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Reproductive Health

Alterations of gene profiles in Leydig-cell-regenerating adult rat testis after ethane dimethane sulfonate-treatment

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Only occupying about 1%–5% of total testicular cells, the adult Leydig cell (ALC) is a unique endocrine cell that produces androgens. Rat Leydig cells regenerate after these cells in the testis are eliminated with ethane dimethane sulfonate (EDS). In this study, we have characterized Leydig cell regeneration and messenger ribonucleic acids (mRNA) profiles of EDS treated rat testes. Serum testosterone, testicular gene profiling and some steroidogenesis-related proteins were analyzed at 7, 21, 35 and 90 days after EDS treatment. Testicular testosterone levels declined to undetectable levels until 7 days after treatment and then started to recover. Seven days after treatment, 81 mRNAs were down-regulated greater than or equal to two-fold, with 48 becoming undetectable. These genes increased their expression 21 days and completely returned to normal levels 90 days after treatment. The undetectable genes include steroidogenic pathway proteins: steroidogenic acute regulatory protein, *Scarb1*, *Cyp11a1*, *Cyp17a1*, *Hsd3b1*, *Cyp11b1* and *Cyp2a1*. Seven days after treatment, there were 89 mRNAs up-regulated two-fold or more including *Pkib*. These up-regulated mRNAs returned to normal 90 days after treatment. *Cyp2a1* did not start to recover until 35 days after treatment, indicating that this gene is only expressed in ALCs not in the precursor cells. Quantitative polymerase chain reaction, western blotting and semi-quantitative immunohistochemical staining using tissue array confirmed the changes of several randomly picked genes and their proteins.

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INTRODUCTION

In rats and mice, Leydig cells are formed as two morphologically and functionally different generations. The first generation, called the fetal Leydig cell, develops in utero from undifferentiated stem Leydig cells (SLCs). In the rat, fetal Leydig cells develop from SLCs between the nascent testis cords, starting on day 12 of gestation, and after birth fetal Leydig cells involute and gradually disappear during puberty.¹ During the pubertal period, SLCs again undergo lineage-specific commitment and give rise to adult Leydig cell (ALC) generation.¹ ALCs, only occupying about 1%–5% of total testicular cells in the mature testis, are unique endocrine cells that produce testosterone for maintaining spermatogenesis and secondary sexual characteristics of males. These cells differentiate from SLCs during puberty.² The cells in the Leydig cell lineage (for example, cells that express 3 β -hydroxysteroid dehydrogenase 1 [3 β -HSD1], encoded by *Hsd3b1*), whose progeny give rise to ALCs, first become apparent by day 11 postpartum in the rat testis.^{3,4} These cells, the progenitor Leydig cells, are spindle-shaped cells that are identified by the expression of luteinizing hormone (LH)

receptor (LH chorionic gonadotropin [LHCGR], encoded by *Lhcgr*)⁵ and 3 β -HSD1.⁶ Progenitor Leydig cells have several testosterone biosynthetic enzymes, including P450 cholesterol side chain cleavage enzyme (CYP11A1, encoded by *Cyp11a1*), 3 β -HSD1 and P450 17 α -hydroxylase/17,20-lyase (CYP17A1, encoded by *Cyp17a1*), but they lack 17 β -HSD3 (17 β -HSD3, encoded by *Hsd17b3*). Because they have higher levels of steroid-metabolizing enzyme 5 α -reductase 1 (SRD5A1, encoded by *Srd5a1*) and 3 α -HSD (3 α -HSD, encoded by *Loc191574*),⁶ progenitor Leydig cells mainly secrete androsterone.⁶ Progenitor Leydig cells gradually enlarge, become round and their proliferative capacity is reduced.⁷ The next identified step in the transition of progenitor Leydig cells to ALCs is the appearance of another intermediate, immature Leydig cells, most commonly seen in the testis during day 35 postpartum.³ Immature Leydig cells are round-shaped and have abundant smooth endoplasmic reticulum. These cells contain cytoplasmic lipid droplets, and secrete high levels of 5 α - and rostanediol because they have 17 β -HSD3 and higher levels of *Srd5a1* and 3 α -HSD.⁶ ALCs are the largest cells among the three stages,

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with abundant smooth endoplasmic reticulum, few lipid droplets, high levels of steroidogenic enzyme activities, and high levels of testosterone secretion due to the shutdown of the expression of *Srd5a1*.⁶ ALCs are the major population of Leydig cells in the sexually mature testis. Testosterone metabolizing enzyme 7α -hydroxylase (CYP2A1, encoded by *Cyp2a1*) becomes expressed only in ALCs, not in their precursor cells in the lineage,⁸ thus ALCs also produce 7α -hydroxytestosterone. The orderly appearance of testosterone biosynthetic and metabolizing enzymes in the Leydig cell lineage becomes the unique biomarkers to identify its components, as well as in progenitor, immature and ALCs.

