Expression of PITX2 Homeodomain Transcription Factor during Rat Gonadal Development in a Sexually Dimorphic Manner

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Key Words

Pitx2 • Transcription • Gonads • Development

Abstract

PITX2, a multifunctional Paired-like homeodomain transcription factor, plays obligatory role during development of organs like heart, brain and pituitary. It regulates differentiation of vascular smooth muscle cells and hematopoietic stem cells. Although we earlier reported the *Pitx2*/PITX2 expression in gonad, but the expression pattern of its different isoforms in mammalian gonads especially during development is still not known. As PITX2 participates in the development of multiple organs and different homeobox genes have been shown to control gonadal functions, we wanted to investigate the role of PITX2 in gonadal development and its function. The objective of our study was to know the expression profile of different *Pitx2*/PITX2 isoforms and its localization throughout the development of gonads. Here we show the temporal and spatial expression pattern of *Pitx2*/PITX2 and its localization throughout the embryonic and postnatal stages of rat gonads. *Pitx2*/PITX2 expression profile reveals the differential

and dimorphic expression pattern of its two isoforms PITX2B2 and-C β throughout the embryonic development stages and also in the postnatal stages, where it becomes more prominent. This is the first report where PITX2 homeodomain transcription factor shows isoform-specific sexually dimorphic expression. In addition, PITX2 localization was found in the embryonic ovarian primordial germ cell clusters and germ cells inside the testicular cords and also in somatic cells. In adults, ovarian granulosa and theca cells as well as germ cells inside the seminiferous tubules in testis express PITX2. All the evidences suggest that the differential expression of PITX2 might be associated with sex-specific embryonic and postnatal gonadal development and the physiological processes.

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Introduction

The role of homeodomain transcription factors in early and late development of different tissues has been shown [1-2]. *Pitx2*, a *paired*-like homeodomain transcription factor, plays an essential role for normal development of multiple organs [3-8], as well as for

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differentiation of vascular smooth muscle cells [9] and hematopoietic stem cells [10-11]. Mice with complete loss of *Pitx2* function die in mid gestation (~ E15) due to cardiac and abdominal defects [6]. Abnormal function of PITX2 has been shown to be associated with different disorders that include Rieger syndrome, dental and craniofacial anomalies, prostate cancer, idiopathic arterial fibrillation, and polycystic ovary syndrome [12-16]. In these cases, PITX2 function is altered due to physiological or mutational defects. *Pitx2* DNA methylation has been utilized as a marker for disease recurrence in breast cancer patients [17]. *Pitx2* is expressed mainly as three mRNA isoforms, i.e. *Pitx2a*, *Pitx2b* and *Pitx2c* in human, rat and mouse. The *Pitx2* genes in human and mouse consist of six exons. *Pitx2a* and *Pitx2b* mRNAs are produced through alternative splicing of the same pre-mRNA. The *Pitx2a* isoform contains exons 1, 2, 5 and 6, whereas *Pitx2b* has an additional exon 3 (138 bp) and a longer N-terminus. *Pitx2c* uses an alternative promoter upstream of exon 4 and so has a unique N-terminus. Moreover, the homeodomain and C-terminus of the protein are encoded by exons 5 and 6 and are similar to all isoforms, i.e. PITX2A, PITX2B and PITX2C in human, rat and mouse. Considering the sequence identity, the existing nomenclature of PITX2A and PITX2B in rat is just reverse to that in mice and human as rat PITX2A (EDL82168.1) and PITX2B (EDL82169.1) show sequence identities with the human and mice PITX2B (hPITX2B is NP_700475.1 and mPITX2B is NP_035228.2) and PITX2A (hPITX2A is NP_700476.1 and mPITX2A is NP_001035969.1) respectively. PITX2C sequence (rPITX2C is EDL 82170.1, mPITX2C is NP_001035967.1 and hPITX2C is NP_000316.2) in all 3 species are however identical.

PITX2 regulates the expression of genes like *LHβ*, *FSHβ*, *GH*, *PRL*, and *GnRH* receptor [18-20]. The cell cycle regulating genes such as *Cyclin D1*, *-D2* and *c-Myc* are also controlled by PITX2 in pituitary [21]. We have previously shown the down regulation of PITX2 in ovary of hypothyroid rat and hypothesized its role to maintain normal ovarian function [22]. It also regulates *Plod2* gene in rat ovary (our unpublished data), suggesting the involvement of PITX2 in ovarian collagen metabolism. The role of PITX2 and its associated factors during mammalian gonadal development is not known. In chicken, *Pitx2* expresses asymmetrically in the left gonad in both sexes from their initial stages of development [23], is involved in the developmental process, and allows proliferation and differentiation of primordial germ cells [24]. But in mammals, where gonads develop

symmetrically, its contribution to early gonadal development remains unclear.

