Differential gene expression and hallmarks of stemness in epithelial cells of the developing rat epididymis

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Abstract
Epididymal development can be subdivided into three phases: undifferentiated, a period of differentiation, and expansion. The objectives of this study were (1) to assess gene expression profiles in epididymides, (2) predict signaling pathways, and (3) develop a novel 3D cell culture method to assess the regulation of epididymal development in vitro. Microarray analyses indicate that the largest changes in differential gene expression occurred between the 7- to 18-day period, in which 1452 genes were differentially expressed, while 671 differentially expressed genes were noted between days 18 and 28, and there were 560 differentially expressed genes between days 28 and 60. Multiple signaling pathways were predicted at different phases of development. Pathway associations indicated that in epididymides of 7- to 18-day old rats, there was a significant association of regulated genes implicated in stem cells, estrogens, thyroid hormones, and kidney development, while androgen- and estrogen-related pathways were enriched at other phases of development. Organoids were derived from CD49f+ columnar cells from 7-day old rats, while no organoids developed from CD49f− cells. Cells cultured in an epididymal basal cell organoid medium versus a commercial kidney differentiation medium supplemented with DHT revealed that irrespective of the culture medium, cells within differentiating organoids expressed p63, AQP9, and V-ATPase after 14 days of culture. The commercial kidney medium resulted in an increase in the number of organoids positive for p63, AQP9, and V-ATPase. Together, these data indicate that columnar cells represent an epididymal stem/progenitor cell population.

Keywords Epididymis · Cell isolation · Genomics · CD49f · Cell signaling · Columnar cells

Introduction
The role of the epididymis in sperm maturation and the acquisition by sperm of both motility and the ability to fertilize has been well established (Elbashir et al. 2021; Jones et al. 2007; Robaire and Hinton 2015). The luminal environment of the epididymis necessary for sperm maturation is created in part by modulation of pH, secretion of sperm-binding proteins by the epididymis, and the presence of the blood-epididymis barrier, which regulates transcellular transport and receptor-mediated transport of selected molecules across the epithelium and their release into the lumen (Cornwall 2009; Cyr et al. 2007; Dube et al. 2007; Dube and Cyr 2012). As such, the development and differentiation of the epididymal epithelium represents a critical aspect of male fertility.

The regulation of the cellular differentiation of the epididymis remains poorly understood. Electron microscopy studies (Hermo et al. 1992; Rodriguez et al. 2002; Sun and Flickinger 1979) led to the suggestion that postnatal epididymal development could be subdivided into three distinct phases in rat: undifferentiated epithelium (birth to day 14), a period of differentiation (days 14 to 44), and a period of expansion (day 44 to adult). In the undifferentiated phase of epididymal development, the epithelium is comprised of small undifferentiated columnar cells (Rodriguez et al. 2002, Sun and Flickinger 1979). These
cells are believed to be the precursors to other cell types of the differentiated epithelium (Rodriguez et al. 2002; Sun and Flickinger 1979). One of the first markers expressed in a subpopulation of columnar cells during early development (days 14–21) is the transcription factor p63 (Gregory and Cyr 2019; Hayashi et al. 2004; Murashima et al. 2015, 2011; Saito et al. 2006). In the differentiated adult epithelium, p63 is expressed exclusively in the basal cells (Gregory and Cyr 2019; Hayashiet al. 2004; Pinel et al. 2019; Saito et al. 2006). This would suggest that the expression of p63 in a subset of small columnar cells represents either basal cells or precursors of basal cells (Gregory and Cyr 2019). During early postnatal development, these cells become associated with the basal region of the epithelium. Morphologically, principal cells become apparent at day 28, while clear cells are evident at day 35 (Hermo et al. 1992; Rodriguez et al. 2002).

Studies by Mandon et al. (2015) suggested that basal cells represent an adult stem cell population. Mou et al. (2016) showed that subcutaneous transplants of KRT5+(cytokeratin 5-positive) murine epididymal basal cells in nude mice resulted in organoids whose cells had differentiated into principal cells. Furthermore, Pinel and Cyr (2021) showed that CD49f-positive (CD49f+) rat basal cells have the ability to self-renew, form epididymal organoids, and can differentiate in vitro, further supporting the notion that these are stem cells. Since basal cells are derived from undifferentiated small columnar cells early in postnatal development, it stands to reason that the undifferentiated cells are also stem cells and that p63+ basal cells likely represent a slowly dividing or quiescent stem cell population. This has been shown in other tissues such as the trachea, for example (Kim et al. 2012; Rock et al. 2010; Roomans 2010). While the timing of the appearance of different cell types in the epididymis is well established, there is little information on the signaling pathways regulating the differentiation of the epithelium.

A large number of studies have examined gene expression during postnatal development in rodent animal models; however, few studies have attempted to predict signaling pathways associated with the phases of epididymal differentiation. Turner et al. (2007) showed that segment-specific gene expression in the adult mouse was lost following efferent duct ligation, indicating an influence of testicular factors. Hedgehog signaling and primary cilia of principal and basal cells appear to play a role in the early development of the epididymis (Girardet et al. 2020). Several studies have shown a role for p-MAPK1/3 signaling in cell proliferation and the regulation of PTEN and SRC (Xu et al. 2018, 2014, 2010), as well as the role of HNF (Browne et al. 2019). Both androgens and estrogens have been shown to play a role in epididymal development and cellular differentiation (Breton et al. 2016; Hejmej and Bilinska 2018; Hess et al. 2021; Ribeiro et al. 2017; Sipila and Bjorkgren 2016).

Nonetheless, we have a very limited understanding of the pathways that are activated at each stage of epididymal development. Furthermore, the characterization of undifferentiated epididymal columnar cells, as well as the signaling pathways implicated in the differentiation of these cells into basal and principal cells, remains to be fully elucidated. The objectives of the present study were to assess gene expression by transcriptomic analyses in the epididymis at different stages of development, using a genomics approach, to predict signaling pathways that may play a role in epididymal cellular differentiation, and to develop a novel in vitro model using CD49f+ columnar cells cultured under 3D conditions.

**Material and methods**

**Animals and tissues collection**

Male Sprague–Dawley rats (7, 18, 28, and 60 days of age) were purchased from Charles River Laboratories, Inc. (Fairfield, NJ). Rats were acclimated for 1 week under constant photoperiod (12-h light/12-h dark) and received food and water ad libitum. At the time of sampling, rats were anaesthetized with CO2 and killed by cervical dislocation or decapitation. Epididymides were dissected from rats under aseptic conditions and either frozen in liquid nitrogen and stored at –86 °C or placed in Dulbecco Modified Eagle’s Medium (DMEM)/HAM F12 culture media containing penicillin (50U/ml) and streptomycin (50 μg/ml) (Sigma-Aldrich, Oakville, ON). All the animal protocols used in this study were approved by the University Animal Care Committee.

**Microarray processing**

Total cellular RNA was isolated from pools of whole epididymides of 7- (n = 3), 18- (n = 4), 28- (n = 4), and 60-day (n = 3)-old rats using the NucleoSpin RNA Plus Kit (Macherey–Nagel, Allentown, PA) according to the manufacturer’s instructions. To remove all traces of contaminating genomic DNA (gDNA), RNA was then DNase-treated (RNase-Free DNase Set; Qiagen) and purified using the RNeasy Mini Kit (Qiagen, Redwood City, CA). The RNA concentration was determined using a NanoDrop One Spectrophotometer (NanoDrop Technologies, Wilmington, DE), and PCR was performed to verify the absence of gDNA. Gene expression profiling was done using a rat oligonucleotide microarray (Affymetrix GeneChip® Rat Gene 2.0 ST Array; Thermo Fisher Scientific, Ottawa, ON), containing 28,407 RefSeq transcripts. Hybridizations were done by Genome Québec’s Innovation Centre (McGill University, Montréal, QC). RNA quality (RNA integrity number higher than 9) was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara,
CA). The GeneChip WT Plus Reagent kit (Thermo Fisher Scientific) was used to reverse transcribe the RNA samples into cDNA, amplify them by in vitro transcription, and convert them into biotin-labeled sense-stranded cDNA. The cDNA was then hybridized to the arrays and stained with a streptavidin–phycoerythrin conjugate, and the arrays were scanned using a GeneChip Scanner 3000 7G (Thermo Fisher Scientific).

**Microarray analysis**

The relative expression level of each transcript was obtained from three or four separate pools of tissues for each age from which RNA was isolated. Raw data were corrected for background, quantile-normalized, and summarized using the RMA (Robust Multi-Chip Analysis) algorithm of the Transcriptome Analysis Console (TAC) Software 4.0.1 (Thermo Fisher Scientific). Analyses were done according to MIAME (minimum information about a microarray experiment) standards (Brazma et al. 2001). Only genes showing an FDR adjusted p-value < 0.05 and an overall FDR F-test < 0.05 were considered for data analysis. Differential expression analysis used eBayes ANOVA method of the LIMMA (Linear Models for Microarray data) package implemented in the TAC software.

Principal component analysis (PCA), hierarchical clustering, and volcano plots were performed with the TAC software. Protein class identification of highly regulated gene (18- vs 7-day comparison) was done using the PANTHER Knowledgebase (Mi et al. 2021). Differentially expressed genes (1.5-fold change) were uploaded to 3 different software programs to analyze the enriched KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways for different age comparisons (day 7 vs day 60, day 18 vs day 7, day 28 vs day 18, and day 60 vs day 28): the DAVID Bioinformatics Resources software (version 6.8; Database for Annotation, Visualization, and Integrated Discovery: Laboratory of Human Retrovirology and Immunoinformatics) (Huang da et al. 2009a, b); g:Profiler (version e101_eg48_p14_d87511f) (Raudvere et al. 2019); and String (version 11.0) (Szklarczyk et al. 2019).

