



Regulatory Architecture of the L β T2 Gonadotrope Cell Underlying the Response to Gonadotropin-Releasing Hormone

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Specialty section:

This article was submitted to
Neuroendocrine Science,
a section of the journal
Frontiers in Endocrinology

Received: 03 August 2017

Accepted: 24 January 2018

Published: 14 February 2018

Citation:

Ruf-Zamojski F, Fribourg M, Ge Y,
Nair V, Pincas H, Zaslavsky E,
Nudelman G, Tuminello SJ,
Watanabe H, Turgeon JL and
Sealfon SC (2018) Regulatory
Architecture of the L β T2
Gonadotrope Cell Underlying the
Response to Gonadotropin-Releasing
Hormone.
Front. Endocrinol. 9:34.
doi: 10.3389/fendo.2018.00034

The L β T2 mouse pituitary cell line has many characteristics of a mature gonadotrope and is a widely used model system for studying the developmental processes and the response to gonadotropin-releasing hormone (GnRH). The global epigenetic landscape, which contributes to cell-specific gene regulatory mechanisms, and the single-cell transcriptome response variation of L β T2 cells have not been previously investigated. Here, we integrate the transcriptome and genome-wide chromatin accessibility state of L β T2 cells during GnRH stimulation. In addition, we examine cell-to-cell variability in the transcriptional response to GnRH using Gel bead-in-Emulsion Drop-seq technology. Analysis of a bulk RNA-seq data set obtained 45 min after exposure to either GnRH or vehicle identified 112 transcripts that were regulated >4-fold by GnRH (FDR < 0.05). The top regulated transcripts constitute, as determined by Bayesian massive public data integration analysis, a human pituitary-relevant coordinated gene program. Chromatin accessibility [assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq)] data sets generated from GnRH-treated L β T2 cells identified more than 58,000 open chromatin regions, some containing notches consistent with bound transcription factor footprints. The study of the most prominent open regions showed that 75% were in transcriptionally active promoters or introns, supporting their involvement in active transcription. *Lhb*, *Cga*, and *Egr1* showed significantly open chromatin over their promoters. While *Fshb* was closed over its promoter, several discrete significantly open regions were found at -40 to -90 kb, which may represent novel upstream enhancers. Chromatin accessibility determined by ATAC-seq was associated with high levels of gene expression determined by RNA-seq. We obtained high-quality single-cell Gel bead-in-Emulsion Drop-seq transcriptome data, with an average of >4,000 expressed genes/cell, from 1,992 vehicle- and 1,889 GnRH-treated cells. While the individual cell expression patterns showed high cell-to-cell variation, representing both biological and measurement variation, the average expression patterns correlated well with bulk RNA-seq data. Computational assignment of each cell to its precise

cell cycle phase showed that the response to GnRH was unaffected by cell cycle. To our knowledge, this study represents the first genome-wide epigenetic and single-cell transcriptomic characterization of this important gonadotrope model. The data have been deposited publicly and should provide a resource for hypothesis generation and further study.

Keywords: L β T2, gonadotrope, gonadotropin-releasing hormone, chromatin accessibility mapping, transcription profiling, single-cell transcriptomics

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) plays a key role in the control of reproduction in mammals. Secreted by the hypothalamus in a pulsatile fashion, GnRH acts *via* its receptor (GnRHR) to trigger the synthesis and release of the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by the pituitary gonadotropes. In turn, the gonadotropins regulate gametogenesis and steroidogenesis in the gonads. The gonadotropins are composed of a common glycoprotein hormone α subunit (CGA) and a specific β subunit (LH β or FSH β). The frequency of GnRH pulse release varies at different stages of reproductive life, e.g., during puberty and the female menstrual cycle. GnRH pulse frequency differentially regulates gonadotropin subunit gene expression and gonadotropin secretion (1). While *Lhb* gene expression is preferentially induced by high-frequency GnRH pulses, low-frequency pulses favor *Fshb* expression (2, 3).

The immortalized L β T2 gonadotrope cells have been used extensively as an *in vitro* model for the study of gonadotropin gene regulation and GnRH signaling. The cell line was developed through targeted tumorigenesis in mice carrying the rat LH β regulatory region linked to the SV40 T-antigen oncogene (4–6). L β T2 cells have some functional characteristics of mature gonadotropes, as they express *Cga*, *Gnrhr*, and *Lhb*. The cell line responds to pulsatile GnRH stimulation by upregulating *Lhb* and *Gnrhr* and secreting LH. In the presence of steroid hormones, L β T2 cells further increase the LH secretory response to GnRH pulses as well as the levels of *Lhb* and *Gnrhr* mRNAs (6). In addition, L β T2 cells induce *Fshb* under either activin A (7, 8) or GnRH pulse stimulation (3), with the level of *Fshb* being influenced by both pulse frequency and average concentration of GnRH (9). While L β T2 cells exhibit an increase in intracellular calcium and exocytosis in response to GnRH stimulation (5, 6), they differ from mature anterior pituitary cells in that they lack a characteristic large-amplitude calcium oscillatory response to GnRH (10). In addition, continuous GnRH stimulation does not induce *Gnrhr* gene expression, which is in contrast with rat pituitary cells (11).

Previous studies in L β T2 cells showed that GnRH activates a complex cell signaling network that rapidly induces the expression of early genes such as *Egr1*, *c-Fos*, and *c-Jun* (12–14), whose products consecutively activate the transcription of gonadotropin subunit

genes. Over the past two decades, a number of studies in the L β T2 cell line have implicated various pituitary factors in gonadotropin subunit gene regulation. These factors include secreted peptides such as bone morphogenetic proteins, pituitary adenylate cyclase-activating polypeptide, growth differentiation factor 9, VGF nerve growth factor inducible (15–19) [for review, see Ref. (20)], as well as transcription factors (TFs) such as AP1 (Fos/Jun heterodimer), SF1, and Egr1 (14, 21–23). Nevertheless, the molecular mechanisms underlying the gonadotrope response to GnRH and the decoding of the GnRH pulse signal are not fully understood.

Recent advances in high-throughput sequencing technologies have enabled researchers to solve key questions about gene regulation both at the chromatin and at the transcriptome levels. Hence, mapping of “open” chromatin regions using the assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) allows the detection of putative DNA regulatory regions that are likely bound by TFs (24, 25). Correlating the transcriptome (measured by RNA-seq) with a map of open chromatin may identify transcriptional regulatory elements that are involved in the GnRH response. Furthermore, as distinct cells within a cell population display significant variations in RNA expression, analysis of cell-to-cell variability of gene expression can deepen our understanding of cell population complexity and transcriptome dynamics by isolating transcriptomic heterogeneity (e.g., cell cycle status) that is concealed in cell population studies [for reference, see Ref. (26)] and providing insight into the cellular variation in gene expression levels and induction. Gel bead-in-emulsion (GEM) Drop-seq is a droplet-based single-cell (SC) RNA-seq method that can profile thousands of individual cells per sample with high sensitivity (27–29).

