GLOBAL GENE EXPRESSION ANALYSES AND DISCOVERY OF NEW RECEPTORS IN BOVINE ANTERIOR PITUITARY

ウシ下垂体前葉における網羅的発現遺伝子解析と新規受容体の発見

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GLOBAL GENE EXPRESSION ANALYSES AND DISCOVERY OF NEW RECEPTORS IN BOVINE ANTERIOR PITUITARY

A Dissertation

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Abstract

The anterior pituitary (AP) receives signals from both the hypothalamus and various peripheral tissues, and secretes the important hormones that control various important functions in multiple organs. The AP is expected to express a large number of genes for various purposes, including paracrine, autocrine, and endocrine roles by the heterogeneous endocrine cells (corticotrophs, gonadotrophs, lactotrophs, somatotrophs, and thyrotrophs) and also the paracrine cells (e.g. folliculostellate cells) and the non-secretory cells (e.g. stromal cells). However, little is known regarding global gene expression in the AP of animals. Deep sequencing of the transcriptome (RNA-seq) via next-generation sequencing (NGS) technology is the most recent and high-throughput type of genetic analysis tool that can be used to determine global gene expression.

I aimed to determine gene expression patterns in the AP of heifers before and after ovulation via RNA-seq to identify new genes and clarify important pathways. Heifers were slaughtered on the estrus day (pre-ovulation; n = 5) or 3 days after ovulation (post-ovulation; n = 5) for AP collection. I randomly selected 4 pre-ovulation and 4 post- ovulation APs, and the ribosomal RNA-depleted poly (A)+RNA were prepared to assemble NGS libraries. The bovine APs expressed 12,769 annotated genes at pre- or post-ovulation. The sum of the reads per kilobase of exon model per million mapped reads (RPKM) values of all transcriptomes were 599,676 ± 38,913 and 668,209 ± 23,690, and 32.2 ± 2.6% and 44.0 ± 4.4% of these corresponded to the AP hormones in the APs of pre- and post-ovulation heifers, respectively. The bovine AP showed differential expression of 396 genes ($P < 0.05$) in the pre- and post-ovulation APs. The 396 genes included two G-protein coupled

receptor (GPCR) genes (*GPR61* and *GPR153*) and those encoding 13 binding proteins. The AP also expressed 259 receptors including GPR173, and other 364 binding proteins. Moreover, ingenuity pathway analysis for the 396 genes revealed ($P=2.4\times10^{-3}$) a canonical pathway linking GPCR to cytoskeleton reorganization, actin polymerization, microtubule growth, and gene expression. Thus, the study clarified the novel genes found to be differentially expressed before and after ovulation and clarified an important pathway in the AP.

Gonadotropin-releasing hormone receptors (GnRHRs) colocalize with insulin and glucocorticoid receptors in lipid rafts of the gonadotroph plasma membrane, where they facilitate downstream signaling. I found that orphan GPR61 and GPR153 are expressed in the AP of heifers, leading us to speculate that the GPRs colocalize with GnRHR in the plasma membrane of gonadotrophs and are expressed at specific times of the reproductive cycle. To test this hypothesis, I examined the coexpression of GnRHR, GPR61 or GPR153, and either luteinizing hormone (LH) β subunit or follicle-stimulating hormone (FSH) β subunit in AP tissue and cultured AP cells by immunofluorescence microscopy. The GPRs were detected in gonadotrophs, with a majority of them being colocalized with GnRHR and the remainder present at other parts of the cell surface or in the cytoplasm. I obtained a strong positive overlap coefficient between the GPRs and GnRHR on the cell-surface of cultured GnRHR-positive AP cells (0.71 ± 0.01 between GPR61 and GnRHR, and 0.75 ± 0.02 between GPR153 and GnRHR). Real-time PCR and western blot analyses found that expression of GPRs was lower ($P < 0.05$) in AP tissues during early luteal phase as compared to other phases. Additionally, the 5'-flanking region of the GPR genes contained several sites with response elements similar to those of estrogen or progesterone. These data suggested that both GPR61 and GPR153 colocalize with

GnRHR in the plasma membrane of gonadotrophs, and their expression changes stage-dependently in the bovine AP.

Gonadotrophs in the AP are important cells; however, the AP has a heterogeneous cell population, and gonadotrophs constitute only 10 to 15% of all cells in the AP and are scattered among other cell types. No methods are currently available for rapidly isolating gonadotrophs from the AP in any species. I developed a method for preparing pure bovine gonadotrophs from a heterogeneous AP cell mixture by magnetic separation and our original antibody against the N terminus of bovine GnRHR. I evaluated the expression of GPR61 and GPR153 in the purified gonadotrophs by western blotting. The purified gonadotrophs express both GPR61 and GPR153, and there was no difference in band size when compared to AP tissues. Non-gonadotrophs cells showed weak or no band.

I evaluated the effect of possible ligand candidate for GPR101 and GPR173 on GnRH induced LH secretion. However, their reported ligand candidates had no effect on LH secretion form the cultured AP cells.

In conclusion, these studies clarified the global gene expression in bovine AP before and after ovulation, and also these studies discovered the new important receptors GPR61 and 153 expressed in gonadotrophs in stage-dependent manner.

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List of Abbreviations

ACTH : Adrenocorticotrophic hormone
ANOVA : Analysis of variance
AP : Anterior pituitary
BLAST : Nucleotide basic local alignment search tool
BLAT : BLAST-like alignment tool
bp : Base pair
BSA : Bovine serum albumin
C : Celsius
cAMP : Cyclic adenosine monophosphate
CCD : Charge-coupled device
cDNA : Complementary deoxyribonucleic acid
CO_2 : Carbon dioxide
CP : Crude protein
Cq : Quantification cycle
DAPI : 4',6-diamidino-2-phenylindole
DM : Dry matter
DMEM : Dulbecco's modified eagle's medium
DNA : Deoxyribonucleic acid
E2 : Estradiol
ECL : Enhanced chemiluminescence
EGR1 : Early growth response protein1
ERE : Estrogen-responsive element
ERK : Extracellular signal-regulated kinase
FBS : Fetal bovine serum
FITC : Fluorescein isothiocyanate
FSH : Follicle stimulating hormone
g : Gravity
G protein : Guanine-nucleotide-binding regulatory proteins
Gs : Stimulatory G protein
GPCR/GPR : G-protein–coupled receptors
GnRH : Gonadotropin releasing hormone

GnRHR : Gonadotropin releasing hormone receptor

GH : Growth hormone

GTP : Guanosine triphosphate

h : Hour

HEPES : 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPG : Hypothalamic-pituitary-gonadal axis

HRP : Horse radish peroxidase

IGF : Insulin like growth factor

IgG : Immunoglobulin G

IPA : Ingenuity Pathways analysis

kDa : Kilo Dalton

kg : Kilogram

LH : Luteinizing hormone

MAP : Molecule activity predictor

MAPK : Mitogen-activated protein kinase

Mcal : Mega calorie

ME : Metabolizable energy

min : Minute

mL : Mililiter

µg : Microgram

µL : Microliter

µm : Micrometer

mRNA : Messenger ribonucleic acid

n : Number

NCBI : National Center for Biotechnology Information

NIDDK : National Institute of Diabetes and Digestive and Kidney Diseases

ng : Nanogram

NGS : Next generation sequencing

nM : Nanomolar

nm : Nanometer

P : Probability

P4 : Progesterone

PBS : Phosphate buffered saline

PCR : Polymerase chain reaction

PFA : Paraformaldehyde

PKA : Protein kinase A

PKC : Protein kinase C

PLSD : Protected least significant difference

PRE : Progesterone-responsive element

PRL : Prolactin

PVDF : Polyvinylidene fluoride

RIN : RNA integrity number

RNA : Ribonucleic acid

RPKM : Reads per kilobase of exon model per million mapped reads

sec : Second

SEM : Standard error of mean

SOLiD : Sequencing by Oligo Ligation Detection

TSH : Thyroid stimulating hormone

vs : Versus

List of Companies Supplying Chemicals or Instruments Used in This Study

Name of company	Address
Agilent Technologies	Santa Clara, CA, USA
Anacyte laboratories UG	Kuhreder, Hamburg, Germany
As One Corporation	Osaka, Japan
Beckman Coulter Inc.	Brea, CA, USA
Bio-Rad	Hercules, CA, USA
Biorbyt	Cambridge, UK
Carl Zeiss	Göttingen, Germany
Fujifilm	Tokyo, Japan
GE Healthcare	Amersham, UK
Genetyx	Tokyo, Japan
Gibco	Grand Island, NY, USA
Ibidi	Planegg, Germany
Kamar Product Inc.	Zionville, IN, USA
Kirkegaard & Perry Laboratories	Baltimore, MD, USA
Leica Microsystems PtyLtd.	Wetzlar, Germany
M. Watanabe & Co.	Tokyo, Japan
Matsunami-Glass	Osaka, Japan
Nacalai Tesque Inc.	Kyoto, Japan
NanoDrop Technologies Inc.	Wilmington, DE, USA
Partec GmbH	Gorlitz, Germany
Peptide Institute Inc.	Osaka, Japan
Pfizer	Tokyo, Japan
Pierce Biotechnology	Rockford, IL, USA
Qiagen	Valencia, CA, USA
SAS Institute, Inc.	Cary, NC, USA
Sakura Fine Technical Co. Ltd.	Tokyo, Japan
Scrum Inc.	Tokyo, Japan
Sigma-Aldrich	St. Louis, MO, USA
Stemcell Technologies	Vancouver, Canada
Sumitomo Bakelite	Tokyo, Japan
Takara Bio Inc.	Shiga, Japan
Thermo Fisher Scientific	Rockford, IL, USA
Toyobo	Tokyo, Japan
Wako Pure Chemicals	Osaka, Japan

CHAPTER I

General Introduction

The AP receives signals from both the hypothalamus and various peripheral tissues, and secretes the important hormones that control various important functions in multiple organs. At least 400 bioactive peptides may be synthesized for various purposes, including endocrine, paracrine, and autocrine roles (Chen et al., 2004) by the heterogeneous endocrine cells (corticotrophs, gonadotrophs, lactotrophs, somatotrophs, and thyrotrophs), the paracrine cells (folliculostellate cells), and the non-secretory cells (stromal cells). For these various roles, the AP is expected to express a large number of genes. However, little is known regarding global gene expression in the AP of animals.

Ovulation and fertilization are the critical steps in successful reproduction. Ovulation is controlled by hormones secreted by the AP, namely LH and FSH. Both hormones also induce important events related to ovulation, such as oocyte maturation, embryo development, and formation and maintenance of the corpus luteum. Peripheral tissues, especially the ovaries, secrete hormones to control gene expression in the AP. On Day 3 after ovulation, when blood concentrations of estradiol and progesterone are low, active pulsatile LH secretion occurs in heifers (Kadokawa and Yamada, 1999). Therefore, we can expect that the expression levels of several genes in the AP on Day 3 after ovulation are different from those in the AP before ovulation.

Deep sequencing of the transcriptome (RNA-seq) via NGS technology is the most recent and high-throughput type of genetic analysis tool that can be used to determine global gene expression. However, only a limited number of previous studies have used this technique for pituitary gene expression. He et al. (2014) used this technique on 200 or 300 whole-pituitary samples (and not just the AP), collected from zebrafish before and after sexual maturation. This was because the size of the

pituitary gland in zebrafish is too small to analyze the genes in the AP alone. RPKM is a method of quantifying gene expression from RNA-seq data by normalizing for total read length and the number of sequencing reads. Few studies have analyzed global gene expression in APs in post-pubertal animals. To the best of our knowledge, only 2 previous studies utilized oligonucleotide microarrays to compare gene expression in APs in dairy cows in the estrus or luteal phases (Kommadath et al., 2010) and in anestrus and cycling postpartum beef cows (Roberts and McLean, 2011). However, NGS methods are becoming increasingly common because of certain advantages relative to microarrays, and RNA-seq has the capacity to identify novel transcript variants and is not limited by the potential for cross-hybridization (Xu et al., 2011).

GnRHRs are present in gonadotroph plasma membrane lipid rafts (Navratil et al., 2009; Wehmeyer et al., 2014; Kadokawa et al., 2014), which are distinct, relatively insoluble regions that have lower density and are less fluid than surrounding membrane (Simons and Tooter, 2000; Head et al., 2014). Lipid rafts facilitate signaling by allowing colocalization of membrane receptors and their downstream signaling components (Simons and Tooter, 2000; Head et al., 2014); as such, they are important pharmacological targets in human medicine (Jaffrès, 2016). Lipid rafts containing GnRHR also harbor insulin receptor (Navratil et al., 2009) and glucocorticoid receptor (Wehmeyer et al., 2014) in the LβT2 clonal murine gonadotroph cell line, thus providing a potential means of integrating neuropeptide and energy homeostasis signals to modulate reproductive function. However, gonadotroph lipid rafts containing GnRHR are also likely to contain other types of receptor that have yet to be identified.

Gonadotrophs in the AP are important cells; however, the AP has a heterogeneous cell population and gonadotrophs constitute only 10 to 15% of all cells in the AP and are scattered among other cell types in rats and humans (Ben-Shlomo et al., 2011). To clarify the mechanisms of action of hormone-secreting AP cells, previous studies have tried to purify specific cell types from heterogeneous AP cell mixtures; however, these attempts have resulted in failure to obtain gonadotrophs in rats (Wynick et al., 1990) or in a low purity of gonadotrophs in sheep (Chen et al., 1994).

We recently developed a highly specific antibody that recognizes the extracellular region at the N terminus of bovine GnRHR (Kadokawa et al., 2014). We could obtain pure bovine gonadotrophs utilizing the antibody for fluorescence-activated cell sorting analysis of bovine AP cell mixture (Kadokawa et al., 2014). However, the slow speed of this method was unsatisfactory, since the system could only process one sample at a time.

The EasySep magnetic separation system (Stemcell Technologies, Vancouver, Canada) is a simple method for the simultaneous isolation and purification of cells using a strong magnet. Recently, microglia were purified from murine brain using the EasySep magnetic separation method (Jose et al., 2015).

