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Involvement of Pitx2, a Homeodomain Transcription Factor, in Hypothyroidism Associated Reproductive Disorders

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Key Words

Pitx2 • Hypothyroid • Ovarian ECM • Granulosa cell • Ovarian dysfunction

Abstract

Hypothyroid-associated reproductive disorders have now become a striking phenomenon worldwide but the molecular mechanism behind these disorders is not fully known. Pitx2 gene encodes homeodomain transcription factor, which regulates Plod2 gene in brain tissue, transactivates gonadotropin genes in pituitary and plays a substantial role in cell growth and proliferation in different tissues. Pitx2 binds to Plod2 promoter and activates this gene in rat ovary. In this report, we show that Pitx2's expression is markedly reduced in hypothyroid ovary as well as in ovarian granulosa cells, which is recovered with T_3-supplementation both *in vitro* and *in vivo* conditions. Reduced Pitx2 expression could decrease Plod2 expression and hence facilitate ovarian ECM degradation. We have also observed similar pattern of expression of Pitx1 and -3 in hypothyroid and T_3-supplemented ovaries. The temporal expression of Pitx2 across the estrous cycle shows that it is expressed through all the 4 phases of the cycle and reaches its maximum in the proestrus phase suggesting its possible role in ovulation followed by luteinization. The present study reveals that the reduced Pitx2 expression in hypothyroid ovary could lead to ovarian dysfunction by modulating the Pitx2-Plod2 interaction, further study will be necessary to unravel the complete regulatory mechanism of Pitx2 in ovarian function.

Introduction

Many structural or functional abnormalities can impair the production of thyroid hormones and cause the clinical state termed as hypothyroidism. Hypothyroidism in the adult animal leads to a number of physiological disorders as total body metabolism depends on thyroid hormone. The hypothalamic-pituitary-thyroid axis (HPT) and the hypothalamic-pituitary-ovarian axis (HPO) are physiologically related, which act together as a unified system in a number of pathological conditions [1]. Thyroid hormone plays vital role in reproduction of both sexes [2-4]. Female fertility depends upon proper execution of ovarian development, regulated maturation of oocytes, proliferation and differentiation of the surrounding somatic cells [5]. Hypothyroidism associated reproductive

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disorders include irregular oestrous cycle [6-8], ovarian atrophy [9], disturbed folliculogenesis, absence of corpora lutea [10], delayed onset of puberty [11], anovulation [12], amenorrhoea or hypermenorrhoea, menstrual irregularity, infertility and increased frequency of repeated abortion [13]. Hypothyroidism impairs reproductive functions in human beings and in experimental animals, though the underlying molecular mechanism of this dysfunction is not clearly known.

T_3 receptor has been identified in mammalian ovary and the role of T_3 in steroidogenesis is reported earlier [14, 15]. In presence of ligand, the TR/co-repressor complex dissociates and is replaced by a co-activator complex that can contain histone acetylase followed by remodelling of chromatin structure and transcriptional activation [16]. T_3 affected genes linked with reproductive function could correlate hypothyroidism and reproductive disorders.

In our earlier report, we have shown that Plod isoforms are down regulated in hypothyroid rat ovarian granulosa cells with the decreased collagen level in hypothyroid rat ovaries. These findings convincingly established that the biosynthesis of major collagens is reduced in hypothyroid ovary and as a consequence the ECM formation is severely affected [17-19]. Plod2 gene encodes procollagen lysyl hydroxylase enzyme that regulates the hydroxylation of lysine residues in procollagens, the first step in collagen biosynthesis. The hydroxylysine residues have two important functions: to act as attachment sites for carbohydrate units and to provide stability to intermolecular cross-links. Cross-links involving hydroxylysine derived aldehydes are more stable than those involving lysine-derived aldehydes [20]. Plod2 gene is under the target of Pitx2 transcription factor in mice brain [20]. The proximal promoter region of Plod2 harbors multiple bicoid elements that bind to Pitx2 in mouse brain [20]. The Pitx isoforms are members of homeodomain transcription factors, which bind both DNA and RNA [21-23]. Three members of this family have been characterized till date, among them Pitx1 and -2 are expressed in the anterior pituitary [24-26] while Pitx3 is not [27]. Pitx1 and -2 regulate the genes like LHβ, FSHβ, GH, PRL and GnRH receptor [28-30] by interacting with cell-restricted factors such as Steroidogenic Factor1 (SF-1), Early Growth Response-1 (Egr-1), the heterodimeric NeuroD1/ Pan1, Tpit and Pit1 [28]. The cell cycle regulating genes such as cyclin D1 and -D2 are also controlled by Pitx2, as well as it regulates Wnt dependent mRNA turn over [31]. Pitx isoforms regulate the expression of other transcription factors, like Hesx1, Prop1, Lhx3 and Gata2 [32].

	T_3 (ng/ml)	TSH (μIU/ml)
Con	2.55± 0.03	0.35 ± 0.04
Con+PTU-T_3	0.51 ± 0.02	3.68 ± 0.04
Con+PTU+T_3	2.31 ± 0.05	0.25 ± 0.05
Con-PTU+T_3	3.71 ± 0.08	0.28 ± 0.07

Table 1. The rats were made hypothyroid as mentioned in the general materials and methods section. The mean serum T_3 and TSH values in hypothyroid rats and T_3-supplemented hypothyroid rats are shown in the table, above.

Pitx2 is an upstream activator of Plod2 gene in rat ovary; moreover induction of Plod2 gene by T_3 requires Pitx2 (our unpublished data). In the present study, we have shown hypothyroidism severely reduces expression level of Pitx2 gene and protein and also the T_3-mediated recovery of Pitx2 gene both in *in vitro* and *in vivo* experimental conditions. The similar kind of expression pattern of other Pitx isoforms namely Pitx1 and -3 have been shown. Down regulation of Plod2 gene, which in turn leads to the loss in ovarian structural integrity, could be due to the reduced Pitx2 expression in hypothyroid ovary. Moreover we have shown the temporal expression pattern of Pitx2 in rat ovary across oestrous cycle, which explores further the role of Pitx2 in ovarian physiology. Therefore, the altered expression of Pitx2 in ovary due to hypothyroidism and its binding with Plod2 promoter evokes pertinent questions regarding its role in hypothyroidism associated reproductive disorders in mammals including human beings.

