Title
Chromatin status and transcription factor binding to gonadotropin promoters in gonadotrope cell lines.

Permalink
https://escholarship.org/uc/item/1f8754ds

Journal
Reproductive biology and endocrinology : RB&E, 15(1)

ISSN
1477-7827

Authors
Xie, Huimin
Hoffmann, Hanne M
Iyer, Anita K
et al.

Publication Date
2017-10-24

DOI
10.1186/s12958-017-0304-z

Peer reviewed
Chromatin status and transcription factor binding to gonadotropin promoters in gonadotrope cell lines

Huimin Xie 1†, Hanne M. Hoffmann 1†, Anita K. Iyer 1,2, Melissa J. Brayman 1,3, Cindy Ngo 1, Mary Jean Sunshine 1 and Pamela L. Mellon 1*

Abstract

Background: Proper expression of key reproductive hormones from gonadotrope cells of the pituitary is required for pubertal onset and reproduction. To further our understanding of the molecular events taking place during embryonic development, leading to expression of the glycoproteins luteinizing hormone (LH) and follicle-stimulating hormone (FSH), we characterized chromatin structure changes, imparted mainly by histone modifications, in model gonadotrope cell lines.

Methods: We evaluated chromatin status and gene expression profiles by chromatin immunoprecipitation assays, DNase sensitivity assay, and RNA sequencing in three developmentally staged gonadotrope cell lines, αT1–1 (progenitor, expressing Cga), αT3–1 (immature, expressing Cga and Gnrhr), and LβT2 (mature, expressing Cga, Gnrhr, Lhb, and Fshb), to assess changes in chromatin status and transcription factor access of gonadotrope-specific genes.

Results: We found the common mRNA α-subunit of LH and FSH, called Cga, to have an open chromatin conformation in all three cell lines. In contrast, chromatin status of Gnrhr is open only in αT3–1 and LβT2 cells. Lhb begins to open in LβT2 cells and was further opened by activin treatment. Histone H3 modifications associated with active chromatin were high on Gnrhr in αT3–1 and LβT2, and Lhb in LβT2 cells, while H3 modifications associated with repressed chromatin were low on Gnrhr, Lhb, and Fshb in LβT2 cells. Finally, chromatin status correlates with the progressive access of LHX3 to Cga and Gnrhr, followed by PITX1 binding to the Lhb promoter.

Conclusion: Our data show the gonadotrope-specific genes Cga, Gnrhr, Lhb, and Fshb are not only controlled by developmental transcription factors, but also by epigenetic mechanisms that include the modulation of chromatin structure, and histone modifications.

Keywords: Epigenetic, DNA accessibility, Histone modification, Gonadotrope development, ChIP Assay, Chromatin

Background

The pituitary arises from the closure of Rathke’s pouch on mouse embryonic day 12 (E12.5), giving rise to five endocrine cell types: gonadotropes, lactotropes, corticotropes, thyrotropes, and somatotropes. The development of these specialized cells depends on a stringent temporal and spatial control of transcription factors [1, 2]. The gonadotropes emerge late in development at ~E16.5, and comprise ~10–20% of pituitary cells in adulthood. The major role of gonadotropes is to regulate puberty and fertility through the synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). FSH and LH are dimeric glycoproteins, formed from an α and a β subunit. The β subunit is distinct for each hormone, and is transcribed from separate genes. Gonadotrope cells can be traced throughout pituitary development by the sequential appearance of early lineage markers. First the mRNA for the common α-subunit of LH and FSH (Cga, glycoprotein hormones also known as αGSU) is detectable at E11.5 in the mouse, followed by the nuclear receptor Steroidogenic Factor 1 (Stf or...
Specification of cell fate is controlled by a combination of transcription factors acting on cis-regulatory elements, as well as epigenetic mechanisms that include the modulation of chromatin structure [4, 5]. Gene activation and repression are specifically regulated through changes in chromatin structure imparted mainly by histone modifications and DNA methylation. Inactive genes typically display condensed chromatin that is resistant to DNaseI digestion, and show histone H3 deacetylation and methylation [6, 7]. Active genes and regulatory elements are often in an open chromatin conformation to facilitate binding of regulatory proteins.

The access of transcription factors to the chromatin requires the relaxation of chromatin, leading to an open conformation and increased sensitivity to DNaseI digestion [8–10]. The regulation of chromatin status is complex and its analysis requires multiple complementary approaches. Open chromatin is often associated with acetylation at histone H3 and tri-methylation at histone H3-lysine 4 (H3K4) [6, 7]. In addition, certain combinations of histone modifications are thought to distinguish different regions within a given gene, i.e., promoters versus enhancers. For example, active promoters show high levels of tri-methylation and low levels of monomethylation at H3K4; whereas enhancers show the opposite methylation pattern at H3K4 [11]. The dynamic nature of chromatin modifications, are key in they role to control chromatin compaction and gene expression. Indeed, histone deacetylases (HDAC) allow histone deacetylation, leading to chromatin compaction [12, 13]. The conversion of chromatin from an inactive to active state at genes involved in differentiation is thought to promote the maturation of progenitor and precursor cells, however the chromatin status changes taking place during gonadotrope maturation are still poorly understood due to the complex cell composition of the pituitary.

Molecular investigation of the regulation of gonadotrope gene expression has been greatly facilitated by the use of well-characterized, cultured cell lines that represent different maturation stages of gonadotropes [14–17]. The αT1–1 cell line represents a progenitor to the gonadotrope and/or thyrotrope lineages [15] and expresses the single common glycoprotein hormone subunit gene, Cga [18]. The immature gonadotrope αT3–1 cell line expresses both Cga and Gnrhr, and the mature gonadotrope LβT2 cell line expresses all four gonadotrope-specific genes Cga, Gnrhr, Lhb, and Fshb [5, 15, 19, 20]. We and others have previously shown that these unique cell lines are excellent model systems for investigating the molecular mechanisms of gonadotrope differentiation [5, 15, 19, 21–26]. Moreover, these cell lines express the known tissue-specific regulators of the four gonadotrope-specific differentiated target genes including Sfi, Lhx3, Pitx1, Runx, Foxl2, and Gata2 [22, 24, 25, 27–31], and their binding sites in the proximal promoters of the gonadotrope-specific genes have been defined either experimentally or bioinformatically [5, 29, 32–34]. These tissue-specific transcription factors play direct roles in regulating the transcription of the gonadotrope-specific target genes, yet the coordinated program of gonadotrope maturation remains to be elucidated [35, 36]. Recent work has begun to address this topic by analyzing the epigenetic regulation of gonadotrope specific genes [5]. To further our understanding of the global chromatin status of the gonadotrope-specific genes during gonadotrope maturation, and obtain a more comprehensive understanding of the changes in chromatin status allowing specific expression of key gonadotrope markers, we investigated chromatin status and its correlation to gonadotrope gene expression in our model pituitary lineage cell lines.