The developmental process of the ALC population can also be mimicked during the regeneration process after ethane dimethane sulfonate (EDS), an alkylating agent that specifically kills ALCs without affecting other testicular cell types.^{9,10} After one intraperitoneal injection of 70 mg kg⁻¹ of EDS, 4–7 days after the treatment all of the ALCs are destroyed.⁹ Three weeks after the treatment, newly formed Leydig cells with 3β -HSD positive staining could be observed within the interstitial area of the testis.¹¹ These newly regenerated Leydig cells are similar to progenitor Leydig cells, which gradually mature into immature Leydig cells 35 days after the treatment and further mature into ALCs after 3 months.¹¹ Although many genes have been identified in Leydig cells, to discover whether they are exclusively or abundantly expressed in Leydig cells is very laborious. One big problem in identifying Leydig cell gene expression in the testis is that over 95% of testicular cells are germ cells in the adult testis.¹² We identified messenger ribonucleic acids (mRNA) levels of Leydig cell-specific genes in the adult rat testis after ALCs were eliminated by EDS.

MATERIALS AND METHODS

Animals and treatments

Thirty-six 90-day-old Sprague-Dawley male rats were purchased from Charles River Laboratories (Wilmington, MA, USA). Thirty rats were injected with EDS and six rats were injected with vehicle control (dimethyl sulfoxide: H₂O, 1:3, *v/v*). Rats were injected intraperitoneally with 70 mg kg⁻¹ of EDS (a gift from Dr. Gray LE Jr. in EPA). Six rats per group per time point were euthanized by inhalation of CO₂ at 0 (0 day group, immediately after EDS injection), 7 (7 days group), 21 (21 days group), 35 (35 days group) and 90 (90 days group) days post-EDS treatment. The time frame is selected based on the previous observations. Seven days post-EDS, all ALCs are destroyed;¹³ 21 days post-EDS, newly formed 3β -HSD positive Leydig cells, which are similar to progenitor Leydig cells could be observed within the interstitial area of the testis;¹³ They gradually mature into immature Leydig cells 35 days after the treatment and further mature into ALCs after 90 days.¹³ Blood samples were collected, centrifuged at 500 × g, and the sera were stored at -20°C for radioimmunoassay (RIA) of testosterone. One testis from each animal was punched holes using a needle and fixed by Bouin's solution for immunohistochemical staining. The contralateral testis from each animal was freshly collected and stored at -80°C for subsequent microarray and quantitative real-time polymerase chain reaction (qPCR) analysis of mRNA levels and western blotting analysis of protein levels. All animal procedures were approved by the Institutional Animal Care and Use Committee of Rockefeller University.

Serum and testicular testosterone measurement

The extraction of testis steroids and the calculation of recovery rate of the extraction were performed as described.¹⁴ Serum and testicular testosterone concentrations were measured with a tritium-based RIA as previously described using testosterone from Sigma (St. Louis,

MO, USA).¹⁵ Inter-assay and intra-assay variations of the testosterone were 7%–8%.

Tissue array and immunohistochemistry

Perfused testes were assembled in five samples per time point in a tissue array and paraffin embedded and 6- μ m thick cross sections were cut, mounted, and dewaxed. The immunohistochemical staining for Leydig cell specific protein CYP11A1, 3β -HSD1 and 11β -HSD1 was performed according to the previous method.^{16–18} In brief, the sections were incubated in primary antibody polyclonal rabbit anti-CYP11A1 (RDI Research Diagnostics, Inc., Flanders, NJ, USA; dilution 1:1000), anti- 3β -HSD (Abcam, CA, USA, dilution 1:1000) and anti- 11β -HSD1 (inhouse made, dilution 1:1000), at 4°C overnight. The following morning the sections were washed in Tris-buffer for 5 min; incubated in secondary goat anti-rabbit antibody (Santa Cruz Biotech, CA, USA) 1:1000 diluted in Tris-buffer supplemented for 30 min; washed in Tris-buffer for 5 min and incubated with DAB/H₂O₂ solution for 10 min. They were then washed for 5 min in distilled H₂O and subsequently dehydrated in serial isopropyl alcohol baths and cover-slipped. In control experiments, sections were incubated with nonimmune rabbit IgG (CYP11A1, 3β -HSD1 and 11β -HSD1).

Determination of Leydig cell number

The above testis were captured using a Nikon Eclipse E800 microscope (Nikon, Inc., Melville, NY, USA) equipped with a 40× objective and a SPOT RT digital camera (model 2.3.0; Diagnostic Instruments Inc., Michigan, IN, USA) interfaced to a computer. The images that were displayed on the monitor represented areas of 0.9 mm² of testis. Interstitial cell numbers were estimated using image analysis software (Image-Pro Plus; Media Cybernetics, Silver Spring, MD, USA). Identification of CYP11A1, 3β -HSD1 and 11β -HSD1 positive cells was based on cytoplasmic staining with their respective antibodies. More than 1000 cells of each type were counted in each testis and adjusted by seminiferous diameters. The numerical index of each of the enumerated cell types was defined as the average number counted per mm² of cross-sectional area. The selection of CYP11A1 and 3β -HSD is based on their expression in Leydig cell lineage,⁶ and selection of 11β -HSD1 is based on its expression in immature and ALCs.¹⁹

Ribonucleic acids extraction

Total RNAs were extracted from five randomly selected testes by homogenization in TRIzol according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Briefly, Testes were homogenized in TRIzol reagent, followed by chloroform extraction. The aqueous supernatant containing the RNA was retained, and the RNA precipitated with isopropanol. RNA pellets were eluted with 70% ethanol, air dried and resuspended in RNase-free water and purified using RNeasy Kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). The RNA concentration and integrity in individual RNA samples were determined using a NanoDrop 2000 spectrophotometer (Fisher Scientific, NJ, USA) and Agilent 2100 bioanalyzer (Santa Clara, CA, USA).

