Molecular Plasticity of Murine Gonadotropes

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Dissertation

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Sen Qiao

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in partial fulfillment of requirements for the degree of

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in

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Submitted by

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Department of Pharmacology and Toxicology

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Zusammenfassung

Gonadotrope Zellen in der anterioren Hypophyse sind innerhalb der Hypothalamus-Hypophysen-Gonaden-Achse von besonderer Bedeutung, da diese die Kommunikation zwischen dem Gehirn und den Gonaden vermitteln und damit eine funktionelle Verbindung innerhalb der Reproduktionsachse darstellen. Neuere Ergebnisse deuten darauf hin, dass gonadotrope Zellen weiblicher Mäuse in Form eines Netzwerkes organisiert sind, das Plastizität zeigt und sich dadurch den veränderten endokrinen Bedingungen unterschiedlicher physiologischer Zustände anpasst. Allerdings ist bisher wenig darüber bekannt, wie sich diese Zellen auf molekularer Ebene den unterschiedlichen hormonellen Bedingungen funktionell anpassen. In dieser Dissertation habe ich eine binäre genetische Strategie benutzt, die das Markieren der gonadotropen Zellen von Mäusen mit einem Fluoreszenzprotein erlaubt. Dieses Mausmodell ermöglichte mir das Anreichern der gonadotropen Zellen unter Verwendung der fluoreszenzaktivierenden Zellsortierung und anschließender mRNA-Sequenzierung. Unter Verwendung dieser Methode habe ich die Expressionsprofile der gonadotropen Zellen unter Berücksichtigung des Geschlechtes und in verschiedenen Entwicklungs- und Hormonstadien analysiert und miteinander verglichen werden. Hierzu habe ich das Transkriptom gonadotroper Zellen aus juvenilen Männchen bzw. Weibchen, aus zyklierenden Weibchen im Diestrus bzw. im Proestrus, und aus laktierenden Weibchen bzw. aus adulten Männchen untersucht. meine Daten zeigen, dass gonadotrope Zellen in jedem analysierten Stadium ein einzigartiges Expressionsprofil mit 100-500 Genen, die nur in einem bestimmten Stadium exprimiert werden, aufweisen. Zudem konnte ich zeigen, dass in diesen Zellen umfangreiche Genexpressionsprofiländerungen mit bis zu 2200 differenziell exprimierten Genen während der Entwicklung und beim Vergleich interschiedlicher Hormonprofile stattfinden. Unter Verwendung der Kyoto Enzyklopädie (Gen und Genomanalyse) konnte ich zeigen, dass von den differentiell exprimierten Genen jene prominent exprimiert waren, die unter anderem im GnRH-Signalweg, im Ca^{2+}-Signalweg und im MAPK-Signalweg eine wichtige Rolle einnehmen. Meine Daten zeigen einen hohen Grad an molekularer Plastizität innerhalb der gonadotropen Zellpopulation.

Neuere Ergebnisse deuten auf einen Zusammenhang zwischen den Transienten Rezeptor Potential (TRP) Kationenkanäle und der Hypophysenphysiologie hin. Bisher ist jedoch nicht bekannt, ob und wie TRP-Kanäle zur Funktion der gonadotropen Zellen beitragen.

Meine Untersuchungen zeigen, dass 14 von 28 TRP-Kanälen, die im gesamten Mausgenom kodiert sind, in den gonadotopen Zellen der Maus exprimiert werden, wobei die höchsten Expressionswerte für TRPC5 bei juvenilen Weibchen gefunden wurden. Die TRP-Kanal-Expression in diesen Zellen weisteine beträchtliche Plastizität auf und hängt sowohl vom Geschlecht als auch vom Entwicklungs- und Hormonstatus des Tieres ab. Wir kombinierten dann verschiedene genetische Strategien, um die TRPC5-Kanalfunktion in den gonadotropen Zellen von juvenilen Weibchen zu charakterisieren. Wir konnten zeigen, dass der TRPC5-Agonist Englerin A ein zytosolisches Ca^{2+}-Signal und einen Zellstrom in diesen Zellen aktiviert, der bei TRPC5-defizienten Mäusen fehlt. Weiterhin konnten wir eine TRPC5-Aktivierung in den gonadotropen Zellen nachweisen, die durch die Stimulation des GnRHR induziert wird. Bisher konnte ich allerdings keine Abnormalitäten in reproduktionsphysiologischen Parametern wie dem Einsetzen der Pubertät wie auch dem Körpergewicht in TRPC5-defizienten weiblichen Mäusen im Vergleich zu den Kontrollen feststellen.

Die Ten-eleven translocation (TET) Enzyme nehmen eine zentrale und komplexe Rolle in der Festlegung der Genexpressionsänderung während der Entwicklung ein. Wir zeigen hier, dass Tet1, Tet2 und Tet3 in murinen gonadotropen Zellen exprimiert werden und dass Tet1 die Expression der luteinisierenden Hormon β-Untereinheit (Lhb) unterdrückt, während Tet2 die Expression des Lhb-Gens verstärkt. Das Tet1-Level sinkt einhergehend mit Zelldifferenzierung und bei Exposition zum regulatorischen GnRH. Unsere Arbeit identifiziert neue regulatorische Elemente und einen neuartigen epigenetischen Signalweg bezüglich der zentralen Reproduktionregulation von Säugetieren.

Zusammenfassend konnte ich zeigen, dass murine gonadotrope Zellen erhebliche Plastizität auf molekularer Ebene aufweisen, um sich den unterschiedlichen physiologischen Bedürfnissen anzupassen. Wir fanden, dass Tet1 und Tet2 unterschiedliche Rollen bei der Regulierung der Expression von Lhb einnehmen. Zudem konnten wir zeigen, dass GnRH den TRPC5-Kanal über den GnRHR aktiviert.

Abstract

Gonadotropes in the anterior pituitary gland are of particular importance within the hypothalamic-pituitary-gonadal axis because they provide a means of communication and thus a functional link between the brain and the gonads. Recent results indicate that female gonadotropes may be organized in the form of a network that shows plasticity and adapts to the altered endocrine conditions of different physiological states. However, little is known about functional changes on the molecular level within gonadotropes during these different conditions. In this study I capitalized on a binary genetic strategy in order to fluorescently label murine gonadotrope cells. Using this mouse model allows to produce an enriched gonadotrope population using fluorescence activated cell sorting to perform mRNA sequencing. By using this strategy, I analyzed and compared the expression profile of murine gonadotropes in different genders and developmental and hormonal stages. I found that gonadotropes taken from juvenile males and females, from cycling females at diestrus and at proestrus, from lactating females, and from adult males each have unique gene expression patterns with approximately 100 to approximately 500 genes expressed only in one particular stage. I also demonstrated extensive gene-expression profile changes with up to 2200 differentially expressed genes when comparing female and male development, juveniles and adults, and cycling females. Differentially expressed genes were significantly enriched in the GnRH signaling, Ca^{2+} signaling, and MAPK signaling pathways by Kyoto Encyclopedia of Genes and Genomes analysis. My data provide an unprecedented molecular view of the primary gonadotropes and reveal a high degree of molecular plasticity within the gonadotrope population.

Recent results have implicated Transient Receptor Potential (TRP) cation channels in pituitary physiology, however, if and how TRP channels contribute to gonadotrope function is not known. I found that 14 out of 28 TRP channels encoded in the mouse genome are expressed in murine gonadotropes with highest expression levels found for TRPC5 in juvenile females. We show that TRP channel expression in these cells exhibits considerable plasticity and that it depends on gender as well as on the developmental and hormonal status of the animal. We then combined different genetic strategies to characterize TRPC5 channel function in gonadotropes from juvenile females. We show that the TRPC5 agonist Englerin A activates a cytosolic Ca^{2+} signal and a whole-cell current in these cells, which is absent in TRPC5-deficient mice. We further show a TRPC5

activation in gonadotropes via stimulation of the GnRHR. However, I also observed normal puberty onset and body weight in TRPC5-deficient female mice.

The Ten-eleven translocation (TET) enzymes play central and complex roles in determining the changing patterns of gene expression during development. We report here that Tet1, Tet2 and Tet3 expressed in murine gonadotropes, and that Tet1 represses the expression of the luteinizing hormone β-subunit (*Lhb*) gene, whereas Tet2 enhances the expression of the *Lhb* gene. Tet1 levels drop with cell differentiation and exposure to the regulatory gonadotropin-releasing hormone. Our work thus exposes new regulatory elements and a novel epigenetic pathway in the central regulation of mammalian reproduction.

In summary, I found that murine gonadotropes show great plasticity to adapt different physiological needs. We found Tet1 and Tet2 played distinct roles in regulating the expression of the gene *Lhb*. We also found a TRPC5 activation in gonadotropes via stimulation of the GnRHR.

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List of abbreviations

°C	degree Celsius
bp	base pair
cDNA	complementary deoxyribonucleic acid
d	day
DAVID	database for annotation, visualization and integrated discovery
DNA	deoxyribonucleic acid
dNTP	deoxynucleotides
eR26-τGFP	eROSA26-τGFP
et al.	*et altera*
FACS	fluorescence activated cell sorting
FPKM	fragment per kilobase of transcript per million fragment
g	grams
gDNA	genomic DNA
GFP	green fluorescent protein
GnRHR	GnRH receptor
GO	gene ontology
GRIC	GnRHR-IRES-CRE
hpg	hypothalamic-pituitary-gonadal
IRES	Internal ribosome entry site
Kb	kilo base
l	litter
M	molar
mg	milligram
Min(s)	minute(s)
ml	milliliter
mM	millimolar
mRNA	messenger ribonucleic acid
nm	nanometer
PBS	phosphate buffered saline

PCOS	polycystic ovary syndrome
PCR	Polymerase chain reaction
pH	potentium hydrogenii
R26-YFP	ROSA26-YFP
RNA	ribonucleic acid
RNA-seq	RNA sequencing
rpm	revolutions per minute
RT	room temperature
Sec(s)	second(s)
TAE	Tris-acetate-EDTA
Tris	Tris-(hydroxymethyl)-aminomethane
V	volume
YFP	yellow fluorescent protein
µg	microgram
µl	microliter
µM	micromolar

1 Introduction

1.1 Hypothalamic–Pituitary–Gonadal (HPG) axis and reproduction

The hypothalamic–pituitary–gonadal (HPG) axis is the key regulator of mammalian reproduction. It consists of three components: the central nervous system, the pituitary and the gonads.

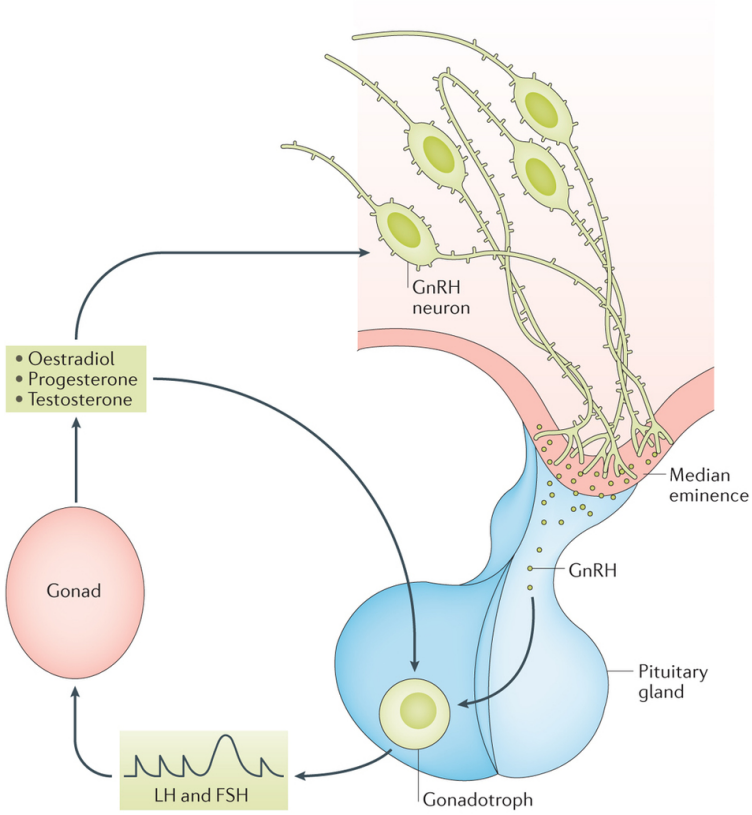

Figure 1 HPG axis

GnRH has been described as the master molecule of reproduction. It is secreted into the hypophyseal portal circulation by GnRH neurons within the hypothalamus. GnRH then acts on its target cells; the gonadotropes in the anterior pituitary. In response to this stimulation, gonadotropes synthesize and release the gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH). These gonadotropins then travel through the blood circulation to the gonads to regulate their function. The gonads in turn secrete sex steroids (among other things) to deliver feedback to the hypothalamus and pituitary levels. Adapted from (Herbison, 2016).

The pituitary gland acts as an interface between the central nervous system and the periphery. A key component in this system is gonadotropin-releasing hormone (GnRH), which is released from the median eminence into the hypophyseal portal circulation, reaching the pituitary and triggering the activation of the gonadotropes (Gore, 2002; Herbison, 2006; Kaiser, 2006). These cells then respond (depending on the pulse frequency) by secreting luteinizing hormone (LH), and/or follicle stimulating hormone (FSH) (Childs, 2006; Haisenleder et al., 1991). The gonads then receive these signals from the pituitary and feedback onto both the central nervous system and the pituitary (Figure 1) (Tilbrook and Clarke, 2001).

1.1.1 GnRH

GnRH is the most important molecule in the HPG axis. GnRH is a 10 amino acids long neuropeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly·NH2). It has three different forms across species: GnRH-1, GnRH-2, GnRH-3 (Gore, 2002). Since only GnRH-1 has been detected in mice, GnRH refers to GnRH-1 in this thesis (Morgan and Millar, 2004) (Figure 2).

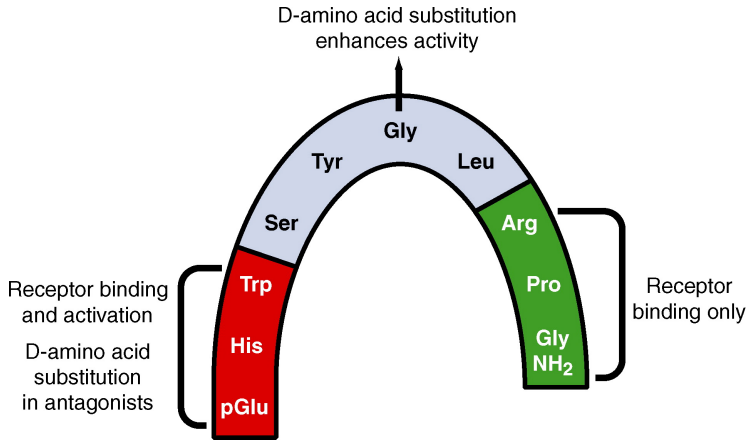

Figure 2 Mammalian GnRH structure model

The red and green amino acids are responsible for receptor binding. GnRH is bent around position 6, glycine. D-amino acids substitution of this position helped the folded status, binding affinity and also reduced the risk of metabolic clearance. Adapted from (Millar and Newton, 2013).

GnRH is produced and secreted by GnRH neurons in the hypothalamus of the murine brain. These neurons are found scattered in the anterior hypothalamus and send projections to the median eminence where GnRH is released. Uniquely, GnRH neurons are born in the

nose and then migrate to the hypothalamus during embryonic development (Merchenthaler et al., 1984; Schwanzel-Fukuda and Pfaff, 1989). Although most GnRH neurons are located in the preoptic area (POA) of the murine hypothalamus, few GnRH neurons can still be detected along their migratory pathway (Silverman, 1994).

Upstream of the GnRH neurons, two distinct population of neurons exert strict control over GnRH neuron activity, both of which express kisspeptin; the most potent stimulator of GnRH neurons (de Roux et al., 2003; Kirilov et al., 2013; Seminara et al., 2003). One population located in the arcuate (ARC) nucleus is thought to regulate GnRH pulse frequency most likely via coordinated intra-network activation (via neurokinin B) and inhibition (via dynorphin) (Navarro, 2013). The other population is located in the anteroventral periventricular nucleus (AVPV) and has been associated with the generation of the LH surge required for ovulation in females (Kinoshita et al., 2005). GnRH neurons however are not only connected to kisspeptin neurons. Transsynaptic tracing has revealed that GnRH neurons are connected to numerous brain regions including the BSTMPM (posteromedial bed nucleus of the stria terminalis) and the PMCO (posterocortical amygdalar nucleus), as well as to the MEA (medial amygdala) (Boehm et al., 2005).

1.1.2 The pituitary gland and gonadotropes

Gonadotropes, which are located in the anterior pituitary, are the target cells of GnRH. The pituitary is one of the endocrine organs, which is located at the base of the murine brain, under the median eminence. It is a key organ to control many physiological events such as body growth, reproduction, and lactation via secreting hormones. It has three different lobes: the anterior lobe, the posterior lobe and the intermediate lobe (Kelberman et al., 2009).

Five different kinds of endocrine cells can be found in the anterior lobe. The corticotrope produces and secretes adrenocorticotropic hormone (ACTH). The lactotrope secretes prolactin (PRL). The somatotrope secretes growth hormone (GH). The thyrotrope secretes thyroid-stimulating hormone (TSH), and the gonadotrope secretes LH or FSH or both. The posterior part of the pituitary contains the axons from hypothalamic neurons and pituicytes. Oxytocin and vasopressin are the two hormones that are made in hypothalamus but

released in the posterior pituitary (Figure 3). The melanotropes located in the intermediate lobe secrete α melanocyte-stimulating hormone (α-MSH).

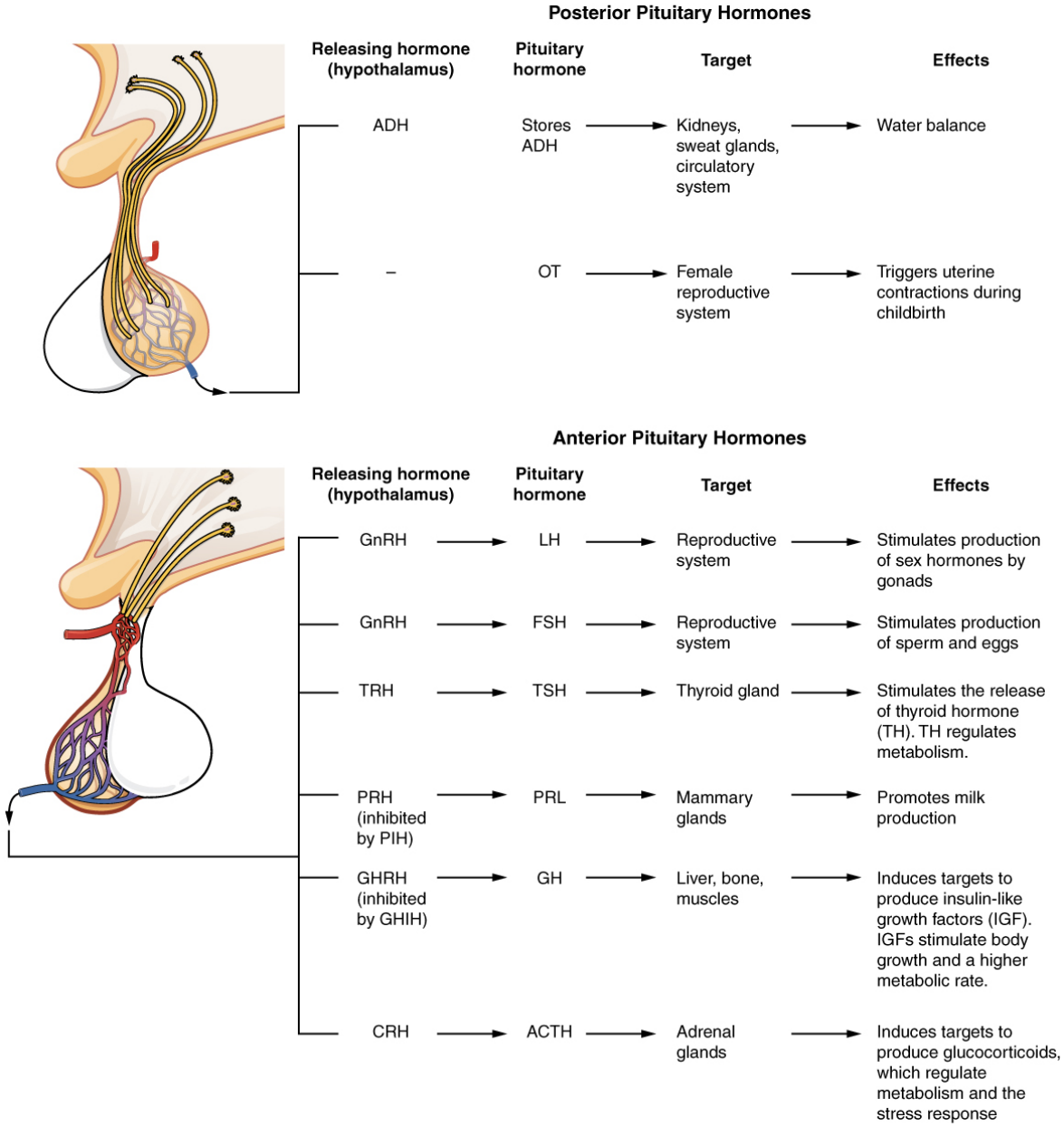

Figure 3 The function of major pituitary hormones

ADH, antidiuretic hormone; OT, oxytocin; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone; PRL, prolactin; GH, growth hormone; GHRH, growth hormone–releasing hormone; GHIH, growth hormone–inhibiting hormone; CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone. Adapted from https://opentextbc.ca/anatomyandphysiology/chapter/17-3-the-pituitary-gland-and-hypothalamus/.

1.1.3 The development of the pituitary and gonadotropes

The three lobes of the pituitary have different embryonic origins. The anterior lobe and intermediate lobe come from Rathke's pouch, which is developed from a thickening of the oral ectoderm. The posterior lobe is derived from neural ectoderm (Rizzoti and Lovell-Badge, 2005).

Gonadotropes compose 5-15% of the pituitary cells. There are four important genes characterizing gonadotropes. They are: the GnRH receptor, *Gnrhr*; the glycoprotein hormone alpha subunit of FSH and LH, *Cga,* which also encodes the alpha subunit of TSH; the beta subunit of LH, *Lhb*; and the beta subunit of FSH, *Fshb.*

During mouse embryonic development, *Cga* is first detected at E11.5 (Ericson et al., 1998). Then *Gnrhr* is detected around E13.5 (Granger et al., 2006). Finally the beta subunits of gonadotropins are detected at E16.5 (*Lhb*), and E17.5 (*Fshb*) (Kelberman et al., 2009) (Figure 4).