Materials and Methods

Animals and treatment

Animal maintenance was performed as described earlier [17]. We divided the rats into two groups i.e. 1) euthyroid and 2) hypothyroid. Rats were made hypothyroid by treatment with propyl thiouracil [PTU, 33]. When the pups were 26 d old, 10 female pups from each hypothyroid and control groups were injected intraperitoneally with pregnant mare's serum gonadotropin (PMSG, Sigma) at a concentration of 10 IU per rat for each experiment. After 48 h of PMSG injection, i.e. at 28 days of age the female pups were dissected and their ovaries were collected, pooled followed by RNA and protein isolation from these tissues. The granulosa cells were isolated from the ovaries and they were pooled to isolate RNA as well as total cellular protein as and when required. For T_3 and TSH measurement, serum from 10 individual rats was collected and the mean value of 10 samples has been shown in table 1. We strictly followed all details of the animal protocol approved

Gene Product	Accession No.	Forward Primer	Reverse Primer	Size of amplicon
rGAPDH	BC059110	5′CAAATGGGGTGATGCTGG TG 3′	5′CATACTTGGCAGGTTTC TCC 3′	512 bp (nt-326-837)
rGAPDH	BC059110	5′GCCATCAACGACCCCTTC 3′	5′AGCCCCAGCCTTCTCCA 3′	237 bp (nt-937-1173)
Pitx2	NM_019334	5′CAGCAGGGAAAGAATGA GGATGTG 3′	5′ATGGATGAGATGGAGT TGGGCG3′	461 bp (nt-322-782)
Pitx2S	NM_019334	5′GCAGGGAAAGAATGAGG ATGTG3′	5′ATGTCTGGGTAGCGGTT TCTC3′	135 bp (nt-324-458)

Table 2. The sequences of the oligonucleotide primers used in semi quantitative RT-PCR along with respective amplicon sizes and accession numbers of different gene products have been listed in the table, above.

by Institutional Animal Ethics Committee during the experiments. The rats were given intraperitoneal T_3 injection at a dose of 15µg/ 100 gm body weight [34]. In both control and hypothyroid sets, the rats were injected from d15 of their birth for 15 consecutive days, then blood was collected and serum samples were prepared from each set. Ovaries from each set were also taken out and total RNA was prepared out of them.

Adult female rats with initial body masses of 200-220 g were taken to observe their estrous cycle. We determined the different phases of the estrous cycle from vaginal smears obtained daily for 2 weeks (two to four cycles) before experiments. Each phase was characterized by a specific number and morphology of cells in the vaginal fluid as revealed by light microscopy. Only animals with a regular estrous cycle (stages are in sequence and the entire cycle lasts 4-5 days) were used for the experiments [35]. Intact female rats in different phases of the estrous cycle were sacrificed to isolate their ovaries and total RNA was prepared from them.

Granulosa cell and hepatocyte isolation

Ovarian granulosa cells were isolated and utilised for subsequent experiments according to the methods described earlier [17]. The granulosa cells were incubated with 100 nM T_3 (Sigma) for 4 hours [36] to get the T_3-treated control and -hypothyroid samples. To get the liver and hepatocyte primary culture, male adult rats (200-250 g) were sacrificed, dissected and isolation was performed using the standard protocol [37]. The isolated granulosa cells and hepatocytes were more than 90% viable as confirmed by trypan blue dye exclusion test.

Immunofluorescence microscopy

Rat ovarian granulosa cells from control and hypothyroid rats and also the cells from human skeletal muscle cells (L6) and cultured rat hepatocytes were fixed on cover glass with PBS containing 4% paraformaldehyde. The cells were permeabilized with 0.1 % TritonX-100 in PBS and then incubated with goat polyclonal anti-Pitx2 antibody (cat# sc-8747, Santa Cruz Biotechnology, Santa Cruz, CA, dilution 1:50) for 3 h followed by incubation with FITC-conjugated secondary antibody (Donkey anti-goat, dilution 1:50, Santa Cruz) for 1 h, with rigorous washing with PBS in between the above steps. 1 µg/ml 4', 6-Diamidino-2-phenylindole (DAPI), was added in each set. The stained cells were observed under a fluorescence microscope (Olympus BX51 microscope, Tokyo, Japan) and the images were captured with cool snap pro camera (Olympus, Tokyo, Japan).

RNA isolation

Total RNA was isolated from rat ovary and ovarian granulosa cells (from control, hypothyroid group, T_3-treated control and hypothyroid rats) using TRI reagent (Sigma) following the standard protocol [17].

Northern hybridization

Northern blotting and hybridization was performed following the methods described earlier [38]. Briefly, 10µg of total ovarian RNA was loaded in each lane of 1% formaldehyde-agarose gel followed by electrophoresis and transfer to nylon membrane (Millipore, MA) by capillary transfer method. Prehybridization was allowed for 2 hours in the buffer containing 6 X SSC with 50% formamide, 1 X Denhardt's solution, 100 µg/ml salmon sperm DNA and 1% SDS at 42°C. Hybridization was carried out in the same condition with the [$^{\alpha-32}$P] dATP-labelled Pitx2 gene fragments for 18 h.Then the membrane was washed for 90 min at 65°C in 2 X SSC containing 0.1% SDS with subsequent three changes of buffer. The hybridized membrane was exposed to Kodak X-ray film and kept at -80°C as per required exposure time. RNA molecular size markers (0.2 kb-6 kb) used in the test was purchased from MBI Fermentas (Hanover, MD).

Reverse Transcription-Polymerase Chain Reaction

First strand cDNA synthesis was performed as mentioned earlier [17, 39]. The PCR was performed with initial denaturation at 94°C for 5 min and then 35 cycles of denaturation at 94°C for 30 sec, annealing at specific temp. for each set of primers for 45 sec, extension at 72°C for 45 sec, final extension at 72°C for 10 min in the last cycle (Perkin-Elmer 9700, Wellesley, MA, USA). The RT-PCR products were cloned and sequenced. Since GAPDH mRNAs are abundant, its amplification reaches the saturation level after 35 cycles. Therefore, we took out GAPDH PCR product after 18th cycle and subjected to gel electrophoresis along with other products. The sequences of the primers used in semi quantitative RT-PCR along with respective amplicon sizes and accession numbers of different gene products have been listed in Table 2.