Therefore, the aims of the first study were to determine gene expression patterns in the AP of heifers before and after ovulation using RNA-seq in order to discover new genes involved in this process and to clarify important gene networks in AP before and after ovulation. In the next study, I evaluated the hypothesis that any differently expressed GPRs colocalize with GnRHR in the plasma membrane of bovine gonadotrophs and that its expression is dependent on estrous stage. Also I developed a new method to obtain pure bovine gonadotrophs from AP using EasySep

and the anti-GnRHR antibody in order to evaluate expression of the differently expressed GPRs in the purified gonadotrophs.

This thesis consists of ten chapters. I explained the background information and main objectives of the study in Chapter I (General Introduction). I reviewed the literatures related to our study in Chapter II. I clarified the novel genes found to be differentially expressed before and after ovulation and clarified an important pathway in the AP (Chapter III). I clarified the co-localization of two differently expressed GPRs, GPR61 and GPR153, with GnRHR and that their expressions are estrus stage dependent in Chapter IV and V. In Chapter VI, I developed a new method for preparing pure bovine gonadotrophs from a heterogeneous AP cell mixture. Then, I confirmed the expression of GPR61 and GPR153 in pure gonadotrophs in Chapter VII. I estimated importance of GPR173 utilizing a ligand candidate, phoenixin, in Chapter VIII. I estimated importance of GPR101 and GPR173 utilizing another ligand candidate, GnRH-(1-5), in Chapter IX. Finally, I discussed the main findings of the present study to conclude in chapter X.

CHAPTER II

Review of Literature

2.1. The importance and structure of HPG axis

Animal reproduction is tightly regulated by the hormones secreted from the hypothalamus, pituitary and gonads, briefly, HPG axis. The HPG axis controls various reproductive functions, which include sexual development, puberty, gametogenesis and pregnancy. Defects in the HPG axis lead to hypogonadotropic hypogonadism, and to induce abnormal reproductive functions and sterility (Larco et al., 2013).

Bovine reproductive functions are regulated by several hormones including GnRH of hypothalamus, LH and FSH of AP, and E2 and P4 of ovaries. The ovaries also secrete follistatin, activin and inhibin that control gene expressions in the AP as well as hypothalamus. Also, blood concentration of each hormone is important. For example, E2 has biphasic effect on GnRH and LH synthesis and secretion depending on its blood concentration: low level has negative-feedback effects, but high level observed during estrus period has positive-feedback effects (Boer et al., 2010). However, little is known regarding global gene expression in each organ in HPG axis, especially for mammalian APs.

2.2. AP is the most important, but complex endocrine organ

The bovine pituitary is an orbicular-ovate structure located at the base of the brain and it constitutes the hub of the endocrine system. Pituitary is comprised of three different lobes: the anterior, posterior, and intermediate lobe. However, anterior part, AP is the most important endocrine organ. AP receives various hypothalamic and peripheral inputs. AP releases various hormones to control downstream endocrine glands. Therefore, AP is the most important organ for controlling growth, metabolism, reproduction, and coping with immune challenges

and stress.

The AP consists of heterogeneous population of highly differentiated endocrine cell types defined by the hormones they secrete (somatotrophs, GH; lactotrophs, PRL; gonadotrophs, LH and FSH; corticotrophs, ACTH; thyrotrophs, TSH) in response to specific hypothalamic releasing hormones. The five type of endocrine cells, corticotrophs, thyrotrophs, somatotrophs, lactotrophs, and gonadotrophs comprise 10-15%, 5%, 50% , 10-15%, and 10-15% of the total AP cells respectively (Ben-Sholmo and Melmed, 2011). However, gonadotrophs increase as three to four fold before the estrus in rats (Childs and Unabia, 2001), because somatotrophs reversibly differentiate to gonadotrophs (Child, 1997). The AP consist also the paracrine cells (e.g. folliculostellate cell which secrete follistatin) and the non-secretory cells (e.g. stromal cells). Furthermore, the number, size, hormonal content of each of five types of AP endocrine cells, and each of the release response to external cues change during the estrus cycle (Lewy et al., 2003). Furthermore, AP has sixth type of endocrine cells, mammosomatotroph, which can role alternatively as somatotroph or lactotroph when needed (Sato et al., 1999).

Therefore, it is important to develop a method to isolate gonadotrophs in order to improve our knowledge.

2.3. The roles and mechanism of gonadotrophs functions

Gonadotrophs play central role in the control of reproductive function. Gonadotrophs are scattered throughout the AP in human (Kaiser, 2011). Size and function of gonadotrophs are various (Childs, 1997; Kadokawa et al., 2014). All types of gonadotrophs actively secrete LH or FSH in estrus period especially (Childs, 1997).

They are either LH- or FSH-monohormonal or bihormonal (Kaiser, 2011; Kadokawa et al., 2014). In the adult male rat pituitary, about 70% of gonadotrophs are bihormonal, and 15% are LH monohormonal, and 15% are FSH monohormonal (Kaiser, 2011). Also bovine gonadotroph are heterogeneous, including bihormonal, LH- and FSH- monohormonal (Kadokawa et al., 2014).

Plasma membrane of gonadotrophs contains lipid-raft microdomains which are essential for GnRHR signaling (Pawson et al., 2005). Lipid rafts of gonadotroph containing GnRHR also harbor insulin receptor (Navratil et al., 2009) and glucocorticoid receptor (Wehmeyer et al., 2014) in the LβT2 clonal murine gonadotroph cell line, thus providing a potential means of integrating neuropeptide and energy homeostasis signals to modulate reproductive function. Lipid rafts are distinct, relatively insoluble regions that have lower density and are less fluid than surrounding membrane (Simons and Tooter, 2000; Head et al., 2014) and facilitate signaling by allowing colocalization of membrane receptors and their downstream signaling components (Simons and Tooter, 2000; Head et al., 2014).

However, gonadotroph lipid rafts containing GnRHR are also likely to contain other types of receptor that have yet to be identified.

2.4. GPCRs

GPCRs are the largest family of membrane receptors, and at least 800 members have been found in human genome (Latorraca et al., 2017). However, ligands for 150 GPCR among the 800 GPCRs have not been clarified (Ngo et al., 2016).

GPCRs are the important pharmacological targets, indeed about 40% of the

human medicine act on GPCRs (Latorraca et al., 2017; Jean-Charles et al., 2017). GPCRs respond to various stimuli such as light, odor, taste, pheromones, hormones, and neurotransmitters. GPCRs stimulate various intracellular signaling pathways including cAMP, inositol triphosphate, diacylglycerol, calcium and enzymes (Latek et al., 2012; Mahoney and Sunahara, 2016). GPCRs also control other membrane proteins including K^+ channel and voltage-gated Ca^{2+} channels (Syrovatkina et al., 2016). Therefore, we need to improve our knowledge for GPCRs in bovine APs.

GnRHR is a most important GPCR on the surface of gonadotrophs (Millar, 2005). GnRHR regulates at least 76 genes in gonadotrophs utilizing various cytoplasmic pathways (Binder et al., 2012). GnRHR coupled Gs activates adenylyl cyclase that increase cAMP, a second messenger that activates PKA. GnRHR coupled Gαq/11 activates increase other 2 second messengers, inositol 1, 4, 5-triphosphate and diacylglycerol. Then, the 2 second messengers increase calcium ions which activates MAPK (Pawson et al., 2005; Melamed et al., 2012).

However, gonadotroph are also likely to contain other types of receptor that have yet to be identified.

2.5. Global gene expression analyses in APs

Few studies have analyzed global gene expression in APs in animals. To the best of our knowledge, only 2 previous studies utilized oligonucleotide microarrays to compare gene expression in APs in dairy cows in the estrus or luteal phases (Kommadath et al., 2010) and in anestrus and cycling postpartum beef cows (Roberts and McLean, 2011).

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RPKM is a method of quantifying gene expression from RNA-seq data by normalizing for total read length and the number of sequencing reads.

NGS methods are becoming increasingly common because of certain advantages relative to microarrays, and RNA-seq has the capacity to identify novel transcript variants and is not limited by the potential for cross-hybridization (Xu et al., 2011). NGS-based approaches have also quickly gained broad applicability in medicine; from genetic diagnosis and disease networks to drug discovery and pharmacogenomics.

IPA is a powerful analysis and search tool that uncovers the significance of omics data and identifies new targets or candidate biomarkers within the context of biological systems. The IPA discerns differences between molecular and cellular functions and canonical pathways of heifers based on millions of findings reported in the literature. The IPA uses a Fisher's exact test to determine whether the input genes are significantly related to pathways as compared to the complete ingenuity knowledge base.

CHAPTER III

(Study I)

Deep Sequencing of the Transcriptome in the AP of Heifers before and after Ovulation

Abstract

I aimed to determine gene expression patterns in the AP of heifers before and after ovulation via RNA-seq to identify new genes and clarify important pathways. Heifers were slaughtered on the estrus day (pre-ovulation; n = 5) or 3 days after ovulation (post-ovulation; n = 5) for AP collection. I randomly selected 4 pre-ovulation and 4 post-ovulation APs, and the ribosomal RNA-depleted poly (A)+RNA were prepared to assemble NGS libraries. The bovine APs expressed 12,769 annotated genes at pre- or post-ovulation. The sum of the RPKM values of all transcriptomes were 599,676 ± 38,913 and 668,209 ± 23,690, and 32.2 ± 2.6% and 44.0 ± 4.4% of these corresponded to the AP hormones in the APs of pre- and post-ovulation heifers, respectively. The bovine AP showed differential expression of 396 genes ($P < 0.05$) in the pre- and post-ovulation APs. The 396 genes included two GPCR genes (*GPR61* and *GPR153*) and those encoding 13 binding proteins. The AP also expressed 259 receptor and other 364 binding proteins. Moreover, IPA for the 396 genes revealed ($P=2.4 \times 10^{-3}$) a canonical pathway linking GPCR to cytoskeleton reorganization, actin polymerization, microtubule growth, and gene expression. Thus, the present study clarified the novel genes found to be differentially expressed before and after ovulation and clarified an important pathway in the AP.

3.1. Introduction

The AP receives signals from both the hypothalamus and various peripheral tissues, and secretes the important hormones that control various important functions in multiple organs. At least 400 bioactive peptides may be synthesized for various purposes, including paracrine, autocrine, and endocrine roles (Chen et al., 2004) by the heterogeneous secretory cells (corticotrophs, gonadotrophs, lactotrophs, somatotrophs, and thyrotrophs) and non-secretory cells. For these various roles, the AP is expected to express a large number of genes. However, little is known regarding global gene expression in the AP of animals.

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is a method of quantifying gene expression from RNA-seq data by normalizing for total read length and the number of sequencing reads. Few studies have analyzed global gene expression in APs in post-pubertal animals. To the best of our knowledge, only 2 previous studies utilized oligonucleotide microarrays to compare gene expression in APs in dairy cows in the estrus or luteal phases (Kommadath et al., 2010) and in anestrus and cycling postpartum beef cows (Roberts and McLean, 2011). However, NGS methods are becoming increasingly common because of certain advantages relative to microarrays, and RNA-seq has the capacity to identify novel transcript variants and is not limited by the potential for cross-hybridization (Xu et al., 2011).

Therefore, the aims of this study were to determine gene expression patterns in the AP of heifers before and after ovulation using RNA-seq in order to discover new genes involved in this process and to clarify important gene networks in AP before and after ovulation.

3.2. Materials and methods

All experiments were performed according to the Guiding Principles for the Care and Use of Experimental Animals in the Field of Physiological Sciences (Physiological Society of Japan) and approved by the Committee on Animal Experiments of the School of Veterinary Medicine, Yamaguchi University.

3.2.1. Animals and treatments

Post-pubertal Japanese Black heifers (30-month-old) were housed in a free-stall barn. Their daily diet included Italian ryegrass hay (84.2% DM, 2.30 Mcal of ME per kilogram of DM [ME·kg^{-1} DM], 13.3% CP) and concentrate (86.6% DM, 3.82 Mcal ME·kg^{-1} DM, 21.3% CP), and were given 23.9 Mcal of ME. The heifers received two intramuscular injection of dinoprost (Pronalgon F; Pfizer) given 11 days apart to control estrous stage. The heifers were slaughtered on the day of estrus (pre-ovulation; n=5) or 3 days after ovulation (post-ovulation; n=5). After the second dinoprost injection, I observed estrous behaviour at least thrice a day for 20 min, based on the reported criteria (Broom and Fraser, 2007) for colour change in heat-mount detectors (Kamar Product Inc.). Briefly, the pre-ovulation heifers behaved restlessly, roamed, discharged mucous, stood to be mounted, and their vulva were sniffed by other heifers. The pre-ovulation heifers were slaughtered within 3 h after confirmation of standing heat. In contrast, the post-ovulation heifers did not show estrous behaviour on the day of slaughtering. I also macroscopically examined the ovaries and uterus immediately after slaughtering to verify the ovulation stage, based on the reported criteria (Miyamoto et al., 2000). The pre-ovulation heifers had a small (5-15 mm) corpus luteum as light yellow to white colour and covered with connective tissues, a large (12-20 mm) dominant follicle, pale pink endometrium, and oedema in the stroma and mucus. In contrast, the post-ovulation heifers reached the ovulation

point with a blood-coloured surface, no dominant follicles, pink to red endometrium, and slight oedema in the stroma. Anterior pituitaries were collected from the head as previously described (Rudolf and Kadokawa, 2014), immediately frozen in liquid nitrogen, and preserved at -80°C until RNA extraction.

3.2.2. Preparation of the transcriptome library for deep sequencing

Total RNA was extracted from the APs of the heifers using an RNeasy Mini kit (Qiagen), and subsequently treated with ribonuclease-free deoxyribonuclease (Qiagen) to eliminate possible genomic DNA contamination. The quality was verified by agarose gel electrophoresis and capillary electrophoresis using a Bioanalyzer (Agilent Technologies). The value of RIN in the samples was >8.0, and absence of genome DNA contamination was confirmed by electrophoresis. Poly(A)+RNA was purified from 3 μg total RNA with the Gene Read Pure mRNA kit (Qiagen) and the concentration of poly(A)+RNA was measured using the Qubit RNA Assay kit (Thermo Fisher Scientific). I randomly selected four pre-ovulation and four post-ovulation heifers. The poly(A)+RNA sample (40.8 ± 4.2 ng) of heifers was fragmented by incubation with RNaseIII for 10 min at 37°C, after which, RNA size was confirmed as being approximately 150 bp using a Bioanalyzer. The purified fragmented RNA was ligated with adaptors using SOLiD Total RNA-Seq kit and reverse-transcribed to cDNA by using ArrayScript Reverse Transcriptase (Thermo Fisher Scientific). After purification with magnetic beads (AMPure, XP, Beckman Coulter Inc.), cDNA was amplified for 15 cycles using Taq polymerase (AmpliTaq, Thermo Fisher Scientific) with a barcode-labelled 3′-PCR primer (SOLiD RNA Barcoding kit Module 1-16; Thermo Fisher Scientific) to differentiate each cDNA sample. The PCR products were purified by PureLink PCR Nano column (Thermo

Fisher Scientific) and their quality was verified using a Bioanalyzer. Each DNA library was determined as approximately 257 bp similar to the molecular mass of the fragmented RNA ligated to the adaptors.