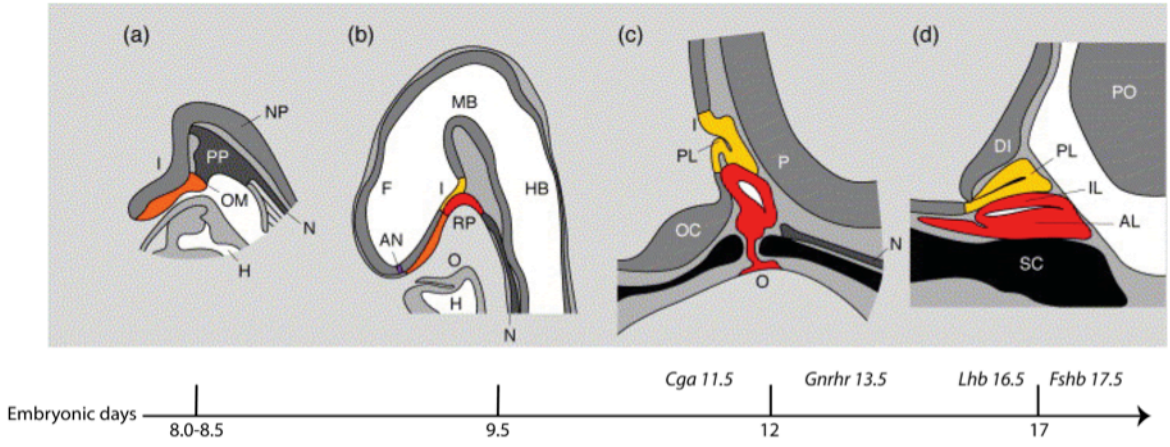

Figure 4 Murine pituitary development and the onset of gonadotrope-specific genes

(a) Oral ectoderm. (b) Rudimentary pouch. (c) Definitive pouch. (d) Adult pituitary gland. I, infundibulum; NP, neural plate; N, notochord; PP, pituitary placode; OM, oral membrane; H, heart; F, forebrain; MB, midbrain; HB, hindbrain; RP, Rathke's pouch; AN, anterior neural pore; O, oral cavity; PL, posterior lobe; OC, optic chiasm; P, pontine flexure; PO, pons; IL, intermediate lobe; AL, anterior lobe; DI, diencephalon; SC, sphenoid cartilage. Adapted from (Kelberman et al., 2009).

1.1.4 The function of the pituitary and gonadotrope

The pituitary needs to adapt to different physiological demands in terms of secreting different or different amount of hormones. Since the pituitary gland is the master gland of hormone control in mammalian species, it must have a great plasticity to adapt to the different physiological demands. Mammalian species experience different developmental stages during their life. Within each developmental stage, there are different physiological events happening. For example, in new-born animals, increasing body weight and size is an important event. At the prepubertal stage, sexual maturation and gender differences are important events. To meet these demands, the pituitary gland needs to secrete a variety of hormones at varying amounts to regulate the hormone target organs.

Out of all different hormone-producing cells, gonadotropes show the most robust plasticity. This is not only because gonadotropes played an important role in sexual maturation, but also these cells secrete a huge variety of different hormones at different levels in a short time (4-5 days in mice) during the estrous cycle in females (Figure 5) (Miller and Takahashi, 2013).

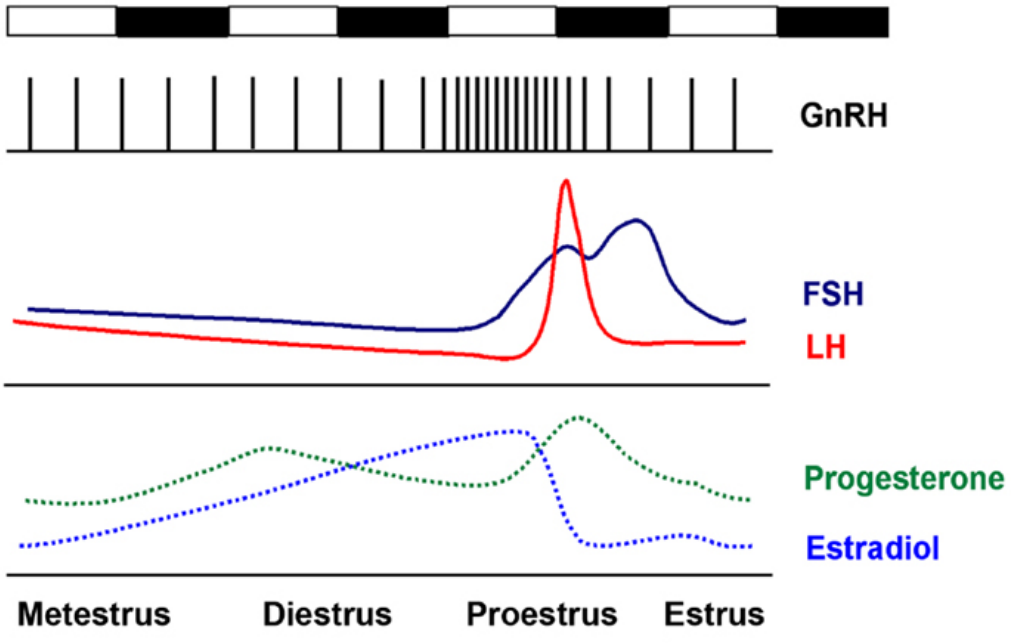

Figure 5 The murine estrous cycle

For the mouse, every estrous cycle lasts on average 4–5 days. Every estrous cycle has four different stages, metestrus, diestrus, proestrus, and estrus. Among them, metestrus and diestrus have low levels of estradiol, progesterone, gonadotropins and low-frequency GnRH pulses. Whereas, starting from proestrus, estradiol

levels are increased, triggering high-frequency GnRH pulses, which in turn drive the LH surge. This hormone surge decreases in the late estrus. Adapted from (Miller and Takahashi, 2013).

1.2 Transient receptor potential (TRP) channels

Transient receptor potential (TRP) channels are a large ion channel family and have been demonstrated to play important functional roles in hormone-secreting cells such as pancreatic β-cells (Sabourin et al., 2015; Skrzypski et al., 2013). 28 known mammalian members of this family share common features such as permeability to cations, six transmembrane segments and sequence homology. They also display diversities such as different activation mechanisms and selective cation permeability (Figure 6). TRP channels have been shown to be mechanistically involved in the conveyance of various stimuli such as sound, touch, light to chemicals, temperature and intracellular messengers (Leinders-Zufall and Boehm, 2014).

After activation, TRP channels contribute to the transfer of cations across the plasma membrane. Via this permeability, they regulate the concentrations of intracellular ion concentrations such as Ca^{2+} and thus membrane potentials to modulate physiological function (Clapham et al., 2001; Venkatachalam and Montell, 2007).

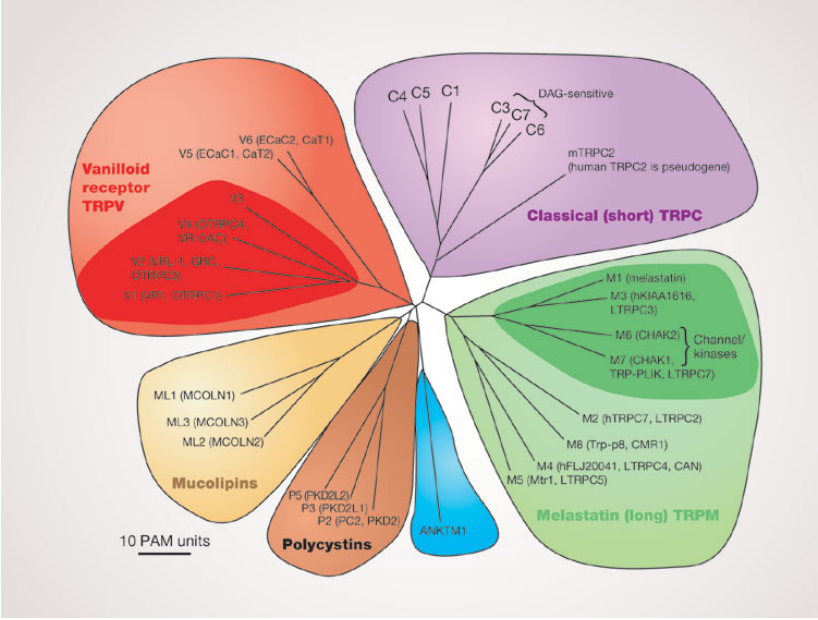

Figure 6 The evolutionary tree of TRP channel family

The total branch distance of the evolutionary distance is shown in point accepted mutations (PAM) units, which is the mean number of substitutions per 100 residues. Adapted from (Clapham, 2003).

1.2.1 TRPC5

TRPC5 is one member of the TRPC subfamily. This gene is located on the X chromosome. It is expressed in different areas of the central nervous system such as in the amygdala, in the cerebellum, and in the hippocampus (Fowler et al., 2007; Meis et al., 2007; Qiu et al., 2011). It also has been detected in peripheral organs such as the kidney, in the liver and the heart. TRPC5 is also expressed in human pituitary cells (Riccio et al., 2002).

TRPC5 can form complexes with itself or with other TRPC subfamily members such as TRPC1 or TRPC4 (Hofmann et al., 2002; Meis et al., 2007; Strubing et al., 2001). TRPC5 can be activated in a variety of ways. It can for example be activated by Gq-coupled receptors, nitric oxide, Ca^{2+} and cold (Okada et al., 1998; Schaefer et al., 2000; Worley et al., 2007). *Trpc5*$^{-/-}$ mice show less anxiety to innately aversive stimuli, but no test has been taken on these mice in terms of reproductive behaviour (Phelan et al., 2013; Riccio et al., 2009; Zimmermann et al., 2011).

1.3 Epigenetic control of gonadotrope function

This part of the introduction is a modified adaption from a manuscript prepared together with our collaborator Prof. Philippa Melamed.

The DNA methylation shapes cellular differentiation by inhibiting gene expression, however the mechanisms of demethylation only emerged with the recent discovery of the ten-eleven translocation (Tet) dioxygenases (reviewed by Wu and Zhang, 2014). The three Tet enzymes catalyze DNA demethylation through conversion of 5-methly cytosine (5mC) into 5-hydroxymethylcytosine (5hmC), which can be followed by further oxidation to 5-formylcytosine and 5-carboxycytosine, or possibly deamination to 5-hydroxyuracil (Figure 7). These modified bases are then excised by thymine DNA glycosylase and replaced by unmodified cytosine (He et al., 2011; Ito et al., 2011; Maiti and Drohat, 2011). However, 5hmC is readily detected in many cell types including neurons, suggesting that it has a low turnover and could function as an epigenetic mark (Kriaucionis and Heintz, 2009). Moreover, it was shown that some 5mC binding proteins do not recognize 5hmC, and that this modification reduces the affinity and activity of DNA methyl transferase (DNMT) 1, while several proteins appear to bind selectively to 5hmC-containing DNA (Hashimoto et al., 2012; Spruijt et al., 2013). 5hmC is characteristically located in the vicinity of

transcription start sites (TSS) as well as active or poised distal regulatory elements, while Tet proteins are found enriched at CpG-rich gene promoters, both suggesting a facilitating role of this modification in transcriptional activation (Song et al., 2011; Xu et al., 2011).

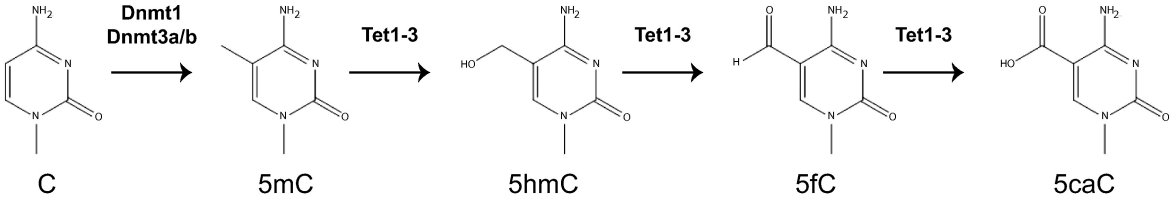

Figure 7 The role of TETs in cytosine modifications

Dnmt1, DNA (cytosine-5)-methyltransferase 1; 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; 5fC, 5-formylcytosine; 5caC, 5-carboxylcytosine. Adapted from (Hill et al., 2014).

The three Tet proteins are expressed differentially in a developmental and cell-specific manner (Ito et al., 2010; Szwagierczak et al., 2010). Tet3 is found only in differentiated cells, and has a unique role in the zygote, but is also found in various adult tissues. In contrast, Tet1 is expressed most highly in pluripotent cells including embryonic stem cells (ESCs), and is down-regulated during differentiation, concomitant with a drop in levels of Oct4. Tet2 is also expressed in ESCs, and transcription of both genes was shown to be regulated in these cells by the pluripotency factors (Etchegaray et al., 2015; Koh et al., 2011; Neri et al., 2015). Despite this, both Tet 1 and 2 are also found in certain differentiated tissues. The distinct functions of these two enzymes during development is not clear, and double knockout (KO) of Tet 1 and 2 led to mice with varied phenotypes, however all the females had notably reduced fertility and small ovaries (Dawlaty et al., 2013). *Lhb* is one of the upregulated genes in pre-iPSC lines after knocking down *Tet1* in these cells (Chen et al., 2013). The hypothalamic gonadotropin-releasing hormone (GnRH) induces expression of LH and FSH already during embryonic development, signaling the initial completion of differentiation of the gonadotrope cells. The effects of GnRH involve activation of a number of cell-specific transcription factors, induction of various histone modifications and a reduction in nucleosome occupancy at the gonadotropin-encoding genes (Jorgensen et al., 2004; Lim et al., 2007; Melamed, 2008; Melamed et al., 2006; Wijeweera et al., 2015). The ability of GnRH stimulated signals to promote a state of active chromatin at these genes is likely crucial not only during embryonic development,

but also at puberty and for their reactivation in seasonal breeders. Notably however, the mechanisms of de-repression were noted to differ for the hormone-specific LH (*Lhb*) and FSH (*Fshb*) subunit genes (Lim et al., 2007).

1.4 RNA-seq

How does the gene expression of gonadotropes change to accommodate the differential requirements of different developmental stages, different hormonal stages and genders? Indeed, is there substantial divergence in the gene expression profile at different stages and genders or are the gonadotropes simply subservient to incoming GnRH pulses? This question is of particular importance to understanding gonadotrope physiology and has yet to be determined. However, with the development of whole transcriptome sequencing it is now possible to answer this important question. RNA-seq (RNA sequencing) is one aspect of next generation sequencing (NGS). NGS is a modern and more efficient method to sequence DNA compared to classical Sanger sequencing. It has proven to be a powerful tool to analyze the whole set of information of all individual transcripts within the cell, the transcriptome, of various cell populations and tissues (Shankar et al., 2012; van Dijk et al., 2014).

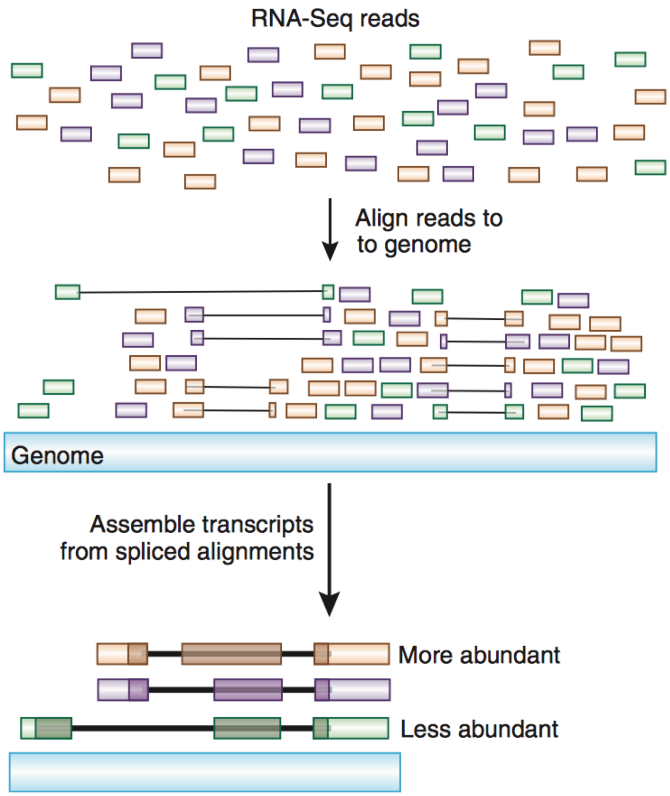

Figure 8 RNA-seq data analysis

Massive short RNA-Seq reads generated by sequencers will be aligned to the genome. Transcripts will then be assembled based on the splicing information after alignment. Adapted from (Haas and Zody, 2010).

Basically, to perform transcriptome analysis via RNA-seq, mRNA will be fragmented before or after reverse transcription. The small DNA fragments will then be amplified and sequenced. The massive data generated by sequencers will then be analyzed to quantify the whole transcriptome using a variety of bioinformatics tools (Figure 8).

1.5 Aims of the present study

Aim 1: Does the gonadotrope gene expression profile change between different developmental stages, hormonal statuses and genders? If so, to what extent does the gene expression profiles differ?

Aim 2: Are TRP channels expressed in gonadotropes? If so, how do they contribute to gonadotrope function?

Aim 3: Are the Tet family members expressed in murine gonadotrope? If so, how do they regulate gonadotrope gene expression?

2 Materials and Methods

2.1 General materials

2.1.1 Enzymes and reagents

Table 1 List of enzymes

Enzymes	**Supplier**
DreamTaq DNA Polymerase	Thermo Fisher scientific (#EP0701)
Taq DNA polymerase	Custom-made

Table 2 List of kits and suppliers

Kits	**Supplier**
Papain Dissociation System	Worthington (#LK003153)
RNeasy Plus Micro Kit	Qiagen (# 74034)
NEBNext® Ultra™ RNA Library Prep Kit for Illumina®	New England Biolabs (#E7530S)
NEBNext Poly(A) mRNA Magnetic Isolation Module	New England Biolabs (#E7490)
RNA 6000 Pico Kit	Agilent (#5067-1513)
Quant-iT^{TM} dsDNA HS Assay Kit	Life Technologies (#Q32851)
SensiFAST™ SYBR® No-ROX One-Step Kit	Bioline (#BIO-72005)

Table 3 List of primers used for RT-qPCR

Gene	Primers (from 5' end to 3' end)
Gria1	FRW: GGGACAACTCAAGCGTCCAGA
	REV: GCAGCCAGTTCCACGCAGTA
Gria2	FRW: AAAGAATACCCTGGAGCACAC
	REV: CCAAACAATCTCCTGCATTTCC
Grem1	FRW: CGTGACAGAGCGCAAGTATCT
	REV: CTCGTGGATGGTCTGCTTCA
Gata2	FRW: ACCTGTGCAATGCCTGTGGG
	REV: TTGCACAACAGGTGCCCGCT
Actb	FRW: AAGGAGATTACTGCTCTGGCTCCTA
	REV: ACTCATCGTACTCCTGCTTGCTGAT
Trpc5	FRW: AGTCGCTCTTCTGGTCTGTCTTT
	REV: TTTGGGGCTGGGAATAATG
Lhb	FRW: CTAGCATGGTCCGAGTACTG
	REV: CTGAGGGCTACAGGAAAGGA
Tet1	FRW: GAGCCTGTTCCTCGATGTGG
	REV: CAAACCCACCTGAGGCTGTT
Tet2	FRW: ACTTCTCTGCTCATTCCCACAGA
	REV: TTAGCTCCGACTTCTCGATTGTC
Tet3	FRW: GAGCACGCCAGAGAAGATCAA
	REV: CAGGCTTTGCTGGGACAATC

Table 4 List of primers used for genotyping

Gene	Mutant allele	Primers (from 5' end to 3' end)
Cre	*GnRHR*	Cre1: GTCGATGCAACGAGTGATGAGGTTCG Cre2: CCAGGCTAAGTGCCTTCTCTACACCTGC
Trpc5	*Trpc5*	FRW1: GACAGCTGAGCTCCCTATTG REV1: CTAGCCTAGACATACAACACAG REV2: CAAGGCCATCAATTACCAGAC
Rosa26	*Rosa26-YFP*	FRW1: CAAAGTCGCTCTGAGTTGTTATC REV1: GCAGAAGGAGCGGGAGAAAT REV2: GCGAAGAGTTTGTCCTCAACC
eRosa26	*eRosa26-τGFP*	FRW1: CAAAGTCGCTCTGAGTTGTTATC REV1: GCAGATGGAGCGGGAGAAAT REV2: GTCCCTATTGGCGTTACTATG

Table 5 General solutions

Chemicals were purchased from Sigma-Aldrich, Merck, Gibco/BRL and Roth.

Ethidiumbromide-staining solution (for electrophoresis)	10 mg/ml ethidiumbromide in TAE buffer.
PBS (20x)	3 M NaCl, 161 mM $Na_2HPO_4.7H_2O$, 39 mM KH_2PO_4, pH 7.4.
TAE buffer (50x) (for gel electophoresis)	2.5 M Tris-acetate, 50 mM EDTA, pH 8.0.
Ear biopsy lysis buffer	50 mM Tris-HCl, pH8,

	1 mM EDTA, 100 mM NaCl, 0.2% NP40, 0.2% Tween.
Tris-HCl (1 M, pH 6.8)	12.1 g Tris base in 100 ml H_2O, adjust pH with concentrated HCl.

2.1.2 Experimental animals

Animal care and experimental procedures were performed in accordance with the guidelines established by the animal welfare committee of the University of Saarland. Mice were kept under a standard light/dark cycle with food and water *ad libitum*. To label gonadotropes, I used the GnRHR-IRES-CRE (GRIC) (Wen et al., 2008) knock-in mouse strain crossed with eROSA26-τGFP (eR26-τGFP) (Wen et al., 2011) or ROSA26-YFP (R26-YFP) (Srinivas et al., 2001) animals. TRPC5 knock-out mice are described in (Xue et al., 2011). All electrophysiological experiments were performed on dissociated gonadotropes prepared from juvenile (three-week-old) GRIC/eR26-τGFP/TRPC5+/+ and GRIC/eR26-τGFP/TRPC5-/- female mice.

2.2 Methods

2.2.1 Genotyping

2.2.1.1 Genomic DNA preparation

Genomic DNA (gDNA) extraction was performed by incubating ear biopsy at 55°C in 100 µl ear lysis buffer supplemented with proteinase K overnight.

2.2.2 PCR

PCR (polymerase chain reaction) was performed in a 50 µl reaction system in 0.2 ml Eppendorf tubes using a thermal cycler (BIO-RAD).

The components of reaction system are as follows:

Table 6 Components for the PCR genotyping system

Components	**Volume**
gDNA template	1.5 µl
PCR buffer (10x)	5.0 µl
dNTPs (25 mM)	0.5 µl
$MgCl_2$ (25 mM)	5.0 µl
Betaine (5 M)	10.0 µl
DMSO	2.5 µl
Primers (10 pM)	1.0 µl for each primer
Taq-Polymerase	1 U
Water, nuclease-free	Add nuclease-free water to make a final volume of 50 µl.

Table 7 Genotyping PCR conditions

Gene	**PCR condition**	**Band size**
Cre	94°C : 5 min	knock-in band: 600 bp
	94°C : 30 sec 60°C : 45 sec 72°C : 90 sec } 36 X	
	72°C : 5 min	
	4°C: ∞	

Trpc5	94°C : 5 min 94°C : 30 sec 65°C : 30 sec -0.5 °C each cycle 72°C : 30 sec } 9X 94°C : 30 sec 60°C : 30 sec 72°C : 30 sec } 24X 72°C : 5 min 4°C: ∞	wild-type band: 192 bp knock-in band: 232 bp (touchdown PCR)
ROSA26	94°C : 5 min 94°C : 35 sec 50°C : 45 sec 72°C : 90 sec } 35 X 72°C : 7 min 4°C: ∞	wild-type band: 610 bp knock-in band: 303 bp
eROSA26	94°C : 5 min 94°C : 35 sec 50°C : 45 sec 72°C : 90 sec } 35 X 72°C : 7 min 4°C: ∞	wild-type band: 610 bp knock-in band: 393 bp

2.2.3 Vaginal cytology

Vaginal smears were taken and examined. Briefly, the proportion of nucleated epithelial cells, anucleated cornified cells and leukocytes was used to determine the stage of the estrous cycle. This was performed daily, at the same time, for two weeks prior to gonadotrope harvesting (Caligioni, 2009).

