Validating A Novel Immortalized Hypothalamic Cell Line For Studying Gene Expression Altered In Diet-Induced Obesity

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Introduction

Obesity is a major and growing health concern in the United States due to its correlation to comorbidities such as diabetes mellitus (type II) and cardiovascular disease. Previous research from our lab has emphasized the importance of several hypothalamic neuropeptides important in energy expenditure, feeding, and activity that may be altered during the development of obesity. The use of in vitro models would assist with characterizing interactions between these genes and neuropeptides due to the complexity of the hypothalamic network.

Adult-derived hypothalamic cell lines provide an advantage to the study of gene expression as they represent a homogenous, clonal population that can be maintained in a controlled environment with fewer uncontrolled variables than in vivo models. Neuronal cell models have been proven to be useful in dissecting the molecular mechanisms of specific neuroendocrine cells in the regulation of energy homeostasis [1]. Validation of these cellular models is of utmost importance, because data from a poorly characterized cell line can lead to the results of entire experiments being termed invalid.

Objective: The primary objective of this study is to validate an immortalized adult-derived mouse hypothalamic cell line, CLU468, as a suitable model for studying gene expression altered in diet-induced obesity.

Methods

Cell culture: Cell culture lysate from the cell line mHypoA-59, or CLU468, was purchased from CELLutions Biosystems, Inc. The lysate was prepared by homogenizing cultured cells in modified RIPA buffer to obtain soluble proteins, and then centrifuged to clarify. Mouse brain tissue was purchased from Pel-Freez Biologicals and came from Swiss Webster mice, 8-10 weeks old and of mixed gender.

RNA Isolation: RNA was isolated from both mouse brain hypothalamic tissue (Pel-Freez; positive control) and cultured CLU468 cells using the RNeasy Mini Kit (Qiagen) for column-based isolation. Tissue samples were homogenized prior to RNA isolation with the aid of TRIzol (Invitrogen) and a Bullet Blender (Next Advance, BBX24).

Real-time Reverse-Transcription Polymerase Chain Reaction (RT-PCR): The expression of target genes was tested using RT-PCR, with samples run at a concentration of approximately 100 ng/µl, measured using a NanoDrop 8000 spectrophotometer (Thermoscientific). Mouse brain RNA was used as a positive control, and RNase-free water (Qiagen) was used as a negative control. Samples were prepared using the LightCycler RNA Amplification Kit: SYBR Green I (Roche) and 10 µM primer mix. Primer sequences (**Table 1**) were generated using MacVector 12 and commercially obtained from Integrated DNA Technologies. RT-PCR was run using the Roche LightCycler Carousel-Based System (LightCycler 1.5) and SYBR Green.

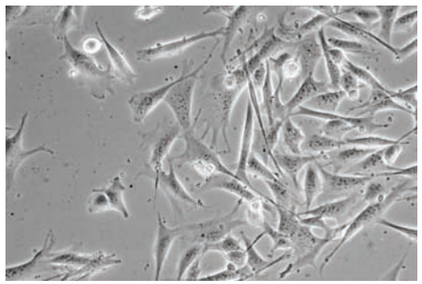

Figure 1: Photomicrograph of adult mouse hypothalamic cells in culture.

Figure 2: Workflow method used for validating gene expression in a cell line.

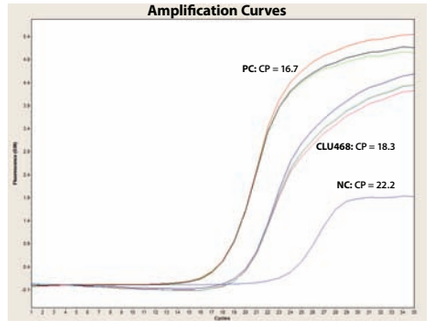

Figure 3: Amplification curves of PCR products for NPY primers. NC, negative control; PC, positive control (mouse brain RNA); CLU468, cell line tested. Crossover point (CP) values are indicated on the graph.

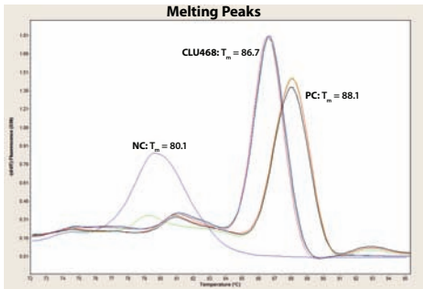

Figure 4: Melting peaks of PCR products for NPY primer. NC, negative control; PC, positive control (mouse brain RNA); CLU468, cell line tested. Melting point (T_m) values (°C) are indicated on the graph.

