Steroid Hormones and Receptors
STEROID HORMONES, NUCLEAR RECEPTORS, AND COLLABORATORS

Quantifying the Protein Levels of All Nuclear Hormone Receptors by Mass Spectrometry

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Nuclear Receptors (NRs) are a family of ligand-activated transcription factors that control the expression of genes involved in a wide range of physiological processes. An atlas detailing the expression of all NRs at the mRNA level was completed in 2006 using quantitative PCR [Bookout et al. Cell 2006]. The comparative measurement of NRs at the protein level, however, has been hindered by the poor quality of commercially available antibodies, as well as the absence of a high throughput method for quantitation. To address this need, we are developing a mass spectrometry-based targeted proteomic assay to quantify the absolute amounts of NR protein in a panel of mouse tissues. NRs were overexpressed in HEK293 cells by transient transfection and protein was isolated. The cell lysates were digested with a combination of trypsin and Lys-C following the Multi-Enzyme Digestion Filter Aided Sample Preparation protocol. The peptides were desalted using an in-house made C18 tip, separated on an EASY-Spray C18 column (75 um x 50 cm, 3Å), and analyzed on a Thermo QExactive HF in Top20 data-dependent acquisition mode. Protein identifications were made using MaxQuant software, and the identifications were mined for members of the NR family. The NR peptides detected were searched against an *in silico* generated list of optimal NR peptides (filtered for uniqueness, length, absence of post translational modifications, and conservation between human and mouse). The matching peptides were validated by parallel reaction monitoring (PRM) and purchased as synthetic isotopes with a heavy terminal arginine or lysine. Peptide linearity, and lower limits of detection (LLOD) were estimated by spiking digests from a C57Bl/6 mouse liver lysate with increasing amounts of the labeled peptides and analyzing by PRM. Peptides that displayed non-linear behavior were excluded for quantitation. The LLOD were between 100 amol and 1.5 fmol on column. A test panel of tissues (cerebrum, hippocampus, cerebellum, liver, spleen, brown/white adipose, and kidney) showed that we could detect endogenous expression of NRs. To date, we have purchased and validated peptides for 44 of the 49 receptors. We used this assay to quantify the changes in NR protein expression in mouse livers in response to 16 hours of fasting. We found significant changes in the nuclear expression of CAR (3.1-fold increase), RXRβ (1.8-fold increase), SHP (3.9-fold decrease) and RARβ (2.0-fold decrease) in the fasted vs. fed state. Increased CAR activity with fasting was further supported by label-free quantitative proteomics on the same lysates which revealed 210 differentially expressed proteins (2-fold change, $p<0.05$), with 61 (29%) identified as known CAR target genes. Once complete, this assay will provide researchers with a robust quantitative tool to investigate changes in NR protein expression that will be widely applicable to endocrine research.

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RNA-Binding Protein Musashi-2 Inhibits Aldosterone Production

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The adrenal cortex is the site of steroid hormone synthesis. These hormones control important physiological processes like metabolism, blood pressure and volume, and sexual characteristic development. While the signaling pathways, transcription factors, and steroidogenic enzymes are well-characterized, surprisingly little is known about the contribution RNA-binding proteins (RBPs). RBPs exert post-transcriptional control by interacting with specific elements within target mRNAs. Here we focus on the RBP, Mushashi-2 (MSI2), which binds to UAG sequences in the 3'UTR of its target transcripts. MSI2 is required for development of steroidogenic tissues which is consistent with its higher mRNA levels in human ovaries and testis. MSI2 also exhibits high expression levels in human adrenal tissue and the immortalized human adrenocortical cell line (H295R). Based on the compelling MSI2 expression pattern, we set out to determine the role of MSI2 on aldosterone production. Depletion of MSI2 using siRNA led to significantly lower aldosterone levels in H295R cells stimulated with AngII. We also employed an orthogonal loss-of-function approach by co-treating cells with AngII and increasing concentrations of Ro-08-2750 (Ro), a direct and selective inhibitor of MSI2-RNA interactions. Ro inhibited aldosterone production in a dose-dependent manner at 1 μM with almost complete inhibition at 5 μM. The molecular mechanism by which MSI2 regulates target RNA translation and/or decay is unknown. Moreover, whether MSI2 acts as a repressor or activator appears to be context dependent. Our goal is to determine the precise molecular mechanism by which MSI2 promotes aldosterone production. Specifically, we will identify MSI2 targets, temporally resolved consequences of MSI2 inhibition, and protein interaction partners. This work will impact our understanding of fundamental principles of RBP-mediated regulation, as well as novel regulatory mechanisms underlying human steroid hormone synthesis. Indeed, Ro (or further optimized compounds) may represent new therapeutic avenues for adrenal disease.

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Safety Analysis of an Oral Testosterone Undecanoate (TU) Formulation Following 2 Years of Administration in Hypogonadal Men

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