2.2.4 Gonadotrope enrichment

Pituitary glands for cell sorting were dissected and pooled from 3-7 GRIC/R26-YFP or GRIC/eR26-τGFP mice. After dissection, glands were cut into small pieces and placed in a solution of papain and DNase in EBSS (Papain Dissociation System, Worthington) and incubated for 30 minutes with gentle agitation at 37 °C. Afterwards, the tissue was dissociated by repeated pipetting with a fire-polished glass pipette. The dissociated cells were then transferred to a 15 ml Falcon tube and centrifuged at 2500 rpm for 5 minutes. The supernatant was then discarded and the cell pellet was resuspended in 1 ml of FACS buffer (0.1% BSA and 1% EDTA in PBS (Qiu et al., 2013)). The cells were then sorted using a BD FACS Aria III (San Jose, USA). Cells were sorted by fluorescence (endogenously expressed YFP/τGFP) with excitation at 488 nm and emission detected at 508 nm. After sorting, fluorescent cells were suspended in RLT buffer from an RNeasy plus Micro kit (Qiagen, Hilden, Germany) for RNA purification.

2.2.5 RNA purification

RNA from sorted or cultured gonadotropes was purified using the RNeasy Plus Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration and integrity of RNA were tested using the Agilent 2100 Bioanalyzer (Santa Clara, USA). RNA samples with an RNA integrity number (RIN) greater than 8 were used to build RNA-seq libraries.

2.2.6 RNA-seq library building and sequencing

Around 400 ng total RNA with RIN number bigger than 8 for each condition was processed to RNA-Seq library construction. The RNA-seq library was built using the NEB Next Ultra RNA Library Prep kit (Ipswich, USA) according to the manufacturer's instructions (Figure 9).

2.2.7 Sequence alignment

Raw reads from the Illumina HiSeq platform were subjected to quality control through FastQC version 0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and then trimmed for library adapters and low quality tails (Q>20) with the cutadapt v1.4.1 (Martin, 2011) wrapper Trim Galore! version 0.3.3 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Trimmed reads were mapped with STAR v2.4.0f1 (Dobin et al., 2013) and the 2-pass procedure described in the documentation of the mouse genome mm10.

2.2.8 Identification of differentially expressed genes

I used Cuffdiff v2.2.1 (Trapnell et al., 2010) to identify differentially expressed genes with the Gencode (Harrow et al., 2006) mouse annotation version M2 as reference. Transcripts with an absolute value of log2 (fold change) larger than 1 and q value below 0.05 were considered to be differentially expressed.

2.2.9 Gene ontology (GO) analysis and pathway analysis

The function of individual genes was analyzed with Database for Annotation, Visualization and Integrated Discovery (DAVID) annotation software. Using DAVID, I identified GO terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways statistically overrepresented in the selected genes compared with the reference genes (all genes). Default settings were used for GO term and pathway analysis. $P<0.05$ were considered statistically significant.

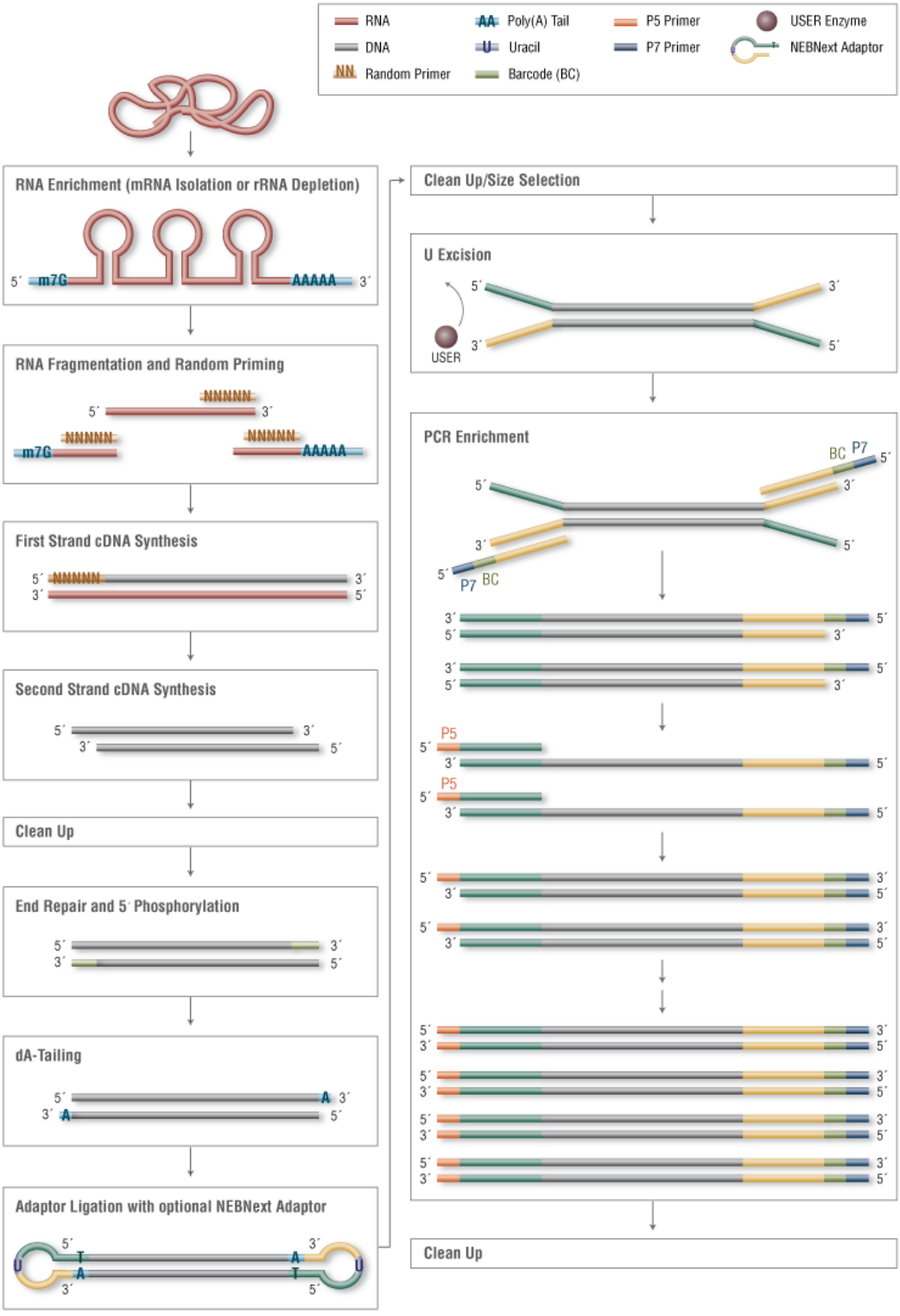

Figure 9 Overview of RNA-seq library building

Briefly, mRNA was enriched using oligo-dT beads and then underwent enzyme-mediated fragmentation, followed by reverse transcription into cDNA using random primers. After this, double stranded cDNA was adaptor-ligated and enriched by PCR using index primers. After this, the library was sequenced in the

Illumina Hiseq2000 platform (San Diego, USA). Adapted from https://www.neb.com/products/e7530-nebnext-ultra-rna-library-prep-kit-for-illumina.

2.2.10 Analysis of TRP channel expression in murine gonadotropes

To identify TRP channels expressed in gonadotropes, I analyzed the FPKM (fragment per kilobase of transcript per million fragment) values for all known TRP channels among these six stages. I considered all TRP channels with FPKM >1 to be expressed. I also analysed differentially expressed genes; those with a fold change in FPKM >2 and q value <0.05 as previously described. The heatmap of TRP channel expression in gonadotropes was generated using the MATLAB clustergram script.

2.2.11 Real-time PCR

For sorted or cultured gonadotropes, purified RNA was stored at -80 °C until use. cDNA synthesis and real-time PCR were performed using the SensiFAST™ SYBR® No-ROX One-Step Kit (Bioline, Taunton, USA) with the CFX-96 real-time PCR Detection System (Bio-Rad, Hercules, USA). The reaction conditions were as follows: 10 min at 45 °C, followed by 2 min at 95 °C and then 40 cycles of 5 s at 95 °C and 20 s at 60 °C. β-actin was used as an endogenous control. Quantified results represent the fold induction of target gene expression using the −2ddCt method (Livak and Schmittgen, 2001). No-template control (reagent alone without template) was included in each assay to detect any possible contamination of the PCR reagents.

2.2.12 Primary gonadotrope cell culture

Pituitaries were harvested from postnatal day six, three-week-old and sixteen-week-old female mice. After dissociation, pituitary cells were resuspended in in DMEM with 10% fetal bovine serum, 10 mM HEPES, 100 U/mL penicillin, and 100 µg/mL streptomycin, then plated on 8-chamber slides or 24-well plates precoated with poly-l-lysine, and kept for 24 hours at 37°C in a humidified incubator with 5% CO_2.

2.2.13 Vaginal opening

Measurements were taken from post-natal day (P) 22 to P42 female mice (with the day of birth being P0). The vaginal opening was taken as an external marker of the onset of puberty.

3 Results

3.1 Molecular plasticity of murine gonadotropes revealed by RNA-seq

3.1.1 Gonadotrope enrichment

In this study, I aimed to analyze the changes in gene expression profile that occur during development, between genders and also in different hormonal stages. Thus for the male development I used three-week-old pre-pubertal mice and 14-week-old post-pubertal mice. I also used these time points to analyze gene expression profile changes in female mouse development and also for comparison between genders. However, as the levels of circulating hormones change during the estrous cycle, for the post pubertal female I took 14-week-old animals in either proestrus (prior to ovulation) or diestrus (after ovulation). I was also interested in understanding to what extent the transcriptional profile of gonadotropes changes during lactation (Figure 10).

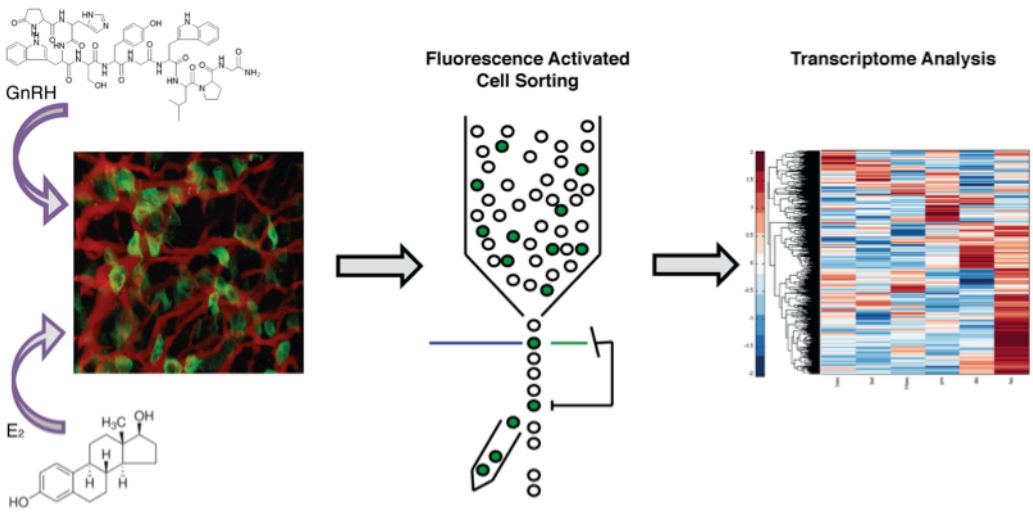

Figure 10 Schematic representation of the process used to attain RNA-seq data from genetically labeled gonadotropes

In order to produce an enriched gonadotrope population for subsequent transcriptome analyses I used FACS to isolate fluorescently labeled gonadotropes from GRIC/eR26-τGFP and GRIC/R26YFP knock-in mouse pituitaries. In a typical FACS session, between

~20,000 and ~30,000 positive cells per pituitary were recovered. However, I did not observe significant changes in the numbers of positive cells per pituitary among all six stages measured, indicating that the proliferation of murine gonadotropes is accomplished before being three-week-old (Figure 11).

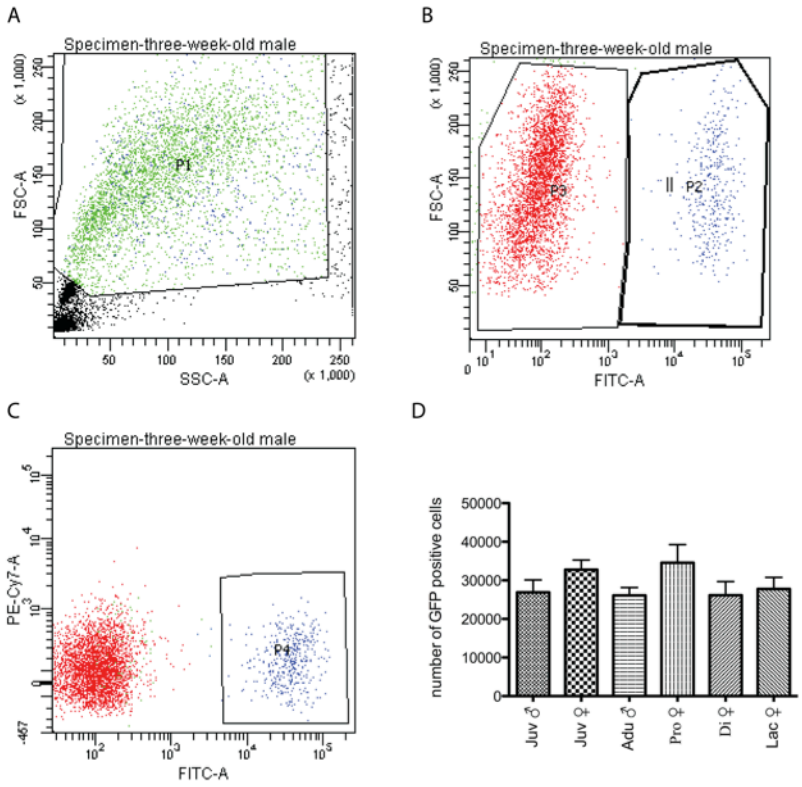

Figure 11 Representative Fluorescence-Activated Cell Sorting (FACS) results for murine gonadotrope enrichment

One example of cell sorting from prepubertal male mice gating strategies of side scatter (SSC) and forward scatter (FSC) (A), GFP signal and FSC (B), GFP signal and the phycoerythrin (PE) signal (C). Numbers of gonadotropes recovered from FACS (D). Three-old-week male (Juv ♂), three-week-old female (Juv ♀), diestrus (Di ♀), proestrus (Pro ♀), lactating (Lac ♀) and 14-week-old male (Adu ♂) (n = 3-6).

3.1.2 Libraries built from single pituitary gained low unique reads level

In order to get transcriptome information of murine gonadotrope at the single pituitary level, I initially built RNA-seq libraries from single prepubertal male or female mice. After sequencing, I found that there was a major complication in the number of duplicated

sequences. Although more than 85% of the reads for each sample mapped uniquely to the mouse genome, the number of unique reads was low. This was especially severe for female samples, with a duplication rate of 97.7% and only 575,999 reads were left over. This was only a 50th. of what was mapped. The male sample had a duplication rate of 89.6% and 4,777,366 reads.

3.1.3 Global view of the murine gonadotrope transcriptome

Next, I pooled pituitaries from 3-7 mice for each individual stage, and then sorted gonadotropes to build RNA-Seq libraries. With 6 experimental groups (juvenile, diestrus, proestrus and lactating females, juvenile and adult males) and 3 independent biological replicates I generated a total of 18 RNA-Seq libraries. Sequencing returned around 90 million reads for each library. I then aligned the reads to the mouse genome mm10 using STAR. Around 93% of the reads were mapped (Table 8).

Table 8 Mapping statistics and number of mice used for each library

Stages	Raw reads	After trimming		Mapped		Number of mice
Di ♀ 1	95439657	91096856	95%	88896513	93%	5
Di ♀ 2	111771842	109403662	98%	104932039	94%	3
Di ♀ 3	114220736	110968126	97%	106964905	94%	4
Adu ♂ 1	80959654	76427621	94%	74113715	92%	7
Adu ♂ 2	103959782	101692716	98%	97754683	94%	3
Adu ♂ 3	130582716	127400028	98%	122381832	94%	3
Lac ♀ 1	112970836	106387285	94%	103213451	91%	3
Lac ♀ 2	133957704	129950274	97%	125564703	94%	6
Lac ♀ 3	111905998	108891694	97%	104346413	93%	7
Pro ♀ 1	67045128	63723808	95%	62235167	93%	5
Pro ♀ 2	136685402	132423030	97%	127771666	93%	4
Pro ♀ 3	105631308	103146562	98%	98258796	93%	4
Juvenile ♀ 1	65090814	61773401	95%	60261384	93%	3
Juvenile ♀ 2	103440346	100745078	97%	96777962	94%	3
Juvenile ♀ 3	137615520	133155900	97%	128451082	93%	3
Juvenile ♂ 1	82741309	79158267	96%	77099838	93%	3
Juvenile ♂ 2	100156670	98597782	98%	94273108	94%	3
Juvenile ♂ 3	160773894	156401594	97%	151158147	94%	3

I then assembled transcripts from the aligned reads. Around 22,000 genes were detected in each sample. Gene abundance within the samples is represented by the fragment per kilobase of transcript per million fragment mapped (FPKM) value. I found that the high-throughput sequencing data within the experimental groups were reproducible and that the Pearson correlation coefficients calculated from the 3 individual RNA-Seq replicates ranged from 0.856 to 0.996 (Figure 12).

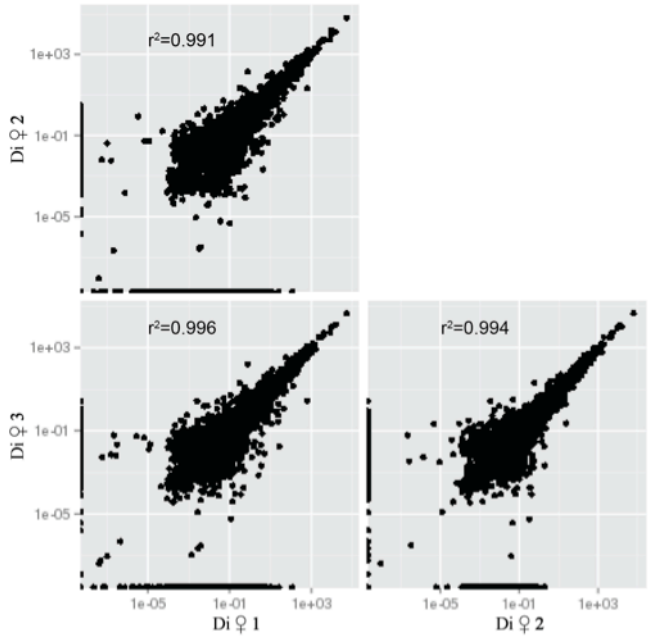

Figure 12 Similarity of three independent diestrus biological replicates

Scatter plots of three biological replicates of diestrus gonadotrope RNA-seq data. FPKM values of individual detected genes from one replicate was plotted against the values of another replicate. (1e-05=0.00001, 1e-01=0.1, 1e+03=1000)

To avoid background fluctuations in gene expression, I considered only genes with FPKM>1 to actually be expressed (Manteniotis et al., 2013). Approximately 60% of the genes had FPKM values >1. Overall, I found that gonadotropes isolated from lactating and proestrus females had a higher proportion of highly expressed genes (FPKM>100) compared to all other samples (Table 9).

Table 9 Distribution of gene expression values among developmental stages examined

Distribution of gene expression values among developmental stages examined					
Stages	FPKM>0	0<FPKM<=1	1<FPKM<=10	10<FPKM<=100	FPKM>100
Juvenile ♀	24085	10716	6170	6451	748
Di ♀	24248	11212	5964	6247	825
Pro ♀	23940	10716	5971	6328	925
Lac ♀	24254	11052	6029	6241	932
Juvenile ♂	24252	10900	6194	6369	789
Adu ♂	23874	10486	6227	6322	839

In order to analyze the number of murine gonadotrope genes that are exclusively expressed at different stages, I performed a Venn analysis including all genes with a FPKM value >1 (Figure 13).

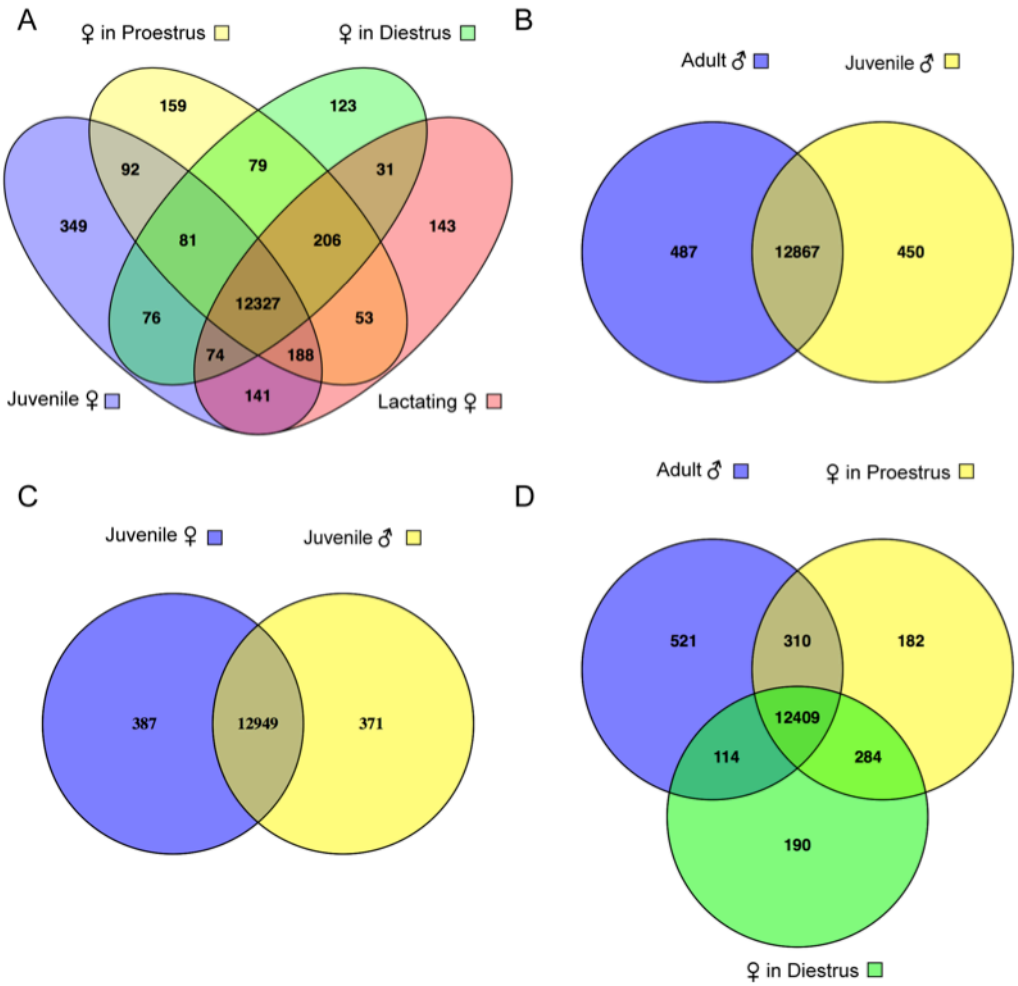

Figure 13 Venn diagram showing exclusively expressed genes

Female development (A), male development (B), between genders in juvenile animals (C) and between genders in adult animals (D). All genes with FPKM > 1 are considered.