Considering the lack of knowledge regarding the role of PITX2 in gonadal development and function, objectives of this study were a) to explore the detailed expression profile of *Pitx2*/PITX2 during gonadal development, b) to identify the cellular expression profile of PITX2 in embryonic and postnatal gonads, and c) to explore if PITX2 expression during embryonic and postnatal gonadal development show difference between the two sexes. Our data shows a comprehensive expression profile and temporal and spatial localization of PITX2 during rat gonadal development in stage-dependent manner. It thus establishes an isoform-specific dimorphic expression of PITX2 in gonads during embryonic and postnatal development in rat.

Materials and Methods

Animals and treatment

Sprague Dawley rats (*Rattus norvegicus*) were procured from the random bred colony of our animal facilities and housed in a well-ventilated and temperature-controlled room supported by diurnal cycle of 12-h light and 12-h darkness schedule. The animals were fed with standard balanced pellet, and drinking water was made available *ad libitum*. Timed pregnancies were induced as healthy sexually mature females mated with fertile males of same strain. The presence of sperm in the vaginal plug on the next day morning was assigned as 0.5 day of gestation (E0.5). All procedures that involved experimental animals were performed in compliance with the regulation of the Institutional Animal Ethics Committee.

Isolation and processing of embryos, embryonic gonads and postnatal gonads

Pregnant mothers were sacrificed by cervical dislocation at E14.5, E16.5, E18.5 and E20.5 days of gestation, and their uterine horns were removed and dissected in ice cold PBS. Embryos from each horn were separated from the deciduous and extra-embryonic membranes and this was followed by fixation and paraffin block preparation. Similarly, their gonads were also isolated at different days of gestational and postnatal stages for RNA isolation, protein preparation and preparation of tissue sections. For each Q-PCR experiment, ovarian and testicular RNAs were isolated at E14.5, E16.5 (n=20 gonads at each day), E18.5 and E20.5 (n=14 gonads at each day) and also at D10 and 30 (n=10 gonads at each day). Similarly, for each western blot experiment same numbers of ovaries and testes were taken as mentioned and protein samples were prepared.

For histological staining (including HE, IHC and IF), whole embryos at embryonic stages E14.5 and 16.5 (n=10 at each stage, male/female) and isolated ovaries and testes at E18.5 and 20.5 (n=6 at each stage) were collected and processed for fixation preparation of paraffin block and section. Similarly, ovaries and testes at postnatal D10 (n=4 each) and D30 (n=4

each) were also collected and processed as mentioned above. These experiments were performed three times using the same number of whole embryos or gonads as mentioned above.

Histology and immunohistochemistry

For histological studies, isolated embryos and gonads were washed in ice cold PBS and this was followed by formalin fixation, processing and paraffin block preparation using standardized protocol. Finally, they were embedded in paraffin blocks. The Parasagittal paraffin sections of 6 μ m thickness were prepared by a microtome (Leica RM2235, Wetzlar, Germany) and were adhered on to the Poly-L-Lysine (Sigma-Aldrich, St. Louis, MO, USA) coated glass slides; IHC and IF assays were then performed using the standard protocol [25]. For IHC, anti-PITX2 antibody (dil 1:500, Chemicon) and the goat anti-rabbit IgG-AP (dil 1:1000, Santa-Cruz) were used as the primary and secondary antibodies respectively. The consecutive tissue sections of mentioned stages were stained with hematoxylin and eosin solution, (Sigma) and the bright field coloured images were captured in a cool-snap pro camera attached with Olympus BX51 microscope (Olympus BX51, Tokyo, Japan). The images of lower magnification were captured in a Canon digital camera attached to the stereomicroscope (Carl Zeiss STEMI 2000C, Gottingen, Germany).

For IF studies, rabbit polyclonal anti- PITX2 (dil 1:200, Chemicon) antibody and goat anti-rabbit IgG-fluorescein (dil 1:400, Chemicon) were used as primary and secondary antibodies respectively. In brief, the slides were de-paraffinized and rehydrated, then blocked in 5% BSA in 1XTBS-T (Tris Buffered Saline containing 0.1% Tween-20) for 30 min, and incubated for 2 h with the primary antibody diluted in 1XTBS containing 0.1% BSA. The slides were then washed and incubated for 1h with the secondary antibody diluted with 1XTBS containing 0.1% BSA. The sections were counterstained with the DNA-specific dye DAPI (10 μ g/ml of TBS, Sigma). The PermaFluor Aqueous Mountant (Immunon, Pittsburgh, PA, USA) was used as the mounting medium. The stained slides were observed under a fluorescence microscope (Olympus BX51) and the images were captured with cool snap pro camera (Olympus). The HE, IHC and IF stainings were performed using at least three different samples for each experiment and images from a representative slide have been shown in the 'figure'. The anti-PITX2 antibody used for the IF and IHC studies can detect all isoforms of PITX2.

