RESEARCH

Platelet-derived growth factor BB stimulates differentiation of rat immature Leydig cells

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Abstract

Platelet-derived growth factor (PDGF) is one family of growth factors that regulate cell growth and differentiation. Rat Leydig cells express PDGF- β receptor (PDGFRB) during pubertal development. However, the mechanism of PDGF in the regulation of Leydig cell development is unclear. In the present study, rat immature Leydig cells were isolated from the testes of 35-day-old Sprague-Dawley rats and treated with 1 and 10 ng/mL of PDGF-BB. After 24 h of treatment, these cells were harvested for genomics profiling and the medium steroids were measured. 1 and 10 ng/mL PDGF-BB significantly increased androgen production by rat immature Leydig cells. Genomics profiling analysis showed that the expression levels of steroidogenic acute regulatory protein (*Star*) were increased by 2-fold. Further analysis showed that *Fos* expression level was increased 2- and 5-fold by 1 and 10 ng/mL PDGF-BB, respectively. In conclusion, PDGF-BB stimulated the differentiation of rat immature Leydig cells via regulating *Star*.

Key Words

- ▶ Platelet-derived growth factor BB
- ▶ rat immature Leydig cells
- ▶ steroidogenic acute regulatory protein
- ▶ Fos

Journal of Molecular
Endocrinology
(2018) 60, 29–43

Introduction

Testosterone stimulates fertility and maintains the secondary sexual characteristics of males at adulthood (Ye *et al.* 2017). The primary source of this hormone in males is the Leydig cell, which is located in the interstitial area of a mammalian testis. Pituitary-secreted luteinizing hormone (LH) and its receptor (LHCGR), cholesterol transportation into the Leydig cell via high-density lipoprotein receptor (SCARB1) and into the mitochondrion via steroidogenic acute regulatory protein (STAR) and androgen-biosynthetic enzymes, including P450 cholesterol side chain cleavage enzyme (CYP11A1), 3 β -hydroxysteroid dehydrogenase 1 (HSD3B1), 17 α -hydroxylase/17,20-lyase (CYP17A1) and 17 β -hydroxysteroid dehydrogenase 3 (HSD17B3), are very critical for the production of testosterone in Leydig cells (Ge & Hardy 2007).

However, the development of adult Leydig cells is also very important for the formation of the male body (Ye *et al.* 2017). The pubertal development of Leydig cells is conceptually divided into four stages: stem, progenitor, immature and adult Leydig cells (Ye *et al.* 2017). In rats, Leydig cells arise from stem Leydig cells (Ge *et al.* 2006), which are first identified in the early postnatal (6-day-old) rat testis (Ge *et al.* 2006) and are shown to have the capability of self-renewing or differentiating into androgen-producing Leydig cell lineage. The first cell seen in the Leydig cell lineage is the spindle-shaped progenitor Leydig cell, which is located adjacent to the stem Leydig cells and appears in the interstitial area around pre-puberty (12 day old) (Hardy *et al.* 1991, Ge & Hardy 1998, Mendis-Handagama & Ariyaratne 2001). The progenitor Leydig cell expresses the Leydig cell lineage

biomarker, LHCGR, SCARB1, STAR, CYP11A1, HSD3B1 and CYP17A1. The progenitor Leydig cell also has the high level of androgen-metabolizing enzyme 5 α -reductase 1 (SRD5A1, gene *Srd5a1*) (Ge & Hardy 1998, Feng *et al.* 2016) and 3 α -hydroxysteroid dehydrogenase (AKR1C9 gene *Akr1c9*) (Ge & Hardy 1998). However, the progenitor Leydig cell lacks the final-step androgen-biosynthetic enzyme (HSD17B3) for testosterone biosynthesis. Thus, the androstenedione formed after the enzyme catalysis of CYP11A1, HSD3B1 and CYP17A1 is metabolized into 5 α -androstenedione by SRD5A1 and further by AKR1C9 into androsterone, which is secreted into the circulation (Ge & Hardy 1998). The progenitor Leydig cell has the high proliferative capacity too, amplifying the Leydig cell number (Ge & Hardy 1997). Around puberty (age, 35 days) in the rat, the progenitor Leydig cell transits into the immature Leydig cell, which is ovoid in shape and contains abundant lipid droplets. The immature Leydig cell has well-developed androgen-biosynthetic enzymes, including HSD17B3, but also has high levels of SRD5A1 and AKR1C9, and therefore, the formation of testosterone by HSD17B3 is metabolized into dihydrotestosterone by SRD5A1 and further into androstenediol (DIOL) by AKR1C9 (Ge & Hardy 1998). The immature Leydig cell has the intermediate proliferative capacity (Ge & Hardy 1997) and can divide once (Hardy *et al.* 1989). The immature Leydig cell matures into the adult Leydig cell around adult stages (56 day old). The adult Leydig cell is round in shape and produces mainly testosterone because the androgen-metabolizing enzyme SRD5A1 in adult Leydig cells is silenced around postnatal day 56 (Ge & Hardy 1998).

Many factors have been reported to regulate the proliferation and differentiation of Leydig cells, including LH, growth factors, cytokines and steroids (see review Chen *et al.* 2009, Ye *et al.* 2017). One family of growth factors is platelet-derived growth factors (PDGFs). PDGFs are disulfide-linked homodimeric proteins. The family of growth factors has been reported to regulate cell proliferation, tissue remodeling, differentiation and growth (Basciani *et al.* 2010).

Members of the PDGF family have been shown to be involved in the development of Leydig cells. PDGF-A and PDGF-B are expressed in the rat testis, forming homodimers of PDGF-AA and PDGF-BB as well as a heterodimer PDGF-AB (Betsholtz *et al.* 2001). In fetal, neonatal and pubertal rat testis, PDGF-A and PDGF-B were found to be present in Sertoli cells (Gnessi *et al.* 1995, Li *et al.* 1997, Uzumcu *et al.* 2002), and in adult rat testis, these two factors were found to be expressed

in both Sertoli and Leydig cells (Gnessi *et al.* 1992). There are at least two PDGF receptors that respond to PDGF stimulation. The PDGF homodimers bind to their corresponding tyrosine kinase receptors, PDGF receptor α (PDGFRA) and PDGF receptor β (PDGFRB), and the PDGF-AB heterodimer binds to both PDGFRA and PDGFRB, thereby activating the unique downstream pathways (Betsholtz *et al.* 2001).