3.2.3. Bead preparation and RNA-sequencing by SOLiD5500

The DNA library was emulsified using SOLiD EZ Bead Emulsifier (Thermo Fisher Scientific) and the products were amplified by emulsion PCR using SOLiD EZ Bead Amplifier (Thermo Fisher Scientific). The bead-combined PCR products were concentrated using SOLiD EZ Bead Enricher (Thermo Fisher Scientific) and bead volumes were measured by NanoDrop. The bead-combined DNA (4.9×10^6 beads) was loaded onto four lanes of a flowchip, with 269,500 beads per lane for the SOLiD5500 system (Thermo Fisher Scientific).

3.2.4. Analysis of read information

Short sequence reads of 75 bp in the XSQ file created by the SOLiD5500 system were assembled and mapped with CLC Genomic Workbench software (ver. 8.5.1, CLC Bio) using Bos_taurus UMD3.1 (NCBI assembly accession GCA_000003055.3) as a reference sequence. RNA-Seq analysis was carried out using parameters as below. For trimming of reads, the ambiguous limit was two, the minimum number of nucleotides in reads was 25, and the quality limit was 0.05. For reads mapping, the number of mismatch was two, number of insertion was three, number of deletion was three, and number of color error was three. The RPKM value was used to represent the expression level for each gene.

3.2.5. Pathway analysis

Globally expressed genes were compared to elucidate important pathways using IPA build version 377306M (Qiagen) utilizing content version 27216297

(release date 16th March, 2016). The IPA discerns differences between molecular and cellular functions and canonical pathways of heifers based on millions of findings reported in the literature, and the software is updated weekly. The IPA uses a Fisher's exact test to determine whether the input genes are significantly related to pathways as compared to the complete ingenuity knowledge base. I selected genes (1) with varying expression ($P < 0.05$) between the pre- and post-ovulation APs, and also (2) with the average RPKM equal to or more than 1 in either or both of the pre- and post-ovulation APs. Then, I used the IPA software, employing the fold change of pre-ovulation expression to post-ovulation expression of the selected genes, on 17th June 2016. The parameters were as default. The MAP function was utilized to predict both upstream and downstream molecules and functions after clarification of canonical pathway.

3.2.6. Real-time PCRs to evaluate differences in the expression of central genes in important pathways

Real-time PCRs were performed to confirm significant differences in the expression of the GPR genes that were differently expressed in the pre- and post-ovulation samples (n = 5 for each group) and the genes involved in the important pathway that was elucidated by the IPA. The cDNA was synthesized from 2 µg of the total RNA per AP in 20-µl reaction mixtures containing random hexamer primers using the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific). **Table 3.1** shows the primers designed for real-time PCR using Primer Express Software V3.0 (Thermo Fisher Scientific) based on the reference sequences. The amount of gene expression was measured in duplicate by real-time PCR analyses with 20 ng cDNA, using CFX96 Real Time PCR System (Bio-Rad) and Power SYBR

Green PCR Master Mix (Thermo Fisher Scientific), together with a 6-point relative standard curve, non-template control, and no reverse-transcription control. Standard 10-fold dilutions of purified and amplified DNA fragments were prepared. Temperature conditions for all genes were as follows: 95°C for 10 min for pre-denaturation; five cycles each of 95°C for 15 sec and 66°C for 30 sec; and 40 cycles each of 95°C for 15 sec and 60°C for 60 sec. Melting curve analyses were performed at 95°C for each amplicon and each annealing temperature to ensure the absence of smaller non-specific products such as dimers. To optimize the real-time PCR assay, serial dilutions of a cDNA template were used to generate a standard curve by plotting the log of the starting quantity of the dilution factor against the C_q value obtained during amplification of each dilution. Reactions with a R^2 >0.98 and efficiency between 95 and 105% were considered optimized. The concentration of PCR products was calculated by comparing C_q values of unknown samples with the standard curve using appropriate software (CFXmanagerV3.1, Bio-Rad). The gene expression levels for each of the eleven genes were normalized to the geometric mean of the expression levels of two house-keeping genes, *GAPDH* (NCBI reference sequence, NM_001034034) and *RANBP10* (NM_001098125). I selected these 2 house-keeping genes from 20 previously reported candidate house-keeping genes (Walker et al., 2009; Rekawiecki et al., 2012) because they had the smallest inter-heifer coefficients of variation of RPKM value.

Table 3.1. Details of the primers used for real-time PCRs

Gene name	Primer	Sequence 5′-3′	Size (bp)
GAPDH	Forward	TGGTGAAGGTCGGAGTGAAC	91
	Reverse	ATGGCGACGATGTCCACTTT	
RANBP10	Forward	CCCAGTCCTACCAGCCTACT	133
	Reverse	CCCCCAGAGTTGAATGACCC	
GPR61	Forward	CATCAACGTGGAGCGCTACTAT	62
	Reverse	GCGTCATTCGCACCTCATAA	
GPR153	Forward	GCTGAGCAACGCCAAGAAG	70
	Reverse	CGACAGGATGAAGGACACCAT	
ACTA2	Forward	TGTCCACCTTCCAGCAGATG	58
	Reverse	GATGGCCCGGCTTCGT	
CDH10	Forward	ACGGCCCCGGATAACG	56
	Reverse	TGTTCCTTGAGCCTTTCATTGA	
ELK1	Forward	AGTACTTCCCCAGCGATGGA	59
	Reverse	AGCAGCTGCAGCAGAAACTG	
GNA14	Forward	GGCGGGAGTACCAACTGTCA	62
	Reverse	GGCGATCCGGTCAATGTC	
LIMK1	Forward	GTGTTCTCGTTCGGGATCGT	59
	Reverse	TCCGGGTCAGCGTTCACT	
LIMK2	Forward	TGCATTCCATGTGTATCATCCA	60
	Reverse	CCAGCTTGATGAGGCAGTTG	
RHOC	Forward	CGCCTGCGGCCTCTCT	59
	Reverse	TCGATGGAGAAGCACATGAGAA	
SEPT5	Forward	CAGCAGGACCGGGAACTG	57
	Reverse	CGTGTTGCTGCCGATAACAG	
WIPF1	Forward	GCTCCCTGCCTGCTTGTG	65
	Reverse	TGGCTGACAAATCGTTAAAGGA	

3.2.7. Data analysis

The statistical significance of differences in RPKM value of each gene between the pre- and post-ovulation heifers (n=4 for each group) was analyzed by non-paired *t*-test. I calculated the average of the RPKM values of pre-ovulation APs and divided it by the average of the RPKM values of the post-ovulation APs (Pre/Post ratio) to investigate the differences in the RPKM value of each gene. The statistical significance of the differences between the gene expression levels of the pre- and post-ovulation heifers, as measured by real-time PCR (n=5 for each group), was analyzed by non-paired *t*-test. The level of significance was set at $P < 0.05$. Data are expressed as mean ± SEM.

3.3. Results

3.3.1. Information from NGS

The number of nucleotides detected in the APs from the pre- and post-ovulation heifers was 12,266 ± 737 and 14,163 ± 1,936 Mbases, respectively. After low quality and ambiguous reads were removed from the detected reads, and high quality reads were mapped on the reference sequences of bosTau7 database, 9.7 ± 0.6 and 11.3 ± 1.6 million read numbers were obtained in the pre- and post-ovulation samples, respectively.

3.3.2. Annotated genes expressed in APs in the pre- and post- ovulation periods

The sum of the RPKM values of all transcriptomes (total RPKM values) was 599,676 ±38,913 and 668,209 ± 23,690 in the APs from the pre-and post-ovulation heifers, respectively. There was no difference between the 2 groups ($P > 0.1$). In total, 12,769 annotated genes (for which the average RPKM was equal to or more than 1 in either or both of the pre- and post- ovulation APs) were expressed in both groups of AP samples put together. There were differences ($P < 0.05$) in the expression level of 396 genes between the pre- and post-ovulation APs. Among the 396 genes, only 31 were more expressed in the post-ovulation AP than the pre-ovulation AP; the remaining 365 genes showed higher expression in the pre-ovulation AP than the post-ovulation AP. The 31 genes that were expressed more after ovulation included *AGXT2* (alanine-glyoxylate aminotransferase 2), *ARG2* (arginase 2), *CDH23* (cadherin-related 23), *DYRK3* (dual specificity tyrosine-Y-phosphorylation regulated kinase 3), *PADI1* (peptidyl arginine deiminase, type I), *PNP* (purine nucleoside phosphorylase), *PNRC1* (proline rich nuclear receptor coactivator 1), *SPAG8* (sperm associated antigen 8), and *TMEM35* (transmembrane protein 35). Among these, 11 genes had only the Ensemble

gene identifier (ENSBTAG), and did not have names.

3.3.3. Transcriptomes of pituitary hormone genes

The sum of the RPKM values of the AP hormone genes (*PRL*, *GH1*, *CGA*, *LHB*, *POMC*, *FSHB*, and *TSHB*) accounted for 32.2 ± 2.6% or 44.0 ± 4.4% of the about 630,000 total RPKM values of all transcriptomes, in the pre-ovulation group and the post-ovulation group, respectively. There were no statistically significant differences in the RPKM of the AP hormone genes between the pre- and post-ovulation heifers **(Table 3.2)**. The RPKM value of *PRL* (prolactin) was the largest among the all genes, including those encoding AP hormones.

3.3.4. Transcriptomes of non-hormonal genes with the highest RPKM values

Table 3.3 and **Table 3.4** show the RPKM values of non-hormonal genes with the highest RPKM values. The RPKM value of *NNAT* (neuronatin) was the largest among the listed genes in both the pre- and post- ovulation APs. Some of the other genes were ribosomal proteins.

3.3.5. Genes of receptors and binding proteins

Bovine APs expressed a total of 259 receptor genes (for which the average RPKM was equal to or more than 1 in all APs in either or both of the pre- and post- ovulation APs). **Table 3.5** shows all of the 11 receptor genes differently ($P < 0.05$) expressed in the pre- and post-ovulation APs. Two orphan GPCRs, *GPR61* and *GPR153,* are listed among the 11 receptor genes. The real-time PCRs verified the difference in *GPR61* and *GPR153* expression levels ($P < 0.01$; **Fig. 3.1**).

There were no significant differences in the RPKM values of steroid hormone receptors and hypothalamic hormone receptors (data not shown).

Table 3.6 shows all 18 orphan GPCRs expressed in bovine APs and the difference in the expression of each GPCR gene. This table does not contain other GPCRs for which the average RPKM values in both the pre- and post-ovulation APs were less than 1.

The bovine AP expressed receptors for insulin, IGF-1 and IGF-2. The bovine AP expressed also 364 binding proteins including IGF binding protein (*IGFBP*) types 2, 3, 4, 5, 6, and 7. **Table 3.7** shows all the 13 binding protein genes differently ($P < 0.05$) expressed in the pre- and post-ovulation APs. This table omitted other binding proteins for which average the RPKM values in both the pre- and post-ovulation APs were less than 1.

3.3.6. Results of IPA analysis

The IPA analysis employed the fold change of 396 genes differently ($P < 0.05$) expressed between the pre- and post-ovulation APs. The results clarified a canonical pathway termed as "Signalling by Rho Family GTPases" **(Fig. 3.2)**. The P-value was 2.4×10^{-3} and the biased Z-score was 2.3. The 236 genes constituted the canonical pathway, and 9 genes [*ACTA2* (actin, alpha 2, smooth muscle, aorta), *CDH10* (cadherin 10), *ELK1* (ELK1, ETS transcription factor), *GNA14* (G protein subunit alpha 14), *LIMK1* (LIM domain kinase 1), *LIMK2* (LIM domain kinase 2), *RHOC* (ras homolog family member C), *SEPT5* (septin5), *WIPF1* (WAS/WASL interacting protein family member 1)] belonging to the pathway were identified in the bovine APs used in this study. **Table 3.8** shows the RPKM values of the 9 genes, and all of the 9 genes were higher in the pre-ovulation APs than post-ovulation APs. The results of the real-time PCRs verified these differences ($P < 0.05$; **Fig. 3.3**).

The IPA software uses the MAP function to predict both upstream and downstream molecules and their functions after clarifying the canonical pathway. The

stimulated functions, as predicted by the MAP function of IPA, were cytoskeleton reorganization, cell trafficking, cytokinesis, cytoskeleton regulation, actin membrane linkage, contraction, membrane ruffling, cell-cell adhesion, microtubule-organizing center orientation, and actin-polymerization. Predicted inhibited functions were actin nucleation, microtubule growth, and actin polymerization. Thus, actin-polymerization was predicted as both stimulated and inhibited function. This canonical pathway also controls gene expression via the *MEK1/2*, *ERK1/2,* and *Elk1* pathway.

Table 3.2. The bovine AP genes in the pre- and post-ovulation periods, arranged as per the Pre/Post ratio. The differences between pre- and post-ovulation APs were not significant in any of the genes listed in the table.

Gene name	Description	Accession number	RPKM value		Pre/Post ratio
			Pre-ovulation	Post-ovulation	
TSHB	thyroid stimulating hormone beta	NM_174205	4249±1564	981±333	4.33
FSHB	follicle stimulating hormone beta	NM_174060	897±519	426±151	2.10
CGA	glycoprotein hormones alpha	NM_173901	25558±7763	15219±2170	1.68
POMC	proopiomelanocortin	NM_174151	3055±513	1889±461	1.62
LHB	luteinizing hormone beta	NM_173930	7081±2009	6783±1974	1.04
GH1	growth hormone 1	NM_180996	38182±15332	56718±15351	0.67
PRL	prolactin	NM_173953	114353±58951	211708±31501	0.54

Table 3.3. The top-10, non-hormonal genes highly expressed in the pre-ovulation period, arranged as per the pre-ovulation RPKM value. The differences between pre- and post-ovulation APs were not significant in any of the genes listed in the table.