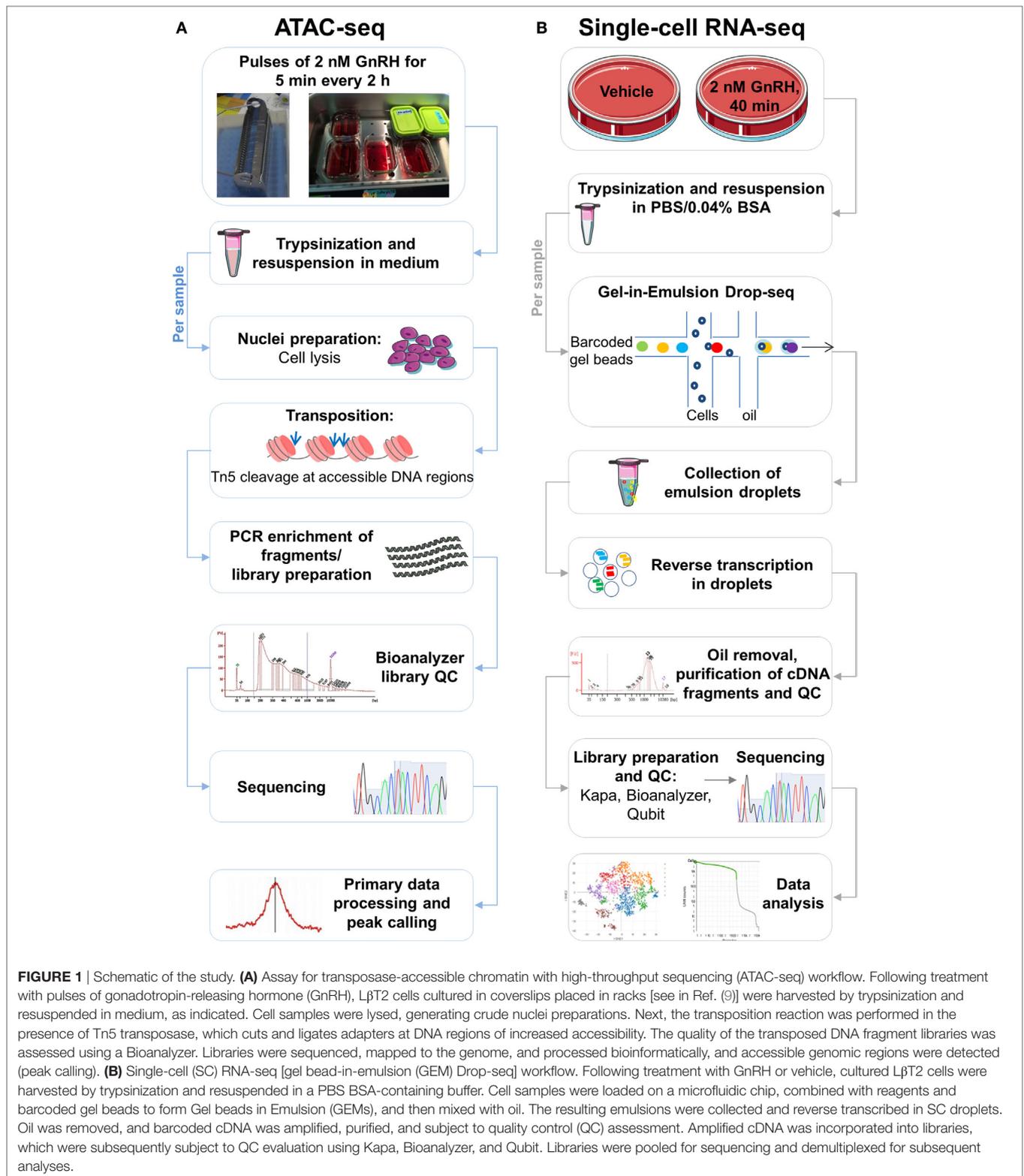
An unbiased epigenomic and SC transcriptomic study of L β T2 gonadotrope cells has not been reported. Here, we generated a global mRNA expression profile and a genome-wide atlas of accessible chromatin in GnRH-stimulated L β T2 cells by analyzing RNA-seq data (30) and ATAC-seq data (Figure 1A), respectively. An integrative analysis of the transcriptome and open chromatin data identified key GnRH-regulated genes along with putative *cis*-regulatory elements. We also analyzed cell-to-cell variability of the transcriptome of GnRH-stimulated L β T2 cells and the effects of cell cycle state on this response using GEM Drop-seq (Figure 1B).

MATERIALS AND METHODS

Cell Culture and Treatment

Gonadotropin-releasing hormone was purchased from Bachem (Torrance, CA, USA). L β T2 cells were obtained from Dr. Pamela

Abbreviations: ATAC-seq, assay for transposase-accessible chromatin with high-throughput sequencing; FSH, follicle-stimulating hormone; GIANT, genome-scale integrated analysis of gene networks in tissues; GEM, gel bead-in-emulsion; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; SC, single cell; TF, transcription factor; TSS, transcription start site.



Mellon (University of California, San Diego, CA, USA). Cells were cultured at 37°C in DMEM (Mediatech, Herndon, VA, USA) supplemented with 10% fetal bovine serum (FBS; Gemini, Calabasas, CA, USA) in a humidified air atmosphere of 5% CO₂.

Cells were frozen in freezing medium containing 70% DMEM, 20% FBS, and 10% DMSO (Sigma) and maintained in liquid nitrogen. Cell line authentication was achieved by comparing our cells with an early passage aliquot of L β T2 cells provided by

Dr. Mellon and used as a standard reference (Idexx Bioresearch, Columbia, MO, USA). Our results confirmed that our L β T2 cells were *Mycoplasma* free, were of mouse origin, and had similar markers as the original cell line aliquot.

Protocols for GnRH stimulation were selected to provide well-characterized response patterns. Hence, early gene responses in L β T2 cells are sensitive to GnRH concentration, while *Fshb* transcript levels respond to variations in both GnRH concentration and pulse patterns (9, 13). Because GnRH stimulation of L β T2 cells was previously shown to induce an early gene program, whose transcripts are upregulated within an hour of GnRH exposure (12, 13), a 40- to 45-min GnRH treatment was used in this study to analyze the transcriptome response to GnRH. For the analysis of chromatin accessibility by ATAC-seq, a pattern of pulsatile GnRH exposure was employed to capture the chromatin state that accompanies maximal *Fshb* induction (9, 18).

Bulk RNA-seq Assay

The generation of the RNA-seq data set that we analyze in this study was previously described (30). Briefly, L β T2 cells were serum starved overnight and stimulated with either 5 nM GnRH or vehicle for 45 min. Each group consisted of four independent replicates. Total RNA (2.5 μ g) from each replicate was sequenced at the Mount Sinai Genomics Core Facility using an Illumina platform (Illumina, Inc., San Diego, CA, USA) and a HiSeq 2000 sequencing system (100-nucleotide length, single read type, multiplexing three samples per lane). The RNA-Seq data are deposited in GEO (GSE42120).