Microarray hybridization and scanning

The RatRef-12 Expression BeadChips for genome-wide expression from Illumina Inc., (San Diego, CA, USA) were used. Each BeadChip contains 21 910 genes that are selected primarily from the NCBI RefSeq database covering the whole rat transcriptome. Probe labeling, hybridization, washing, and scanning were performed according to the manufacturer's instructions using the Illumina Total Prep kit (Applied Biosystems, Foster City, CA, USA) as reported.²⁰ First

strand cDNA was synthesized in a total volume of 20 μ l with the supplied reagents. The complete first strand product was used for 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 during this step, resulting in a biotinylated complementary RNA (cRNA) probe. Probe integrity was verified using the Agilent 2100 bioanalyzer. Labelled cRNA (750 ng) was hybridized to the array overnight at 58°C in a total volume of 30 μ l of hybridization buffer, followed by posthybridization stringency washing and scanning.

Microarray data analysis

Scanned microarray expression data were imported into BeadStudio (Illumina, San Diego, CA, USA) for normalization, preliminary analysis and filtering. Average normalization without background subtraction was used, 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) on each array and to call differentially expressed genes at each of the developmental stages (defined as $P < 0.05$ after false discovery rate correction). Normalized data from BeadStudio was filtered to exclude genes not expressed in the testis (i.e. data from probes that were classed as "absent" in all samples). Of the 21 910 genes were present in the data based on which further analyses were carried out. In BeadStudio, linear plots compared between groups were made, and Heatmap profiling was generated. The data were further imported into Microsoft Access 2007 (Microsoft Corporation, Redmond, WA, USA), and queries were generated to find Leydig cell-specific genes.

Biological pathway analysis

For the characterization of the biological processes, Gene MicroArray Pathway Profiler 2.1 (GenMAPP2.1, San Francisco, CA, USA) software was used to produce lists of significantly ($P < 0.05$) regulated pathway and GenMAPP2.1 was used to create a map of signal 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 qPCR.

Quantitative real-time polymerase chain reaction

Levels of specific mRNA species were measured by qPCR using the SYBR method following reverse transcription of isolated RNA. Briefly, first strand synthesis and qPCR were performed as described previously.²¹ qPCR was carried out in the 20- μ l volume using a 96-well plate format using the SYBR Green PCR Core Reagents purchased from Applied Biosystems (Foster City, CA, USA). Primer titration was performed with the concentration of 300 nmol L⁻¹. Fluorescence was detected using an ABI 7700 system (PE Applied Biosystems, Carlsbad, CA, USA). Each sample was run in duplicate and in parallel with no template controls. The relative mRNA levels of targeted genes were normalized to ribosomal protein S16 (*Rps16*, internal control gene) by using a standard curve method. All primers in this 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: 11 β -HSD1 (*Hsd11b1*), *Cyp2a1*, fatty acid binding protein 3 (*Fabp3*), *Hsd3b1*, proteolipid protein (*Plp*), sugar-phosphate/phosphate exchanger family (*Slc37*). The primers used are described in Supplemental Table 1.

Western blot analysis

Testes were homogenized and boiled in equal volumes of sample loading buffer, a Tris-Cl buffer (pH 6.8), containing 20% glycerol,

5% sodium dodecyl sulfate, 3.1% dithiothreitol, and 0.001% bromophenol blue. Homogenized samples (50 μ g protein) were electrophoresed on 10% polyacrylamide gels containing sodium dodecyl sulfate. Proteins were electrophoretically transferred onto nitrocellulose membranes, and after 1 h exposure to 5% nonfat milk to block nonspecific binding, the membranes were incubated with a rabbit polyclonal anti-11 β -HSD1 (custom antibody, dilution 1:1000),¹⁸ 3 β -HSD1 (Abcam, San Francisco, CA, USA, dilution 1:2000), CYP11A1 and FABP3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA dilution 1:1000), CYP11A1 and steroidogenic acute regulatory protein (STAR) (kindly donated by Dr. D.M. Stocco, Texas Tech Health Sciences Center, Lubbock, TX, USA, dilution 1:1000 ~ 5000), and actin β (ACTB, Cell signaling technology, Danvers, MA, USA, dilution 1:1000) antibodies. The membranes were then washed and incubated with a 1:5000 dilution of goat anti-rabbit antiserum that was conjugated to horseradish peroxidase. The washing step was repeated, and immunoreactive bands were visualized by chemiluminescence using a kit (ECL, Amersham, Arlington Heights, IL, USA).

Statistical analysis

The data were analyzed by one-way analysis of variance followed by planned comparisons with the Sidak adjustment of the P value, as an estimation of the experimental error rate. All data are expressed as means \pm standard error of the mean (s.e.m.). Differences were regarded as significant at $P < 0.05$.

