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# Epigenetic regulation of alternative promoters and enhancers in progenitor, immature, and mature gonadotrope cell lines

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ABSTRACT

Gonadotrope cell identity genes emerge in a stepwise process during mouse pituitary development. *Cga*, encoding for the α-subunit of TSH, LH, and FSH, is initially detected at E11.5 followed by *Gnrhr* and steroidogenic factor *Sf1* at E13.5, specifying cells engaged in a gonadotrope cell fate. *Lhb* and *Fshb* appear at E16.5 and 17.5, respectively, typifying differentiated gonadotrope cells. Using the αT1-1, αT3-1 and LβT2 cell lines recapitulating these stages of gonadotrope differentiation, DNA methylation at *Gnrhr* and *Sf1* was investigated. Regulatory regions were found hypermethylated in progenitor αT1-1 cells and hypomethylated in differentiated LβT2 cells. Abundance of RNA polymerase II together with active histone modifications including H3K4me1, H3K4me3, and H3K27ac were strictly correlated with DNA hypomethylation. Analyses of epigenomic modifications and chromatin accessibility were further extended to *Isl1*, *Lhx3*, *Gata2*, and *Pitx2*, highlighting alternative usages of specific regulatory gene domains in progenitor αT1-1, immature αT3-1, and mature LβT2 gonadotrope cells.



## 1. Introduction

Ontogenesis of the pituitary gland involves the differentiation of six endocrine cell types, the somatotrope, lactotrope, corticotrope, melanotrope, thyrotrope, and gonadotrope lineages, all originating from a common primordium, the adenohypophyseal placode that initiates at embryonic day (E) 7.5 in mouse (Rizzoti and Lovell-Badge, 2005). From this stage differentiation gradually occurs and extends until birth. In neonates, the six endocrine cell lineages are well differentiated in the pituitary. During the developmental period, the glycoprotein hormone alpha subunit (*Cga*) is the first endocrine gene that can be detected at E11.5 (Ericson et al., 1998; Kelberman et al., 2009). This gene encodes for the alpha subunit common to TSH, LH and FSH. Appearance of their specific beta subunits is markedly delayed, *Tshb*, *Lhb* and *Fshb* being detected at E14.5, E16.5, and E17.5, respectively (Kelberman et al., 2009). Regarding the gonadotrope lineage, two other marker genes arise around E13.5, the GnRH receptor (*Gnrhr*) and the steroidogenic factor 1 (*Nr5a1* hereafter referred to as *Sf1*) genes, within a time window extending from the emergence of *Cga* to that of *Lhb* and *Fshb* (Ingraham et al., 1994; Aubert et al., 1985; Granger et al., 2004, 2006). These data emphasize three major steps in the emergence of genes of gonadotrope cell identity: the *Cga* expressing cells, which fate may be gonadotrope or thyrotrope, the cells expressing *Cga*,

*Abbreviations:* *Gnrhr*, gonadotropin releasing hormone receptor gene; *Cga*, glycoprotein hormone α-subunit gene; *Lhb*, luteinizing hormone β-subunit gene; *Fshb*, follicle stimulating hormone β-subunit gene; *Sf1*, steroidogenic factor 1 (*Nr5a1*) gene; *Lhx3*, LIM homeobox 3 gene; *Isl1*, Isl LIM homeobox 1 gene; *Pitx2*, paired-like homeodomain transcription factor 2 gene; *Gata2*, GATA-binding protein 2 gene; H3K4me1, histone H3 monomethylated on lysine 4; H3K4me3, histone H3 trimethylated on lysine 4; H3K27ac, histone H3 acetylated on lysine 27; Pol II, RNA polymerase II phosphorylated on serine 5; E, embryonic day; TSS, transcription start site; CNS, evolutionary conserved non-coding sequences; CTCF, CCCTC-binding factor.

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*Gnrhr* and *Sf1*, which are likely engaged in a gonadotrope cell fate and finally the cells that express *Cga*, *Gnrhr*, *Sf1*, *Lhb* and *Fshb*, which can be considered as mature gonadotrope cells. In addition, several transcription factors involved in the expression of genes of gonadotrope cell identity (Roberson et al., 1994; Steger et al., 1994; Sloop et al., 1999; West et al., 2004; McGillivray et al., 2005; Susa et al., 2009; Wu et al., 2010; Lo et al., 2011; Schang et al., 2013) arise prior to *Cga*, *Sf1* and *Gnrhr* such as ISL1 at E8.5, LHX3 at E9.5 and GATA2 at E10.5 (Ericson et al., 1998; Dasen et al., 1999).

Different mouse gonadotrope cell lines have been generated by directed oncogenesis using the SV40 T antigen under the control of *Cga* and *Lhb* 5′ flanking sequences (Windle et al., 1990; Alarid et al., 1996; Thomas et al., 1996; Turgeon et al., 1996). This strategy led to the generation of cell lines representative of discrete stages of development. Among them the αT1-1, αT3-1, and LβT2 cell lines, which display clearly distinct developmental phenotypes. The αT1-1 cells express only *Cga* similarly to bipotential precursor cells that give rise *in vivo* to the gonadotrope and thyrotrope lineages. The αT3-1 cells also express *Gnrhr* and *Sf1* and are thus likely derived from cells engaged in a gonadotrope cell fate. LβT2 cells express all marker genes, including *Lhb* and, under stimulation, *Fshb* (Graham et al., 1999; Pernasetti et al., 2001; Lannes et al., 2015). These three cell lines have been considered representative of progenitor (αT1-1), immature (αT3-1), and mature (LβT2) gonadotrope cells (Xie et al., 2015). We took advantage of these cell lines to investigate the epigenetic modifications that could affect specific genes at different stages of differentiation. Epigenetic analyses were focused on DNA methylation and histone modifications.