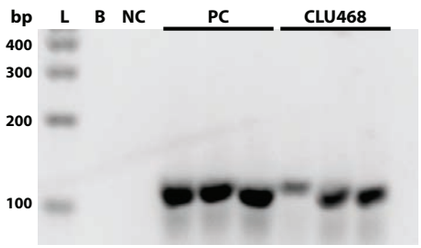

Figure 5: Visualization of PCR products. L, 100 bp ladder; B, blank; NC, negative control; PC, positive control; CLU468, cell line tested. As expected, both the cell line and the mouse brain control show a PCR product band at approximately 133 bp.

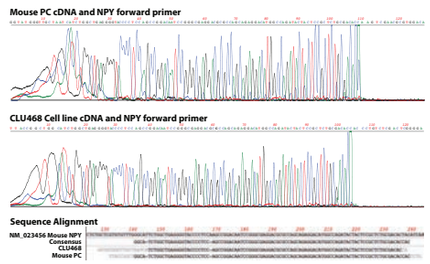

Figure 6: Sequencing results from the University of Minnesota Genomics Center. Colors correspond to nucleotide bases. Top: positive control trace; Center: CLU468 cell line trace; Bottom: Alignment with reference sequence, confirming product is NPY.

Gel Electrophoresis: PCR products were visualized by running a gel electrophoresis (Mini-Sub Cell GT Cell electrophoresis, BioRad) on a 3% agarose gel (Lonza NuSieve 3-1; TBE, BioRad). Gels were prepared using SYBR green (Invitrogen) in a 1:10,000 dye dilution. Gels were run at 100 V for 45-60 minutes and then visualized using a Gel Logic 220 Probe and Molecular Imaging Software (Carestream). A PCR ladder (Sigma) was used to determine the location of the PCR product size.

DNA Sequencing: Samples were prepared using the MinElute PCR Purification Kit (Qiagen). DNA sequencing was performed by the University of Minnesota Genomics Center using the Sanger di-deoxy terminator method. Sequences were aligned to reference using MacVector, and sequence identity was confirmed using NCBI BLAST.

References

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Table 1: Mouse primer sequences.

Accession Number	Gene	Primer	Nucleotide Sequence (5'-3')	Size (bp)
NM_008084	**GAPDH**	**Glyceraldehyde-3-phosphate dehydrogenase**		**116**
	Forward	GAPDH F1	GACATCAAGAAGGTGGTGAAGCAG	24
	Reverse	GAPDH R1	AAGGTGGAAGAGTGGGAGTTGC	22
NM_198959	**Ox1R**	**Orexin Receptor 1**		**161**
	Forward	OX1R 1F	GCGATTATCTCTACCCGAAGC	21
	Reverse	OX1R 1R	CAGGGACAGGTTGACAATG	19
NM_198962	**Ox2R**	**Orexin Receptor 2**		**120**
	Forward	OX2R 3F	AATCCCACGGACTATGACGACG	22
	Reverse	OX2R 3R	GAGAGCCACAACGAACACGATG	22
NM_023456	**NPY**	**Neuropeptide Y**		**133**
	Forward	NPY 3F	GGACTGACCCTCGCTCTATC	20
	Reverse	NPY 3R	AGTGTCGCAGAGCGGAGTAG	20

Results

Preliminary data confirms the presence of at least two genes important in energy expenditure, feeding and activity in a new adult mouse hypothalamic cell line, CLU468. These genes include orexin 1 receptor (Ox1R) and neuropeptide Y (NPY). Specifically, these genes are important in obesity-induced hypothalamic dysregulation. The orexins, acting via G protein-coupled receptors Ox1R and orexin 2 receptor (Ox2R) [2], have been shown to reciprocally interact with hypothalamic neurons producing several proteins including NPY and pro-opiomelanocortin (POMC), stimulating and inhibiting feeding, respectively [3]. NPY is one of the most abundant brain peptides and has been shown to play an important role in the neural regulation of food intake [4, 5].

The expression of both Ox1R and NPY was confirmed through RT-PCR and gel electrophoresis. PCR data was analyzed using LightCycler Software and the Qualitative Detection and T_m (melting temperature) reports. The expected bands for the gene products, at approximately 161 bp for Ox1R and 133 bp for NPY, were visible during gel electrophoresis. However, the Ox1R PCR products also exhibited a band near 250 bp. DNA Sanger Sequencing confirmed the identity of the NPY gene in both the positive control and cell line samples.

Future Work

Ongoing work will consist of investigating the presence of nine other genes important in feeding, activity, and energy expenditure using the same methods described here. These genes will include prepro-orexin, Ox2R, pro-opiomelanocortin (POMC), leptin, peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α), and multiple hypoxia inducible factors (HIF-1α, HIF-1β, HIF-2α, and HIF-2β). Our data will be used to determine if the cell line CLU468 warrants further investigation for studies evaluating hypothalamic function, and whether this cell line may be used as a suitable model for studying gene interactions in obesity-induced hypothalamic dysregulation.

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