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Microtranscriptome regulation by gonadotropin-releasing hormone

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ABSTRACT

Gonadotropin-releasing hormone (GnRH) regulates biosynthesis in the pituitary gonadotrope via a complex signaling and gene network. Small non-coding microRNAs (miRNA) can play important roles in gene expression. We investigated the microtranscriptome in the mouse LβT2 gonadotrope cell line using microarray, single molecule coincidence detection assays, hairpin real time PCR and LNA (locked nucleic acid) primer-extension PCR. Expression of nearly 200 miRNAs were detected by array and a panel of 101 hairpin real time PCR assays. Within this broad family of expressed miRNAs, GnRH induced upregulation of two miRNA products of the same primary transcript, miR-132 and miR-212, a result confirmed by single molecule, hairpin and LNA assays. Induction peaked 6 h after GnRH exposure and showed no significant frequency sensitivity. Bioinformatics analysis was used to predict potential targets of each of these GnRH-regulated miRNAs. These findings suggest the importance of the microtranscriptome in gene control in the gonadotrope and implicate miR-132 and miR-212 in the regulation of GnRH-stimulated biosynthetic response.

1. Introduction

Gonadotropin-releasing hormone (GnRH) mediates the hypothalamic control of gonadotropin gene induction and biosynthesis in the pituitary gonadotrope. GnRH binds to a high affinity heptahelical G-protein coupled receptor on the gonadotrope membrane and modulates a variety of signaling cascades, including inositol phosphate signaling, calcium mobilization, protein kinase C activation, and various phosphorylation cascades including the mitogen activated protein kinases ERK, p38 MK, and JNK (Ruf et al., 2003; Ruf and Sealfon, 2004). These intracellular signaling changes modulate a layered gene network consisting of dozens of immediate early genes and secondary genes. The initial wave of GnRH-activated genes encodes transcription factors that converge to regulate the gonadotropin genes, as well as regulatory proteins that feed back to the signaling pathway. Understanding the mechanisms by which this complex information transfer system integrates data about GnRH frequency and other extracellular signals to control reproductive timing and competency requires clarifying both the topology (connections) and the global dynamics (regulatory changes over time) of the components of the network.

Genomics studies have characterized the overall changes in mRNA expression in the gonadotrope induced by GnRH (Lawson et al., 2007; Wurmbach et al., 2001; Yuen et al., 2002). However, little is known about the expression and regulation of an important, more recently recognized class of genes, those encoding microRNAs (miRNA). miRNAs are small, approximately 22-nucleotide gene products that are increasingly recognized to serve, like transcription factors, as the basis for a combinatorial code that contributes to the regulation of expression of specific genes and proteins (Hobert, 2008). miRNAs hybridize with complementary 3′-UTR mRNA sequences leading to their recruitment into specialized protein complexes that mediate mRNA degradation, sequestration or translational repression (Williams, 2008). Given the complex orchestration of biosynthetic regulation in the gonadotrope that is necessary for normal reproductive physiology, it is important to study the role of miRNAs in these regulatory processes.

Because the study of miRNAs and the technologies for their measurement are relatively new, it is valuable to compare results using different methodologies. In order to obtain a reliable assessment of miRNA expression and regulation, we utilized four different approaches to measure miRNAs in the LβT2 gonadotrope cell line. We identify expression of a large number of miRNAs in these cells and demonstrate using multiple assay technologies that GnRH

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induces the selective regulation of two miRNAs that are the product of the same gene.

2. Materials and methods

2.1. Cell culture and RNA sample preparation

LβT2 gonadotrope cells (Turgeon et al., 1996) obtained from Pamela Mellon (University of California, San Diego) were maintained at 37 °C in 5% CO_2 in humidified air in DMEM (Mediatech, Herndon, VA) containing 10% fetal bovine serum (Gemini, Calabasas, CA). 40–50 million cells were seeded in 15 cm dishes and medium was replaced 24 h later with DMEM containing 25 mM HEPES (Mediatech) and glutamine and 10% charcoal stripped fetal bovine serum. On the next day, the cells were treated with 100 nM GnRH (Bachem, Torrance, CA) or vehicle and were returned to the CO_2 incubator for 3 h before harvesting for microarray and real time PCR experiments. For pulsing experiments, cells were incubated with 100 nM GnRH or vehicle for 10 min, washed once with DMEM, and incubated further with DMEM for 20, 50 or 110 min (for 30 min, 1 or 2 h pulse frequencies). Cycles of GnRH or vehicle treatment were repeated and six replicate samples from each treatment were collected after 6 and 18 h. Total RNA was isolated using mirVana miRNA Isolation Kit (ABI/Ambion, Austin, TX) according to the manufacturer's protocol.