Both PDGF-A/PDGFRA and PDGF-B/PDGFRB are essential for the development of Leydig cells (Schmahl *et al.* 2008). Adult Leydig cells did not differentiate in the adult knockout mice, which lacked expression of the *Pdgfra* (Gnessi *et al.* 1995). Knockout of *Pdgfra* gene led to few Leydig cells in fetal mouse testis (Brennan *et al.* 2003). Leydig cell conditional knockout of *Pdgfra* or *Pdgfrb* also led to a significant reduction of Leydig cell number in the fetal testis (Schmahl *et al.* 2008). However, the role of PDGF-BB in the pubertal development of Leydig cells is still not clear. Our objective of the present study was to analyze the possible regulatory effects of PDGF-BB on the Leydig cell lineage. To do so, we isolated rat immature Leydig cells and treated them with PDGF-BB *in vitro*. We identified a series of downstreamed genes that were regulated by PDGF-BB.

Materials and methods

Chemicals

Rat PDGF-BB was purchased from R&D Systems Inc. PDGFRB tyrosine kinase inhibitor IV (521233, also called PKI) was purchased from Calbiochem. LH (USDA-bLH-B-6) was provided by USDA Animal Hormone Program (Beltsville, MD, USA). Testosterone was purchased from Steraloids Inc (Newport, RI, USA), and [1,2,6,7,16,17-³H(N)]-testosterone was purchased from PerkinElmer. Testosterone antibody was from MP Biomedicals (Solon, OH, USA). The RatRef-12 Expression BeadChip for genome-wide expression was purchased from Illumina Inc (San Diego, CA, USA).

Animals

Sixty 28-day-old male Sprague-Dawley rats (Laboratory Animal Center, Wenzhou Medical University, Wenzhou, China) were raised in a 12-h darkness/light cycle temperature at 23 $\pm$ 2°C, and relative humidity of 45%–55%. Water and food were accessed *ad libitum*. After one-week adjustment, rats (35-day-old) were used for the isolation

of immature Leydig cells. All studies were approved by the Wenzhou Medical University's Animal Care and Use Committee and were performed in accordance with the Guide for the Care and Use of Laboratory Animals.

Leydig cell isolation

Immature Leydig cells were purified as previously described (Ge & Hardy 1998). Immature Leydig cells were used because they have been reported to express *PDGFRB*, and they have all testosterone biosynthetic and metabolizing enzymes (Ge & Hardy 1998). In brief, the removed testis was perfused with a collagenase solution via the testicular artery and digested with the M-199 medium containing collagenase and DNase (Sigma-Aldrich) for 15 min. Then, the suspension was filtered through 100 μ m 2-layer nylon mesh and the cells were separated under a Percoll gradient (Sigma-Aldrich). The cells with a density of 1.07–1.088 g/mL were collected and washed. Purified cells were evaluated by histochemical staining for HSD3B1 activity, with 0.4 mM etiocholanolone as the steroid substrate and NAD⁺ as a cofactor (Payne *et al.* 1980). The purity of immature Leydig cells was $\geq 95\%$.

Immature Leydig cell culture

To test the effects of PDGF-BB on androgen biosynthesis, isolated immature Leydig cells were seeded at each well of a 12-well plate. Cells were cultured in the presence of different concentrations of PDGF-BB (0, 1 or 10 ng/mL) alone or plus LH (10 ng/mL) or 22R-hydroxycholesterol (22R, 2 μ M) in 1:1 DMEM:F-12 culture medium containing 0.1% bovine serum albumin in 5% O₂ and 34°C for 24 h, and this culture system causes very low oxidative stress of Leydig cells (Weissman *et al.* 2005). 22R was used because immature Leydig cells are permeable to 22R, which was served as the substrate of CYP11A1 to judge its enzyme activity (Ge & Hardy 1998). 1 or 10 ng/mL PDGF-BB were added to immature Leydig cells, and the cells were cultured for 24 h. After that, the media were collected for analysis of androgen. In another set of experiment, PDGF-BB (10 ng/mL) and its inhibitor PKI (20 nM) were used alone or in combination. 20 nM PKI was used because it had selective inhibition for PDGFRB (IC₅₀ = 4.2 nM) over PDGFRA (IC₅₀ = 45 nM) (Ho *et al.* 2005). Medium testosterone and androstenediol from 24-h culture media were measured, and then the levels after treatment were compared with the control (no treatment, basal).

Thymidine incorporation

Immature Leydig cells were labeled with [³H]thymidine (DuPont-New England Nuclear, Boston, MA, USA) at 1 Ci/mL (specific activity 104.7 Ci/mmol) during the last 2 h of incubation as described (Ge & Hardy 1997). After labeling, the Leydig cells were washed twice with PBS and harvested by centrifugation at 750 g. Leydig cells were lysed in 0.5 mL hyamine hydroxide (ICN Radiochemicals, Irvine, CA, USA), and radioactivity was measured in a liquid scintillation counter (PerkinElmer).

RNA extraction

Total RNAs were extracted from immature Leydig cells using the TRIzol Kit according to the manufacturer's instructions (Invitrogen). Briefly, rat immature Leydig cells were collected after treatment with PDGF-BB (0, 1 or 10 ng/mL) for 24 h and dissolved in TRIzol and homogenized in a glass homogenizer, and total RNAs were extracted. RNA pellets were eluted with 70% ethanol and air dried. Total RNAs were resuspended in the RNase-free water and purified using the RNeasy Kit (Qiagen) according to the manufacturer's instructions. The RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and the RNA integrity in individual RNA samples were analyzed with Agilent 2100 Bioanalyzer (Santa Clara, CA, USA).