Gene name	Description	Accession number	RPKM value		Pre/Post ratio
			Pre-ovulation	Post-ovulation	
NNAT	neuronatin	NM_001201324	5203±856	8435±1976	0.62
SCG2	secretogranin II	NM_174176	5120±1725	3253±539	1.57
EEF1A1	eukaryotic translation elongation factor 1 alpha 1	NM_174535	4445±700	4016±457	1.11
MT-ND3	mitochondrially encoded NADH dehydrogenase 3	NC_012920	4048±1350	3950±1495	1.02
COX3	cytochrome c oxidase subunit III	YP_209211	3976±989	3219±892	1.24
COX1	cytochrome c oxidase subunit I	YP_209207	3468±854	2760±444	1.26
ATP6	ATP synthase F0 subunit 6	YP_209210	3440±937	3052±922	1.13
RPL10	ribosomal protein L10	NM_174760	2528±216	2665±342	0.95
SCG5	secretogranin V	NM_001045998	2527±358	2516±202	1.00
RPLP1	ribosomal protein large P1	NM_001025340	2508±337	3004±359	0.83

Table 3.4. The top-10, non-hormonal genes highly expressed in the post-ovulation period, arranged as per the post-ovulation RPKM value. The differences between pre- and post-ovulation APs were not significant in any of the genes listed in the table.

Gene name	Description	Accession number	RPKM value		Pre/Post ratio
			Pre-ovulation	Post-ovulation	
NNAT	neuronatin	NM_001201324	5203±856	8435±1976	0.62
EEF1A1	eukaryotic translation elongation factor 1 alpha 1	NM_174535	4445±700	4016±457	1.11
MT-ND3	mitochondrially encoded NADH dehydrogenase 3	NC_012920	4048±1350	3950±1495	1.02
SCG2	secretogranin II	NM_174176	5120±1725	3253±539	1.57
COX3	cytochrome c oxidase subunit III	YP_209211	3976±989	3219±892	1.24
ATP6	ATP synthase F0 subunit 6	YP_209210	3440±937	3052±922	1.13
RPLP1	ribosomal protein large P1	NM_001025340	2508±337	3004±359	0.83
RPL19	ribosomal protein L19	NM_001040516	2288±385	2840±376	0.81
COX1	cytochrome c oxidase subunit I	YP_209207	3468±854	2760±444	1.26
RPL10	ribosomal protein L10	NM_174760	2528±216	2665±342	0.95

Table 3.5. The receptor genes expressed differently (P <0.05) between pre- and post-ovulation periods, arranged as per the Pre/Post ratio. The table only shows genes for which the RPKM was equal or more than 1 in all APs in either or both of the pre- and post- ovulation group.

Gene name	Description	Accession number	RPKM value		P-value	Pre/Post ratio
			Pre-ovulation	Post-ovulation		
SCARA3	scavenger receptor class A, member 3	XM_002689483	8.06±1.24	3.02±0.50	<0.01	2.67
CRHR1	corticotropin releasing hormone receptor	NM_174287	12.85±2.75	4.95±1.02	<0.05	2.60
GPR153	G protein-coupled receptor 153	XM_005217164	8.50±1.16	3.68±0.92	<0.05	2.31
CX3CR1	chemokine (C-X3-C motif) receptor 1	NM_001102558	4.89±0.67	2.56±0.46	<0.05	1.91
P2RY6	pyrimidinergic receptor P2Y, G-protein coupled, 6	NM_001192295	2.20±0.25	1.18±0.04	<0.01	1.86
GPR61	G protein-coupled receptor 61	NM_001038571	2.85±0.38	1.66±0.22	<0.05	1.72
CELSR2	cadherin, EGF LAG seven-pass G-type receptor2	NM_001192931	4.60±0.55	2.80±0.43	<0.05	1.64
RARA	retinoic acid receptor, alpha	NM_001014942	11.13±1.40	7.03±0.76	<0.05	1.58
IL27RA	interleukin 27 receptor, alpha	NM_001098028	37.25±2.95	25.71±1.23	<0.05	1.45
NR1D1	nuclear receptor subfamily 1, group D, member 1	NM_001078100	14.83±0.78	11.55±0.97	<0.05	1.28
OGFR	opioid growth factor receptor	NM_001077019	7.80±0.25	6.23±0.48	<0.05	1.25

Table 3.6. The 18 orphan GPCRs identified in AP during pre- or post-ovulation period. The table only shows genes for which the RPKM was equal or more than 1 in all APs in both of the pre- and post- ovulation groups.

Gene name	Accession number	RPKM value		P-value	Pre/Post ratio
		Pre-	Post-		
GPR6	NM_001100307	1.69±0.61	3.41±0.67	>0.05	0.50
GPR19	NM_001101214	2.82±0.26	3.63±0.40	>0.05	0.78
GPR61	NM_001038571	2.85 ±0.38	1.66±0.22	<0.05	1.72*
GPR63	XM_005210862	2.86±0.16	4.03±1.37	>0.05	0.71
GPR68	NM_174329	2.17 ±0.50	1.42±0.14	>0.05	1.53
GPR75	NM_001205061	1.96±0.34	1.20±0.29	>0.05	1.64
GPR85	NM_001075150	5.14±0.62	5.21±0.93	>0.05	0.99
GPR89	NM_001076970	9.81±1.92	7.60±1.01	>0.05	1.29
GPR107	NM_001099164	14.34±3.37	9.24±2.33	>0.05	1.55
GPR108	NM_001075333	20.41±2.53	18.23±2.42	>0.05	1.12
GPR137	NM_001113295	17.74±1.20	16.56±1.61	>0.05	1.07
GPR137B	NM_001191481	7.10 ±0.61	5.73±1.18	>0.05	1.24
GPR153	XM_005217164	8.50 ±1.16	3.68±0.92	<0.05	2.31*
GPR158	NM_001206442	3.85±0.93	2.27±0.56	>0.05	1.70
GPR161	NM_001077066	3.37±0.84	2.60±0.50	>0.05	1.30
GPR162	NM_001076217	10.08±1.15	11.06±0.75	>0.05	0.91
GPR173	NM_001015604	8.08±0.60	5.34±1.15	>0.05	1.51
GPR180	NM_001164031	5.88±1.09	6.06±1.50	>0.05	0.97

* $P < 0.05$: significant difference between the pre- and post-ovulation stages.

Table 3.7. The binding protein genes expressed differently (P <0.05) between the pre- and post-ovulation periods, arranged as per the Pre/Post ratio. The table only shows genes for which the RPKM was equal or more than 1 in all APs in either or both of the pre- and post- ovulation group.

Gene name	Description	Accession number	RPKM value		P-value	Pre/Post ratio
			Pre-ovulation	Post-ovulation		
RLBP1	retinaldehyde binding protein 1	NM_174451	7.39±1.91	2.02±0.73	<0.05	3.66
GNA14	guanine nucleotide binding protein(G protein), alpha 14	NM_174323	2.97±0.61	1.12±0.29	<0.05	2.64
DMTN	dematin actin binding protein	NM_001034431	38.69±5.05	20.58±2.53	<0.05	1.88
RBFOX3	RNA binding protein, fox-1 homolog (C. elegans) 3	NM_001075537	1.59±0.07	0.90±0.01	<0.01	1.78
RAC2	ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding	NM_175792	3.04±0.17	1.72±0.16	<0.01	1.77
N4BP3	NEDD4 binding protein 3	NM_001205742	2.27 ±0.30	1.30±0.18	<0.05	1.75
MAP3K7IP1	TGF-beta activated kinase 1/MAP3K7 binding protein 1	NM_001102057	10.49±1.62	6.27±0.59	<0.05	1.67
CREB3L4	cAMP responsive element binding protein 3-like 4	XM_015462516	1.76±0.15	1.08±0.21	<0.05	1.62

TAPBP	TAP binding protein (tapasin)	NM_001045885	77.93±9.65	50.99±3.10	<0.05	1.53
CIRBP	cold inducible RNA binding	NM_001034278	57.72±4.20	39.15±5.35	<0.05	1.47
RBBP9	RB binding protein 9, serine hydrolase	NM_001083424	3.75±0.15	2.62±0.23	<0.01	1.43
MYBBP1A	MYB binding protein (P160) 1a	XM_010815952	11.07±0.76	8.66±0.49	<0.05	1.28
DRG2	developmentally regulated GTP binding protein 2	NM_001014865	11.70±0.55	9.95±0.40	<0.05	1.18

Table 3.8. The 9 genes consisting the "Signalling by Rho Family GTPases", arranged in alphabetical order of the gene name.

Gene name	Description	Accession number	RPKM value		P-value	Pre/Post Ratio
			Pre-ovulation	Post-ovulation		
ACTA2	actin, alpha 2, smooth muscle, aorta	NM_001034502	63.84±17.52	18.14 ±2.60	<0.05	3.52
CDH10	cadherin 10	NM_001076266	1.03±0.35	0.11± 0.03	<0.05	9.21
ELK1	ELK1, member of ETS oncogene family	NM_001191236	7.03± 0.53	5.62 ±0.15	<0.05	1.25
GNA14	guanine nucleotide binding protein (G protein), alpha 14	NM_174323	2.97±0.61	1.12 ±0.29	<0.05	2.64
LIMK1	LIM domain kinase 1	NM_001206904	10.72 ±0.91	7.87 ±0.33	<0.05	1.36
LIMK2	LIM domain kinase 2	NM_001038098	20.99 ±1.21	16.19 ±0.98	<0.05	1.30
RHOC	ras homolog family member C	NM_001046138	33.78±3.85	21.38± 2.85	<0.05	1.58
SEPT5	septin 5	NM_001076371	32.07 ±1.20	25.25 ±1.41	<0.05	1.27
WIPF1	WAS/WASL interacting protein family member 1	NM_001076923	2.88 ±0.63	1.18± 0.20	<0.05	2.43

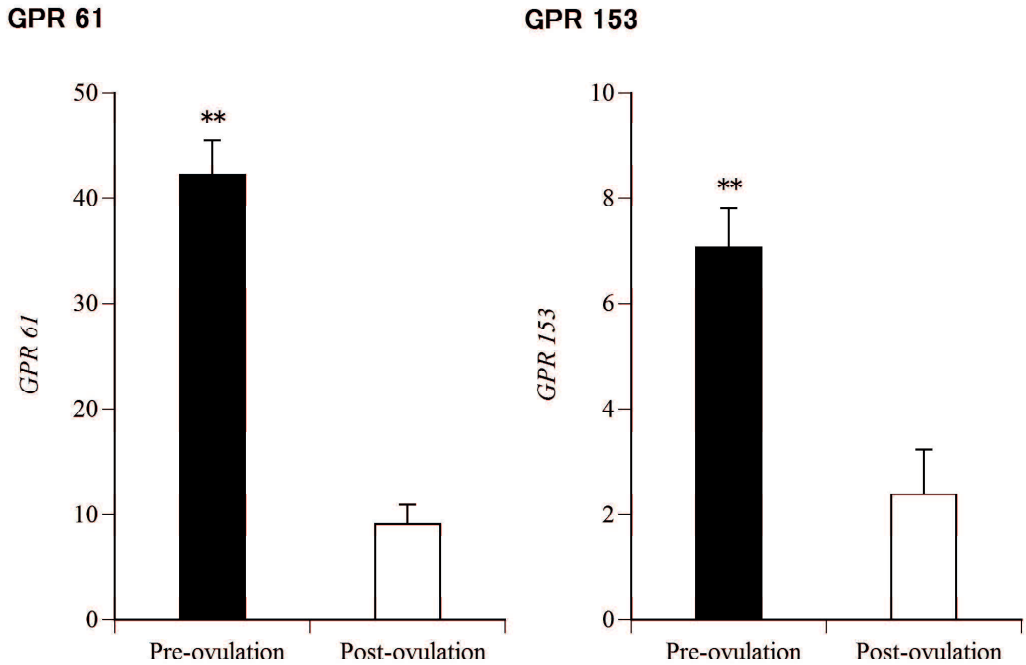


Fig. 3.1. The mean ±SEM (n = 5 for each stage) of *GPR 61* and *GPR 153* expression (measured by real time PCR) in bovine AP, before and after ovulation. Gene expression levels were normalized to the geometric means of two housekeeping genes, *GAPDH* and *RANBP10*.