RESULTS

Regeneration of Leydig cells and testosterone levels after ethane dimethane sulfonate treatment

The testis weights of rats before and 7, 21, 35, and 90 days after EDS treatment were 1.199 \pm 0.028 (mean \pm s.e.m.), 1.102 \pm 0.046, 0.820 \pm 0.015, 0.992 \pm 0.052, and 1.110 \pm 0.035 g, respectively. Compared to the pretreatment value, the testis weights of rats 21 and 35 days after EDS treatment were reduced ($P < 0.01$), while those at day 7 and 90 post-EDS were not significantly changed. Leydig cells were identified by the histochemical staining of CYP11A1. Seven days after EDS treatment, there were no Leydig cells present in the rat testis (Figure 1b) compared to normal 90-day-old rat testis which showed many ALCs (Figure 1a). Twenty-one days post-EDS treatment, some spindle-shaped progenitor Leydig cells were formed (Figure 1c), and at day 35 many immature Leydig cells were formed (Figure 1d). At day 90, Leydig cells were fully regenerated (Figure 1e). In the tissue array, Leydig cell specific proteins CYP11A1, 3 β -HSD1 and 11 β -HSD1 were further immunohistochemically stained and their positive cells were counted. The changes of Leydig cell numbers containing its specific protein CYP11A1, 3 β -HSD1 and 11 β -HSD1 per testis were shown in Figure 2. At days 21 and 35, *Cyp11a1* and 3 β -HSD1 positive cells were regenerated to about 16% and 35% of normal levels, respectively, while 11 β -HSD1 positive cells were delayed to recover, indicating that this protein is expressed in more mature stage of Leydig cell lineage. Since testosterone is synthesized primarily by Leydig cells in the testis, 7 days post-EDS treatment, serum and testicular testosterone levels decreased to almost undetectable levels (Figure 3a and 3b). Serum and testicular testosterone levels gradually increased from day 21 to 90 (Figure 3a and 3b). The almost undetectable serum and testicular testosterone level at day 7 was correlated with the disappearance of ALCs. The increase of testosterone level mirrors the appearance of CYP11A1-positive and 3 β -HSD1-positive cells.

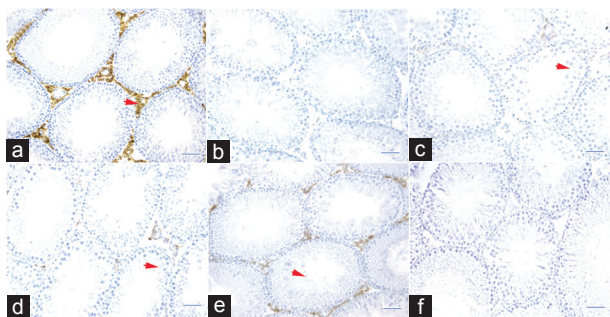


Figure 1: Immunohistochemical staining of CYP11A1 protein in Leydig cells after ethane dimethane sulfonate (EDS) treatment. (a) day 0 (insert is the high magnification); (b) day 7; (c) day 21; (d) day 35; (e) day 90 after EDS treatment; (f) Pre-immune control staining. Bars = 50 μ m. Brown staining of CYP11A1 indicates Leydig cells (red arrowhead).

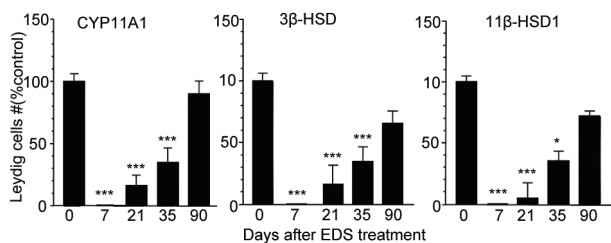


Figure 2: Cell number by staining with CYP11A1, 3 β -HSD and 11 β -HSD1 after ethane dimethane sulfonate (EDS) treatment. The asterisk denotes a difference at * $P < 0.05$ and *** $P < 0.001$ compared to control (0 day after EDS).

Profiling of gene changes after ethane dimethane sulfonate treatment

The Illumina RatRef-12 expression BeadChips was used to determine the changes of mRNA expression levels before (0 day after EDS), 7, 21, 35 and 90 days post-EDS treatment. A genome-wide expression containing 21,910 probes were analyzed. Of these probes, 15,973 probes were detected in the control group. The heatmap showed the expression changes after EDS treatment (Figure 4). The heatmap lists three subsets of gene profiles, with the first and second sets of genes whose levels were higher and went to undetectable levels 7 days post-EDS treatment and reappeared at day 21, as well as the third set of genes whose levels were lower and went up at day 7 and then fell back to normal levels at day 21. We further compared the gene changes between the 0 and 7 day group. We identified 129 genes that were down-regulated two-fold or more, among them 81 gene expressions being undetectable or barely detectable (Supplemental Table 2) and 48 genes being down-regulated ($P < 0.05$, Supplemental Table 3). Of 81 genes that were undetectable or barely detectable, the expression levels of 48 genes were undetectable (below or near negative control base line, Figure 5a, blue dots). Of these 129 genes, 88%, 91%, 95% were recovered at 21, 35 and 90 days post-EDS treatment, respectively (Figure 5b–5d, blue dots). We also identified 89 (6 known and 81 unknown) genes that were up-regulated two-fold or more at day 7 (Figure 5a, red dots and Supplemental Table 4). Of these 89 genes, 87%, 91%, and 99% were recovered at 21, 35 and 90 days post-EDS treatment, respectively (Figure 5b–5d, red dots). Twenty one days post-EDS treatment, there were also 17 newly up-regulated genes (Figure 5b, cyan dots and Supplemental Table 5). At day 35, there were also 26 newly up-regulated genes compared to 21 days group (Figure 5c, black dots and Supplemental Table 6). These up-regulated genes returned to control levels at day 90. Thirty five days