Methods

Cell culture

To establish how chromatin status on key gonadotrope genes changes during gonadotrope cell maturation, we studied three model immortalized mouse gonadotrope cell lines αT1–1, αT3–1, and LβT2, as well as two control cell lines, the mouse thyrotrope cell line TαT1, and the mouse fibroblast cell line, NIH3T3 (ATCC). All cell lines were cultured in DMEM with 4.5% glucose (Mediatech Inc., Herndon, VA), 10% fetal bovine serum (Gemini Bio, West Sacramento, CA), and 1× penicillin-streptomycin (Life Technologies, Inc./Invitrogen, Grand Island, NY) in 5% CO2 at 37 °C. Cells were seeded on 10 cm dishes (Nunc, Roskilde, Denmark) and harvested at subconfluence. For hormone treatment of LβT2 cells, cells were serum-starved for 16 h in DMEM containing 4.5% glucose, 1x penicillin-streptomycin (Life Technologies, Inc./Invitrogen, Grand Island, NY) in 5% CO2 at 37 °C. Cells were then treated for 4 h with 100 ng/ml GnRH (Sigma-Aldrich, St. Louis, MO) ± 25 ng/ml activin (Calbiochem, La Jolla, CA) before they were harvested for the DNase sensitivity assay.

DNase sensitivity assay

Actively transcribed chromatin is characterized by being in an open conformation, allowing easy access of transcription factors to their binding sites. This open chromatin increases the sensitivity of the chromatin to DNaseI. To establish to what degree the gonadotrope promoters of model gonadotrope cell lines would increase their sensitivity to DNaseI treatment during maturation we performed a DNase sensitivity assay. DNase sensitivity assay was performed as previously described [37]. In brief, αT1–1, αT3–1, LβT2, and NIH3T3 cells were lysed in hypotonic buffer and nuclei were isolated.
by centrifugation at 2200 g for 5 min at 4 °C. Intact nuclei were resuspended in 1X DNase I Reaction Buffer (Promega, Madison, WI) containing 2% glycerol. Equal amounts of nuclei were added to increasing quantities of DNaseI (Promega) in 1X DNase Reaction Buffer, ranging from 0 units (U) to 7.5 U, and incubated at 37 °C for 5 min. Treated nuclei were lysed in Nuclei Lysis Buffer followed by RNase A and Proteinase K digestion. Genomic DNA was then isolated by extraction twice with phenol/chloroform/isoamyl alcohol and once with chloroform. DNA was ethanol precipitated and resuspended in Tris-EDTA buffer followed by 55 °C incubation for one hour. qPCR was performed using SYBR Green supermix and an iQ5 real-time PCR machine (BioRad). Primer sequences are detailed in Table 1. Forty ng of DNA from each treatment condition was quantitated relative to a standard curve of dilutions of undigested DNA. Data from each primer set were normalized to the active gene, Actb, or to the vehicle treatment as stated in the figure legends [37].

Chromatin immunoprecipitation (ChIP)
To evaluate transcription factor binding to key gonadotrope gene promoters, we performed ChIP assays. ChIP assays were performed as previously described [38]. Chromatin was sonicated to an average length of 300–500 bp using a Branson Sonifier 250 (Branson Ultrasonics Corp., Danbury, CT). Antibodies recognizing specific histone modifications were: anti-acetyl-Histone-H3 (06–599; Millipore, Temecula, CA), anti-trimethyl-Histone H3-Lys4 (07–923; Millipore), anti-dimethyl-Histone H3-Lys9 (ab1220; Abcam), all of which are ChIP-grade antibodies. To recognize phosphorylated polymerase, ChIP-grade anti-RNA polymerase II CTD repeat YSPTSPS (phospho S5) (ab5131; Abcam, Cambridge, MA), was used. Immunoprecipitated DNA and DNA from input chromatin were analyzed for sequences of interest by qRT-PCR using promoter-spanning primers shown in Table 1. For ChIP assays comparing αT1–1, αT3–1, and LβT2 chromatin, the percentage of enrichment of antibody signal over IgG was calculated for each primer set. IgG was the same species and source as the comparison antibody. Then, values for activating chromatin marks were normalized to those for the inactive gene Gnrh1. For repressive chromatin marks, values were normalized to the highly active gene Actb [37–39].

Association of transcription factors by ChIP
Antibodies were ChIP grade or previously validated for ChIP assays: anti-LHX3 (L2202, US Biological, MA) [25], anti-PITX1 (sc-18,922X, Santa Cruz, ChIP grade). Immunoprecipitated DNA was analyzed for sequences of interest by qRT-PCR using promoter-specific primers shown in Table 1. For ChIP assays comparing αT1–1, αT3–1, and LβT2 chromatin, the percentage of enrichment of antibody signal over IgG was calculated for each primer set. Values were then normalized to those for the inactive gene Gnrh1 [37, 38].

RNA sequencing
To establish if gene expression levels in the studied cell lines correlated with chromatin status, we performed RNA sequencing. RNA was isolated from αT1–1, αT1–3, LβT2, αT1, and NIH3T3 cells using TRIzol® (Invitrogen, Grand Island, NY), as per the manufacturer’s instructions, and treated with Turbo DNA-free DNase.