Sex determination of embryos

XY and XX gonads are morphologically indistinguishable until testis cords are formed in male at approximately E14.5. Therefore, the embryos were randomly processed for paraffin block followed by section preparation and their sex was determined by PCR analysis using rat *Sry*-specific oligonucleotide primers. Genomic DNA was extracted from paraffin sections using DEXPAT™ Kit [DNA Extraction from Paraffin embedded tissue, TaKaRa, Shinga, Japan] and

subjected to PCR analysis. The Rat *Sry* (NM_012772) primer sequences are Forward; 5'-GCC TTA CAG AAG CCG AAA A-3' and Reverse; 5'-GTA GGT TGT TGT CCC ATT GC-3' that amplify 192 bp PCR product at annealing temperature 58 °C.

RNA isolation, cDNA preparation, Tqman Q-PCR analysis

For RNA isolation, embryos were dissected in presence of cold DEPC-treated PBS under a Stereomicroscope (Carl Zeiss STEMI 2000C). Ovaries and testes from E14.5, 16.5, 18.5 and 20.5 embryos and also from the rats at D10 and 30 were isolated and stored in RNAlater solution (Ambion, Austin, TX, USA) at -80°C until further use. Total RNA was isolated from ovary and testis using TRI Reagent (Sigma) following the manufacturer's instructions. cDNAs were synthesized from 1 μ g each of ovarian or and testicular RNAs using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. The Taqman gene expression assay primers and probes specific to rat *Pitx2b* and *-c* and β -*Actin* (Assay ID- Rn00571000_m1, Rn01521836_m1 and part no- 4352931E respectively) were purchased from Applied Biosystems (Foster City, CA, USA). The gene expression assay was performed using Taqman Master Mix (Applied Biosystems) according to the manufacturer's instructions. In brief, this consisted of initial holding at 50°C for 2 min, then holding at 95°C for 10 min followed by 40 cycles of amplification at 95°C for 10 sec and at 60°C for 1 min in 96-well format PCR plate in a 7500 Real-Time PCR System (Applied Biosystems). The PCR reaction mixture 20 μ l consisted of: 2 μ l of cDNA template (100 ng), 1 μ l of 20X TaqMan® Gene Expression Assay, 10 μ l of 2X TaqMan® Gene Expression Master Mix and 7 μ l of RNase-free water. Relative quantification of gene expression data analysis in fold change was calculated using the $2^{-\Delta\Delta C_T}$ method, where $\Delta\Delta C_T = \Delta C_T(\text{sample}) - \Delta C_T(\text{calibrator})$, and ΔC_T is the C_T of target gene subtracted from the C_T of the housekeeping gene. The gene expression level of *Pitx2c* at E14.5 ovary /testis was taken as 1.

Western blot analysis

The protein samples were prepared from the ovary and testis at different embryonic days (E14.5, 16.5, 18.5 and 20.5) and postnatal days (D10 and 30). The protein samples were prepared according to the protocol described earlier [22] and quantified using Lowry method. For differential study equal amounts of proteins were resolved on 12% SDS-polyacrylamide gel and transferred onto Immobilon-P membranes (Millipore). The membrane was then incubated overnight with 5% blocking solution (TBST with 5% non-fat dried milk, Biorad), washed twice with TBST, and incubated with rabbit polyclonal anti-PITX2-antibody (Chemicon, 1:1000 dil) or mouse monoclonal β -ACTIN antibody (Santa Cruz, 1:1000 dil). Immunoreactive bands were visualized by reacting goat anti-rabbit or anti-mouse AP-conjugated secondary antibody (Santa Cruz, 1:4000 dil in 5% blocking solution) with the substrates NBT/BCIP. The anti-PITX2 antibody used for western blotting, detects all isoforms of PITX2.

Statistical analysis

For IHC and IF analysis, the experiments were performed three times and the representative images are presented. The Q-PCR and Western detection data were expressed as the mean $\pm$ SE, and statistical analyses were performed by *Student's t-test*. Here P-value<0.05 was considered to be significant. The Q-PCR and western blot experiments were repeated at least three times in three different samples unless otherwise stated.

Results

Epitope mapping followed by western detection with PITX2 antibody to show the expression of different isoforms of PITX2

Rat *Pitx2* gene sequences are submitted according to the isoforms and variants in NCBI GenBank database, where complete sequence of *Pitx2a*, *-2b* and *Pitx2v1*, *-v2* are available (GenBank accession nos. AJ222971.1, AJ222972.1 and NM_001042505.1, NM_019334.2 respectively) while *Pitx2c* (GenBank accession no. EF519321.1) is available as partially submitted sequence. To know the sequence identity among *Pitx2a*, *-b*, *-c* and *Pitx2v1*, *-v2* CDS, we performed CLUSTAL W-based multiple sequence alignment (CLUSTAL W MSA). The alignment result shows that *Pitx2v2* is same as *Pitx2b* since they show 100% sequence identity with each other, whereas *Pitx2v1* shows sequence identity with both *Pitx2a* and partial *Pitx2c* sequence (Fig. 1A). But, based on the current literature [26], and our MSA shows that the GenBank accession no. AJ222971.1 submitted as rat *Pitx2a* is actually *Pitx2c* or *Pitx2v1*. We were unable to identify the actual rat *Pitx2a* in NCBI GenBank database searches. Similarly, CLUSTAL W MSA analysis of protein sequences shows that PITX2V2 is actually PITX2B and PITX2V1 is actually PITX2C, since they show 100% sequence identity with each other (Fig. 1B).