Gene Product	Accession No.	Forward Primer	Reverse Primer	Size of amplicon
rGAPDH	BC059110	5′GCCATCAACGACCCCTTC 3′	5′AGCCCCAGCCTTCTCCA 3′	237 bp (nt-937-1173)
Pitx1	NM_053624	5′CACCGCCGCCACACGAC 3′	5′GCACCAAGCCGCTGAACT G 3′	444 bp (nt-374-817)
Pitx2S	NM_019334	5′GCAGGGAAAGAATGAGG ATGTG3′	5′ATGTCTGGGTAGCGGTTT CTC3′	135 bp (nt-324-458)
Pitx3	NM_019247	5′CTCCACACCCTCCGCTTC C3′	5′TCCCGCTTCCGCCACTTG 3′	295 bp (nt-131-425)

Table 3. The sequence of the oligonucleotide primers used in QPCR along with respective amplicon sizes and accession numbers of different gene products have been listed in the following table.

Real Time quantitative reverse transcription PCR (QPCR)

The QPCR was performed on iCycler (Bio-Rad Laboratories, Hercules, CA, USA) machine using DyNAmo SYBR Green QPCR Kit, (Finnzyme, Finland) following the manufacturers instructions. In short, 2 µg of total RNA isolated from ovaries and granulosa cells of control and experimental rats were reverse transcribed as described above followed by QPCR. The condition of this QPCR was as follows: initial denaturation step (95°C for 5 min) and cycling step [denaturation at 94°C for 15 sec, annealing at specific temp for each set of primers for 30 sec, extension at 72°C for 30 sec, repeated for 35 cycles] followed by melt curve analysis (45-90°C). An internal control GAPDH was amplified in separate tubes. The C_T value was calculated as mentioned below. Data from three determinations (mean ± SD) are expressed as fold changes compared to normal rats [40]. The sequence of the primers used in QPCR along with respective amplicon sizes and accession numbers of different gene products have been listed in Table 3**.**

Western Blotting

The ovaries of control and hypothyroid rats were lysed in lysis buffer (150 mM NaCl, 500 mM Tris, 10 mM EDTA) supplemented with protease inhibitors (1µg/ml aprotinin, 1µg/ml pepstatin, 1µg/ml leupeptin, 1mM PMSF, 1µg/ml trypsin inhibitor) and 1% Triton X-100. It was then centrifuged at 5,000 rpm for 10 min at 4°C. The supernatant was collected and taking an aliquot protein concentration was estimated and then resolved on a 12% sodium dodecyl sulfate polyacrylamide gel [39] and transferred onto Immobilon-P membranes (Millipore Corp). 50 µg of total protein from each sample were loaded on each lane. The membrane was then incubated with 5% blocking solution (Tris Buffered Saline [TBS] containing 0.1% Tween-20, 5% non-fat dried milk) for 2 hrs, washed twice with TBS-T (TBS containing 0.1% Tween-20) and then incubated for 4 hrs with goat polyclonal anti Pitx2-antibody (cat no# sc-8747**,** 1:500 dilution), mouse anti-actin antibody (cat no# sc-8432, 1:1000 dilution). The antibodies were purchased from Santa Cruz Biotechnology and were diluted in 5% blocking solution during detection. Immunoreactive bands were visualized by reacting alkaline phosphatase-labelled secondary anti goat (1:1000 in 5% blocking solution) and anti mouse antisera (1:2000 dilution) respectively with the substrate NBT/BCIP [39].

Sequencing and analysis

Sequencing of the plasmid DNA and the PCR products was performed by ABI Prism Automatic DNA Sequencer (Perkin Elmer). Sequence alignment and data analysis was done through BLAST search from NCBI GenBank. [41].

C_T value calculation

In QPCR, relative quantification can be done by comparative C_T method. In this method arithmetic formulae are used to calculate relative expression, compared with a calibrator, which can for instance be a control (non-treated) sample. The amount of target normalized to an endogenous housekeeping gene and relative to the calibrator, is then given by $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T$ (sample) - ΔC_T (calibrator), and ΔC_T is the C_T of target gene subtracted from the C_T of the housekeeping gene [40].

Statistical analysis

All data are expressed as the mean ± SD, and statistical analysis was performed by *Student's t-test*. $P<0.05$ was considered to be significant. The experiments were repeated at least three times in duplicate unless otherwise stated. To make the variance independent of the mean, statistical analysis of real-time PCR data was performed after logarithmic transformation.

Results

Pitx2 expression is decreased in hypothyroid condition

Experiments were performed to find out whether the expression level of Pitx2 gene is affected in hypothyroid condition. The results show a significant decrease in the Pitx2 expression level in hypothyroid

