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HNF1 regulates critical processes in the human epididymis epithelium

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Abstract

The luminal environment of the epididymis participates in sperm maturation and impacts male fertility. It is dependent on the coordinated expression of many genes encoding proteins with a role in epithelial transport. We identified *cis*-regulatory elements for critical genes in epididymis function, by mapping open chromatin genome-wide in human epididymis epithelial (HEE) cells. Bioinformatic predictions of transcription factors binding to the regulatory elements suggested an important role for hepatocyte nuclear factor 1 (HNF1) in the transcriptional program of these cells. Chromatin immunoprecipitation and deep sequencing (ChIP-seq) revealed HNF1 target genes in HEE cells. In parallel, the contribution of HNF1 to the transcriptome of HEE cells was determined by RNA-seq, following siRNA-mediated depletion of both HNF1 α and HNF1 β transcription factors. Repression of these factors caused differential expression of 1892 transcripts (902 were downregulated and 990 upregulated) in comparison to a non-targeting siRNA. Differentially expressed genes with HNF1 ChIP-seq peaks within 20kb were subject to gene ontology process enrichment analysis. Among the most significant pathways associated with down-regulated genes were epithelial transport of water, phosphate and bicarbonate, all critical processes in epididymis epithelial function. Measurements of intracellular pH (pHi) confirmed a role for HNF1 in regulating the epididymis luminal environment.

Keywords

Epididymis epithelium; *cis*-regulatory elements; HNF1; transcriptional network

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Conflict of interest statement

None declared.

Introduction

The luminal environment in the epididymis is established and maintained by cellular processes in the epithelial layer lining the duct. These functions provide a milieu that is critical for sperm maturation, protection and storage, and hence male fertility. Many genes encode proteins that are required for the normal properties of the luminal fluid, which include ionic concentration, pH, and protein/peptide composition. For example, mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), cAMP-activated chloride channel, which is highly expressed in the male genital duct epithelium, are associated with male infertility (1-4). The network of transcription factors that coordinate expression of important epithelial transport proteins in the human epididymis is not fully characterized, though several mouse models provide clues. For example infertility of male mice that lack the Foxi1 transcription factor is attributed to misregulation of its target genes encoding the H⁺-ATPase proton pump, carbonic anhydrase II and the chloride/bicarbonate transporter pendrin (SLC26A4), all of which contribute to luminal fluid composition (5). Similarly, infertility in Estrogen Receptor (ESR1)-null mice is attributed to loss of regulation of a key target gene, sodium/hydrogen exchanger 3 (NHE3), in efferent ductules (6). However, many other transcription factors that are pivotal to normal epididymis epithelium function remain to be characterized. We identified hepatocyte nuclear factor 1 (HNF1) as a key epididymis epithelial transcription factor by bioinformatic analysis of genome-wide open chromatin in immature epididymis epithelial cells. The HNF1 motif was over-represented in these cells (2,7) and we show here that it is also over-represented in adult human caput epididymis epithelial (HEE) cells (8). HNF1 α and HNF1 β recognize the same DNA consensus sequence, which they occupy as homodimers or heterodimers. Both factors share a high degree of homology in their DNA binding and dimerization (N-terminus) regions but have a more divergent C-terminal transactivation domain. HNF1 β is essential for the generation of a functional urogenital tract in mice (9), and genital tract abnormalities are evident in humans with recessive mutations in HNF1 β (10,11). Though the functions of HNF1 α and HNF1 β are well studied in epithelia from several other tissues (liver, kidney, intestine, and pancreas, reviewed in (12) their role in the adult epididymis epithelium remains unexplored. The human epididymis has 3 functionally distinct regions: the head (caput), body (corpus) and tail (cauda) with different transcriptomes (13-16). The caput and cauda luminal environments are probably the most relevant for sperm maturation (17,18). Here we investigate the role of HNF1 in regulating gene expression and important biological processes in the caput epididymis epithelium using primary adult HEE cells (8). Following siRNA-mediated depletion of HNF1 α and HNF1 β we determined the contribution of these factors to the caput HEE cell transcriptome. Next, we integrated these data with genome-wide occupancy of HNF1 in caput epithelial cells, identified by ChIP-seq. Together these datasets reveal multiple novel HNF1-target genes and pathways of direct relevance to epididymis function and sperm maturation.