Assay for Transposase-Accessible Chromatin with High-Throughput Sequencing

Assay for transposase-accessible chromatin with high-throughput sequencing was performed as previously described (25) on two replicate samples of L β T2 cells treated with 2 nM GnRH pulses every 2 h for a duration of 6 h and 45 min (4 pulses in total; cells harvested 45 min after last pulse) in a high-throughput GnRH pulse system (9). Briefly, 4,500 cells were washed with cold PBS at 4°C and lysed for 10 min at 4°C. Cell pellets were resuspended in the transposase reaction mix [5 μ l 2 \times TD buffer, 0.5 μ l transposase (Illumina) and 4.5 μ l nuclease-free water] and incubated at 37°C for 30 min. DNA from the transposase reaction was purified with a DNA Clean & Concentrator-5 Kit (Zymo Research). PCR amplification was performed using Nextera PCR primers. The optimal number of cycles was determined *via* quantitative real-time PCR (qPCR) to stop the amplification before saturation. Libraries were purified with AMPure beads and then quantified using a KAPA Library Quantification Kit (Kapa Biosystems) and High-Sensitivity DNA Bionalyzer kit (Agilent) and sequenced on an Illumina HiSeq 2500 to >164M reads with 50 bp read length, paired-end. The ATAC-seq data are deposited in GEO (GSE102480).

GEM Drop-seq Assay

Gel bead-in-emulsion Drop-seq was performed as described [10 \times Genomics, Pleasanton, CA, USA (29)]. Briefly, L β T2 cells

were seeded at 350,000 cells per well in 12-well plates for 48 h and treated on day 3 with either 2 nM GnRH or vehicle for 40 min. Cells were then trypsinized and resuspended in medium before being washed and resuspended in 1 \times PBS/0.04% BSA. Following filtration of the cell suspension, cells were counted on a Countess instrument, and viability was assessed to be above 90% using Trypan Blue. Final concentration was set at 1,000 cells/ μ l in 1 \times PBS/0.04% BSA. As a starting point, ~8,000 cells from each sample were loaded into the fluidics chip. Reverse transcription was performed in the emulsion, and cDNA was amplified for 12 cycles before library construction. Quality control (QC) and quantification of the amplified cDNA were assessed using the High-Sensitivity DNA Bioanalyzer kit. Library quality control and quantification were evaluated. The SC data set is deposited in GEO (GSE102480).

Quantification and QC of RNA and Libraries

RNA concentrations were determined with Quant-iT RiboGreen RNA reagent (Invitrogen, Carlsbad, CA, USA) using a fluorescence microplate reader (SpectraMax M3, Molecular Devices, Sunnyvale, CA, USA). RNA quality was assessed by determining the RNA Integrity Number using Bioanalyzer.

Library QC and quantification were assessed using Nanodrop, Qubit (fluorometric quantitation, ThermoFisher Scientific), Kapa (quantification, Kapa Biosystems), High-Sensitivity DNA Bioanalyzer kit (Agilent), and qPCR of selected genes.

Quantitative Real-time PCR

Following total RNA isolation, 1 μ g of RNA was reverse transcribed with the Affinity Script reverse-transcriptase (Agilent, Santa Clara, CA, USA). Next, samples were diluted 1:20 in molecular biology grade H₂O (Cellgro, Manassas, VA, USA). SYBR Green qPCR assays were performed (40 cycles) in an ABI Prism 7900HT thermal cycler (Applied Biosystems, Foster City, CA, USA) using 5 μ l of cDNA template and 5 μ l of master mix containing the specific primers for the targeted gene, Platinum[®]Taq DNA polymerase, and the required qPCR buffer, following the manufacturer's recommendations. Three technical qPCR replicates were run for each biological replicate. Results were exported as cycle threshold (Ct) values, and Ct values of target genes were normalized to that of *Rps11* in subsequent analysis. Data were expressed as arbitrary units by using the formula, $E = 2,500 \times 1.93^{(\text{rps11 CT value} - \text{gene of interest CT value})}$, where E is the expression level in arbitrary units. Primer sequences were as previously described (9, 12).

Bulk RNA-seq Data Analysis

The RNA-seq data generated about 36–45 million reads per sample. The RNA-seq reads were aligned using STAR (31) v2.5.1b with the mouse genome (GRCm38 assembly) and gene annotations (release M8, Ensembl version 83) downloaded from the <https://www.encodegenes.org/web> site. 91–93% of the reads were uniquely mapped to the mouse transcriptome. The matrix counts of gene expression for all eight samples were computed by featureCounts v1.5.0-p1 (32). Differentially expressed genes (5% FDR and at least 2 log₂ fold change) were identified using the

voom method (33) in the Bioconductor (34) package Limma (35). When comparing the bulk RNA-seq analysis with SC RNA-seq or ATAC-seq data, the transcripts per million (TPM) computed by RSEM (36) was used for the comparison.

ATAC-seq Data Analysis

Primary data analysis involved read mapping *via* bowtie2 (37) to GRCm38 (mm10), followed by duplicate read removal and peak calling *via* MACS2 (38) with parameters “-g dm -nomodel -shiftsize 100 extsize 200.” For examining correspondence between chromatin accessibility and gene expression (Figure 4A), all peaks were first annotated to the nearest gene, and the most significant peak score for each gene was selected.

SC RNA-seq Data Analysis

Single-cell RNA-seq data were processed using the Cell Ranger pipeline v1.3, which provides a data matrix of expression for all genes and all cells. Differentially expressed genes were analyzed using the sSeq method (41), as implemented in the R package cellrangerRkit v1.1. The cell phase computation for the single cells follows the ideas described in the Supplementary Material of the study by Macosko et al. (28) with our own customized R script implementation. A schematic of the cell phase score computation is described in Figure 6A. The t-SNE analysis (42, 43) was performed using the implementation from the Cell Ranger pipeline.

RESULTS

Transcriptome Profiling of L β T2 Cells

To characterize the transcriptome response to GnRH in L β T2 cells for comparison with the chromatin accessibility and SC studies described below, we analyzed an RNA-seq experiment in which L β T2 cells were exposed to either GnRH (5 nM) or vehicle for 45 min [(30); n = 4 for each group]. We identified 112 differentially expressed genes relative to the control (>4-fold at FDR < 0.05, see Figure 2A; Figure S1 in Supplementary Material and Table S1 in Supplementary Material), including a large number of early gene transcripts that are known to be regulated by GnRH in gonadotropes, such as *Egr1*, *Fos* (c-Fos), and *Jun* (c-Jun) (12, 45, 46). As is evident from the asymmetrical expression change versus significance volcano plot (Figure 2B), the majority of regulated transcripts were upregulated at this time point. The plot highlights the most highly upregulated genes, which include *Jun*, *Fos*, *Fosb*, *Egr1*, *Egr2*, *Egr3*, *Egr4*, *Nr4a3*, and *Nr4a1*.