Microarray hybridization and scanning

The RatRef-12 Expression BeadChip containing 21,910 rat genes was used. Genes are picked up from the NCBI RefSeq database to cover the whole rat transcriptome. Three groups of samples were used: 0, 1, and 10 ng/mL PDGF-BB-treated immature Leydig cells. Four replicates per group were performed. Probe labeling, hybridization, washing and scanning were performed using the Illumina Total Prep Kit (Applied Biosystems) as previously described (Griffin *et al.* 2010). First strand of cDNA was synthesized in a total volume of 20 μ L with the supplied reagents. The complete first strand product was used for the second strand synthesis, followed by column purification. The purified product was then used for *in vitro* transcription using T7 polymerase. Biotin-16-dUTP was incorporated and the biotinylated complementary RNA (cRNA) probe as prepared. The probe integrity was verified using the Agilent 2100 Bioanalyzer. Labeled cRNA (750 ng) was hybridized to the array chip overnight at 58°C in a total volume of 30 μ L of hybridization buffer, followed by

post-hybridization stringency washing. The chip was scanned in the NextSeq 550 System (Illumina).

Microarray data analysis

Microarray data analysis was performed as previously described (Wang et al. 2016). Briefly, after scanning, the microarray data were imported into the BeadStudio software (Illumina) for normalization, preliminary analysis and filtering. The background subtraction was performed, and the Illumina custom error model was used to generate present/absent calls for each probe ('present' defined as $P < 0.01$ for signal detection) and to call differentially expressed genes (defined as $P < 0.05$ after false discovery rate correction). For each array, all probe sets were normalized to a mean signal intensity value of 100. Normalized data from BeadStudio was filtered to exclude genes not expressed in the Leydig cells (i.e. data from probes that were classed as 'absent' in all samples). All of the 21,910 genes were present in the data based on which further analyses were carried out. The data were further imported into Microsoft Access 2010, and queries to find the increased and decreased genes after PDGF-BB treatment when compared to the control were generated to find the expression levels.

Biological pathway analysis

Biological pathway analysis was performed as previously described (Wang et al. 2016). The Gene MicroArray Pathway Profiler 2.1 (GenMAPP2.1, San Francisco, CA, USA) software was used to find the biological pathway, and GO pathway was generated according to the software developer's instruction. The GenMAPP2.1 was used to create a map of signal pathways for the potential pathways. We imported our statistical results into the program and illustrated biological pathways containing differentially expressed genes. The results of the differential gene expression profile were validated by RT-qPCR.

Quantitative real-time PCR (RT-qPCR)

Briefly, first-strand synthesis of DNA and RT-qPCR were performed as previously described (Lin et al. 2009). RT-qPCR was carried out in a 20 μ L volume in a 96-well plate using the SYBR Green PCR Kit from Applied Biosystems. Primer titration was performed with the concentration of 300 nM. Fluorescence was detected using the ABI 7700 system (PE Applied Biosystems). Each sample was run in duplicate and in parallel with no template

controls. The relative mRNA levels of targeted genes were adjusted to house-keeping gene, ribosomal protein S16 (*Rps16*), as the internal control. *Rps16* in Leydig cells has been used as the internal control in many studies because it showed consistent expression (Ge et al. 2005, Zhang et al. 2015). Furthermore, in this microarray, the values for *Rps16* levels in 0, 1 and 10 ng/mL PDGF-BB groups were 40 ± 3 , 50 ± 6 , and 48 ± 4 , respectively, and there were no significant changes between groups. The Ct value was read and the levels of the target mRNAs were calculated using the standard curve method as previously described (Wang et al. 2016). All primers in the present study were designed by Primer 3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). Forward and reverse primers were placed in different exons to minimize the effects of possible DNA contamination. These genes are luteinizing hormone receptor (*Lhcgr*), scavenger receptor class B member 1 (*Scarb1*), *Star*, *Cyp11a1*, *Hsd3b1*, *Cyp17a1*, *Hsd17b3*, *Srd5a1*, *Akr1c9*, insulin-like 3 (*Insl3*), sulfotransferase 1A1 (*Sult1a1*) and cyclin D1 (*Ccnd1*). The primers were listed in Supplementary Table 1 (see section on supplementary data given at the end of this article).

Western blotting analysis

Immature Leydig cells after PDGF-BB treatment were homogenized and lysed. Protein concentrations in samples were measured using the Bio-Rad Protein Assay Kit (cat# 500-0006; Bio-Rad) according to manufacturer's protocol. Bovine serum albumin was used as the protein standard. Samples (50 μ g protein) were boiled in equal volumes of sample loading buffer, containing 20% glycerol, 5% sodium dodecyl sulfate, 3.1% dithiothreitol and 0.001% bromophenol blue. Samples were electrophoresed on 10% polyacrylamide gels containing sodium dodecyl sulfate. Proteins were electrophoretically transferred onto nitrocellulose membrane. After one-hour exposure to 5% non-fat milk to block nonspecific binding, the membranes were incubated with the primary antibodies (1:1000 dilution) against LHCGR (Santa Cruz), CYP11A1 (Santa Cruz), CYP17A1 (Santa Cruz), STAR (Pterosaur Biotech, Hangzhou, Zhejiang, China) and actin β (ACTB, Cell Signaling Technology). The membranes were then washed and incubated with a 1:1000 dilution of anti-rabbit or anti-goat antiserum (R&D Systems) that was conjugated to horseradish peroxidase. The washing step was repeated, and immunoreactive bands were visualized by chemiluminescence using an ECL kit (Amersham). The density was scanned by ImageJ software.

Medium testosterone and androstenediol measurement

The medium testosterone and androstenediol were fractionated using Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden) column chromatography as previously described (Ge & Hardy 1998). The elution system was chloroform-butane-ethanol (50:50:1, v/v/v) saturated with distilled water. Clear separation of testosterone and androstenediol in this system was confirmed using radiolabeled steroids (Ge & Hardy 1998). The recovery rates following extraction and column separation were $75.6 \pm 4.2\%$ for testosterone and $87.1 \pm 2.1\%$ for DIOL, and these were used to correct the final concentration of testosterone and DIOL measured by RIA. Separated medium testosterone and androstenediol concentrations were measured with a tritium-based RIA as previously described (Cochran *et al.* 1981). Inter-assay and intra-assay variations of the androgen were 7–8%.

Statistics

All data are expressed as means $\pm$ S.E.M. Data were subjected to analysis by one-way ANOVA test followed by ad hoc Tukey multiple comparisons to identify significant differences between the tested group and the control. Normalized signal values of microarray data were

transformed to the log base 10, and an ANOVA analysis was performed. Differences were regarded as significant at $P < 0.05$.