First I compared the transcriptomes of female gonadotropes at different hormonal stages (i.e. juvenile vs. diestrus vs. proestrus vs. lactating) (Figure 13A). I found that 12,327 genes were expressed in these cells independent of the hormonal stage (please note that the Venn analysis presented in Figure 13 does not distinguish between potentially different relative expression levels of individual genes). 349 genes were exclusively expressed in juvenile female gonadotropes compared to the adult female stages. 63 of these 349 genes were expressed with FPKM>2 and 13 genes with FPKM>5 in juvenile females. 159 genes

(35 genes with FPKM>2, 7 genes with FPKM>5) were expressed in gonadotropes isolated in proestrus but not in gonadotropes prepared from juvenile, diestrus or lactating females. 123 genes (25 genes with FPKM>2, 7 genes with FPKM>5) were exclusively expressed in diestrus and 143 genes (27 genes with FPKM>2, 8 genes with FPKM>5) were selectively found in gonadotropes from lactating mice. Taken together, these data reveal a remarkable molecular plasticity of these cells and suggest a hormonal stage-specific molecular female gonadotrope signature.

Comparing all four individual female stages analyzed in this study, I found that 12,693 (12,327 + 81 + 79 + 206) genes were commonly expressed in diestrus and proestrus females whereas 492 (92 + 159 + 188 + 53) genes were expressed in proestrus but not in diestrus gonadotropes (Figure 13A). Vice versa, 304 (123 + 31 + 76 + 74) genes were expressed in diestrus but not in proestrus. 206 genes were expressed in adult females (i.e. commonly found in proestrus as well as in diestrus and during lactation) but not in juveniles (Figure 13A). Furthermore, a total of 794 genes (159 + 79 + 123 + 206 + 31 + 53 + 143) corresponding to 3.5% of the entire murine gonadotrope transcriptome were expressed in at least one of the adult female stages but not in juveniles indicating major molecular reprogramming of these cells through reproductive maturation. Comparing the transcriptomes of adult female gonadotropes, I found 160 (81 + 79) genes specifically in cycling but not in lactating animals (Figure 13A). Vice versa, 284 (141 + 143) genes were only found in lactating but not in adult cycling animals (i.e. either in proestrus or in diestrus). Finally, 610 (92 + 159 + 76 + 81 + 79 + 123) genes were exclusively expressed in cycling females either in proestrus or in diestrus but not in lactating mice.

Comparing the transcriptomes of adult and juvenile male gonadotropes, I found 487 genes (107 genes with FPKM>2, 26 genes with FPKM>5) to be expressed in adults but not in juveniles and 450 genes (83 genes with FPKM>2, 16 genes with FPKM>5) in juveniles but not in adults (Figure 13B). Comparing juvenile gonadotropes across genders, I found 387 genes (84 genes with FPKM>2, 27 genes with FPKM>5) in females but not in males and 371 genes (64 genes with FPKM>2, 21 genes with FPKM>5) in males only (Figure 13C). 656 (190 + 284 + 182) genes were exclusively found in cycling females and 521 genes (122 genes with FPKM>2, 30 genes with FPKM>5) in adult males only (Figure 13D).

3.1.4 Quantitative changes in gonadotrope gene expression

Next I analyzed quantitative changes in gonadotrope gene expression at different hormonal stages. To do this, I selected genes with a greater than two-fold change, $q<0.05$ and FPKM >1 in at least one condition. Totally, 2168 differentially expressed genes were identified. Interestingly, I found relatively few changes in gene expression between juvenile animals of both genders and adult male samples. This is in stark contrast to samples derived from cycling females, which revealed a substantially higher number of differentially expressed genes when compared with samples derived from male and female juvenile and adult male animals (Figure 14).

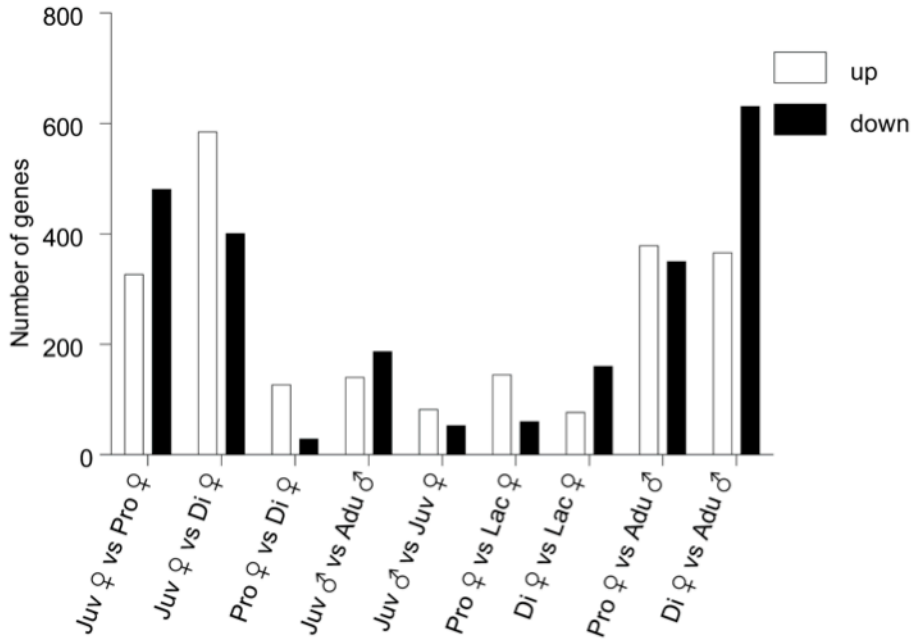

Figure 14 Overview of differentially expressed genes across different comparisons

Number of DEGs (differentially expressed genes) obtained from different comparisons across six stages.

During female reproductive maturation, I found 327 genes to be upregulated and 481 genes to be downregulated in gonadotropes from juvenile females compared to gonadotropes from females in proestrus. 585 upregulated genes and 401 downregulated genes were identified in juvenile vs. diestrus females. In cycling adults, 127 genes were upregulated and 28 genes were downregulated comparing proestrus and diestrus.

Comparing cycling with lactating adults, 145 upregulated genes and 60 downregulated genes were identified in proestrus vs. lactating and 77 upregulated genes and 160 downregulated genes were found comparing gonadotrope transcriptomes from diestrus and lactating animals.

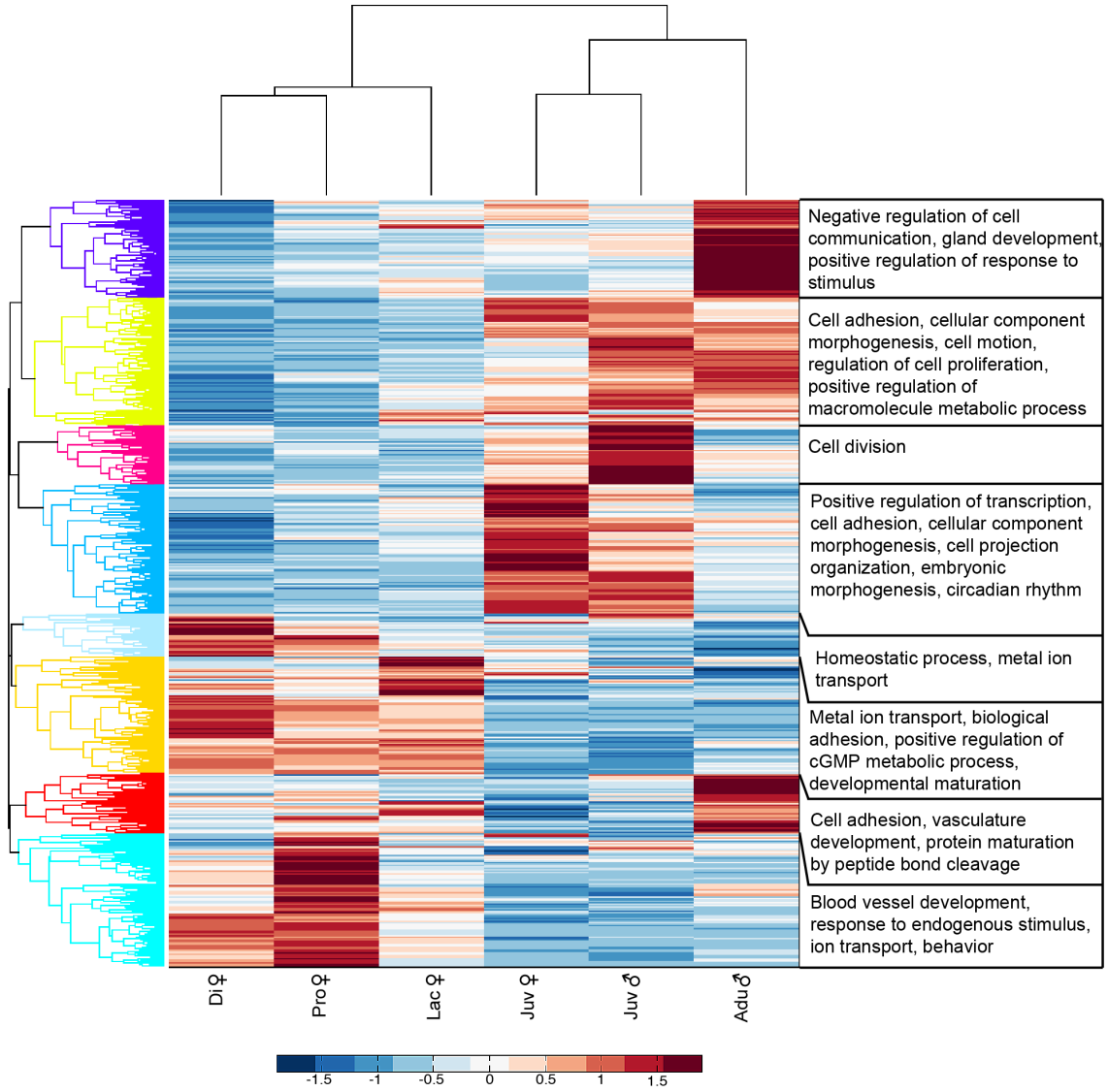

Figure 15 Heat map and gene ontology analysis of 2168 differentially expressed genes

Heat map of differentially expressed genes in at least one of nine comparisons. The GO terms enriched in the eight clusters are shown.

During male reproductive maturation, I found 140 upregulated genes and 187 downregulated genes in juvenile male vs. adult males. Between gender comparisons identified 82 upregulated and 53 downregulated genes in juvenile males vs. juvenile females, 379 upregulated and 350 downregulated genes in proestrus females vs. adult males and 366 upregulated and 631 downregulated genes comparing diestrus females and adult males.

I found that the genes Meg3 (maternally expressed 3), Rnase1 (ribonuclease, RNase A family, 1), Gm4149 (predicted gene 4149), Cdkn1c (cyclin-dependent kinase inhibitor 1C), Serpine2 (serine or cysteine peptidase inhibitor, clade E, member 2), Hspa1b (heat shock protein 1B), Grem1 (gremlin 1) are highly expressed (FPKM>100) in at least one experimental group. In particular, Grem1 and Rnase1 were highly expressed in multiple comparisons, highlighting these genes as interesting targets for understanding gonadotrope physiology. Interestingly, two paternally imprinted (Peters, 2014) and maternally expressed genes, Meg3 (Miyoshi et al., 2000) and Cdkn1c (Matsuoka et al., 1996), are among the most highly expressed genes. With regard to Meg3, multiple miRNAs have been proposed to be encoded in the long transcript (Tierling et al., 2006).

I then performed a hierarchical clustering of all 2168 differentially expressed genes (Figure 15). Gene ontology (GO) analysis of the eight identified clusters revealed that adult male gonadotropes are enriched in the GO terms negative regulation of cell communication and gland development when compared with other stages. Juvenile male gonadotropes are enriched in GO terms cell division and regulation of cell proliferation. Juvenile female gonadotropes are enriched in the GO term regulation of transcription. Adult female gonadotropes are enriched in GO terms ion transport, homeostatic process and blood vessel development.

Next, using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis tool of the Database for Annotation, Visualization and Integrated Discovery (DAVID) annotation software, I performed a pathway enrichment analysis of all 2168 differentially expressed genes. Several interesting pathways were found to be differentially regulated such as “GnRH signaling”, “Ca^{2+} signaling”, “MAPK signaling” and “neuroactive ligand-receptor interaction” (Figures 16 and 17). In order to visualize the changes in gene expression within these enriched pathways, I generated heat maps showing the change in expression. Interestingly, in all four pathways analyzed, juvenile and adult male samples

had similar gene expression patterns, whereas samples from lactating and cycling mice had similar patterns, however samples from lactating mice had less upregulated genes compared to all other samples (Figures 16 and 17).

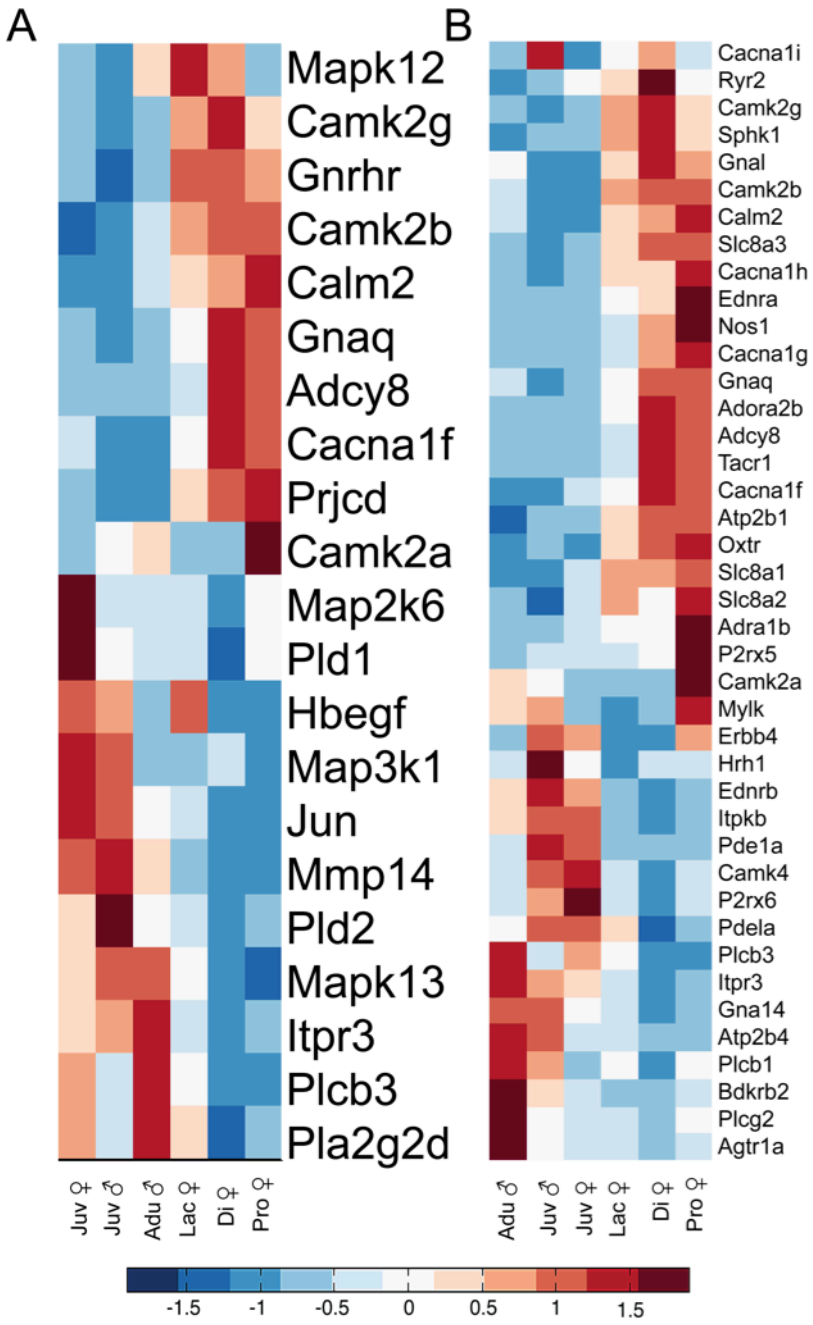

Figure 16 Heatmaps of differentially expressed genes involved in "GnRH signaling" (A) and "Ca^{2+} signaling" (B)

Data are expressed as mean FPKM, standardized, and visualized using the MATLAB "clustergram" script. Red: relatively high expression; blue: relatively low expression.

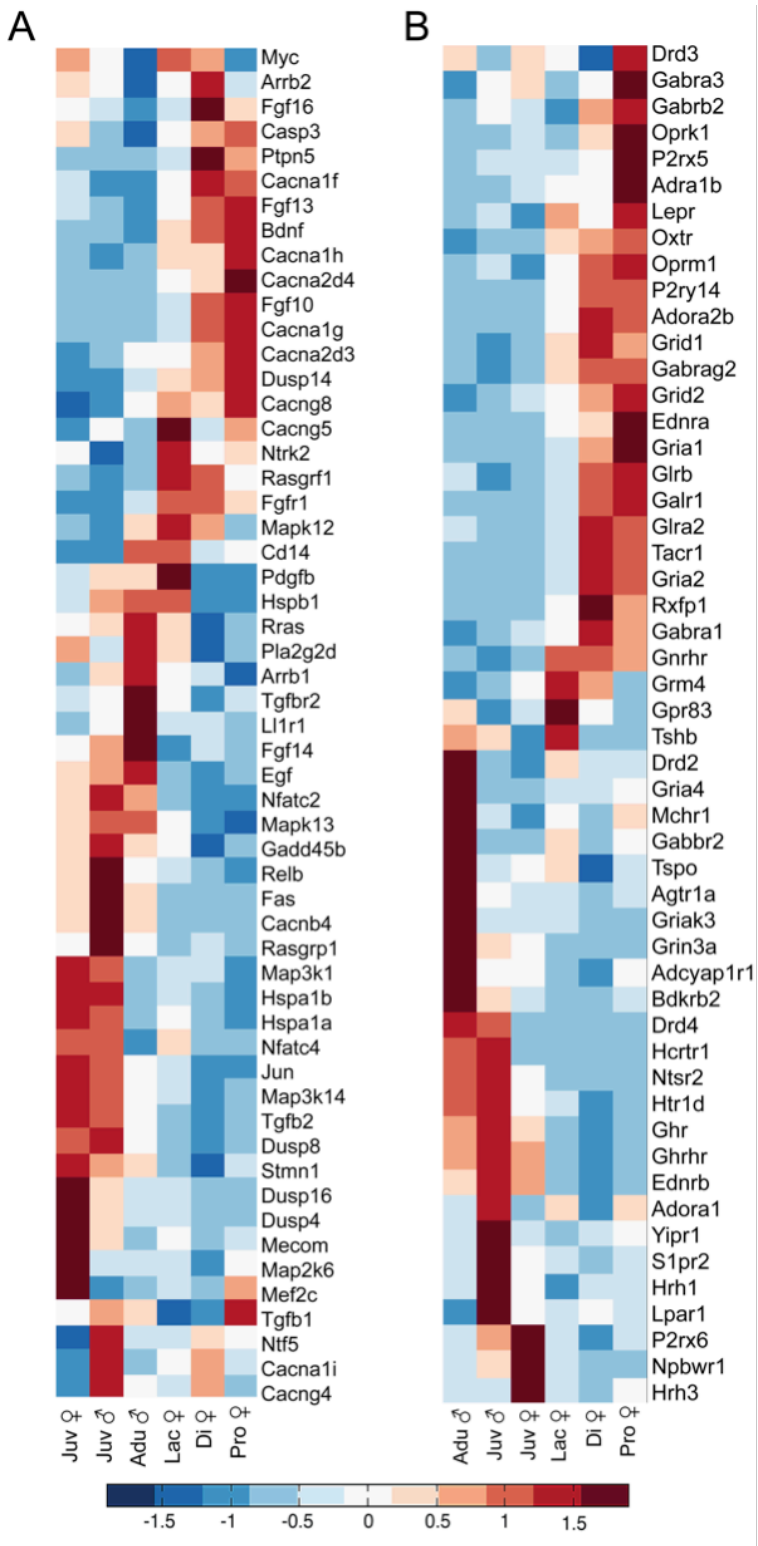

Figure 17 Heatmaps of differentially expressed genes involved in "MAPK signaling" (A) and "neuroactive ligand-receptor interaction" (B)

Data are expressed as mean FPKM, standardized, and visualized using the MATLAB "clustergram" script. Red: relatively high expression; blue: relatively low expression.

3.1.5 Individual comparisons

After functional analysis of all differentially expressed genes, I next proceeded to make individual comparisons in order to further understand the extent of transcriptional profile changes between different groups.

3.1.5.1 Gonadotropes harvested from prepubertal mice

135 genes were significantly differentially expressed between gonadotropes from juvenile male and female mice. Out of these genes, 82 were upregulated and 53 were downregulated. The top upregulated genes were the ubiquitously transcribed tetratricopeptide repeat gene, Y chromosome, calcium binding protein 7, G protein-coupled receptor 101, dopamine receptor 4, lysine (K)-specific demethylase 5D, vasoactive intestinal peptide receptor 1 (Table 10).

Table 10 Top 20 differentially expressed genes between male and female prepubertal gonadotropes

Gene	Sample 1	Sample 2	FPKM 1	FPKM 2	log2(fold_change)	*p* value	*q* value
Kdm5d	three-week-old.female	three-week-old.male	0	5.41718	inf	5.00E-05	0.00178848
Uty	three-week-old.female	three-week-old.male	0.00156897	8.57018	12.4153	5.00E-05	0.00178848
Drd4	three-week-old.female	three-week-old.male	0.0641815	8.75079	7.09111	0.0023	0.0408346
Gm5560	three-week-old.female	three-week-old.male	0.448204	13.7477	4.93889	5.00E-05	0.00178848
Vipr1	three-week-old.female	three-week-old.male	0.0836713	1.22121	3.86743	0.00025	0.00708146
Spcs2-ps	three-week-old.female	three-week-old.male	0.550188	7.68279	3.80363	0.0002	0.00589277
Gpr101	three-week-old.female	three-week-old.male	1.48248	13.0723	3.14043	5.00E-05	0.00178848
Sowahd	three-week-old.female	three-week-old.male	0.171117	1.4538	3.08677	0.00135	0.0273381
Hcrtr1	three-week-old.female	three-week-old.male	0.68902	5.65779	3.03762	5.00E-05	0.00178848
Cabp7	three-week-old.female	three-week-old.male	0.532153	3.82377	2.84508	0.00055	0.0134068
Xist	three-week-old.female	three-week-old.male	27.1235	0.0495176	-9.09739	5.00E-05	0.00178848
Umod	three-week-old.female	three-week-old.male	2.65194	0.217387	-3.60871	5.00E-05	0.00178848
Akr1cl	three-week-old.female	three-week-old.male	4.04648	0.588307	-2.78202	0.00015	0.004614
Klk1	three-week-old.female	three-week-old.male	13.0528	1.90977	-2.77289	5.00E-05	0.00178848
Ddit4l	three-week-old.female	three-week-old.male	26.1503	5.99505	-2.12498	5.00E-05	0.00178848
I830012O16Rik	three-week-old.female	three-week-old.male	3.2921	0.776798	-2.0834	5.00E-05	0.00178848
Rnase1	three-week-old.female	three-week-old.male	16.8584	4.03759	-2.06191	5.00E-05	0.00178848
Aox2	three-week-old.female	three-week-old.male	2.14567	0.525422	-2.02988	5.00E-05	0.00178848
Vcan	three-week-old.female	three-week-old.male	13.5675	3.48061	-1.96274	0.00035	0.00931834
Cdkn1c	three-week-old.female	three-week-old.male	206.231	55.4477	-1.89506	5.00E-05	0.00178848

GO analysis demonstrated that most upregulated genes were enriched in ion transport and chemical homeostasis. KEGG analysis showed these genes were involved in pathways such as neuroactive ligand-receptor interaction and MAPK signaling pathways (Figure 18). The top downregulated genes were ribonuclease, RNase A family, 1 (pancreatic), inactive X specific transcripts, uromodulin, cyclin-dependent kinase inhibitor 1C (P57), kallikrein 1, versican (Table 10). GO analysis showed that most downregulated genes were enriched in cell adhesion and organ development. KEGG analysis demonstrated that these genes

were involved in pathways such as cell adhesion molecules (CAMs) and axon guide pathways (Figure 18).