Materials and Methods

Cell culture

Human epididymis tissue was obtained with Institutional Review Board approval from three patients aged 22, 27 and 32 years, undergoing inguinal radical orchiectomy for a clinical diagnosis of testicular cancer. None of the epididymides had extension of the testicular cancer, and all were placed in normal saline solution until further processing. Epithelial cells from the caput were isolated and established in culture as described previously (8). The majority of these cells express CFTR protein (8), consistent with principal cells (19).

RNAi-mediated dual-knockdown of HNF1-alpha and HNF1-beta

Cells at 40-50% confluence were transfected with a non-targeting control siRNA (40nM, sc-37007) or both HNF1alpha- and HNF1beta- targeting siRNA (20nM each, sc-35567 and sc-37928, Santa Cruz Biotechnology (SCB)) using Lipofectamine RNAiMax reagent (Life Technologies (LT)). At 72h post transfection, the cells were washed in PBS and immediately harvested for RNA extraction using TRIzol® (LT) or for whole cell lysate using NET Buffer (20). When required, cells were subjected to cellular fractionation to isolate cytoplasmic and nuclear fractions as previously described (21).

RNA-seq

RNA libraries were prepared from 2 µg of total RNA from three replicates (from one donor), of control- and HNF1-siRNA transfected cells. RNA quality was confirmed by Nanodrop measurement of OD 260/280 and 260/230 ratios. RNA-seq libraries were prepared using the TruSeq RNA Sample Preparation Kit v2 per the manufacturer's Low-Throughput protocol (Illumina). The libraries were sequenced on Illumina HiSeq2500 machines. Data was analyzed using TopHat and Cufflinks (22). All data are deposited at GEO (<http://www.ncbi.nlm.nih.gov/geo/Accession> # pending).

DNase-seq

DNase-seq libraries were prepared from DNase I-digested caput HEE cells from two individual donors and sequenced separately on an Illumina Hi-Seq machine as previously described (23,24). DNase peak calls from these two libraries were combined (GEO: GSE Accession # pending) and used for further analysis. Comparative analysis of DNase-seq data used five cell types (FibroP, GM12878, K652, HepG2 and HUVEC) (25) from the Encyclopedia of DNA Elements Consortium (<http://genome.ucsc.edu/ENCODE/>). All genome data coordinates refer to the hg19 genome and are available at GEO:GSE30227 for the 5 cell types and GSE Accession # pending for the caput data. Data analysis was performed as described in (7,24,26). DNase-seq peaks from the caput HEE cells that were not seen in any other data sets were designated caput-selective sites, while those that overlapped in all cell types were called caput-ubiquitous sites.

ChIP-seq

Chromatin was prepared from caput HEE cells as described previously (27). ChIP-seq for HNF1 was performed in 2 biological replicates (donors) by standard protocols (28). 10ug of

HNF1 antibody [Santa Cruz Biotechnology (SCB) sc-8986x] and $\sim 2 \times 10^7$ cells, and libraries were prepared as described previously (28). Libraries were sequenced on Illumina HiSeq2500 machines. FASTQ files were aligned to the hg19 version of the human genome using Bowtie (29) and ChIP-seq peaks were identified using HOMER (30) with a false discovery rate (FDR) of 0.1%. Peaks from both replicates were intersected using BEDTools and only the 10,480 sites found in both replicates were used for further analysis. The ChIP-seq data sets are available on GEO (GSE: Accession # pending). Identification of transcription factor motifs in the data set and peak annotation based on the nearest gene were also performed using HOMER. GO terms enriched among the nearest genes in the ChIP-seq data were determined using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (31,32).

Real-time quantitative PCR (RT-qPCR)

The TaqMan® reverse transcription kit (LT) was used to make cDNA from total RNA and RT-qPCR then used to measure gene expression levels (primers in Suppl. Table S-I).

Western Blotting

Protein lysates were separated on SDS/PAGE and western blotting performed as described previously (20) using antibodies specific for HNF1 (sc-8986, 1:400), Histone H3 (ab1791, Abcam, 1:5000) and β -tubulin (T4026, Sigma, 1:8000).

