GAPDH products were shown as a loading control. C, Effect of hypothyroidism in the expression of Plod-1, -2, and -3 mRNA transcripts in control and hypothyroid rat ovarian granulosa cells as measured by real-time RT-PCR. Data are presented as fold changes from normal levels by analyzing the CT numbers corrected by CT readings of corresponding internal β-actin controls. Data from four determinations (mean ± SEM) are expressed in all experiments as fold changes, compared with normal rat. *, $P < 0.001$. D, Expression of Plod protein in control, hypothyroid, and T_3-injected hypothyroid rat ovaries. Fifty micrograms of total ovarian proteins from each sample were fractionated on 10% SDS-polyacrylamide gel, transferred onto polyvinyl difluoride membrane and subjected to immunodetection with either rabbit anti-Plod or mouse anti-β-actin antibody (as an internal control). The lanes, indicating Con, Hypo, and T_3-treated, representing the protein loaded was isolated from control, hypothyroid, and T_3 injected-hypothyroid animals respectively. E, Immunohistochemical localization of Plod in rat ovary. Five-micrometer-thick paraffin-embedded ovarian sections were deparaffinized by dipping into xylene for 20 min. The sections were dehydrated by passing through graded alcohol, blocked in 2% BSA, and stained with polyclonal anti-Plod antibody raised in our laboratory. Alkaline phosphatase-conjugated antirabbit-IgG was employed as second antibody. Color development due to immunoreaction by 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indoyl-phosphate, 4-toluidine salt was visualized throughout the ovarian section (magnification, ×40); color was absent in absence of primary antibody (negative control). The pixel densities of the bands (A, B, and D) were quantified with ImageJ software (NIH) and have been represented as relative arbitrary units considering the control value as 1. All the experiments were performed three times in duplicate and the mean ± SD values have been shown. *, $P < 0.05$.