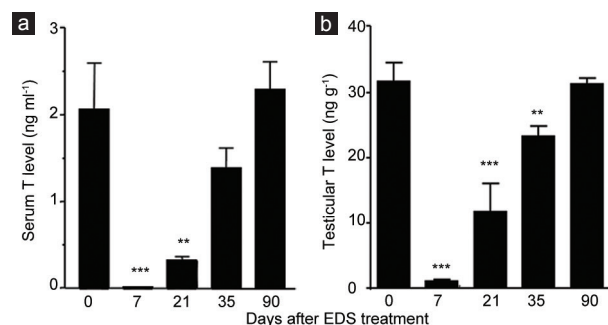


Figure 3: Serum and testicular testosterone (t) level after ethane dimethane sulfonate (EDS) treatment. (a) serum T; (b) testicular T. The asterisk denotes a significant difference at ** $P < 0.01$ and *** $P < 0.001$ compared with control (0 day after EDS).

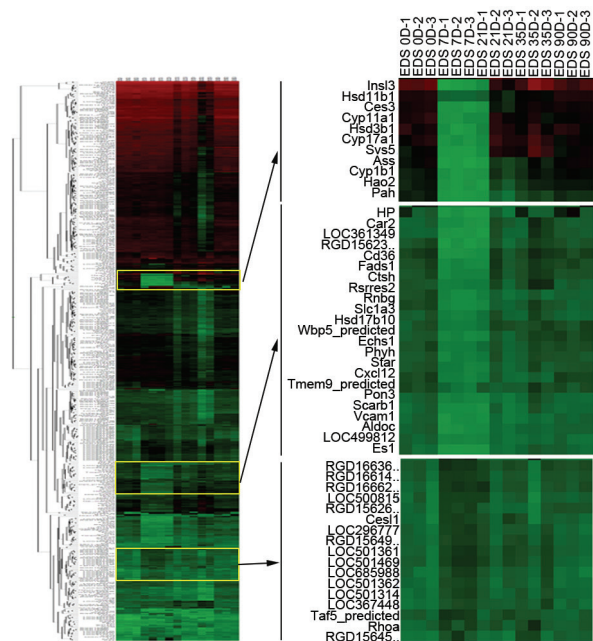


Figure 4: Heatmap analysis of gene profiling in the testes collected before and after ethane dimethane sulfonate (EDS) treatment. Testes were collected at 0 (EDS 0), 7 (EDS 7), 21 (EDS 21), 35 (EDS 35) and 90 (EDS 90) days after EDS treatment. Each group has three samples, designated by “–1, –2 and –3.”

after EDS, we observed many genes were down-regulated more than two-fold, and these genes returned to control levels 90 days post-EDS treatment (Figure 5d, lime green dots).

Major pathways in adult Leydig cells

Using GenMAPP 2 and MAPPFinder 2, we discovered several metabolic pathways that were specific to ALCs. As shown in Figure 6a and Supplemental Table 2, steroidogenesis-related genes (*Cyp11a1*, *Hsd3b1*, *Cyp17a1*, *Cyp2a1* and cytochrome 1 member b1 [*Cyp11b1*]) were exclusively found in ALCs. At day 7, all these genes were down-regulated to undetectable levels. Of these genes, only *Cyp2a1* was not restored until 90 days after EDS treatment (Figure 6a). We also observed that many metabolism-related genes were exclusively present in Leydig cells. One of the pathways of which was the mitochondrial fatty acid β -oxidation pathway (Figure 6b). Four genes (*Decr1*, *Drcl*, *Echs1*, *Acaa2*) were abundantly expressed in ALCs.