Table 1 Primers used for qRT-PCR for ChIP and DNase sensitivity assays

| Primer | Name | Sequence (5′-3′) | Product length | Chromosome Localization | Start | End |
|--------|------|-----------------|----------------|------------------------|-------|-----|
| Actb-F | GGGCCAGGCCAGGCTTCTTATGCTTGCACCAAAAGGCTTCTAAAT | 181 | 5 | 142,905,689 | 142,905,869 |
| Actb-R | CGAACATCAAGAGACAACAAAAGGCTTCTATA | | | | |
| Fshb-F | GGTGTGCTGCACATATACAGATCGGTGG | 280 | 2 | 107,059,594 | 107,059,873 |
| Fshb-R | GACATCGTTGCTGCTACTCAGCTCTGTT | | | | |
| Gnhr-F | CACCGAGGTGTTGCAATATTACATTACAAATCTAAG | 227 | 14 | 67,745,100 | 67,745,326 |
| Gnhr-R | CCTGTTTGGATTGGAATGTTCAAAATGGAGTTCCCTCTC | | | | |
| Cga-F | GAAAAAGCGCAATATGTCCTC | 193 | 4 | 34,893,533 | 34,893,725 |
| Cga-R | GTCTCCACGTGGCAGCATTAAG | | | | |
| Lhb-F | CGAGTGTGACGCGCAATATTACCACTGG | 218 | 7 | 45,420,767 | 45,420,984 |
| Lhb-R | GCCGCTACATCTTTACCTGG | | | | |
| Gnhr-F | ATCCAGAGATACAGGGGCAGTCCACTC | 202 | 5 | 86,197,672 | 86,197,873 |
| Gnhr-R | AGGCGTATGACAGATAGGAAAAAGGAAG | | | | |

Localization and product length (bp) from the UCSC Genome Browser using the Mouse Dec. 2011 (GRCm38/mm10) Assembly.
The RNA integrity (RNA Integrity Number ≥ 9) and quantity was determined on the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). cDNA libraries (n = 2) were created using the TruSeq™ RNA Sample Prep-v2 (Illumina, San Diego, CA), using the manufacturer’s low-throughput protocol. Indexed samples were mixed at equal concentrations, four samples per lane, and sequenced using the HiSeq 2000 sequencer (Illumina). The resulting sequences were aligned to the mouse genome using the Illumina Consensus Assessment of Sequence and Variation (CASAVA) software program. qRT-PCR, sequencing, and alignment were performed by the UCSD BIOGEM Core facility supported by NIH grants P30 DK063491 and P30 CA023100. Thirty-six bp, single end sequencing of the five cell lines generated an average of 3.6 GB of sequencing data per sample with the following characteristics: NIH3T3: 852.2 Mbp, 23.67 million reads and passing filter % (PF%) of 75.6; αT1–1: 933.2 Mbp, 25.92 million reads and PF% of 75.2; LβT2: 897.0 Mbp, 24.92 million reads and PF% of 67.8; and TaT1: 887.4 Mbp, 24.65 million reads and PF% of 70.7. 

Statistical analysis
All experiments were repeated independently at least three times. In the figures, the error bars represent the SEM. Data were analyzed by one or two-way ANOVA in GraphPad Prism 7 (La Jolla, CA). For all analyses, the result was considered significant if P ≤ 0.05.

Results
Chromatin density on gonadotrope-specific genes during development
To confirm that the immortalized pituitary cell lines expressed the key pituitary lineage markers and provide direct quantitation of expression levels, we performed RNA sequencing. We found that all five of the cell lines expressed high levels of β-actin (Fig. 1a, Actb). As expected, the common gonadotrope hormone α subunit, Cga, was expressed in all the gonadotrope lineage cell lines, as well as the thyrotrope cell line, TaT1 [15, 18], but not in NIH3T3 cells (Fig. 1a). The immature (αT3–1) and mature (LβT2) pituitary cell lines both expressed Gnrhr, whereas the gonadotrope marker, Lhb, was only expressed by LβT2 cells. The thyrotrope cell line TaT1 [18] was the
only cell line studied expressing the thyrotrope marker
Tshb, and did not express any of the gonadotrope-specific
genes, other than the common α subunit, Cga (Fig. 1a).
We did not detect Fshb in LβT2 cells, although these cells
are known to express this mRNA as detected by the more
sensitive method of qRT-PCR. Fshb is found at low levels
that can be induced by activin treatment [19, 40, 41].
NIH3T3 cells were used as a negative control for pituitary
gene expression (Fig. 1a).

To determine if chromatin status correlates with
expression of gonadotrope genes, we investigated the
chromatin status by DNasel sensitivity assay [42]. We
focused on chromatin accessibility of Cga, Gnrhr, Lhb,
and Fshb in the gonadotrope lineage cell lines αT1–1,
αT3–1, and LβT2, which represent different stages of
gonadotrope development. NIH3T3 cells were used as a
negative control. Actb (β-actin) is ubiquitously expressed
in all the above cell lines, reflecting an open chromatin
status, and was therefore used to normalize the DNasel
data (Fig. 1a and b, Actb). Three classes of chromatin
accessibility have been determined by this assay: closed
(repressed), poised, and open (active) [43, 44]. One of
the characteristics of chromatin is how dynamic its con-
formation is, and its capacity to compact and unfold.
Thus, degrees of compaction and opening of the DNA
are possible. Normalizing our data to the highly tran-
scribed Actb allowed us to determine the relative DNasel
sensitivity of the studied gonadotrope genes. To reveal
the degree of accessibility of the DNA, we used increas-
ing amounts of DNasel. As expected, we found that Cga,
Lhb, and Gnrhr were much less sensitive to DNasel
treatment than Actb (Fig. 1b), which correlated with
gene expression levels (Fig. 1a). Unexpectedly, Fshb was
sensitive to DNasel treatment, although to a lesser ex-
tent than Actb (Fig. 1b). However this did not correlate
with gene transcription (Fig. 1a), supporting the import-
ance of using more than one approach to study chroma-
tin status to obtain a complete image of chromatin
accessibility to transcription factors. Cga is the earliest
gonadotrope expressed gene (E11.5 in the mouse em-
byro) and has high expression in the gonadotrope pro-
genitor αT1–1 cell line (Fig. 1a). Consistent with this,
we found an open chromatin structure in the promoter
region of Cga in this cell line (Fig. 1b, αT1–1). The chroma-
tin states of all other later chromatin modifications of
gonadotrope cell lines were repressed as evidenced by
their structures being closed and resistant to DNasel
deradation (Fig. 1b, αT1–1, Fshb, Lhb and Gnrhr). The
immature gonadotrope cell-line αT3–1 expresses both
Cga and Gnrhr (Fig. 1a). In agreement with this, we
determined the chromatin state of these genes to be
open (Fig. 1b, αT3–1). Lhb and Fshb are not expressed
in the αT3–1 cells and have closed chromatin in this cell
(Fig. 1a). Our data show that the chromatin of Lhb and
Fshb started to shift from closed to poised in the LβT2
cells (Fig. 1b, LβT2, Two-way ANOVA, followed by
Sidak’s multiple comparison, p > 0.01 at 7.5 Units of
dNasel). The only partial opening of Lhb and Fshb in
LβT2 cells is not surprising, as cells in culture are not in
the tridimensional environment they experience in vivo
and do not receive hormonal stimulation, as gonado-
tropes would in vivo, allowing them to fully activate
expression of Lhb and Fshb.