2.2. miRNA microarray printing, sample labeling and processing

LβT2 cells were treated with 100 nM GnRH or vehicle for 3 h and total RNA was isolated using mirVana miRNA Isolation Kit. Small RNAs (<40 nucleotides) were size-selected by running through a flashPAGE fractionator (ABI/Ambion) with a starting quantity of 100 μg of total RNA. The small RNAs were precipitated overnight with 10 μg of glycogen (ABI/Ambion) as carrier and resuspended in 15 μl of DEPC-water for microarray analysis. A total of 350 oligonucleotides (mirVana miRNA probe set 1564V1, ABI/Ambion) complimentary to known miRNA sequences were printed in triplicate on SuperEpoxy glass slides (ArrayIt, Sunnyvale, CA) using a BioRobotics microGRID II arrayer (Digilab, Ann Arbor, MI). Two identical arrays were printed on each slide. Four control biosynthesized oligos (Control 1–4) were supplied and included in the array for labeling and hybridization quality control. Control 1 oligo which recognized the spiked-in control miRNA served as a positive control; whereas Control 2–4 oligos were derived from prokaryotic sequences and served as negative controls. Several anti-sense probes containing complementary sequence of known miRNAs were also included in the library and printed for additional hybridization quality control. A dye-swap assay was performed. The paired dye-swapped targets were hybridized onto the two arrays on a microarray slide. Three microliters of the PAGE-purified small RNA solution (equivalent to 20 μg of total RNA) spiked with miRNA positive control were labeled with either Cy-3 or Cy-5 (Amersham/GE Healthcare, Piscataway, NJ) using mirVana miRNA Labeling Kit. Hybridization and post-processing were carried out following the manufacturer's instructions (mirVana miRNA Probe Set Kit). A raised-edge coverslip (Lifter-Slips, Erie Scientific) was used to improve hybridization efficiency. The fluorescence image was captured using a ScanArray HT scanner (PerkinElmer, Waltham, Massachusetts) and analyzed using ImaGene 6.0 (BioDiscovery, El Seguno, CA). The images were initially examined manually and the spots with non-specific brightness were flagged. Empty spot (signal <16), negative spot (signal <0) or spot whose signal intensity is less than 2 standard deviation of background intensity for both channels was flagged automatically by the program. The success of sample labeling and hybridization were evaluated with the control spots on the microarray where Control 1 spots showed strong signal intensity, and Control 2–4 spots as well as the mouse anti-sense spots were flagged as well as by consistency of results obtained with dye swapping. The actual signal intensity for each spot was corrected by subtracting its local median background intensity and then was log based 2 transformed. The global signal intensity was normalized by using a robust locally linear normalization function (Lowess) comparing the results obtained with each of the two fluorescence channels within each subgrid. All flagged spots were excluded from further analysis. Median signal intensity of each miRNA probe was calculated from the triplicates on an array. Non-flagged spots represented the expression of the corresponding miRNAs.

2.3. Single molecule miRNA detection analysis

A published protocol developed by US Genomics (USG, Boston, MA) was followed as described (Chan et al., 2004; Neely et al., 2006). Briefly, a pair of LNA–DNA chimeric oligonucleotide probes, labeled with either Oyster 556 or Oyster 656, was designed for each target miRNA. In each of the miRNA assays, 1 nM probes were hybridized with 2 μg total RNA from LβT2 cells in the presence of 0.5 μl of RNase inhibitor (USB Corporation, Cleveland, OH) and 1× USG hybridization buffer for 2 h at 55 °C. A final concentration of 1.3 nM of DNA quencher probes, containing A-quenchers (Exiqon, Woburn, MA) and synthetic miRNA templates (Integrated DNA Technologies, Coralville, IA) were added and the quenching reaction was allowed to proceed for 1 h at 40 °C. The samples were diluted 10-fold in USG dilution buffer A before assay using a Trilogy 2020 Single Molecule Analyzer (US Genomics). Macromolecules were streamed through a microfluidic channel with a confocal laser-induced fluorescence detector (Fig. 1A). Single molecules of target miRNA