In DAVID, calculations of enrichment scores were based on Expression Analysis Systematic Explorer (EASE scores < 0.1), which is a modified Fisher exact p-value (Hosack et al. 2003). In g:Profiler, only annotated genes were selected. Pathways were identified when consistently enriched using these three software programs and with a FDR p-value corrected for multiple testing using the Benjamini–Hochberg procedure and applying a significance threshold of p < 0.05.

The enrichment of GO annotations in the biological processes and clustering analyses of non-redundant terms were analyzed with Metascape (Zhou et al. 2019) for differentially expressed genes with a twofold change in expression (day 18 vs day 7, day 28 vs day 18, and day 60 vs day 28). Heatmaps were generated using the Heatmapper software (Babicki et al. 2016) without selecting any cutoff in gene expression levels or statistical tests.

Membership search of different terms (stem cell, androgen, estrogen, cell junction, frizzled, immune system, epidermal growth factor, thyroid hormone, kidney differentiation) using Metascape was applied to the following ontologies: GO biological processes, KEGG pathway, Reactome gene sets, and WikiPathways. Binary 1/0 results for each gene in the input list for differentially expressed genes with a two-fold change in expression (day 18 vs day 7, day 28 vs day 18, and day 60 vs day 28) were summarized. The number of genes in the input gene list (percentage relative to the background) associated with the membership was compared to the number of genes in the background associated with the membership, relative to the same background (whole genome) to generate a p-value.

**Epididymal organoids/isolation of CD49f-positive cells**

Epididymal organoids were derived from total cells or from Cd49f-positive cells isolated from epididymides of 7-day-old rats, following a protocol previously described for isolating basal cells from adult (48-day old) rats and establishing basal cell organoids (Mandon et al. 2015; Pinel and Cyr 2021). Briefly, 7-day-old male rats were euthanized; epididymides were removed under aseptic conditions and placed immediately into pre-warmed DMEM containing antibiotics (penicillin (50U/ml) and streptomycin (50 μg/ml) (Sigma-Aldrich)). Epididymides were trimmed from surrounding connective tissue and fat and digested in a 37 °C water bath with gentle agitation in the same medium containing collagenase (type I, 2 mg/ml; Gibco, Thermo Fisher Scientific). Two rounds of digestion with fresh medium were performed for 50 min each, followed by a final digestion period of 10 min. After allowing the digested fragments to sediment to the bottom of the flask, most of the medium was removed and replaced with a Trypsin–EDTA solution and incubated for 10 min at 37 °C with gentle agitation. Addition of FBS stopped the reaction, and the suspension was then transferred to sterile 50-ml centrifuge tubes and centrifuged for 5 min at 100 g. The supernatant was removed and discarded, and the cell pellet resuspended in fresh cold DMEM medium containing antibiotics. This suspension was then sequentially filtered through 100-, 70-, and 40-μm nylon filters, followed by a viability test and cell counts using Trypan blue. For total cells organoid cultures, the suspension was cultured in medium/Matrix drops as described below.

The suspension was again centrifuged at 300 g, 4 °C for 5 min, and the resulting supernatant was carefully removed. The pellet was placed on ice and incubated with cold MACS...
buffer (PBS pH 7.2, 25 mM EDTA, 5% BSA) containing antibody (1 µg per 1 x 10⁶ cells). Cells were incubated with MACS buffer and anti-rat CD49f antibody (integrin-α6; Bio-Rad/Serotec) for 20 min on ice, followed by three centrifugations (10 min, 300 g, 4 °C) and washes. Anti-mouse magnetic beads (Miltenyi Biotec, San Diego, CA) were then added and incubated with the cellular suspension for 15 min on ice. An additional series of three centrifugations and washes were performed, followed by magnetic separation of cells, according to the manufacturer’s instructions (Miltenyi Biotec). The cellular suspension containing cells bound to magnetic beads-antibody complexes were then passed through MS separation columns (Miltenyi Biotec) in the presence (negative fraction, Cd49f) or absence (positive fraction, CD49f⁺) of a magnetic field. Aliquots of cells from each fraction were mixed with Trypan blue, and cell counts of all fractions were performed in duplicate. Cellular suspensions were then incubated with appropriate volumes of Advanced DMEM/Matrigel (1:1 mixture) on ice. Drops of cell suspensions/Matrigel (Corning, Glendale, AZ; 20,000 cells per drop) were placed on pre-warmed 24-well plates (Sarstedt, Montreal, QC) or 8-chamber glass slides (Lab-Tek, Thermo Fisher), allowed to set upside down for 30 min in a 37 °C incubator, and then covered with warmed medium containing appropriate supplements. The organoid cultures were grown in a 32 °C, 5% CO₂ incubator, and 50% of the medium was replaced every 2 days until the end of the experiment.

Expansion and differentiation of organoids

Organoids were grown in medium containing appropriate supplements for up to 14 days. For the initial period (6 days) of expansion, epididymal organoid medium was composed of Advanced DMEM/F12, heparin (4 µg/ml), Glutamax (1X), HEPES (10 mM), and EGF (10 ng/ml; Thermo Fisher); Tocopherol (200 ng/ml), A8301 (250 nM), N-acetylcysteine (1.25 mM), cAMP (10 ng/ml), hydrocortisone (80 µg/ml), and DHT (10 nM; Sigma-Aldrich); and bFGF (10 ng/ml; Sino Biological, Wayne, PA). The medium was sterilized through a 0.22-µm filter prior to the addition of DHT and stored at 4 °C. For differentiation, the following components were added to the medium: Cholera toxin (100 ng/ml; Sigma Aldrich), ROCK inhibitor Y27632 (10 µM), R-spondin (500 ng/ml), and Noggin (100 ng/ml; PeproTech, Cranbury, NJ). Medium (50%) was replaced every 2 days until the end of the experiment.

In a series of parallel experiments, organoids derived from CD49f⁺ cells were cultured in growth medium, as described above, for 6 days. On day 6, this growth medium was replaced with medium plus supplements for differentiation, as described above, or with commercially available STEMdiff basal kidney medium, designed for kidney organoids, supplemented with STEMdiff SG supplements (STEMCELL Technologies, Vancouver, BC) and with dihydrotestosterone (DHT), which we know from previous work is required for epididymal cells (Mandon et al. 2015). After 36–40 h, this medium was replaced with STEMdiff basal kidney medium plus STEMdiff DM supplements and DHT, and organoids were cultured for an additional week until day 14 of the experiment. Multiple wells of organoids were grown under both types of media, and experiments were repeated twice.

Quantitation of organoid size

In order to assess the growth of specific organoids over time, a Cytaion 5 Imaging Multi-Mode Reader (BioTek, Winooski, VT) was used. Beginning on day 1, randomly selected defined coordinates of the organoids in multiple wells were monitored and tracked throughout the period of culture (14 days). A minimum of 4 fields per drop, and 4 drops per plate, were viewed and photographed at multiple time points throughout the experiment.

To quantify size and numbers of organoids, as well as number of organoids stained with a cell-specific marker, organoids were stained with fluorescent antibodies and counted, according to standard protocols. Briefly, medium was gently removed by aspiration from each well, and organoid culture drops rinsed with PBS. They were then fixed with ice-cold methanol for 20 min at −20 °C or 4% paraformaldehyde (PFA) for 15 min at room temperature. The choice of fixative was dependent upon the nature of the target antigen (membrane-bound, cytoplasmic, or nuclear localization), as well as upon the manufacturer’s recommendations. Organoids were washed three times with TBS (Tris-buffered saline) containing 0.3 M glycine (Thermo Fisher) for 10 min, permeabilized with 0.3% Triton X-100 (Thermo Fisher) in PBS for 15 min and washed three times with TBS-Tween (0.1%) in 0.3 M glycine (TBS-T-glycine). All subsequent washes were done using TBS-T-glycine. Blocking was performed with a solution containing 5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and 3% BSA in TBS-T-glycine for

Immuno-fluorescence

Immuno-fluorescent staining of organoids using cell-specific markers was performed at days 7 and 14 of each experiment, according to standard protocols. Briefly, medium was gently removed by aspiration from each well, and organoid culture drops rinsed with PBS. They were then fixed with ice-cold methanol for 20 min at −20 °C or 4% paraformaldehyde (PFA) for 15 min at room temperature. The choice of fixative was dependent upon the nature of the target antigen (membrane-bound, cytoplasmic, or nuclear localization), as well as upon the manufacturer’s recommendations. Organoids were washed three times with TBS (Tris-buffered saline) containing 0.3 M glycine (Thermo Fisher) for 10 min, permeabilized with 0.3% Triton X-100 (Thermo Fisher) in PBS for 15 min and washed three times with TBS-Tween (0.1%) in 0.3 M glycine (TBS-T-glycine). All subsequent washes were done using TBS-T-glycine. Blocking was performed with a solution containing 5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and 3% BSA in TBS-T-glycine for
90 min. Immunolabeling was done using different primary antibodies (Supplemental Table I) diluted in blocking solution and incubated overnight at 4 °C. Organoids were then washed in TBS-T-glycine and incubated at room temperature for 90 min with a donkey anti-rabbit Rhodamine Red-conjugated or a donkey anti-mouse AF488-conjugated secondary antibody (3.1 µg/ml; Jackson ImmunoResearch Laboratories) containing Hoechst dye (1.0 µg/ml; Biotium, Scarborough, ON). Following incubation with the secondary antibody, slides were washed again three times with TBS-T-glycine and a final wash in TBS only. Slides were mounted in Fluoromount (Southern Biotech, Birmingham, AL). Organoids incubated without primary antibody were used as a negative control. Immunofluorescence was examined under a Zeiss LSM 780 confocal microscope. Images were processed using the Zen software (Oberkochen, Germany).

