Integrated bioinformatics analysis reveals significant genes associated with cryptorchidism (CO)

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Abstract

**Objective:** we performed a comprehensive bioinformatics analysis to identify potential genes and signaling pathways associated with cryptorchidism.

**Materials and Methods:** This study aimed to identify genes significantly associated with cryptorchidism (CO), between the LE and ORL gubernaculum groups at GD17 and GD19 stages, using a GSE57924 dataset. We identified other DEGs which were changed to up-regulation or down-regulation between the groups by adjusting the cut-off ($|\text{log2 fold change (FC)}| \geq 0.5$ and adjusted $p < 0.05$). We subjected the identified DEGs to gene ontology (GO) and pathway enrichment analyses, using an online database DAVID, then employed Cytoscape v3.7.1 to construct a protein-protein interaction network.

**Results:** We detected functional hub genes using MCODE. Overall, we identified 147 DEGs between the LE gubernaculum group and ORL gubernaculum group, of which 33 genes were up-regulated (21 genes always down-regulated and 13 genes from down-regulated in GD17 to up-regulated in GD19) while 96 was down-regulated (27 genes always down-regulated and 69 genes from up-regulated in GD17 to down-regulated in GD19). Gene Ontology (GO) revealed that cell adhesion and extracellular matrix were the most abundant biological processes and Cellular Components, whereas the ECM-receptor interaction was the most significant pathway. Analysis of hub genes in the PPI network revealed that 7 genes including Igfbp5, Cyr61, Lamb1, Lamc1, Cp, Fn1, and Ltbp1, were significantly associated with CO.

**Conclusions:** we successfully identified 7 significant markers that provide valuable insights into the molecular mechanism of cryptorchidism. Of note, one of the genes (Fn1) was verified significant differential expression gene in human testicular cancer.

1. Introduction

Incidence of cryptorchidism in male infants was recently estimated to be about 2-5% at birth (Bradshaw et al., 2014). However, this incidence usually drops to 1-2% at the age of 1 year (Berkowitz et al., 1993), necessitating surgical intervention to correct the condition (Hutson & Thorup, 2015). Numerous reports have revealed the multiple factors that cause cryptorchidism. These include maternal hormonal abnormalities or endocrine disorders, mothers’ medication during pregnancy, parental genetic factors, exposure of both parents to adverse environmental conditions, assisted reproduction, age of the mother, and the number of pregnancies (Barthold et al., 2016; Gurney et al., 2017a). However, only two studies have demonstrated the link between exposure of fetuses to elevate levels of endogenous estrogen with cryptorchidism (Bartel, 2004; McGlynn et al., 2005). Androgens have been shown to play a critical role in the development of the testicles in the fetus (Mizuno et al., 2007; Mizuno et al., 2011). While the human choriionic glandular hormone (hCG) is part of the principal hormones that regulate the early development of testis. Interestingly, the level of hCG in the placenta may indicate a decrease in testosterone, thus it has been applied in cryptorchidism treatment therapies, achieving favorable outcomes (Chedane et al., 2014; Scott et al., 2009).
Genetic variation is another important factor in cryptorchidism development. To date, more than 14 gene mutations, namely AHR, AR, ARNT2, AXINI, CYPI7A1, ESRI, HOXAI0, INSL3, RXFP2 (LGR8 or GREAT208.210), NRII2, PDE4B211, NR5A1 (SFI), SPAG5, and STRBP2, have been associated with the pathogenesis of human cryptorchidism (Gurney et al., 2017a; Salemi et al., 2016; Salemi et al., 2013; Toft et al., 2016; Wada et al., 2006). The protein hormone insulin-3 (INSL3) is the most significant factor that regulates the dropping of the testis into the scrotum. Previous studies have shown that knocking out INSL3 causes cryptorchidism in mice, affirming its role in cryptorchidism. Other studies have demonstrated that INSL3 is significantly downregulated in the blood of cryptorchidism infants relative to normal subjects (Ferlin et al., 2003).

Cryptorchidism is one of the major risk factors for testicular cancer. In fact, the risk of cancer is significantly lower in contralateral than in ipsilateral testis (cryptorchidism testicular cancer RR: 6.33, 95% CI 4.30-9.31; contralateral testis RR 1.74, 95% CI 1.01–2.98), indicating that cryptorchidism and testicular cancer share common risk factors (Akre et al., 2009). Recent studies have concluded that testicular defects acquired in the intrauterine environment may be related to progression of cryptorchidism, as well as the risk of tumor formation in the testis of cryptorchidism. Thus, the risk of testicular cancer increases later in life.