Intracellular pH measurements

Caput HEE cells were cultured in collagen-I coated 96-well plates (black walled, clear bottom, Corning). At 2 days post confluence, cells were loaded for 30 min with 2.5 μ M 2', 7'-bis-(2- carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM, Life Technologies] pH-sensitive fluorescent dye, in 1 $\times$ Hanks' Balanced Salt Solution (HBSS) solution at pH 7.4 and 37°C. Cells were washed twice at 37°C and bathed for up to 13 min in Ringers buffer (5 mM glucose, 5 mM potassium gluconate, 1 mM calcium gluconate, 1 mM MgSO₄, 10 mM HEPES, 2.5 mM NaH₂PO₄, 130 mM NaCl, 25 mM NaHCO₃, pH 7.4). Repetitive dual fluorescence readings were recorded in a SpectraMax M5 fluorescence plate reader (Molecular Devices) at 37°C every 20/30 seconds (excitation 444nm and 488nm, emission 535nm). 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) was used at 10- 200 μ M. Fluorescence ratio values were converted to pH_i by comparison with values from a calibration curve performed by equilibrating BCECF-loaded cells in 145mM KCl containing 10 μ M nigericin (Sigma) at pH 6.5, 7.0, 7.5, 8.0 and 8.4.

Statistical analysis

Data are expressed as mean $\pm$ SD. GraphPad Prism 6 software was used to analyze statistical differences between experimental groups using students t-test and values of P<0.05 were considered significant.

Results

HNF1 is predicted to have an important role in adult human epididymis epithelial (HEE) cells

We first used bioinformatics tools to ask whether HNF1 likely regulates important biological processes in adult HEE cells, particularly within the caput. This hypothesis arose from our previous observations on immature HEE cells (2), in which the HNF1 motif was over-represented in open chromatin (7). Cis-acting regulatory elements controlling gene expression are frequently found within regions of open chromatin, which coincide with DNase I hypersensitive sites (DHS). To investigate further the potential role of HNF1, we inspected genome-wide DNase I-hypersensitivity data in adult caput HEE cells (33). To identify caput-selective sites, we compared the DHS from caput HEE cells with DHS from five different human cell types, generated by the ENCODE consortium as described previously for the immature HEE cells (7) and recorded the non-overlapping sites. Of the total 128,573 sites in caput HEE cells, 37,565 (~29 %) were caput-selective and 31,248 (~24 %) were ubiquitous, being found in all the other ENCODE cell lines.

Next we used the Clover application (34) to search for over-represented transcription factor binding motifs within caput HEE intergenic open chromatin peaks (Suppl. Table S-II). We chose to focus on these sites rather than promoters as our previous analyses in several cell types showed that cell-specific transcription factor occupancy was more evident here (7,26). Within these categories, data were analyzed in three groups: all DHS, caput-selective DHS and ubiquitous DHS. The HNF1 binding motif was among the most over-represented in caput HEE-specific intergenic sites, thus reinforcing the predicted importance of HNF1 in the biology of these cells.

HNF1 shows differential expression along the human epididymis epithelium

Many functional pathways in the human epididymis show regional distribution, with the caput epithelium being distinct from both the corpus and cauda, which are more similar (16). HNF1 α and HNF1 β protein levels in caput, corpus and cauda HEE cells were analyzed by western blot (Fig. 1A). HNF1 α (90 kDa) was only evident in caput HEE cells. HNF1 β (70 kDa) was much more abundant than HNF1 α in the caput HEE cells but was also seen at much lower levels in corpus or cauda cells (Fig. 1A). These results were confirmed in HEE cells cultured from three donors (not shown). Cellular fractionation of the caput HEE cells showed that HNF1 β was more abundant in the nuclear fraction than the cytoplasm (Fig. 1B) consistent with its role as an important transcription factor in these cells.

Genome wide occupancy of HNF1 in Caput HEE cells

Having established that HNF1 α/β are expressed in caput HEE cells and are predicted to occupy *cis*-regulatory elements in these cells we next used ChIP-Seq to examine the genome-wide binding of HNF1 in caput HEE cells from two donors.