Chromatin modifications of gonadotrope-specific genes
during development
Numerous modifications of chromatin and histones allow
for successful recruitment of phosphorylated RNA poly-
merase II (phospho-Pol II), which is required to initiate
transcription [10]. To determine if the opening of the
chromatin in these cell lines correlates with a change in
the acetylation and methylation signatures on the histones
binding to the promoters and allows occupation with
phospho-Pol II, we next performed ChIP assays. We used
antibodies recognizing the activating histone modification
marks histone H3 acetylation (H3Ac), and H3K4 tri-
methylation (H3K4Me3, Fig. 2), as well as recruitment of
phospho-Pol II [38, 44]. Although, Cga is highly expressed
by all three gonadotrope lineages (Fig. 1a), and the pro-
moter is sensitive to DNasel treatment (Fig. 1b), the active
histone marks in all three cell lines were surprisingly little
enriched as compared to Gnrh1, despite recruitment of
phospho-Pol II and active transcription (Fig. 1a and 2a).
This is unexpected and is possibly due to the chosen re-
region of study of the Cga promoter. In agreement with
both transcription levels of Gnrhr, and its promoter’s sen-
sitivity to DNasel treatment, the Gnrhr regulatory region
went from possessing very few active histone marks in
αT1–1, to highly enriched in these marks in αT3–1 and
LβT2 cells (Fig. 2). In agreement with our findings using
the DNasel sensitivity assay, which found the Fshb pro-
moter to be in a rather closed conformation (Fig. 1b), this
promoter exhibits only one of the chromatin marks of an
open promoter (H3Ac), but does not bind phosphorylated
polymerase II (Fig. 2). Finally, the chromatin status of Lhb
is enriched in H3Ac and recruits phosphorylated polymer-
ase II to the regulatory region somewhat in αT3–1 and
more strongly in LβT2, which correlated with transcrip-
tion in LβT2 cells where it also exhibits enrichment of
H3K4Me3 (Fig. 1a and 2).

To assess the epigenetic role of repression, we per-
formed ChIP assays to analyze the repressive histone
modifications H3K9Me2 and H3K27Me3 [45]. We found
that one or both modifications were reduced with devel-
opmental maturation on the promoters of Gnrhr and Lhb
(Fig. 3). Gnrhr loses both repressive marks in αT3–1 and
LβT2 cells, while Lhb has progressively reduced H3K27Me3 from αT1–1 to αT3–1 and finally LβT2 and
trends toward lower H3K9Me2 in LβT2 cells (Fig. 3). Whereas no changes were observed on the Fshb promoter (Fig. 3).

Sequential recruitment of developmental pituitary transcription factors to gonadotrope-specific promoters
Opening of the chromatin, along with activating histone modifications, promotes access to the chromatin of transcription factors. It has previously been shown that the known gonadotrope regulatory proteins PITX2 (Pitx2), SF1 (Nr5r1), and LHX3 (Lhx3) control expression of the mature gonadotrope markers Lhb, Fshb, and Gnrhr [25, 46, 47]. Using RNA sequencing, we show that these transcription factors are expressed in the gonadotrope lineage and thyrotrope cell lines, but not in NIH3T3.

Fig. 2 Active histone modifications and recruitment of phospho-Pol II to gonadotrope-specific genes in gonadotrope cells. ChIP analysis using antibodies specific to (a) H3Ac, (b) phospho-Pol II, and (c) H3K4Me3 in αT1–1, αT3–1, and LβT2 cells. Immunoprecipitated chromatin from αT1–1, αT3–1, and LβT2 cells was subjected to qRT-PCR. Data are presented as fold enrichment relative to IgG, with values normalized to Gnrh1 and expressed as mean ± SEM (n = 4). Statistical analysis by one-way ANOVA followed by a Tukey post-hoc, * p < 0.05, ** p < 0.01, *** p < 0.001, as indicated by bar. n.s.: non-significant.

Fig. 3 Histone modifications of inactive chromatin in αT1–1, αT3–1, and LβT2 cells. ChIP analysis was performed using antibodies specific to H3K27Me3, and H3K9Me2. Immunoprecipitated chromatin from αT1–1, αT3–1, and LβT2 cells was subjected to qRT-PCR. Data are presented as fold enrichment relative to IgG, with values normalized to Actb and expressed as mean ± SEM. Statistical analysis by one-way ANOVA followed by a Tukey post-hoc, * p < 0.05, ** p < 0.01, *** p < 0.001, as indicated by bar. n.s.: non-significant.
cells (Fig. 4a). Appropriately, the gonadotrope-specific transcription factor SF1 is not expressed in thyrotropes (TαT1) or in the gonadotrope progenitor cells (αT1–1). To test whether the gonadotrope-specific regulatory factors, LHX3 and PITX1, are associated with regulatory regions of gonadotrope promoters in any of the studied cell lines, we performed a ChIP analysis using LHX3 and PITX1 antibodies. ChIP analysis revealed that both transcription factors can bind on the gonadotrope terminal target genes (Fig. 4b, c). LHX3 is able to bind the promoter of Cga and Gnhr, but neither Lhb nor Fshb exhibit binding by this method, at any of the stages of development (Fig. 4b, LHX3). PITX1 binds the gonadotrope-specific promoters Cga and Lhb, but not Gnhr nor Fshb in this assay (Fig. 4c). This suggests the program of differentiation in these cells is not regulated simply by deacetylated histones [48], but more likely requires a timed balance of transcription factors, co-factors, histone modifications, DNA methylation, and/or networks to control the developmental gonadotrope gene expression program.