The epitope mapping suggests that the PITX2 antibody (chemicon) was raised from the epitope (DPSKKKRQRRQRTHF) that is present in PITX2A, -B, and -C isoforms of rat, human and mouse (Fig. 1C), and hence it is predicted that the antibody would cross-react with each PITX2 isoform of these mammals. To validate this hypothesis, western immunoblot was performed with proteins of different cell or tissue lysates of human, rat and mice origin. The protein samples of rat and mouse were taken from adult ovary, testis, pituitary and hind limb muscle, whereas for human, SKOV-3 ovarian cancer cell lysate was taken for western blot.

All samples were subjected to SDS-PAGE followed by western detection with PITX2 antibody. The immunoblot result suggests that this antibody cross reacts with all PITX2 isoforms in human SKOV-3, rat L6 and rat pituitary cell lysate and these are PITX2A, -B1, -B2, -C α and -C β (Fig. 1D, 1F and 1G respectively) according to the previous nomenclature by Lamba et al. [26]. But in CHO cell extract, where PITX2 does not express, no cross reactivity was observed (data not shown), and this shows the specificity of the signals. In rat embryonic ovary and testis it detects four PITX2 isoforms (i.e. PITX2B1, -B2, -C α and -C β ; Fig. 1E), but only PITX2B2 and -C β in adult rat ovary and testis (Fig. 1F), suggesting that PITX2B2 and -C β are the predominant isoforms that express in adult rat gonads. Predominant expression of PITX2B2 and -C β , and faint expression of PITX2C α and -B1 were found in embryonic gonads. However, we couldn't exclude or rule out the expression of PITX2A isoform in rat gonads as our western blot data show the PITX2A is negligibly expressed in rat embryonic gonads (Fig. 1G, lane 2) only when considerably excessive amount of protein extracts are loaded in western blot experiments.

To further investigate whether the antibody used can detect PITX2 isoforms in mice and also to know the predominant isoforms that express in adult mice gonads as in rat, western immunodetection was performed using whole tissue lysate of gonads and hind limb muscle of rat and mice. The result shows that as in rat PITX2B2 and -C β are also the isoforms that express in adult mice gonads in a sexually dimorphic manner (Fig. 1H). In hind limb muscle of both rat and mice, PITX2B2 and -C β are the isoforms that express, with B2 expressing at significantly higher level compared to -C β . Interestingly, in rat, both isoforms resolve little faster than that in mouse.

Expression of Pitx2 isoforms throughout the rat gonadal development in a sexually dimorphic pattern

To elucidate the *Pitx2* gene expression profile, ovaries and testes were isolated from embryos at different days of gestation and from postnatal rat gonads, followed by RNA isolation. The real time quantitative RT-PCR (Q-PCR) was then performed using *Pitx2* isoform-specific taqman assay primers along with β -*Actin* taqman primer as an internal control. The Q-PCR (Fig. 2A, B) data indicates that *Pitx2b* and *-c* express at the embryonic days (E14.5, 16.5, 18.5 and 20.5) and postnatal days (D10 and 30) in both gonads. Although there is no