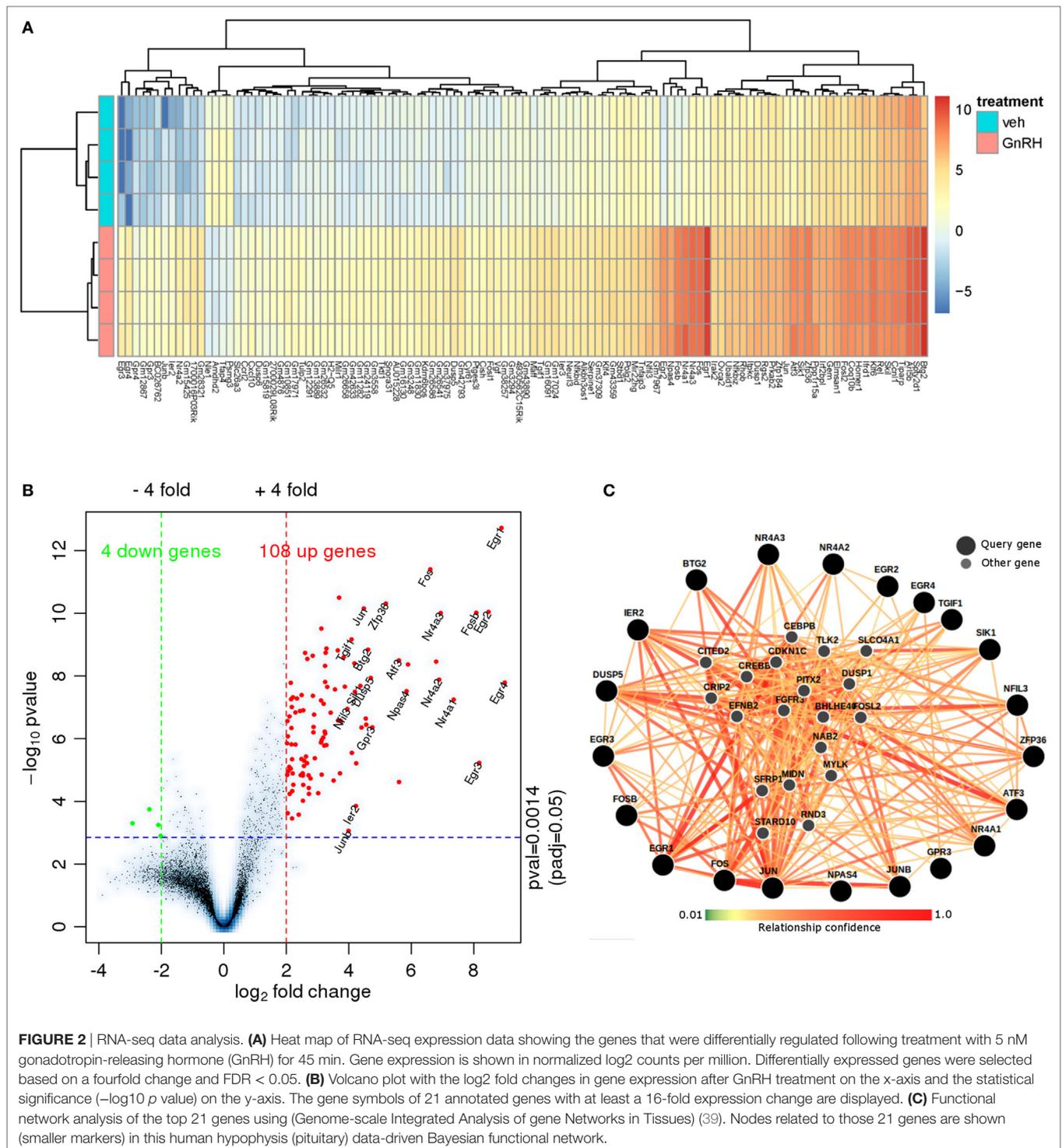
We investigated the functional relationships of the 21 highest fold-change annotated GnRH-regulated genes in the context of a human pituitary gene network using Genome-scale Integrated Analysis of gene Networks in Tissues [GIANT; Figure 2C; (39)]. GIANT uses a massive public data compendium to infer tissue-specific data-driven functional gene–gene relationships. The output of a GIANT analysis is a graph indicating the strength of the functional relationship of each pair of the input genes in the pituitary, as well as the inference of additional highly related genes. The edges connecting the nodes indicate the statistical strength of the evidence for that relationship in the human pituitary. Notably,

the highest regulated genes form highly interrelated subgroups (e.g., *Jun/Fos/Ier2*), and many highly related inferred genes are regulated by GnRH (e.g., *Dusp1*, *Nab2*) or known to be central to key gonadotropin developmental or regulatory processes [e.g., *Pitx2*; Table S2 in Supplementary Material; (12, 14, 47–50)]. Included among the statistically significant gene enrichment set were MAPK signaling and SMAD protein signaling (Table S3 in Supplementary Material). Overall, this analysis reveals a coordinated gene program activated by short-term GnRH exposure.

Genome-Wide Mapping of Chromatin Accessibility in L β T2 Cells

To map open chromatin regions, we carried out ATAC-seq in L β T2 cells treated with GnRH (Figure 1A). ATAC-seq uses the hyperactive Tn5 transposase, loaded with adapters for high-throughput DNA sequencing, to integrate into regions of accessible chromatin. The resulting DNA fragments, generated from locations of open chromatin, are amplified, sequenced, and computationally mapped to the genome to obtain a genome-wide accessibility landscape (24, 25). The ATAC-seq libraries and the sequence data showed a characteristic ~200 bp size distribution periodicity (Figure 3A), reflecting individual nucleosome occupancy patterns and confirming specific transposase activity and assay accuracy. Primary data analysis (see Materials and Methods) identified more than 58,000 statistically significant regions of open chromatin (peaks). The open chromatin map was found to be reproducible across independent samples and libraries. We note that, while determining whether chromatin accessibility changes with GnRH exposure is an important question, the present study was intended only to provide a baseline analysis of open chromatin structure. Performing a reliable comparative analysis of changes in specific regions with GnRH stimulation would require assaying a large number of samples, which was not feasible for this investigation.

We examined the 2,000 most prominent open chromatin regions (showing the highest peak scores; see Materials and Methods) with respect to their location relative to annotated genomic features. Approximately 75% of these peaks were located in immediate gene promoters or introns, which is consistent with open chromatin in the proximal regions of transcriptionally active genes (Figure 3B). Focusing on the sequence fragments in genomic areas flanking the transcription start sites (TSS, –3 to 3 kb), we observed a distribution highly preferential to the regions in close vicinity to the TSS (Figure 3B). We next examined chromatin state in the proximal promoter regions of several key genes (Figure 3C). While open chromatin peaks were detected at genes that are either constitutively expressed in L β T2 cells (*Gnrhr*) and/or regulated by GnRH (*Lhb*, *Cga*, and *Egr1*), chromatin was closed at the *Tshb* gene that is not expressed in gonadotropes. To gain insight into the overall transcriptional regulatory state of these cells, we used the HOMER tool to determine TF binding motifs showing enrichment among all open chromatin regions (40). The enrichment results show extremely high statistical significance (see Table S4 in Supplementary Material), reflecting the high power of this global analysis. Finding highly enriched global binding motifs for the TFs Smad2 and Six6, which have



been implicated in *Fshb* gene expression (51, 52), suggests that this analysis is likely to have generated useful leads for further study. In these high-resolution ATAC-seq data, we were also able to observe TF footprints, which create a notch in a region of otherwise open chromatin due to the presence of a bound TF. As an example, **Figure 3D** shows a notch detected in the otherwise open *Jun* promoter that precisely matches an SP1 consensus site.