**P < 0.01: significant difference compared to post-ovulation.

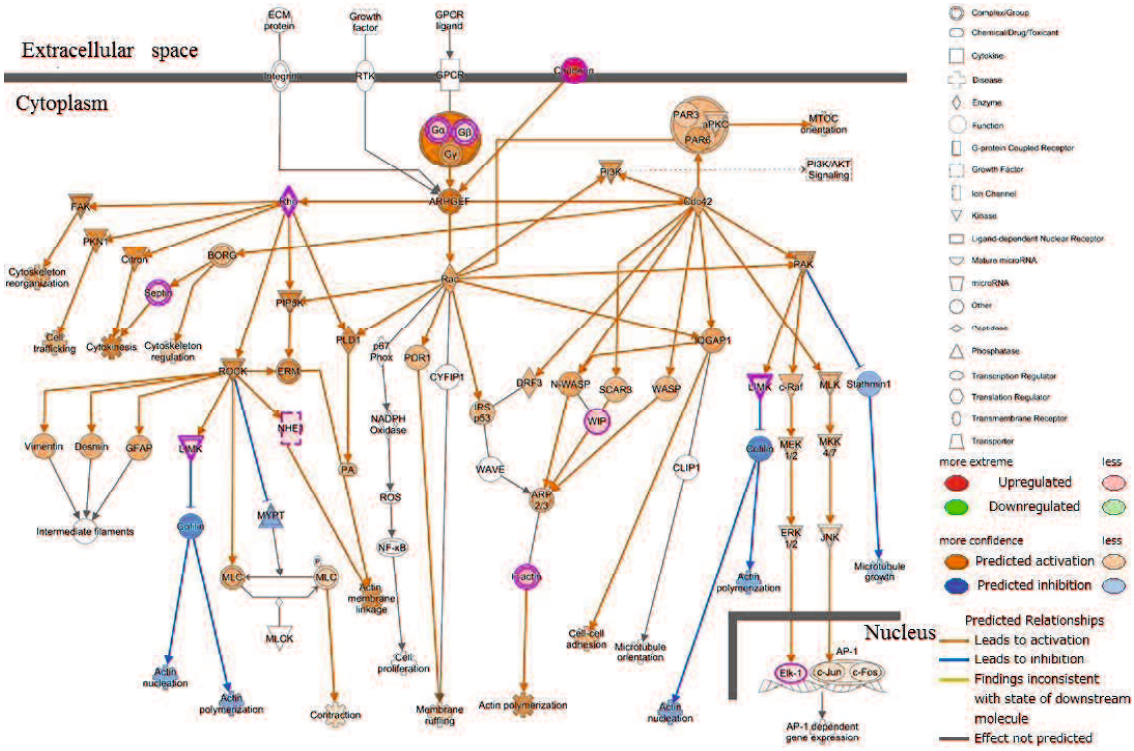
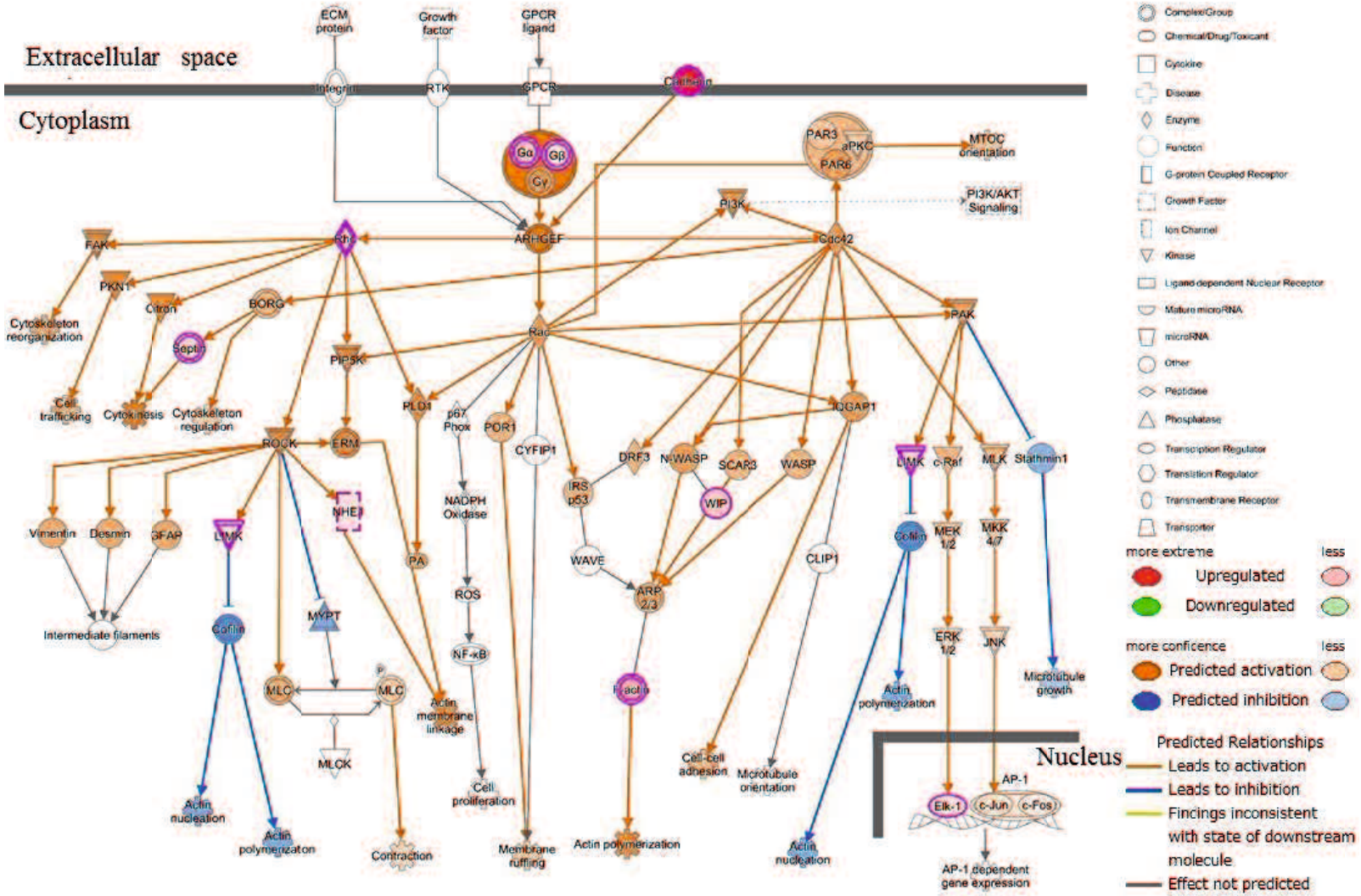


Fig. 3.2. The subcellular location of genes in the canonical pathway, termed as "Signalling by Rho Family GTPases", as determined via IPA analysis to compare transcriptomes in the APs of pre- and post-ovulation heifers. Note that the IPA software shows gene names as normal font and not italics. The solid lines and dotted line indicate direct or indirect biological relationship between genes, respectively. The bold grey lines indicate the border between the extracellular space, cytoplasm, and nucleus. The purple indicates the upregulated genes identified in this experiment. This figure includes the results of molecule activity predictor (MAP), and the predicted stimulated genes are shown in orange, and the predicted inhibited genes are shown in blue. In total, 236 genes constituted the canonical pathway, and 9 of these genes (*ACTA2*, *CDH10, ELK1*, *GNA14*, *LIMK1*, *LIMK2*, *RHOC*, *SEPT5*, and *WIPF1*) were identified in the bovine APs used in this study. In this figure, F-actin represents *ACTA2*, Cadherin represents *CDH10*, Gα represents *GNA14*, LIMK represents both *LIMK1* and *LIMK2*, Rho represents *RHOC*, Septin5 represents *SEPT5*, and WIP represents *WIPF1*.


Extracellular space
Cytoplasm
ECM protein
Growth factor
GPCR ligand
Integrin
RTK
GPCR
Cadherin
Gα
Gβ
Gγ
PAR3
aPKC
PAR6
MTOC orientation
PI3K
PI3K/AKT Signaling
Rho
ARHGEF
Cdc42
FAK
PKN1
Citron
BORG
Cytoskeleton reorganization
Septin
Rac
PAK
PIP5K
Cell trafficking
Cytokinesis
Cytoskeleton regulation
PLD1
p67 Phox
POR1
IQGAP1
ROCK
ERM
CYFIP1
DRF3
N-WASP
SCAR3
WASP
LIMK
c-Raf
MLK
Stathmin1
IRS p53
WIP
Vimentin
Desmin
GFAP
LIMK
NHE1
NADPH Oxidase
Cofilin
MEK 1/2
MKK 4/7
PA
WAVE
CLIP1
ROS
ARP 2/3
ERK 1/2
JNK
Intermediate filaments
Cofilin
MYPT
NF-κB
F-actin
Actin polymerization
Microtubule growth
MLC
MLC
Actin membrane linkage
MLCK
Cell-cell adhesion
Microtubule orientation
Nucleus
AP-1
Actin nucleation
Cell proliferation
Elk-1
c-Jun
c-Fos
Actin nucleation
Actin polymerization
Contraction
Membrane ruffling
Actin polymerization
AP-1 dependent gene expression
Complex/Group
Chemical/Drug/Toxicant
Cytokine
Disease
Enzyme
Function
G-protein Coupled Receptor
Growth Factor
Ion Channel
Kinase
Ligand dependent Nuclear Receptor
Mature microRNA
microRNA
Other
Peptidase
Phosphatase
Transcription Regulator
Translation Regulator
Transmembrane Receptor
Transporter
more extreme
less
Upregulated
Downregulated
more conficence
less
Predicted activation
Predicted inhibition
Predicted Relationships
Leads to activation
Leads to inhibition
Fincings inconsistent with state of downstream molecule
Effect not predicted

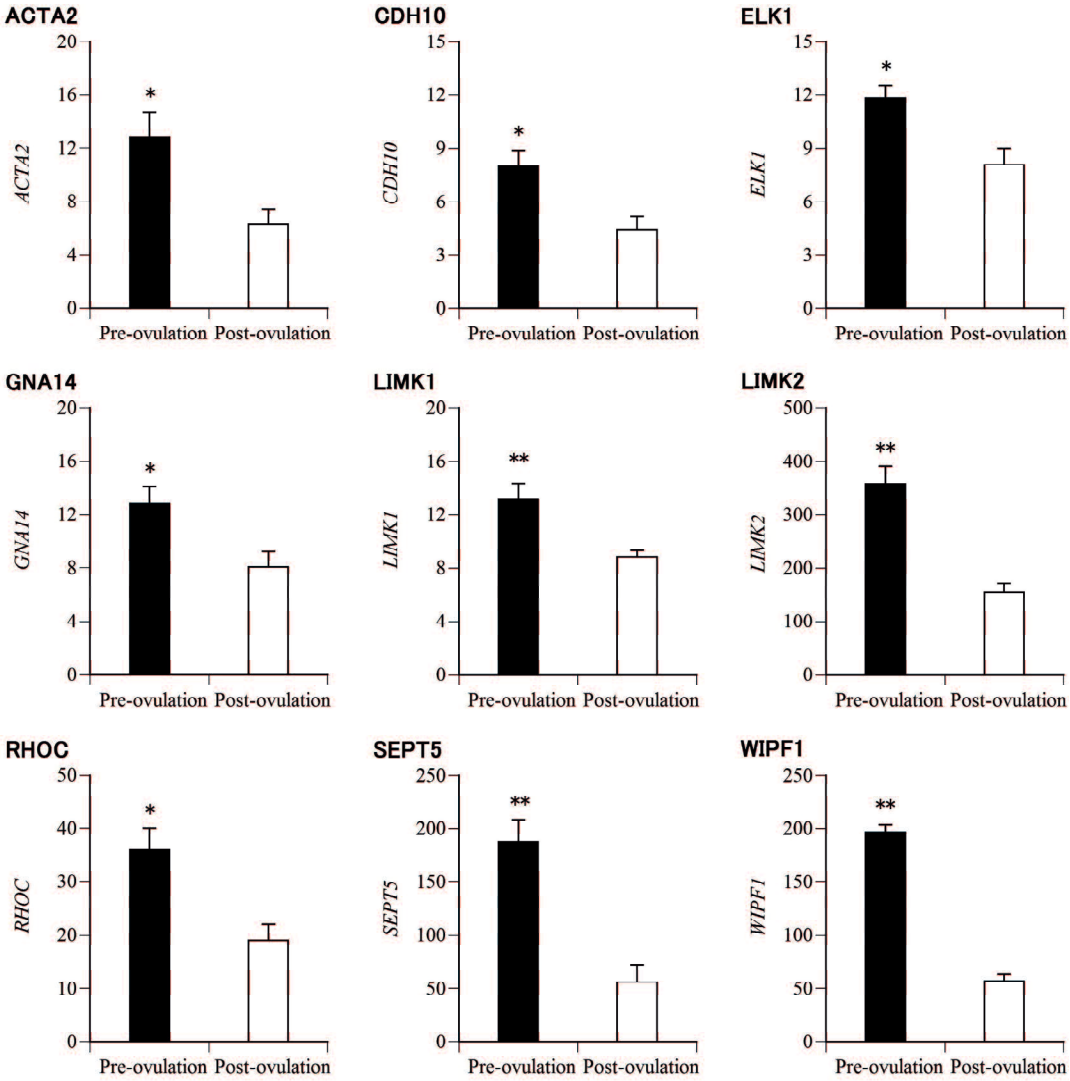


Fig. 3.3. The mean ±SEM (n = 5 for each stage) of the expression levels of 9 genes (*ACTA2*, *CDH10*, *ELK1*, *GNA14*, *LIMK1*, *LIMK2*, *RHOC*, *SEPT5*, and *WIPF1*) comprising the "Signalling by Rho Family GTPases" (measured by real time PCR) in bovine AP, before and after ovulation. Gene expression levels were normalized to the geometric means of two housekeeping genes, *GAPDH* and *RANBP10*.

*P < 0.05: significant difference compared to post-ovulation.

**P < 0.01: significant difference compared to post-ovulation.

3.4. Discussion

The present study showed that bovine APs expressed 12,769 annotated genes. Cattle genome contains a minimum of 22,000 genes, with a core set of 14,345 orthologs shared among seven mammalian species including humans (Bovine Genome Sequencing and Analysis Consortium, 2009). Therefore, about 58% genes are expressed in bovine AP, suggesting the importance of AP.

Up to 32.2% or 44.0% of total RPKM values corresponded to AP hormones in this study. He et al. (2014) reported that a similar percentage (44.1%) of the total RPKM values corresponded to eight major AP hormones in fish (including somatolactin, which is absent in the bovine genome) in the whole pituitary from postpubertal zebrafish. Therefore, AP needs to synthesize several mRNAs for its primary role, namely secreting AP hormones.

The RPKM value of *PRL* was the highest among the all genes including AP hormone genes in this study. Freeman et al. (2000) reported that *PRL* is expressed in both lactotrophs and mammosomatotrophs, and these cells comprise up to 50% of the cellular population in the AP. Prolactin has a broad range of functions in the body, apart from its defining role in promoting lactation (Grattan, 2015). The present study used non-lactating heifers; therefore, the high RPKM value of *PRL* suggested multiple important roles of prolactin.

As expected, AP was found to express many receptors and binding proteins in this study. However, the roles of most genes listed in Tables 3.5 and 3.7 are not well elucidated. For example, murine gonadotroph-like cells express *CRHR1* (Seasholtz et al., 2009), but the functions of CRH have not been clarified yet. *RARA* in Table 3.5 is retinoic acid receptor alpha, and *RLBP1* in Table 3.7 is retinaldehyde binding protein 1. Retinoic acid regulates GnRH release and gene expression in the rat hypothalamic

fragments (Cho et al., 1998). Vitamin A deficient rats have higher serum FSH and LH concentration than controls (Huang et al., 1985). It has been established that vitamin A and retinoic acid are very important for reproduction (Clagett-Dame and Knutson, 2011); however, their roles in the pituitary remain to be clarified.