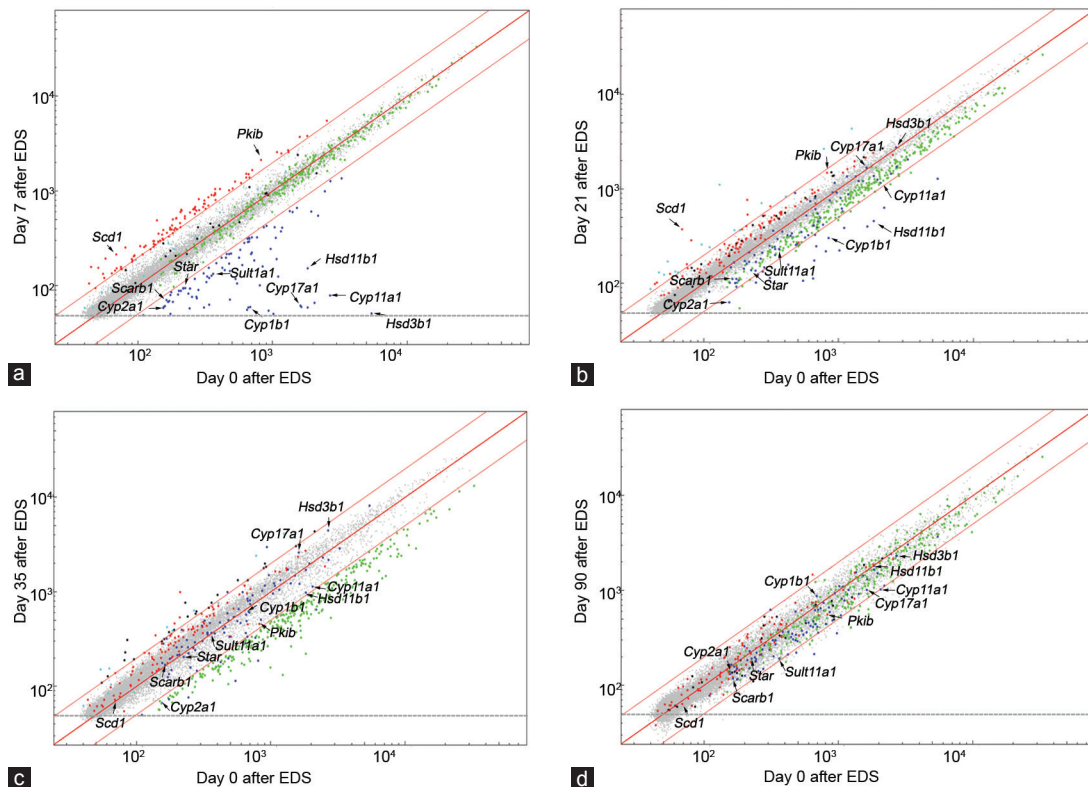


Figure 5: Linear plotting of gene profiles of testes after ethane dimethane sulfonate (EDS) treatment against 0 day group. (a) 7 days; (b) 21 days; (c) 35 days and (d) 90 days versus control (0 day after EDS). In panel (a–d) blue dots indicate genes that were down-regulated by greater than or equal to two-fold at day 7; red dots up-regulated by greater than or equal to two-fold at day 7; green dots down-regulated by greater than or equal to two-fold at day 90. At (b and c) cyan dots indicate genes that were up-regulated by greater than or equal to two-fold at day 21 compared to control (EDS 0); and dark dots indicate genes that were up-regulated by greater than or equal to two-fold at day 35 compared to 21 days group. Dashed line represents the noise line (which was shown by negative control).

Comparison of microarray with quantitative polymerase chain reaction data

We selected three sets of genes: exclusively expressed (*Hsd3b1*, *Cyp11a1*, *Cyp2a1*), abundantly expressed (*Fabp3*) and maybe equally expressed compared to other testicular cell types (*Slc37* and *Plp*) and performed qPCR analysis. We found similar trends of changes of these genes after EDS treatment (Figure 7), suggesting that the microarray analysis is reliable.

Identification of 3 β -HSD1, CYP11A1, STAR, 11 β -HSD1 and FABP3 proteins

We performed Western blotting to identify the protein changes of 3 β -HSD1, CYP17A1, STAR, 11 β -HSD1, and FABP3 in rat testes. As shown in Figure 8, all the proteins were parallel with changes of their mRNA levels. As indicated in gene expression levels FABP3 was barely detectable at day 7.

DISCUSSION

In the present study, the Leydig cell regeneration model was used to identify the genes exclusively or abundantly expressed in the Leydig cell lineage in rat testes. Several pathways were found to be exclusively or predominantly present in ALCs. The selection of the time frame for progenitor, immature and ALCs after Leydig cell regeneration post-EDS is based on the previous observations.^{8,11} Leydig cells were identified by the histochemical staining of CYP11A1. Seven days post-EDS, there were no Leydig cells present in the rat testis compared to normal 90-day-old rat testis as shown in the present study. Twenty-one days

post-EDS, some spindle-shaped progenitor Leydig cells were formed as shown by the staining of CYP11A1 and 3 β -HSD1 without 11 β -HSD1 staining, and at day 35 many immature Leydig cells were formed as shown by the staining of 11 β -HSD1 and CYP11A1 (in the present study, Figure 1d). At day 90, Leydig cells were fully regenerated because the expression of CYP2A1 was shown in our previous study.⁸