Real-time reverse transcription polymerase chain reaction

Real-time RT-PCR assays were performed on rat tissue total RNA to validate the microarray analyses and on total RNA extracted from organoids as described previously (Mandon et al. 2015). Total cellular RNA was isolated from organoid cultures as follows: medium was removed from each well and rinsed gently with PBS. Dispase (1 mg/ml, Gibco, Thermo Fisher) was added to each well and incubated at 37 °C until the Matrigel was dissociated. The resulting suspensions were collected, and additional dispase was used to rinse all the wells and collect any residual cells. This was followed by a series of three PBS washes and centrifugation (300 g) steps. The resulting cell pellets were resuspended in RNA extraction lysis buffer (Macherey–Nagel) and stored at -80 °C. Total RNA was extracted using the NucleoSpin RNA Plus kit (Macherey–Nagel) following the manufacturer’s instructions. Concentration and purity of the RNA was assessed using a NanoDrop (Thermo Fisher)

An aliquot of RNA (250 ng from tissues or 20 to 50 ng from organoids) was reverse transcribed using qScript® cDNA SuperMix (Quanta BioSciences, Beverly, MA), and real-time PCR was performed using the PerfeCTa TM SYBR Green SuperMix (Quanta Biosciences). Real-time PCR was done using a Rotor-Gene RG3000 (Corbett Life Science, Mortlake, NSW, Australia). Primers were designed using Primer Blast software (National Center for Biotechnology Information, NIH, Bethesda MD) and Oligo Primer Analysis Software (Molecular Biology Insights, Cascade, CO). The sequences of the primers are listed in Supplemental Table II. Standard curves using appropriate cDNA were created for all genes examined and used to calculate relative expression levels. Relative mRNA levels of target genes of interest were normalized to the Atp5pb mRNA or 18S rRNA, for microarray validation or organoid experiments, respectively. All samples were done in triplicate.

Flow cytometry

Frozen samples of total epididymal cells from 10-day-old rats were thawed on ice and gently resuspended in DMEM medium containing 10% FBS. Cells were centrifuged (300 g; 7 min). The supernatant was discarded, and the pelleted cells were suspended in FACS buffer for counting (0.1% BSA in 1× PBS). Cells were distributed as 700,000 cell aliquots into 1.5 ml tubes and centrifuged (300 g; 7 min) and resuspended in blocking buffer (FACS buffer containing 2% BSA and 10% normal donkey serum). Cells were incubated on ice for 30 min. A mouse monoclonal anti-rat CD49f antibody conjugated with Alexa Fluor® 647 (CD49F; 0.5 µg/µl; Bio-Rad Laboratories, Mississauga, ON) was then added to the cells, and the mixture was incubated on ice for 1 h in the dark. Cells were washed two times with 1 ml of FACS buffer and resuspended in 350 µl of FACS buffer containing Hoechst dye (0.05 µg/ml; Biotium) and incubated on ice for 10 min. Cells were then washed once with 1 ml of FACS buffer, resuspended in 350 µl of FACS buffer and transferred to FACS tubes. Flow cytometric analyses were done using an LSR Fortessa and Cell Quest Pro software (BD Biosciences, Franklin Lakes, NJ).

Statistics

Each experiment used in organoid culture and analyses contained a minimum of three replicates and was repeated at least twice unless otherwise indicated. Statistical analyses were done using SigmaPlot (version 12.5, USA). Values are presented as mean ± SEM. Statistical significance was calculated using a Student’s t-test and/or Mann–Whitney U-test, as applicable; p-values ≤ 0.01 were considered significant.

Results

Gene expression profiling of epididymides from 7-, 18-, 28-, and 60-day-old rats

In order to identify specific genes associated with epididymal columnar cells, microarray analyses were used to compare gene expression profiles in epididymides of 7-, 18-, 28-, and 60-day-old rats. Rat oligonucleotide microarrays containing 28,407 RefSeq transcripts were used to assess gene expression levels. Box plot analyses of the arrays indicated that the overall signal between arrays was similar and that the data from the different arrays could be normalized to assess gene expression in epididymis from rats of different ages (Fig. 1a). Three-dimensional component analyses indicated that the expression data within an age group was similar between biological replicates but that there existed a marked difference in gene expression between different
Fig. 1  Microarray data of 7-, 18-, 28-, and 60-day-old rat epididymides RNA transcripts. a Box plot visualization of distribution of intensities for each sample. The blue boxes represent the CEL files of raw intensities and the red boxes, the background corrected, normalized, and summarized CHP files following RMA. b Principal component analysis of summarized CHP data performed on a maximum number of 5000 data points evenly distributed for each microarray sample. c Heatmap and hierarchical cluster analysis of 1009 genes differentially expressed (twofold change) in the epididymis of 7-, 18-, 28-, and 60-day-old rats. Genes expressed at levels above the average are represented in red, below average in blue, and average in black.
ages, indicating that the gene expression profiles were different between epididymides of different ages (Fig. 1b). Unsupervised hierarchical clustering using the Euclidean distance between objects and the complete linkage method between clusters indicated specific changes in gene expression at different age-dependent phases of epididymal development, with clearly identifiable genes that either increased or decreased as a function of age (Fig. 1c). Volcano plots comparing gene expression between consecutive sampling ages indicated both significantly increased and decreased gene expression patterns (Fig. 2a–c). The largest change occurred between the 7- and 18-day period, in which a total of 1452 differentially expressed genes (twofold change) were observed (Fig. 2a), while 671 differentially expressed genes were noted between days 18 and 28 (Fig. 2b) and 560 differentially expressed genes between days 28 and 60 (Fig. 2c).

In the day 18 to day 7 comparison, 1084 genes were shown to be specifically modulated, whereas 297 and 331 transcripts were specifically regulated in the 28- vs 18-day and 60- vs 28-day analyses, respectively (Fig. 2d). The number of upregulated genes by a 2-, 3-, 4-, or fivefold change was always higher than the number of downregulated genes in every comparison (18- to 7-; 28- to 18-; 60- to 28-day comparisons), except for the twofold change regulated genes when day 18 was compared to day 7 (787 downregulated genes; 665 upregulated genes) (Fig. 2e). Remarkably, of the 1452 differentially expressed genes between days 7 and 18, 107 genes were upregulated by a factor of fivefold or more. The 40 coding transcripts showing the highest degrees of differential expression are presented in Supplementary Table III. Grouping of the 107 upregulated genes by protein class using the PANTHER web resource for classification indicated that the majority of transcripts showing an increased expression are associated with defense/immunity, enzymes, transporters and channels, protease inhibitors, and carrier and scaffold proteins (Fig. 2f).

Gene expression profiles between days 18 and 28 indicated 344 upregulated and 327 downregulated genes. Of these, there were 103 genes that were highly upregulated by a factor of at least fourfold, with 71 genes upregulated by a factor of at least fivefold. Only 16 genes were downregulated by a factor of at least fourfold and 8 of them by a factor of at least fivefold (Fig. 2e). Highly regulated coding transcripts are presented in Supplemental Table IV.

Comparison of the expression profiles between 28 and 60 days revealed 406 upregulated genes, while 154 genes were downregulated. Of these, 82 were highly upregulated with increases of 4- to fivefold, while only 11 genes were highly downregulated (Fig. 2e). Coding transcripts of highly regulated genes are presented in Supplemental Table V. Upregulated genes during the 28 to 60 day transition included defensins (Ribeiro et al. 2016), lipocalins (Fouchecourt et al. 2003), and a transcript encoding a protein of the specialized mitochondria present in the midpiece of the sperm flagella, SMCP (sperm mitochondria-associated cysteine-rich protein) (Kleene et al. 1994).

**Enriched genes grouped according to their biological processes**

Genes that were differentially expressed between subsequent sampling ages were grouped according to their biological processes and clustered using Metascape (Zhou et al. 2019). While the data show that multiple processes are activated or repressed between ages, it is interesting to note that several highly significant biological processes were consistently identified between different ages, including regulation of biological processes, cell metabolism, and various immune-related functions. Likewise, clusters of genes such as defensins and defensin-related genes were present at all ages (Figs. 3 and 4). It is noteworthy that comparisons in gene expression profiles between days 18 and 7 indicated clusters of genes implicated in urogenital, vascular, skeletal, and skin development, as well as in renal system processes, suggestive of common genes implicated in development (Fig. 3a).

**Genes implicated in cell–cell interaction**

Cellular junctions play an essential role in development and the formation of the blood-epididymis barrier (Cyr et al. 2018; Dube and Cyr 2012). Dynamic changes in the expression of cadherins (adherens junctions), tight junction proteins (claudins, occludin, and tight junction proteins) and connexins (gap junctions) have been previously shown to be regulated in epididymal development. While specific genes implicated in these functions vary with development, it is noteworthy that several genes implicated in cell adhesion are highly expressed in epididymides of day 7 rats (CDH1, CDH3, CDH5, CDH11, CDH13, and CDH16) and remain elevated until day 60 (Fig. 5a). Interestingly, CLDN4, CLDN23, occludin, TJP2, and TJP3 show an increased expression at day 18, when the tight junctions of the blood-epididymis barrier become impermeable (Fig. 5d). This also coincides with an increase in gap junction proteins GJB5 (Cx31.1) and GJB3 (Cx31). GJA1 (Cx43) was highly expressed throughout development (Fig. 5d). Microarray validation by qPCR of CLDN6, GJB1, and GJA1 showed similar results to those obtained by microarrays (Fig. 6 c, h).