In the present study, we performed a comprehensive bioinformatics analysis to identify potential genes and signaling pathways associated with cryptorchidism. Firstly, we downloaded the GSE57924 expression dataset, which contains LE gubernaculum and ORL gubernaculum samples groups, from the National Center for Biotechnology Information (NCBI) (Hadziselimovic et al., 2009), and identified differentially expressed genes. Thereafter, we exposed the differentially-expressed genes (DEGs) to Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and protein-protein interactions (PPI) analysis, to identify potential genetic biomarkers and related pathways associated with cryptorchidism. We also elucidated the underlying molecular mechanisms of cryptorchidism.

2. Materials And Methods

Gubernaculum is a specialized intermediate mesoderm derivative, whose development is stimulated by Leydig cells' hormones insulin 3 (INSL3) and androgens. These hormones target receptors in the developing rat embryo during the swelling stage (GD16 and GD19), which mainly occurs between the 16th and 19th day of the embryonic period. If the fetal rat exposure to antiandrogens inhibits both gubernacular enlargement and testicular descent at this stage (Husmann & McPhaul, 1991; Spencer et al., 1991; Welsh et al., 2008). we screened a database containing wild-type (wt) Long Evans (LE) gubernaculum and ORL gubernaculum, which is one of the strains with inherited cryptorchidism, and provided a platform for studying cryptorchidism (Barthold et al., 2008; Barthold et al., 2006), at two stages of GD17 and GD19. It has been performed that cryptorchidism in the fetal gubernaculum was associated with muscle patterning defects and altered hormonal signaling (Barthold et al., 2014). A detailed description of the study is presented using a flowchart in Figure 1.
2.1 Data download and processing

We screened the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) and downloaded GSE57924 datasets as well as their clinical information. These datasets were generated using the GPL1355 (Affymetrix Rat Genome 230 2.0 Array platform) platform, which was used to screen for DEGs. All sequence data had been subjected to quality control, background correction, normalization, and logarithmic conversion, prior to their downloading, while their platform information was used to transform probe identification numbers (IDs) into gene symbols. Specifically, gene expression value of the probe with the largest p-value was used for subsequent bioinformatics analysis when multiple probes corresponded to a gene symbol. Consequently, we constructed an expression matrix, with columns rows denoted sample and gene names, respectively.

2.2 Identification of DEGs

DEGs were identified using the “LIMMA” package in R (Ritchie et al., 2015) between the cryptorchidism and normal groups in the GSE57924 dataset. The resulting genes were analyzed using on a cut-off value of |log2 fold change (FC)| ≥ 1 and adjusted p-value < 0.05, to identify upregulated and downregulated ones. In addition, |log2 fold change (FC)| ≥ 0.5 and adjusted p-value< 0.05 were used to identify up-regulated and down-regulated DEGs between the LE and ORL gubernaculum groups, at GD17 and GD 19 stages.

2.3 Functional enrichment analyses

To elucidate the possible role of the DEGs, we performed Gene Ontology (GO) was performed targeting biological processes, cell composition, and molecular function (Ashburner et al., 2000). Additionally, we employed the Kyoto Encyclopedia Gene and Genome Database (KEGG) to explain the functions and characteristics of biologically-generated organic systems (Kanehisa & Goto, 2000). Furthermore, we adopted an online tool DAVID (http://david.abcc.ncifcrf.gov/) for annotation and visualization of DEGs. Finally, genes with a false discovery rate (FDR) <0.05 were considered statistically significant(Huang et al., 2007).

2.4 Integrate protein-protein interaction (PPI) network, modular analysis

DEGs were uploaded to the STRING database (https://string-db.org/) and PPI network generated as previously described (Szklarczyk et al., 2015). The interaction score of the data was set at >0.4, and was defined as the threshold of the selected genes of the PPI network. These genes were then visualized using the Cytoscape software. Moreover, we employed molecular complex detection (MCODE), a plug-in of the Cytoscape software, to select and cluster the significant genes (Bader & Hogue, 2003; Shannon et al.,
2003). Then, the plug-in MCODE was used to classify significant genes into clusters, which were highly correlated gene clusters in the PPI network.