Fig. 1. Analysis of variation in rat *Pitx2*/PITX2 isoforms, and epitope mapping followed by western validation of the PITX2 specific antibody in rat, human and mice. (A) CLUSTAL W generated Multiple Sequence Alignment (CLUSTAL W MSA) shows the variation in the m-RNA CDS region of *Pitx2a*, *-b* and *-c* isoforms and their homology with variant wise nomenclature *Pitx2 variant1* and *variant2* of rat. The available NCBI GenBank accession number and gene nomenclature are shown with each sequence. The rat *Pitx2c* CDS sequence partially available in NCBI GenBank and termed here as *rPitx2c-per* (r, rat). (B) CLUSTAL W MSA shows the variation in the N-terminal region of rat PITX2A, -B and -C isoforms and their homology with PITX2 variant1 and variant2 of rat. The available NCBI Protein Bank accession number and nomenclature are shown with each sequence. (r, rat). (C) CLUSTAL W MSA of rat PITX2A, -B and -C protein epitope alignment with other orthologues from human and mice showing the conserved nature of 15 amino acid long epitope sequence. The epitope is indicated by box containing the colour case letters from where the antibody was raised. The NCBI Protein Bank accession number and nomenclature of isoforms are shown with each sequence. (r, rat; m, mice; h, human). (D) Immunoblot analysis of PITX2 isoforms expressed in SKOV-3 ovarian cell line detects all spliced variants of PITX2. (Are PITX2A, -B1, -B2, -C α and -C β , lane 1 and 2). (E) Immunoblot analysis of rat embryonic ovary and testis protein shows expression of four PITX2 isoforms which correspond to PITX2B1, -B2, -C α and -C β (lane 1; Ovary and lane 2; Testis, are highlighted from lane 6 and 7 of Fig. 3A image). (F) Immunoblot analysis of PITX2 isoforms expressed in adult rat gonads (lane 1; Testis and lane 2; Ovary) and in L6 rat muscle fibroblast cell line (lane 3). (G) Immunoblot analysis of PITX2 isoforms expressed in adult rat pituitary compared to embryonic rat gonads. (H) Comparative analysis of PITX2 isoforms expressed in rat versus mice gonads and muscle. Immuno-detection shows only two specific bands of PITX2 which correspond to PITX2B2 and -C β ; mice muscle (lane 1), mice ovary (lane 2) mice testis (lane 3), rat muscle (lane 4), rat ovary (lane 5) and rat testis (lane 6). PITX2B2 and -C β in both gonads show sexually dimorphic level of expression with significantly higher expression level of B2 in ovary and of C β in testis of both animals (compare lane 2-3 and lane 5-6). In hind limb muscle of both rat and mice, PITX2B2 expresses in significantly higher level compared to PITX2C β (lanes 1 and 4). Interestingly, in rat, both isoforms migrate slightly faster than their orthologue mice isoforms. Both adult mice and rat were of 90 days old. In the experiments of Fig. 1 D-H, whole cell/tissue lysates were subjected to electrophoresis on 12% SDS-PAGE followed by immuno-detection with PITX2 specific antibody.

Fig. 2. Comparative gene expression profile of *Pitx2b* and *c* in rat gonads at embryonic and postnatal stages. Total RNAs from ovary (O) and testis (T) were isolated at embryonic days E14.5, 16.5, 18.5, and 20.5, and at postnatal days, D10 and 30 followed by Taqman Q-PCR using rat *Pitx2b* and *Pitx2c* gene-specific primers and probes. Fig 2(A) and (B) show the Q-PCR results of these two isoform with ovarian and testicular RNAs respectively. The data are represented by relative gene expression in fold change calculated by $2^{-\Delta\Delta C_T}$ method. The C_T -values of *Pitx2* gene are normalized from the C_T -value of the housekeeping gene β -Actin. The gene expression level of *Pitx2c* at E14.5 ovary/testis was considered as 1. The embryonic and the postnatal developmental days of ovary and testis have been mentioned. The experiments were performed three times and the mean $\pm$ SE value have been shown, *, $P < 0.05$.

significant difference in their expression in male and female gonads during developmental stages, their

Fig. 3. Comparative PITX2 protein isoform expression profile in embryonic and postnatal rat gonads. Whole tissue proteins from the embryonic (E14.5, 16.5, 18.5, 20.5) and postnatal gonads (D10, 30) were isolated as mentioned in methods section. The protein samples were subjected to 12% SDS-PAGE followed by western immuno-detection using PITX2 specific antibody. Fig. 3A shows that the PITX2 isoforms that express throughout the embryonic gestational days at E14.5, 16.5, 18.5 and 20.5 rat gonads are predominantly PITX2B2 and -Cβ whereas there with relatively lower level expression are PITX2B1 and Cα (Fig. 3A bands above -B2). Fig. 3B shows that PITX2B2 and -Cβ are the isoforms expressed at postnatal D10 and 30 gonads. The pixel densities of the individual isoforms were measured using ImageJ software (NIH, USA) and are shown below the specific lanes; respective isoforms are indicated with arrow. Below each bar diagram, the day and the tissue types have been mentioned. The experiments were performed three times and the mean $\pm$ SE value have been shown, *, $P < 0.05$. 'O'-Ovary and 'T'-Testis.

expression profile drastically changes in a sexually dimorphic manner at postnatal stages (Fig. 2A, B).

Fig. 4. Localization of PITX2 at different embryonic developmental stages of ovary. The whole embryo at E14.5 (Fig. 4A) and E16.5 (Fig. 4B) and the developing ovary at E18.5 (Fig. 4C) and E20.5 (Fig. 4D) are shown here. The paraffin sections were subjected to HE staining as well as IHC and IF studies with PITX2-specific antibody. In Fig. 4A, the HE-stained whole embryonic section shows the location of different developing organs along with ovary. In these sections, IHC study shows that the ovary has specific localization of PITX2 as marked by dotted line. This region has been shown in higher magnification in next

column by IHC and IF studies using PITX2-specific antibody. In higher magnification, the PGC shows specific localization of PITX2 (arrow head). Similarly Fig. 4B and 4C show specific localization of PITX2 in E16.5 and E18.5 ovary on whole embryo sections as well as in higher magnification while, Fig. 4D shows its localization at E20.5. For the sake of brevity, images of the selected days are furnished to highlight the pattern of developmental expression. The Negative Control images represent the staining in presence of secondary antibody but without PITX2 antibody. Scale bars: black, 1 mm; red, 100 μ m; yellow, 50 μ m; pink, 20 μ m. 'Ov'-Ovary, 'M'-Mesonephros, Arrow indicates PGC.