In many tissues, WNT signaling has been shown to play an important role in the proliferation and differentiation of undifferentiated progenitor and/or adult stem cells. Transcripts for several members of the WNT gene family were noted early in epididymal development (Fig. 5g). These included WNTs 4, 4.1, 5a, 6, and 9b. With the exception
of WNT 5a, other Wnt members decreased after day 18 (Fig. 5g). Members of the WNT receptor family, Frizzle (FZD), were also differentially expressed during development, with FZD 2, 4, and 10 being more highly expressed at day 7. FZD6 mRNA levels stayed fairly constant at all ages (Fig. 5e). Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5), an R-Spondin-dependent plasma membrane protein linked to Wnt signaling, was also expressed predominantly at day 7 (Fig. 5h). LGR5 has been shown to be a robust marker of adult stem cells in various tissues (Leung et al. 2018). qPCR analyses of Wnt9b and Fzd10 were used to confirm microarray data (Fig. 6g, i).

**Cell signaling pathways**

Predicted signaling pathways using 1.5-fold differentially expressed genes were assessed using three separate software programs: DAVID (Huang da et al. 2009a, b), STRING (Szklarczyk et al. 2019), and g:Profiler (Raudvere et al. 2019). KEGG-enriched pathways consistently identified by all three different software programs are indicated in Supplemental Table VI. The data show that many more pathways were predicted when comparing gene expression in the 18- versus 7-day-old rats. It is interesting to note that basal cell carcinoma signaling is identified, as this is the period of basal cell development in the epididymis. Comparisons of genes between days 7 and 60 also predict pathways associated with cell signaling, cell cycle, and p53 signaling, which may point to genes implicated in early columnar cell proliferation.

Membership analysis using Metascape shows that the differentially expressed genes (twofold change) between days 18 and 7 are specifically enriched for the term “stem cell” and “thyroid hormone.” Throughout the development (day 18 vs day 7, day 28 vs day 18, and day 60 vs day 28), the regulated genes were enriched for the term “estrogen.” The “androgen” membership was associated with differentially expressed genes from days 28 to 60 and days 18 to 28, while the search for “kidney differentiation” shows that this term is enriched in the differentially expressed genes from days 7 to 18 and days 18 to 28 (Table 1).

**Columnar cell organoids**

Microarray analyses clearly indicate the substantial changes that occur during epididymal development between days 7 and 18. The ability of these cells to form organoids in 3D cultures was assessed. Enzymatic digestion of epididymides from 7-day-old rats into single cells cultured under 3D conditions in Matrigel resulted in the formation of sphere-like structures within 24 to 48 h of culture (Fig. 7a–a’’’). These continued to proliferate and expand in size. All of the organoids that were generated from single columnar cells expressed the stem cell marker LGR5 (Fig. 7b’’’), identified in the microarray analysis. After 14 days of culture, a small portion (<10%) of these organoids displayed immunostaining for Aquaporin 9 (AQP9), a marker of principal cells, or p63, a basal cell marker (Fig. 7c’, c’’’).

**Isolation of columnar cells**

Previous studies indicated that MACS isolation of basal cells with CD49f antibody resulted in highly enriched basal cell population (Mandon et al. 2015). Immunostaining of epididymal sections from day 7 rats indicated an intense immunostaining of CD49f at the base of the epithelium. While there are no clearly identifiable basal cells in epididymides of day 7 rats, columnar cells appear to be positive for CD49f (Fig. 7d–d’’’). Isolation of CD49f+ cells using MACS and culturing the cells under 3D conditions in Matrigel indicated that only CD49f+ cells had the ability to form organoids (Fig. 7e–f’’’’). Analysis by FACS of CD49f+ cells in day 10 epididymides suggest that there is a single population of CD49f+ cells, and unlike other tissues of the urogenital tract (Santos et al. 2019), we did not observe CD49fhigh and CD49flow cells (Fig. 7g–g’’’).

Coordinate positioning of cultured Cd49f+ columnar cells and following them over time confirmed that these organoids were derived from a single cell (Fig. 8a). Several markers of kidney development (Osr1, Lhx1, Wt1, Sall1) were also expressed in the epididymis and were significantly decreased at day 18 as compared with day 7. Signaling molecules and receptors implicated in kidney cell differentiation (Wnt9b, Wnt6, Wnt5a, Wnt2b, Wnt9a, RARα, and Acvr1) showed a greater than 1.5-fold decrease at day 18 as compared to day 7 (Fig. 8b). We cultured CD49f+ cells from epididymides of day 7 rats in a culture medium previously used for growing and differentiating organoids derived from basal cells, as well as in a commercial medium used to differentiate kidney organoids, to which was added 10 nM of DHT, previously reported to be essential for the differentiation of basal cells.
BIOLOGICAL PROCESSES

- **a**: 
  - GO:0071840: cell component organization/biogenesis
  - GO:0032502: developmental process
  - GO:0050896: response to stimulus
  - GO:0048519: negative regulation of biological process
  - GO:0008152: metabolic process
  - GO:0022414: reproductive process
  - GO:0048518: positive regulation of biological process
  - GO:0007810: behavior
  - GO:0023052: signaling
  - GO:0009875: growth
  - GO:0050728: regulation of biological process
  - GO:0048511: rhythmic process
  - GO:0008153: cellular process
  - GO:0001906: cell killing
  - GO:0043473: pigmentation
  - GO:0098754: detoxification

- **a'**: 
  - GO:0051704: multi-organism process
  - GO:008152: metabolic process
  - GO:0048519: negative regulation of biological process
  - GO:002376: immune system process
  - GO:0065007: biological regulation
  - GO:0051179: localization
  - GO:0040011: locomotion
  - GO:0022414: reproductive process
  - GO:0009987: cellular process
  - GO:0032501: multicellular organismal process
  - GO:0023052: signaling
  - GO:0001906: cell killing
  - GO:0032502: developmental process
  - GO:0050896: response to stimulus
  - GO:0098754: detoxification
  - GO:0048511: rhythmic process
  - GO:0071840: cell component organization/biogenesis

- **a'**: 
  - GO:0048519: negative regulation of biological process
  - GO:0051704: multi-organism process
  - GO:008152: metabolic process
  - GO:0065007: biological regulation
  - GO:002376: immune system process
  - GO:0071840: cell component organization/biogenesis
  - GO:0050896: response to stimulus
  - GO:0022414: reproductive process
  - GO:0009987: cellular process
  - GO:0032501: multicellular organismal process
  - GO:0023052: signaling
  - GO:0001906: cell killing
  - GO:0007810: behavior
  - GO:0032502: developmental process
Epididymides of 7-day-old rats are comprised of undifferentiated short columnar cells. Transcripts expressed at day 7 are heavily enriched with genes implicated in the cell cycle and stem cell signaling, supporting the previously reported morphological observations that the epithelium is largely undifferentiated at this age. Our microarray data demonstrate that numerous genes were highly expressed almost exclusively at day 7, including some of the gap junction proteins, cadherins (e.g., CDH3, CDH5, and CDH6), claudin-6 (CLDN6), a tight junction protein, and multiple proteins implicated in WNT signaling (WNT9b, Wnt6, LGR5, FZD2, and FZD10). CDH2 and CDH3 expression in undifferentiated columnar cells is interesting. These genes have been shown to be expressed in stem cells of the mammary gland, osteoblasts, chondrocytes, pro-myoocytes and others (Alimperti and Andreadis 2015; Patil 2021; Ranjan et al. 2021). Curiously, following the differentiation of basal cells, these genes become localized specifically to basal cells. LGR5 has been reported as a marker of adult stem cells in multiple tissues, including the intestine, bronchioles, mammary gland, and prostate (Barker and Clevers 2010; de Visser et al. 2012; Leung et al. 2018; Schuijers and Clevers 2012; Seishima et al. 2019).

Cellular differentiation of the epididymis is accompanied by dramatic changes in gene expression as the epithelium proliferates and differentiates. The largest changes in gene expression occurred between days 7 and 18, during which 1452 genes were differentially expressed, with 787 genes being downregulated and 665 genes being upregulated. Downregulated genes at day 18 vs day 7 represented the major group of genes of the microarray analyses, showing the unique signature of the columnar cells.

Crispl (cysteine-rich secretory protein 1) and serpina16 (serine peptidase inhibitor, Clade A member 16) genes, also known as rat HongrES1 (Ni et al. 2009; Zhou et al. 2008), showed the highest increase in mRNA levels between days 7 and 18 with over 95-fold increase, while Bin2a (betagalactosidase-like protein), the third highest, showed a 63-fold increase (Supplemental Table III). All three genes have been shown to be androgen-responsive in the epididymis (Brooks et al. 1986; Zhen et al. 2009; Zhou et al. 2008). Other androgen-regulated genes such as Lcn5 (lipocalin 5), Adam7 (a disintegrin and metallopeptidase domain 7), DEFB1 (defensin beta 1), AQP9, Spink13 (serine peptidase inhibitor, Kazal type 13), and several DEFB (Hu et al. 2014; Ma et al. 2013) were also highly upregulated from 7 to 18 days (Lareyre et al. 2000; Ma et al. 2013; Oh et al. 2005; Oliveira et al. 2005; Palladino et al. 2004), suggesting that early development is strongly influenced by androgens, even though androgen levels have been reported as being low at this age (Scheer and Robaire, 1980). Furthermore, while transcripts for the androgen receptor increase only slightly by day 18, there is a tenfold increase in type 2 4-ene-5-alpha-reductase (SRD5a) (Viger and Robaire 1995, 1996). While
expression levels of many of these genes further increase by day 28, the results show that androgen regulation in the epididymis begins at these early stages of development, prior to the presence of morphologically distinct principal cells (Sun and Flickinger 1979).