This finding is consistent with the known role of SP1 in mediating induction of this gene (53).

To examine the relationship of the epigenetic landscape and the global pattern of mRNA expression, we compared the ATAC-seq and RNA-seq data. While the GnRH treatment conditions used for ATAC-seq versus RNA-seq experiments differed, they were both compatible with early gene induction. Indeed,

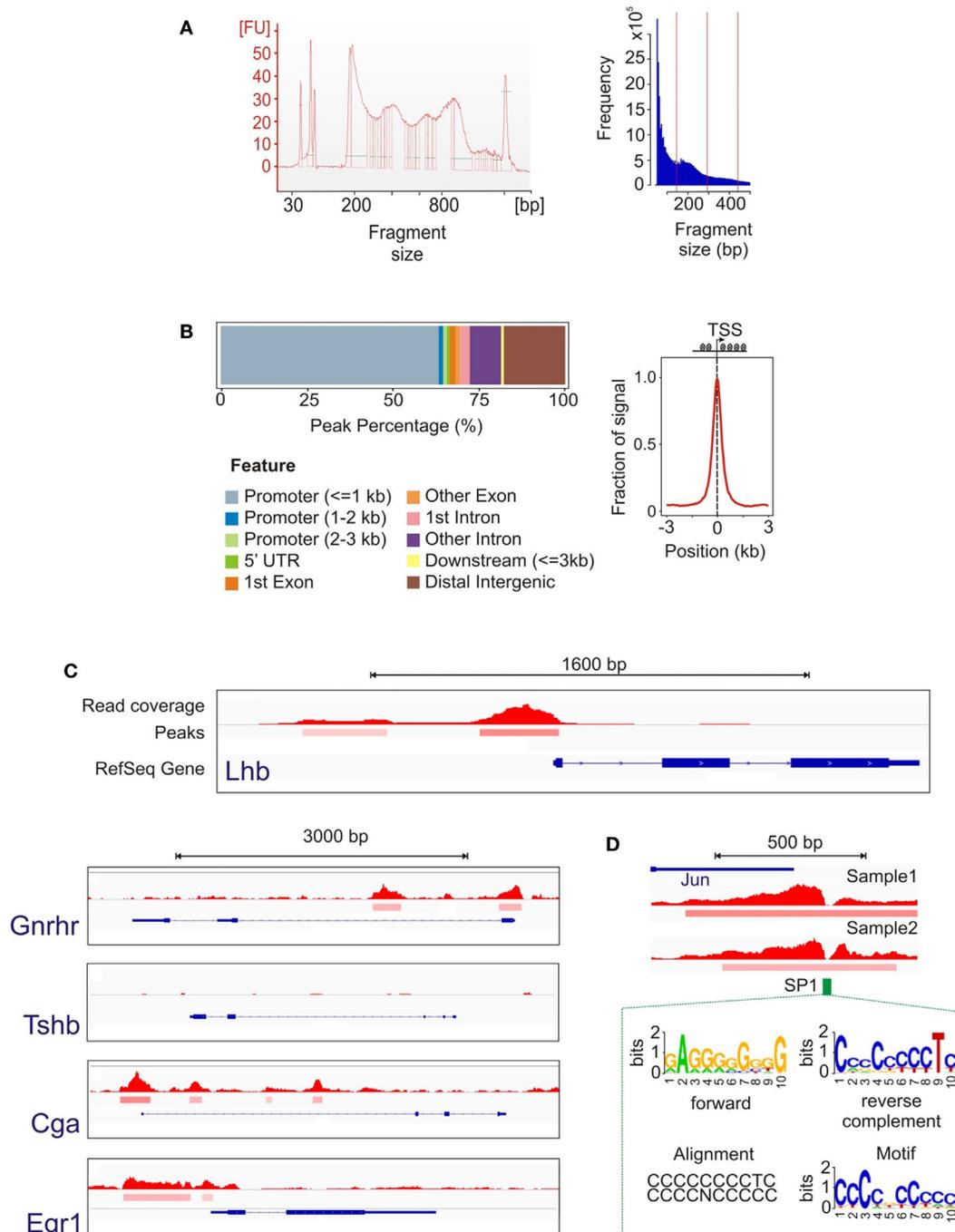


FIGURE 3 | Assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) data analysis. **(A)** Library and sequence fragment size distribution showing a periodicity characteristic of the effect of nucleosome binding. **(B)** Position of open chromatin peaks with respect to genomic features shows their genome-wide distribution are concentrated around the active transcription start sites (TSS), indicating that TSS are enriched for open chromatin. **(C)** Representative loci of genes showing the open chromatin signal. The top track represents the read coverage (density of sequence reads) at each location. The middle peak track indicates genomic regions that achieve statistical significance for chromatin accessibility. The bottom track shows the RefSeq gene annotation (intron/exon locations, transcription direction, etc.). As expected, accessible chromatin segments were detected around the promoter regions for *Lhb*, *Gnrhr*, *Cga*, and *Egr1*, but not for *Tshb*. **(D)** Example of transcription factor binding identification based on the footprint analysis of the open chromatin signal. The notch in the open chromatin region corresponds to the location of a consensus SP1 site. The HOMER analysis suite utilized (40) calculates a data-driven motif model from our ATAC-seq peak data, shown in forward and reverse configurations, and then associates this motif with known motifs. In this example, the known SP1 consensus motif is nearly identical to the data-driven motif.