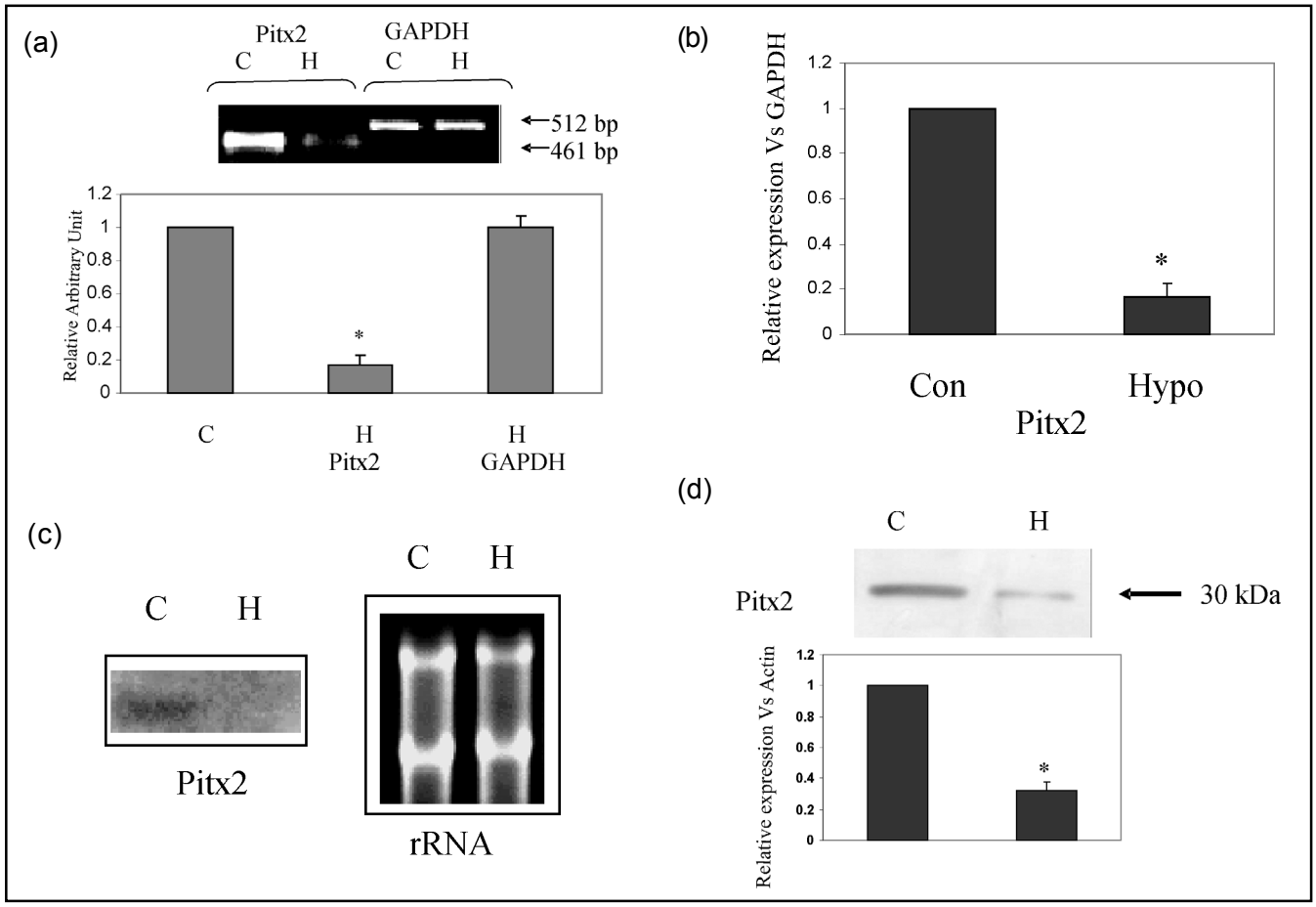

Fig. 1. Expression of Pitx2 in control and hypothyroid rat ovaries. Ovarian RNAs from control (C) and hypothyroid (H) rats were isolated and were subjected to RT-PCR and QPCR using gene specific primers of Pitx2 and GAPDH. The RT-PCR products are shown in (a) and the fold difference in QPCR has been shown in (b). In Fig.a the amplified product sizes are indicated with arrows. The isolated RNAs were electrophoresed on agarose gel and Northern hybridized with radio-labeled Pitx2 cDNA fragment as probe. Fig (c) shows the hybridized band and the ethidium bromide stained rRNA bands have been shown as loading control. Total proteins isolated from control and hypothyroid rat ovaries were immunodetected with goat polyclonal anti-Pitx2 antibody (d), which was normalized over the expression of actin (here mouse monoclonal actin antibody was used). The molecular weights of the respective proteins have been indicated with arrow. In Fig 1a-d, C and H represent the samples isolated from control and hypothyroid rats respectively. Below the panel of Fig 1a and d, scanning data of the respective band intensities have been shown, where pixel value of each band was calculated by the ImageJ software. The above experiments were performed three times in duplicate and the scanning data is the mean ± SD. *P < 0.05.

condition compared to control as shown by RT-PCR (Fig 1a), QPCR (Fig 1b) and Northern hybridisation (Fig 1c). Western immunoblotting was conducted to find out whether the change in Pitx2 gene expression is reflected in its protein level or not. Ovarian proteins of control and hypothyroid rats were isolated and subjected to SDS-PAGE followed by transfer onto the PVDF membrane and western immunodetection. Western immunoblot with anti-Pitx2 antibody showed a marked decrease of Pitx2 under hypothyroid condition compared to control (d), whereas expression of actin remained unaltered (here the expression of Pitx2 is normalized over that of actin). The same experiment was performed with the proteins isolated from ovarian granulosa cells and the Pitx2 protein significantly decreased in the hypothyroid condition compared with control (data not shown).

Localization of Pitx2 in ovarian granulosa cell

To localize the Pitx2 protein in the ovarian granulosa cells, immunocytochemistry was performed with anti-Pitx2 antibody followed by FITC-tagged secondary antibody. The data confirms the presence of Pitx2 in the nucleus of ovarian granulosa cells isolated from the control rat (Fig 2a). The intensity of the fluorescence in granulosa