### 2.5 Verified the hub gene using online tools

Herein, we searched for existing drugs or compounds of the module genes, as the potential targets, using the Drug-Gene Interaction Database (DGIdb: http://www.dgidb.org), which is a database based on more than 30 trusted sources that support searching, browsing, and filtering information about gene-drug interactions. As cryptorchidism causing infertility and testicular cancer, we were further verified using the Gene Expression Profiling Interactive Analysis database (GEPIA: http://gepia.cancer-pku.cn/index.html).

### 3. Results And Discussion

#### 3.1. Identification of DEGs

We constructed an expression matrix DEGs, then applied cut-off criteria of $|\log_2 \text{fold change (FC)}| \geq 1$ and an adjusted p-value $< 0.05$ to reveal a total of 48 DEGs between GD17 and GD 19 stages. Among them, 21 and 27 were up-regulated and down-regulated, respectively. Moreover, 82 DEGs were differentially expressed up-regulation or down-regulation between the GD17 and GD 19 stages ($|\log_2 \text{fold change (FC)}| \geq 0.5$ and the adjusted p-value $< 0.05$), among which 13 DEGs were down-regulated at GD17 stage and up-regulated in GD19 stage, whereas 69 DEGs were up-regulated in GD17 stage and down-regulated in GD19 stage. Subsequently, the top 200 DEGs were used to generate a volcano plot using R (Figure 2A & B).

#### 3.2. Functional enrichment analysis

Functional analysis of biological processes revealed that the DEGs were enriched in “cell adhesion”. With regards to cellular components, the genes were enriched in “proteinaceous extracellular matrix”, “extracellular matrix”, “basal lamina”, “basement membrane”, “extracellular exosome”, and “laminin-10 complex”. Moreover, the genes were enriched in “ECM-receptor interaction”, and “Focal adhesion” using KEGG pathway analysis (Figure 3 & Table 2).

#### 3.3 Identification of hub genes

We used the screened DEGs to construct a protein-protein interaction network (PPI), using the STRINGS database, then visualized it in Cytoscape software (Figure 4A). MCODE, a plug-in of the Cytoscape, revealed 2 clusters, with the first comprising 7 nodes, namely Igfbp5, Cyr61, Lamb1, Lamc1, Cp, Fn1, and Ltbp1, and 21 edges (Figure 4B).
IGFBP-5 is one of the family of the insulin-like growth factors binding proteins (IGFBPs) that contains 6 IGFBPs. IGFBP proteins contain N domain, link domain, and C domain. IGFBP-5 inhibits degradation of IGF-1 and regulate IGF-1 receptor binding (Beattie et al., 2006; Mukherjee et al., 2008). Its N-terminal domain contains IGF-1 and caveolin binding sites (Ravid et al., 2008). Previous studies have shown that IGFBP-5 is located in the ECM of tissues, where it directly binds to a variety of ECM proteins, including laminin, fibronectin, and thrombospondin, among others (Abrass & Hansen, 2010; Jones et al., 1993; Nam et al., 2000; Nam et al., 1997). Moreover, IGFBP-5 is expressed in many tissues of the human body, including lungs, bones, muscles, testes, ovaries, and kidneys, among others, although this expression is differential across various developmental stages and species (Schneider et al., 2002). Functionally, IGFBP-5 plays important role in regulation of cell proliferation, apoptosis, migration, and transcription, and has also been associated with cell differentiation, senescence, and cancer development (Abrass et al., 1997; Duan & Allard, 2020). Results of the present study revealed differential expression of several genes, including Myog, Tnnt2, Fst, et al., in both LE and ORL gubernaculum, which may have an effect on muscle development, cytoskeleton function, and androgen regulation pathways (Barthold et al., 2008).

Fibronectin-1 (Fn1) present on the cell surface, extracellular fluid, and connective tissue, where it interacts with collagen, fibrin, and integrin. Fn1 occurs in three forms, namely cellular Fn1, plasma Fn1, and fetal Fn1 (Singh et al., 2010). And contains several ligand-binding domains that allow it to activate a series of signal transduction pathways, thereby regulating a variety of cellular processes, including cell adhesion and differentiation, proliferation, and migration (Hu et al., 2019). To date, nothing is known regarding the role of Fn1 in development and decline of a fetus's testis. However, Fn1 has been found to regulate development of the cardiovascular system in the fetus (Chen et al., 2015; Wang & Astrof, 2016).

