Molecular and Cellular Endocrinology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

PLAG1 expression and target genes in the hypothalamo-pituitary system in male mice

Almas R. Juma[a], Nathan E. Hall[b], Joanne Wong[a], Jemma G. Gasperoni[a], Yugo Watanabe[a], Akashdeep Sahota[a], Pauliina E. Damdimopoulou[c], Sylvia V.H. Grommen[a], Bert De Groef[a,*]

[a] Department of Physiology, Anatomy and Microbiology, La Trobe University, Bundoora, Victoria, 3086, Australia
[b] Department of Biochemistry and Genetics and La Trobe Institute for Molecular Sciences, La Trobe University, Bundoora, Victoria, 3086, Australia
[c] Department of Clinical Sciences, Intervention and Technology, Karolinska Institute, Karolinska University Hospital, 14183, Huddinge, Sweden

ARTICLE INFO

Keywords:
GnRH
Gonadotrope
Hypothalamus
LH
Pituitary gland
PLAG1

ABSTRACT

Knockout of pleomorphic adenoma gene 1 (PLAG1) in mice results in reduced fertility. To investigate whether PLAG1 is involved in reproductive control by the hypothalamo-pituitary system in males, we determined PLAG1 expression sites and compared gene expression between hypothalami and pituitary glands from *Plag1* knockout and wildtype animals. Abundant expression of PLAG1 was detected throughout the pituitary gland, including gonadotropes and somatotropes. The hypothalamus also contained a large number of PLAG1-expressing cells. PLAG1 was expressed in some gonadotropin-releasing hormone neurons, but not in kisspeptin neurons. Gene ontology analysis indicated upregulation of cell proliferation in both structures, and of cholesterol biosynthesis in the hypothalamus, but functional confirmation is required. Expression levels of pituitary gonadotropins and gonadotropin-releasing hormone receptor, and of brain gonadotropin-releasing hormone and kisspeptin mRNA were unaffected in knockout mice. We conclude that PLAG1 deficiency does not have a major impact on the reproductive control by the hypothalamo-pituitary system.

1. Introduction

Pleomorphic adenoma gene 1 (PLAG1) is a zinc finger transcription factor that regulates cell proliferation through its target genes, which include insulin-like growth factor 2 and other growth factors (Voz et al., 2000, 2004). PLAG1 is mainly known for its involvement in the development of various types of tumors, but also plays a role in normal growth in humans, mice and various farm animal species (Juma et al., 2016; Fink et al., 2017; Habib et al., 2018). In addition, PLAG1 is thought to be important in reproduction, since male and female mice with targeted disruption of both alleles of *Plag1* show reduced fertility (Hensen et al., 2004). Recently, we showed that in *Plag1* knockout (KO; *Plag1*$^{-/-}$) males, at least part of this reproductive phenotype is due to significantly reduced sperm output and sperm motility (Juma et al., 2017).

Besides a role in the functioning of the gonads, PLAG1 could also affect reproductive function at the level of the pituitary gland and/or the hypothalamus. The hypothalamus secretes gonadotropin-releasing hormone (GnRH), which stimulates gonadotropes in the anterior pituitary gland via the GnRH receptor (GnRHr) to synthesize and release follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These hormones are crucial for reproduction in both sexes. In males, they directly regulate testicular androgen synthesis by stimulating the Leydig cells, and spermatogenesis via effects on the Sertoli cells (Rey et al., 2013). The activity of GnRH neurons is under the control of various other neurons, most notably kisspeptin (KiSS1) neurons (Pinilla et al., 2012). Defects in the pituitary gland and/or hypothalamus can affect hormone production, which may then alter the function of downstream organs.