Peaks of HNF1 occupancy were seen at 17,328 and 12,823 sites in the two replicas, and of these, 10,480 peaks were shared in both datasets and were used for further analysis. As we observed for several other tissue-specific transcription factors (28,35) the majority of the

HNF1 peaks localized within intergenic regions (48%) and intronic regions (47%) rather than at promoter (3%) regions (Fig. 2A). Next, we overlapped the HNF1 ChIP-seq peaks in caput HEE cells with the open chromatin data, generated by DNase-seq. HNF1 occupied sites showed increased DNase hypersensitivity, which confirms that HNF1 binds to open chromatin (Fig. 2B). A *de novo* motif analysis was performed in a 200bp window centered on the ChIP-seq peaks with HOMER, revealing in addition to the HNF1 motif (70%), motifs for activator protein 1 (AP-1) (24%), Homeobox-containing protein 1 (HMBOX1) (17%) and V-Ets Avian Erythroblastosis Virus E26 Oncogene Homolog (ERG) (18%) among others (Fig. 2C).

Despite its inaccuracies, we then used the nearest gene method to identify potential targets of the HNF1 ChIP-seq peaks. A gene ontology process enrichment analysis by DAVID was then applied to the gene list (31,32). The top 13 most significant processes are shown in (Fig. 2D) and a more extensive list is shown in Suppl. Table S-III. Among the most significant processes are those relating to plasma membrane function, which include ion channels, exchangers and many other genes encoding proteins that are critical for establishing and maintaining the epididymis luminal environment. These include GO: 0044459 (plasma membrane part; $P = 4.4 \times 10^{-21}$), GO:0031226 (intrinsic component of plasma membrane, $P = 5.3 \times 10^{-14}$) and GO:0005887 (integral component of plasma membrane, $P = 3.1 \times 10^{-13}$). More specific processes include ion transport (GO:0006811, $P = 2.3 \times 10^{-3}$) and potassium transport (GO:0030955, $P = 1.2 \times 10^{-5}$). Also significant are known HNF1-regulated processes such as urogenital tract development and tube morphogenesis (GO:0001655, $P = 2 \times 10^{-7}$ and GO:0035295, $P = 1.7 \times 10^{-10}$), and enzyme linked receptor protein/kinase intracellular signaling pathways (GO:0007167, $P = 5.3 \times 10^{-13}$ and GO:0007169, $P = 9.2 \times 10^{-11}$) that regulate the expression of genes involved in cellular responses. Of interest are cellular responses such as cell proliferation and apoptosis (GO:0042127, $P = 2.8 \times 10^{-11}$ and GO:0010941, $P = 7.7 \times 10^{-6}$) and cell migration (GO: 0048870, $P = 1.2 \times 10^{-9}$ and GO:0016477, $P = 5.9 \times 10^{-9}$).

HNF1 ChIP followed by qPCR was used to validate the ChIP-seq results (Fig. 2E). We chose five genes with HNF1 ChIP-seq peaks either at their promoter, within introns or in nearby intergenic regions and measured HNF1 enrichment over IgG. These included solute carrier family 4 (anion exchanger) members -2 and -3 (SLC4A2, promoter and SLC4A3, intergenic), SLC4 sodium bicarbonate cotransporter member 4 (SLC4A4, intron 1), PDZ domain containing 1 (PDZK1, intergenic) and polycystic kidney and hepatic disease 1 (PKHD1, promoter) (Fig. 2E). In all cases we observed at least 3 fold enrichment over IgG.

The role of HNF1 in regulating the transcriptome of Caput HEE cells

To investigate the contribution of HNF1 in controlling gene expression in caput cells we performed siRNA-mediated depletion of HNF1 α and HNF1 β together, followed by RNA-seq analysis. Three replicas of caput cells were transfected with the specific siRNAs or with a non-targeting control siRNA. Efficacy of the siRNA-mediated reduction in HNF1 protein (~62% for HNF1 α and ~80% for HNF1 β) is shown by western blot in Fig. 3A,B. RNA-seq libraries were generated for each replica and six libraries sequenced together on one lane of a HiSeq 2500, yielding $\sim 2.6\text{-}2.9 \times 10^7$ reads per sample (Suppl. Table S-IV). A Multi-

Dimensional Scaling plot shows that the control- and HNF1-siRNA-treated samples clustered together as two distinct groups (Suppl. Fig. S2). RNA-seq data were analyzed by TopHat and Cufflinks (22) to obtain estimates of the expression levels of transcripts. HNF1 α /HNF1 β -depletion in caput HEE cells differentially regulated the expression of 1892 transcripts of which 902 were repressed and 990 were activated, by at least 1.4-fold (FPKM 0.3) (Suppl. Table S-V).

