untitled protocol

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Abstract

Helicase-like transcription factor is regulated by alternative mRNA splicing. Global deletion of Hltf causes perinatal lethality, i.e. 75% die of hypoglycemia <24 hours postpartum. In heart, the full-length Hltf isoform is a transcriptional regulator of *Hif-1α* that regulates transport systems. Thus, we tested the hypothesis that *Hltf* deletion in placenta either caused or exacerbated neonatal hypoglycemia via *Hif-1α* regulation of nutrient transporters. RNA-seq data analyses of the placentome identified significant changes in transcript expression and *splicing.* The iPathwayGuide tool was used for gene ontology (GO) analysis of biological processes, molecular functions and cellular components. The Elim pruning algorithm identified hierarchical relationships. The methylome was evaluated by Methyl-MiniSeq Epiquest analysis. GO analysis with DAVID identified gene enrichment within biological processes. Protein expression was visualized with immunohistochemistry. Although two Hltf mRNA isoforms are quantifiable in most murine tissues, only the truncated Hltf isoform is expressed in placenta. iPathwayGuide analysis identified targets − 157 genes of 11,538 total genes with measured expression – of the encoded Hltf protein. *Hltf* deletion altered transcription of trophoblast lineage-specific genes, and increased transcription of the *Cxcr7* (p=0.004) gene whose protein product is a co-receptor for human and simian immunodeficiency viruses. Concomitant increased Cxcr7 protein was identified with immune labeling. *Hltf* gene deletion had no effect on transcription or site-specific methylation patters of *Hif-1α*, the major glucose transporters, or System A amino acid transporters. There was no measureable evidence of uteroplacental dysfunction or fetal compromise. iPathGuide analysis revealed Hltf suppresses cytolysis (10/21 genes; p-value 1.900e-12; p-value correction: Elim pruning; GO:019835) including the perforin-granzyme pathway in uterine natural killer cells. Our findings 1) identify a functional link between alternative splicing of Hltf and immunosuppression at the feto-maternal interface, and 2) underscore the importance of differential splicing analysis to identify functional diversity in the transcription factor under investigation.

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Protocol

Reagents and Kits

Step 1.

Sigma-Aldrich was the source of anti-HLTF (HPA 015284 to human HLTF aa 164-300). Abcam was the source of anti-HLTF (ab183042 to human HLTF aa 950-C-terminus), anti-protocadherin gamma (pan) - C-terminal (ab187186) and anti-perforin (ab180773) antibodies. Novus Biologicals was the source of anti-Cxcr7 (MAB42273). DBA lectin (L6533), diaminobenzidine (D12384), and Harris Modified Hematoxylin (HHS16) were purchased from Sigma-Aldrich for use with Streptavidin, peroxidase conjugate (189733) from MilliporeSigma. Biotinylated goat-anti-mouse (BA-9200) and goat anti-rabbit (BA-1000) IgG antibodies and the ABC-enzyme complex were purchased from Vector Laboratories, Inc. DNeasy Blood & Tissue Kit (69506) was purchased from Qiagen for isolation of genomic DNA from tail biopsies. SequalPrep™ Long

PCR Kit with dNTPs (A10498) was purchased from ThermoFisher Scientific. PCR primers were synthesized by Midland Certified Reagent Company. OmniPur agarose (2120) was purchased from Calbiochem division of EMD4Biosciences, and MetaPhor® agarose (50181) was purchased from Lonza Rockland, Inc. Promega was the source of agarose gel markers (G171A, G173A, and G176A). ZR Genomic DNA-Tissue MidiPreps (D3110) were purchased from Zmyo Research for use in conjunction with Agilent DNA Chips (5067-1522) and DNA 12000 reagents on the Agilent 2100 bioanalyzer.

Hltf null mice

Step 2.

Global Hltf null mice were developed in collaboration with genOway (Lyon, France) as previously described and backcrossed into the C57BL/6J genomic background for 10 generations. These mice were used throughout the study. For a limited comparison study, the Hltf deletion was bred into the recombinase activating gene 2 (Rag2)/common gamma (IL2rg) double knockout background, i.e. mice lacking lymphocytes (NK-, T- B-; alymphoid). Briefly, stock female [*Hltf*$^{+/+}$*/Rag2*$^{-/-}$*/Il2rg*$^{-/-}$] mice from Taconic (4111-F) were crossed with our male [*Hltf*$^{-/-}$*/Rag2*$^{+/+}$*/Il2*$^{+/y}$*]* mice to produce the F1 generation in which all males had the genotype *Hltf*$^{+/-}$*/Rag2*$^{+/-}$*/Il2*$^{-/y}$. These mice were intercrossed with stock female mice [*Hltf*$^{+/+}$*/Rag2*$^{-/-}$*/Il2rg*$^{-/-}$] to produce F2 mice fixed for the Il2rg null allele (homozygous for all offspring). Select F2 mice [*Hltf*$^{+/-}$*/Rag*$^{-/-}$*/Il2rg*$^{-/-}$ x *Hltf*$^{+/-}$*/Rag*$^{-/-}$*/Il2rg*$^{-/y}$] homozygous for Rag2$^{-/-}$ and heterozygous for Hltf$^{+/-}$ were intercrossed to yield F3 triple knockout [*Hltf*$^{-/-}$*/Rag2*$^{-/-}$*/Il2rg*$^{-/y}$ or *Hltf*$^{-/-}$*/Rag2*$^{-/-}$*/Il2rg*$^{-/-}$] and control [*Hltf*$^{+/+}$*/Rag2*$^{-/-}$*/Il2rg*$^{-/-}$or *Hltf*$^{+/+}$*/Rag2*$^{-/-}$*/Il2rg*$^{-/y}$] mice. Triple knockout and control mice are immune compromised as they completely lack T cells, B cells, and natural killer cells. These mice are bred and maintained in sentinel-monitored, bioBubble™-husbandry conditions.