Laminin Subunit Beta 1 (Lamb1) and Laminin Subunit Gamma 1 (Lamc1) belong to the extracellular matrix glycoproteins family, which are the main non-collagen component of the basement membrane. These glycoproteins have been associated with a variety of biological processes, including cell adhesion and differentiation, signal transduction, and metastasis et al. (Ye et al., 2019). Specifically, Lamb1 regulates expression of β-galactosidase during development of mouse testes and ovaries (Li & Gudas, 1997). On the other hand, Lamc1 encodes an extracellular matrix protein (laminin-γ1), which is found in the early embryogenesis of embryo and extraembryonic basement membrane. Functionally, it binds to cells through a high-affinity receptor, thereby regulating cell attachment, migration and organization by interacting with other extracellular matrix components during embryonic development. Basement membranes (BMs) prevent the EMT in Embryoid bodies (EB) and the premature differentiation of primitive ectoderm to the mesoderm, indicating that BM is important in controlling the gastric urinary system in mammals. (Fujiwara et al., 2007). Cao et al showed that EBs derived from mouse embryonic stem cells lacking the laminin γ1 chain could not deposit BM, hence failed to epithelialize primitive ectoderm. Moreover, it had been showed that MiR-183 binds laminin gamma (Lamc1) can inhibit embryo implantation (Cao et al., 2020).

Latent transforming growth factor-β binding protein-1 (Ltbp-1) is an extracellular protein with similar structure to fibrin, belonging to matrix glycoprotein family, that stores cytokines outside the cell.
Functionally, it controls transforming growth factor-β (TGF-β), which is essential for signal transduction, and activates potential growth factors. Ltbp-1 can independently control transcription initiation (Koski et al., 1999). Ceruloplasmin (Cp), a glycoprotein in serum characterized by copper-containing (Cu) protein that binds to most of Cu, is mainly produced by the liver (Healy & Tipton, 2007; Linder, 2016). Previous studies have shown that Glycosyl phosphatidylinositol-anchored ceruloplasmin is expressed in Sertoli cells and in concentrated in the detergent-insoluble membrane fraction (Fortna et al., 1999). Herman et al. demonstrated that CP was expressed in germ and Sertoli cells, where it regulated function of copper-binding proteins, especially copper transporters, copper transport ATPase, and SOD1 (a copper-dependent antioxidant), to ensure bioavailability of copper in germ cells and prevent copper toxicity (Herman et al., 2020).

Cysteine-rich 61/CCN1 (Cyr61) belongs to the CYR61/CTGF/NOV (CCN) protein family (Jun & Lau, 2011). It is secreted as a stromal cell protein, and can directly bind to various integrin receptors and heparan sulfate proteoglycans, thereby regulating many cell functions in a cell type and background-dependent manner (Jun & Lau, 2011; Kim et al., 2015; Kuonen et al., 2012). Mounia et. found that an increase in the copy number of VAMP7, as well as upregulation of estrogen-responsive genes, including ATF3, CYR61, and CTGF, disrupted development of the male genitourinary system, such as cryptorchidism (Tannour-Louet et al., 2014).

### 3.4 Verified the hub gene using online tools

The 7 genes gathered were analyzed using the Drug-Gene Interaction Database. We have found that 3 genes are targeting 17 potential existing drugs, and we have found that some drugs have been used in the treatment of cryptorchidism and have achieved certain effects, such as Estradiol, Danazol et al (Figure 5A&Table 3). Cryptorchidism is a known risk factors for testicular cancer. Report to men with normal testes, men with a history of cryptorchidism are more likely to develop testicular cancer (Cook et al., 2010; Lip et al., 2013). So that, there are common risk factors between cryptorchidism and testicular cancer. Genes that increase the likelihood of testicular cancer may be related to cryptorchidism (Gurney et al., 2017b). The Fn1 gene was verified significant differential expression gene in the human using the Gene Expression Profiling Interactive Analysis database (Figure 5B). While numerous studies have shown that Fn1 expression is closely associated with formation and development of tumors as well as disease prognosis. Cryptorchidism is a common cause of testicular cancer. For example, a previously found that FN1 was overexpressed in testicular cancer, especially in non-seminoma cell carcinoma, relative to normal testicular tissues (Bo et al., 2019).