DNA methylation is an epigenetic modification mostly correlated with gene repression (Schübeler, 2015). It is notably involved in long-term mono-allelic repression including X-chromosome inactivation and genomic imprinting. DNA methylation occurs in vertebrate genomes on cytosine at CpG dinucleotides symmetrically on both DNA strands. This epigenetic modification is inherited via the action of DNA methyl transferase I and associated factors. DNA methylation is widespread in mammalian genomes except within CpG islands. These regions are genomic domains extending from 200 up to 500 bp that are highly enriched in C and G nucleotides resulting in elevated density of CpG dinucleotides. Cytosine residues within such genomic domains are rarely methylated. CpG islands are present in the vicinity of numerous gene promoters. *Sf1*, *Isl1* and *Lhx3* are examples of genes containing CpG islands flanking (*Sf1*) or overlapping (*Isl1*) their promoter regions. This contrasts with the *Gnrhr*, *Cga* and *Fshb* promoters that are A-T rich and display low density of CpG dinucleotides.

In addition to DNA methylation, post-translational modifications of histones contribute to either active or repressive chromatin configurations. Chromatin immunoprecipitation (ChIP) assays followed by high-throughput sequencings have generated genome-wide chromatin data sets that led to the establishment of general principles. These rules link some histone modifications to gene activity or inactivity (Barski et al., 2007; Ernst et al., 2011). Acetylated sites on histone H3 including lysine 9, 14, and 27 (H3K27Ac) are correlated with transcriptional activity as well as trimethylation of lysine 4 of histone H3 (H3K4me3). Furthermore, histone modifications act in combinatorial fashion to delimit distinct functional gene domains such as active, weak or inactive promoters and enhancers (Ernst et al., 2011). Thereby, active promoters, which carry phosphorylated polymerase II on Ser 5 (Pol II) at the transcription start site (TSS), are further preferentially associated with both H3K4me3 and H3K27ac while active enhancers are marked by the dual presence of histone H3 monomethylated on lysine 4 (H3K4me1) and H3K27ac. Inactive promoters fail to exhibit H3K4me3 and H3K27ac marks while inactive enhancers may be characterized by the sole presence of the H3K4me1 (Creyghton et al., 2010). These specific associations of chromatin marks are predictive of the activity states of promoters and enhancers in a given cell type.

In this study, the DNA methylation state of genomic regulatory domains of *Gnrhr* and *Sf1* were determined using bisulfite DNA sequencing. Modifications of histone H3 including H3K4me3, H3K27ac, and H3K4me1 together with the presence of Pol II were assessed using specific antibodies in ChIP assays. Such analyses were extended to genes encoding transcription factors involved in the gonadotrope-specific expression of *Gnrhr* such as *Isl1*, *Lhx3*, and *Gata2* as well as to *Pitx2*, which is primarily implicated in the activation of *Sf1* gonadotrope enhancer (Shima et al., 2008). DNase I sensitivity assays were further performed to evaluate accessible chromatin conformation at promoters and enhancers (Thurman et al., 2012). Our data showed that DNA methylation state together with specific histone modifications were well correlated with *Gnrhr* and *Sf1* expression levels in the aforementioned cell lines. Interestingly, gene promoters and enhancers were differentially implemented depending on the cell line to achieve developmental stage-specific levels of gene expression.

## 2. Materials and methods

### 2.1. Materials and antibodies

Chemical products were obtained from Sigma-Aldrich (Sigma-Aldrich, Lyon, France) unless otherwise indicated. Antibodies against H3K4me3 (ab8580), H3K4me1 (ab8895), H3K27ac (ab4729) and Pol II (ab5131) were from Abcam (Cambridge, UK).

### 2.2. Cell cultures

Mouse αT1-1, αT3-1 and LβT2 cells, generously provided by Dr. P. Mellon (University of California, La Jolla, CA), and corticotrope AtT20 cells obtained from the American Type Culture Collection were maintained in monolayer cultures using high glucose DMEM supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin at 37 °C in humidified 5% CO2, 95% air.

### 2.3. Bisulfite conversion of genomic DNA

Bisulfite conversion was performed essentially as described in the Schumacher's guide (Schumacher, 2009). Six micrograms of *Hin*dIII restricted genomic DNA were dissolved in water to a final volume of 50 μl and 25 μl of 0.9 N NaOH, 25 mmol l−1 EDTA were added. The sample was incubated at 95 °C for 3 min to denature the DNA strands. A total of 500 μl of 500 mg/ml sodium bisulfite, 8 mmol l−1 hydroquinone, 0.3 N NaOH, 0.5 mg/ml Trolox, 1 mmol l−1 tetraethylammoniumchloride, 300 mmol l−1 guanidine hydrochloride, pH 5.0 was added and the sample was then heated at 95 °C for 30 s, followed by four successive steps at 58 °C for 20 min, 40 min, and two 60 min steps, each separated by a 10 s step at 95 °C. The bisulfite converted DNA was then purified on nucleospin columns (Macherey-Nagel, Düren, Germany) and desulfonated by adding 11 μl of 3 N NaOH to 100 μl DNA in water followed by a 20 min incubation at 42 °C. DNA was then ethanol precipitated and re-suspended in water. Amplification of bisulfite converted DNA was performed using the hotstart PlatinumTaq DNA Polymerase (Thermo Fisher Scientific/Invitrogen, Illkirch, France). The resulting PCR products were then inserted into pGEM-T vector (Promega) and transfected into bacteria. At least 10 clones per cell line were selected and sequenced to determine the state of CpG methylation. C to T (or G to A on the antisens strand) conversion outside the CpG sites reached 99.8% efficiency.