Results

PDGF-BB stimulates androgen production in immature Leydig cells

Immature Leydig cells are the cells at the intermediate stage during pubertal development of Leydig cells (Ge & Hardy 1997, 1998), and they primarily produced androstenediol (Ge & Hardy 1998). We investigated the effects of PDGF-BB on androgen production of rat immature Leydig cells. As shown in Fig. 1, PDGF-BB stimulated basal production of testosterone (Fig. 1A) or androstenediol (Fig. 1B) or testosterone plus androstenediol at 10 ng/mL (Fig. 1C). However, PDGF-BB did not affect the androgen production of rat immature Leydig cells under the stimulation of LH and 22R (Fig. 1D, E, F, G, H and I).

To dissect whether PDGF-BB exerts its action via PDGFRB, we used a PDGFRB inhibitor, PKI. 20 nM PKI was used because it selectively inhibited PDGFRB ($IC_{50} = 4.2$ nM) over PDGFRA ($IC_{50} = 45$ nM) (Ho *et al.* 2005). PKI (20 nM) alone did not affect androgen production of immature Leydig cells. However, it reversed PDGF-BB-stimulated testosterone (Fig. 2A), androstenediol (Fig. 2B)

Figure 1

Effects of PDGF-BB on basal, LH- and 22R-hydroxycholesterol (22R)-stimulated androgen production in rat immature Leydig cells. Immature Leydig cells were cultured without (basal) or with 10 ng/mL LH (LH-stimulated) or 2 μ M 22R for 24 h. Panel A, B and C, basal condition; panel D, E and F, LH-stimulated condition; panel G, H and I, 22R-stimulated condition. Mean $\pm$ S.E.M., $n = 8$. *, ** indicate significant difference at $P < 0.05$ and $P < 0.01$, respectively, when compared to the control (PDGF-BB, 0 ng/mL).

Figure 2
Effects of PDGF-BB and PDGFRB tyrosine kinase inhibitor IV (PKI) on basal androgen production in rat immature Leydig cells. Leydig cells were cultured in the basal condition with PKI (20 nM) or PDGF-BB (10 ng/mL), or both (20 nM PKI + 10 ng PDGF-BB, PKI + PDGF-BB) for 24 h. Panel A, testosterone (T); panel B, androstenediol (DIOL); panel C, T + DIOL. Mean $\pm$ S.E.M., $n=8$. Identical letters between two groups indicate no significant difference at $P<0.05$.

Figure 3
Effects of PDGF-BB and PDGFRB tyrosine kinase inhibitor IV (PKI) on thymidine incorporation into rat immature Leydig cells. Immature Leydig cells were cultured in the basal condition with PKI (20 nM) or PDGF-BB (1 ng/mL P1; 10 ng/mL, P10), or both (20 nM PKI + 10 ng PDGF-BB, PKI + P10) for 24 h. Mean $\pm$ S.E.M., $n=3$. Identical letters between two groups indicate no significant difference at $P<0.05$.

and testosterone plus androstenediol (Fig. 2C). This indicates that PDGF-BB exerts its action via PDGFRB.

PDGF-BB stimulates Leydig cell DNA synthesis

Previous studies showed that immature Leydig cells were capable of proliferating (Ge & Hardy 1997). PDGF-BB is also a possible growth factor to stimulate cell proliferation (Odeh *et al.* 2014). We measured immature Leydig cell DNA synthesis using ³H-thymidine incorporation. As shown in Fig. 3, PDGF-BB (10 ng/mL) stimulated thymidine incorporation. To dissect whether PDGF-BB exerts its action via PDGFRB, we treated the cells with PKI alone or together with PDGF-BB (10 ng/mL). PKI alone did not affect thymidine incorporation, but it reversed PDGF-BB-stimulated thymidine incorporation (Fig. 3). This indicates that PDGF-BB exerts its proliferation-stimulating action via PDGFRB.

Microarray analysis reveals PDGF-BB-mediated Leydig cell steroidogenesis

We examined the effects of PDGF-BB on Leydig cell gene expression levels using microarray analysis of transcriptome. We analyzed a genome-wide expression containing 21,910 probes, of these probes, 9466 probes were detected in the control (2 fold about the noise background). Of 9466 probes, 10 ng/mL PDGF-BB treatment

upregulated 197 gene expression by ≥ 2 fold, in which 57 genes were also upregulated by 1 ng/mL PDGF-BB. Of the increased genes, we found that *Star* was significantly increased by 1.9- and 2.1-fold by 1 and 10 ng/mL PDGF-BB, respectively. 10 ng/mL PDGF-BB decreased 238 gene expression by ≥ 2 fold, in which 58 genes were also decreased by 1 ng/mL PDGF-BB. Of decreased genes, *Akr1c9* and *Amhr2*, which are biomarkers in the earlier stage of Leydig cells and significantly reduced during the maturation of immature Leydig cells were decreased by ≥ 2 fold.

RT-qPCR verification

To validate microarray gene analysis, we used RT-qPCR verified the gene expression levels and specifically examined the effects of PDGF-BB on the expression levels of steroidogenesis-related and proliferation-related genes in rat immature Leydig cells, including *Lhcgr*, *Scarb1*, *Star*, *Cyp11a1*, *Hsd3b1*, *Cyp17a1*, *Hsd17b3*, *Srd5a1*, *Akr1c9* and

Sult1a1 in different sets of samples. We also analyzed Leydig cell maturation biomarker *Insl3* and cell proliferation biomarker *Ccnd1*. As shown in Fig. 4, PDGF-BB upregulated *Star*, *Hsd17b3* and *Insl3* expression levels at ≥ 1 ng/mL. At 1 ng/mL PDGF-BB also increased expression of *Cyp11a1* and *Srd5a1*, but it did not increase their expression levels at 10 ng/mL. PDGF-BB at the higher concentration (10 ng/mL) significantly increased *Ccnd1* expression level. PDGF-BB concentration-dependently decreased the expression levels of *Akr1c9* and *Sult1a1* but did not affect *Scarb1*, *Hsd3b1* and *Cyp17a1* expression levels. These data were in agreement with the changes of these genes in the microarray dataset. For example, in RT-qPCR data, 1 and 10 ng/kg PDGF-BB increased expression of *Star* by 2.78- and 2.45-fold, respectively while in microarray data they did by 1.9- and 2.1-fold, respectively. In RT-qPCR data, 1 and 10 ng/kg PDGF-BB decreased the expression of *Akr1c9* by 4- and 7.7-fold, respectively while in microarray data they did by 2- and 6-fold, respectively. These data indicate that PDGF-BB specifically regulates some gene expression levels.