Overall, these genes correlated with cryptorchidism, as well as infertility and testicular cancer caused by cryptorchidism. Despite cancer datasets for the humanized cryptorchidism not being easily accessible, we successfully identified significant markers that provide valuable insights into the molecular mechanism of cryptorchidism.
Declarations

Acknowledgments:

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Data Availability:


Ethical approval:

This article does not contain any studies with human participants or animals performed by any of the authors. Informed consent No informed consent because this study does not contain any human or animal participants.

Authors’ Contributions:

Each author has contributed to the research process. Conceived and designed the experiments: HLr, FXm; Wrote the manuscript and analyzed data: HLr, FXm. Critically revised, and completed/corrected the manuscript, and approved the final draft: GZj, LJf, LFI, TJp.

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spermatogenesis [Journal Article; Review]. *INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES*, 21(23) http://doi.org/10.3390/ijms21239053


development through altered estrogen action [Journal Article; Research Support, N.I.H., Extramural].

*NATURE MEDICINE*, 20(7), 715-724. http://doi.org/10.1038/nm.3580


http://doi.org/10.1172/JCI34241


**Tables**

*Table 1 The DEGs in LE gubernaculum vs ORL gubernaculum group of Rattus norvegicus*
DEGs
gene names

Up-regulated in GD17 and GD19
Ctn3, LOC100125362, Agtrap, Cp, Nefl, Pxmp4, Nefm, Igfbp5, Ephx2, Egfr, Rnf34, Mfsd10, Cped1, Homer2, Dtna, Alx1, Cpt1b, Lmod3, Trdn, Gsta1, LOC102552654

Ephx2, Egfr, Rnf34, Mfsd10, Cped1, Homer2, Dtna, Alx1, Cpt1b, Lmod3, Trdn, Gsta1, LOC102552654

Down-regulated in GD17 and GD19
Ano3, Zp2, Chma5, Alyref, LOC102552284, Retsat, Ttc19, Rrp9, Lin7c, Hpgd, Naglu, Pet100, Avpr1a, Ogn, Ppp1r3b, Fahd1, Slc15a2, Rock2, Ilf3, Mfhas1, Rab27b, Abca1, Cldn11, Lrrn3, Ccn1, Cxcl13, Mcpt2

Down-regulated in GD17 and up-regulated in GD19
Bcs1l, Coa7, Lix1, Fabp4, Ptrhd1, Mlf1, Tatdn3, Ttc32, Ca8, Coq5, Acot13, Hist1h2af, Tmem121

DEGs: differentially expressed genes; GD17: gestational day 17; GD19: gestational day 19.

Table 2. The significant GO and KEGG enrichment terms of DEGs.

<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
<th>Category</th>
<th>Count</th>
<th>FDR*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0005578</td>
<td>proteinaceous extracellular matrix</td>
<td>CC</td>
<td>16</td>
<td>7.72974E-09</td>
</tr>
<tr>
<td>GO:0031012</td>
<td>extracellular matrix</td>
<td>CC</td>
<td>16</td>
<td>7.72974E-09</td>
</tr>
<tr>
<td>GO:0005605</td>
<td>basal lamina</td>
<td>CC</td>
<td>7</td>
<td>1.06295E-07</td>
</tr>
<tr>
<td>GO:0005604</td>
<td>basement membrane</td>
<td>CC</td>
<td>9</td>
<td>7.29920E-06</td>
</tr>
<tr>
<td>GO:0070062</td>
<td>extracellular exosome</td>
<td>CC</td>
<td>34</td>
<td>0.003018399</td>
</tr>
<tr>
<td>GO:0043259</td>
<td>laminin-10 complex</td>
<td>CC</td>
<td>3</td>
<td>0.004433392</td>
</tr>
<tr>
<td>GO:0007155</td>
<td>cell adhesion</td>
<td>BP</td>
<td>10</td>
<td>0.042500244</td>
</tr>
<tr>
<td>rno04512</td>
<td>ECM-receptor interaction</td>
<td>KEGG</td>
<td>10</td>
<td>1.23524E-06</td>
</tr>
<tr>
<td>rno04510</td>
<td>Focal adhesion</td>
<td>KEGG</td>
<td>11</td>
<td>0.000113692</td>
</tr>
</tbody>
</table>

GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; FDR: false discovery rate; CC: Cellular Component; BP: Biological Process.