Of the 787 downregulated genes in the 7 to 18-day period, 41 were downregulated by a factor of fivefold or more. The transcription factor FOS (FBJ osteosarcoma oncogene), which has been shown to be androgen-repressed (Shankar et al. 2016), displayed the highest degree of downregulation, 14.5-fold. In contrast to the upregulated genes associated with androgen regulation in the epididymis, there was no obvious pattern of regulation with the most highly downregulated genes.

Interestingly, several imprinted genes were downregulated at 18 days: DLK1 (delta-like non-canonical Notch ligand 1) with a fold change of \(-7.75\), the GNAS complex locus with a fold change of \(-6.79\), and IGF2 (insulin-like growth factor 2) with a fold change of \(-6.26\). DLK1 regulates cell differentiation and developmental processes through Notch and other signaling pathways (Traustadottir et al. 2019). Igf-2 is almost exclusively expressed in the embryo of rodents (Chao and D’Amore 2008). AGTR2 (angiotensin II receptor, type 2), which is a basal cell marker, was also decreased (fold change of \(-4.8\), (Shum et al. 2008)), as was Osr1 (odd-skipped related transcription factor 1; fold change of \(-4.35\)), a transcription factor implicated in normal development and organogenesis of the heart and kidney (Wang et al. 2005). Numerous V-ATPases were found to be upregulated at day 18 (Fig. 6b). The development of clear cells in the epididymis, which display high levels of V-ATPase, does not occur until day 35 of development, suggesting that at this age, V-ATPase may have other roles in epididymal cell differentiation (Rodriguez et al. 2002). Interestingly, V-ATPases have been shown to be implicated in cell differentiation in other tissues (McGuire et al. 2017; Sun-Wada and Wada 2015).

During the period of cell differentiation, days 18 and 28, our results indicated 344 upregulated and 327 downregulated genes. During this phase of development, the tall columnar cells of the epididymis differentiate into principal and narrow cells and, in rats, the blood-epididymis barrier becomes impermeable (Agarwal and Hoffer 1989; Rodriguez et al. 2002). It has been suggested that the formation of the blood-epididymis barrier is completed at postnatal day 21 (Agarwal and Hoffer 1989). Gene expression data do not support an increase in tight junctional proteins (CLDNS, Occludin) at this age. In other species, such as mice and mink, the blood-epididymis barrier is formed prior to birth (Guan et al. 2005; Pelletier 1994). Cyr et al. (1999) noted that occludin was expressed and localized to the plasma membrane of the embryonic epididymis by embryonic day 13.5. It is, therefore, possible that the CLDNS and the development of the impermeability of the rat blood-epididymis barrier are regulated by posttranscriptional factors.

Among the most highly expressed genes, epididymis-specific Gpx5 (glutathione peroxidase 5), an androgen- and testicular factors-regulated gene (Drevet et al. 1998), showed the highest increase, with a fold change of 62.61. Rat Gpx5 transcripts were previously detected at day 32 by RT-PCR, before the appearance of the protein in the epididymis at day 44, just prior to puberty and the entry of sperm in the epididymis (Williams et al. 1998). Other sperm-binding molecules and androgen-regulated genes also showed increased transcripts levels, including multiple defensins, Crisp4, and Pate (prostate and testis expressed) proteins (Hu et al. 2014; Jalkanen et al. 2005; Turunen et al. 2011). These data support the notion of continuous androgen regulation of gene expression from early development through to adulthood. The gamma-aminobutyric acid (GABA-A) receptor pi (GABRP) was decreased by 9.48-fold. This receptor subunit has been shown to be implicated in airway epithelial progenitor cell differentiation (Wang et al. 2021). WNT9b (wingless-type MMTV integration site family, member 9B), CLDN6 (claudin-6) and CFTR (cystic fibrosis transmembrane conductance regulator) were also downregulated from 18 to 28 days, with a fold change of \(-6.25\), \(-4.22\), and \(-3.19\), respectively.

There were 406 upregulated and 154 downregulated genes between days 60 and 28. During this phase of development, sperm first enter the epididymis and will be present throughout the epididymis (Robaire and Hermo 1988). During this period of time, androgen levels will also reach adult levels at approximately day 42 (Scheer and Robaire 1980). It is, therefore, not surprising that many of the genes that were significantly upregulated included sperm-binding proteins such as defensins (Ribeiro et al. 2016), lipocalins (Fouchecourt et al. 2003), and sperm mitochondria-associated cysteine-rich protein (SMPC) (Kleene 1989; Kleene et al. 1994; Legare et al. 2017). Furthermore, gene expression data indicate that among the genes that are most highly expressed, there are a number of cystatins (Cornwall et al. 2019; Do et al. 2019). Cystatins are implicated in the formation an amyloid matrix in the epididymis. Amyloids bind to spermatozoa, and it has been suggested that these play a role in sperm maturation (Cornwall et al. 2019).

Pathway associations using Metascape analyses indicated that in epididymides of 7- to 18-day-old rats, there was a significant enrichment of regulated genes implicated in stem
cells, estrogen, thyroid hormones, and kidney development. This is consistent with previous observations that have reported the differentiation of small epididymal columnar cells into other cell types, as well as implicating a role for estrogens and thyroid hormones in epididymal development (Anbalagan et al. 2010; De Paul et al. 2008; Hess et al. 2021; St-Pierre et al. 2003; Sun and Flickinger 1979). Similarities between epididymal and renal development may be related to the common embryonic mesonephric origin of both tissues. The influence of estrogens on the epididymis appeared to extend throughout all phases of development and cell differentiation (Hess 2000; Hess et al. 2021). Unlike estrogens, androgen-dependent genes displayed significant changes between days 28 and 18 and 60 and 28. However, some androgen-dependent epididymal genes showed considerably increased gene expression between days 28 and 18 and may represent highly sensitive transcriptional activation of certain genes to low levels of androgens. Surprisingly, there were no significant associations in cell junctions, epidermal growth factor, or immune function genes. While many of these genes showed differential expression, it is possible that only a few key specific genes are altered during development or that these occur in a segment-specific manner and were not detected with the current experimental protocol using the entire epididymis.

Immune cells of the epididymis, such as dendritic cells and macrophages, can produce inflammatory cytokines implicated in the immune response. Transcript levels for the cytokines IL1 alpha and beta, IL6, IL10, IL12, and IL20, as well as for interferon gamma and tumor necrosis factor, were expressed at low levels throughout development. Expression of the tryptophan-depleting enzymes (indoleamino 2,3-dioxygenases (Ido1 and Ido2) increased during epididymal development. These enzymes are recognized to be implicated in the regulation of the equilibrium between the immune response and self-tolerance (Jrad-Lamine et al.)

Fig. 5 Heatmaps of families of genes expressed during development. a–h Heatmaps were generated using the Heatmapper software without any cutoff in gene expression levels or statistical tests. Heatmaps of genes implicated in adherens junctions (cadherins; a), gap junctions (connexins; b, c), tight junction proteins (d), Frizzle receptors (e), V-ATPase (f), Wnt signaling (Wnt proteins, WNT signaling; g, h), and genes implicated in immune function (i) are shown.
Transforming growth factor-beta (TGFß) ligands 1, 2, and 3 were expressed at similar levels at day 7 in the epididymis. TGFb1 mRNAs showed a constant level of expression throughout development, while TGFb2 and 3 mRNA levels decreased slightly. The TGFb pathway has been shown to be implicated in the immunotolerance to sperm in mice (Pierucci-Alves et al. 2018).

Innate immune receptors to pathogens, Toll-like receptors (TLRs) 1 to 11 are expressed in the epididymis (Liman et al. 2019; Munipalli et al. 2019; Rodrigues et al. 2008). Microarray data indicated that TLR3 and 5 were the most highly expressed in the epididymis. TLR3 has the highest level of expression throughout the development, and TLR5 increased with age. TLR8 and 9 were not probed by the microarray. Nuclear factor kappa B subunits 1 and 2 (NFkB1 and NFkB2) were detected at all time points of epididymal development used in this study. The alpha inhibitor (NFkBia) was also highly expressed at days 7 and 18 and decreased at days 28 and 60 (Gregory and Cyr 2014).

A highlight of the microarray data results is that the largest changes in gene expression were observed between the day 7 and day 18 rat epididymides. Furthermore, data indicated that epididymides from day 7 rats shared common properties with stem cells in terms of gene expression, predicted pathway analyses, and gene membership analyses. To further characterize the potential stem cell functions of columnar cells and determine whether or not these could be used to develop an in vitro model, a 3D organoid cell culture was performed. Dispersed epididymal cells from day 7 rats...
were able to rapidly form organoids and differentiate into basal and principal cells in vitro.

MACS separation of CD49f+ cells from day 7 epididymides indicated that these cells were responsible for the formation of organoids. This was similar to our previous studies in which basal stem cells were isolated from adult rats and cultured under 3D conditions (Mandon et al. 2015; Pinel and Cyr 2021). In urogenital cells, it has been reported that progenitor cells are also CD49f+; however, their stem cell properties are attributed exclusively to a subpopulation of cells that display a high expression of CD49f+ (Santos et al. 2019). Flow cytometry of epididymal columnar cells did not show any distinct populations of CD49f+high or CD49f+low cells suggesting a single population of cells in epididymides of young rats. CD49f+ cells could differentiate into basal, principal, and clear cells. The ability of these cells to differentiate is similar to what was shown for basal cells from adult rats and mice (Mandon et al. 2015; Mou et al. 2016; Pinel and Cyr 2021).