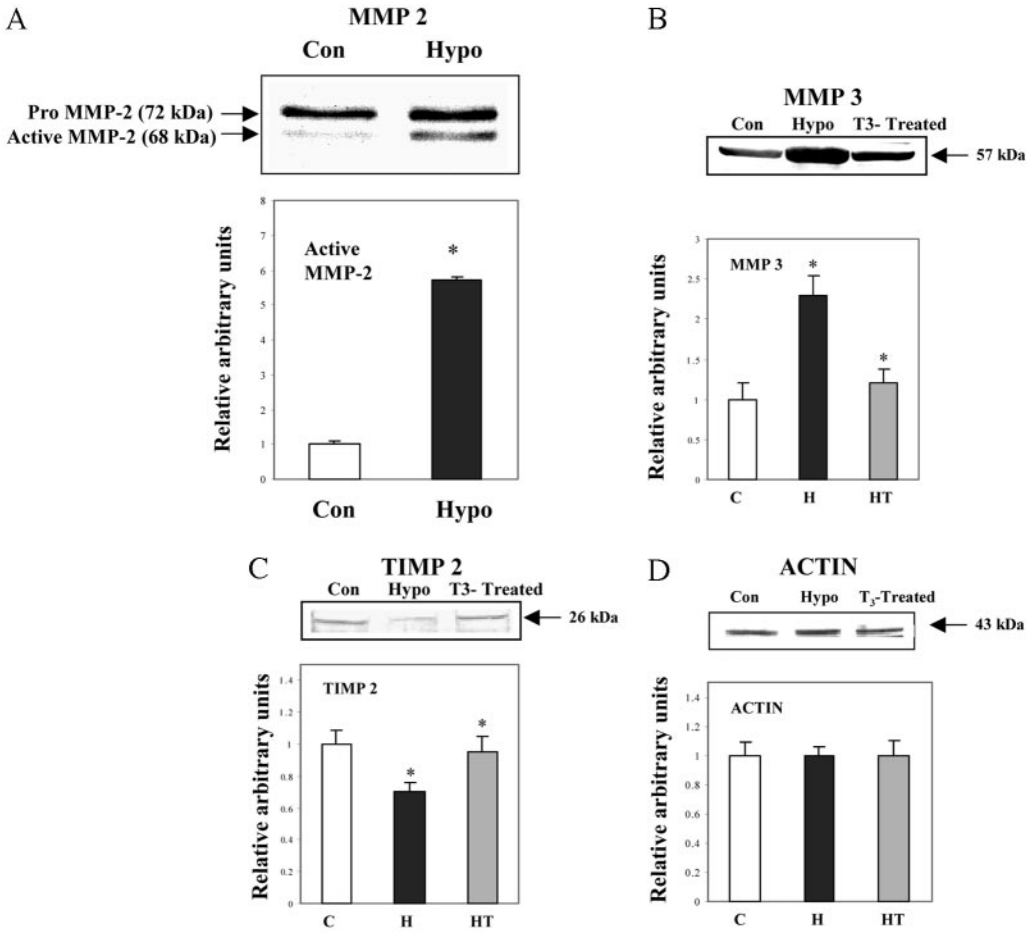

FIG. 6. Effect of hypothyroidism on the expression of MMP-2, MMP-14, MMP-3, and TIMP-2 in hypothyroid rat ovary. A–C, Differential expression of MMP-2, MMP-3, and TIMP-2 expression in the hypothyroid ovary by Western blot analysis. Thirty micrograms of protein from each set were electrophoresed on 10% SDS-polyacrylamide gel, transferred onto polyvinyl difluoride membrane and subjected to Western blot using rabbit anti-MMP-2 antibody, goat anti-MMP-3 antibody, and rabbit anti-TIMP-2 antibody, respectively. Western immunoblotting with antimouse antiactin antibody was performed to show equal loading on each lanes (D). The lane Con represents the protein isolated from the control rat ovary; Hypo represents the protein isolated from the hypothyroid rat ovary. E–H, Semiquantitative RT-PCR data of MMP-2, 3, and 14 and TIMP-2, respectively, which was performed with the respective gene-specific primers using ovarian RNA. RT-PCR with GAPDH primers was performed to show equal loading in each lane (I). The RNAs used for the RT-PCR were isolated from control (C), hypothyroid (H), and T_3-injected hypothyroid ovaries (HT). The pixel densities of each band (A–I) were quantified with ImageJ software (NIH). The control values of the band intensities obtained were represented as 1 relative arbitrary unit, and those of the hypothyroid RNA or HT samples have been represented by the same relative arbitrary unit, compared with the control value. All experiments were performed three times in duplicate and the mean ± SD values have been shown. *, $P < 0.05$.

and females, the ultimate result is reproductive failure. However, proper information on its cause and the molecular basis of the pathophysiological mechanism is yet to be known. The current study is an attempt to address this question at the molecular level.

The ovary is a very dynamic organ in which follicles and corpora lutea continually grow and regress. Cell migration, movement, division, specialization, differentiation, and death are the processes occurring continuously in this organ; the ECM participates in all of these. ECM is extremely important for the follicular development. It helps follicular fluid formation, filters soluble materials, and provides rigid or elastic mechanical support for tissues. In addition, nutrients and hormones and other extracellular signals are often required to traverse the matrix to reach the target cells (43).