Support for a potential role for PLAG1 in the hypothalamus or pituitary gland comes from earlier chicken and mouse studies. *PLAG1* was identified among nine upregulated genes in the hypothalamo-pituitary system of two high-producing chicken layer strains compared to low-producing strains (Shiue et al., 2006; Chen L.-R. et al., 2007). *PLAG1* expression in the hypothalamo-pituitary system was positively correlated with several laying traits, such as number of eggs to 50 weeks of age and laying rate after first egg. A negative correlation was found between hypothalamo-pituitary *PLAG1* expression levels and oviposition lag within clutch and average pause length (Chen C.-F. et al., 2007). Furthermore, the anterior pituitary gland is one of few organs that show high *Plag1* mRNA expression in adult mice (Hensen et al., 2004).

* Corresponding author. La Trobe University, School of Life Sciences, Bundoora, VIC, 3086, Australia.
E-mail address: b.degroef@latrobe.edu.au (B. De Groef).

https://doi.org/10.1016/j.mce.2018.07.009
Received 27 April 2018; Received in revised form 28 June 2018; Accepted 23 July 2018

The aim of this study was to investigate the role of PLAG1 in the male pituitary gland and hypothalamus by determining its expression sites and its target genes, the latter through transcriptome comparisons between wildtype (WT; *Plag1*$^{+/+}$) and KO male mice, and to determine whether the fertility issues seen in male KO mice relate to impaired pituitary or hypothalamus control in the hypothalamo − pituitary − gonadal axis.

2. Materials and methods

2.1. Animals

Mice were kept in individually ventilated cages with food and water *ad libitum*. The generation of *Plag1* KO mice was described earlier (Hensen et al., 2004); in these mice, the entire *Plag1* coding sequence has been replaced by the *lacZ* reporter gene, resulting in temporal and spatial expression of β-galactosidase in place of PLAG1, and therefore allowing the use of X-gal staining to determine PLAG1 expression sites. Ear or tail clips were used to genotype the mice by PCR as previously described (Hensen et al., 2004). This study was carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th edition, 2004) of the National Health and Medical Research Council. All animal procedures were approved by the Animal Ethics Committee of La Trobe University (AEC13-08).

2.2. X-gal staining

For X-gal staining, adult (> 2 months old) KO, heterozygous (HET; *Plag1*$^{+/-}$), and WT male mice (n = 5 per genotype) were euthanized by CO_2 asphyxiation. Brains and pituitary glands were dissected and further processed for cryosectioning as described by Juma et al. (2017). Pituitary glands and brains were cut into 7-µm and 30-µm cryosections, respectively. X-gal staining, postfixation and mounting were performed as described before (Juma et al., 2017).

2.3. Combined X-gal staining and immunohistochemistry

First, X-gal staining was performed as above (n = 3). Immunostainings were done as described by Juma et al. (2017). Pituitary sections were incubated overnight at 4 °C with polyclonal rabbit anti-mouse growth hormone (GH) or rabbit anti-mouse LHβ antibody (both purchased from Dr A F Parlow, National Hormone and Peptide Program, USA), each diluted 1:8000 in phosphate-buffered saline (PBS) with 4% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) Triton X-100. Brain sections were incubated overnight at 4 °C with polyclonal rabbit anti-mouse GnRH (Abcam, Cambridge, UK; catalog number ab5617; RRID: AB_304986) or rabbit anti-mouse KiSS1 (Merck Millipore, Darmstadt, Germany; catalog number AB9754; RRID: AB_2296529), each diluted 1:1000. The slides were further incubated for 1 h at 4 °C with biotinylated goat anti-rabbit IgG (1:200; Dako) and streptavidin − horseradish peroxidase (1:400; Dako). The signal was visualized with 3,3′-diaminobenzidine tetrahydrochloride hydrate.