Given the common origin of embryonic development of the epididymis and the kidney, as well as the fact that epididymides from day 7 rats express numerous genes associated with kidney development, we compared culture media developed for adult epididymal basal cells with a commercially available medium designed for kidney organoids (Freedman et al. 2015; Pinel and Cyr 2021). Although the organoids cultured in both media displayed similar morphology and appearance, there were differences in the proportion of sizes of organoids (40–80 μm vs ≥ 80 μm). Staining of these organoids with various epididymal cell type markers was also comparable between the two groups in terms of correct localization of these markers but revealed significant differences between the percentage of organoids stained for a particular marker (Aqp 9, p63, and V-ATPase). Comparison of organoids cultured in the control medium versus the kidney medium revealed that there was at least a two-fold greater percentage of organoids staining for these markers in the organoids grown in kidney medium. These results, taken together with the microarray data, suggest that the kidney medium favored a greater tendency towards cellular differentiation, as compared to the control medium.

The widespread immunostaining for V-ATPase observed in undifferentiated organoids derived from CD49f+ epididymal cells from day 7 rats suggests that as the epididymal epithelium develops postnatally, many cells destined for further differentiation express this protein. Whether or not this is a transient type of a previously undescribed cell, or rather a stage of basal-type cells, is unknown. Numerous V-ATPases were also upregulated between days 7 and 18. There are reports in the literature regarding the expression of V-ATPase (various isoforms) as a function of differentiation in multiple tissues and/or cell types. Wissel et al. (2018) have reported a V-ATPase-NOTCH regulatory loop in *Drosophila* neural stem cells or neuroblasts. They and others suggest that V-ATPase and NOTCH act in multiple stem cell lineages during nervous system and adult gut development. Although various subunits or isoforms of V-ATPase family members are implicated in many cellular processes (McGuire et al. 2017; Sun-Wada and Wada 2015), the authors of the *Drosophila* studies suggest that the V-ATPase complex is required for self-renewal of the neuroblast stem cells. Furthermore, they report that it is among the first and most significantly downregulated complex in differentiating daughter cells and that it is necessary for maintaining NOTCH pathway activity.

V-ATPase (multiple isoforms) is associated with luminal acidification, which is critical in the epididymis and other tissues (Breton and Brown 2013; Shum et al. 2011). This luminal acidification is necessary for preserving spermatozoa in a quiescent state. However, multiple isoforms of V-ATPase in the epididymis have been reported in the literature (Pietrement et al. 2006), which prompts the question of whether or not these multiple isoforms have different functions. V-ATPase subunits can interact with some enzymes that are part of the glycolytic pathway in mammalian cells (Breton and Brown 2007). Intriguingly, another group has examined the roles of glucose metabolism, fatty acid oxidation, and reactive oxygen species (ROS) in the equilibrium between quiescence and self-renewal of adult stem cells (Mohammad et al. 2019). The authors point out that low ROS is a hallmark of adult stem cells. This would align with a role for V-ATPase in maintaining pH and protecting cells from ROS damage, as well as promoting self-renewal of stem cells. Nonetheless, the fact that organoids derived from basal cells of adult rats also expressed V-ATPase to a significant degree (Pinel and Cyr 2021), as well as organoids cultured in either epididymal basal cell medium or the commercial kidney medium, further supports the notion that the expression of V-ATPase is, at the very least, an indicator of

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**Table 1** Membership analysis using Metascape software showing differentially expressed genes by at least twofold between days 18 and 7 specifically enriched for the specific terms listed in the table. * indicates significant difference

| Search term          | day 18 vs day 7 | day 28 vs day 18 | day 60 vs day 28 |
|----------------------|-----------------|------------------|------------------|
|                      | p-value         | p-value          | p-value          |
| Stem cell            | 0.00092*        | 0.54             | 0.42             |
| Androgen             | 0.47            | 0.016*           | 0.0000019*       |
| Estrogen             | 0.00014*        | 0.00011*         | 0.000064*        |
| Cell junction        | 0.99            | 0.76             | 0.98             |
| Frizzled             | 0.53            | 0.81             | 1.0              |
| Immune system        | 1.0             | 1.0              | 1.0              |
| Epidermal growth factor | 0.97         | 0.61             | 0.51             |
| Thyroid hormone      | 0.021*          | 0.34             | 0.28             |
| Kidney differentiation| 0.0025*         | 0.021*           | 0.16             |
self-renewal of at least a subset of stem cells and perhaps also suggests a transient phase of cellular differentiation. The role of V-ATPase in these organoids is intriguing and remains to be explored in future studies.

In conclusion, the development of the epididymal epithelium and subsequent cellular differentiation into multiple specialized cell types is a complex, highly coordinated process which relies on a variety of cues from the...
Fig. 8 Organoids derived from CD49f+ cells. a Organoids derived from single CD49f+ epididymal epithelial cells were cultured and followed over a 10-day period (scale bar = 300 µm). b Expression of kidney differentiation markers present in epididymides of 7-day-old rats versus day 18. c–c′′′ CD49f+ cells were cultured with an expansion medium during 6 days (c–c′) and for additional 8 days in either a control epididymal basal cell differentiation culture medium (d–d′′′) or a commercial kidney stem cell medium (e–e′′′) (scale bar = 200 µm).

f–f′ Quantification of the number of organoids according to size indicated that there was a consistent and significantly larger proportion of organoids ranging between 40 and 80 µm in size (f) as well as a corresponding lower proportion of organoids > 80 µm (f′) in organoids grown in the commercial kidney as compared with the control medium. **p < 0.01
physiological environment as well as from numerous signaling pathways. We have identified multiple key signaling pathways involved in the various stages of epididymal epithelial development and differentiation. Information from our microarray and PCR analyses highlight the genes which play major roles at specific time points during development. Furthermore, we have developed a new in vitro model of epididymal organoids, derived from undifferentiated Cd49f-positive columnar cells. The development of these organoids provides us with an additional tool to further elucidate the differentiation of the epididymal epithelium. This organoid model may also serve as a tool to further examine factors which may be implicated in male infertility, as well as having potential use in toxicological studies. Finally, we have provided evidence of the stem cell-like properties of these cells and have provided methods for both proliferation as well as differentiation of these columnar cells into other cell types. We believe that these data significantly advance our understanding of epididymal epithelial development and differentiation.

Fig. 9 Differentiation of CD49f⁺ cell-derived organoids. (a–b‴) Immunofluorescent staining of organoids derived from single CD49f⁺ epididymal columnar cells of 7-day-old rats and cultured for 14 days. Immunostaining for p63, AQP9, and V-ATPase is shown in cells cultured in control epididymal basal cell medium (a–a‴) versus the commercial kidney medium (b–b‴) (scale bar = 10 µm). c Quantification of p63, AQP9 and V-ATPase positive organoids. Data show a significant increase in immunostaining in organoids cultured in the commercial kidney medium. * p < 0.05. d–d‴ RT-PCR analyses of mRNA levels for p63 (d), AQP9 (d′), and V-ATPase (d″) in organoids cultured for 14 days in control epididymal basal cell medium versus the commercial kidney medium. There was a significant increase in both p63 and AQP9 mRNA levels, while mRNA levels of the ATP6V1B2 isoform of V-ATPase was not different between culture media. * p < 0.05
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Data availability The gene expression data from transcriptomic analyses have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) and are accessible through GEO accession number GSE193653. Other data are available from the corresponding author.

Declarations

Ethics approval All the animal protocols used in this study were approved by the Institut National de la recherche Scientifique (INRS) University Animal Care Committee according to the guidelines of the Canadian Council on Animal Care.

Informed consent All participants have given their verbal consent regarding their participation in this study and submission of this article.

Conflict of interest The authors declare no competing interests.