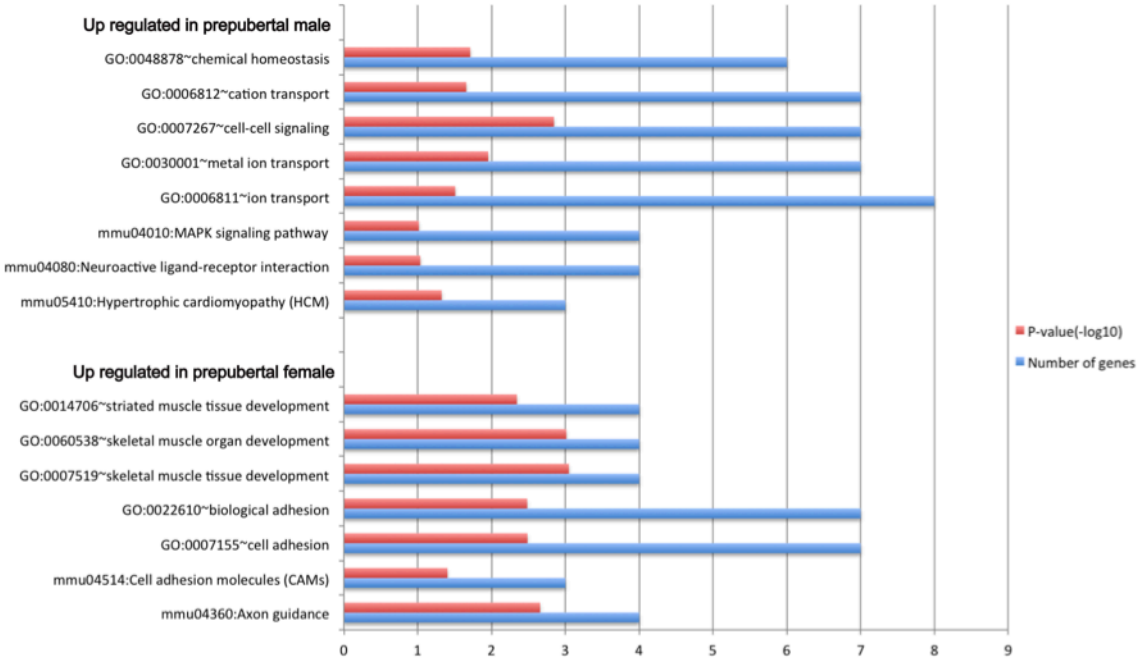

Figure 18 Top GO terms and pathways enriched from differentially expressed genes between male and female prepubertal gonadotropes

3.1.5.2 Gonadotropes harvested from cycling female mice

155 genes were significantly differentially expressed between gonadotropes from proestrus and diestrus female mice. Out of these genes, 127 were upregulated and 28 were downregulated. The top upregulated genes were phosphatidic acid phosphatase type 2 domain containing 1A, FK506 binding protein 6, contactin 5, aquaporin 1, seizure related gene 6, melanocortin 2 receptor accessory protein 2, sushi domain containing 3, serine (or cysteine) peptidase inhibitor, clade E, member 2, potassium voltage-gated channel, subfamily H (eag-related), member 1, nuclear protein 1 (Table 11). GO analysis showed that most upregulated genes were enriched in ion transport and biological adhesion. KEGG analysis showed that these genes were involved in pathways such as neuroactive ligand-receptor interaction and tight junction (Figure 19). The top downregulated genes were maternally expressed 3, glutamate receptor, metabotropic 4, sciellin, actinin alpha 2 (Table 11). No significant GO or KEGG pathway was enriched because of the limited number of genes.

Table 11 Top 20 differentially expressed genes between diestrus and proestrus gonadotropes

Gene	Sample 1	Sample 2	FPKM 1	FPKM 2	log2(fold_change)	*p* value	*q* value
Susd3	diestrus	proestrus	0.222301	3.17575	3.83651	0.0001	0.00326321
Cntn5	diestrus	proestrus	0.21285	2.03724	3.25871	0.0025	0.0434573
Ppapdc1a	diestrus	proestrus	0.754339	5.85787	2.95709	0.00015	0.004614
Sez6	diestrus	proestrus	4.24942	30.9332	2.86382	5.00E-05	0.00178848
Fkbp6	diestrus	proestrus	0.644295	3.9712	2.62378	0.00065	0.0153588
Aqp1	diestrus	proestrus	0.231255	1.22852	2.40936	0.0006	0.0143905
Mrap2	diestrus	proestrus	3.81615	19.9371	2.38527	5.00E-05	0.00178848
Serpine2	diestrus	proestrus	60.4273	311.748	2.36711	5.00E-05	0.00178848
Nupr1	diestrus	proestrus	1.75374	8.91955	2.34654	5.00E-05	0.00178848
Kcnh1	diestrus	proestrus	0.210758	1.06097	2.33172	0.00075	0.0171948
Gm10714	diestrus	proestrus	1.44901	0.00192549	-9.55562	0.0002	0.00589277
Gm8113	diestrus	proestrus	1.49066	0.364111	-2.0335	0.001	0.0216047
Meg3	diestrus	proestrus	382.907	94.1676	-2.02369	0.00105	0.0224568
Hist2h4	diestrus	proestrus	1.07398	0.267989	-2.00272	0.00225	0.0401676
Snhg11	diestrus	proestrus	21.5176	5.43637	-1.9848	5.00E-05	0.00178848
Actn2	diestrus	proestrus	3.58616	0.940493	-1.93095	0.0001	0.00326321
Scel	diestrus	proestrus	2.30988	0.617507	-1.90329	0.0002	0.00589277
Gm21781	diestrus	proestrus	1.94035	0.526499	-1.88182	5.00E-05	0.00178848
Gm10406	diestrus	proestrus	2.47254	0.685407	-1.85096	0.00275	0.046597
Grm4	diestrus	proestrus	1.1633	0.349897	-1.73323	0.00135	0.0273381

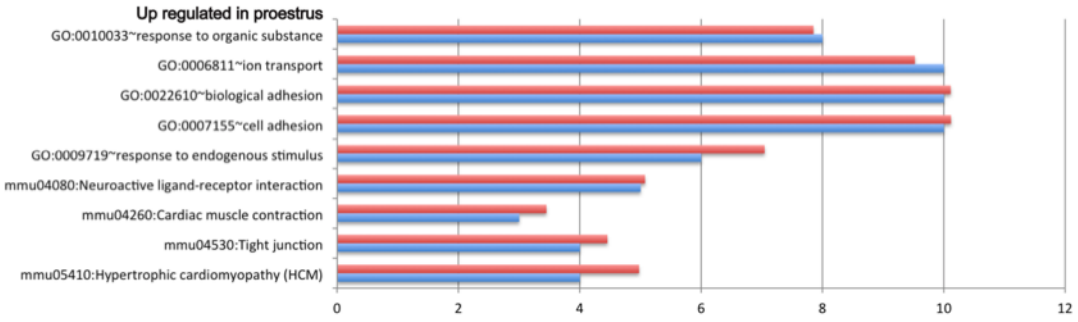

Figure 19 Top GO terms and pathways enriched from upregulated genes in proestrus

3.1.5.3 Gonadotropes harvested from lactating and proestrus mice

205 genes were significantly differentially expressed between gonadotropes from proestrus and lactation mice. Out of these genes, 145 were upregulated and 60 were downregulated. The top upregulated genes were interphotoreceptor matrix proteoglycan 1, ribonuclease, RNase A family, 1 (pancreatic), purinergic receptor P2X, ligand-gated ion channel, 5, dipeptidylpeptidase 10, potassium voltage-gated channel, subfamily Q, member 5, contactin 5, KiSS-1 metastasis-suppressor (Table 12). GO analysis showed that most upregulated genes were enriched in ion transport and focal adhesion. KEGG analysis

showed these genes were involved in pathways like neuroactive ligand-receptor interaction and Ca^{2+} signaling pathways (Figure 20). The top downregulated genes were glutamate receptor, metabotropic 4, claudin 4, otoferlin, receptor-interacting serine-threonine kinase 3, G protein-coupled receptor 83 (Table 12). GO analysis showed that most downregulated genes were enriched in regulation of apoptosis and cation transport. KEGG analysis showed these genes were involved in pathways like MAPK signaling and taste transduction pathways (Figure 20).

Table 12 Top 20 differentially expressed genes between proestrus and lactation gonadotropes

Gene	Sample 1	Sample 2	FPKM 1	FPKM 2	log2(fold_change)	*p* value	*q* value
Gm8034	lactating	proestrus	0	24.7435	inf	0.00055	0.0134068
Ccdc147	lactating	proestrus	0.0845312	1.41115	4.06124	5.00E-05	0.00178848
Kiss1	lactating	proestrus	0.275473	3.94838	3.84128	5.00E-05	0.00178848
Impg1	lactating	proestrus	0.191203	2.70443	3.82214	0.0001	0.00326321
E330020D12Rik	lactating	proestrus	0.179936	2.14322	3.57423	5.00E-05	0.00178848
Cntn5	lactating	proestrus	0.267241	2.03724	2.9304	0.0003	0.00823277
Kcnq5	lactating	proestrus	0.143954	1.0882	2.91827	0.0017	0.0325213
Dpp10	lactating	proestrus	3.42174	25.1131	2.87564	5.00E-05	0.00178848
P2rx5	lactating	proestrus	0.339244	2.47589	2.86755	5.00E-05	0.00178848
Rnase1	lactating	proestrus	34.3779	240.309	2.80534	5.00E-05	0.00178848
Olfr315	lactating	proestrus	2.45298	0.323343	-2.9234	0.00125	0.0257336
C1ra	lactating	proestrus	3.64831	0.61571	-2.5669	5.00E-05	0.00178848
Ripk3	lactating	proestrus	1.71577	0.328924	-2.38303	0.0008	0.0181052
Cldn4	lactating	proestrus	30.0796	5.92667	-2.34349	5.00E-05	0.00178848
Otof	lactating	proestrus	1.98442	0.416382	-2.25274	5.00E-05	0.00178848
Hspa1b	lactating	proestrus	37.3655	7.97812	-2.22759	5.00E-05	0.00178848
Gpr83	lactating	proestrus	1.67943	0.370256	-2.18137	0.0001	0.00326321
Aif1l	lactating	proestrus	28.0773	6.44771	-2.12255	5.00E-05	0.00178848
Hspa1a	lactating	proestrus	36.9432	8.68324	-2.089	5.00E-05	0.00178848
Grm4	lactating	proestrus	1.47339	0.349897	-2.07414	0.00025	0.00708146

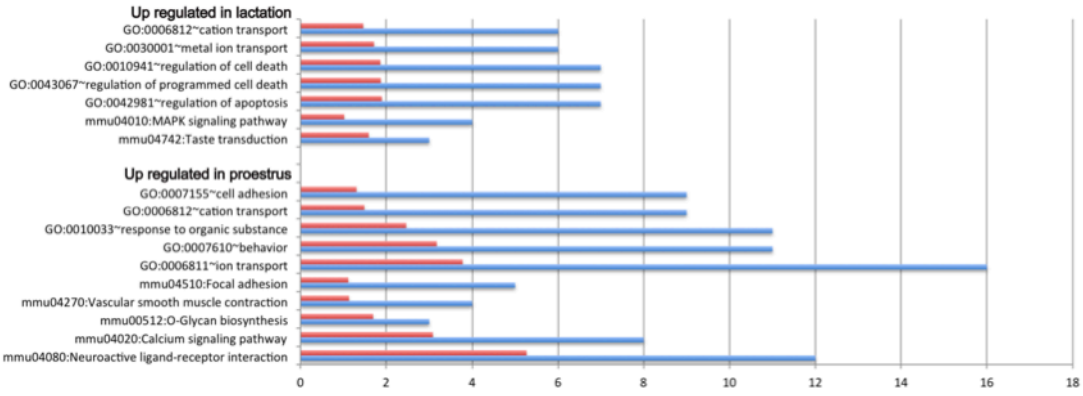

Figure 20 Top GO terms and pathways enriched from differentially expressed genes between proestrus and lactation gonadotropes

3.1.5.4 Gonadotropes harvested from lactating and diestrus mice

237 genes were significantly differentially expressed between gonadotropes from diestrus and lactating mice. Out of these genes, 77 were upregulated and 163 were downregulated. The top upregulated genes were tumor necrosis factor (ligand) superfamily, member 11,

protein tyrosine phosphatase, non-receptor type 5, potassium voltage-gated channel, subfamily Q, member 5, interphotoreceptor matrix proteoglycan 2, interphotoreceptor matrix proteoglycan 1 (Table 13). GO analysis showed that most upregulated genes were enriched in ion transport and positive regulation of cell communication. KEGG analysis showed these genes were involved in pathways such as neuroactive ligand-receptor interaction pathway (Figure 21). The top downregulated genes were serine (or cysteine) peptidase inhibitor, clade F, member 1, insulin-like growth factor binding protein 2, T cell receptor alpha constant, ribonuclease P 25 subunit, claudin 4, sushi domain containing 3, allograft inflammatory factor 1-like (Table 13). GO analysis showed most downregulated genes were enriched in cell adhension and embryonic organ development. KEGG analysis showed these genes were involved in pathways like MAPK, cytokine-cytokine receptor interaction and tight junction pathways (Figure 21).

Table 13 Top 20 differentially expressed genes between diestrus and lactation gonadotropes

Gene	Sample 1	Sample 2	FPKM 1	FPKM 2	log2(fold_change)	*p* value	*q* value
Impg2	lactating	diestrus	0.0720095	1.55664	4.43411	0.00125	0.0257336
Gm8113	lactating	diestrus	0.122197	1.49066	3.60867	5.00E-05	0.00178848
Col5a1	lactating	diestrus	0.238972	2.27124	3.24857	5.00E-05	0.00178848
Gm13404	lactating	diestrus	0.173144	1.34679	2.95948	0.0001	0.00326321
Kcnq5	lactating	diestrus	0.143954	1.10154	2.93585	0.0016	0.0310931
Impg1	lactating	diestrus	0.191203	1.43718	2.91006	0.00145	0.0288571
Gm4149	lactating	diestrus	95.9395	652.339	2.76543	5.00E-05	0.00178848
Tnfsf11	lactating	diestrus	1.27454	8.2373	2.6922	5.00E-05	0.00178848
Snhg11	lactating	diestrus	3.40921	21.5176	2.65801	5.00E-05	0.00178848
Ptpn5	lactating	diestrus	6.67142	37.0803	2.47459	5.00E-05	0.00178848
Serpina3c	lactating	diestrus	3.63379	0.452817	-3.00448	5.00E-05	0.00178848
Susd3	lactating	diestrus	1.75116	0.222301	-2.97772	0.00035	0.00931834
Rpp25	lactating	diestrus	1.75271	0.231422	-2.92099	0.00185	0.0347322
Trac	lactating	diestrus	4.46492	0.619719	-2.84895	0.00215	0.0388388
Aif1l	lactating	diestrus	28.0773	4.37432	-2.68228	5.00E-05	0.00178848
Gm26917	lactating	diestrus	17.7341	2.88748	-2.61865	5.00E-05	0.00178848
Serpinf1	lactating	diestrus	8.19462	1.53454	-2.41688	0.0012	0.0249397
Igfbp2	lactating	diestrus	26.843	5.12877	-2.38786	5.00E-05	0.00178848
Cldn4	lactating	diestrus	30.0796	5.86651	-2.35821	5.00E-05	0.00178848
Phex	lactating	diestrus	3.84178	0.76904	-2.32064	5.00E-05	0.00178848

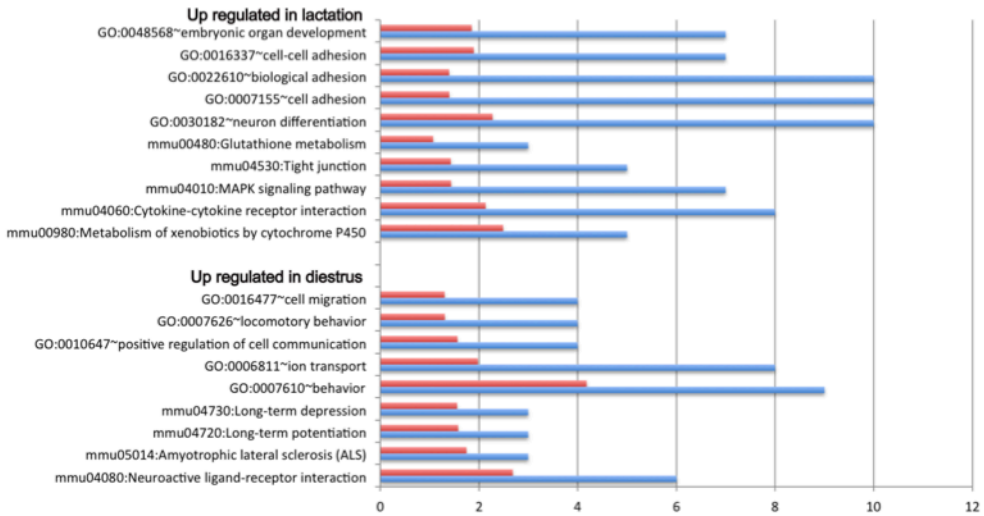

Figure 21 Top GO terms and pathways enriched from differentially expressed genes between diestrus and lactation gonadotropes

3.1.5.5 Gonadotropes harvested from prepubertal female and proestrus mice

808 genes were significantly differentially expressed between gonadotropes from proestrus and prepubertal female gonadotropes. Out of these genes, 481 were upregulated and 327 were downregulated.

The top upregulated genes were relaxin/insulin-like family peptide receptor 1, protein tyrosine phosphatase, non-receptor type 5, dopamine beta hydroxylase, KiSS-1 metastasis-suppressor, interphotoreceptor matrix proteoglycan 1, aldo-keto reductase family 1, member C14 (Table 14). GO analysis showed most upregulated genes were enriched in ion transport and cell adhesion. KEGG analysis showed these genes were involved in pathways such as Ca^{2+} signaling, cytokine-cytokine receptor interaction pathways (Figure 22).

Table 14 Top 20 differentially expressed genes between gonadotropes from proestrus and prepubertal female gonadotropes

Gene	Sample 1	Sample 2	FPKM 1	FPKM 2	log2(fold_change)	*p* value	*q* value
Prss29	three-week-old.female	proestrus	0	2.58316	inf	5.00E-05	0.00178848
Gm24363	three-week-old.female	proestrus	0	1.86178	inf	5.00E-05	0.00178848
Gm17689	three-week-old.female	proestrus	0	1.00905	inf	5.00E-05	0.00178848
Kiss1	three-week-old.female	proestrus	0.00314772	3.94838	10.2927	0.00105	0.0224568
Rxfp1	three-week-old.female	proestrus	0.00360196	1.47905	8.68167	0.00015	0.004614
Akr1c14	three-week-old.female	proestrus	0.0435409	13.3385	8.25901	5.00E-05	0.00178848
Impg1	three-week-old.female	proestrus	0.0130981	2.70443	7.68982	0.0013	0.0265595
Ptpn5	three-week-old.female	proestrus	0.205843	23.5977	6.84096	5.00E-05	0.00178848
Siglec15	three-week-old.female	proestrus	0.05446	5.80754	6.73659	0.00065	0.0153588
Dbh	three-week-old.female	proestrus	0.0366915	3.50211	6.57663	0.0004	0.0103989
Gpr101	three-week-old.female	proestrus	1.48248	0.0202698	-6.19254	5.00E-05	0.00178848
Edn2	three-week-old.female	proestrus	69.6568	4.05171	-4.10366	5.00E-05	0.00178848
Khdc1a	three-week-old.female	proestrus	2.30072	0.151561	-3.92412	5.00E-05	0.00178848
Tc2n	three-week-old.female	proestrus	9.19381	0.676143	-3.76526	5.00E-05	0.00178848
Hspa1b	three-week-old.female	proestrus	101.185	7.97812	-3.6648	5.00E-05	0.00178848
Grem1	three-week-old.female	proestrus	2.38813	0.197313	-3.59733	5.00E-05	0.00178848
Vgll2	three-week-old.female	proestrus	3.69694	0.329992	-3.48583	0.00055	0.0134068
Fgg	three-week-old.female	proestrus	3.11277	0.285328	-3.44751	5.00E-05	0.00178848
Plch1	three-week-old.female	proestrus	7.43361	0.717428	-3.37316	5.00E-05	0.00178848
Stac	three-week-old.female	proestrus	7.28569	0.74126	-3.29701	0.00015	0.004614

The top downregulated genes were gremlin 1, G protein-coupled receptor 101, fibrinogen gamma chain, src homology three (SH3) and cysteine rich domain, endothelin 2 (Table 14). GO analysis showed most downregulated genes were enriched in regulation of transcription and cell adhesion. KEGG analysis showed these genes were involved in pathways such as notch signaling, ECM-receptor interaction, and GnRH signaling pathways (Figure 22).

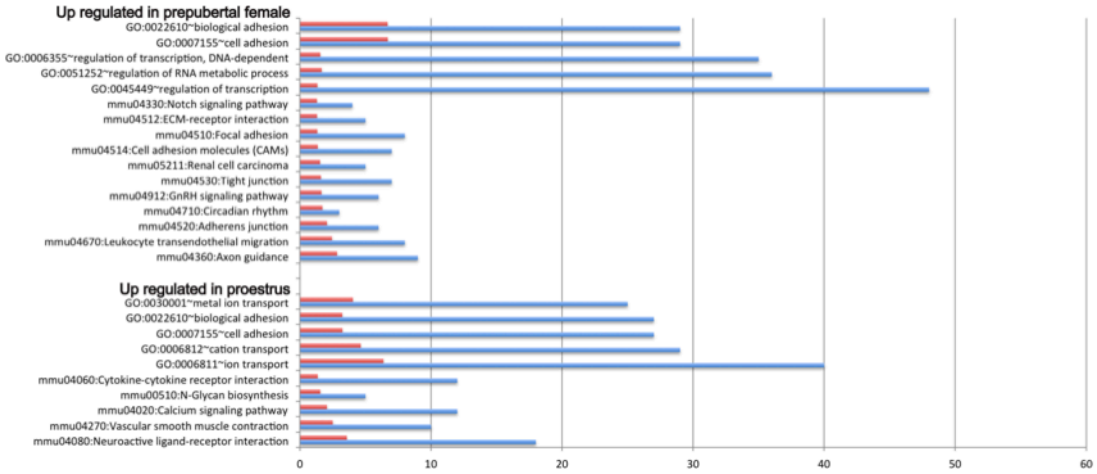

Figure 22 Top GO terms and pathways enriched from differentially expressed genes between gonadotropes from proestrus and prepubertal female gonadotropes

3.1.5.6 Gonadotropes harvested from prepubertal female and diestrus mice

986 genes were significantly differentially expressed between gonadotropes from diestrus and prepubertal female gonadotropes. Out of these genes, 401 were upregulated and 585 were downregulated.

The top upregulated genes were relaxin/insulin-like family peptide receptor 1, protein tyrosine phosphatase, non-receptor type 5, cyclic nucleotide gated channel alpha 3, solute carrier family 14 (urea transporter), member 1, KiSS-1 metastasis-suppressor, adenylate cyclase 8, sialic acid binding Ig-like lectin 15, ldo-keto reductase family 1, member C14 (Table 15). GO analysis showed most upregulated genes were enriched in ion transport and intracellular signaling cascade. KEGG analysis showed these genes were involved in pathways such as neuroactive ligand-receptor interaction, Ca^{2+} signaling pathway and MAPK signaling pathways (Figure 23).