To extend this further, we asked if the final differentiation of the gonadotrope lineage cells required GnRH and/or activin treatment for the proper expression of mature gonadotrope genes. We serum starved LβT2 cells for 16 h then treated them for 4 h with 100 ng/ml GnRH ±25 ng/ml activin and assayed the chromatin status of the Lhb promoter for DNase sensitivity. Indeed, activin with or without GnRH treatment promoted Lhb promoter opening (Fig. 4d, Two-way ANOVA compared to vehicle, $p > 0.05$ for activin at 2 and 5 units of DNaseI, and $p > 0.01$ for activin + GnRH at 2 and 5 units of DNaseI, $p < 0.05$ when comparing activin to activin + GnRH). Lhb is known to be induced by either GnRH or activin [23]. In contrast, these treatments did not change chromatin opening of the already opened Cga and Gnhr promoters and could not change the chromatin status of Fshb (data not shown).

**Discussion**

Elucidation of the molecular and cellular mechanisms underlying pituitary development and cellular specification is critical to our understanding of reproduction and infertility. Herein, we used three immortalized pituitary cell lines, which have been shown to model many aspects of developing gonadotropes [15, 20, 49]. It is clear that epigenetic regulation of chromatin is key in correct gene expression and cellular maturation. In this study, we present data showing that pituitary gonadotrope cell lines reveal epigenetic programming that allows the sequential expression of pituitary hormone genes in differentiation (Fig. 5).

---

**Fig. 4** Pituitary transcription factor accessibility in developing gonadotrope cell lines. **a** Expression levels of transcription factors in NIH3T3, αT1–1, αT3–1, LβT2, and TαT1 cell lines ($n = 2$). ChIP assays in αT1–1, αT3–1, and LβT2 cells were performed using antibodies specific to the transcription factors **b** LHX3 and **c** PITX1. Immunoprecipitated chromatin was subjected to qRT-PCR. Data are presented as fold enrichment relative to IgG, with values normalized to Gnhr1 and expressed as mean ± SEM. Statistical analysis by one-way ANOVA followed by a Tukey post-hoc, *$p < 0.05$, **$p < 0.01$, as indicated by bar. n.s.: non-significant. **d** LβT2 cells were treated with GnRH and/or activin ($n = 3$), before DNA from nuclei were treated with increasing concentrations of DNaseI and analyzed by qRT-PCR with primers specific to Lhb. Amplicon quantities were normalized to untreated samples (Vehicle), and data presented as the mean fraction of DNA remaining relative to vehicle.
The anterior pituitary has five different endocrine cell types: gonadotropes, lactotropes, corticotropes, thyrotropes, and somatotropes. Until now, no efficient method has been established to isolate live gonadotropes for in vitro cell culture. We have previously used the Ribotag technique, which allows selection of ribosome-associated mRNAs of specific cell-types, to isolate gonadotrope mRNAs and process them for qRT-PCR [50, 51]. However, as we were interested in comparing effects of hormone treatments and transcription factors on chromatin status, we performed this study in model gonadotrope cell lines. We used the developmentally staged gonadotrope cell lines, progenitor α T1 – 1, immature α T3 – 1, and mature LβT2 cell models to address the epigenetic regulation status of gonadotrope-specific genes (Cga, Gnhr, Fshb, and Lhb) during gonadotrope maturation.

Studies of the chromatin state of Cga shows that it is open at a very early stage represented by αT1–1 cells, while Gnhr chromatin opens at an immature developmental stage represented by αT3–1. These correlate directly with recruitment of phospho-Pol II and mRNA expression (Fig. 5). The Lhb and Fshb genes only begin to show relaxation of chromatin status in the more developmentally mature cell lines, αT3–1 and LβT2. This opening of the chromatin correlates with the appearance of positive, and loss of negative, histone modifications, which correlates with specific gene expression. We found that both Gnhr and Lhb, though not Fshb, have progressive increases in phospho-Pol II, H3Ac and/or H3K4Me3, as well as a loss of H3K27Me3 and/or H3K9Me2 from the progenitor αT1–1 and immature αT3–1, to the mature LβT2 (Fig. 5). This partial correlation between chromatin state and gene transcript levels was recently described in αT1–1, αT3–1, and LβT2 cells, where Laverriere et al. were unable to detect Fshb and found the Lhb promoter to be equally sensitive to DNaseI treatment in αT1–1, αT3–1, and LβT2 cells [5]. Interestingly, despite lack of Fshb transcription in the mouse fibroblast cell line, NIH3T3, our DNaseI sensitivity assay found the Fshb regulatory region to be relatively sensitive to DNaseI. DNaseI Chip-seq of NIH3T3 cells has been done previously and is available on the USCS genome browser (consulted July 25, 2017). The deposited data set was generated using a different DNaseI protocol from ours, using a single DNaseI concentration, and showed the Fshb and Lhb regulatory regions to be in relatively closed conformations. However, by comparing chromatin status and transcription factor occupancy of
the Fshb regulatory region of numerous studies deposited on the USCS genome browser, we found the upstream region of Fshb to be able to bind transcription factors including REST (repressor element-1 silencing transcription factor), which is a known repressor of transcription [52]. Thus, it is very likely that the DNasel sensitivity of the Fshb regulatory region, in NIH3T3 cells in our assay, shows some degree of opening. In addition, approximately 1 Kb upstream of the transcriptional start site of Fshb is a SINE repeat [53], which can allow expression in many different cell lines, and thus could confer a DNasel sensitive region upstream of the Fshb gene. Interestingly, a SINE repeat has been found within intron 1 of the pig Fshb gene, which affects Fshb expression and reproductive function in pigs [54]. It will therefore be of interest in future studies to address the role of the SINE repeats upstream of the mouse Fshb transcription start site, to determine if these also impact Fshb expression in gonadotrope cell lines.