Figure 4

Effects of PDGF-BB on gene expression levels in rat immature Leydig cells. Immature Leydig cells were cultured in the basal condition with PDGF-BB (1 and 10 ng/mL) for 24 h. Three sets of genes were selected: genes with increased expression (*Star*, *Hsd17b3*, *Insl3*, *Cyp11a1*, *Srd5a1*, and *Ccnd1*), and genes with reduced expression (*Akr1c9*, *Sult1a1*, and *Lhcgr*) as well as unchanged genes (*Scarb1*, *Hsd3b1*, and *Cyp17a1*). Mean $\pm$ S.E.M., $n=4$. *, **, *** indicate significant difference at $P<0.05$, $P<0.01$, and $P<0.001$, respectively, when compared to the control (PDGF-BB, 0 ng/mL).

Figure 5

Effects of PDGF-BB on protein expression levels in rat immature Leydig cells. Immature Leydig cells were cultured in the basal condition with PDGF-BB (1, 10 ng/mL) for 24 h. LHCGR, STAR, and HSD17B3 protein levels were measured by Western blot. Panel A, Western blot images; panel B, C and D, quantitative data. Mean $\pm$ S.E.M., $n=4$. *, *** indicate significant difference at $P<0.05$ and $P<0.001$, respectively, when compared to the control (PDGF-BB, 0 ng/mL).

Effects of PDGF-BB on Leydig cell proteins

We examined some specific protein (LHCGR, STAR and HSD17B3) levels in Leydig cells after PDGF-BB treatment (Fig. 5). We found that STAR ($P<0.001$) and HSD17B3 ($P<0.05$) levels were significantly increased by 8.4-fold and 2.9-fold, respectively, at ≥ 1 ng/mL PDGF-BB, and LHCGR level was significantly decreased by 2-fold at 10 ng/mL PDGF-BB ($P<0.05$). This was in agreement with the trend of their mRNA levels (e.g., *Lhcgr* was decreased by 2.4 fold by 10 ng/mL PDGF-BB).

Major pathways of PDGF-BB

We presented the genes with ≥ 4 -fold alteration in Table 1 (increased expression of genes) and Table 2 (decreased expression of genes). These genes were listed according to the GO analysis for biological pathway by Gene MicroArray Pathway Profiler 2.1. The increased expression of genes include *cell structure proteins* (*Myo1b*, *Sgca*, *Add2*), *extracellular matrix proteins* (*Lsmp*, *Col8a1*, *Mmp3*, *Igsf4c*), *growth factors and their receptors* (*Npy1r*, *Il1r1*, *Igf1r*, *Ucn2*, *Ednrb*, *Adora2b*, *Fst*, *Inhba*, *Gal*, *Gap43*, *Npw*), *intracellular signaling proteins* (*Phlda1*, *Wisp1*, *Serpinb2*, *Arhgdg*, *Rgs4*, *Egr1*, *Dusp6*, *Serinc2*, *Egr2*, *Ier2*, *Sh2b2*, *Dclk2*), *metabolism-related proteins* (*Upp1*, *Usp13*, *Cyp24a1*, *Akr1c18*, *Cpe*, *Rrm2*, *Enpp1*, *Ptgis*, *Serpine1*, *Mthfd1l*, *Car2*), *transcription factors* (*Maf*, *Runx1*, *Etv4*, *Hmga1*, *Fos*), *transporters* (*Slc17a1*, *Slc1a5*, *A2m*). The decreased genes

include *cell structure proteins* (*LOC287167*, *Eln*), *growth factors and their receptors* (*Lgals5*, *Ccl20*, *Cfb*, *Cxcl11*, *Cxcl10*, *Cxcr7*, *Amhr2*, *Ccl12*, *Ccl11*, *Il18bp*), *intracellular signaling proteins* (*Pla2g2a*, *Ifit1*, *Ifit3*, *Tnmt2*, *Ubd*, *Omd*, *Gzmb*, *Defb52*, *Gbp5*, *Mx2*, *Mx1*, *Rgnef*, *Slfn3*), *metabolism-related proteins* (*Akr1c1*, *Akr1c9*, *Oas1a*, *Maob*, *Enpp2*, *Sult1a1*, *Cyp4f4*).

Using GenMAPP2, we discovered several pathways that were specific to the regulation of PDGF-BB. As shown in Fig. 6 for steroidogenesis pathway, the expression level of *Star* was significantly increased and the expression levels of *Akr1c9* and *Sult1a1* were significantly decreased. The expression levels of other steroidogenic enzymes were not changed (Supplementary Table 2).

The expression levels of cholesterol biosynthesis pathway (*Hmgcs1*, *Idil*, *Fdps*, *Sqle*, *Cyp51*, *Sc4mol*, and *Dhcr7*) were decreased by 2–4 fold (Fig. 7). This indicates that after PDGF-BB treatment the de novo cholesterol biosynthesis is reduced.

Discussion

In the present study, we found that PDGF-BB positively regulated androgen production of rat immature Leydig cells by increasing the expression of *Star* and *Hsd17b3*, and decreasing the expression of *Akr1c9* and *Sult1a1*. This finding is novel.

Previous studies demonstrated that all cells in the Leydig cell lineage had PDGFRB (Ge et al. 2006,

Table 1 The expression levels of genes that were increased by PDGF-BB.