To the best of our knowledge, this is the first report of the two orphan receptors, *GPR 61* and *GPR153*, expressed in the AP of any animal species. Although the ligand(s) and functions of GPR61 are unknown, it is known to associate with the Gs protein (Takeda et al., 2003; Toyooka et al., 2009) and to stimulate ERK signalling in neurons (Hossain et al., 2016). It was also reported to regulate cyclic AMP in Chinese hamster ovary cells (Martin et al., 2015). Both ERK and cAMP pathways are important for GnRH-induced LH secretion in bovine gonadotrophs (Nakamura and Kadokawa, 2015; Nakamura et al., 2015; Rudolf and Kadokawa, 2016). These findings implied that GPR61 was expressed on the surface of bovine gonadotrophs. In addition, GPR153 is an orphan receptor that is widely expressed in the brain, including in the hypothalamus arcuate nucleus and pituitary (Shreedharan et al., 2011). Thus, further studies are required to clarify whether GPR61 and GPR153 are expressed in gonadotrophs, and whether their expression levels change based on the estrous stage. Furthermore, recent studies have indicated that GnRHR co-localized with insulin and glucocorticoid receptors in lipid rafts on the gonadotroph plasma membrane, where they facilitated downstream signalling (Navratil et al., 2009; Wehmeyer et al., 2014). Therefore, further studies are required to clarify whether GPR61 and GPR153 are localized on the lipid raft with the GnRH receptor in gonadotrophs.

The “Signalling by Rho Family GTPases” is a cytoplasmic pathway that mediates for any GPCR bound any ligand. The pathway was not reported in the 2

previous studies that used oligonucleotide microarray analysis for bovine APs (Kommadath et al., 2010; Roberts and McLean, 2011). The MAP function of IPA predicted the following stimulated functions: cytoskeleton reorganization, cell trafficking, cytokinesis, cytoskeleton regulation, actin membrane linkage, contraction, membrane ruffling, cell-cell adhesion, microtubule-organizing center orientation, and actin-polymerization. It also predicted the following inhibited functions: actin nucleation, microtubule growth, and actin polymerization. *ACTA2* and other actin genes encode the cell cytoskeleton. Both cell cytoskeleton and microtubule are very important parts of gonadotrophs for the secretion of LH and FSH (Adams and Nett, 1979; Anderson, 1996; Watanabe et al., 2014). GnRH regulates the morphology and migration of the immortalized LbetaT2 gonadotroph-like cells via the pathway regulating cytoskeletal reorganization (Godoy et al., 2011). The protein encoded by *RhoC,* Ras homolog family member C, may have important roles in cytoskeleton protein reorganization by regulating the polymerized actin to control secretory granule exocytosis in AP cells in response to extracellular signals (Cussac et al., 1996). The LIM kinases, encoded by *LIMK1* and *LIMK2*, mediate the action of estrogen for regulating the dynamics of actin (Yuen et al., 2011). WIPF1 is an actin-binding protein that regulates actin polymerization (Anton and Jones, 2006). Although the role of the *CDH10* product, cadherin-10, in the pituitary has not yet been studied, it is known to be important for cell-cell adhesion in the construction of the blood-brain barrier (Williams et al., 2005). The product of *SEPT5*, septin 5, is a member of the septin GTPase family and is involved in exocytosis in post mitotic neurons (Asada et al., 2010). Therefore, the canonical pathway may control AP hormone secretion.

I next investigated the specific AP cells that contained the “Signalling by Rho Family GTPases” pathway. Because this study used a heterogeneous cell mixture, in

which a large number of GPCRs were expressed, it was impossible to further clarify the mechanisms. However, the canonical pathway also includes ELK1. Utilizing ELK1, the activated GnRHR enhances the expression of Egr-1 (Mayer et al., 2008), which binds to the promoter region of *LHB* and increases *LHB* expression in gonadotrophs (Topilko et al., 1998; Buggs et al., 2006). The *GNA14* product, Gα(14), is a G protein that can interact with different classes of receptors to regulate phospholipase C (Ho et al., 2001), which is important for the release of LH in nonhuman primate pituitary (Luque et al., 2011). Therefore, the canonical pathway may exist in the gonadotrophs.

The blood levels of estradiol and progesterone are low on day 3 after ovulation, when active pulsatile LH secretion occurs in heifers (Kadokawa and Yamada, 1999). Only 31 genes showed higher expression in the post-ovulation AP than the pre-ovulation AP, whereas about 12 times that number (365) of genes was expressed more in the pre-ovulation AP than the post-ovulation AP. The low concentrations of estradiol and progesterone on day 3 after ovulation may be the direct or indirect cause of this difference. The 31 genes that were expressed more after ovulation included *PNRC1*, *SPAG8*, *AGXT2*, and *ARG2*. The product of *PNRC1*, proline rich nuclear receptor coactivator 1, interacts with estrogen receptor α (Byrne et al., 2013), and a single nucleotide polymorphism in this gene influences egg weight-related and hatchability traits in ducks (Chang et al., 2012). The product of *SPAG8*, sperm associated antigen 8, enhances the transcription of the cAMP response element modulator (Wu et al., 2010), which may influence the estrogen-mediated negative feedback on GnRH neurons (Kwakowsky et al., 2012). The product of *AGXT2*, alanine-glyoxylate aminotransferase 2, was recently found to have several novel functions through genomic and metabolomic studies; it also has a unique role in the

intersection of key mitochondrial pathways (Rodionov et al., 2014). The product of *ARG2*, arginase 2, reduces the activity of NOS in genital tissues (Kim et al., 2004); NOS is known to control the secretion of LH and FSH in the pituitary gland (McCann et al., 2003). Thus, these 4 genes may contribute to the synthesis and secretion of LH and FSH in gonadotrophs. However, the roles of the remaining 27 genes, which are expressed more in post-ovulation AP, are unclear. Furthermore, 11 genes still do not have names. Therefore, further studies are required to clarify the roles of these 31 genes.

The roles of most genes listed in tables 3.3 and 3.4 are unclear. For example, the *NNAT* product, neuronatin, is expressed in prenatal and postnatal rat pituitary (Kanno et al., 2016). However, the role of neuronatin in the adult pituitary has not been clarified yet. The *SCGII* product, secretogranin II, may be a paracrine factor secreted from lactotrophs to stimulate gonadotropin release (Zhao et al., 2010). Moreover, estradiol decreases *SCGII* expression in rat pituitary cells (Anouar and Duval, 1992). Therefore, bovine lactotrophs may express *SCGII* under the control of estradiol to stimulate gonadotropin secretion. *SCG5* encodes neuroendocrine protein 7B2 and is expressed in various types of AP cells, including gonadotrophs (Marcinkiewicz et al., 1993). GnRH injection increased blood 7B2 concentration in women, but the role of increased 7B2 levels has not been clarified yet (Natori et al., 1989). Therefore, these genes may contribute to AP hormone synthesis and secretion in bovine AP.

In conclusion, the present study clarified the novel genes differentially expressed in the pre- and post-ovulation stages as well as an important pathway in the AP. Silent heat, delayed ovulation, and luteal hypoplasia are common problems in domestic animals; however, little is known regarding the underlying

pathophysiological mechanisms. Therefore, further studies are required to clarify the roles of most of these genes that show an increase or decrease in expression after ovulation.

CHAPTER IV

(Study II)

Heifers Express GPR61 in Gonadotrophs in Stage-Dependent Manner

Abstract

GnRHRs colocalize with insulin and glucocorticoid receptors in lipid rafts of the gonadotrophs plasma membrane, where they facilitate downstream signaling. The study I found that GPR61 is expressed in the AP of heifers, leading us to speculate that GPR61 colocalizes with GnRHR in the plasma membrane of gonadotrophs and is expressed at specific times of the reproductive cycle. To test this hypothesis, I examined the co-expression of GnRHR, GPR61, and either LHβ subunit or FSHβ subunit in AP tissue and cultured AP cells by immunofluorescence microscopy. GPR61 was detected in gonadotrophs, with a majority of them being colocalized with GnRHR and the remainder present at other parts of the cell surface or in the cytoplasm. I obtained a strong positive overlap coefficient (0.71 ± 0.01) between GPR61 and GnRHR on the cell-surface of cultured GnRHR-positive AP cells. Real-time PCR and western blot analyses found that expression was lower ($P < 0.05$) in AP tissues during early luteal phase as compared to pre-ovulation or mid- or late luteal phases. Additionally, the 5'-flanking region of the *GPR61* gene contained several sites with response elements similar to those of estrogen or progesterone. These data suggested that GPR61 colocalizes with GnRHR in the plasma membrane of gonadotrophs, and its expression changes stage-dependently in the bovine AP.

4.1. Introduction

GnRHRs are present in gonadotroph plasma membrane lipid rafts (Navratil et al., 2009; Wehmeyer et al., 2014; Kadokawa et al., 2014), which are distinct, relatively insoluble regions that have lower density and are less fluid than surrounding membrane (Simons and Tooter, 2000; Head et al., 2014). Lipid rafts facilitate signaling by allowing colocalization of membrane receptors and their downstream signaling components (Simons and Tooter, 2000; Head et al., 2014); as such, they are important pharmacological targets in human medicine (Jaffrès, 2016). Lipid rafts containing GnRHR also harbor insulin receptor (Navratil et al., 2009) and glucocorticoid receptor (Wehmeyer et al., 2014) in the LβT2 clonal murine gonadotroph cell line, thus providing a potential means of integrating neuropeptide and energy homeostasis signals to modulate reproductive function. However, gonadotroph lipid rafts containing GnRHR are also likely to contain other types of receptor that have yet to be identified.

The study I discovered that GPR61 significantly expressed higher during pre-ovulation period. GPR61 is an orphan receptor that is widely expressed in the brain, including in the hypothalamus and pituitary (Lee et al., 2001; Nambu et al., 2011), although its function and ligand(s) are unknown. GPR61 associates with Gs protein (Takeda et al., 2003; Toyooka et al., 2009) and stimulates ERK signaling in neurons (Hossain et al., 2016). It was also found to inhibit cAMP-dependent reporter (luciferase) gene expression induced by forskolin in Chinese hamster ovary cells that are transiently co-transfected with plasmids containing the reporter and GPR61 (Martin et al., 2015). The ERK and cAMP pathways inhibit and stimulate GnRH-induced LH secretion, respectively, in bovine gonadotrophs (Nakamura et al., 2015;

Nakamura and Kadokawa, 2015; Rudolf and Kadokawa, 2016). These findings imply that GPR61 is expressed at the surface of bovine gonadotrophs.

We previously developed a guinea pig polyclonal antibody that recognizes the extracellular region of GnRHR (anti-GnRHR) (Kadokawa et al., 2014) and used this for immunofluorescence detection of GnRHR in bovine gonadotroph lipid rafts (Kadokawa et al., 2014; Pandey et al., 2016). In this chapter, I tested the hypothesis that GPR61 colocalizes with GnRHR in the plasma membrane of bovine gonadotrophs and that its expression is dependent on reproductive stage.

4.2. Materials and methods

4.2.1. Antibodies

I previously determined using the SOSUI v.1.11 algorithm that bovine GPR61 protein (451 amino acids; accession number AAX31376.1 in NCBI reference bovine sequences) contains seven hydrophobic transmembrane domains linked by hydrophilic extracellular and intracellular loops (Hirokawa et al., 1998; http://harrier.nagahama-i-bio.ac.jp/sosui/). Bovine GPR61 has 96% homology to the human protein (451 amino acids; NCBI accession number AAH67464.1). I used a rabbit polyclonal anti-GPR61 antibody against the extracellular region of human GPR61 (ORB183901, Biorbyt) whose peptide antigen corresponds to amino acids 4–18 (SPIPQSSGNSSTLGR) in the N-terminal extracellular domain of human GPR61. This sequence has 100% homology to amino acids 4–18 in the N-terminal extracellular domain of bovine GPR61 but not to other proteins in the bovine genome based on sequences retrieved from DNA Data Bank of Japan/GenBank/European Bioinformatics Institute Data Bank using the protein basic local alignment search tool.

I used a guinea pig polyclonal anti-bovine GnRHR antibody against the N-terminal extracellular domain (corresponding to amino acids 1–29; MANSDSPEQNENHCSAINSSIPLTPGSLP) (Kadokawa et al., 2014) as well as a mouse monoclonal anti-LHβ antibody (clone 518-B7; Matteri et al., 1987) for immunohistochemical analysis of AP tissue and cultured AP cells. This antibody does not cross-react with other pituitary hormones (Iqbal et al., 2009).

Finally, I used a mouse monoclonal anti-FSHβ antibody (clone A3C12) that does not cross-react with other pituitary hormones (Borromeo et al., 2004) for

immunohistochemical analysis of AP tissue; it was not used in cultured AP cells owing to weak labeling.

4.2.2. AP sample collection for protein or RNA analysis

AP tissue was collected from the head of adult (26 months old) non-pregnant healthy Japanese Black heifers in pre-ovulation [day 19 to 21 (day 0 = day of estrus); n = 5], early luteal (day 2 to 5; n = 8), mid-luteal (day 8 to 12; n = 9), or late luteal (day 15 to 17; n = 7) phase, as determined by macroscopic examination of the ovaries and uterus (Miyamoto et al., 2000). Samples were obtained at a local abattoir as previously described (Rudolf and Kadokawa, 2014) and immediately frozen in liquid nitrogen and stored at −80°C until RNA or protein extraction.