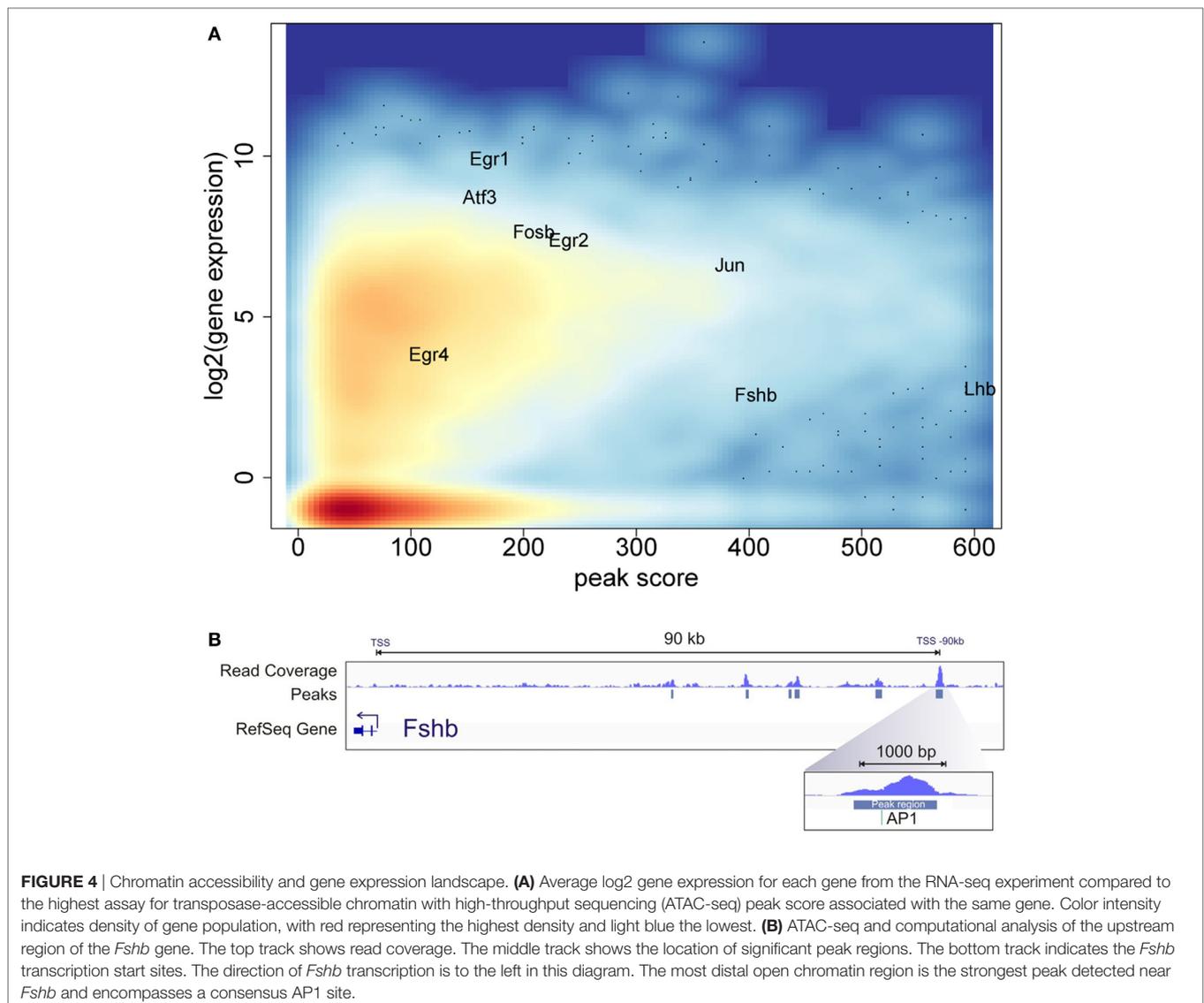
previous analysis of temporal responses of the early genes *Fos* and *Egr1* in response to GnRH pulse stimulation demonstrated that they are highly expressed within 40 min after the fourth pulse (9). In this analysis, we identified the open chromatin peak showing the highest ATAC-seq peak score associated with each annotated gene. This estimate of chromatin accessibility for each gene was plotted against its level of expression following GnRH treatment using the RNA-seq data (Figure 4A). The results show a general pattern of increased chromatin accessibility for expressed genes. Genes found to be highly induced by GnRH (e.g., *Egr1*, *Egr2*, *Egr4*, *Jun*, *Fosb*, *Atf3*) tend to show high chromatin accessibility.

The gonadotropin subunit genes *Lhb* and *Fshb* showed very high levels of chromatin accessibility at specific locations. The *Lhb* area of high accessibility, shown in Figure 3C, is centered around the proximal promoter. In contrast, *Fshb* does not show statistically significant open chromatin in the region of the proximal promoter. Notably, several significant open chromatin peaks were

found between -40 and -90 kb upstream of the *Fshb* TSS. The segment of highest chromatin accessibility, at -90 kb, contains a binding motif for AP1, a known *Fshb* regulator [Figure 4B; (14)]. This pattern raises the possibility that this region may represent a distal *Fshb* enhancer.

SC Transcriptome Analysis of L β T2 Cells and Characterization of Early Gene Response to GnRH

To investigate cell-to-cell variability in gene expression in the gonadotrope response to GnRH, we performed a SC transcriptome analysis of L β T2 cells exposed to either GnRH or vehicle for 40 min using GEM Drop-seq (see Figure 1B). This assay measures the entire transcriptome in thousands of individual cells from each sample. Specifically, we sought to assess cell-to-cell heterogeneity with respect to the individual cell response to GnRH.



We started with ~8,000 L β T2 cells in each sample. The resulting SC RNA-seq libraries all exhibited the expected electropherogram traces on the Agilent Bioanalyzer High Sensitivity Chip, thus passing the QC assessment. In addition, qPCR assays of several early response genes in the library provided further evidence that our SC RNA-seq libraries were suitable for the detection of individual gene expression and regulation (data not shown). We sequenced to a depth of ~300 million reads in the SC libraries from each sample (vehicle- and GnRH-treated L β T2 cells). We obtained good sequence data for 1,992 vehicle-treated and 1,889 GnRH-treated cells, with ~98,000 mean reads/cell and >4,000 median genes/cell above detection threshold. Analysis of the GEM Drop-seq data demonstrated even coverage, with the slow drop-off of expression/cell in the top nearly 2,000 cells being similar in untreated versus GnRH-treated cells (Figure 5A). These features are indicative of a high-quality SC transcriptome data set.

A heat map of selected genes expressed in all 3,881 analyzed cells revealed a global pattern of differential expression between GnRH-treated and vehicle-treated cells (Figure 5B). We identified 95 differentially expressed genes that included known GnRH-regulated immediate-early genes such as *Egr1*, *Fos*, *Fosb*, *Jun*, *Btg2*, *Junb*, and *Nr4a1* (Table S5 in Supplementary Material). Notably, we observed high cell-to-cell heterogeneity in response to GnRH, and several GnRH-treated cells even exhibited a gene expression pattern similar to that of untreated cells (Figure 5B; Figure S2 in Supplementary Material). Comparison of SC gene expression measurements averaged across two randomly selected subgroups of 996 cells highlighted the high reproducibility and consistency of the data, as the correlation coefficient was >0.99 in either vehicle- or GnRH-treated cells (Figure 5C). To further evaluate the performance of the digital transcriptome, we compared Drop-seq gene expression measurements averaged across all SC with the bulk RNA-seq measurements analyzed above, in either vehicle- or GnRH-treated cells. Despite the SC and bulk sequencing data sets coming from different experiments, the aggregated Drop-seq data showed high correlation with the bulk RNA-seq data (correlation coefficient >0.90; Figure 5D). The high cell-to-cell variation in the response to GnRH and in gene expression levels results from a combination of technical measurement variation and true cell-to-cell biological variation. To gain a sense of the degree of cell expression measurement resulting from technical noise, we modeled the technical measurement variation by a Poisson distribution [(54); Figure 5E]. Notably, at high levels of expression, the contribution of technical noise is relatively small and supports the presence of high levels of true biological cell-to-cell expression variation in these cells.

Analysis of Cell Cycle Dependence of the SC Transcriptome of GnRH-Stimulated L β T2 Cells

One limitation in many studies of response to GnRH performed by our group and others in L β T2 cells is that the effects of cell division on GnRH-induced gene regulation are not controlled. L β T2 cultures comprise asynchronously dividing cells. A SC transcriptome experiment provides the possibility of identifying the

cell cycle stage of each individual cell and determining whether cell cycle stage influences the response to GnRH. Previous studies in yeast indicate that cell cycle has global effects on protein and RNA synthesis, thus affecting the transcriptional activity (55–57). RNA levels are controlled by transcriptional bursting that can vary during different cell cycle stages (58).