The top downregulated genes were SAM domain, SH3 domain and nuclear localization signals, 1, endothelin 2, guanine nucleotide binding protein, alpha transducing 3, G protein-coupled receptor 101, neurexophilin 4, Fras1 related extracellular matrix protein 2, neuropilin 2 (Table 15). GO analysis showed most downregulated genes were enriched in regulation of RNA metabolic process and biological adhesion. KEGG analysis showed these genes were involved in pathways such as cell adhesion molecules (CAMs), tight junction and GnRH signaling pathways (Figure 23).

Table 15 Top 20 differentially expressed genes between diestrus and prepubertal female gonadotropes

Gene	Sample 1	Sample 2	FPKM 1	FPKM 2	log2(fold_change)	*p* value	*q* value
Gpr101	diestrus	three-week-old.female	0.0118853	1.48248	6.96269	0.00055	0.0134068
Phex	diestrus	three-week-old.female	0.76904	23.3208	4.92242	5.00E-05	0.00178848
Vgll2	diestrus	three-week-old.female	0.164687	3.69694	4.48853	0.0002	0.00589277
Nrp2	diestrus	three-week-old.female	0.640843	14.3377	4.4837	5.00E-05	0.00178848
Edn2	diestrus	three-week-old.female	3.68032	69.6568	4.24236	5.00E-05	0.00178848
Samsn1	diestrus	three-week-old.female	0.103516	1.94388	4.23101	0.0001	0.00326321
Gnat3	diestrus	three-week-old.female	0.141076	2.59533	4.20138	0.0007	0.0162884
Nxph4	diestrus	three-week-old.female	0.311793	5.58659	4.16331	5.00E-05	0.00178848
Frem2	diestrus	three-week-old.female	0.0993312	1.64971	4.05383	5.00E-05	0.00178848
Serpina3c	diestrus	three-week-old.female	0.452817	7.08104	3.96696	5.00E-05	0.00178848
Rxfp1	diestrus	three-week-old.female	2.52153	0.00360196	-9.4513	0.00015	0.004614
Kiss1	diestrus	three-week-old.female	1.75306	0.00314772	-9.12135	0.00095	0.0207831
Akr1c14	diestrus	three-week-old.female	8.01585	0.0435409	-7.52434	5.00E-05	0.00178848
Ptpn5	diestrus	three-week-old.female	37.0803	0.205843	-7.49297	5.00E-05	0.00178848
Siglec15	diestrus	three-week-old.female	8.91152	0.05446	-7.35433	0.00045	0.0114492
Cnga3	diestrus	three-week-old.female	5.59823	0.0604624	-6.53279	5.00E-05	0.00178848
Slc14a1	diestrus	three-week-old.female	19.7392	0.222638	-6.47022	5.00E-05	0.00178848
Adcy8	diestrus	three-week-old.female	31.3514	0.377052	-6.37762	5.00E-05	0.00178848
C130060K24Rik	diestrus	three-week-old.female	14.5534	0.192157	-6.24293	5.00E-05	0.00178848
Gm14216	diestrus	three-week-old.female	19.7751	0.383479	-5.68839	5.00E-05	0.00178848

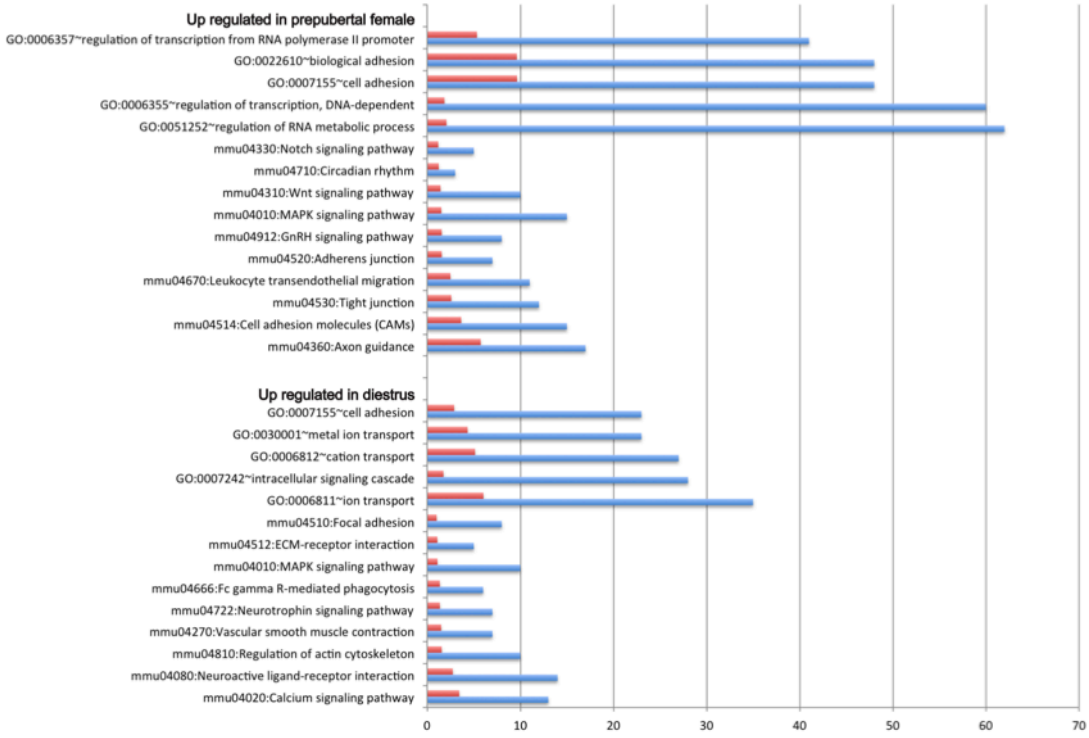

Figure 23 Top GO terms and pathways enriched from differentially expressed genes between diestrus and prepubertal female gonadotropes

3.1.5.7 Gonadotropes harvested from adult male and diestrus mice

997 genes were significantly differentially expressed between gonadotropes from diestrus female and adult male mice. Out of these genes, 366 were upregulated and 631 were downregulated.

The top upregulated genes were ribonuclease, RNase A family, 1 (pancreatic), inactive X specific transcripts, corticotropin releasing hormone binding protein, tumor necrosis factor (ligand) superfamily, member 11, protein tyrosine phosphatase, non-receptor type 5, solute carrier family 14 (urea transporter), member 1 (Table 16). GO analysis showed most upregulated genes were enriched intracellular signaling cascade, biological adhesion, and ion transport. KEGG analysis showed these genes were involved in pathways such as neuroactive ligand-receptor interaction, Ca^{2+} signaling pathway and MAPK signaling pathways (Figure 24).

The top downregulated genes were homeo box gene expressed in ES cells, gremlin 1, G protein-coupled receptor 101, T cell receptor alpha constant, neuropilin 2, serine (or cysteine) peptidase inhibitor, clade A, member 3C, sushi domain containing 3, insulin-like growth factor binding protein 1, peptidyl arginine deiminase, type I (Table 16). GO analysis showed most downregulated genes were enriched in regulation of cell proliferation and cell adhesion. KEGG analysis showed these genes were involved in pathways such as cytokine-cytokine receptor interaction, neuroactive ligand-receptor interaction and wnt signaling pathways (Figure 24).

Table 16 Top 20 differentially expressed genes between diestrus female and adult male mice gonadotropes

Gene	Sample 1	Sample 2	FPKM 1	FPKM 2	log2(fold_change)	*p* value	*q* value
Igkv4-58	diestrus	fourteen-week-old.male	0	2.75576	inf	5.00E-05	0.00178848
Grem1	diestrus	fourteen-week-old.male	0.177511	108.479	9.25529	5.00E-05	0.00178848
Gpr101	diestrus	fourteen-week-old.male	0.0118853	6.3696	9.06588	0.00055	0.0134068
Serpina3c	diestrus	fourteen-week-old.male	0.452817	27.5572	5.92736	5.00E-05	0.00178848
Hesx1	diestrus	fourteen-week-old.male	0.117249	4.19983	5.16268	5.00E-05	0.00178848
Nrp2	diestrus	fourteen-week-old.male	0.640843	20.4749	4.99774	5.00E-05	0.00178848
Padi1	diestrus	fourteen-week-old.male	0.167016	4.71543	4.81933	5.00E-05	0.00178848
Trac	diestrus	fourteen-week-old.male	0.619719	17.1116	4.78721	5.00E-05	0.00178848
Igfbp1	diestrus	fourteen-week-old.male	0.106384	2.68951	4.65999	0.0001	0.00326321
Susd3	diestrus	fourteen-week-old.male	0.222301	5.5536	4.64284	5.00E-05	0.00178848
Ptpn5	diestrus	fourteen-week-old.male	37.0803	0.0674581	-9.10245	5.00E-05	0.00178848
Xist	diestrus	fourteen-week-old.male	36.5427	0.0700468	-9.02705	5.00E-05	0.00178848
Slc14a1	diestrus	fourteen-week-old.male	19.7392	0.0521405	-8.56445	5.00E-05	0.00178848
C130060K24Rik	diestrus	fourteen-week-old.male	14.5534	0.0558923	-8.02449	5.00E-05	0.00178848
Crhbp	diestrus	fourteen-week-old.male	31.1457	0.14546	-7.74227	0.00015	0.004614
Tnfsf11	diestrus	fourteen-week-old.male	8.2373	0.101319	-6.34519	5.00E-05	0.00178848
Rnase1	diestrus	fourteen-week-old.male	96.6989	1.28448	-6.23424	5.00E-05	0.00178848
Asic2	diestrus	fourteen-week-old.male	8.53333	0.12812	-6.05754	5.00E-05	0.00178848
Gm12248	diestrus	fourteen-week-old.male	3.57644	0.059333	-5.91355	5.00E-05	0.00178848
Gm14216	diestrus	fourteen-week-old.male	19.7751	0.343359	-5.84782	5.00E-05	0.00178848

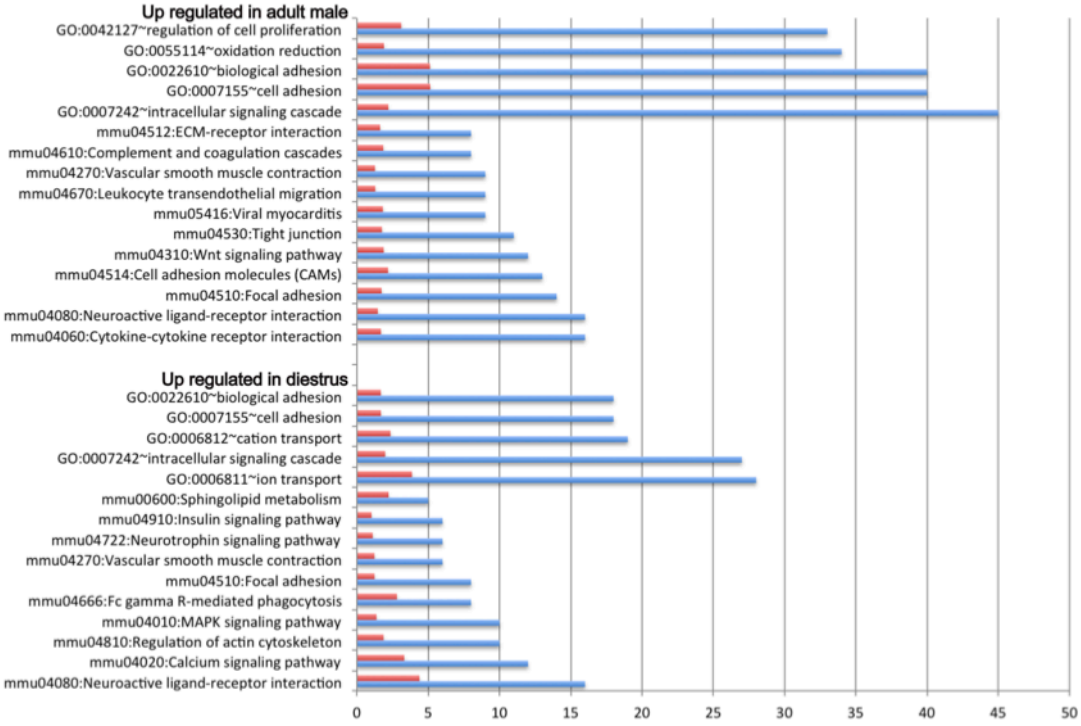

Figure 24 Top GO terms and pathways enriched from differentially expressed genes between diestrus female and adult male mice gonadotropes

3.1.5.8 Gonadotropes harvested from prepubertal and adult male mice

327 genes were significantly differentially expressed between gonadotropes from prepubertal male and adult male gonadotropes. Out of these genes, 140 were upregulated and 187 were downregulated.

The top upregulated genes were mainly predicted genes (Table 17). GO analysis showed that most upregulated genes were enriched in positive regulation of RNA metabolic process and neuron development. KEGG analysis showed these genes were involved in pathways like tight junction and ECM-receptor interaction (Figure 25).

The top downregulated genes were myocilin, gremlin 1, alpha-2-macroglobulin, heat shock transcription factor 4, immunoglobulin joining chain, hyaluronan and proteoglycan link protein 1, aldo-keto reductase family 1, member C14 (Table 17). GO analysis showed most downregulated genes were enriched cell adhesion, ion transport. KEGG analysis showed these genes were involved in pathways like cytokine-cytokine receptor interaction and focal adhesion (Figure 25).

Table 17 Top 20 differentially expressed genes between prepubertal and adult male gonadotropes

Gene	Sample 1	Sample 2	FPKM 1	FPKM 2	log2(fold_change)	*p* value	*q* value
Gm5560	fourteen-week-old.male	three-week-old.male	0.409976	13.7477	5.06751	0.0001	0.003263
Gm5796	fourteen-week-old.male	three-week-old.male	0.195723	5.78049	4.88431	5.00E-05	0.001788
Gm3752	fourteen-week-old.male	three-week-old.male	0.268266	5.15853	4.26522	0.0004	0.010399
Gm3005	fourteen-week-old.male	three-week-old.male	0.216659	3.39812	3.97124	5.00E-05	0.001788
Gm3591	fourteen-week-old.male	three-week-old.male	0.146155	1.96021	3.74544	0.00075	0.017195
Gm2974	fourteen-week-old.male	three-week-old.male	0.168633	2.16032	3.67929	0.0004	0.010399
Gm10406	fourteen-week-old.male	three-week-old.male	0.565467	6.70978	3.56875	5.00E-05	0.001788
Gm8281	fourteen-week-old.male	three-week-old.male	0.756394	8.69247	3.52256	5.00E-05	0.001788
Gm3164	fourteen-week-old.male	three-week-old.male	0.431056	4.81328	3.48107	5.00E-05	0.001788
Gm3558	fourteen-week-old.male	three-week-old.male	0.104517	1.12476	3.4278	0.00165	0.031815
Grem1	fourteen-week-old.male	three-week-old.male	108.479	6.00746	-4.17452	5.00E-05	0.001788
Irx6	fourteen-week-old.male	three-week-old.male	2.55081	0.169836	-3.90875	5.00E-05	0.001788
Bpifa1	fourteen-week-old.male	three-week-old.male	11.2944	0.943642	-3.58122	5.00E-05	0.001788
Igj	fourteen-week-old.male	three-week-old.male	1.00527	0.10143	-3.30902	0.00155	0.030402
Hsf4	fourteen-week-old.male	three-week-old.male	2.93048	0.298533	-3.29517	5.00E-05	0.001788
Snhg11	fourteen-week-old.male	three-week-old.male	5.98119	0.616682	-3.27784	5.00E-05	0.001788
Myoc	fourteen-week-old.male	three-week-old.male	15.3856	1.61301	-3.25376	5.00E-05	0.001788
A2m	fourteen-week-old.male	three-week-old.male	3.96179	0.417375	-3.24674	5.00E-05	0.001788
Hapln1	fourteen-week-old.male	three-week-old.male	1.39093	0.152223	-3.1918	0.00015	0.004614

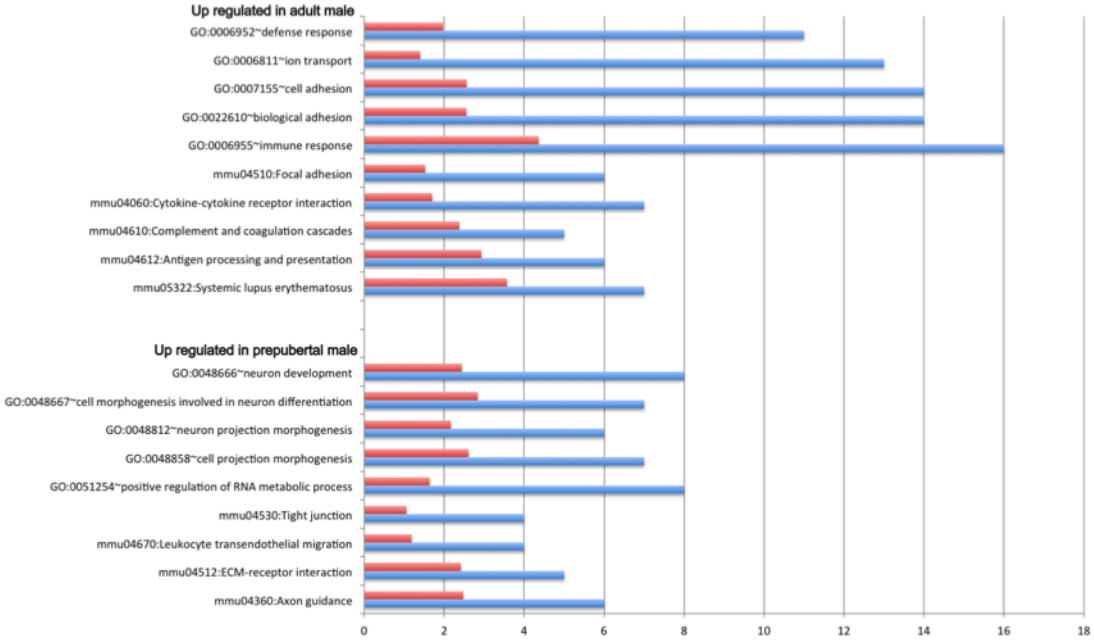

Figure 25 Top GO terms and pathways enriched from differentially expressed genes between prepubertal and adult male gonadotropes

3.1.5.9 Gonadotropes harvested from adult male and proestrus mice

729 genes were significantly differentially expressed between gonadotropes from proestrus female and adult male gonadotropes. Out of these genes, 379 were upregulated and 350 were downregulated.

The top upregulated genes were ribonuclease, RNase A family, 1 (pancreatic), inactive X specific transcripts, corticotropin releasing hormone binding protein, reelin, uromodulin

(Table 18). GO analysis showed most upregulated genes were enriched in ion transport, intracellular signaling cascade, and biological adhesion. KEGG analysis showed these genes were involved in pathways such as neuroactive ligand-receptor interaction and Ca^{2+} signaling pathway (Figure 26).

The top downregulated genes were contactin associated protein-like 4, gremlin 1, G protein-coupled receptor 101, neuropilin 2, gastrin releasing peptide (Table 18). GO analysis showed most downregulated genes were enriched in regulation of cell proliferation and biological adhesion. KEGG analysis showed these genes were involved in pathways such as cell adhesion molecules (CAMs), Wnt signaling and cytokine-cytokine receptor interaction (Figure 26).

Table 18 Top 20 differentially expressed genes between proestrus and adult male gonadotropes

Gene	Sample 1	Sample 2	FPKM 1	FPKM 2	log2(fold_change)	*p* value	*q* value
Grem1	proestrus	fourteen-week-old.male	0.197313	108.479	9.10272	5.00E-05	0.001788
Gpr101	proestrus	fourteen-week-old.male	0.02027	6.3696	8.29573	5.00E-05	0.001788
Miat	proestrus	fourteen-week-old.male	0.085069	1.76551	4.37531	5.00E-05	0.001788
Cntnap4	proestrus	fourteen-week-old.male	0.170637	3.5195	4.36637	5.00E-05	0.001788
Cilp	proestrus	fourteen-week-old.male	0.176329	3.21792	4.18979	5.00E-05	0.001788
Serpina3c	proestrus	fourteen-week-old.male	1.98609	27.5572	3.79442	5.00E-05	0.001788
Grik3	proestrus	fourteen-week-old.male	1.27777	17.377	3.76548	5.00E-05	0.001788
Fam159b	proestrus	fourteen-week-old.male	0.241383	2.9989	3.63503	0.00015	0.004614
Grp	proestrus	fourteen-week-old.male	1.40776	16.9685	3.59139	5.00E-05	0.001788
Nrp2	proestrus	fourteen-week-old.male	1.71909	20.4749	3.57414	5.00E-05	0.001788
Slc14a1	proestrus	fourteen-week-old.male	13.5773	0.052141	-8.02458	5.00E-05	0.001788
C130060K24Rik	proestrus	fourteen-week-old.male	13.6513	0.055892	-7.93217	5.00E-05	0.001788
Xist	proestrus	fourteen-week-old.male	16.0052	0.070047	-7.83601	5.00E-05	0.001788
Crhbp	proestrus	fourteen-week-old.male	29.5886	0.14546	-7.66827	0.00015	0.004614
Rnase1	proestrus	fourteen-week-old.male	240.309	1.28448	-7.54756	5.00E-05	0.001788
Umod	proestrus	fourteen-week-old.male	13.6181	0.147145	-6.53214	5.00E-05	0.001788
Asic2	proestrus	fourteen-week-old.male	10.8529	0.12812	-6.40443	5.00E-05	0.001788
Reln	proestrus	fourteen-week-old.male	29.585	0.407455	-6.18208	5.00E-05	0.001788
Gm12248	proestrus	fourteen-week-old.male	4.10558	0.059333	-6.11261	5.00E-05	0.001788

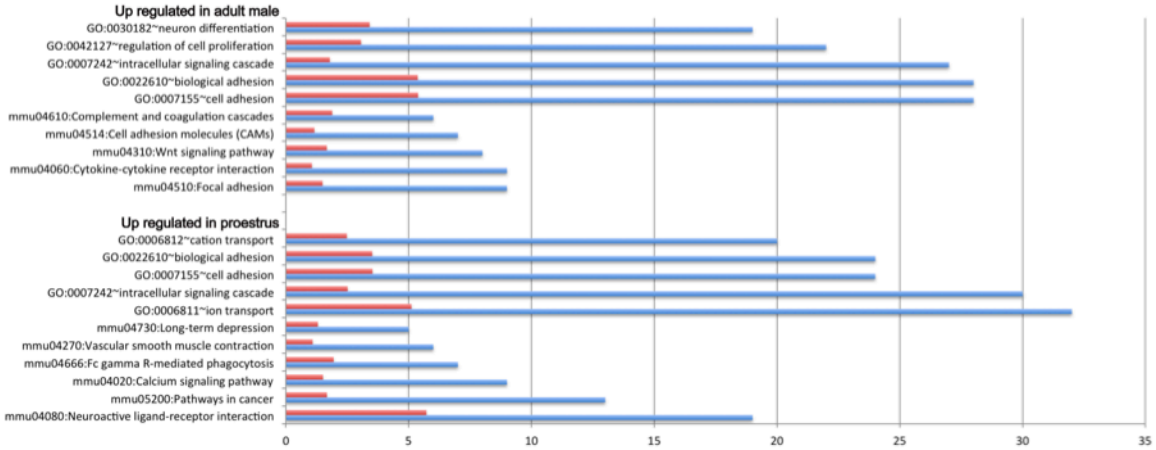

Figure 26 Top GO terms and pathways enriched from differentially expressed genes between proestrus and adult male gonadotropes

3.1.6 Transcription factors

I also compared the differentially expressed genes with the mouse transcription factor database and found 138 transcription factors to be differentially expressed. The bHLH (basic helix-loop-helix), homeobox, TF_bZIP (Basic-leucine zipper), and zf-C2H2 (C2H2 zinc fingers) families of transcription factors were the most represented families with 9, 13, 8 and 19 genes, respectively. The most highly differentially regulated transcription factors are shown in Figure 27.