In postnatal D10 and 30 ovaries, *Pitx2b* expresses at much higher level (around 6-fold) compared to *Pitx2c*. However, in postnatal testis (D10 and 30), the situation is just reversed; here *Pitx2c* expression is significantly higher (about 6-fold) than that of *Pitx2b*.

Expression of PITX2 isoforms throughout the rat gonadal development in a sexually dimorphic pattern

The western immunoblot experiments were performed with embryonic ovarian and testicular proteins isolated at different stages of development, i.e. on E14.5, 16.5, 18.5, and 20.5 (Fig. 3A), and with postnatal ovary and testis at D10 and 30 (Fig. 3B). The results show the expression of four PITX2 isoforms PITX2B1, -B2, -C α and -C β during rat embryonic gonadal development.

Among these isoforms only PITX2B2 and -C β express predominantly, whereas, PITX2B1 and -C α express faintly in both gonads (Fig. 3A and 1D, bands above -C β), which suggests the stage-specific expression pattern of different PITX2 isoforms in gonads. The PITX2B2 isoform equally expresses in ovary and testis at E14.5 and as gonadal development progresses, i.e. E16.5 onwards its significant down-regulation was observed especially in testis which is maintained in postnatal stages too (Fig. 3A, B, upper bands). The PITX2C β isoform shows no such significant changes during embryonic development in both gonads, but shows significant up-regulation at postnatal testis (Fig. 3A, B, lower band). Interestingly, when the expression of these isoforms was considered as ovary versus testis at postnatal stages, a clear sexually dimorphic expression pattern was observed. In ovary PITX2B2 expresses at

Fig. 5. Localizaion of PITX2 at different embryonic developmental stages of testis. Whole embryo at E14.5 (Fig. 5A) and E16.5 (Fig. 5B) and isolated testis from embryos at E18.5 (Fig. 5C) and E20.5 (Fig. 5D) were processed for subsequent HE, IHC and IF studies. At E14.5 (Fig. 5A), the HE stained whole embryonic section shows the location of different developing organs with testis. The IHC and IF study in consecutive section shows the localization of PITX2 in testis as marked by dotted line and the higher magnification of images show its localization in PGCs. Similarly, at E16.5 (Fig. 5B), E18.5 (Fig. 5C) and E20.5 testis (Fig. 5D) shows the localization of PITX2 in male germ cells inside the seminiferous tubules/testicular cords on whole embryonic- and isolated testicular section. For the sake of brevity, images of the selected days are furnished to highlight the pattern of developmental expression. The Negative Control images represent the staining in presence of secondary antibody but without PITX2 antibody. Scale bars: black, 1 mm; red, 100 μ m; yellow, 50 μ m; pink, 20 μ m. 'T'-Testis, 'M'-Mesonephros, 'Tc'- Testicular cords (arrow), Arrow head indicates PGC.

For the sake of brevity, images of the selected days are furnished to highlight the pattern of developmental expression. The Negative Control images represent the staining in presence of secondary antibody but without PITX2 antibody. Scale bars: black, 1 mm; red, 100 μ m; yellow, 50 μ m; pink, 20 μ m. 'T'-Testis, 'M'-Mesonephros, 'Tc'- Testicular cords (arrow), Arrow head indicates PGC.

Fig. 6. Localization of PITX2 at postnatal ovary and testis. The ovary (Fig. 6A) and testis (Fig. 6B) were isolated at D10 and D30 followed by paraffin block preparation, sectioning and subsequent IHC studies. In Fig. 6A, the right upper and lower panels show the localization of PITX2 in the differentiating ovarian follicles at D10 and 30 respectively. At D30, PITX2 intra-follicular localization was observed especially in granulosa (arrows) and theca cells (arrow heads). In Fig. 6B the right upper and lower panels show the localization of PITX2 in the spermatogonia (arrow head) and primary spermatocytes (arrows) of seminiferous tubules at D10 and D30 testis respectively. Consecutive sections of each sample were stained in hematoxylin and eosin and are shown in the left panel. For the sake of brevity, images of the selected days are furnished to highlight the pattern of developmental expression. The Negative Control images represent the staining in presence of secondary antibody but without PITX2 antibody. Scale bars: red, 100 μ m; yellow, 50 μ m; black, 15 μ m. In Fig. 6A, arrows in upper panel shows growing follicles, arrows in lower panel -granulosa cells, arrowhead -theca cells. In Fig. 6B, arrow shows spermatocytes, arrowhead -spermatogonia, asterisks -intertubular blood vessels.