Next, to validate the RNA-seq data, RT-qPCR was used to measure transcript levels in independent samples of HNF1 α / β or negative control siRNA-treated caput HEE cells (Fig. 3C). Of particular relevance to the role of HNF1 in coordinating ion transport processes in the epididymis, were genes encoding ion channels and exchangers. We first confirmed the repression after HNF1 α / β depletion of genes involved in bicarbonate transport: *SLC4A2* ($P < 0.01$) and *SLC4A3* ($P < 0.001$), *SLC4A4* ($P < 0.001$) and *SCL4A7* ($P < 0.01$). We then examined transcript levels of genes involved in water reabsorption: aquaporin -1 -9 and -11 (*AQP1*, $P < 0.0001$ and *AQP11*, $P < 0.01$, *AQP9* was not significantly repressed). Next, we evaluated genes involved in other epithelial transport process, which were significantly repressed by HNF1 α / β depletion: solute carrier family 26 (anion exchanger), Member 11 (*SLC26A11*, $P < 0.01$), transient receptor potential cation channel, subfamily V, member 4 (*TRPV4*, $P < 0.001$), solute carrier organic anion transporter family, member 4C1 (*SLCO4C1*, $P < 0.0001$), ATPase, Na⁺/K⁺ transporting, alpha 1 polypeptide (*ATP1A1*, $P < 0.01$); ATPase, H⁺ transporting V0 subunit e2 (*ATP6V0E2*, and $P < 0.001$), PDZ domain containing 1 (*PDZK1*, $P < 0.0001$). Finally, other genes with diverse functions in the caput HEE cells that were predicted from the RNA-seq data to be down-regulated after HNF1 α / β knockdown: polycystic kidney and hepatic disease 1 (*PKHD1*, $P < 0.01$), annexin A4 (*ANXA4*, $P < 0.001$) and Defensin, beta 1 (*DEFB1*, $P < 0.0001$). These data suggest the RNA-seq data are robust and confirm the important role of HNF1 α / β in regulating genes involved in the controlling the luminal environment in the caput epididymis.

Identification of direct target of HNF1 in caput HEE cells

Next we intersected the Entrez Gene ID for genes with HNF1 ChIP-seq peaks within 20kb of the locus and those that were differentially expressed following HNF1 α / β depletion. These 433 genes were then subjected to a gene ontology pathway analysis by DAVID (31,32). Among the 271 downregulated genes following HNF1 depletion were many within functionally relevant processes (Suppl. Table S-VI). These included epithelial transport processes (GO:0006811, $P = 9.5 \times 10^{-6}$ and GO:0055085, $P = 1.1 \times 10^{-4}$) that regulate the epididymal luminal environment. Also important to epididymis epithelial function were cell adhesion processes (GO:0007155, $P = 4.2 \times 10^{-5}$ and GO:0022610, $P = 4.3 \times 10^{-5}$). In contrast, among the 162 upregulated genes following HNF1 depletion were many with important roles in cellular responses such as proliferation (GO:0008284, $P = 2.2 \times 10^{-4}$ and GO:0042127, $P = 1.1 \times 10^{-3}$), cell migration (GO:0030335, $P = 1.1 \times 10^{-5}$ and GO:0051272, $P = 2.1 \times 10^{-5}$) and responses to hypoxia (GO:0070482, $P = 1.3 \times 10^{-3}$) (Suppl. Table S-VII).

Since we and others showed that the majority of tissue-specific transcription factor binding sites that coincide with peaks of open chromatin, lie outside of gene promoters, in intronic and intergenic regions (7,24,26,35), it was relevant to examine HNF1 occupancy at a

number of specific loci in caput HEE cells. The location of HNF1 ChIP-seq peaks at the genomic loci of four functionally important genes that were differentially expressed following HNF1-knockdown are shown in Fig. 4. Arrows mark the HNF1 ChIP-seq peaks near the promoter of *SLC4A2* (Fig. 4A), 20 kb downstream of the *SLC4A3* gene (Fig. 4B), in introns 1, 6 and 20 of the *SLC4A4* gene (Fig. 4C) and in the promoter and intron 4 of the *PKHD1* gene (Fig. 4D arrows). Also shown on each panel are the relevant DNase-Seq data.