As expected, Cga, which is highly transcribed in all three cell lines, did not have significant differences between the cell lines in histone modifications or phospho-Pol II occupation. Although we and others [5] did not detect Fshb mRNA in any of the three studied cell lines without using highly sensitive qRT-PCR or activin treatment, Fshb does start to gain H3Ac histone modifications in LβT2 cells as well. Since Fshb is detected at low levels, and only using qRT-PCR in LβT2 cells [55], it is not unexpected that histone modifications may reflect a less active status (Fig. 5). We believe that the low expression of Fshb in LβT2 cells is due to the relatively simple milieu in which the cells are maintained, in comparison to the broad range of stimuli and hormones received by gonadotropes in vivo. The low expression of Fshb is possibly mediated by HDAC. It was previously shown that a GnRH treatment or inhibition of HDACs in αT3–1 cells allowed rapid expression of both Lhb and Fshb [56]. This confirms that αT3–1 cells possess all of the required transcriptional machinery to initiate expression of Lhb and Fshb, but removal of HDACs, particularly HDAC4 from these promoters is required to actively transcribe Lhb and Fshb [56]. Based on this, we hypothesized that hormonal treatment of LβT2 cells would allow opening of the chromatin of these genes. Thus, we treated LβT2 cells with activin ± GnRH. Indeed, activin significantly promoted Lhb promoter opening in LβT2 cells with or without GnRH, however we were unable to detect opening of the Fshb promoter in any of the studied conditions (not shown). As GnRH alone was inefficient in opening the chromatin of the Lhb promoter, perhaps due to the tonic as opposed to pulsatile treatment or the single time point of a 4-h exposure. GnRH does induce Lhb gene expression but may act without further opening the chromatin.

Prior studies have shown that an increase in GnRH pulse frequency favors Lhb expression [57, 58], while a decrease favors Fshb gene expression [59–61]. To further our understanding of chromatin status of gonadotrope genes, and their response to hormone treatment, it will be of interest to expand the presented studies using Chip-seq, which allows the study of enhancers as well as other regulatory regions, in combination with ATAC-seq, a technique which has been shown to be more precise than H3K27Ac in identifying active promoters [62].

To establish the relationship between molecular and epigenetic mechanisms in gonadotropes, we investigated the accessibility of the transcription factors, LHX3, and PITX1, to bind gonadotrope-specific gene promoters in the studied cell lines [63–67]. Interestingly, the regulatory proteins PITX1/2, SF1, and LHX3 were all present in the intermediate gonadotrope cell line (αT3–1), and some even in αT1–1 (PITX1/2, and LHX3). By comparing chromatin status and transcription factor access to the chromatin, our data indicate that LHX3 is important in early and maintained expression of Cga. In agreement with previous studies [25, 31], the opening of the Gnrhr promoter in αT3–1 cells allowed increased association of LHX3 with the promoter, whereas the increased opening of the Lhb promoter correlated with recruitment of PITX1 (Fig. 5) [68]. In our hands, the Fshb promoter was in a rather closed configuration, not allowing detectable association of transcription factors with this gene.

**Conclusion**

Our data show exciting new evidence of gonadotrope-specific chromatin changes taking place during development using immortalized model gonadotrope cell lines. Although the cell lines utilized remain an in vitro system, representative of developing gonadotrope cell lines, we show a progressive opening of the gonadotrope-specific gene promoters, Cga, Gnrhr, Lhb, and Fshb in these model cell lines and demonstrate that these promoters are not only controlled by developmental transcription factors, but also by epigenetic mechanisms that include the modulation of chromatin structure, and histone modifications.

**Abbreviations**

ACTb: Beta actin; Cga: Glycoprotein hormones or αSNU; CHIP: Chromatin immunoprecipitation; E: Embryonic day; FSH: Follicle-stimulating hormone; Fshb: FSH beta subunit; GnRH: Gonadotrope-releasing hormone; GnRHR: Gonadotropin-releasing hormone receptor; H3Ac: H3 acetylation; H3K4: Histone H3-lysine 4; H3K4me3: H3K4 trimethylation; HDAC: Histone deacetylases; LH: Luteinizing hormone; Lhb: LH beta subunit; phospho-Pol II: phosphorylated polymerase II; SF1: Steroidogenic Factor 1 or Nr5a1

**Acknowledgements**

The authors thank Crystal Trang for her assistance with this work and Dr. Dorota Skowronska-Krawczyk for assistance with UCSC databases.
Funding
This work was supported by NIH grants R01 HD085267, R01 HD072754, and R01 HD020377 (to P.L.M.) and by NICHD/NIH P50 HD012303 as part of the National Centers for Translational Research in Reproduction and Infertility (P.L.M.). P.L.M. was partially supported by P30 DK063947, P42 ES101337, and P30 CA023100. H.X. was partially supported by T32 HD007203 and F32 HD070579. H.M.H. was partially supported by K99 HD084759. A.K.J. was partially supported by F32 HD058427 and T32 DK007494. M.J.B. was partially supported by F32 HD058460 and T32 HD007203. Funding sources had no role in this work.

Availability of data and materials
The datasets generated and analyzed during the current study are accessible through GEO Series accession number GSE104513 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE104513).

Authors’ contributions
H.X., H.M.H., and P.L.M. wrote the manuscript. H.X., A.K.J., M.J.B., H.M.H., and P.L.M. designed the experiments, analyzed the data and discussed the results. H.X., A.K.J., M.J.B., and N.S. performed the experiments. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details
1Department of Reproductive Medicine, Center for Reproductive Science and Medicine, University of California, 9500 Gilman Drive La Jolla, San Diego, CA 92039-0674, USA. 2Illumina Inc, 5200 Illumina Way, San Diego, CA 92122, USA. 3Tabor-Ley and Gardner LLP, 402 West Broadway, Suite 2100, San Diego, CA 92101, USA.