Gene symbol	Gene name	PDGF-BB (ng/mL)		
		0	1	10
Structure protein				
Myo1b	Myosin Ib	9±1	14±3 (2)	47±4 (5)*
Sgcg	Sarcoglycan gamma	7±2	14±2 (2)	33±2 (5)*
Add2	Adducin 2	7±1	12±3 (2)	26±3 (4)*
Extracellular matrix				
Lsmp	Limbic system-associated membrane protein	4±2	14±1 (3)	40±7 (10)*
Col8a1	Procollagen type VIII α1	12±4	31±6 (3)	96±6 (8)*
Mmp3	Matrix metalloproteinase 3	996±63	1625±38 (2)	5046±69 (5)*
Igsf4c	Immunoglobulin superfamily, member 4C	29±2	34±1 (1)	127±6 (4)*
Growth factors and receptors				
Npy1r	Neuropeptide Y receptor Y1	1±2	4±3 (4)	50±6 (50)*
Il1rl1	Interleukin 1 receptor-like 1	47±12	190±16 (4)	557±18 (12)*
Igf1bp3	Insulin-like growth factor binding protein 3	188±29	310±8 (2)	1104±25 (7)*
Ucn2	Urocortin 2	5±4	17±2 (3)	37±3 (7)*
Ednrb	Endothelin receptor type B	301±5	588±18 (2)	1539±34 (5)*
Adora2b	Adenosine A2B receptor	21±2	40±5 (2)	73±3 (4)*
Fst	Follistatin	37±11	31±4 (1)	148±7 (4)*
Inhba	Inhibin beta-A	14±2	23±2 (2)	57±1 (4)*
Gal	Galanin	22±3	37±3 (2)	89±3 (4)*
Gap43	Growth associated protein 43	69±2	96±1 (1)	274±7 (4)*
Npw	Neuropeptide W	39±3	46±2 (1)	142±4 (4)*
Intracellular signaling				
Phlda1	Pleckstrin homology-like domain family A1	3±1	7±2 (2)	39±2 (15)*
Wisp1	WNT1 inducible signaling pathway protein 1	5±2	12±2 (2)	84±2 (15)*
Serpinb2	serine peptidase inhibitor, clade B, member 2	43±4	66±5 (2)	314±8 (7)*
Arhgdig	Rho GDP dissociation inhibitor (GDI) gamma	20±1	49±2 (2)	141±5 (7)*
Rgs4	Regulator of G-protein signaling 4	43±12	97±5 (2)	267±20 (6)*
Egr1	Early growth response 1	475±27	737±33 (2)	2859±60 (6)*
Dusp6	Dual specificity phosphatase 6	111±2	126±3 (1)	614±20 (6)*
Serinc2	Serine incorporator 2	117±12	214±6 (2)	557±15 (5)*
Egr2	Early growth response 2	317±29	301±7 (1)	1466±33 (5)*
Ier2	Immediate early response 2	297±17	442±11 (2)	1202±17 (4)*
Sh2b2	SH2B adaptor protein 2	34±1	78±4 (2)	133±4 (4)*
Dclk2	Doublecortin-like kinase 2	18±4	44±2 (2)	67±4 (4)*
Metabolism				
Upp1	Uridine phosphorylase 1	764±64	1430±26 (2)	4332±131 (6)*
Usp13	Ubiquitin specific protease 13	9±1	38±4 (4)	50±2 (5)
Cyp24a1	Cytochrome P450, subfamily 24	8±2	18±3 (2)	44±3 (5)*
Akr1c18	Aldo-keto reductase family 1, member C18	9±1	25±2 (3)	44±1 (5)*
Cpe	Carboxypeptidase E	12±2	34±5 (3)	52±4 (4)
Rrm2	Ribonucleotide reductase M2	168±11	289±10 (2)	640±38 (4)*
Enpp1	Phosphodiesterase 1	532±121	1324±48 (2)	2000±37 (4)*
Ptgis	Prostaglandin I2	525±68	1133±88 (2)	2170±72 (4)*
Serpine1	Serine peptidase inhibitor, clade E, member 1	323±28	504±12 (2)	1195±25 (4)*
Mthfd1l	Methylenetetrahydrofolate dehydrogenase 1	46±11	107±4 (2)	165±4 (4)*
Car2	Carbonic anhydrase II	156±2	200±7 (1)	550±1 (4)*
Transcription factors				
Maf	Monocyte to macrophage differentiation	117±7	213±6 (2)	810±16 (7)*
Runx1	Runt related transcription factor 1	85±10	202±17 (2)	511±10 (6)*
Etv4	PREDICTED: ets variant gene 4	6±2	8±1 (1)	35±5 (5)*
Hmga1	High mobility group AT-hook 1	23±6	43±2 (2)	117±1 (5)*
Fos	FBJ murine osteosarcoma viral oncogene	66±1	102±3 (2)	315±17 (5)*
Bcl2	Bcl2, bcl-2-like	54±8	102±2 (2)	191±10 (4)*
Transporters				
Slc17a1	Solute carrier family 17	12±2	21±3 (2)	56±1 (5)*
Slc1a5	Solute carrier family 1	128±10	196±6 (2)	504±23 (4)*
A2m	Alpha-2-macroglobulin	19±3	32±2 (2)	70±1 (4)*
Unknown proteins				
RGD1560268	Similar to AT motif-binding factor	4±2	19±2 (6)	25±1 (7)*
RGD1562464	Similar to hypothetical protein A030011M19	6±2	6±2 (1)	31±1 (5)*
LOC498829	PREDICTED: Ab2-143	262±92	718±38 (3)	1038±38 (4)*
RGD1562920	PREDICTED: similar to Aig1 protein	163±12	252±7 (2)	596±7 (4)*
RGD1564216	PREDICTED: similar to Myoferlin	41±4	64±6 (2)	152±3 (4)*
RGD1305534	PREDICTED: similar to C21ORF5	17±2	33±4 (2)	64±3 (4)*

() Fold changes compared to 0 ng/PDGF-BB.

*Indicates significant difference between 1 and 10 ng/mL PDGF-BB groups at $P < 0.05$.

Table 2 The expression levels of genes that were decreased by PDGF-BB.