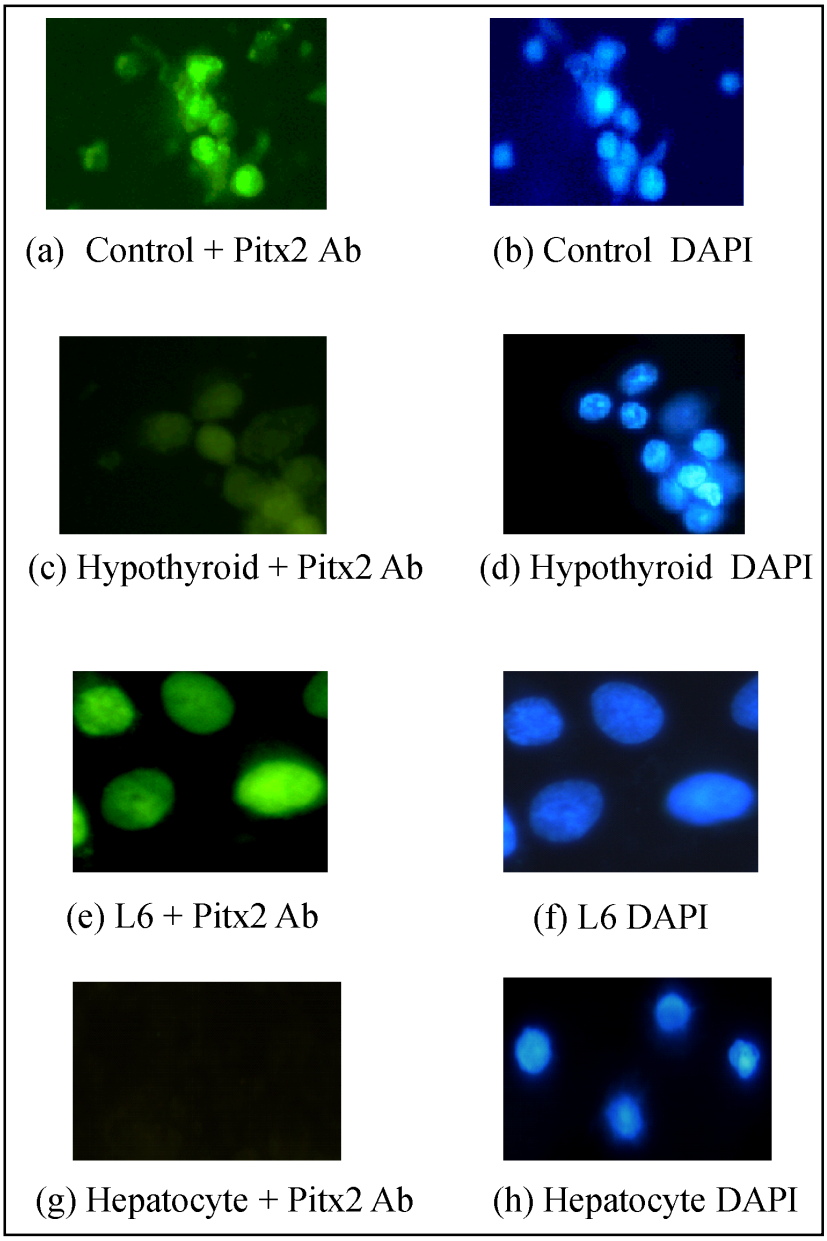

Fig. 2. Cellular distribution of Pitx2 in the rat ovarian granulosa cells. Immunocytochemistry was performed with the granulosa cells isolated from control and hypothyroid rat ovaries, using Pitx2 antibody and FITC linked secondary antibody and the cells from each set were also stained with DNA-specific dye, i.e. DAPI. Similarly immunocytochemistry was also performed in L6 cell line (human skeletal muscle) and in rat hepatocytes. The granulosa cells isolated from euthyroid rat ovaries were treated with Pitx2 antibody (a) and the cells from hypothyroid animals were treated with Pitx2 antibody (c). However, L6 cells were treated with Pitx2 Ab (e) and hepatocytes were treated with (g) Pitx2 Ab; each set was then treated with FITC-linked secondary antibody. The respective DAPI stained cells have been shown as 'b' (control granulosa cells), 'd' (hypothyroid granulosa cells), 'f' (L6 cells), and 'h' (hepatocytes).

cells isolated from the hypothyroid rats (Fig. 2c) was significantly less compared to that in control set, which gives a clear indication of less expression of Pitx2 in hypothyroidism. Pitx2 is known to express in skeletal muscle, whereas in hepatocytes it does not. Therefore, these two cells were chosen as positive and negative control respectively in our experiment. The data shows the presence of Pitx2 in the nucleus of L6 cells when incubated with Pitx2 Ab (Fig 2e). However, no fluorescence was detected in hepatocyte in presence of Pitx2 Ab (Fig 2g) confirming the specificity of the signals obtained. The DAPI stained cells corresponding to Fig 2a, c, e and g are represented as 2b, d, f and h respectively.

In vitro and in vivo effect of T_3 on Pitx2 gene expression

The significant decrease in Pitx2 expression in hypothyroidism suggests a definite role of T_3 in regulating the expression of this gene. To show the *in vitro* effect of T_3 on Pitx2 gene expression, ovarian granulosa cells were isolated from control and hypothyroid rats, incubated in presence or in absence of T_3 as described in the methods section. RNA was isolated from the ovarian granulosa cells and RT-PCR as well as QPCR was performed with the gene specific primers of both Pitx2 and GAPDH. The results of RT-PCR (Fig 3a) and QPCR (Fig. 3b) show that Pitx2 gene expression was significantly