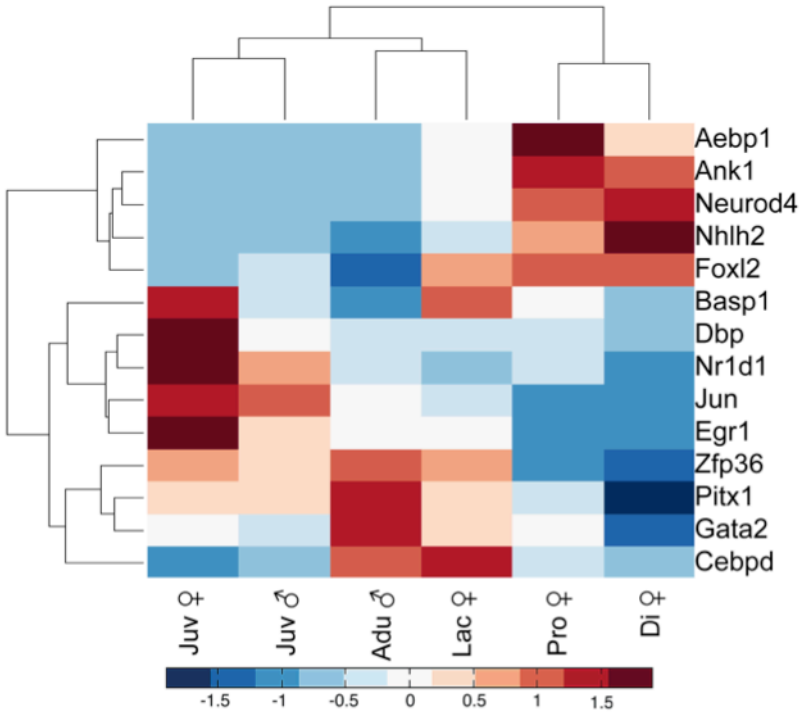

Figure 27 A two-way hierarchical clustering dendrogram of the top changed transcription factors

Data are expressed as mean FPKM, standardized, and visualized using the MATLAB "clustergram" script. Red: relatively high expression; blue: relatively low expression. Transcription factors with FPKM>100 in at least one experimental group are considered.

3.1.7 Validation of the RNA-seq data

Finally, to independently confirm the validity of the RNA-seq data, four genes Gria1 (Glutamate receptor 1), Gria2 (Glutamate receptor 2), Grem1 (gremlin 1) and Gata2

(GATA binding protein 2) were selected based on their complex regulation including those with high (FPKM>100) and low (FPKM<10) expression levels for examination by real-time RT-PCR. I found that the RNA-seq data for these genes were highly similar to those obtained by RT-qPCR (Figure 28).

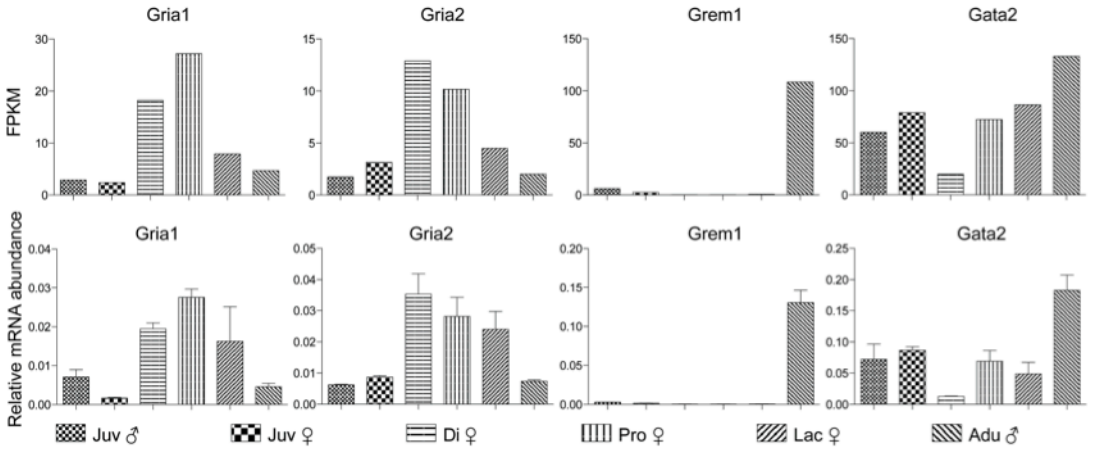

Figure 28 Comparison of gene expression data obtained by RNAseq and by RT-qPCR

Expression level of selected genes in murine gonadotropes were measured by RT-qPCR (lower row). Gene expression was normalized to the reference gene, Actb (n = 3). FPKM values were obtained from RNA-seq data (upper row). Abbreviations: Gria1: Glutamate receptor 1, Gria2: Glutamate receptor 2, Grem1: gremlin 1 and Gata2: GATA binding protein 2, Actb: actin, beta.

3.2 The function of TRPC5 in murine gonadotropes

3.2.1 TRP channel plasticity in murine gonadotropes

Transient receptor potential (TRP) cation channels have been proven to play multiple roles in hormone secreting cells such as pancreatic β cells. Some TRP channels were detected in rat pituitary cells, however little is known about the function of TRP channels in mouse pituitary hormone secreting cells. After performed RNA-seq, I first asked whether TRP channels are expressed in these cells. I found that 14 out of 28 TRP channels encoded in the mouse genome were expressed in gonadotropes with FPKM values (representing gene abundance within the samples (Trapnell et al., 2010)) >1 in at least one experimental group. Out of these genes, *Mcoln3* (encoding TRPML3), *Mcoln1* (encoding TRPML1), *Pkd2* (encoding TRPP1) and *Trpc5* were expressed at high levels with FPKM values >10 in at

least one stage. *Trpv2* and *Trpc4* displayed higher expression levels in males whereas *Pkd2* and *Trpm3* showed higher expression levels in gonadotropes isolated from cycling females. Conversely, in juveniles, *Trpc5* and *Trpm4* were expressed at higher levels (Figure 29).

Next I analyzed quantitative changes in individual TRP channel gene expression in gonadotropes at different hormonal stages. To do this, I selected genes with a greater than two-fold change, $q<0.05$ and FPKM >1 in at least one condition. I found five TRP channels (Trpv2, Trpc4, Trpc5, Trpc6 and Trpa1) to be differentially expressed employing these criteria. Taken together, these data demonstrate, that TRP channel expression in murine gonadotropes exhibits considerable plasticity and depends on gender as well as on the developmental and hormonal status of the animal (Table 19). As *Trpc5* was found to be the most highly expressed TRP channel of these five genes, I independently validated its expression using RT-qPCR. I found that the expression pattern determined via RT-qPCR was highly similar to that obtained by RNA-sequencing. I therefore decided to focus our functional analyses on TRPC5 in gonadotropes from juvenile females (Figure 29).

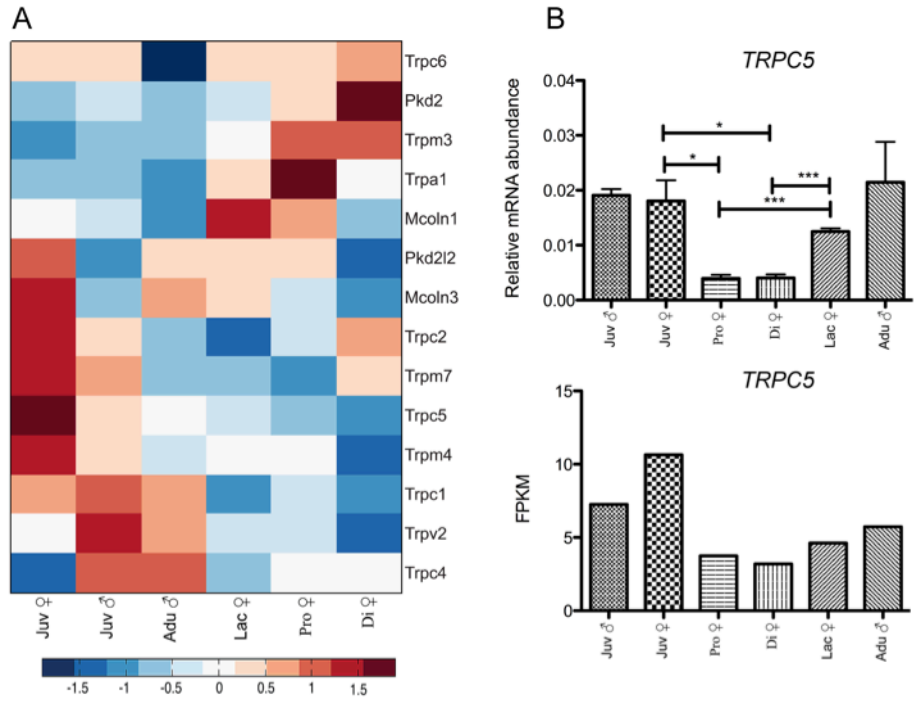

Figure 29 Heat map of Trp channel expression in murine gonadotropes and validation of Trpc5 expression data obtained from RNA-seq by RT-qPCR

(red=relatively high expression; blue=relatively low expression). Juv ♂, 3-old-week male; Juv ♀, 3-week-old female; Di ♀, diestrus; Pro ♀, proestrus; Lac ♀, lactating female; Adu ♂, 14-week-old male. Pkd2=Trpp1,

Pkd2l2=Trpp3, Mcoln1=Trpml1, Mcoln3=Trpml3. B. Validation of Trpc5 expression data obtained from RNA-seq by RT-qPCR. Data were normalized to the mRNA level of Actb (beta-actin; n=3). FPKM values were obtained from RNA-seq data.

Table 19 List of differentially expressed TRP channels in gonadotropes between different genders, hormonal stages and developmental stages

Gene	Compared stages	FPKM values
Trpa1	Pro ♀ *vs* Juv ♂	1.17 *vs* 0.18
	Juv ♀ *vs* Pro ♀	0.12 *vs* 1.17
Trpc4	Lac ♀ *vs* Juv ♂	2.87 *vs* 5.78
	Juv ♀ *vs* Juv ♂	2,00 *vs* 5.78
	Juv ♀ *vs* Adu ♂	2,00 *vs* 5.41
Trpc5	Juv ♀ *vs* Pro ♀	**10.64** *vs* 3.75
	Di ♀ *vs* Juv ♂	3.19 *vs* 7.26
Trpc6	Di ♀ *vs* Adu ♂	1.08 *vs* 0.19
	Pro ♀ *vs* Adu ♂	1.01 *vs* 0.19
Trpv2	Di ♀ *vs* Juv ♂	1.34 *vs* 3.13

Significant comparison or comparisons for individual genes are shown with their corresponding FPKM values. The FPKM with value bigger than 10 is marked with bold numbers. Juv ♂, 3-old-week male; Juv ♀, 3-week-old female; Di ♀, diestrus; Pro♀, proestrus; Lac ♀, lactating female; Adu ♂, 14-week-old male. The FPKM with value bigger than 10 is marked with bold letter.

I then collaborated with Dr. Andreas Beck to investigate to function of TRPC5 in gonadotropes from juvenile females. I did the genotyping for the experimental mice and prepared the primary cultured pituitary cells. Dr. Andreas Beck performed all the Ca^{2+} imaging and electrophysiological recordings. The results are summarized as follows. We showed that the TRPC5 agonist Englerin A activated a cytosolic Ca^{2+} signal and a whole-cell current in gonadotropes, which is absent in TRPC5-deficient mice. We further showed that the Englerin A-activated TRPC5-dependent Ca^{2+} signal was mediated by Ca^{2+} influx both via TRPC5 and via L-type voltage-gated Ca^{2+} channels, activated by the depolarization through TRPC5-mediated cation influx. Finally, we demonstrated that the GnRH-mediated net depolarization was significantly reduced in gonadotropes isolated from TRPC5-deficient mice.

3.2.2 Normal puberty onset and body weight in TRPC5-deficient female mice

Since TRPC5 gained highest expression level in juvenile gonadotropes among all the four female stages, I asked whether TRPC5 play an important role in puberty onset and body weight of female mice. Therefore, I measured vaginal opening and body weight of TRPC5 knock-out mice and control mice. I found TRPC5 knock-out female mice showed normal puberty onset and body weight compared with control mice (Figure 30).

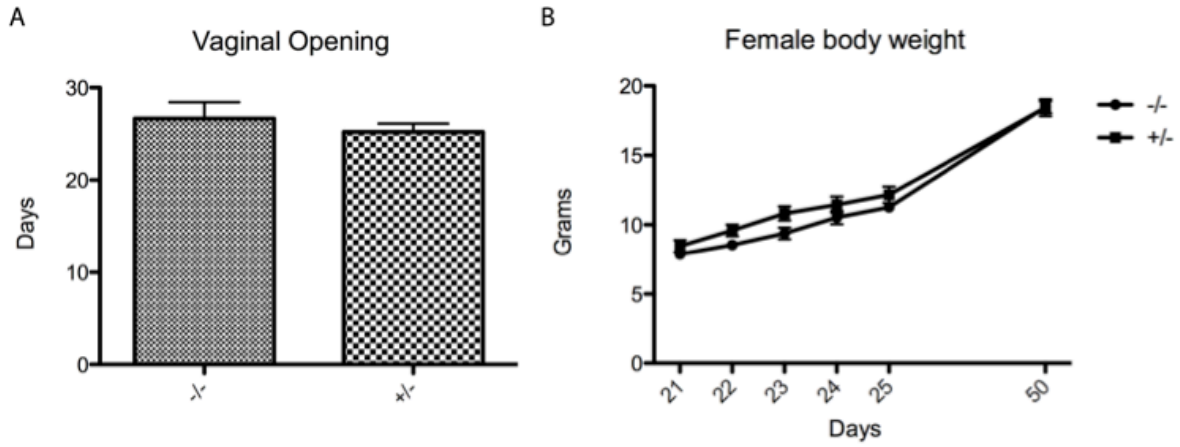

Figure 30 Vaginal opening days and body weight in female TRPC5$^{+/-}$ or TRPC5$^{-/-}$ mice

Days of vaginal opening (A) and weight gain (B) female TRPC5$^{+/-}$ or TRPC5$^{-/-}$ littermate mice (n = 3 for each group). All data showed as mean ± SEM.

3.3 The function of Tet1 and Tet2 in murine gonadotropes' diffferentiation

3.3.1 The Tet family is expressed in gonadotropes at diverse levels

DNA methylation at cytosine bases is one of the critical factors controlling gene expression. Therefore, DNA methylation pattern is tightly linked to cell differentiation. The Tet enzymes play central and complex roles in determining the changing patterns of gene expression during development. After performing RNA-seq of murine gonadotropes at different stages, I asked whether members of Tet family are expressed in gonadotropes and what is their expression pattern. Tet1, Tet2 and Tet3 were indeed expressed in murine gonadotropes at all six stages (FPKM>1), although they showed low expression levels

(FPKM<10). I further confirmed the detection of RNA-seq by mapping the raw reads to the murine genome via IGV (Integrative Genomics Viewer). I found that all exons of Tet1, Tet2 and Tet3 are covered by reads. However, I also found that there were less reads covering exon1 of Tet1 compared with other exons. Moreover, there were more reads covering the region nearby exon2 than exon1, suggesting a potential exon that has not yet been discovered, which was consistent with Prof. Philippa Melamed's group's findings (Figure 31).

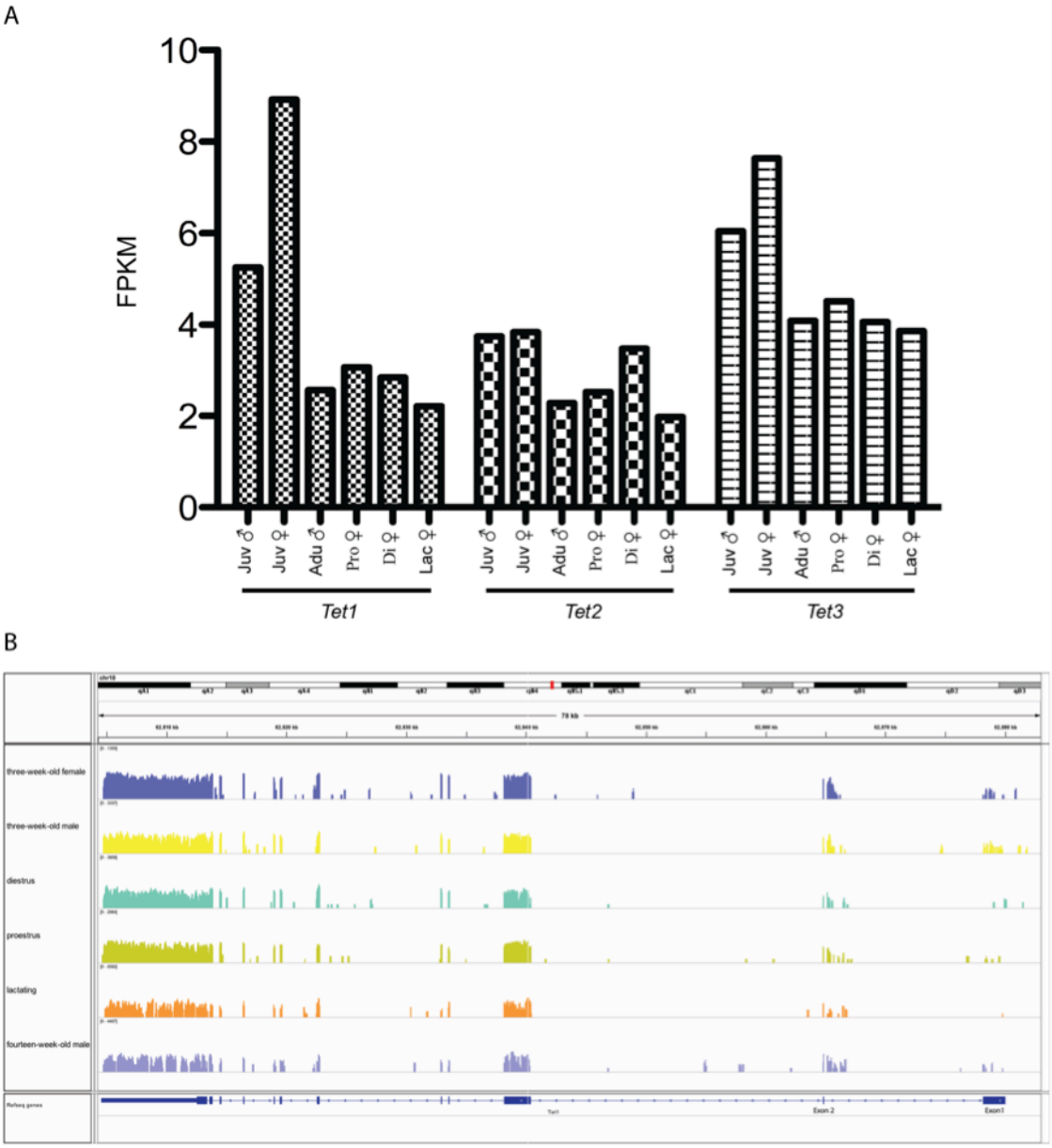

Figure 31 Tet family's expressions in murine gonadotropes

A: FPKM values of Tet1, Tet2 and Tet3 among all six stages examined. B: Visualization of aligned reads in Tet1 loci of all six stages examined.

To further investigate the function of Tet family members in murine gonadotropes, I collaborated with Prof. Philippa Melamed's group. The results are summarized as follows. They measured Tet family expression levels via RT-qPCR between mature (8-14 weeks old mice) gonadotropes and immature (postnatal day 6 mice) gonadotropes in which the gonadotrope population is expanding.

Tet1 showed a much higher expression level in immature gonadotropes, while levels of Tet2 and Tet3 did not differ between stages. Although the absolute mRNA levels of Tet2 and 3 in the primary gonadotropes are 2-2.5 fold higher than Tet1 in immature mice, this ratio increases to 9-13 fold in mature mice, revealing a clear shift in the relative expression of these enzymes during gonadotrope development and differentiation.

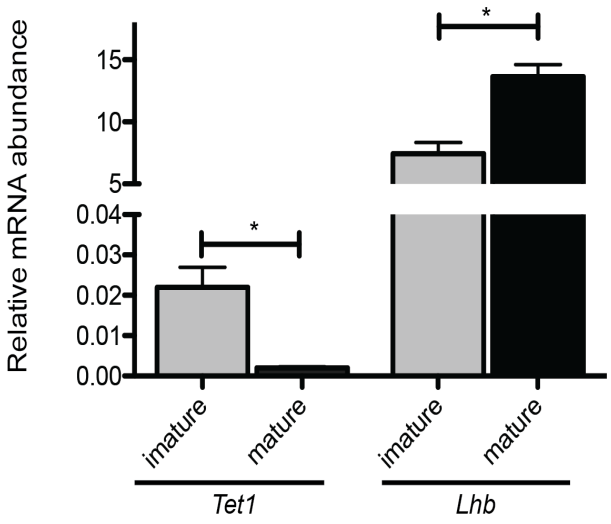

Figure 32 Tet1 negatively correlates with Lhb expression in gonadotropes

RT-qPCR analyses in primary gonadotropes from immature (postnatal day 6) female or mature (14-week-old) female mice. *Lhb* and *Tet1* levels are shown relative to *Actb* (n=3). *: P<0.05, **: P<0.01.

3.3.2 The expression of *Tet1* and *Tet2* is regulated by GnRH

We also observed that Tet1 negatively correlates with Lhb expression in gonadotropes (Figure 32). Also in gonadotrope cell lines, *Tet1* levels were much lower in the more fully differentiated LβT2 cells in which *Lhb* is expressed abundantly, than in the poorly differentiated αT3-1 cells in which *Lhb* is barely expressed. Given that the relative levels of *Tet* gene expression shifted in accordance with a change in gonadotrope differentiation, we considered that GnRH, which is the major regulator of gonadotrope differentiation, might mediate part of its effect during development and gonadotrope differentiation by altering the expression of these enzymes.

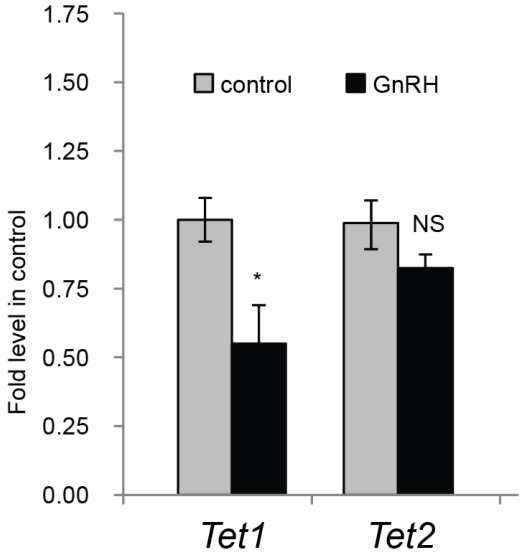

Figure 33 Tet1's expression in gonadotropes is regulated by GnRH

Tet1 (A) and Tet2 (B) levels following GnRH treatment were assessed by RT-qPCR and were shown relative to levels in non-treated cells: mean ± SEM.

Indeed, GnRH introduced a reduction in Tet1 mRNA levels in the primary cells, with the strongest effect seen in LβT2 cells. Tet2 responded to GnRH with a minor increase in αT3-1 cells, but a decrease in LβT2 cells and no clear response in the primary cells (Figure 33). Thus activation of Lhb expression during development and in response to GnRH is associated with a reduction in Tet1 expression, and a consistently clear change in Tet1: Tet2 ratio, suggesting a distinct function of the two Tet enzymes in these cells, and a

repressive effect for Tet1. Tet3, did not show any changes in expression levels either with gonadotrope differentiation or after exposure to GnRH, so we considered it likely plays less of a role in regulating gonadotropin gene expression and focused the remainder of this study on the roles of Tet1 and Tet2.