Received: 28 March 2017 Accepted: 4 October 2017
Published online: 24 October 2017

References
1. Simmons DM, Voss JW, Ingraham HA, Holloway JM, Brodie RS, Rosenfeld MG, et al. Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other classes of transcription factors. Genes Dev. 1990;4:695–711.
2. Scalke KM, Rosenfeld MG. Pituitary development: regulatory codes in mammalian organogenesis. Science. 2002;295:2231–5.
3. Japón MA, Rubinstein M, Low MJ. In situ hybridization analysis of anterior pituitary hormone gene expression during fetal mouse development. J Histochem Cytochem. 1994;42:1117–25.
4. Hsieh J, Gage FH. Chromatin remodeling in neural development and plasticity. Curr Opin Cell Biol. 2005;17:664–71.
5. Lavermiere JN, LHote D, Tabouy L, Schang AL, Querat B, Cohen-Tannoudji J. Epigenetic regulation of alternative promoters and enhancers in progenitor, immature, and mature gonadotrope cell lines. Mol Cell Endocrinol. 2016;434:250–65.
6. Li B, Carey M, Workman JL. The role of chromatin during transcription. Cell. 2007;128:707–19.
7. Sims RJ 3rd, Nishioka K, Reinberg D. Histone lysine methylation: a signature for chromatin function. Trends Genet. 2003;19:629–39.
8. Gross DS, Garrard WT. Nuclear hypersensitive sites in chromatin. Annu Rev Biochem. 1988;57:159–97.
9. Shewchuk BM, Asa SL, Cooke NE, Liebhaber SA. Pit-1 binding sites at the somatotrope-specific DNase I hypersensitive sites I, II of the human growth hormone locus control region are essential for in vivo hGH-N gene activation. J Biol Chem. 1999;274:35725–33.
10. Venkatases S, Workman JL. Histone exchange, chromatin structure and the regulation of transcription. Nat Rev Mol Cell Biol. 2015;16:178–89.
11. Heintzman ND, Stuart RK, Hon G, Fu Y, Ching CW, Hawkins RD, et al. Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. Nat Genet. 2007;39:331–8.
12. Hirsch N, Nakashima K, Kusakbara T, Mejia E, Gage FH. Histone deacteaylsation inhibition-mediated neuronal differentiation of multipotent adult neural progenitor cells. Proc Natl Acad Sci U S A. 2004;101:16659–64.
13. Siebzehnrull FA, Buslei R, Eyupoglu IY, Seufert S, Hahnen E, Blumcke I. Histone deacteaylsation inhibitors increase neuronal differentiation in adult forebrain precursor cells. Exp Brain Res. 2007;176:672–8.
14. Windle JJ, Weiner R, Mellon PL. Cell lines of the pituitary gonadotrope lineage derived by targeted oncogenesis in transgenic mice. Mol Endocrinol. 1990;4:597–603.
15. Alarid ET, Windle JJ, Whyte DB, Mellon PL. Immunolization of pituitary cells at discrete stages of development by directed oncogenesis in transgenic mice. Development. 1996;122:3319–29.
16. Lew D, Brady H, Klausing K, Yagihama K, Theill LE, Stauber C, et al. GFI-1 promoter-targeted immortalization of a somatotrophic progenitor cell results in dwarfism in transgenic mice. Genes Dev. 1993;7:683–93.
17. Pemasetti F, Spady TJ, Hall SB, Rosenberg SB, Givens ML, Anderson S, et al. Pituitary tumorigenesis targeted by the ovine follicle-stimulating hormone beta-subunit gene regulatory region in transgenic mice. Mol Cell Endocrinol. 2003;203:169–83.
18. Yusta B, Alarid ET, Gordon DF, Ridgway EC, Mellon PL. The thyrotropin beta-subunit gene is repressed by thyroid hormone in a novel thyrotrope cell line, MtGtT cells. Endocrinology. 1998;139:4476–82.
19. Pemasetti F, Vasiliev VV, Rosenberg SB, Bailey JS, Huang H-J, Miller WL, et al. Cell-specific transcriptional regulation of FSHβ by activin and GnRH in the LβT2 pituitary gonadotrope cell model. Endocrinology. 2001;142:2284–95.
20. Graham KE, Nusser ND, Low MJ. LβT2 gonadotroph cells secrete follicle stimulating hormone (FSH) in response to activin A. J Endocrinol. 1999;162R1–5.
21. Bailey JS, Rave-Harel N, Coss D, McGilivray SM, Mellon PL. Activin regulation of the follicle-stimulating hormone beta-subunit gene involves Smads and the TALE homeodomain proteins Pbx1 and Prep1. Mol Endocrinol. 2004;18:1158–70.
22. Barnhart KM, Mellon PL. The orphan nuclear receptor, steroidogenic factor-1, regulates the glycoprotein hormone alpha-subunit gene in pituitary gonadotropes. Mol Endocrinol. 1994;8:876–85.
23. Coss D, Thackray KG, Deng CX, Mellon PL. Activin regulates luteinizing hormone beta-subunit gene expression through smad-binding and homeobox elements. Mol Endocrinol. 2005;19:2610–23.
24. Corpuz PS, Lindaman LL, Mellon PL, Coss D, Foxl2 is required for activin induction of the mouse and human follicle-stimulating hormone beta-subunit genes. Mol Endocrinol. 2010;24:1037–51.
25. McGilivray SM, Bailey JS, Ramanez R, Kirkwood BJ, Mellon PL. Mouse GnRH receptor gene expression is mediated by the LHX3 homeodomain protein. Endocrinology. 2005;146:2180–5.
26. Xie H, Hoffmann HM, Meadows JD, Mayo SL, Trang C, Leming SS, et al. Homeodomain proteins SIX1 and SIX6 regulate gonadotropin-specific genes during pituitary development. Mol Endocrinol. 2015;29:842–55.
27. Steger DJ, Hecht JH, Mellon PL. GATA-binding proteins regulate the human gonadotropin α-subunit gene in placenta and pituitary gland. Mol Cell Biol. 1994;14:5592–602.
28. Rosenberg SB, Mellon PL. An Otx-related homeodomain protein binds an LHβ promoter element important for activation during gonadotrope maturation. Mol Endocrinol. 2002;16:1280–98.
29. Harris AN, Mellon PL. The basic helix-loop-helix-leucine zipper transcription factor USF1 is a key regulator of SF-1 gene expression in pituitary gonadotropes and steroidogenic cells. Mol Endocrinol. 1998;12:724–7.