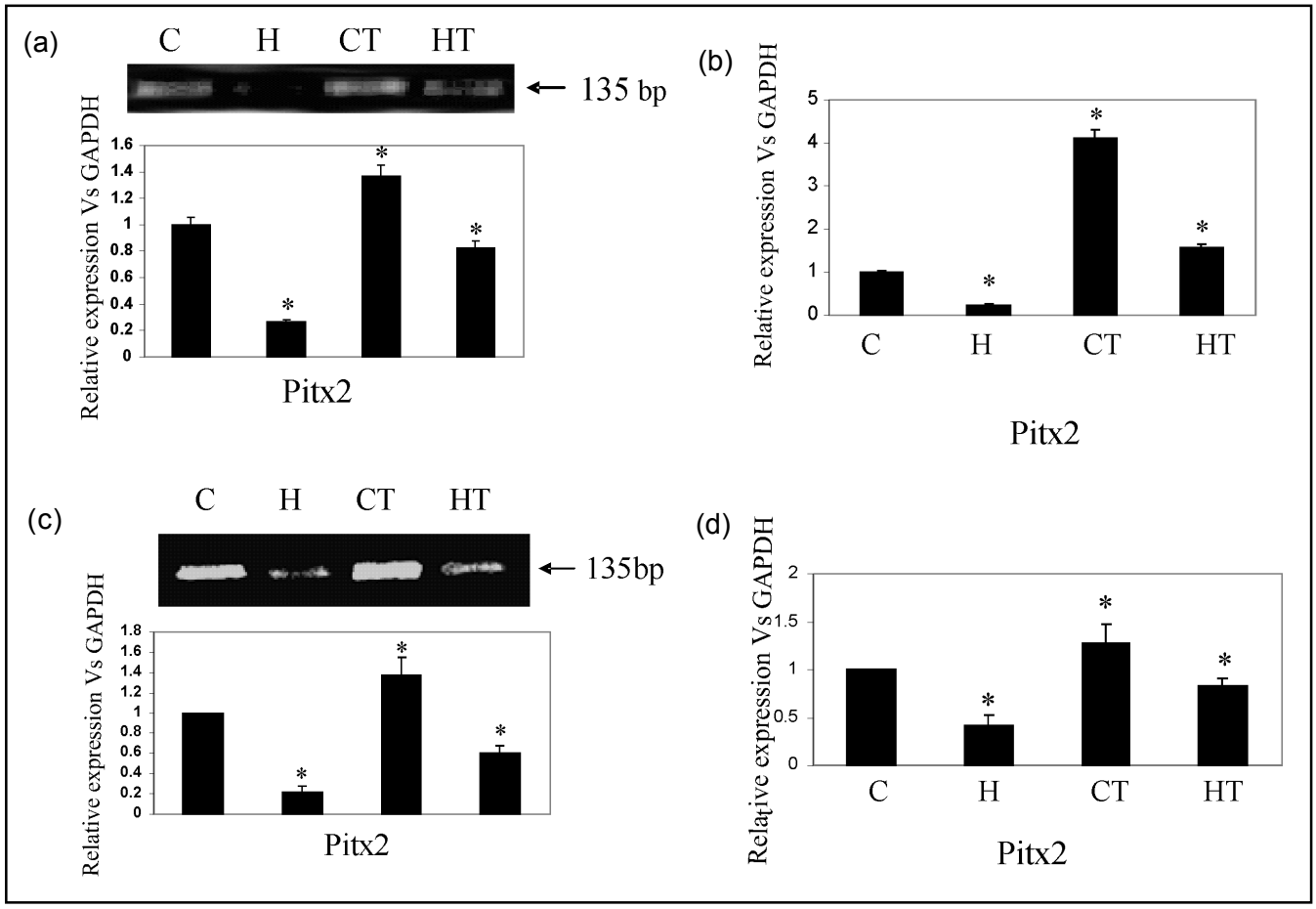

Fig. 3. *In vitro* and *in vivo* effect of T_3 on Pitx2 expression. RNAs were isolated from control (C), hypothyroid (H), T_3-treated control (CT) and T_3-treated hypothyroid (HT) ovarian granulosa cells to observe *in vitro* effect of T_3 on Pitx2 gene expression. Using these RNAs, RT-PCR was performed with the gene specific primers of Pitx2 (Fig. a) and the respective product sizes are indicated with arrow. The QPCR was performed using the gene specific primers of Pitx2 and GAPDH and represented in Fig b. The expression level of Pitx2 is normalized over that of the GAPDH and the comparative expression is indicated as change in 'fold' in the Y-axis. Similarly, for *in vivo* studies, RT-PCR was performed with the gene specific primers of Pitx2 (Fig. c) and the respective product sizes are indicated with arrow. QPCR was also performed with gene specific primers of Pitx2 and GAPDH and the expression level of Pitx2 is normalized over that of the GAPDH and the comparative expression is indicated as change in 'fold' in the Y-axis (Fig. d). In Fig 'a' and 'c', the expression of Pitx2 has been normalized with that of GAPDH gene expression. The scan data of the bands obtained as pixel value using the Image J software are shown as a bar diagram below the results of RT-PCR (Fig a and c). The above experiments (a-d) were performed three times in duplicate and the scanning data is the mean ± SD, *P < 0.05.

decreased in hypothyroid condition compared to control, whereas its expression was increased to a significant extent upon T_3 addition in the culture medium of control granulosa cells. However, the reduction in expression of Pitx2 due to hypothyroidism was restored upon addition of T_3 in the granulosa cell culture medium isolated from the hypothyroid rat ovaries. This suggests that T_3 has an influential role on Pitx2 gene expression in *in vitro* cell culture system.

To obtain the *in vivo* effect of T_3 on Pitx2 gene expression, the rats were injected with T_3 as described in methods section. Similar to the *in vitro* effect, Pitx2 gene expression was increased in T_3-injected control and -hypothyroid ovaries compared to that in the ovaries of control and hypothyroid rats respectively as shown by RT-PCR (Fig 3c) and QPCR data (Fig 3d). The expression of Pitx2 both *in vivo* and *in vitro* as obtained by RT-PCR has been normalized by GAPDH gene expression.

Pitx2 expression pattern in rat ovary across oestrous cycle

Both RT-PCR and QPCR were performed with total RNA isolated from different stages of rat ovaries across the oestrous cycle. Both RT-PCR (Fig. 4a) and QPCR

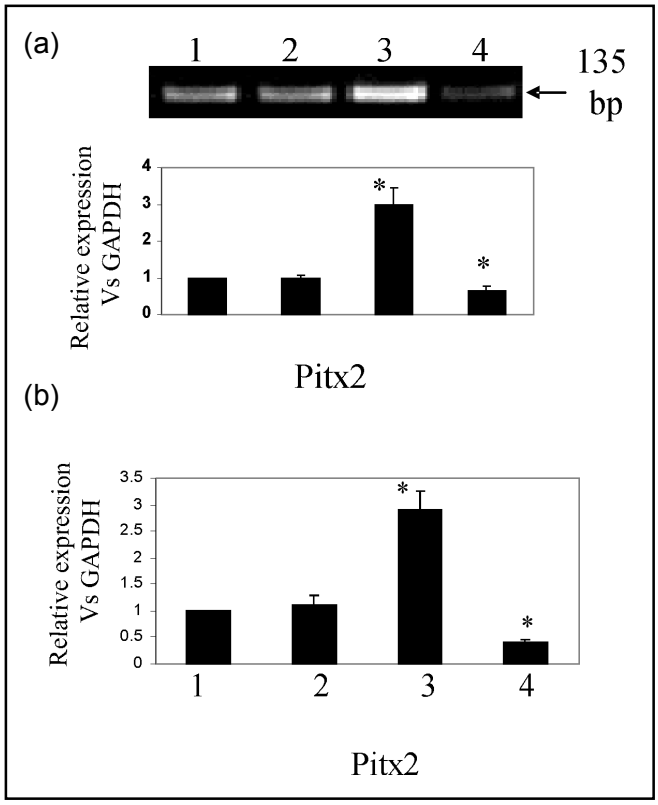

Fig. 4. Pitx2 expression pattern in rat ovary across the estrous cycle. Both RT-PCR (Fig. a) and QPCR (Fig. b) were performed with total RNA isolated from rat ovaries across oestrous cycle. Fig. (a) shows Pitx2 expression level at metestrus (lane 1), diestrus (lane 2), proestrus (lane 3) and at oestrous (lane 4) stages respectively. The expression of Pitx2 has been normalized with that of GAPDH and shown in the figure. In Fig. a, respective product sizes are indicated with arrow and below the panel, scanning data of the respective band intensities have been shown, where pixel value of each band was calculated by the ImageJ software. Fig. (b) shows the QPCR data where expression pattern of Pitx2 has been shown in the same order as mentioned in Fig. (a). In QPCR, the expression level of Pitx2 is normalized over that of the GAPDH and the comparative expression is indicated as change in 'fold' in the Y-axis. The experiments were performed three times in duplicate and the scanning data is the mean ± SD, *P < 0.05.