We detected 81 genes whose expression levels were undetectable and 48 genes whose expression levels were significantly down-regulated greater than or equal to two-fold in the rat adult testis 7 days post-EDS. Of these 129 genes, 14 genes or their protein products have been reported exclusively in rat ALCs by enzyme assay, immunohistochemical staining, and *in situ* hybridization performed by our and other laboratories. These 14 genes include vascular cell adhesion molecule 1,²² vitronectin (*Vtn*),²³ insulin-like 3,²⁴ platelet derived growth factor receptor α ,²⁵ carboxylesterase,²⁶ 7-dehydrocholesterol reductase,²⁷ epoxide hydrolase 2,²² *Cyp1b1*,²⁸ 17 β -HSD10 (*Hsd17b10*),²⁹ *Cyp2a1*,^{8,30} *Cyp11a1* (Figure 7) and *Cyp17a1*,^{31,32} *Hsd3b1*,³² and *Star*.³³ Another five genes have also been confirmed to be abundantly present in ALCs, including gap junction protein, alpha 1 (*Gja1*),³⁴ mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase,³⁵ peripheral-type benzodiazepine receptor,³⁶ *Hsd11b1*,¹⁸ and *Fabp3* (Figure 7). Using this approach, we identified several pathways that were predominantly present in rat ALCs. One confirmed pathway in the present and previous study is the testosterone biosynthetic pathway (*Cyp11a1*, *Hsd3b1*, *Cyp17a1*), which was exclusively present in ALCs. Our immunohistochemical staining using tissue array also confirmed that CYP11A1 and 3 β -HSD1 are exclusively located in Leydig

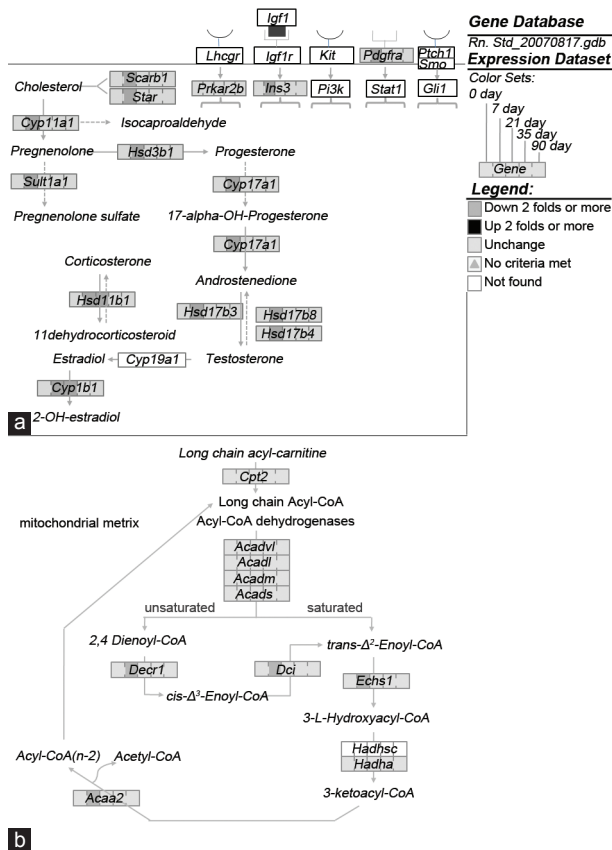


Figure 6: GenMAPP analysis of steroidogenic pathway (a) and mitochondrial fatty acid β -oxidation pathway (b) of rat testis after ethane dimethane sulfonate (EDS) treatment. Grey color shows down-regulation compared to control at $P < 0.05$ (0 day after EDS).

cells. The pathway was confirmed in the present study (Figures 5, 7 and 8) and previous studies.^{17,37} The levels of *Cyp2a1* were very low even at day 35 post-EDS treatment, indicating that this mRNA is exclusively expressed in ALCs and not in progenitor and immature Leydig cells. *Cyp2a1* has been shown to be exclusively present in Leydig cells and is involved in testosterone metabolism by 7α -hydroxylation.³⁰ We have identified that *Cyp2a1* was only present in mature ALCs in the Leydig cell lineages.⁸ It has been found that *Cyp2a1* disappeared day 7 and recovered until 90 days post-EDS treatment.⁸ *Cyp2a1* not only metabolizes testosterone, but also regulates 11β -HSD1 catalysis.⁸

We also identified that the gene expressions of enzymes for fatty acid β -oxidation (especially for unsaturated fatty acid) are predominantly or exclusively present in ALCs. Four enzymes (*Acad*, *Echs1*, *Hadh* and *Acaa2*) are responsible for long-chain saturated fatty acid oxidation. Among them, *Echs1* completely disappeared, and *Acaa2* was reduced to barely detectable level at day 7 (Figure 5a). Two additional enzymes (*Decr1* and *Dci*) are responsible for unsaturated acid oxidation, and both were reduced two-fold more. This indicates that fatty acid β -oxidation is required for energy in ALCs, which requires large amount of cofactor nicotinamide adenine dinucleotide phosphate for steroidogenesis. Fatty acid transporters including CD36 and FABP3 were predominantly expressed in ALCs, indicating that these proteins are very important for the transportation of fatty acids into ALCs for fatty acid β -oxidation.