* Terms that do not meet the FDR < 0.05 conditions are not shown
Table 3. The significant GO and KEGG enrichment terms of respectively up-regulated and down-regulated DEGs.

<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
<th>Category</th>
<th>Count</th>
<th>FDR*</th>
</tr>
</thead>
<tbody>
<tr>
<td>always down-regulated and from up-regulated to down-regulated DEGs</td>
<td>GO:0031012 extracellular matrix</td>
<td>CC</td>
<td>16</td>
<td>8.21E-11</td>
</tr>
<tr>
<td></td>
<td>GO:0005578 proteinaceous extracellular matrix</td>
<td>CC</td>
<td>16</td>
<td>8.21E-11</td>
</tr>
<tr>
<td></td>
<td>GO:0005605 basal lamina</td>
<td>CC</td>
<td>7</td>
<td>1.55E-08</td>
</tr>
<tr>
<td></td>
<td>GO:0005604 basement membrane</td>
<td>CC</td>
<td>9</td>
<td>6.25E-07</td>
</tr>
<tr>
<td></td>
<td>GO:0043259 laminin-10 complex</td>
<td>CC</td>
<td>3</td>
<td>0.00259676</td>
</tr>
<tr>
<td></td>
<td>GO:0070062 extracellular exosome</td>
<td>CC</td>
<td>27</td>
<td>0.00455593</td>
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<tr>
<td></td>
<td>GO:0007155 cell adhesion</td>
<td>BP</td>
<td>10</td>
<td>0.0055008</td>
</tr>
<tr>
<td></td>
<td>GO:0016477 cell migration</td>
<td>BP</td>
<td>8</td>
<td>0.02461004</td>
</tr>
<tr>
<td></td>
<td>rno04512 ECM-receptor interaction</td>
<td>KEGG</td>
<td>10</td>
<td>7.17E-08</td>
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<tr>
<td></td>
<td>rno04510 Focal adhesion</td>
<td>KEGG</td>
<td>10</td>
<td>6.40E-05</td>
</tr>
<tr>
<td></td>
<td>rno05146 Amoebiasis</td>
<td>KEGG</td>
<td>6</td>
<td>0.00888117</td>
</tr>
<tr>
<td></td>
<td>rno04151 PI3K-Akt signaling pathway</td>
<td>KEGG</td>
<td>9</td>
<td>0.00888117</td>
</tr>
<tr>
<td></td>
<td>rno05222 Small cell lung cancer</td>
<td>KEGG</td>
<td>5</td>
<td>0.01991411</td>
</tr>
</tbody>
</table>

always up-regulated and from down-regulated to up-regulated DEGs

GO and KEGG pathway enrichment terms can't available with FDR < 0.05

GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; FDR: false discovery rate; CC: Cellular Component; BP: Biological Process.