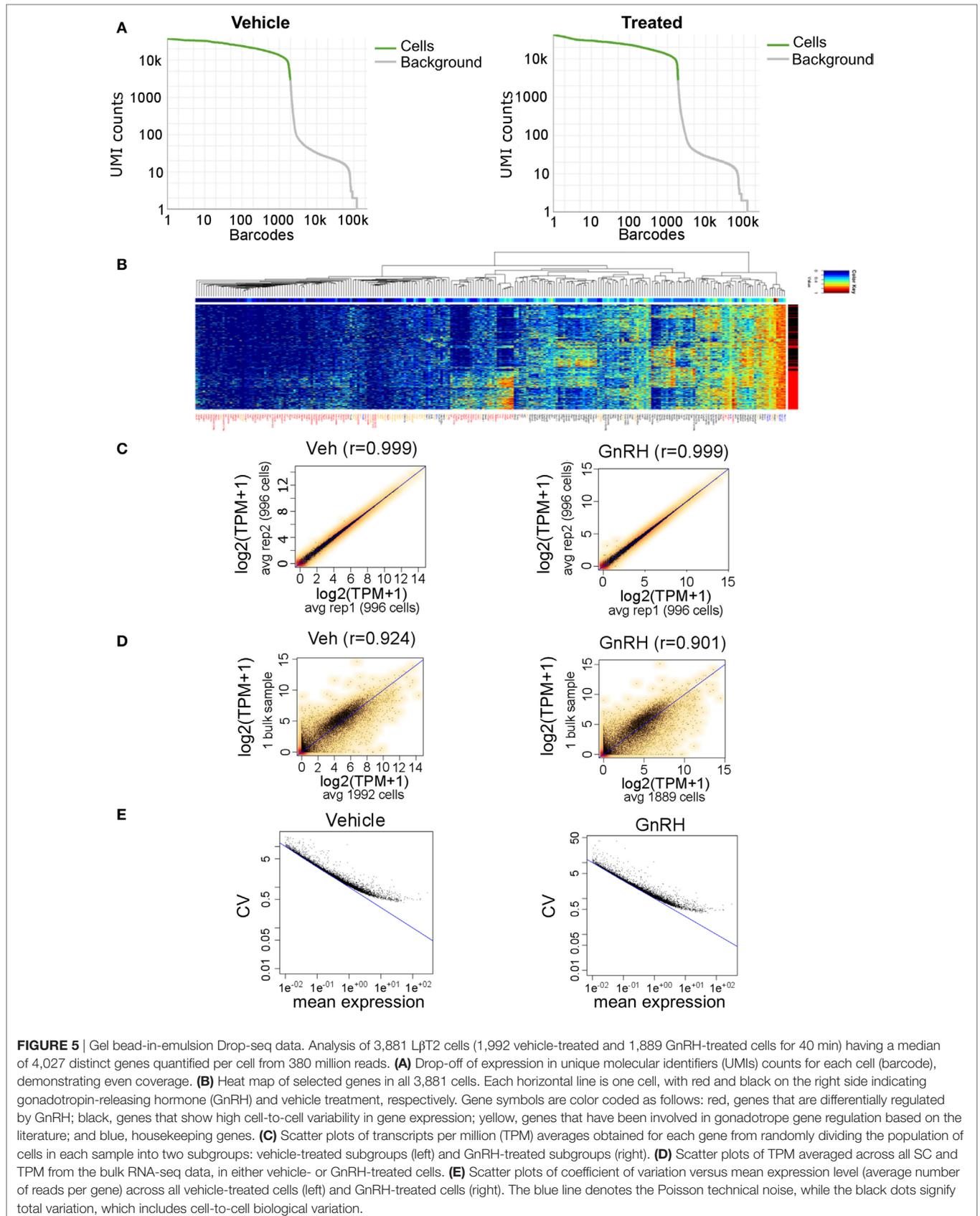
We examined cell cycle state of L β T2 cells as well as the influence of cell cycle phase on gene expression. Comparison of each cell's average expression with gene sets known to be enriched in one of the five cycle phases [G1/S, S, G2/M, M, and M/G1; Table S6 in Supplementary Material; for reference, see Ref. (44)] enabled alignment of each cell by the cell cycle stage (Figures 6A,B). Consistent with this alignment, a gene associated with the M cycle phase (*Cenpf*) exhibited the highest expression at the M phase and high expression at the G2/M phase (Figure 6C). In contrast, *Cenpf* was poorly expressed at G1/S and moderately expressed at S and M/G1. Likewise, the expression levels of other genes known to be associated with a specific phase of the cell cycle (e.g., *Pcna* with G1/S and S, *Top2a* with both S and G2/M) were highest at that cell cycle phase (Figure S3 in Supplementary Material). On the other hand, immediate-early genes induced by GnRH (e.g., *Egr1*, *Fos*, *Junb*) and other GnRH-regulated genes (e.g., *Gdf9*) showed no significant expression change with cell cycle phase (Figure 6C). The overall cell distribution with respect to cell cycle phase was comparable in GnRH-treated cells versus control (vehicle-treated cells) cells (data not shown). Additional analysis showed that the five cycle phases were represented throughout all analyzed cells, with individual cells partly forming clusters based on their cell cycle phase (Figure 6D).

DISCUSSION

In this study, we analyzed the global transcriptional, epigenetic, and single-cell transcriptional landscapes of the L β T2 gonadotrope cell line using RNA-seq data, ATAC-seq data and GEM Drop-seq data, respectively. To our knowledge, this represents the first genome-wide epigenetic characterization and the first SC transcriptome study performed in any gonadotrope experimental system. Our results provide insight into the global transcriptional regulatory processes of these cells and provide data sets and hypotheses to guide further work in this field.

A recent characterization of epigenetics in gonadotrope models reported CpG methylation, DNase hypersensitivity, and histone modification at regions of several specific genes in several cell lines, including L β T2 cells (59). Their finding of open chromatin correlating with expression at the specific genes investigated corresponds with our genome-wide characterization of this relationship. The global ATAC-seq epigenetic approach that we have pursued opens the avenue to entirely unexpected discovery, such as the putative new *Fshb* enhancer identified as highly open chromatin (see Figure 4B). Further study is needed to evaluate the functional role of this novel putative enhancer.