The microarray analysis may not be able to completely detect the changes of all Leydig cell-specific gene expression. In this regard, in

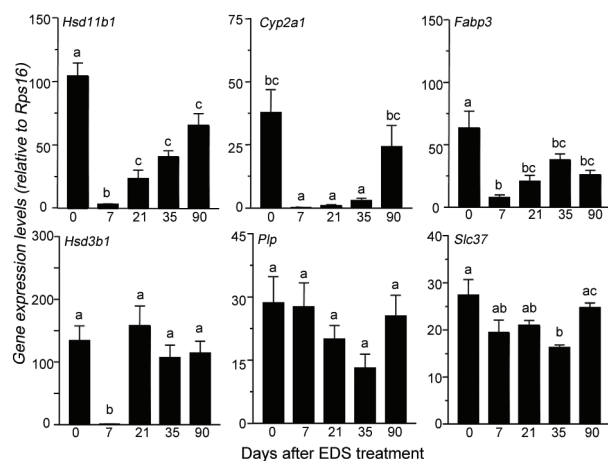


Figure 7: Gene expression levels in rat testis after ethane dimethane sulfonate (EDS) treatment. Testes were collected at 0, 7, 21, 35 and 90 days after EDS treatment. Mean \pm standard error of the mean, $n = 5$. The identical letters indicate that there is no significant difference between two groups at $P < 0.05$.

the present study we did not find difference of LH receptor (*Lhcgr*) expression levels 7 days post-EDS compared with control (before EDS treatment). Since *Lhcgr* is a Leydig cell-specific gene, the elimination of ALCs should eliminate the expression of this gene. The unchanged *Lhcgr* levels of rat testes 7 days post-EDS could be contributed by the differential expressions of splicing *Lhcgr* variants, which have been reported in the EDS-treated testis.³⁸ Seven days post-EDS, the level of 1.8 kb variant of *Lhcgr* was increased while the levels of other transcripts (2.4–6.8 kb variants) of *Lhcgr* disappeared. The microarray cannot detect this change possibly due to the probes, which can hybridize with both 1.8 kb variant and other transcripts of *Lhcgr*. We cannot rule out the changes of testosterone-dependent genes expressed in peritubular myoid, Sertoli cells, and even germ cells. Further study is required to aid this model to show the differential expressions of these testicular genes.

We also identified 89 genes that were up-regulated two-fold or more at day 7 post-EDS treatment. However, the localization of these mRNAs is unclear. In the future, *in situ* hybridization will be used to identify the location of the expressions of these genes. These genes include protein kinase (cyclic adenosine monophosphate [cAMP]-dependent, catalytic) inhibitor β (*Pkib*). *Pkib* encodes a protein that is a member of the cAMP-dependent protein kinase inhibitor family.^{39,40} This protein may interact with the catalytic subunit of cAMP-dependent protein kinase and act as a competitive inhibitor, thus stimulating cell growth.⁴⁰ *Pkib* returned to normal level 21 days after EDS treatment. *Scd1* encodes a rate-limiting enzyme SCD1 that catalyzes the conversion of saturated long-chain fatty acids into monounsaturated fatty acids.⁴¹ *Scd1* did not return to the normal level until 35 days after EDS treatment (Figure 5c). The function of *Scd1* in SLCs is still unknown. There are many unknown genes which were up-regulated at day 7.

Seventeen genes were newly up-regulated at day 21 compared with day 7 (Figure 5b, cyan dots). These up-regulated genes returned to control levels at day 90. These up-regulated genes may refer to the specific genes in progenitor Leydig cells. Thirty five days after EDS, there were also 26 newly up-regulated genes compared to the 21 days group. These up-regulated genes returned to control levels 90 days after EDS, possibly referring to the specific genes in immature Leydig cells.

While EDS exclusively targets Leydig cells, the cell-to-cell talk in the testis is highly complex, some genes are not differentially expressed

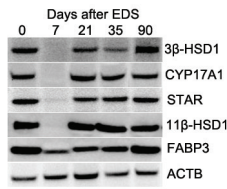


Figure 8: Western blotting shows the protein levels of 3 β -HSD1, CYP11A1, 11 β -HSD1, STAR, FABP3 which were compared to β -actin (ACTB). At day 7 postethane dimethane sulfonate (EDS) treatment, 3 β -HSD1, CYP17A1, 11 β -HSD1, *Star* protein levels went to undetectable levels while FABP3 level was greatly reduced. The levels of these proteins returned to normal level 90 days post-EDS treatment.

by absence of Leydig cells. Many other genes are expressed in other testis cells; for example, TIMP1 and *Gjal* are expressed by Sertoli cells, and *Vtn* is expressed in spermatocytes. It has been reported that the EDS treatment of adult rats caused the temporary germ cell degeneration in the testes due to the loss of testicular testosterone and that the kinetics of disappearance of germ cells and their regeneration broadly followed the changes in Leydig cell population.⁴² Therefore, this EDS model cannot rule out the changes of the expression levels of these germ cell genes. Further study is required to aid this model to show the differential expressions of testicular genes.

In summary, using gene expression profiling in the EDS-treated Leydig cell regeneration model, we identified many genes were significantly altered after the elimination of ALCs.

AUTHOR CONTRIBUTIONS

YFZ carried out the animal study and western blotting and drafted the manuscript; KMY and WZ carried out the microarray assay and analysis; YFG did the enzyme assay; CMS performed histochemical staining; YHC, QQL, YL and ZJS participated in the design of study and helped manuscript revising; RSG designed the study, performed the statistical analysis and drafted the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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Supplementary information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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