Collagens, a large family of glycoproteins, are the structural building blocks of tissues and are the major component of the ECM. Collagen biosynthesis requires a large number of posttranslational modifications. One of the important steps in collagen biosynthesis is hydroxylation of lysine residues, which provide attachment sites for glycosylated hydroxylysine residues. Plod regulates the first step, *i.e.* hydroxylation of lysine residue. Plod, a peripheral membrane protein within the endoplasmic reticulum, catalyzes the hydroxylation of lysine in collagens and more than 15 other proteins (18). Hydroxylysine residue appears to play a critical role in the type IV collagens of basement membranes because mutations in the gene for the only Plod present in certain nematodes have been found to be embryonic lethal (19). Plod-1, -2, and -3 have been recently shown to be coregulated together with total collagen synthesis (22). There are several isoforms of the Plod gene in mouse, rat, and human (21, 44, 45). In our present study, we have shown that all the Plod isoforms are down-regulated in hypothyroid rat

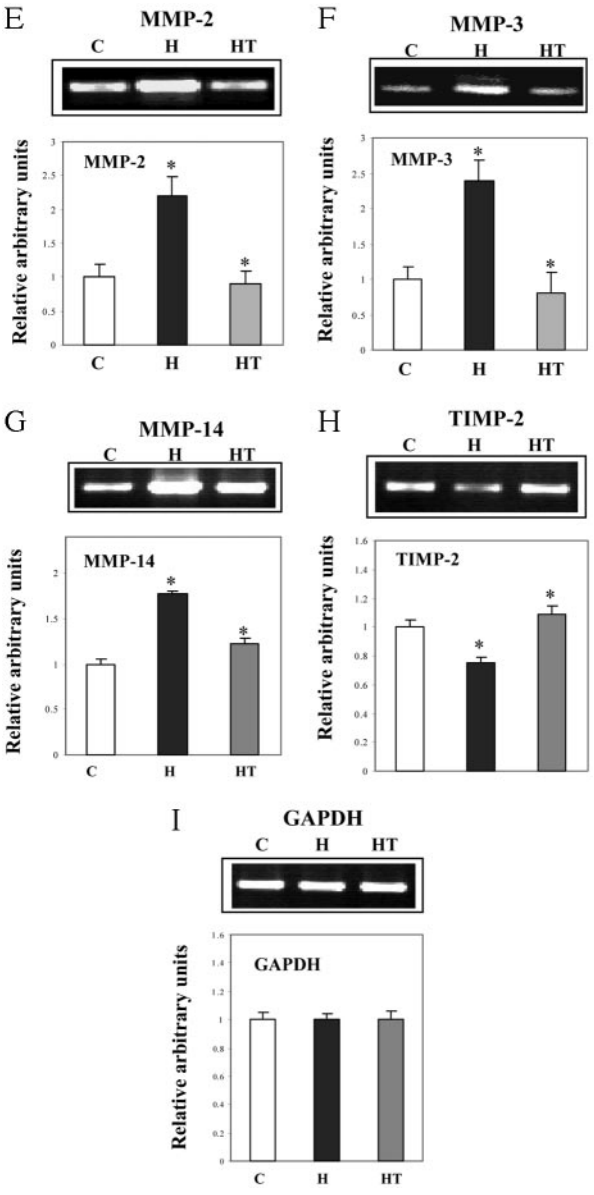

FIG. 6. *Continued*

ovary as evident from RT-PCR, real-time PCR, and Northern hybridization data. The results of the present studies also documented a reduction of collagen I and III in hypothyroid ovary as confirmed by Western immunoblot. These findings convincingly establish that the biosynthesis of major collagens is reduced in hypothyroid ovary, and as a consequence the ECM formation is severely affected. It has also been shown that in both granulosa cells and the residual ovaries, the expression pattern of these Plod isoforms were similar, *i.e.* they are down-regulated in hypothyroid condition and their expression was increased when T_3 was added back. The same pattern of expression was observed in the level of collagen in ovary also. So the expression of Plod isoforms was always increased in hypothyroid ovary on T_3-addback; concomitantly the collagen I and III was also increased. The reduction of collagens may occur because procollagen lysyl hydroxylation was decreased due to the low abundance of the Plod isoforms in hypothyroid ovary. This is the first report of down-regulation of Plods in the ovary due to hypothyroidism, which may result in less hydroxylation of the procollagen lysine residues.