HNF1 depletion alters intracellular pH in caput HEE cells

Our RNA-seq data following HNF1 α/β depletion showed that several anion exchanger genes are direct targets of HNF1. Since bicarbonate transporters are known to regulate intracellular pH (pHi), we measured the pHi in caput HEE cells transfected with control or HNF1 α/β specific siRNAs. First, the high K⁺/nigericin method was applied to calibrate the pHi of the cells and the pH values of calibration were set as 6.5, 7.0, 7.5, 8.0 and 8.4. We recorded the ratios of intracellular fluorescence intensity of different emission wavelengths, which represent the pHi values, and made the linear calibration (Fig. 5A). Depletion of HNF1 significantly ($P < 0.05$) reduced pHi by 0.05 pH units (Fig. 5B, inset shows effective HNF1 knockdown by western blot). A similar reduction in pHi was also observed in caput HEE cells exposed to the anion exchanger inhibitor, DIDS (200 μ M, Fig. 5C).

Discussion

Combining genome-wide occupancy data for transcription factors, with transcriptome analysis is a compelling approach to unravel transcriptional networks governing tissue-specific gene expression. We took this route to decipher the mechanisms controlling the differentiated function of the human epididymis epithelium, which has a pivotal role in male fertility. By using a combination of open chromatin mapping and bioinformatic predictions of transcription factor occupancy we identified multiple transcription factors that likely contribute to the transcriptional network in these cells. HNF1 α/β were particularly relevant factors to pursue based on their role in the kidney and genital tract (reviewed in (12)). Though in an analysis of gene expression in intact epididymis tissue segments by microarray (14), neither HNF1 α nor HNF1 β showed a regional distribution, this likely reflects the minor contribution of the epithelium to the whole tissue gene expression profile. We previously showed that HNF1 β is much more abundant than HNF1 α throughout the epididymis epithelium, though both show strong regional expression (16). Here, we confirm by western blot that HNF1 β is much more abundant than HNF1 α in caput HEE cells and so our data primarily relate to the functions of HNF1 β .

A bioinformatic analysis of open chromatin in caput HEE cells identified predicted binding sites not only for HNF1, but also for Runt-related transcription factor 1 (RUNX1), TEA Domain Family Member 1 (TEAD1), SMAD4, E74-Like Factor 3 (ELF3), and paired box 2 and 8 (PAX2 and PAX8). RUNX1 is the α subunit of the core binding factor (CBF) complex that binds many enhancers and promoters, though it has mainly been studied in hematopoiesis (reviewed in (36)). TEAD1 is involved in the Hippo signaling pathway, which dictates organ size (37). SMAD4 is an important downstream effector of TGF- β signaling that regulates many cellular functions (38) including blood epididymis barrier

permeability (39). ELF3 is thought to participate in epithelial cell differentiation and tumorigenesis (reviewed in (40)). PAX2 and PAX8 are required for initiation of mesenchymal-epithelial transition and tube formation during urogenital tract development, (41,42). We recently showed that PAX2 coordinates gene expression in a human epididymis epithelial cell line (24). Of interest, PAX2 and PAX8 are, like HNF1, more abundant in the caput HEE cells than in corpus or cauda cells (16). HNF1 is part of the transcription factor complex that regulates expression of the *CFTR* gene in intestinal epithelial cells (43,44). It probably has a similar role in the caput epithelium, since we observe HNF1 ChIP-seq peaks overlapping known *cis*-regulatory elements in the locus.

To date, most of the literature on HNF1 is focused on the impact of its binding to gene promoters. However, our data show that the majority of HNF1 binding sites in caput HEE cells are intronic or intergenic. This implies an important role for HNF1 in regulating gene expression through distal *cis*-regulatory elements. Consistent with the open chromatin data discussed above, our HNF1 ChIP-seq data reveal that peaks of HNF1 occupancy are also enriched for other transcription factor binding motifs, which are potentially relevant to epididymis function. These include AP-1, HMBX1, ERG, CCAAT/enhancer-binding protein (C/EBP)-like protein and RUNX2. HNF1 was previously reported to cooperate with AP1 (C-FOS/C-JUN) in liver injury (45). HMBX1 is phylogenetically related to HNF1 as both are homeobox-containing transcription factors (46). The role of the ETS family factor ERG is less clear in the caput HEE cells, though chromosomal translocations that generate several fusion proteins with ERG are known to occur in prostate cancer (47).