Gene symbol	Gene name	PDGF-BB (ng/mL)		
		0	1	10
Cell structure				
LOC287167	Globin, alpha	212±62	35±8 (–6)	7±1 (–27)*
Eln	Elastin	62±14	44±4 (–1)	12±1 (–5)*
Cytokines, growth factors and receptors				
Lgals5	Lectin, galactose binding, soluble 5	502±207	45±5 (–11)	23±1 (–21)*
Ccl20	Chemokine ligand 20	373±151	47±7 (–8)	21±3 (–17)*
Cfb	Complement factor B	4882±1595	1103±88 (–4)	326±11 (–15)*
Cxcl11	Chemokine ligand 11	1926±976	244±13 (–8)	110±14 (–7)*
Cxcl10	Chemokine (–C–X–C motif) ligand 10	1921±748	598±114 (–3)	344±4 (–6)*
Cxcr7	Chemokine (–C–X–C motif) receptor 7	306±20	196±8 (–1)	59±4 (–5)*
Amhr2	Anti-Mullerian hormone receptor 2	80±20	27±2 (–3)	15±6 (–5)*
Ccl12	Chemokine (–C–C motif) ligand 12	116±52	35±1 (–3)	25±3 (–4)*
Ccl11	Chemokine (–C–C motif) ligand 11	542±118	220±9 (–2)	122±9 (–4)*
Il18bp	Interleukin 18 binding protein	196±53	89±14 (–2)	45±3 (–4)*
Intracellular signaling				
Pla2g2a	Phospholipase A2, group IIA	1969±809	246±4 (–4)	24±3 (–79)*
Ifit1	IFN-induced/tetratricopeptide repeats1	356±113	129±38 (–3)	25±2 (–14)*
Ifit3	IFN-induced/tetratricopeptide repeats 3	257±106	76±21 (–3)	19±1 (–13)*
Tnnt2	Troponin T type 2	165±69	25±7 (–4)	16±3 (–10)*
Ubd	Ubiquitin D	2856±1274	638±93 (–4)	284±18 (–10)*
Omd	Osteomodulin	81±8	29±2 (–3)	8±1 (–9)*
Gzmb	Granzyme B	98±25	32±4 (–3)	11±3 (–8)*
Defb52	Defensin beta 52	156±73	20±3 (–7)	19±5 (–8)
Gbp5	Guanylate nucleotide binding protein 5	292±122	67±7 (–4)	37±3 (–8)*
Mx2	Myxovirus resistance 2	1265±517	374±25 (–3)	165±6 (–7)*
Mx1	Myxovirus resistance 1	1562±601	495±110 (–3)	293±11 (–5)*
Rgnef	Rho-guanine nucleotide exchange factor	89±12	44±2 (–2)	17±1 (–5)*
Slfn3	Schlafen 3	822±389	308±36 (–2)	199±10 (–4)*
Metabolism				
Akr1c11	Aldo-keto reductase 1, member C like1	62±9	43±3 (–1)	8±2 (–7)*
Akr1c9	3-Alpha-hydroxysteroid dehydrogenase	1507±338	657±24 (–2)	241±4 (–6)*
Oas1a	2'-5' Oligoadenylate synthetase 1A	190±71	57±9 (–3)	29±2 (–6)*
Maob	Monoamine oxidase B	74±9	50±6 (–1)	14±1 (–5)*
Enpp2	Ectonucleotide pyrophosphatase	1469±485	466±36 (–4)	356±19 (–4)
Sult1a1	Sulfotransferase family, cytosolic, 1A1	948±76	561±17 (–2)	232±3 (–4)*
Cyp4f4	Cytochrome P450, family 4, subfamily f4	82±14	48±2 (–2)	20±1 (–4)*
Unknown proteins				
LOC498795	Coiled-coil domain containing 3	92±24	39±5 (–2)	6±1 (–18)*
RGD1559565	EF hand domain containing 1	55±7	22±2 (–2)	4±2 (–13)*
LOC497812	Hypothetical gene supported	124±38	80±40 (–1)	13±2 (–9)*
RGD1564008	Similar to dapper 1	203±39	81±5 (–2)	26±2 (–7)*
RGD1563645	Similar to granzyme C	231±27	103±3 (–2)	38±3 (–6)*
RGD1309362	Similar to interferon-inducible GTPase	514±102	175±10 (–2)	89±9 (–6)*
LOC312688	Ubiquitin specific protease UBP43	121±53	55±8 (–2)	22±3 (–5)*
RGD1310093	Hypothetical protein FLJ11354	116±42	40±2 (–3)	22±5 (–5)*
LOC497841	Hypothetical gene NM_016994	145±34	76±6 (–2)	28±3 (–5)*
RGD1563091	Similar to OEF2	157±45	62±10 (–2)	33±4 (–5)*
Plac8_predicted	Placenta-specific 8	2103±494	1041±70 (–2)	475±11 (–4)*
LOC500297	Similar to env precursor	211±33	148±6 (–1)	49±3 (–4)*
RGD1561444	Similar to RIKEN cDNA 9530077C05	118±10	86±9 (–1)	29±4 (–4)*

() Fold changes compared to 0ng/mL PDGF-BB.

*Indicates significant difference between 1 and 10ng/mL PDGF-BB groups at $P < 0.05$.

Schmahl *et al.* 2008, Bergeron *et al.* 2011, Odeh *et al.* 2014). Using the knockout mice with Leydig cell specific knockout of PDGFRB, it was demonstrated that PDGFRB was important for maintenance of Leydig cell number

at adulthood (Schmahl *et al.* 2008). In the present study, we demonstrated that rat immature Leydig cells are responsive to PDGF-BB. During the maturation of immature into adult Leydig cells, the expression levels

Figure 6

Steroidogenesis pathway in at immature Leydig cells after PDGF-BB treatment. *Star* level was significantly increased by 2 fold. *Akr1c9* and *Sult1a1* levels were significantly decreased by 2-fold. PDGF0, PDGF1 and PDGF10 represent the doses of 0, 1 and 10 ng/mL.

of *Star* and androgen-biosynthetic enzymes (*Cyp11a1*, *Hsd3b1*, *Cyp17a1*, and *Hsd17b3*) are increased while the androgen-metabolizing enzymes (*Srd5a1*, *Akr1c9*, *Sult1a1*) are decreased (Ge & Hardy 1998, Ge et al. 2005, Zhang et al. 2013). Indeed, we found that after 24 h of treatment PDGF-BB concentration-dependently increased the expression levels of *Star* and *Hsd17b3* while decreased that of *Akr1c9* and *Sult1a1* (Fig. 6 and Tables 1 and 2).