4.2.3. Protein extraction and western blot analysis of GPR61

I extracted protein from AP tissue and performed western blotting using the previously described method (Kadokawa et al., 2014). The extracted protein sample (16.7μg of total protein in 18.75μl) was mixed in 6.25μl of 4x Laemmli sample buffer (Bio-Rad) containing 10% (v/v) β-mercaptoethanol, then boiled for 3 min at 100 °C. Boiled protein samples (15 μL; 10 μg of total protein) were then loaded on a polyacrylamide gel along with a molecular weight marker (Precision Plus Protein All Blue Standards; Bio-Rad), and four samples (2.5, 5, 10, and 20 μg total protein for each of five randomly selected AP samples diluted with protein extraction reagent) were resolved by electrophoresis on sodium dodecyl sulfate polyacrylamide gels at 100 volt for 90 min. Proteins were then transferred to PVDF membranes Immunoblotting was performed with the anti-GPR61 antibody (1:25,000 dilution) after blocking with 0.1% Tween 20 and 5% non-fat dry milk for 1h at 25 °C. Incubation with the primary antibody was performed overnight at 4 °C. Protein bands

were visualized using an ECL-Prime chemiluminescence kit (GE Healthcare). The signal intensity of protein bands was quantified using MultiGauge v.3.0 software (Fujifilm). The intensities of the GPR61 band for 20-, 10-, 5-, and 2.5-μg AP protein samples were set as 100%, 50%, 25%, and 12.5%, respectively, and the intensity of other samples was calculated as a percentage of these standards using MultiGauge software. After antibodies were removed from the PVDF membrane with stripping solution (Nacalai Tesque Inc.), the membrane was used for immunoblotting with the anti-β-actin mouse monoclonal antibody (A2228, 1:50,000 dilution; Sigma-Aldrich).The intensities of the β-actin band for 20-, 10-, 5-, and 2.5-μg AP protein samples were set as 100%, 50%, 25%, and 12.5%, respectively, and the intensity of other samples was calculated as a percentage of these standards using MultiGauge software. GPR61 expression level was normalized to that of β-actin in each sample.

4.2.4. RNA extraction, cDNA synthesis, and real time PCR

Total RNA was extracted from the sample using RNAiso Plus (Takara Bio Inc.) according to the manufacturer's protocol. The extracted RNA samples were treated with ribonuclease-free deoxyribonuclease (Toyobo) to eliminate possible genomic DNA contamination. The concentration and purity of each RNA sample were evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.) to ensure the A_{260}/A_{280} nm ratio was in the acceptable range of 1.8–2.1. Electrophoresis of total RNA followed by staining with ethidium bromide was performed to verify the mRNA quality of all samples, and the 28S:18S ratios were 2:1. The GPR61 primers and procedure are same as detailed in chapter III.

4.2.5. Triple immunofluorescence analysis of AP tissue

Nett et al. (1987) reported that the concentrations of LH and GnRHR in AP were greater during the luteal phase in heifers than during the immediate post-estrus period. Therefore, AP tissue was obtained from post-pubertal Japanese Black heifers in the middle luteal phase (n = 3, 26 months of age) were stunned using a captive bolt pistol, and were then exsanguinated by a throat cut. Within 5 min of slaughter, the heads were placed on ice. Within 15 min of slaughter, pituitaries had been dissected at the midline, and the APs were separated from the posterior pituitaries, cut into small blocks (<0.8 cm thick), and fixed in 4% paraformaldehyde at 4 ^{o}C for 16 h. After fixation, the blocks were placed in PBS containing 30% sucrose diluted in PBS until infiltrated with sucrose. The blocks were then frozen in an embedding medium (Tissue-Tek OCT compound; Sakura Fine technical Co. Ltd.) and maintained at − 80^{o}C until sectioning into 15- μm sagittal sections using a cryostat (CM1900, Leica Microsystems PtyLtd.) and mounted on slides (MAS coat Superfrost, Matsunami-Glass). The sections were treated with 0.3% Triton X-100 in PBS for 15 min, then, incubated with 0.5 mL of PBS containing 10% nor-mal goat serum (Wako Pure Chemicals) for blocking for 1 h. Incubation with a cocktail of primary antibodies [guinea pig anti-GnRHR antibody, 1:1,000; rabbit polyclonal anti-GPR61 antibody, 1:1,000; and either mouse monoclonal anti-LHβ or anti-FSHβ antibody (both at 1:1,000)] for 12 h at 4°C was followed by incubation with a cocktail of fluorochrome-conjugated secondary antibodies (4 μg/mL Alexa Fluor 488 goat anti-rabbit IgG, 4 μg/mL Alexa Fluor 546 goat anti-mouse IgG, and 4 μg/mL Alexa Fluor 647 goat anti-guinea pig IgG (all from Thermo Fisher Scientific) and 1 μg/mL of DAPI (Wako Pure Chemicals) for 2 h at room temperature. The stained sections were covered with a slip using a mountant and were observed by confocal microscopy (LSM710; Carl Zeiss)

equipped with a diode laser 405nm, argon laser 488nm, HeNe laser 533nm, and HeNe laser 633nm. Images obtained by fluorescence microscopy were scanned with a 40× or 63× oil-immersion objective and recorded by a CCD camera system controlled by ZEN2012 black edition software (Carl Zeiss). The optical resolution in the Z-axis (optical slice) was more than 0.6 μm and less than 4.6 μm. GnRHR, GPR61, and LHβ or FSHβ localization was examined in confocal images of triple-immunolabeled specimens. To verify the specificity of the signals, I included several negative controls in which the primary antiserum had been omitted or pre-absorbed with 5 nM antigen peptide synthesized by Scrum Inc. or in which normal guinea pig IgG was used instead of the primary antibody. Percentages of single- and double-labeled GPR61- and GnRHR-positive cells were determined from 12 representative confocal images per pituitary gland.

4.2.6. AP cell culture and immunocytochemical analysis of cells

APs were obtained from post-pubertal Japanese Black heifers in the middle luteal phase for fixation (n = 5, 26 months of age). Enzymatic dispersal of the AP cells was performed using a previously described method (Suzuki et al., 2008) and confirmation of cell viability of greater than 90% was determined via Trypan blue exclusion. Total cell yield was 19.8 $\times 10^6 \pm$ 0.8 $\times 10^6$ cells per pituitary gland. The dispersed cells were then suspended in DMEM (D1152, Sigma-Aldrich) containing 1× nonessential amino acids (Thermo Fisher Scientific), 100U/mL penicillin, 50μg/mL streptomycin, 10% horse serum (Gibco), and 2.5% FBS (Thermo Fisher Scientific). The cells (2.5×10^5cells/mL, total = 0.15 mL per lane) were cultured in the culture medium at 37 °C in 5% CO_2 for 82 h, using a microscopy chamber (μ-Slide VI 0.4, Ibidi). I cultured the AP cells for 82 h (3.5 days) for 2 reasons. The first reason was that in our preliminary study, I observed that if I cultured for a shorter time,

almost all AP cells were washed off from the microscopy chamber during the staining and washing steps of immunocytochemistry. The second reason was that various previous publications by other groups and ours on LH, GH, and prolactin (Hashizume et al., 2003; Kadokawa et al., 2008; Hashizume et al., 2009; Kadokawa et al., 2014; Nakamura et al., 2015) have described culturing for 3.5 days.

I fixed and treated the cultured cells by using either (1) 4% PFA fixation for 3 min and 0.1% Triton X-100 treatment for 1 min (PFA-Triton method) or (2) CellCover (Anacyte laboratories UG) for fixation, instead of 4% PFA, for 2 min, and no Triton X-100 treatment (CellCover method). For both these methods, the cells were blocked with 10% normal goat serum for 30 mins. These methods were described previously (Kadokawa et al., 2014). For the PFA-Triton method, the fixed cells were incubated with 0.1 mL of the same cocktail of primary antibodies for 2 h at room temperature. Incubation with Triton X-100 allowed both anti-GnRHR and anti-GPR61 antibodies to bind to target proteins in the cytoplasm and at the cell surface. For the CellCover method, the fixed cells were incubated with only guinea pig anti-GnRHR and rabbit polyclonal anti-GPR61 antibodies (both 1:1,000) for 2 h at room temperature. Since the cells were not treated with Triton X-100, the antibodies bound only to the extracellular domains of the respective receptors in most cells, although some cytoplasmic labeling occurred in broken cells. For both PFA-Triton and CellCover methods, cells were washed four times with PBS and then incubated with 0.15 mL of fluorochrome-conjugated secondary antibody cocktail and 1 μg/mL DAPI for 2 h at room temperature. After four washes, the cells were visualized by confocal microscopy and fluorescence micrographs and differential interference contrast images were obtained on a single plane. Signal specificity was confirmed using negative controls in which the primary antiserum was omitted or pre-absorbed with 5

nM antigen peptide, or in which the normal guinea pig IgG replaced the primary antibody.

4.2.7. Analysis of the GPR61 gene 5'-flanking region

The 5000-nucleotide sequence of the 5'-flanking region of the *GPR61* gene (chromosome 3: 34023462–34028461) was obtained using the online Ensembl (www.ensembl.org) and BLAT Search Genome program (http://genome.ucsc.edu) (Cow Jun. 2014, Bos_taurus_UMD_3.1.1/bosTau8). The sequence was analyzed using Genetyx software v.13 (Genetyx) for the presence of consensus response element sequences for estrogen—i.e., ERE (5'-GGTCANNNTGACC-3'), ERE-like sequence (5'-TGACCCCTGGGTCA-3') (Gruber et al., 2004), and half ERE (GGTCA, TGACC, or TGACT) (Liu et al., 1995), as well as for progesterone—i.e., PRE (5'-G/A G G/T AC A/G TGGTGTTCT-3') (Geserick et al., 2005) and half PRE (5'-TGTTCT-3') (Tsai et al., 1988).

4.2.8. Data analysis

The statistical significance of differences in GPR61 mRNA or protein expression were analyzed by one-factor ANOVA followed by *post-hoc* comparisons using Fisher's PLSD test using StatView version 5.0 for Windows (SAS Institute, Inc.). The level of significance was set at $P < 0.05$. Data are expressed as mean ±SEM.

4.3. Results

4.3.1. Relationship between GPR61 expression and estrous stage in bovine AP tissue

The ANOVA revealed a significant effect of estrous stage on GPR61 mRNA ($P < 0.05$; **Fig. 4.1**) and protein ($P < 0.05$; **Fig. 4.2**) expression: the level was lower in the AP of heifers in early luteal as compared to other phases of the reproductive cycle. The GPR61 band size was around 23 kDa by western blot analysis. Such band was not observed in the negative control membranes for which the primary antiserum had been omitted or pre-absorbed with antigen peptide or for which the normal guinea pig IgG had been used instead of the primary antibody.

4.3.2. Immunofluorescence analysis of GnRHR and GPR61 expression in bovine AP tissue

Coexpression of LHβ or FSHβ with GnRHR and GPR61 in bovine AP tissue was investigated by immunohistochemistry. LHβ and GPR61 showed colocalization, with the latter strongly expressed at the plasma membrane of LHβ-/GnRHR-positive and LHβ-negative/GnRHR-positive cells in each pituitary gland **(Fig. 4.3)**. FSHβ and GPR61 were also colocalized, with the latter strongly expressed at the plasma membrane of FSHβ-/ GnRHR-positive and FSHβ-negative/GnRHR-positive cells in each pituitary gland **(Fig. 4.4)**. Majority of the GPR61 colocalized with GnRHR in gonadotrophs. Percentages of single- and double-labeled GPR61- and GnRHR-positive cells were determined from 12 representative confocal images per pituitary gland. In each pituitary gland, there was an average of 51.3 ± 3.8 GnRHR-positive cells, 47.7 ± 1.7 GPR61-positive cells, and 36.0 ± 2.3 double-positive cells; 70.5% ± 4.7% of GnRHR-positive cells were GPR61-positive, whereas 75.4% ± 2.9% of GPR61-positive cells were GnRHR-positive.

4.3.3. GPR61 and GnRHR aggregate on the surface of cultured AP cells

GPR61 and GnRHR aggregated on the surface of LHβ-/GnRHR-positive and LHβ-negative/GnRHR-positive cultured AP cells prepared by the PFA-Triton method **(Fig.4.5)**. In the AP cells prepared by the CellCover method, GPR61 aggregated on the surface of GnRHR-positive cells **(Fig. 4.6)**. The overlap coefficient between GPR61 and GnRHR was 0.71 ± 0.01 on the cell surface of cultured AP cells.

4.3.4. ERE, PRE, and similar sequences in the 5'-flanking region of bovine GPR61 gene

The 5'-flanking region of the bovine *GPR61* gene was analyzed for EREs, PREs, and similar sequences **(Table 4.1)**. Although there were no ERE nor PRE sequences, one ERE-like, 25 half ERE, and three PRE-like sequences were identified.

Table 4.1. Analysis of ERE, ERE-like, half ERE, PRE, and PRE-like sequences in the 5000-nucleotide of 5'-flanking region of *GPR61* gene

Response element		Number of sites	Position*
ERE	5'-GGTCANNNTGACC-3'	0	
ERE-like	5'-TGACCCCTGGGTCA-3'	1	134
Half ERE	5'-GGTCA-3'	6	143, 314, 463 ,2138, 4531, 4629
Half ERE	5'-TGACC-3'	6	134, 391, 1269, 2660, 2699, 4236
Half ERE	5'-TGACT-3'	13	21, 306, 362, 792, 2546, 2554, 2956, 3524, 3532, 3641, 3681, 4617, 4678
PRE	5'-G/A G G/T AC A/G TGGTGTTCT-3'	0	
PRE-like	5'-TGTTCT-3'	3	3516, 3877, 4157

*Nucleotide 5000 upstream of the start codon is defined as nucleotide 1.