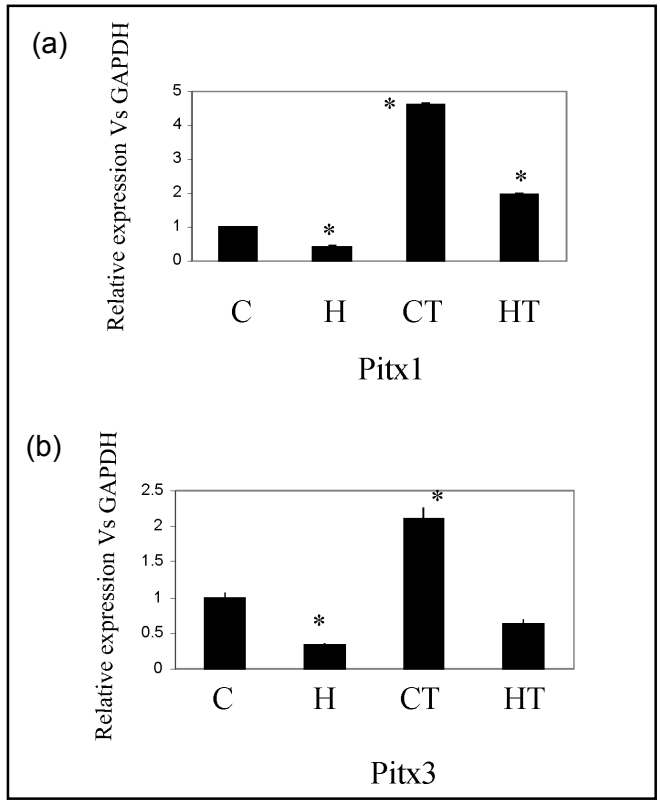

Fig. 5. Pitx1 and -3 are present in rat ovary and the effect of T_3 over their expression. QPCR was performed using RNAs isolated from control (C), hypothyroid (H), T_3-incubated control (CT) and from T_3-incubated hypothyroid (HT) ovarian granulosa cells. QPCR result with Pitx1 and Pitx3 gene specific primers is shown in Fig a and Fig b respectively. In QPCR the expression level of Pitx1 and -3 are normalized over that of the GAPDH and the comparative expression is indicated as change in 'fold' in the Y-axis. The sequences of the primers used for Pitx1 and -3 amplification, have been mentioned in materials and methods section. The experiments were performed three times in duplicate and the scanning data is the mean ± SD, *P < 0.05.

(Fig. 4b) data show similar expression pattern of Pitx2 in metestrus and diestrus stages. In proestrus stage, Pitx2 expression level was significantly higher compared to metestrus and diestrus stages, whereas at the oestrous stage its expression was reduced to the normal level (lane 4). The expression of Pitx2 has been normalized over GAPDH gene expression and presented in Fig. 4a. The QPCR data (Fig. 4b) shows the similar pattern of Pitx2 expression level across the stages of oestrous cycle as was obtained in the RT-PCR result. The above data shows the temporal expression pattern of Pitx2 in ovary across estrous cycle.

Effect of T_3 on Pitx1 and -3 expressions

Oligonucleotide primers for Pitx1 and -3 were synthesised (as mentioned in methods section) to perform QPCR using total RNA isolated from control, hypothyroid, T_3-incubated control and -hypothyroid rat ovarian granulosa cells respectively. QPCR data shows that like Pitx2, Pitx1 (Fig 5a) and -3 (Fig. 5b) isoforms were

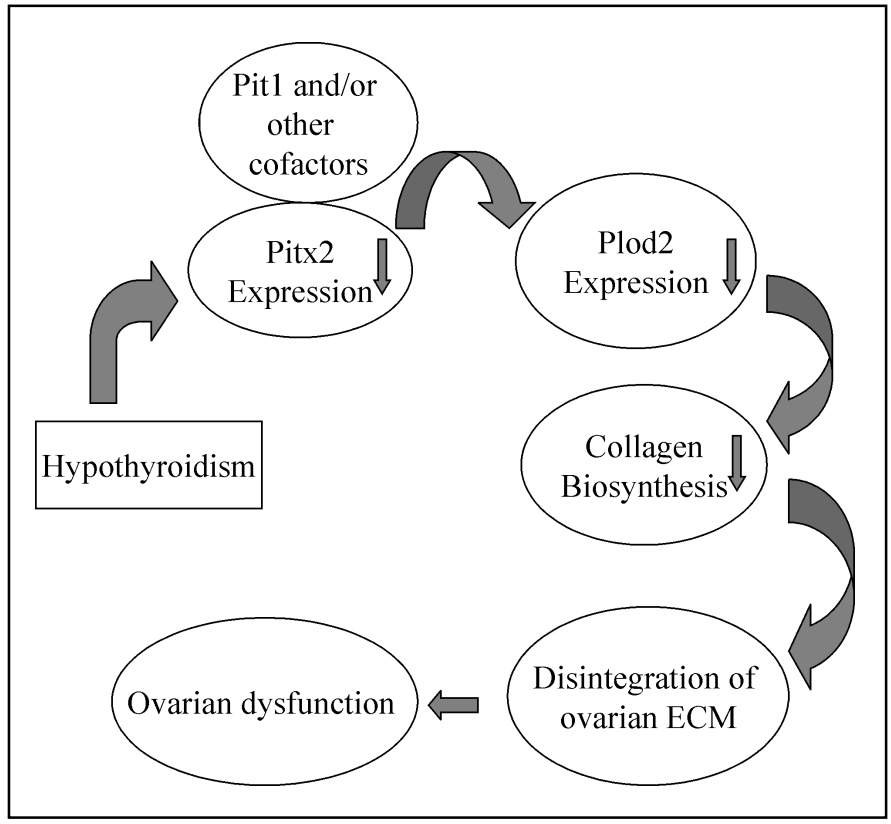

Fig. 6. Schematic representation of a hypothetical model to show the possible involvement of Pitx2 in disintegration of ovarian ECM and subsequent ovarian dysfunction in hypothyroid condition. In this scheme, upward arrows indicate up regulation of gene or protein expression and downward arrows indicate down regulation of the same.

markedly reduced in hypothyroid condition compared to control. Their expression levels were significantly increased in T_3-incubated control and -hypothyroid ovarian granulosa cells. This data suggests that like Pitx2, Pitx1 and -3 expressions are also affected by T_3.