During follicular development, continual remodeling of the follicular wall occurs as it enlarges. These processes of matrix turnover require discrete control because the final outcome is an expansion of the matrix, not a total degradation (46). The precise mechanism by which this occurs is poorly understood, although involvement of simultaneous degradation and synthesis of the matrix may not be ruled out. There is a major role of different MMPs in maintaining the dynamics of the matrix. The increased level of MMP-2, MMP-3, and MMP-14 and the decreased level of TIMP-2 in hypothyroid rat ovary, both at the mRNA and protein level, indicate that hypothyroidism enhances the degradation of ECM by up-regulating MMPs and down-regulating TIMP-2 in the rat ovary. It has already been shown that the expression of TIMP-1 is increased by T_3 in human granulosa cells (33), whereas we have demonstrated in this report that TIMP-2 expression is decreased in hypothyroid condition. It has also been shown that on T3 add-back to the hypothyroid rats the expression of MMPs was decreased and that of TIMP-2 was increased. Although function of Plods and MMPs are inversely related in maintaining the status of collagen in a normal tissue, the present study shows that both of their expressions is affected due to hypothyroidism; Plods are down-regulated, whereas MMPs are up-regulated. The outcome of these two events is virtually same, *i.e.* the reduction of collagen in the tissue. Down-regulation of Plod causes inhibition of new collagen formation, whereas up-regulation of MMPs enhances the degradation of already existing collagens; the cumulative effect might be the cause of disintegration of ECM. The combinatorial effect of these two events might cause the overall disturbances of collagen status and also the ovarian structure leading to the improper follicular development and cell-to-cell interaction that may affect the normal reproductive function. When T_3 is injected, there is a clear indication of the recovery of collagens as well as ECM in the hypothyroid rat ovary, although the overall structure of the ovary was not recovered completely. Because we injected T_3 for 15 d only, more recovery in the follicular structure may be visible after long-term treatment with T_3. At this point in time, it is difficult to ascertain whether these two events (collagen formation and ECM degradation) are controlled by the same or by different mechanisms, although the net result was the destabilization of the ovarian matrix.

In this report we have shown that the expression of Plod isoforms are not restricted to any particular type of cells in the ovary; rather they are expressed both in granulosa and theca cells. We have further shown that the differential expression of Plod-2 due to hypothyroidism does not show the same kind of expression in all the tissues. Due to hypothyroidism, Plod-2 expression is down-regulated in the ovary but significantly increased in the heart, lung, and kidney. We did not find any significant change in Plod-2 expression of the gene in the hypothyroid brain tissue. Further study in this direction will be helpful to know the regulation of tissue-specific expression of Plod isoforms with respect to thyroid hormone.