30. Breen KM, Thackray VG, Coss D, Mellon PL. Runt-related transcription factors impair activin induction of the follicle-stimulating hormone beta-subunit gene. Endocrinology. 2010;151:2669–80.
31. Schang AL, Granger A, Querat B, Beauch S, Cohen-Tannoudji J, Lavermiere JN. GATA2-induced silencing and LHX-homeodomain protein-induced activation are mediated by a bi-functional response element in the rat GnRH receptor gene. Mol Endocrinol. 2013;27:74–91.
32. Zubair M, Ishihara S, Oka S, Okumura K, Morohashi K. Two-step regulation of Ad4BP/SF-1 gene transcription during fetal adrenal development: initiation by a Hox-Pbx1-Prep1 complex and maintenance via autoregulation by Ad4BP/SF-1. Mol Cell Biol. 2006;26:4111–21.
33. Pick L, Schier A, Affolter M, Schmidt-Glensink T, Gehring WJ. Analysis of the fzd upstream element: germ layer-specific enhancers are independently autoregulated. Genes Dev. 1990;4:1224–39.
34. Shima Y, Zubair M, Komatsu T, Oka S, Yokoyama C, Tachibana T, et al. Pituitary homeobox 2 regulates adrenmedullin expression in the pituitary-gonadotrope through interaction with the intronic enhancer. Mol Endocrinol. 2008;22:1633–46.
35. Halvorson LM, Kaiser UB, Chin WW. Stimulation of luteinizing hormone beta gene promoter activity by the orphan nuclear receptor, steroidogenic factor-1. J Biol Chem. 1996;271:6645–50.
36. Coss D, Hand CM, Yaphockun KK, Ely HA, Mellon PL. p38 mitogen-activated protein kinase is critical for synergistic induction of the FShbeta gene by gonadotropin-releasing hormone and activin through augmentation of c-Fos induction and Smad phosphorylation. Mol Endocrinol. 2007;21:3071–86.
37. Lim S, Luo M, Koh M, Yang M, bin Abdul Kadir MN, Tan JH, et al. Distinct mechanisms involving diverse histone deacetylases repress expression of the two gonadotropin beta-subunit genes in immature gonadotropes, and their actions are overcome by gonadotropin-releasing hormone. Mol Cell Biol. 2007;27:4105–20.
38. Burger LL, Haisenleder DJ, Aylor KW, Marshall JC. Regulation of intracellular signaling cascades by GnRH pulse frequency in the rat pituitary: roles for CaMK II, ERK, and JNK activation. Biol Reprod. 2008;79:947–53.
39. Haisenleder DJ, Burger LL, Walsh HE, Stevens J, Aylor KW, Shupnik MA, et al. Pulsatile gonadotropin-releasing hormone stimulation of gonadotropin subunit transcription in rat pituitaries: evidence for the involvement of Jun N-terminal kinase but not p38. Endocrinology. 2008;149:139–45.
40. Dallin AC, Haisenleder DJ, Otolano GA, Ellis TR, Marshall JC. The frequency of gonadotropin-releasing-hormone stimulation differentially regulates gonadotropin subunit messenger ribonucleic acid expression. Endocrinology. 1989;125:917–24.
41. Haisenleder DJ, Katt JA, Otolano GA, el Gevery MR, Duncan JA, Dee C, et al. Influence of gonadotropin-releasing hormone pulse amplitude, frequency, and treatment duration on the regulation of luteinizing hormone (LH) subunit messenger ribonucleic acids and LH secretion. Mol Endocrinol. 1988;2:338–43.
42. Papavassiliou SS, Zmeili S, Khoury S, Landefeld TD, Chin WW, Marshall JC. Gonadotropin-releasing hormone differentially regulates expression of the genes for luteinizing hormone alpha and beta subunits in male rats. Proc Natl Acad Sci U S A. 1988;83:4026–9.
43. Buenrostro JD, Giresi PG, Zaba LC, Chang HY, Greenleaf WJ. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. Nat Methods. 2013;10:1213–8.
44. Mullen RD, Colvin SC, Hunter CS, Savage JJ, Walvoord EC, Banggou AP, et al. Roles of the LHX3 and LHX4 LIM-domain factors in pituitary development. Mol Cell Endocrinol. 2007;290:9–5.
45. Tremblay JJ, Lanctot C, Drouin J. The pan-pituitary activator of transcription, Ptx1 (pituitary homeobox 1), acts in synergy with SF-1 and Pit1 and is an upstream regulator of the Lim-homeodomain gene Lim3/llh3. Mol Endocrinol. 1998;12:424–81.
46. Sheng HZ, Zhadano AB, Mosinger BI, Fuji T, Bertuzzi S, Grinberg A, et al. Specification of pituitary cell lineages by the LIM homeobox gene Lhx3. Science. 1996;272:1004–7.
47. Charles MA, Mortensen AH, Potok MA, Camper SA. Pitx2 deletion in pituitary gonadotropes is compatible with gonadal development, puberty, and fertility. Genesis. 2008;46:507–14.
48. Tremblay JJ, Drouin J. Egri-1 is a downstream effector of GnRH and synergizes by direct interaction with Ptx1 and SF-1 to enhance luteinizing hormone beta gene transcription. Mol Cell Endocrinol. 1999;195:567–76.
49. Fortin J, Lamba P, Wang Y, Bernard DJ. Conservation of mechanisms mediating gonadotropin-releasing hormone 1 stimulation of human luteinizing hormone beta subunit transcription. Mol Hum Reprod. 2009;15:77–87.
50. Cederberg RA, Smith JE, McDonald EA, Lee C, Perkins AR, White BR. Activity of the porcine gonadotropin-releasing hormone receptor gene promoter is partially conferred by a distal gonadotrope specific element (GSE) within an upstream enhancing region, two proximal GSEs and a retinoic X receptor binding site. Reprod Biol Endocrinol. 2015;13:45.
51. Jacobs SB, Coss D, McGillivray SM, Mellon PL. Nuclear factor Y and steroidogenic factor-1 physically and functionally interact to contribute to cell-specific expression of the mouse Follicle-stimulating hormone-beta gene. Mol Cell Endocrinol. 2003;17:87–93.