Analysis of SC transcriptome in vehicle- and GnRH-treated cells demonstrates, for the first time, that there is no influence of cell cycle stage on the gene response to GnRH. The SC variability observed at the level of basal gene expression and gene induction by GnRH is high and largely not explained by the technical



variation to which SC transcriptome analysis is prone (60). High levels of SC expression and gene induction variation in LβT2 cells could be anticipated based on previous studies of response

variation and noise scale of selected transcripts (61, 62). The cause of this high level of variation in expression and response to GnRH in LβT2 cells and whether it accurately models the expression and

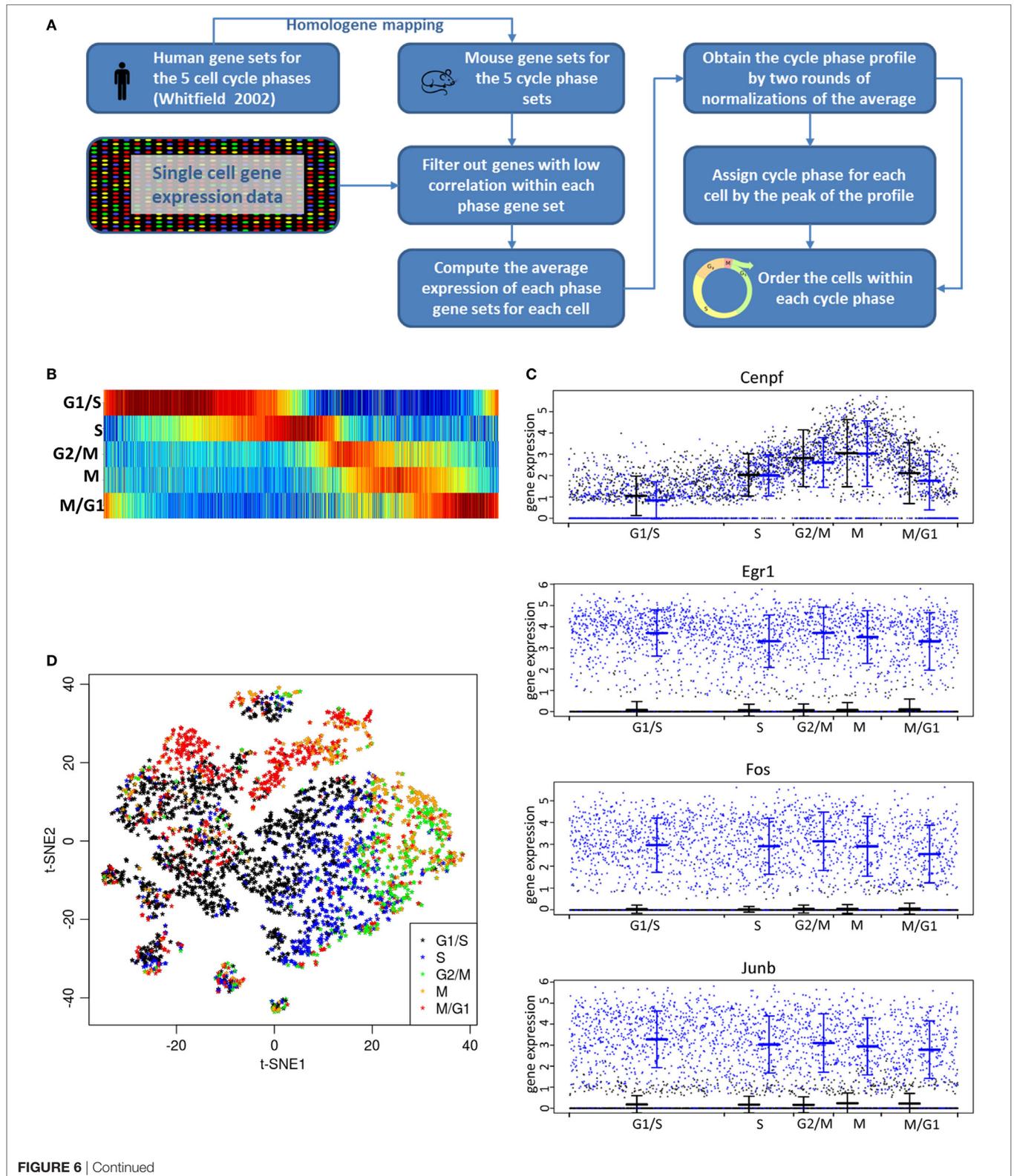


FIGURE 6 | Analysis of effects of cell cycle. **(A)** Flowchart diagram of the cell cycle analysis method. The score computation is based on the Supplemental Material from the study by Macosko et al. (28) with the cell cycle genes taken from their Table S2 in Supplementary Material mapped to mouse gene using Homologene. The gene sets for the five cell cycle phases (G1/2, S, G2/M, M, and M/G1) were determined by comparing cells that were arrested at different cell cycle phases (44). **(B)** Each of the 3,881 (GnRH- and vehicle-treated) cells on the x-axis is aligned by the cell cycle progression. Each vertical line represents one cell with the color code indicating low (dark blue) to high (dark red) score assignment to the cell cycle phase. All cells are ranked by cell cycle progression according to the steps depicted in **(A)**. The five cell cycle phases are indicated by the labels on the horizontal lines. **(C)** Single-cell expression of individual transcripts in relationship to cell cycle phase. Shown is the expression [\log_2 (TPM + 1)] of a gene associated with the mitotic (M) phase of cell cycle (*Cenpf*) and of GnRH-regulated genes (*Egr1*, *Fos*, and *Junb*). X-axis indicates cell cycle progression, as derived from **(B)**. The mean and SD for vehicle- (in black) and GnRH-treated cells (in blue) are shown at each cell cycle phase. Note that the expression level of any gene in these cells at any point in the cell cycle can be accurately determined without experimental cell cycle synchronization. **(D)** t-SNE plot of all 3,881 cells based on global expression of all genes (see Materials and Methods). The color of each cell indicates the cell cycle phase as determined in **(B)**. The fact that cells are clustered according to the cell cycle phase indicates that the primary cause of gene expression change is the cell cycle phase change.

response patterns of the intact mouse gonadotrope are unknown. While recapitulating many properties of a mature gonadotrope, L β T2 cells are a transformed cell line generated by tumorigenesis. It is conceivable that the heterogeneity of these cells has been augmented by the process of transformation (63, 64). Cell-to-cell variation in expression and response to stimulation can also result from normal biological variation and stochastic mechanisms (65). Thus, alternatively, this line may faithfully reflect SC expression and response variation and represent an accurate model of the behavior of the primary gonadotrope.

Individual gonadotropes largely function as a SC processor in controlling the reproductive axis and understanding the role of cell-to-cell variation in the engineering of this system is a relevant question to approach through SC biology. Evaluation of chromatin accessibility variation at the SC level [SC ATAC-seq (66, 67)] is also an interesting area for future investigation. The generation of high-quality global ATAC-seq chromatin accessibility and SC GEM Drop-seq transcriptome data should provide a useful resource for the research community.

AUTHOR CONTRIBUTIONS

FR-Z and MF designed and performed research, analyzed, and interpreted data; YG, EZ, GN, ST, and HW contributed analytic

tools and analyzed data; VN performed research and analyzed data; HP interpreted data and drafted the work; JT analyzed and interpreted data; SC conceived research, analyzed data, and drafted the work. All authors drafted or revised the work critically and approved the final version to be submitted.

ACKNOWLEDGMENTS

Sequencing for the ATAC-seq and GEM Drop-seq assays were performed at the Genomics and Epigenomics Core Facility of Weill Cornell Medical College. qPCR assays were performed at the Quantitative PCR CoRE at the ISMMS.

FUNDING

This work was supported by the National Institutes of Health Grant DK46943.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://www.frontiersin.org/articles/10.3389/fendo.2018.00034/full#supplementary-material>.

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