3.3.3 Tet1 and Tet2 have different effects on *Lhb* gene expression

To test whether either Tet1 and/or Tet2 alter Lhb gene expression, Prof. Philippa Melamed's group first transiently inhibited Tet2 expression in αT3-1 cells by transfecting a shRNA targeting Tet2, which led to a ~50% decrease in Lhb mRNA levels. Conversely, over-expression of the Tet2 catalytic domain led to an increase in Lhb mRNA levels, supporting a positive regulatory role for Tet2 on Lhb transcription. In contrast with the positive correlation between Tet2 levels and Lhb expression, Tet1 levels appeared to be inversely correlated. To explore whether Tet1 negatively regulates Lhb expression, they performed stable knockdown (KD) of Tet1 which led to a reduction in Tet1 mRNA levels by ~80 %, and a ~2.4 increase in Lhb mRNA levels. Taking together, we found that Tet1, Tet2 and Tet3 are expressed in murine gonadotropes, and that Tet1 represses the expression of the luteinizing hormone β-subunit (*Lhb*) gene, whereas Tet2 enhances the expression of the *Lhb* gene.

4 Discussion

4.1 Gonadotrope plasticity

In this part of the study I took advantage of a binary genetic approach, the Cre-loxP system, to fluorescently label gonadotropes. I then used a cell sorting strategy to enrich the gonadotropes based their endogenous fluorescence. After acquiring a purified population of cells, I performed RNA-seq in order to attain whole transcriptome information from gonadotropes from six different developmental stages. By using this high throughput technology, I analyzed the plasticity of gonadotropes at the whole genome scale level. I compared the molecular difference of gonadotropes during development, the extent of sexual dimorphism in juvenile and adult animals, and also the extent of changes in gene expression that occur throughout the estrous cycle and during lactation. I found around 22000 genes expressed in murine gonadotropes in all six stages. I also found that murine gonadotropes have unique gene signatures with approximately 100 to approximately 500 genes expressed specifically in one particular stage. Furthermore, I identified approximately 2200 differentially expressed genes when comparing females and males during development, juvenile and adult gender differences, and cycling females. Moreover, these differential expressed genes are enriched in the GO terms like cell adhesion, blood vessel development, ion transport, and vasculature development, and in pathways such as GnRH signaling, Ca^{2+} signaling, MAPK signaling, and neuroactive ligand-receptor from functional analysis. Besides this, I also found that gonadotropes have unique transcriptional factor signatures.

Our data demonstrate that juvenile animals of both genders were most similar in terms of their gene expression profile. In addition, adult male animals were also relatively similar to juveniles. In contrast, the expression profiles of gonadotropes from cycling adult females were substantially divergent from that in the juvenile female. These results suggest that the gonadotropes in cycling adult females undergo substantial changes in expression profile, possibly to meet the requirements of the estrous cycle. However, even between proestrus and diestrus, there are substantial differences in gene expression patterns. I found that there are substantially more genes upregulated in proestrus in comparison with diestrus.

Our results have revealed substantially divergent gene expression profiles in gonadotropes in different genders and across developmental and hormonal stages. In each stage

analyzed, between approximately 100 to approximately 500 genes were expressed only in one particular stage. Among them, I not only found known gender-specific genes such as Xist (inactive X specific transcript), Uty (ubiquitously transcribed tetratricopeptide repeat gene, Y chromosome), and Kdm5d (lysine [K]-specific demethylase 5D) but also identified several new gender-specific genes in gonadotropes such as Galr1 (galanin receptor 1) and Gpr101 (G protein coupled receptor 101). The differential regulation of Gpr101 stands out as being particularly interesting because it has recently been discovered that a cleavage product of GnRH ($GnRH_{1–5}$) can bind and activate this receptor (Cho-Clark et al., 2014).

I identified ~2200 differentially expressed genes among individual comparisons. Among the differentially expressed genes, I found several that have already been well studied in gonadotropes such as Egr1 (early growth response 1) (Witham et al., 2013), Foxl2 (forkhead box L2) (Herndon and Nilson, 2015), Jun (Jun oncogene) (Binder et al., 2012) and Gata2 (GATA binding protein 2) (Charles et al., 2006). Among them, Tran *et al.* reported that gonadotrope-specific Foxl2 knock out mice had impaired fertility and FSH synthesis. Moreover, when this gene was ablated together with Smad4 in gonadotropes, FSH production is essentially halted and female mice are sterile. These findings seem to correspond with our data, which show that Foxl2 had a moderate expression value in male gonadotropes but high expression in adult female gonadotropes (Fortin et al., 2014; Tran et al., 2013). However, I also identified several new target genes that have not been well studied in gonadotropes so far.

One potentially interesting gene encodes fibroblast growth factor receptor 1 (FGFR1) which has recently been demonstrated to be involved in reproductive disease (Miraoui et al., 2011). Different mutations of this gene have been identified in human patients and are associated with different reproductive phenotypes such as Kallmann syndrome (KS) (18), normosmic idiopathic hypogonadotropic hypogonadism (nIHH) (Raivio et al., 2009) and hypothalamic amenorrhea (Caronia et al., 2011). Our data show that Fgfr1 is expressed in murine gonadotropes but interestingly has a higher expression level in adult females. While significant efforts have been made to examine the role of Fgfr1 in GnRH neurons (Hu et al., 2013), so far very little work has been done to examine the role of this gene in gonadotropes.

I also found that the transcription factor Sox2 (Sex-determining region Y (SRY) box 2) is expressed in juvenile gonadotropes but barely expressed in adult samples. It has been shown previously that Sox2 positive cells can differentiate into all hormone producing cells in the pituitary (Andoniadou et al., 2013). These data raise the possibility that some juvenile gonadotropes may in fact be progenitor cells and merits further investigation. Cldn4 (Claudin 4) is a member of the claudin family and found in tight junctions (Neesse et al., 2012). Our data show that this gene is differentially expressed in gonadotropes consistent with a role in gonadotrope organization at different stages.

Among the top differentially expressed genes I found that Serpine2 is highly expressed in gonadotropes at all six stages analyzed in this study but has relatively lower FPKM values in juvenile female and diestrus gonadotropes. Serpine2 is a serine protease inhibitor (Bouton et al., 2012) and its dramatic change in expression level could signify a necessity to inhibit degradation of specific proteins in all stages except juvenile and diestrus females. Former studies have shown that Serpine2 deficient male mice have a marked impairment in fertility from the onset of sexual maturity (Murer et al., 2001). Several following studies have investigated this gene's role in the gonads (Lu et al., 2013; Zhang et al., 2007), but our result suggests that the reproductive deficit in these mice may also be at the level of the pituitary. Gremlin1 is only highly expressed in adult male gonadotropes and has been reported to inhibit the transforming growth factor beta (TGFβ) signaling pathway via antagonistic interaction with bone morphogenetic proteins (BMPs) (Benazet et al., 2009), meanwhile BMP2 has been studied to participate in regulating follicle-stimulating hormone beta (FSHβ) transcription (Wang et al., 2014). Taken together, these data raise the possibility that Gremlin1 may also play a role in FSHβ synthesis in adult male gonadotropes.

The top three common gender differentially expressed genes were Gpr101, Xist, Rnase1. Gpr101 and Xist are also exclusive expressed genes as mentioned above. Rnase1 is one of the pancreatic-type of secretory ribonucleases. No study to date has linked this particular gene with gonadotropes or even the pituitary. Its main biological function is enzymatically-digesting bacteria or degrading pathogenic RNA (Beintema, 1990; Sorrentino et al., 2003). Our study suggests that this gene may play a sexually dimorphic role in gonadotropes, since it is expressed at a substantially higher level in female gonadotropes (especially during proestrus) compared to males.

Ptpn5, Gpr101, Edn2 were the top differentially expressed genes during female development. Ptpn5 attained a remarkably high expression level in adult female gonadotropes. PTPN5, also known as STEP, is a protein tyrosine phosphatase. It consists of two major isoforms: a membrane-associated $STEP_{61}$ and a cytosolic $STEP_{46}$ (Lombroso et al., 1993). Many efforts have been put to study the role of this gene in synaptic plasticity. However, this gene has also been proposed to be one of the estradiol/estrogen receptor alpha regulated genes (Kim et al., 2011). Edn2 (Endothelin 2) is a one of the secretory vasoconstrictive peptides. Its expression is induced in the ovary during ovulation; meanwhile mice treated with antagonists of endothelin receptors had a reduction in the number of released oocytes. This increased expression was abolished in progesterone receptor knock-out mice (Palanisamy et al., 2006). However the expression pattern of this gene in our study (much higher in prepubertal female gonadotropes) suggests that this gene maybe play an important role in the development of gonadotropes.

The gene expression signatures of prepubertal and adult male gonadotropes are similar to each other. Few genes are differentially expressed. The majority of top upregulated genes in prepubertal male gonadotropes are predicted genes. The top upregulated genes in adult male gonadotropes are Grem1，Bpifa1 and Myoc. BPIFA1, a BPI family protein, has been found to be involved in bacterial infection (Liu et al., 2013). Myoc encodes a secretory protein myocilin. Mutations of this gene in human beings have been reported to be associated with glaucoma (Takamoto and Araie, 2014). Both Bpifa1 and Myoc have not been studied in the murine gonadotropes. Our study is the first time to suggest these genes may participate in the development of murine gonadotrops.

Lactation is a unique physiological stage in female reproduction. Lactotropes are a population of cells in the pituitary gland well studied in terms of plasticity. Besides lactotropes, gonadotropes have also been reported to demonstrate cellular plasticity during lactation in terms of the relationship to the vasculature. Kcnq5, Impg1, Cldn4 are the top common differentially genes in the comparisons of cycling and lactation female gonadotropes. Kcnq5 (Potassium voltage-gated channel subfamily KQT member 5) belongs to the KCNQ potassium channel family. This voltage-gated potassium channel was suggested to play a role in photoperiod-transduction of ovine pituitary (Dupre et al., 2008). Mutations of Impg1 (Interphotoreceptor matrix proteoglycan 1) have been reported to be associated with vitelliform macular dystrophies (Manes et al., 2013). Cldn4 is one

constituent of intercellular tight junction complexes (Neesse et al., 2012). The significantly increased expression of this gene in gonadotropes from lactating mice raised the possibility that tight junctions may be involved in this particular physiological event in female reproduction.

When comparing between different stages, cell adhesion, blood vessel development, ion transport and vasculature development are the most represented GO terms across different comparisons. These GO terms are particularly interesting, as it has been previously shown that gonadotropes show spatial plasticity in the female during development (Alim et al., 2012). The pituitary gland is highly vascularized and many gonadotropes were in contact with blood vessels and showed extensions in the direction of the vessels. This could increase the secretory potential of gonadotropes (Alim et al., 2012).

An important physiological mechanism that is not yet fully understood is the coupling of GnRH pulse frequency to preferential LH/FSH release. It is known that high GnRH pulse frequency results in high LH pulse frequency, whereas low GnRH pulse frequency results in low LH pulse frequency and the preferential release of FSH (Wildt et al., 1981). High LH release is associated with polycystic ovary syndrome (PCOS) in humans (McCartney et al., 2002; Venturoli et al., 1988). One hypothesis regarding the pathogenesis of PCOS is that it may be caused by androgen exposure in utero (Abbott et al., 1998; Barnes et al., 1994). Consistent with this, some reproductive symptoms of PCOS can be experimentally reproduced in both monkeys (Abbott et al., 1998) and mice (Caldwell et al., 2014; Moore et al., 2015) by androgen exposure *in utero* despite differences between the menstrual cycle and the estrous cycle. Since I have now revealed extensive changes in gene expression throughout the estrous cycle, it is tempting to speculate that these changes may also underlie preferential LH/FSH release. Analysis of the functional role of the differentially expressed genes between proestrus and diestrus may thus provide a new mechanistic insight into GnRH/gonadotropin release coupling, potentially revealing novel approaches to manipulate the HPG axis.

In conclusion, our data reveal an unexpectedly high degree of genetic plasticity within the gonadotrope population. Substantial changes in gene-expression profile were found between each gender, developmental stage and hormonal stage analyzed. Even between proestrus and diestrus, only one to two days apart, the gene expression profile had changed dramatically. These data are not only suprising but also bring the physiological relevence

of research performed on cell lines and cultured gonadotropes in to question, as these models are unlikely to undergo these dramatic gene expression changes *in vitro*. These data provide a comprehensive basis for analysis of target genes using conditional knockout mice. Several of the genes found in this study have already been analysed using conditional knockout mouse models. As previously discussed, conditional knockout of Foxl2 exclusively in gonadotropes has already been shown to be detrimental to gonadotrope function and fertility. However, there are other genes such as Smad2 (that I found to be moderately expressed in gonadotropes of all stages) that, when conditionally knocked out in gonadotropes, have no detrimental effects. Therefore, it is important to note that only with the use of conditional knockout mouse models will it be possible to delineate the role (and relative importance) of the many potential targets highlighted in this study. However, these data provide a strong basis for further studies by highlighting which genes and pathways are active in gonadotropes and how they change through development, between genders and in response to different hormonal stages.

4.2 TRPC5 and gonadotropes

I also analyzed TRP channel plasticity in murine gonadotropes. I found that gonadotropes at different stages showed preferential TRP channel expression. Gonadotropes harvested from prepubertal female mice had more highly expressed TRP channels compared to other stages. Out of these differentially expressed channels, Trpc5 is the most highly expressed. Next, by using a combination of Ca^{2+} imaging, electrophysiology and phenotypic analysis of Trpc5 knockout mice, we uncovered a functional role for Trpc5 channels in murine gonadotropes and found that this channel is indirectly activated in gonadotropes via stimulation of the GnRHR.

Previous studies showed that TRPC5 is predominantly expressed in the central nervous system (Okada et al., 1998; Philipp et al., 1998), and that TRPC5, activated via G protein-coupled neuronal receptors, has an essential function in innate fear (Riccio et al., 2009), reduced pilocarpine-induced seizures (Phelan et al., 2013) and an impaired cold sensation (Zimmermann et al., 2011). However, nothing was known so far about expression and function of TRPC5 within gonadotropes. Our study revealed one function of TRPC5 in murine gonadotropes. Although we did not observe abnormal puberty onset in TRPC5 knock-out mice, we did find a reduced Ca^{2+} response when challenging gonadotropes with

GnRH in the absence of Trpc5. Moreover the current introduced by GnRH in gonadotrope was significantly reduced in the absence of Trpc5. Furthermore, although there was no significant alteration in pubertal onset in these mice, further reproductive phenotypic analysis is required to determine whether fertility is affected in these animals.

TRP channels can be activated by different or even distinctly unrelated stimulus. Their main function has been studied in two categories. One is their response to different external sensory stimuli such as temperature, light, or even touch (Caterina et al., 1997; Liedtke et al., 2003; Panda et al., 2005; Qiu et al., 2005). The other is modulating intracellular Ca^{2+} signals (Minke, 2006). Individual TRP channels have unique characteristics. The TRP channel plasticity we revealed here in this study suggests that gonadotropes at different stages have a ability to respond to different physiological demands and different ion channel compositions.

A substantial amount of data available about Ca^{2+} responses or Ca^{2+} signaling cascades of gonadotropes to GnRH has been gained in tumor cell lines. Since gonadotropes consist of only a small portion of the whole pituitary, it is not convenient to identify gonadotropes by the challenging with GnRH in dissociated pituitary cells. By use our GRIC/eR26-τGFP mouse model we overcame this limitation. The typical Ca^{2+} response pattern in gonadotropes to higher GnRH concentration (> 1 nM) is biphasic in response: an initial rapid increase of Ca^{2+} and the following prolonged Ca^{2+} plateau. However, detailed information of which channel or molecular participate in this Ca^{2+} signaling is missing. One of most interesting findings of this study is that Trpc5 contributes to the Ca^{2+} plateau in the gonadotropes in the response to GnRH.

4.3 Tet1, Tet2 and gonadotropes

Together with Prof. Philippa Melamed's group, we have shown that Tet 1 and Tet 2 regulate gonadotrope development through their distinct effects on the Lhb subunit gene. Accordingly, their relative levels change with gonadotrope differentiation and their exposure to GnRH. Such key regulatory roles for these enzymes in determining LH expression might well explain the reduced fertility in the double Tet 1/2 KO mice (Dawlaty et al., 2013).

Previous observations suggested that Tet enzymes act as protectors of CpGs, whereby they can oxidate aberrantly methylated CpGs to maintain regulatory activity during development (Hon et al., 2014; Lu et al., 2014; Williams et al., 2011). Tet2 was found at higher levels in LβT2 cells than in αT3-1 cells, suggesting that, to exert its positive effect on Lhb gene expression, Tet2 likely keeps the proximal promoter free of 5mC for binding of key transcription factors and the general transcription machinery.

In contrast to Tet2, Tet1 inhibits Lhb expression, and its levels are negatively correlated with gonadotrope differentiation. Repressive effects of Tet 1 on gene expression have been reported, and its knockdown in ESCs increased expression of the genes whose promoters it occupies (Williams et al., 2011; Wu et al., 2011). Accordingly, Tet1-bound promoters are often occupied by the polycomb repression complex 2 (PRC2), and Tet1 was suggested to facilitate PRC2 binding, associate with the Sin3A complex and/or help recruit the MBD3/NuRD repressor complex to repress gene expression (Williams et al., 2011; Wu et al., 2011; Yildirim et al., 2011).

This study provides further weight to the idea that distinct genomic regions can be utilized differently at various stages of development or in different tissues (Levine, 2010; Melamed et al., 2016). Furthermore, whatever its precise role in the development of murine gonadotrope, we have shown that distinct-regulation of Tet1 and Tet2 might play an important role in Lhb expression and thus that these two enzymes are also important for the differentiation completion of gonadotropes and the realization of reproductive competence.

4.4 Future experiments

4.4.1 Transcriptome analysis of embryonic and newborn gonadotropes

Our study was mainly focused on the changes between prepubertal and adult gonadotropes. However, there is another interesting perspective to the development of murine gonadotropes; the embryonic stages. Our cell counting results also indicated that gonadotropes stop expanding before the earliest stage I analyzed in this study. Three marker genes of gonadotropes (GnRH receptor, *Fshb* and *Lhb*) are initially expressed at different embryonic stages; with GnRH receptor first expressed. It will be extremely important to investigate the transcriptome of gonadotropes at these stages. The mouse

model used in this study was the GRIC/eR26-τGFP mouse model. In this mouse model, expression of green fluorescent protein is triggered by the initial expression of GnRH receptor. This opens up the possibility to enrich gonadotropes at all the embryonic stages I am interested in since the GnRH receptor is the first of three gonadotrope marker genes to be expressed. One possible challenge regarding this future plan will be the limited number of embryonic gonadotropes. To overcome this I could either pool additional mice into one biological sample or take advantage of new RNA-seq library building methods to study the transcriptome of embryonic gonadotropes at either the single animal or even single cell level.

4.4.2 Specific manipulation of genes of interest in murine gonadotropes

I have uncovered numerous interesting genes that could be important to the function of gonadotropes. The ideal way to investigate the function roles of these genes is to specifically ablate or to overexpress these genes specifically in murine gonadotropes.
However, one substantial limitation is the time and cost required to generate novel mouse strains with Cre-dependent alleles if they are not currently available. In addition, phenotypic and mechanistic analyses will require substantial time and manpower. Another challenge of future experimental design is selecting which interesting gene to further analyze. Judging the importance of genes in murine gonadotropes based completely upon the FPKM value is inadvisable. In the extreme case, a limited number of molecules may be sufficient physiologically. Moreover, in this study, I put a lot of emphasis on differentially expressed genes. The majority of the genes remained not significantly changed. Therefore, careful consideration must be required before embarking on long-term and potentially very costly research projects.

4.4.3 Analysis of other TRP channel functions in murine gonadotropes

In our study, we only analyzed the function of Trpc5 in murine gonadotropes. However, we found multiple TRP channels to be expressed or even to be differentially expressed in murine gonadotropes at different stages. It is also important to study the roles these channels in murine gonadotropes. Recent endeavors have been made within the Transregional Collaborative Research Centre 152 (TRR 152) to establish a TRP “Zoo” (platform project Z02) to provide researchers with conditional TRP alleles as well as TRP-specific Cre-driver mouse lines. The generation of novel conditional TRP-knockout mouse

strains will provide new opportunities in the near future to further unravel the physiological role of TRP channels within gonadotropes.

Moreover, in this study we utilized a global knockout mouse model to study the role of Trpc5 in gonadotropes. A more elegant approach would be the use of a conditional knockout mouse model. This would have the distinct advantage that any phenotype of these mice would be the result of Trpc5 ablation exclusively in cells expressing the GnRH receptor, rather than potential compound effects of *Trpc5* knockout in other cell types such as GnRH and Kisspeptin neurons.

4.4.4 Analysis of the function of Tet1 and Tet2 in embryonic gonadotropes

In our study, we analyzed the function of Tet1 and Tet2 in new born and adult mice. It will be extremely interesting to study the roles of these genes in embryonic stages since epigenetic control is critical in tissue development. Moreover, we performed our study in cell lines and primary cultured cells. It will be our next step to study these genes *in vivo*.

5 Summary

In summary, I found that gonadotropes taken from juvenile males and females, from cycling females at diestrus and at proestrus, from lactating females, and from adult males each have unique gene expression patterns with approximately 100 to approximately 500 genes expressed only in one particular stage. I also demonstrate extensive gene-expression profile changes with up to approximately 2200 differentially expressed genes when comparing female and male development, juveniles and adults, and cycling females. Differentially expressed genes were significantly enriched in the GnRH signaling, Ca^{2+} signaling, and MAPK signaling pathways by Kyoto Encyclopedia of Genes and Genomes analysis. Our data provide an unprecedented molecular view of the primary gonadotropes and reveal a high degree of molecular plasticity within the gonadotrope population.

From the RNA-seq data, I demonstrated that TRP channel expression in murine gonadotropes exhibits considerable plasticity and depends on gender as well as on the developmental and hormonal status of the animal. Out of all the detected TRP channels, TRPC5 was found to be the most highly expressed. We found that the TRPC5 agonist Englerin A activates a cytosolic Ca^{2+} signal and a whole cell current in genetically labeled gonadotropes which is absent in TRPC5-deficient mice. We also found a TRPC5 activation in gonadotropes via stimulation of the GnRHR. However we also observed normal puberty onset and body weight in TRPC5-deficient female mice.

We also report here that Tet1, Tet2 and Tet3 are expressed in murine gonadotropes, and that Tet1 represses the expression of the luteinizing hormone β-subunit (Lhb) gene, whereas Tet2 enhances the expression of Lhb gene. Tet1 levels drop with cell differentiation and exposure to the regulatory gonadotropin-releasing hormone.

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8 List of Publications

1.Qiao, S., K. Nordstrom, L. Muijs, G. Gasparoni, S. Tierling, E. Krause, J. Walter and U. Boehm (2016). "Molecular Plasticity of Male and Female Murine Gonadotropes Revealed by mRNA Sequencing." Endocrinology **157**(3): 1082-1093.

2. Andreas Beck, Viktoria Götz, Sen Qiao, Petra Weissgerber, Veit Flockerzi, Marc Freichel and Ulrich Boehm. “Functional characterization of Transient Receptor Potential (TRP) Channel C5 in female murine gonadotropes.” In revision.

3. Yahav Yosefzon, Anna Tsukerman, Cfir David, Lilach Pnueli, Sen Qiao, Ulrich Boehm and Philippa Melamed. “Repression of a novel truncated Tet1 isoform is required for the central activation of reproduction.” In revision.