References

Agarwal A, Hoffer AP (1989) Ultrastructural studies on the development of the blood-epididymal barrier in immature rats. J Androl 10:425–431
Alimperti S, Andreadis ST (2015) CDH2 and CDH11 act as regulators of stem cell fate decisions. Stem Cell Res 14:270–282
Anbalagan J, Sashi AM, Vengatesh G, Stanley JA, Neelamohan R, All. Nucleic Acids Res 44:W147-153
Barker N, Clevers H (2010) Leucine-rich repeat-containing G-protein-coupled receptors as markers of adult stem cells. Gastroenterology 138:1681–1696
Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC, Gaasterland T, Glenisson P, Holstege FC, Kim IF, Markowitz V, Matses JC, Parkinson H, Robinson A, Sarkans U, Schulze-Kremer S, Stewart J, Taylor R, Vilo J, Vingron M (2001) Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat Genet 29:365–371
Breton S, Brown D (2007) New insights into the regulation of luminal acidification by the V-ATPase. Biol Reprod 76:1034–1044
Breton S, Ruan YC, Park YJ, Kim B (2016) Regulation of epithelial function, differentiation, and remodeling in the epididymis. Asian J Androl 18:3–9
Brooks DE, Means AR, Wright EJ, Singh SP, Tiver KK (1986) Molecular cloning of the cDNA for two major androgen-dependent secretory proteins of 18.5 kilodaltons synthesized by the rat epididymis. J Biol Chem 261:4956–4961
Brouwne JA, Leir SH, Yin S, Harris A (2019) Transcriptional networks in the human epididymis. Andrology 7:741–747
Chao W, D’Amore PA (2008) IGF2: epigenetic regulation and role in development and disease. Cytokine Growth Factor Rev 19:111–120
Cornwall GA (2009) New insights into epididymal biology and function. Hum Reprod Update 15:213–227
Cornwall GA, Do HQ, Hewetson A, Muthusubramanian A, Myers C (2019) The epididymal amyloid matrix: structure and putative functions. Andrology 7:603–609
Cyr DG, Dufresne J, Gregory M (2018) Cellular junctions in the epididymis, a critical parameter for understanding male reproductive toxicology. Reprod Toxicol 81:207–219
Cyr DG, Gregory M, Dubé E, Dufresne J, Chan PT, Hermo L (2007) Orchestration of occludins, claudins, catenins and cadherins as players involved in maintenance of the blood-epididymal barrier in animals and humans. Asian J Androl 9:463–475
Cyr DG, Hermo L, Egenberger N, Merteitne C, Trasler JM, Laird DW (1999) Cellular immunolocalization of occludin during embryonic and postnatal development of the mouse testis and epididymis. Endocrinology 140:3815–3825
De Paul AL, Mukdisi H, Pellizas CG, Montesinos M, Gutierrez S, Susperreguy S, Del Rio A, Maldonado CA, Torres AI (2008) Thyroid hormone receptor alpha 1-beta 1 expression in epididymal epithelium from euthyroid and hypothyroid rats. Histochem Cell Biol 129:631–642
de Visser KE, Ciampricotti M, Michalak EM, Tan DW, Speksnijder EN, Hau CS, Clevers H, Barker N, Jonkers J (2012) Developmental stage-specific contribution of LGR5(+)-cells to basal and luminal epithelial lineages in the postnatal mammary gland. J Pathol 228:300–309
Do HQ, Hewetson A, Myers C, Khan NH, Hastert MC, F MH, Latham MP, Wylie BJ, Sutton RB, Cornwall GA, (2019) The Functional mammalian CRES (cystatin-related epididymal spermaticogenic) amyloid is antiparallel beta-sheet rich and forms a metastable oligomer during assembly. Sci Rep 9:9210
Drevet JR, Lareyre JJ, Schwaab V, Vernet P, Dufaure JP (1998) The PEA3 protein of the Ets oncogene family is a putative transcriptional modulator of the mouse epididymis-specific glutathione peroxidase gene gpx5. Mol Reprod Dev 49:131–140
Dubé E, Chan PT, Hermo L, Cyr DG (2007) Gene expression profiling and its relevance to the blood-epididymal barrier in the human epididymis. Biol Reprod 76:1034–1044
Dubre E, Cyr DG (2012) The blood-epididymis barrier and human male fertility. Adv Exp Med Biol 763:218–236
Elbashir S, Magdi Y, Rashed A, Henkel R, Agarwal A (2021) Epididymal contribution to male infertility: an overlooked problem. Andrologia 53:e13721
Fouchecourt S, Lareyre JI, Chaurand P, DaGue BB, Suzuki K, Ong DE, Olson GE, Matusik RJ, Capriolo RM, Orgebin-Crist MC (2003) Identification, immunolocalization, regulation, and postnatal development of the lipocalin EP17 (epididymal protein of 17 kilodaltons) in the mouse and rat epididymis. Endocrinology 144:887–900
Freedman BS, Brooks CR, Lam AQ, Fu H, Morizane R, Agrawal V, Saad AF, Li MK, Hughes MR, Werff RV, Peters DT, Lu J, Baccei A, Siedlecki AM, Valerius MT, Musunuru K, McNagny KM, Steinman TI, Zhou J, Lerou PH, Bonventre JV (2015)
Modelling kidney disease with CRISPR-mutant kidney organoids derived from human pluripotent epithelial spheroids. Nat Commun 6:8715

Girardet L, Bernet A, Calvo E, Soulet D, Joly-Beauparlant C, Droit A, Cyr DG, Belleau C (2020) Hedgehog signaling pathway regulates gene expression profile of epididymal principal cells through the primary cilium. FASEB J 34:7593–7609

Gregory M, Cyr DG (2014) The Blood-Epididymis Barrier and Inflammation Spermagenesis 4:e979619

Gregory M, Cyr DG (2019) Effects of prostaglandin E2 on gap junction protein alpha 1 in the rat epididymis. Biol Reprod 100:123–132

Guo X, Inai T, Shibata Y (2005) Segment-specific expression of tight junction proteins, cadmu-2 and -10, in the rat epididymal epithelium. Arch Histol Cytol 68:213–225

Hayashi T, Yoshinaga A, Ohno R, Ishii N, Kamata S, Yamada T (2004) Expression of the p63 and Notch signaling systems in rat testes during postnatal development: comparison with their expression levels in the epididymis and vas deferens. J Androl 25:692–698

Hejmej A, Bilinska B (2018) The effects of flutamide on cell-cell junctions in the testis, epididymis, and prostate. Reprod Toxicol 81:1–16

Hermo L, Barin K, Robaire B (1992) Structural differentiation of the epithelial cells of the testicular efferent duct system of rats during postnatal development. Anat Rec 233:205–228

Hess RA (2000) Oestrogen in fluid transport in efferent ducts of the male reproductive tract. Rev Reprod 5:84–92

Hess RA, Sharpe RM, Hinton BT (2021) Estrogens and development of the rete testis, efferent ductules, epididymis and vas deferens. Differentiation 118:41–71

Holschbach C, Cooper TG (2002) A possible extratubular origin of epididymal basal cells in mice. Reproduction 123:517–525

Hosack DA, Dennis G Jr, Sherman BT, Lane HC, Lempicki RA (2003) Identifying biological themes within lists of genes with EASE. Genome Biol 4:R870

Hu SG, Zou M, Yao GX, Ma WB, Zhu QL, Li XQ, Chen ZJ, Sun Y (2014) Androgenic regulation of beta-defensins in the mouse epididymis. Reprod Biol Endocrinol 12:76

da Huang W, Sherman BT, Lempicki RA (2009a) Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 37:1–13

da Huang W, Sherman BT, Lempicki RA (2009b) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Proto 4:44–57

Jalkanen J, Huhtaniemi I, Poutanen M (2005) Discovery and characterization of new epididymis-specific beta-defensins in mice. Biochim Biophys Acta 1730:22–30

Jones RC, Dacheux J, Nixon B, Ecroyd HW (2007) Role of the epididymis in sperm competition. Asian J Androl 9:493–499

Jrad-Lamine A, Henry-Berger J, Gourbeyre P, Damon-Soubeyrand C, Jones RC, Dacheux JL, Nixon B, Ecroyd HW (2007) Role of the Toll-like receptors 2, 4, 5 and 9 in the epididymis and vas deferens of a adult tom cats. Theriogenology 68:23–41

Ma L, Yu H, Ni Z, Hu S, Ma W, Chu C, Liu Q, Zhang Y (2013) Spink13, an epididymis-specific gene of the Kazal-type serine protease inhibitor (SPINK) family, is essential for the acrosomal integrity and male fertility. J Biol Chem 288:10154–10165

Mandon M, Herlo M, Cyr DG (2015) Isolated rat epididymal basal cells share common properties with adult stem cells. Biol Reprod 93:115–130

McGuire C, Stranks L, Cotter K, Forzag M (2017) Regulation of V-ATPase Activity Front Biosci (landmark Ed) 22:609–622

Mehraj V, Ramendra R, Isnard S, Dupuy FP, Ponte R, Chen J, Kema I, Jenabia MA, Costiniuk CT, Lebouche B, Thomas R, Cote P, Leblanc R, Baril JG, Durand M, Chartrand-Lefebvre C, Tremblay C, Ancuta P, Bernard NF, Sheppard DC, Routy JP, Montreal Primary HIVIS, Canadian HIV, Aging Cohort Study G (2020) Circulating (1>3)-beta-D-glucan is associated with immune activation during human immunodeficiency virus infection. Clin Infect Dis 70:222–241

Mi H, Ebert D, Maragamujan A, Mills C, Albou LP, Mushahayama T, Thomas PD (2021) PANTHER version 16: a revised family classification, tree-based classification tool, enhancer regions and extensive API. Nucleic Acids Res 49:D394–D403

Mohammad K, Dakik P, Medkour Y, Mitrofanova D, Titorenko VI (2019) Quiescence entry, maintenance, and exit in adult stem cells. Int J Mol Sci 20:2158

Mou H, Vinarsky V, Tata PR, Brauzaskus K, Choi SH, Crooke AK, Zhang B, Solomon GM, Turner B, Bipler H, Harrington J, Lapey A, Channick C, Keyes C, Freund A, Artandi S, Mense M, Rowe S, Engelhardt JF, Hsu YC, Rajagopal J (2016) Dual SMAD signaling inhibition enables long-term expansion of diverse epithelial basal cells. Cell Stem Cell 19:217–231

Munipalli SB, Mounika MR, Aisha J, Yenugu S (2019) Tlr1-13, Nod1/2 and antimicrobial gene expression in the epididymis and testes of rats with alloxan-induced diabetes. Andrologia 51:e13437

Murashima A, Kishigami S, Thomson A, Yamada G (2015) Androgens and mammalian male reproductive tract development. Biochim Biophys Acta 1849:163–170

Murashima A, Miyagawa S, Ogino Y, Nishida-Fukuda H, Araki K, Matsumoto T, Kaneko T, Yoshinaga K, Yamamura K, Kurita T, Kato S, Moon AM, Yamada G (2011) Essential roles of androgen signaling in Wollfian duct stabilization and epididymal cell differentiation. Endocrinology 152:1640–1651

Ni Y, Zhou Y, Chen WY, Zheng M, Yu J, Li C, Zhang Y, Shi QX (2009) HongReES1, a cauda epididymis-specific protein, is involved in capacitation of guinea pig sperm. Mol Reprod Dev 76:984–993

Oh J, Woo JM, Choi E, Kim T, Cho BN, Park Y2, Kim YC, Kim DH, Cho C (2005) Molecular, biochemical, and cellular characterization of epididymal ADAMs, ADAM7 and ADAM28. Biochem Biophys Res Commun 331:1374–1383

Oliveira CA, Carnes K, Franca LR, Herlo M, Hess RA (2005) Aquaporin-1 and -9 are differentially regulated by oestrogen in the efferent ductule epithelium and initial segment of the epididymis. Biochim Biophys Acta 1730:22–30