Genotyping

Step 3.

At weaning, PCR screening reactions were used to authenticate the *Hltf* null vs wild type genotype. The Rag2 genotyping PCR protocol was adapted from bkeelab.bsd.uchicago.edu/Rag2.pdf**.** Sexing of fetal mice associated with each placenta was achieved by inspection of genital tubercles, i.e. male newborn mice have a pigmented spot over the scrotum, and validated by PCR analysis of the Sry sex-determining region of the Y chromosome and myogenin (Myog) of the X chromosome in DNA isolated from fetal tail biopsies.

Microscopy

Step 4.

For histological evaluation, each uterine horn was cut between implantation sites. Placentae were peeled away from their attachment to the decidua leaving a residual mesometrial myometrium landmark, emersion-fixed in formalin-based fixatives, cut mid-sagittally and paraffin embedded cut-face down. A mid-sagittal plane is the preferred orientation for visualization of the lymphocyte-rich mural micro domain otherwise known as the mesometrial lymphoid aggregate of pregnancy characterized by an abundance of immune competent uNK cells. Tissue blocks were serially sectioned (4 µm). Two sections were placed on each slide and deparaffinized before staining. Beginning with the first slide, sections on every fifth slide were stained with hematoxylin and eosin (H&E) and evaluated by light microscopy. Sections on alternate slides were stained for amylase-resistant periodic acid Schiff (PAS) positive granules in uNK cells, Dolichos biflorus (DBA) lectin reaction to a glycoconjugate containing N-acetyl D-galatosamine terminal sugar moiety in the plasma membrane and granules of uNK cells, or immunostained (Hltf, protocadherin gamma,

Cxcr7, perforin1). The placement of two tissue sections per slide facilitated the use of one section for positive staining, and the companion section for negative (minus primary antibody) control staining. Primary antibodies were used at the following concentrations Hltf (1:100 for HPA015284; 1:50 for ab183042), protocadherin gamma (1:5), Cxcr7 (1:10) and perforin1 (1:25). The secondary antibody was either biotinylated goat anti-mouse or goat anti-rabbit (1:200) depending upon the species in which the primary antibody was generated.

Uterine NK cells were counted (double-blind) in duplicate cross-sections from the middle of three different placentae from immunocompetent wild type and Hltf null mice. The fetal-to-placental-weight ratio was calculated from 14 control and 19 null values. Litter size was calculated from 37 null, 31 control, and 26 triple null values. All statistical comparisons were made with GraphPad Prism v7.02 (significance, $p<0.05$).

Placentome (RNA-seq)

Step 5.

Individual samples [1 placenta/sample x 5 biological replicates for test and control littermate female mice = 10 total samples] were flash frozen and sent to Otogenetics Corp. (Norcross, GA) for RNA-seq assays. Paired-end 100 nucleotide reads were aligned (mapped, averaged 53.68%) to genomic assembly mm10 (Table S1) and analyzed using the platform provided by DNAnexus, Inc. (Mountain View, CA) to generate an unbiased gene expression analysis report of RNA-seq; alternative splicing analysis of Hltf; mutation/RNA-editing analysis and parallel comparison of expression profiles between null and control samples. The power in detecting alternative splicing was dramatically increased by paired-end sequencing relative to single-end sequencing. FPKM (fragments per kilobase of transcript per million mapped reads) were mapped against mm10 with Tophat (V1.3.3) to obtain .bam mapping files that were input into Cufflinks for transcript assembly. Cuffdiff (V 1.3.0), part of the Cufflinks package, uses the alignment reads for rigorous statistical comparison of two conditions (null, control) and five replicates for each condition. Data were imported into iPathwayGuide (Advaita Corporation 2017) a next-gen pathway analysis tool. Standard enrichment parameters (0.6, $p<0.05$) were used. iPathwayGuide sets a default minimum threshold log fold change (logFC) of 0.6 for inclusion.

Methyl-MiniSeq Epiquest analysis

Step 6.

Individual samples [1 placenta/sample x 3 biological replicates for null and control mice = 6 total samples] were flash frozen prior to DNA isolation and purity assessment using the Agilent Bioanalyzer. Genomic DNA was sent to Zymo Research (Irvine, CA) for Methyl-MiniSeq Epiquest analysis. Genomic DNA was subjected to DNA fragmentation, endo modification, adaptor addition, bisulfite conversion, and limited amplification. Nine data sets were generated for each sample: one each for CpG islands, gene promoters, and gene bodies (according to USCS browser GCRm38/mm10 assembly annotations) in CpG, CHG, and CHH contexts.

Samples were grouped based on similarity for the top 100 differentially methylated CpG, CHG, and CHH sites covered in the assay. These genes were subjected to gene ontology analysis with DAVID (Database for Annotation, Visualization and Integrated Discovery) v6.7 to analyze gene enrichment within biological processes. DAVID analysis is agnostic to the direction of methylation change. It included sites that were significantly different for either hypo- or hypermethylation. Only GO terms for CpG sites with a count of at

least 9 genes and an enrichment score greater than 5 are reported.