It was of interest to compare our HNF1 ChIP-seq and RNA-seq data in adult caput HEE cells with gene expression data from other tissues. In the kidney, HNF1 targets include genes encoding the transmembrane calcium permeable cation channel (polycystic kidney disease 2 (*PKD2*)) which is involved in calcium signaling and the receptor-related protein, fibrocystin (*PKHD1*) (48); also other transport proteins (FXFD Domain Containing Ion Transport Regulator 2 (*FXFD2*) (49) and solute carrier family 17 (Organic Anion Transporter), Member 1 (*SLC17A1*) (50)). We identified peaks of HNF1 occupancy in *PKD2* (intronic) and *PKHD1* (promoter), genes that are mutated in autosomal dominant- and autosomal recessive- forms of polycystic kidney disease, respectively (48,51,52). We also showed that depletion of HNF1 significantly repressed *PKHD* expression. The *FXFD2* and *SLC17A1* genes encode the γ -subunit of the sodium/potassium-transporting ATPase (Na^+/K^+ -ATPase) and the sodium-dependent phosphate transport protein 1 (NPT1), respectively, both of which show HNF1 occupancy peaks at their promoters and are down regulated in HNF1-depleted caput HEE cells. Another ion transporter, which we showed previously to be HNF1-regulated in intestinal cells, is CFTR (43,44,53). In caput HEE cells both intronic (1 and 11, legacy nomenclature) and intergenic (-44 kb and + 15.6 kb) HNF1 ChIP-seq peaks were seen at the *CFTR* locus, corresponding to known *cis*-regulatory elements for the gene (reviewed in (35)).

Our data show that HNF1 regulates many genes contributing to ion and water transport across the epididymis epithelium. These genes express bicarbonate transporters at both the basal and apical surfaces of the epithelium that facilitate a low bicarbonate and acidic lumen to preserve sperm motility (reviewed in (54) and (18)). As examples, *SLC4A2* and *SLC4A3*

encode two Na⁺-independent anion exchangers (AE2 and AE3, respectively) that mediate electroneutral chloride/bicarbonate exchange. *SLC4A4* encodes the electrogenic (NBCe1) sodium bicarbonate cotransporter, while PDZK1 encodes NHERF3, a scaffolding protein that mediates the localization and activity several exchangers and ion channels. These include the SLC26A6 chloride/bicarbonate exchanger, which also transports oxalate and sulfate, and CFTR (55,56). Consistent with these data are our measurements of pH_i after HNF1 α/β depletion, which indicate a subset of anion exchangers in the caput HEE cells are HNF1-regulated bicarbonate transporters.

Relatively high potassium levels in the epididymal lumen are thought to facilitate sperm quiescence (57). In caput HEE cells, we identify both intronic and intergenic HNF1 binding sites at multiple genes encoding potassium channels. Of note, in addition to *FXYD2* (discussed above) is *ATP1A1*, which encodes the α subunit of the Na⁺/K⁺-ATPase, has nearby HNF1 ChIP-seq peaks and is downregulated in HNF1-depleted caput HEE cells. ATP1A1 maintains Na⁺ and K⁺ electrochemical gradients across epithelia, for both osmoregulation and the sodium-coupled transport of a variety of organic and inorganic molecules.

Phosphate levels are elevated in the vas deferens (24mM) (58). In caput HEE cells, we identified HNF1 binding sites in the promoters of solute carrier family 17 member A1 (*SLC17A1*) and family 34 member A2 (*SLC34A2*) and in the first intron of family 20 member A2 (*SLC20A2*). These genes encode sodium-phosphate transporters. *SLC17A1* and *SLC34A2* are downregulated in HNF1-depleted caput cells, consistent with reduced renal reabsorption of phosphate in HNF1-deficient mice (59).