Palladino MA, Powell JD, Korah N, Herlo M (2004) Expression and localization of hypoxia-inducible factor-1 subunits in the adult rat epididymis. Biol Reprod 70:1121–1130
Pelletier RM (1994) Blood barriers of the epididymis and vas deferens act asynchronously with the blood barrier of the testis in the mink (Mustela vison). Microsc Res Tech 27:333–349

Pierucci-Alves F, Midura-Kielia MT, Fleming SD, Schultz BD, Kielia PR (2018) Transforming growth factor beta signaling in denudoric tissue is required for immunotolerance to sperm in the epididymis. Front Immunol 9:1882

Pietrement C, Sun-Wada GH, Silva ND, McKee M, Marshansky V, Brown D, Futai M, Breton S (2006) Distinct expression patterns of different subunit isoforms of the V-ATPase in the rat epididymis. Biol Reprod 74:185–194

Pinel L, Mandon M, Cyr DG (2019) Tissue regeneration and the epididymal stem cell. Andrology 7:618–630

Pinel L, Cyr DG (2021) Self-renewal and differentiation of rat epididymal basal cells using a novel in vitro organoid model. Biol Reprod 105:987–1001

Ranjan M, Lee O, Cottone G, Mirzaei Mehrabad E, Spike BT, Zeng Z, Yadav S, Chatterton R, Kim JJ, Clare SE, Khan SA (2021) Progerosterone receptor antagonists reverse stem cell expansion and the paracrine effectors of progerosterone action in the mouse mammary gland. Breast Cancer Res 23:8

Raudvere U, Kolberg L, Kuzmin I, Arak T, Adler P, Peterson H, Vilo J (2019) g:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). Nucleic Acids Res 47:W191–W198

Ribeiro CM, Ferreira LG, Thimoteo DS, Smith LB, Hinton BT, Avellar MC (2017) Novel androgen-induced activity of an antimicrobial beta-defensin: regulation of Wolffian duct morphogenesis. Mol Cell Endocrinol 442:142–152

Ribeiro CM, Silva EJ, Hinton BT, Avellar MC (2016) beta-defensins and the epididymis: contrasting influences of prenatal, postnatal, and adult scenarios. Asian J Androl 18:323–328

Robaire B, Hinton BT (2015) The Epididymis. In: Plant T, Zeleznik A (eds) Knobil and Neill’s reproductive physiology. Academic Press, New York, pp 691–771

Robaire B, Hermo L (1988) Diferent ducts, epididymis, and vas deferens: structure, functions and their regulation. In: Neill JK, E (ed) The Physiology of Reproduction. Raven Press, pp 999–1080

Rock JR, Randell SH, Hogan BL (2010) Airway basal stem cells: a perspective on their roles in epithelial homeostasis and remodeling. Dis Model Mech 3:545–556

Rodrigues A, Queiroz DB, Honda L, Silva EJ, Hall SH, Avellar MC (2008) Activation of toll-like receptor 4 (TLR4) by in vivo and in vitro exposure of rat epididymis to lipopolysaccharide from Escherichia Coli. Biol Reprod 79:1135–1147

Rodriguez CM, Kim Y.J.; Hinton, B.T. (2002) The development of the epididymis. In: Robaire BH, B.T. (ed) The Epididymis: From Molecules to Clinical Practice. Kluwer Academic/Plenum Publishers, New York, pp 251–268

Roomans GM (2010) Tissue engineering and the use of stem/progenitor cells for airway epithelial repair. Eur Cell Mater 19:284–299

Saito K, Kawakami S, Okada Y, Takazawa R, Koga F, Kageyama Y, Kihara K (2006) Spatial and isoform specific p63 expression in the male human urogenital tract. J Urol 176:2268–2273

Santos CP, Lapi E, Martinez de Villarreal J, Alvaro-Espinosa L, Fernandez-Barral A, Barbachano A, Dominguez O, Laughney AM, Megtas D, Munoz A, Real FX (2019) Urothelial organoids originating from Cd49f(high) mouse stem cells display Notch-dependent differentiation capacity. Nat Commun 10:4407

Scheer H, Robaire B (1980) Steroid delta 4–5 alpha-reductase and 3 alpha-hydroxy steroid dehydrogenase in the rat epididymis during development. Endocrinology 107:948–953

Schuijers J, Clevers H (2012) Adult mammalian stem cells: the role of Wnt, Lgr5 and R-spondins. EMBO J 31:2685–2696

Seishima R, Leung C, Yada S, Murad KBA, Tan LT, Hajihamoohideen A, Tan SH, Itoh H, Murakami K, Ishida Y, Nakamizo S, Yoshikawa Y, Wong E, Barker N (2019) Neonatal Wnt-dependent Lgr5 positive stem cells are essential for uterine gland development. Nat Commun 10:5378

Shanker E, Song K, Corum SL, Bane KL, Wang H, Kao HY, Danielpour D (2016) A signaling network controlling androgenic repression of c-Fos protein in prostate adenocarcinoma cells. J Biol Chem 291:5512–5526

Shum WW, Da SN, Belleaune C, McKeever M, Brown D, Breton S (2011) Regulation of V-ATPase recycling via a RhoA- and ROCKII-dependent pathway in epididymal clear cells. Am J Physiol Cell Physiol 301:C31–C43

Shum WW, Da Silva N, McKeever M, Smith PJ, Brown D, Breton S (2008) Transepithelial projections from basal cells are luminal sensors in pseudostratified epithelia. Cell 135:1108–1117

Sipila P, Bjorkgren I (2016) Segment-specific regulation of epididymal gene expression. Reproduction 152:R91–99

St-Pierre N, Dufresne J, Rooney AA, Cyr DG (2003) Neonatal hypothyroidism alters the localization of gap junctional protein connexin 43 in the testis and messenger RNA levels in the epididymis of the rat. Biol Reprod 68:1232–1240

Sun-Wada GH, Wada Y (2015) Role of vacuolar-type proton ATPase in signal transduction. Biochim Biophys Acta 1847:1166–1172

Sun EL, Flickinger CJ (1979) Development of cell types and of regional differences in the postnatal rat epididymis. Am J Anat 154:27–55

Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, Simonovic M, Doncheva NT, Morris JH, Bork P, Jensen LJ, Mering CV (2019) STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res 47:D607–D613

Traustadottir GA, Lagoni LV, Ankerstjerne LBS, Bisgaard HC, Jensen CH, Andersen DC (2019) The imprinted gene Delta like non-canonical Notch ligand 1 (Dlk1) is conserved in mammals, and serves a growth modulatory role during tissue development and regeneration through Notch dependent and independent mechanisms. Cytokine Growth Factor Rev 46:17–27

Turner TT, Johnston DS, Finger JN, Jelinsky SA (2007) Differential gene expression among the proximal segments of the rat epididymis is lost after efferent duct ligation. Biol Reprod 77:165–171

Turunen HT, Sipila P, Pujianto DA, Damdimopoulos AE, Bjorkgren J, Hubianiemi I, Poutanen M (2011) Members of the murine Pate family are predominantly expressed in the epididymis in a segment-specific fashion and regulated by androgens and other testicular factors. Reprod Biol Endocrinol 9:128

Viger RS, Robaire B (1995) Steady state steroid 5 alpha-reductase messenger ribonucleic acid levels and immunocytochemical localization of the type 1 protein in the rat testis during postnatal development. Endocrinology 136:5409–5415

Viger RS, Robaire B (1996) The mRNAs for the steroid 5 alpha-reductase isozymes, types 1 and 2, are differentially regulated in the rat epididymis. J Androl 17:27–34

Wang A, Zhang Q, Wang Y, Li X, Li K, Li Y, Wang J, Li L, Chen H (2021) Inhibition of Gabrp reduces the differentiation of airway epithelial progenitor cells into goblet cells. Exp Ther Med 22:720

Wang Q, Lan Y, Cho ES, Maltby KM, Jiang R (2005) Odd-skipped related 1 (Odd 1) is an essential regulator of heart and urogenital development. Dev Biol 288:582–594

Williams K, Frayne J, Hall L (1998) Expression of extracellular glutathione peroxidase type 5 (GPX5) in the rat male reproductive tract. Mol Hum Reprod 4:841–848

Wissel S, Harzer H, Bonnay F, Burkard TR, Neumann RA, Knoblach JA (2018) Time-resolution transcriptomics in neural stem cells identifies a v-ATPase/Notch regulatory loop. J Cell Biol 217:3285–3300

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Xu B, Turner SD, Hinton BT (2018) Alteration of transporter activities in the epididymides of infertile initial segment-specific Pten knockout mice. Biol Reprod :99:536–545
Xu B, Washington AM, Hinton BT (2014) PTEN signaling through RAF1 proto-oncogene serine/threonine kinase (RAF1)/ERK in the epididymis is essential for male fertility. Proc Natl Acad Sci U S A 111:18643–18648
Xu B, Yang L, Lye RJ, Hinton BT (2010) p-MAPK1/3 and DUSP6 regulate epididymal cell proliferation and survival in a region-specific manner in mice. Biol Reprod 83:807–817
Zhen W, Li P, He B, Guo J, Zhang YL (2009) The novel epididymis-specific beta-galactosidase-like gene Glb1l4 is essential in epididymal development and sperm maturation in rats. Biol Reprod 80:696–706
Zhou Y, Zheng M, Shi Q, Zhang L, Zhen W, Chen W, Zhang Y (2008) An epididymis-specific secretory protein HongrES1 critically regulates sperm capacitation and male fertility. PLoS ONE 3:e4106
Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, Benner C, Chanda SK (2019) Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. Nat Commun 10:1523

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