*Terms that do not meet the FDR < 0.05 conditions are not shown

Table 4 The special information of drugs and international genes
<table>
<thead>
<tr>
<th>Number</th>
<th>Gene</th>
<th>Drug*</th>
<th>Interaction</th>
<th>Drug information</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lamb1/Fn1</td>
<td>Ocriplasmin</td>
<td>cleavage (inhibitory)</td>
<td>no available</td>
</tr>
<tr>
<td>2</td>
<td>Cp</td>
<td>Penicillamine</td>
<td>n/a</td>
<td>specific antirheumatic agents, penicillamine and similar agents, immunosuppressive agents, myelosuppressive agents</td>
</tr>
<tr>
<td>3</td>
<td>Cp</td>
<td>Nicotine</td>
<td>n/a</td>
<td>agonist, antagonists &amp; inhibitors, oct1 inhibitors, oct2 inhibitors</td>
</tr>
<tr>
<td>4</td>
<td>Cp</td>
<td>Hydroxyurea</td>
<td>n/a</td>
<td>antisickling agents, antineoplastic agents, cytochrome p-450 enzyme inhibitors</td>
</tr>
<tr>
<td>5</td>
<td>Cp</td>
<td>Calcium</td>
<td>n/a</td>
<td>nutraceutical, calcium salts, calcium, agonists</td>
</tr>
<tr>
<td>6</td>
<td>Cp</td>
<td>Vincristine</td>
<td>n/a</td>
<td>antineoplastic agents, cardiotoxic antineoplastic agents, neurotoxic agents</td>
</tr>
<tr>
<td>7</td>
<td>Cp</td>
<td>Progesterone</td>
<td>n/a</td>
<td>contraceptives, bcrg /abcg2 inhibitors, oct1 inhibitors, oct2 inhibitors, progesterone, antagonists &amp; inhibitors</td>
</tr>
<tr>
<td>8</td>
<td>Cp</td>
<td>Anakinra</td>
<td>n/a</td>
<td>antirheumatic agents, recombinant protein, disease-modifying antirheumatic agents, agents reducing cytokine levels</td>
</tr>
<tr>
<td>9</td>
<td>Cp/Fn1</td>
<td>Methyldopa</td>
<td>n/a</td>
<td>antihypertensive agents, growth substances, agents for treatment of hemorrhoids and anal fissures for topical use</td>
</tr>
<tr>
<td>10</td>
<td>Cp</td>
<td>Estradiol</td>
<td>n/a</td>
<td>hormone replacement agents, estradiol, agonists</td>
</tr>
<tr>
<td>11</td>
<td>Cp</td>
<td>Dexrazoxane</td>
<td>n/a</td>
<td>cardiotonic agents, cytoprotective agent, immunosuppressive agents, miscellaneous therapeutic agents</td>
</tr>
<tr>
<td>12</td>
<td>Cp</td>
<td>Ampicillin</td>
<td>n/a</td>
<td>penicillins with extended spectrum</td>
</tr>
<tr>
<td>13</td>
<td>Cp/Fn1</td>
<td>Zinc Chloride</td>
<td>modulator /ligand</td>
<td>basic ointments and protectants, agents for treatment of hemorrhoids and anal fissures for topical use</td>
</tr>
<tr>
<td>14</td>
<td>Cp</td>
<td>Ferrous Glycine Sulfate</td>
<td>n/a</td>
<td>iron preparations, iron bivalent, oral preparations</td>
</tr>
<tr>
<td>15</td>
<td>Cp</td>
<td>Isoproterenol</td>
<td>n/a</td>
<td>bronchodilator agents, cardiotonic agents, adrenergic, inhalants</td>
</tr>
<tr>
<td>16</td>
<td>Cp</td>
<td>Danazol</td>
<td>n/a</td>
<td>antiendometriosis agent, antagonadotropins and similar agents, androgens, thyroxine-binding globulin inhibitors, cytochrome p-450 cyp3a4 inhibitors</td>
</tr>
</tbody>
</table>
CP: Ceruloplasmin; Lamb1: Laminin Subunit Beta 1; Fn1: Fibronectin-1.

* drugs are regulatory approval status

**Figures**

**Figure 1. Flowchart of this study**

**Figure 1**

See image above for figure legend.
Figure 2. Data processing and identification of DEGs. (A) Volcano plot and Heatmap of the top 200 DEGs between GD17-LE and GD17-ORL gubernaculum groups (|logFC|>1 and adjust P<0.05). (B) Volcano plot and Heatmap of the top 200 DEGs between GD19-LE and GD19-ORL gubernaculum groups (|logFC|>1 and adjust P<0.05). (C) Upregulated and downregulated DEGs in the GD17 and GD19 groups (|logFC|>1 and adjust P<0.05). (D) Positive and negative changes of the log2FC of DEGs in GD17 and GD19 (|logFC|>0.5 and adjust P<0.05).

Figure 2

See image above for figure legend.
Figure 3. Gene ontology (GO) and KEGG enrichment terms of DEGs

See image above for figure legend.
Figure 4. A PPI network of the DEGs. (A) A PPI network of all significant DEGs between the LE and ORL gubernaculum groups. (B) Gene clusters were selected using plus-in MCODE of cytoscape in the PPI networks. A total of 7 hub genes were identified in the first cluster.

Figure 4

See image above for figure legend.
Figure 5. Verified of the module gene. (A) Chord plot for the connection between 20 drugs and 3 genes using DGIdb. (B) The Fn1 was significant differential expression gene between testicular germ cell tumors and normal using GEPIA (log2 fold change (FC)) ≥ 2 and adjusted p-value < 0.05).

Figure 5

See image above for figure legend.