The *Star* mRNA (Fig. 4) and STAR protein (Fig. 5) levels were remarkably increased. STAR mediates the rate-limiting step in the androgen biosynthesis in the Leydig cells, in which it serves the carrier to transport cholesterol, the substrate for steroidogenesis, from the Leydig cell mitochondrial outer membrane into the inner membrane (Zhou et al. 1991, Lemaire et al. 2006), where CYP11A1 cleaves the cholesterol side chain to form pregnenolone. There are many results to point to the crucial role of

STAR in regulating steroidogenesis (reviewed in Thomas et al. 1988). Many signaling pathways, including protein kinase A (PKA), protein kinase C, and nuclear receptor, are involved in the regulation of transcription of *Star* gene (reviewed in De Jong et al. 2002). The *Star* gene is transcriptionally regulated via the concerted action of multiple proteins that bind directly or indirectly to the DNA regulatory elements (Manna et al. 2003, 2004a,b). Transcriptional activation by cAMP signaling after LH-stimulation is mediated through the interaction of the cAMP-response element (CRE)-binding protein (CREB) with CRE-like elements including an overlapping CRE2/AP-1 motif (Fos/Jun) in the *Star* gene promoter because the promoter lacks the classic conserved CRE (TGACGTCA) (Manna et al. 2003, 2004a,b). The CREB/activating transcription factor (ATF) and Fos/Jun interact with themselves or each other to form selective dimers

Cholesterol Biosynthesis

Figure 7

Cholesterol biosynthesis pathway of immature Leydig cells after PDGF-BB treatment. Cholesterol biosynthesis genes (*Hmgcs1*, *Idi1*, *Fdps*, *Sqle*, *Cyp51*, *Sc4mol*, and *Dhcr7*) were decreased by 2–4 fold. PDGF0, PDGF1 and PDGF10 represent the doses of 0, 1 and 10 ng/mL.

that bind to closely related CRE and AP-1 sequences and induce transcriptional responses. CREB binds to DNA as a homodimer and it is activated after phosphorylation at Ser133 of CREB by several kinases including PKA (Clem et al. 2005). Fos members bind to DNA as heterodimers with Jun proteins (Wagner 2001). Expression of Fos is regulated by many extracellular signal factors. FOS has been demonstrated to bind to the CRE2/AP-1 motif alone transactivating basal *Star* gene transcription. In the present study, we demonstrated that *Fos* expression level was increased 2 fold and 5 fold by 1 and 10 ng/mL PDGF-BB, respectively (Table 1). This suggests that PDGF-BB increases *Star* expression via increasing the *Fos* expression. Interestingly, when PDGF-BB together with LH treatment was performed, androgen production was not changed. The exact mechanism is not unclear. We speculated that LH already maximally increased androgen

production, which was 5 fold compared to the basal level and this may mask the stimulatory effects of PDGF-BB.

We also found that the significant decrease of *Akr1c9* even at 1 ng/mL PDGF-BB. *Akr1c9* encodes AKR1C9 (3 α -hydroxysteroid dehydrogenase), which catalyzes the metabolism of dihydrotestosterone, a potent androgen, into androstenediol, a weak androgen. The expression level of *Akr1c9* is the highest in the progenitor Leydig cells and gradually declines and reaches the lowest level in adult Leydig cells (Shan et al. 1995, Ge & Hardy 1998). This indicates that PDGF-BB promotes the development of immature Leydig cells. The regulation of *Akr1c9* expression is far from clear in Leydig cells. A previous study demonstrated that both LH and androgen can increase *Akr1c9* expression (Shan et al. 1995). Interestingly, liver *Akr1c9* gene promote region contains AP-1 site (Lin & Penning 1995), whether FOS can regulate via this

site awaits for a further investigation in the future. In our study, we also found that *Sult1a1* was significantly reduced. SULT1A1 is an enzyme that catalyzes the sulfate conjugation of many steroid hormones, neurotransmitters, drugs, and xenobiotic compounds.

At the higher concentration (10 ng/mL), PDGF-BB also stimulated the proliferation of rat immature Leydig cells as evidence of increased incorporation of thymidine into these cells (Fig. 3). Interestingly, PDGF-BB is a potent stimulator of the mitosis of stem Leydig cells (Li et al. 2016). In the present study, the *Ccnd1* expression level was significantly increased by 10 ng/mL PDGF-BB, indicating that this growth factor can regulate the cell proliferation via increasing expression of CCND1. PDGF-BB has been reported to increase *Ccnd1* expression in mesenchymal stem cells for their proliferation (Gharibi et al. 2012).

There are not many studies about the effects of PDGF-BB on steroidogenic cells. One study showed that PDGF-BB inhibited the activity of SRD5A1 and HSD3B1 in cultured Leydig cells from (immature Leydig cells) 25-day-old rat testis (Murono & Washburn 1990), while another study showed it increased LH-stimulated T production *in vitro* (Loveland et al. 1993, Risbridger 1993). PDGF-BB also inhibited stem Leydig cell commitment into the Leydig cell lineage in rats by reducing testosterone production after the stem Leydig cells were cultured in the presence of LH and ITS (Odeh et al. 2014). However, in the present study, we clearly demonstrated that PDGF-BB alone stimulated androgen production in rat immature Leydig cells (Fig. 1). This indicates that the effects of PDGF-BB on Leydig cell development depending on the stages of Leydig cells. The pathway of PDGF-BB stimulated steroidogenesis is not clear until the present study, in which we demonstrated that it increased the expression levels of *Star* and *Hsd17b3*. PDGF-BB might have significant physiological action on Leydig cell development since Leydig cell conditional 1 knockout of *Pdgfrb* in mice led to a significant reduction of Leydig cell number in the fetal testis (Schmahl et al. 2008). However, the exact concentration of PDGF-BB in the pubertal rat testis is still not known. Furthermore study is required to identify its physiological level.

In conclusion, the present study demonstrated that PDGF-BB regulated the steroidogenesis of immature Leydig cells by increasing the expression levels of *Star* and steroidogenic enzyme gene *Hsd17b3*.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

The present study was supported by NSFC (81730042 to R S G, 81601266 to X L), Zhejiang Provincial NSFC (LY15H310008 to R S G).

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Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-17-0222>.

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Received in final form 19 November 2017

Accepted 24 November 2017