Finally, we consider water transport in caput HEE cells. The aquaporin family of water channels facilitates water reabsorption in the male reproductive tract. Although the efferent ducts reabsorb the majority of the luminal fluid (60) water reabsorption continues along the proximal epididymis. Our RNA-seq data suggest that AQP1 and 9 are the major aquaporins in caput HEE cells, and both genes encoding these proteins have promoter or nearby HNF1 ChIP-seq peaks. AQP1 is required for fluid reabsorption in the renal proximal tubule (61), however, the contribution of both AQP1 and AQP9 to epididymis function remains unclear since male AQP1- and AQP9-null mice are both fertile (6,62,63).

To conclude, our genome wide data sets reveal the pivotal importance of HNF1 β in the coordinated biology of human caput epididymis epithelial cells. They reveal multiple genes and functional processes that are regulated directly by this transcription factor, and open new avenues of investigation to better understand epididymis function and its contribution to ensuring male fertility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Open chromatin in caput human epididymis epithelial (HEE) cells predicted a key role for HNF1 β .
- HNF1 ChIP-seq identified direct target genes of this transcription factor in HEE cells.
- RNA-seq after HNF1 α/β depletion defined their contribution to the HEE transcriptome.
- HNF1 has an important role in regulating epithelial water and solute transport in caput HEE cells.
- Intracellular pH values show HNF1 contributes to the control of epididymis luminal environment.

Figure 1. Regional HNF1 α and HNF1 β expression in HEE cells

(A) Western blot of caput, corpus and cauda cell lysates shows that both HNF1 α (90 kDa) and HNF1 β (70 kDa) are most abundant in caput cells. (B) Cellular fractionation shows HNF1 α and HNF1 β are mainly in nuclei of caput cells.

Figure 2. HNF1 occupancy genome-wide in primary human caput HEE cells

(A) Percentage of HNF1 peaks within intergenic, intronic, promoter and other sites (including exons, 5'UTRs and 3'UTRs). (B) Caput DNase-seq tag counts measured from the center of the HNF1 peaks. (C) *De novo* motif analysis within HNF1 ChIP-seq peaks. (D) Gene ontology analysis by DAVID on the nearest genes to each HNF1 peak. (E) ChIP-qPCR validation of HNF1 binding to 5 sites identified by ChIP-seq, with values relative to IgG control.

Figure 3. HNF1-depletion reveals its role in epididymis epithelial function

(A) Efficacy of siRNA-mediated depletion of HNF1 in caput HEE cells shown by western blot of cell lysates probed with an antibody detecting HNF1 α (90 kDa) and HNF1 β (70 kDa). Negative control siRNA (NC). (B) Densitometry of western blot in A). (C) RT-qPCR validation of differentially expressed genes in HNF1-siRNA-depleted and non-targeting siRNA treated cells. Data are normalized to β -2 microglobulin (mean $\pm$ SD, $n = 3$) in comparison with negative control siRNA (NC, black bars, HNF1 α/β siRNA white bar). * $P < 0.01$, ** $P < 0.001$ and *** $P < 0.0001$ versus NC siRNA-transfected cells.

Figure 4. Identification of novel *cis*-regulatory elements coinciding with HNF1 ChIP-seq peaks in HNF1-regulated genes

Caput HEE open chromatin identified by DNase-seq are shown above each gene, together with HNF1 ChIP-seq peaks. Arrows denote open chromatin site coinciding with HNF1 ChIP-seq peaks (A) *SLC4A2* locus, promoter site. (B) *SLC4A3* locus, intergenic site DHS 20 kb downstream. (C) *SLC4A4* locus, sites in the promoter and within introns 6 and 20. (D) *PKHD1* locus, sites in the promoter and intron 5.

Figure 5. Effect of HNF1 knockdown on intracellular pH (pHi) of caput HEE cells
 (A) pHi calibration of caput HEE cells. (B) Effect of HNF1 depletion on pHi. Efficacy of siRNA-mediated depletion of HNF1 in BCECF-loaded cells (B, inset). (C) Effect of DIDS on pHi. BCECF-loaded cells were incubated in Ringers buffer containing 130mM NaCl for 5min. Repetitive dual fluorescence readings (excitation 444nm and 488nm, emission 535nm) were recorded and converted to pHi. Representative traces are shown. Each point represents the mean $\pm$ S.E.M. ($n = 3$). * $P < 0.05$, versus NC siRNA-transfected cells.