2.4. RNA isolation, library construction and RNA-seq

Pituitary glands (n = 3 of each WT, HET and KO) and brains (n = 3 of each WT and KO) from male mice aged 5 weeks were collected and stored in RNA*later* at −80 °C. Each brain was cut coronally at the level of the optic chiasm and rostrally of the pons. Then two lateral sagittal cuts and one dorsal axial cut were made to remove the cortex portion, leaving an area comprising the thalamus, hypothalamus and a small part of the midbrain, hereafter referred to as 'hypothalamus'. RNA isolation, library construction and RNA-sequencing proceeded as described by Juma et al. (2017). Briefly, total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), treated with DNase I using the TURBO DNA-*free* kit (Invitrogen), and purified using the Zymo RNA Clean & Concentrator-5 (Zymo Research Corporation, Irvine, CA, USA). RNA quality was determined using the Agilent RNA 6000 Nano kit on an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA); RNA Integrity Number (RIN) values ranged from 7.9 to 8.8 for the pituitary samples and from 7.0 to 9.0 for the hypothalamus samples. The RNA concentration was measured using the Qubit RNA HS Assay kit on the Qubit 3.0 Fluorometer (Invitrogen) and an equal starting quantity of 150 ng of total RNA for each of the pituitary samples and 1000 ng for the hypothalamus samples underwent poly(A) selection.

Nine or six sequencing libraries (for pituitary and hypothalamus, respectively) were constructed with the TruSeq Stranded mRNA LT − Set A (Illumina, San Diego, CA, USA). Library quality was determined using an Agilent DNA 1000 kit on the Agilent 2100 Bioanalyzer, and the concentration was determined using the Qubit dsDNA HS Assay kit on the Qubit 3.0 Fluorometer. The average fragment size for each library was ~250 bp. Prior to sequencing, the nine individually indexed libraries were pooled in equimolar quantities, and sequenced using 150-bp paired-end sequencing on a single lane of an Illumina NextSeq 500, generating approximately 36–42 million, 30–34 million, and 42–45 million 150-bp paired-end reads for the WT, HET and KO pituitary libraries, respectively, and approximately 32–48 million and 40–65 million for the WT and KO hypothalamus libraries, respectively. The raw data were submitted to the NCBI Sequence Read Archive, accession number PRJNA358071.

2.5. RNA-seq data analysis

Analysis of RNA-seq data was performed as described before (Juma et al., 2017). The raw sequencing data were initially assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The paired-end reads were merged to a single read using PEAR (Zhang et al., 2014), with 97% of reads properly overlapping. The resulting high-quality single reads were mapped to the mouse genome (version mm10) using TopHat2 version 2.1.0 (Kim et al., 2013) with Bowtie2 version 2.2.6 (Langmead and Salzberg, 2012). Expression counts were determined for each gene using htseq-count and the standard mm10 gene annotations. Standard differential expression analysis with a false discovery rate (FDR) cut-off of 0.05 was performed using the edgeR program as implemented in Degust (http://www.vicbioinformatics.com/degust). GOrilla (Eden et al., 2007, 2009) and PANTHER (Mi and Thomas, 2009; Mi et al., 2013) gene ontology (GO) analysis tools were used to identify biological pathways that were enriched and to categorize genes according to GO terms in the different data sets.

2.6. Quantitative RT-PCR

The same RNA samples as those used for RNA-seq were reverse transcribed, subjected to qPCR and analyzed as described by Juma et al. (2017). Quantitative PCR was used to determine expression levels of hypophyseal *Fshb, Lhb, Gh, Cenpe, Top2a, Plagl1*; hypothalamic *Gnrh, Kiss1, Top2a*; and, in both tissues, the reference gene *Actb*, whose expression levels were shown to be unchanged by *Plag1* knockout in the transcriptome study. Primer sequences are listed in Supplementary Table S1.

2.7. Serum hormone measurement

Serum was collected from seven WT males (aged between 5 and 36 weeks) and eight KO males (aged between 5 and 54 weeks). Serum LH concentrations were measured using a discontinuous radioimmunoassay on 40-µl duplicates, as described by Itman et al. (2015). Serum samples were measured in a single assay, the lowest and highest limits of detection being 0.087 ng/ml and 16.09 ng/ml, respectively. The intra-assay variation was 8.8%. Serum FSH concentrations were