Consecutive sections of each sample were stained in hematoxylin and eosin and are shown in the left panel. For the sake of brevity, images of the selected days are furnished to highlight the pattern of developmental expression. The Negative Control images represent the staining in presence of secondary antibody but without PITX2 antibody. Scale bars: red, 100 μ m; yellow, 50 μ m; black, 15 μ m. In Fig. 6A, arrows in upper panel shows growing follicles, arrows in lower panel -granulosa cells, arrowhead -theca cells. In Fig. 6B, arrow shows spermatocytes, arrowhead -spermatogonia, asterisks -intertubular blood vessels.

significantly higher level compared to PITX2C β , but in testis the situation was just reversed, suggesting a

criss-cross pattern of expression of two isoforms as mentioned in gonad.

Localization of PITX2 in rat gonads at different stages of embryonic development

To investigate the localization profile of PITX2 in female gonad, embryonic sections containing ovarian tissue at different days of gestation were prepared and subjected to IHC and IF studies (Fig. 4). The bipotent gonad starts differentiating into ovary and/or testis from day 14.5 of gestation. At E14.5 and 16.5, the whole embryos were processed and sections were prepared, whereas at E18.5 and 20.5 the isolated ovaries were processed for section preparation followed by HE, IHC and IF studies. The IHC data suggests that PITX2 expresses at the initiation of the ovarian development, i.e. from E14.5 (Fig. 4A), and its expression maintained in ovaries, through E16.5 (Fig. 4B), E18.5 (Fig. 4C) and E20.5 (Fig. 4D). The view at lower magnification (HE and IHC) of whole embryo (Fig. 4A, B, 1st and 2nd panels from left) shows the position of developing ovaries at E14.5 and 16.5 (dotted box). At a higher magnification the figure shows the exact area or cell types of PITX2 localization in ovaries, where developing PGCs show specific localization of PITX2 (arrows) at different days of embryonic development, although some somatic cells also express the same during development.

Similarly, the embryonic sections containing male gonad were prepared from the same stages of development as mentioned before. PITX2 was localized in the male PGCs (arrows) in the testicular cords (arrow heads) during early developmental stages at E14.5 (Fig. 5A), E16.5 (Fig. 5B), E18.5 (Fig. 5C) and E20.5 (Fig. 5D). As in ovary, lower magnifications of the images were shown to indicate the position of the testicular structure (5A, B, 1st and 2nd panels from left, dotted box). The IHC and IF data at higher magnification show the localization of PITX2 in specific areas or cell types in male gonads at different embryonic stages, suggesting its localization in the PGCs and also in some somatic cells. Similarly, as in ovary, the whole embryos at E14.5 and 16.5 and the isolated testes at E18.5 and -20.5, were processed for section preparation followed by HE, IHC and IF studies. Along with gonads, expression of PITX2 was markedly visible even in many developing tissues like pituitary, eye, mandible, brain, lungs, limbs, and somites with a varied expression profile.

Localization of PITX2 in rat gonads during neonatal and postnatal development

To show the PITX2 localization in rat gonads at postnatal stages, gonads were collected at D10 and 30 and then HE/IHC staining was performed. At D10, PITX2

was localized in the differentiating ovarian follicles (Fig. 6A, upper panel, arrows). At D30 (Fig. 6A, lower panel), intra-follicular localization of PITX2 was observed especially in granulosa (arrows) and theca cells (arrow heads). Similarly, in testis at D10 and 30 (Fig. 6B, upper and lower panel respectively), PITX2 localizes predominantly in the spermatogonia (arrow head) and primary spermatocytes (arrows) inside the seminiferous tubules. It was also expressed in the inter-tubular blood vessels (asterisks) and peri-tubular smooth muscles, but to a lesser extent.

Discussion

The development of the mammalian gonad during embryogenesis is exceptional because the bipotent gonad either leads to testis or ovary. The gonads are complex organ composed of multiple cell types, and multiple molecular signaling cascades are involved during their differentiation and development. To know the molecular mechanisms of gonadal differentiation, development and function, it is important to have knowledge of the function of genes that are involved and especially expressed in a sexually dimorphic manner in gonads. In our study, we found that PITX2, a homeobox transcription factor, shows isoform-specific sexually dimorphic expression pattern during gonadal development, at both mRNA and protein level. *Pitx2* plays an important role in the development of organs like heart, brain, pituitary, lungs, eyes and mandible and in differentiation of vascular smooth muscle cells. Altered PITX2 expression leads to several developmental and physiological disorders [12-16]. The expression of *Pitx2* has been identified in avian gonads [23-24, 27], where it follows asymmetric expression pattern in both sexes of chicken from the initiation of their development [23]. But its involvement in the development and morphogenesis of mammalian gonads, where the gonad develops symmetrically, is completely unknown. To unravel its role, the very first step was to perform a detailed study of its temporal and spatial expression pattern and its localization in rat gonads during the embryonic and postnatal developmental stages. The CLUSTAL W MSA analysis data, current literature and our gene and protein expression data suggests that in case of rat *Pitx2v2* is *Pitx2b* and *Pitx2v1* is *Pitx2c* (Fig. 1), similarly, PITX2V2 is PITX2B and PITX2V1 is PITX2C. Further, all 5 isoforms of PITX2 express in human SKOV-3 cell line, in rat pituitary, and in rat L6 muscle cell line, and according to published nomenclature, these are PITX2A,

-B1, -B2, -C α and -C β (Fig. 1D, F and G [26]). Interestingly, in gonads of adult rat and mice, a significant sexually dimorphic expression pattern of two isoforms was observed (Fig. 1H), with little difference in molecular weight between two species.