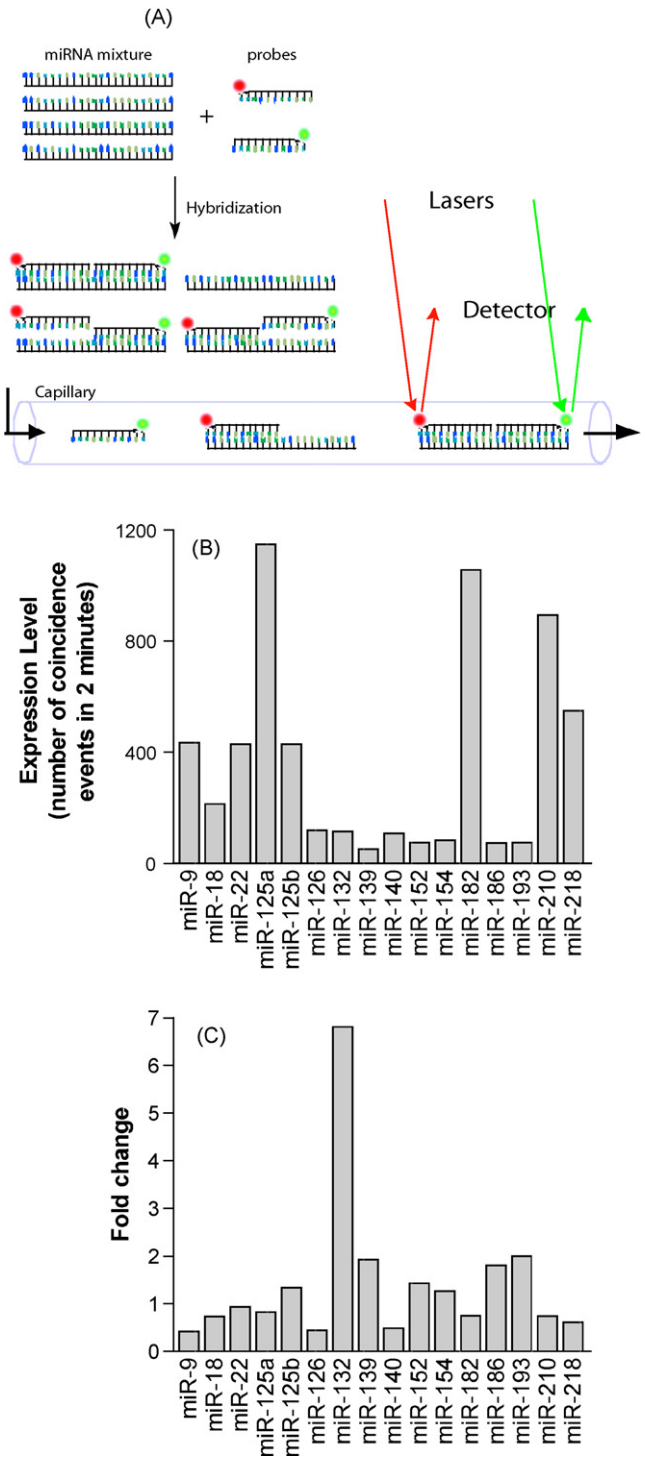

Fig. 1. (A) Schematic of single molecule detection assay. A pair of LNA–DNA chimeric oligonucleotide probes complementary to specific miRNA targets were labeled with either Oyster 556 (green) or Oyster 656 (red). The labeled probes were hybridized with miRNA samples from LβT2 gonadotropes, and then were passed through a microfluidic capillary for confocal laser-induced fluorescence detection. Coincidence events of the green and red signals, which represent a single miRNA target molecule, were counted. (B) Expression level of 16 miRNAs in control LβT2 cells determined by single molecule detection assay. (C) Change in miRNA levels in LβT2 cells following 3 h GnRH treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)