Discussion

Thyroid dysfunction is very common in women and has unique consequences related to menstrual cyclicity and reproduction. Even mild hypothyroidism can increase rates of miscarriage and fetal death and may also have adverse effects on later cognitive development of the offspring [42]. Thyroid function and reproductive function have many interactions, the mechanism of which is not fully understood [43]. A wide range of reproductive disorders such as irregular menstruation and frank infertility is found in women with hypothyroidism. Most of the research work done so far has focused on steroid and gonadotropin hormone profiles [44]. However, its cause and the molecular basis of the pathophysiological mechanism are yet to be ascertained. Several transcription factors have already been reported to play crucial role in regulating significant genes, whose mutant forms may cause impaired follicular growth and differentiation, immature oocyte formation, meiotic arrest of oocytes, ovulatory problem and also impaired steroidogenesis [45, 46]. In an endeavor to find out the molecular basis of hypothyroidism associated ovarian dysfunction, we performed several experiments in hypothyroid rat model. The serum T_3 and TSH levels were measured in hypothyroid and T_3-supplemented rats, which showed that the rats were truly hypothyroid.

Ovary is a very dynamic organ where follicles and corpora lutea continually grow and regress and ovarian ECM is extremely important for the follicular development, helps follicular fluid formation, filters soluble materials; provides rigid or elastic mechanical support for tissues [47]. Collagen biosynthesis requires a large number of post-translational modifications. One of the important steps in collagen biosynthesis is hydroxylation of lysine residues, which provide attachment sites for glycosylated hydroxylysine residues. Plod2 gene encodes procollagen lysyl hydroxylase enzyme that regulates the hydroxylation of lysine residues in procollagens, the first step in collagen biosynthesis.

Pitx2 is an upstream activator of Plod2 gene in brain tissue [20] and in ovary (our unpublished result). In this report we show that Pitx2 is down regulated in hypothyroid ovary, supported by RT-PCR, QPCR, Northern hybridization, immunocytochemistry and immunoblotting

data. Therefore, we postulate that the reduced expression of Pitx2 may be responsible for the down-regulation of Plod2 gene in hypothyroid ovary. Both *in vitro* (in ovarian granulosa cells) and *in vivo* (in ovary) studies suggest that T_3 has definite role in Pitx2 gene expression. T_3 up regulates Pitx2 gene expression in normal rat ovary and T_3 supplementation recovers its reduced expression in total ovary. Since Pitx1 and -2 share binding specificities to transactivate their target genes [28], we do not exclude the possibility that these isoforms might also be involved in the regulation of Plod2 gene. *In vitro* studies show like Pitx2, similar type of influence of T_3 on Pitx1 and -3 expressions was found in ovarian granulosa cells. Since hypothyroidism reduces the expression of all Pitx isoforms, the chance of rescuing Plod2 expression by other isoforms seems to be very less. However, the target genes of Pitx1 and -3 in ovary and their regulatory pathways could be studied in future. It is noteworthy that Rieger syndrome causing Pitx2 mutant failed to induce Plod2 gene [20]. It is compatible with our hypothesis that suggests, down regulation of Pitx2 affects Plod2 gene expression. However, in αSUnul mice, the expression of Pitx2 was unaltered when treated with T_4 [48] in pituitary tumor. We propose that the T_3-influenced altered expression of Pitx2 in ovary could be due to the tissue-specific regulation of thyroid hormone.

The temporal expression pattern of Pitx2 in rat ovary across estrous cycle suggests that it could play role in the follicular development as Pitx2 is remarkably increased in proestrus phase compared to other phases. In the proestrus phase, when the progesterone level is low [49] and the specific follicles destined to ovulate among others, Pitx2 expression is the maximum. In the estrus phase, when ovulation occurs and estrogen level reaches at its maximum [49], Pitx2 expression is remarkably less. In the metestrus and diestrus phases, when estrogen level is less and progesterone levels begin to rise and the corpora lutea predominates, Pitx2 expression is maintained at the low level. This suggests that Pitx2 expression across the estrous cycle is modulated by steroid hormones. In *in vitro* studies with human ovarian cell line (SK-OV3), we have found altered expression of Pitx2, which was decreased in the higher dose of estrogen supplementation in the culture medium (data not shown). This result is compatible to the *in vivo* effect of estrogen in ovarian Pitx2 gene expression as already discussed here. There may be other factor(s) regulating the expression of Pitx2 in ovary and further study will unravel these regulatory mechanisms.

The possible involvement of Pitx2 in regulation of Plod2 gene expression and the consequent ECM disintegration in hypothyroid ovary has been schematically represented in a hypothetical model (Fig 6). In this schematic representation, the association of Pitx2 with its cofactor Pit1 (our unpublished data) has also been implicated in regulation of ovarian function. Pitx2 may regulate so far unidentified genes, apart from Plod2, in ovary and that may affect steroidogenesis, ovarian development and folliculogenesis in hypothyroid condition. Pitx2 is necessary for initiating expansion of Rathke's pouch and maintaining expression of the foetus-specific transcription factors, like Hesx1 and Prop1 [50]. At later stages Pitx2 is necessary for specification and expansion of the gonadotropes. Thus the reduced Pitx2 in hypothyroid ovary not only facilitate disintegration of ovarian structure but also could impair ovarian growth and its functions and there is enough scope of further extensive studies in this specific area.

Abbreviations

Pitx (Pituitary Homoeobox Gene); PTU (Propyl Thio Uracil); PMSG (Pregnant Mare Serum Gonadotropin); LH (Luteinizing Hormone); FSH (Follicle Stimulating Hormone); TSH (Thyroid Stimulating Hormone); PRL (Prolactin), T_3(Triiodothyronine); GAPDH (Glyceraldehyde 3-phosphate dehydrogenase); FITC (Fluorescein Isothiocyanate); DAPI-4' (6-Diamidino-2-phenylindole); ECM (Extra cellular Matrix); TRI-reagent (Tripure RNA Isolation reagent); RT-PCR (Reverse Transcriptase Polymerase Chain Reaction); SSC-(Standard Saline Citrate); SDS (Sodium Dodecyl Sulphate).

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