The Q-PCR profile shows that both *Pitx2b* and -*c* isoforms are expressed during embryonic and postnatal development in both gonads. The extent of differential expression of *Pitx2b* and -*c* is remarkable in postnatal gonads and a 'vice-versa' type of expression pattern is observed. Here *Pitx2c* expression level was about 6-fold higher in postnatal testis than *Pitx2b* but the pattern is just reversed in ovary (Fig. 2B). Similarly, PITX2B2 and -C β are the two predominant isoforms of PITX2 that express in adult gonads whereas three other isoforms PITX2A, -B1 and -C α express comparatively lesser in embryonic gonads (Fig. 1 E,G and Fig. 3A). At E14.5 in both gonads, PITX2B2 expresses evenly but down regulates significantly and progressively in testis from E16.5 onwards till E20.5 (Fig. 3A). In postnatal stages also, this expression pattern prevails, i.e. higher and lower expression levels of B2 in ovary and testis respectively at both D10 and 30 (Fig. 3B). The C β isoform, however, is more or less unchanged in both gonads during embryonic development (Fig. 3A), but up-regulated more than 5 fold than B2 in postnatal testis D10 and 30 (Fig. 3B). Hence, there is a biased expression of B2 and C β in ovary and testis respectively, where B2 shows sexually dimorphic expression during embryonic development and B2 along with C β show it at postnatal stages to a higher extent. These data again strongly suggests that two predominant PITX2 isoforms are differentially expressed in embryonic and postnatal stages and more importantly their expression is sexually dimorphic as shown by gene and protein expression profiles. The mRNA and protein levels of both isoforms show similar expression profile in postnatal gonads. However, at embryonic stages the mRNA, of *Pitx2b* and -*c* is not differentially expressed in ovary versus testis but at protein level significant change in expression is observed which might be due to the alternative mRNA splicing and alternative translation initiation.

Considering significant differences at the expression level of different PITX2 isoforms in gonads, we speculate that sex-specific variation might occur in gonads in their post-transcriptional and translational modification. We therefore hypothesize that the different isoforms might play sex- and stage-specific dose dependent role in embryonic and adult gonads like regulation of gonadal

cell type specification, cell lineage determination, cell cycle regulation and involvement in mitotic or meiotic processes during embryonic development and after birth. In histological studies, we observed very specific and robust localization of PITX2 in both gonads at E14.5, the day when the ovary or testis starts differentiating from its bipotent state. In whole embryo section (E14.5 and E16.5), the expression and localization of PITX2 is clearly visible in PGCs of gonads as shown by IHC and IF studies (Fig. 4-5). Gradually at E18.5 and 20.5, PITX2 localization was found restricted to PGCs in ovary (Fig. 4, arrows) and in testicular cords (Fig. 5, arrow heads and arrows respectively). In postnatal (D10 and 30) rats, PITX2 localizes in granulosa and theca cells of ovary (Fig. 6A) and in the germ cells inside the seminiferous tubules of testis (Fig. 6B). Therefore, PITX2 expresses in primordial germ cells when they start differentiating at embryonic developmental stages and consistently maintains the expression, however at adult stages the expression was observed in germ cells and also in some somatic cells.

In summary, we have shown that, 1) *Pitx2*/PITX2 transcription factor expresses throughout the embryonic development as well as in adult gonads, but their different isoforms express in a sexually dimorphic manner in both gonads 2) between two predominant isoforms, only PITX2B2 shows sexually dimorphic expression during embryonic developmental stages, 3) during postnatal stages, both PITX2 and -C β isoforms show sexually dimorphic expression pattern, but in a criss-cross manner in male and female gonads respectively 4) the PITX2 is localized in the PGCs of gonads at embryonic stages, and 5) in postnatal ovary, PITX2 localizes chiefly in granulosa and theca cells in growing follicles; in postnatal testis, it localizes in the germ cells inside seminiferous tubule and also in the somatic cells. Our data suggests that two isoforms of PITX2 may play essential and sex-specific roles in the regulation of reproductive functions during embryonic and postnatal development.

Abbreviations

E (Embryonic); D (Day postnatal); NBT (Nitro blue tetrazolium); BCIP (5-Bromo-4-chloro-3 indolyl phosphate); FITC (Fluorescein Isothiocyanate); HE (Hematoxylin and Eosin); IHC (Immunohistochemistry); IF (Immunofluorescence); O (Ovary); T (Testis); TC (Testicular Cord); M (Mesonephros); PGC (Primordial germ cell); V/v (Variant).

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