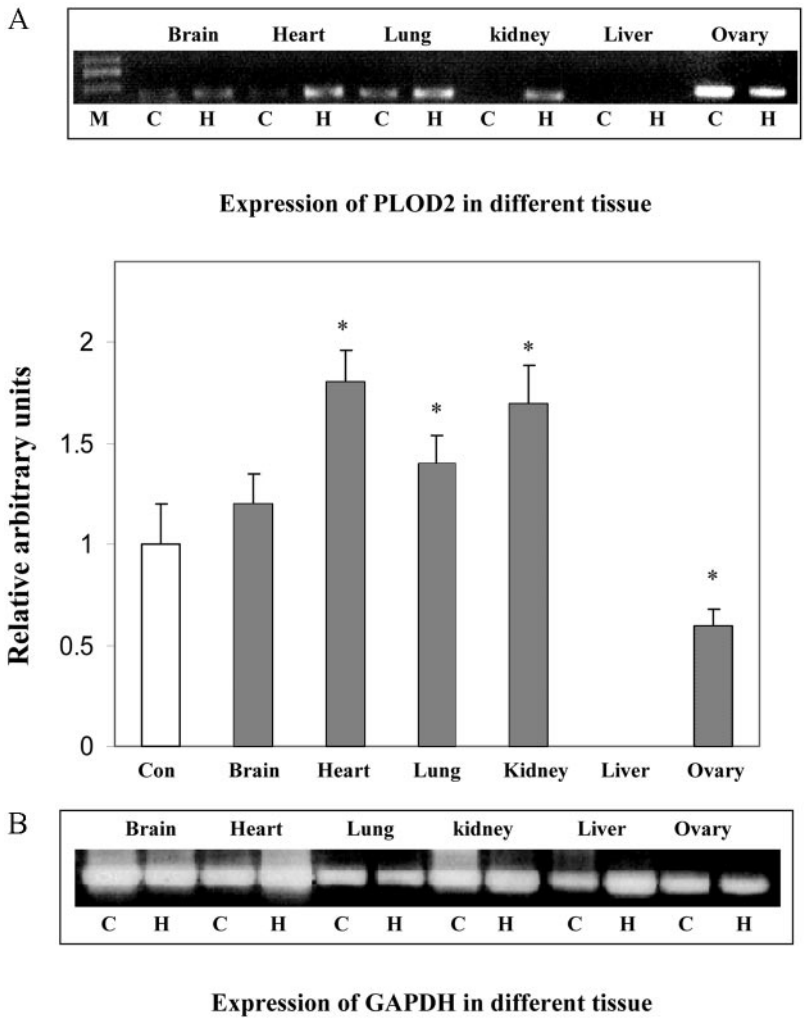

FIG. 7. Effect of hypothyroidism of Plod gene expression in different tissue. Total RNAs were isolated from different tissues, *i.e.* brain, heart, lung, kidney, liver, and ovary of control and hypothyroid rats, and RT-PCR was performed using Plod-2 gene-specific primers. A, Plod-2 gene is up-regulated in heart, lung, and kidney, whereas no significant change was observed in brain tissue. The expression of Plod-2 gene was down-regulated in hypothyroid ovary. The expression of GAPDH was used as a loading control (B). The experiment was performed three times in duplicate, and the mean ± SD values are shown, *, $P < 0.005$.

The relationship between the hypothyroid-induced reproductive malfunction and the reduction of collagen synthesis as well as the matrix disintegration seems to remain an interesting area for further research. This lacuna notwithstanding, it is evident that collagens have some other important regulatory functions. Some collagens form scaffolds that keep cells in place within the tissues, connect tissues within the organ, and facilitate attachment and migration of cells. Ovarian granulosa and theca cells are the sites of steroidogenesis, and the interaction between these two cells is extremely important for the synthesis of steroids. ECM proteins play very important roles in holding and keeping the cells in proper position. Hence, disintegration of ECM may affect the steroidogenesis process. It has also been indicated that collagen can directly serve as a ligand for receptor tyrosine kinases, and, as a consequence of binding to the receptor, a cascade of phosphorylation is induced in the cells (23, 46, 47). So this information and our current observations strongly suggest that there is a definite link between ECM disintegration and the irregular growth of ovarian follicle leading to reproductive disorders.

Acknowledgments

The skillful technical assistance of Mr. Prabir Kumar Dey and Mr. Swapan Mandal (Indian Institute of Chemical Biology) is gratefully acknowledged. We also acknowledge Drs. Malabika Datta, Arun Bandyopadhyay, Asoke Das Gupta, and S. N. Kabir (Indian Institute of Chemical Biology, Kolkata) and Dr. Satinath Mukhopadhyay (Institute of Postgraduate Medical Education and Research, Kolkata) for stimulating discussion. We also acknowledge the contributions of Mr. Sasi Mukherjee for his assistance in revising the manuscript.

Received November 3, 2004. Accepted March 28, 2005.

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This work was supported by the Council of Scientific and Industrial Research (CSIR) (India) and CSIR fellowship and contingency grant (to S.K.S. and P.G.).

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