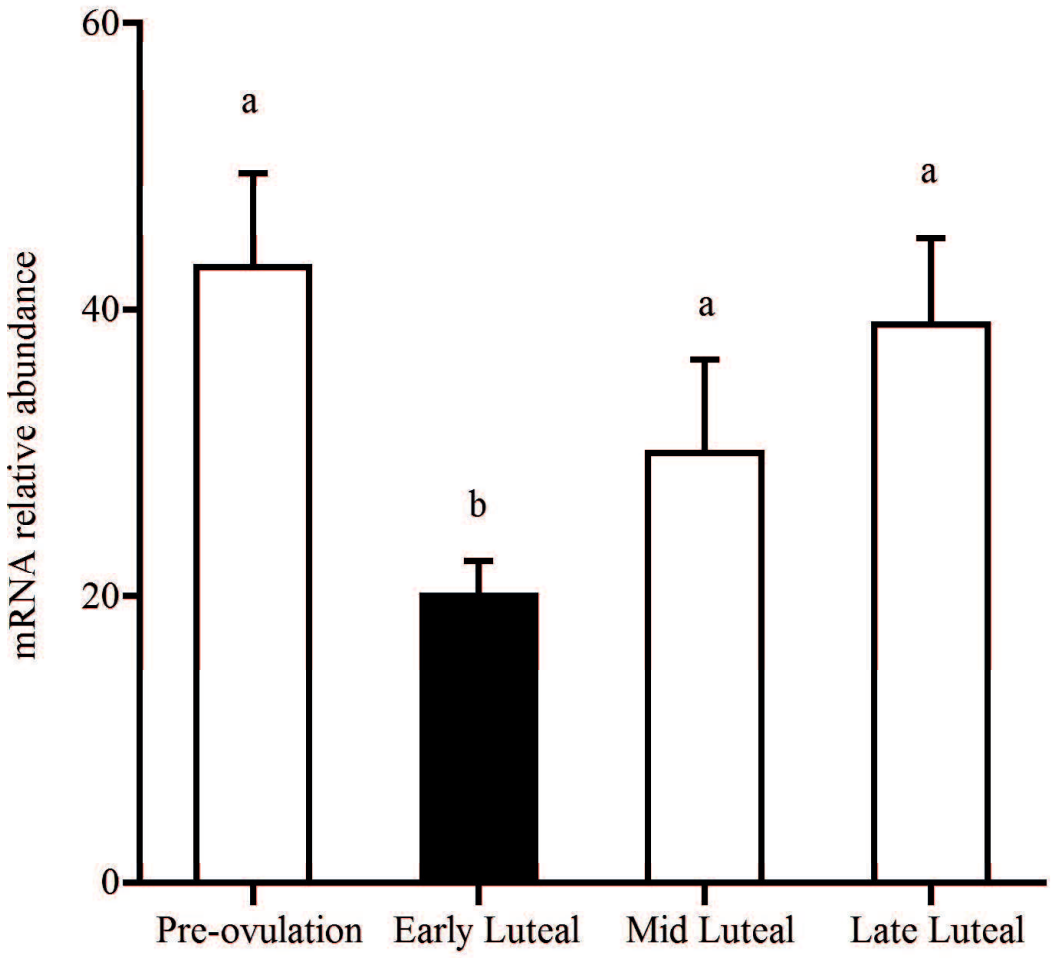


Fig. 4.1. Relative *GPR61* mRNA levels (mean ± SEM) in bovine AP during pre-ovulation [day 19 to 21 (day 0 = day of estrus); n = 5], early luteal (day 2 to 5; n = 8), mid-luteal (day 8 to 12; n = 9), or late luteal (day 15 to 17; n = 7) phases, as determined by real-time PCR. Data were normalized to the geometric mean of *GAPDH* and *RAN-binding protein* (*RANBP10*) levels. Letters (a *vs*. b) indicate significant differences ($P < 0.05$) among phases.

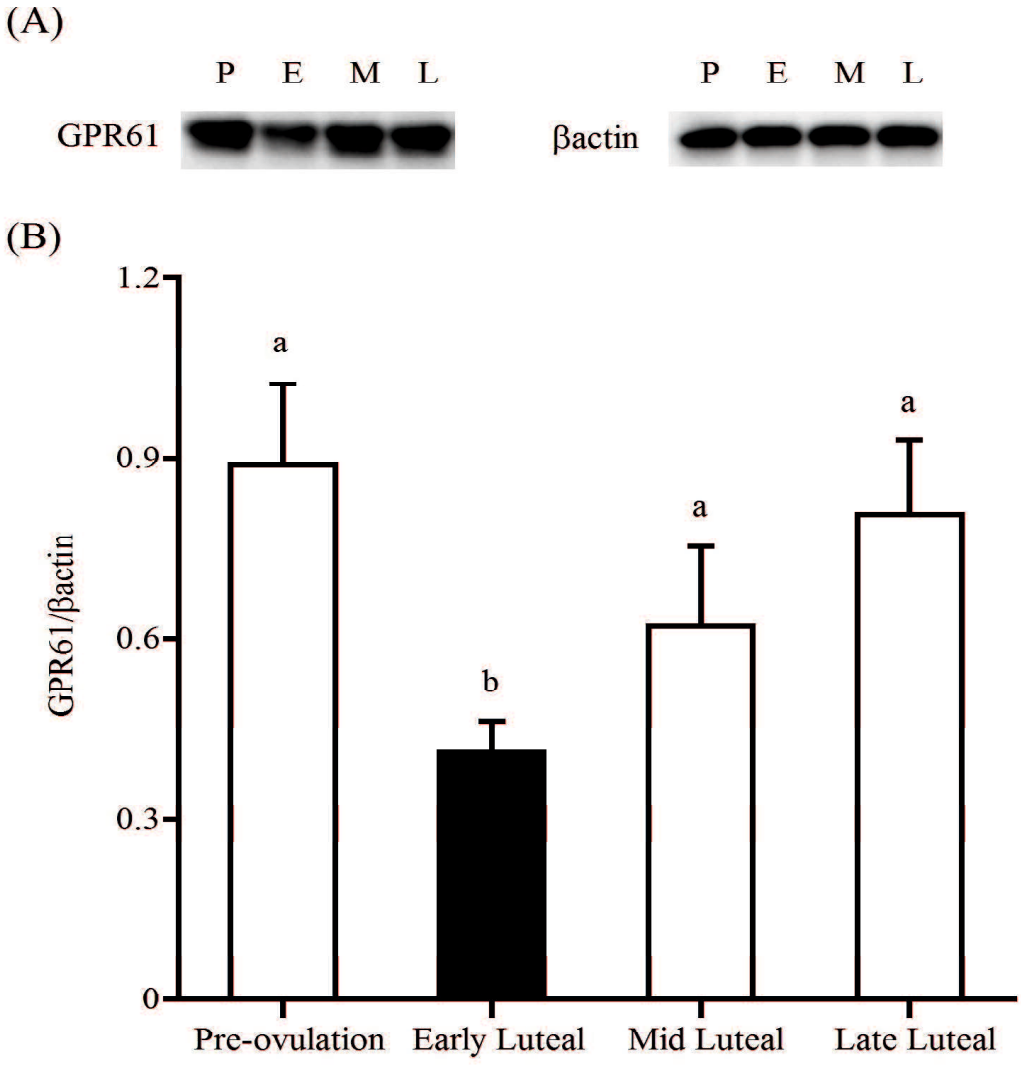


Fig. 4.2. (A) Representative GPR61 and β-actin protein expression in bovine AP tissue obtained during the pre-ovulation (P), early luteal (E), mid-luteal (M), or late luteal (L) phases, as detected by western blotting. (B) GPR61 protein expression normalized to that of β-actin in bovine AP tissue obtained during pre-ovulation (n = 5), early luteal (n = 8), mid-luteal (n = 9), and late luteal (n = 7) phases. Letters (a *vs*. b) indicate significant differences ($P < 0.05$) among phases.

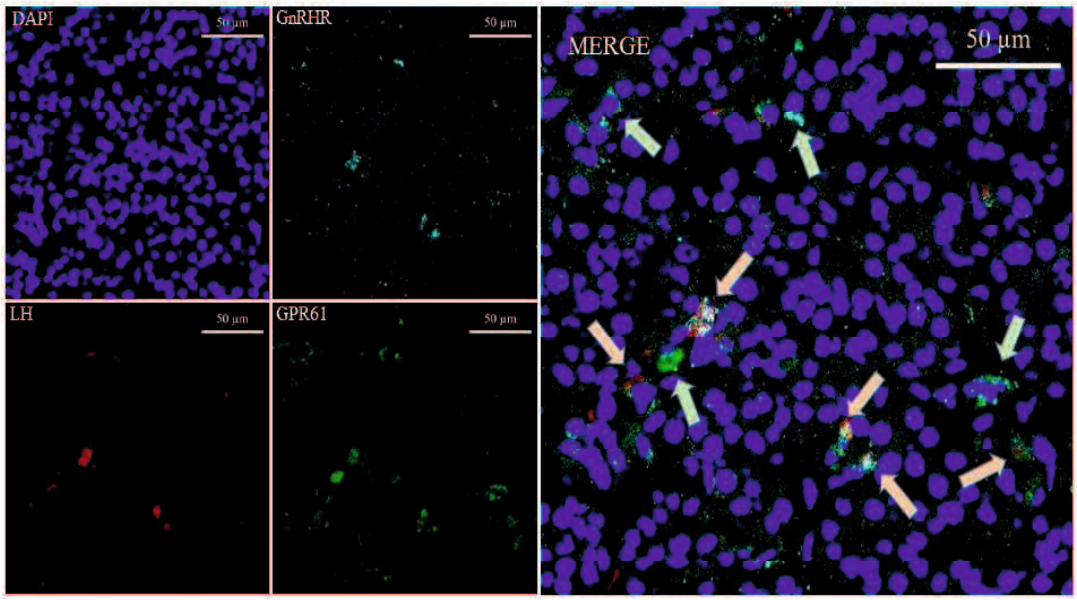


Fig. 4.3. Immunofluorescence analysis of LHβ (red), GnRHR (light blue), and GPR61 (green) in bovine AP tissue by laser confocal microscopy; nuclei were counterstained with DAPI (dark blue). Yellow indicates colocalization of LHβ and GPR61. High GPR61 expression was observed at the plasma membrane of LHβ-/GnRHR-positive cells (red arrows) and LHβ-negative/GnRHR-positive cells (green arrows). Majority of the GPR61 colocalized with GnRHR in gonadotrophs. Scale bars, 50 µm.

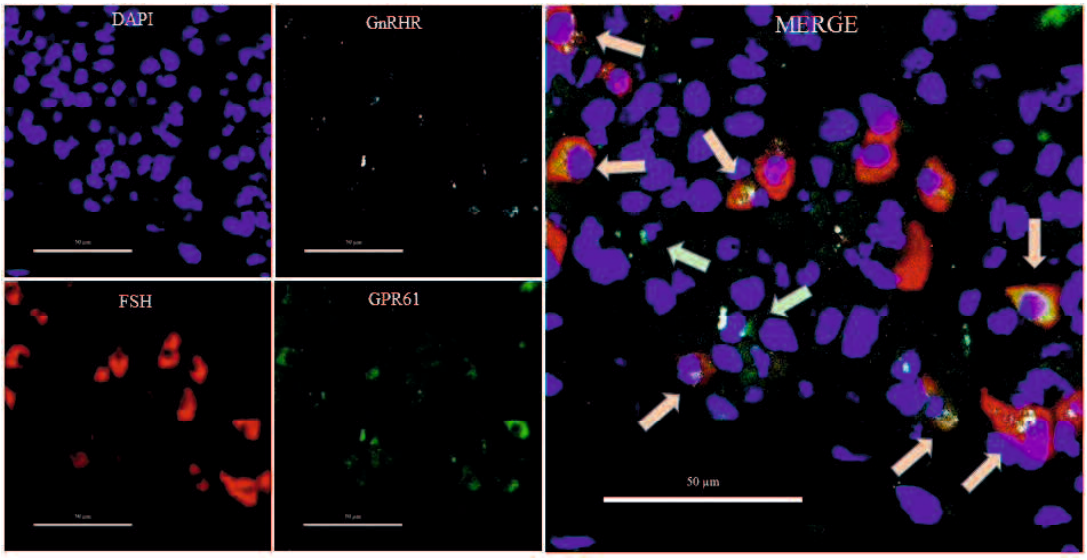


Fig. 4.4. Immunofluorescence analysis of FSHβ (red), GnRHR (light blue), and GPR61 (green) in bovine AP tissue by laser confocal microscopy; nuclei were counterstained with DAPI (dark blue). Yellow indicates colocalization of FSHβ and GPR61. High GPR61 expression was observed at the plasma membrane of FSHβ-/GnRHR-positive cells (red arrows) and FSHβ-negative/GnRHR-positive cells (green arrows). Majority of the GPR61 colocalized with GnRHR in gonadotrophs. Scale bars, 50 µm.

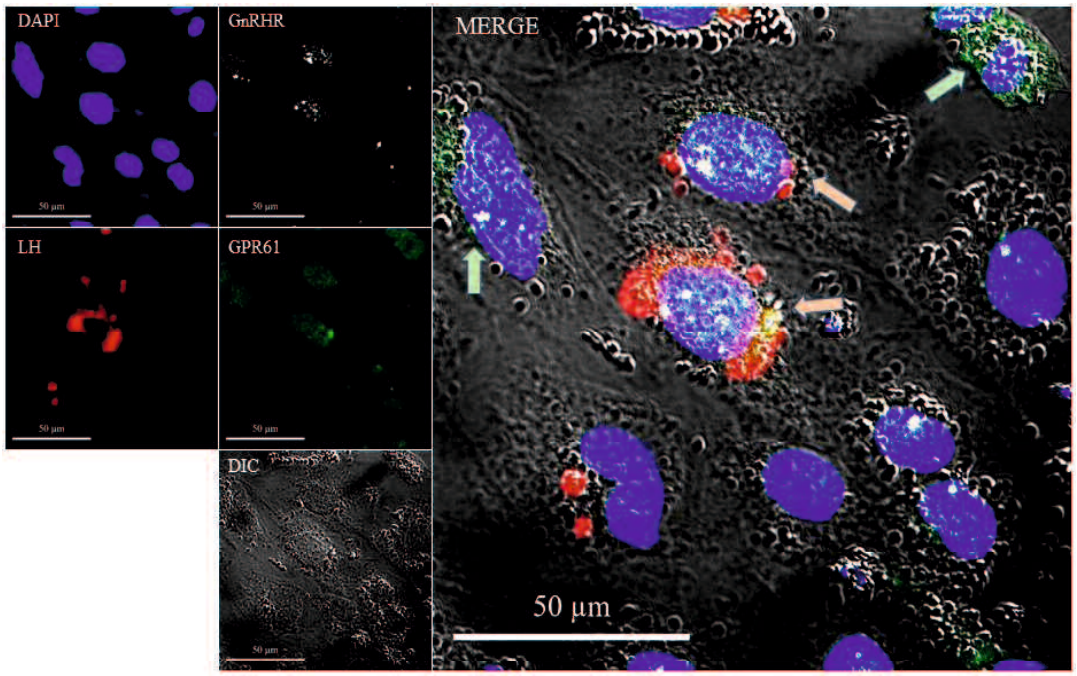


Fig. 4.5. Immunofluorescence analysis of GPR61 and GnRHR aggregation at the surface of cultured bovine AP cells treated with Triton X-100. Images of LHβ (red), GnRHR (light blue), and GPR61 (green) expression were captured by laser confocal microscopy; nuclei were counterstained with DAPI (dark blue). Scale bars, 50 µm.

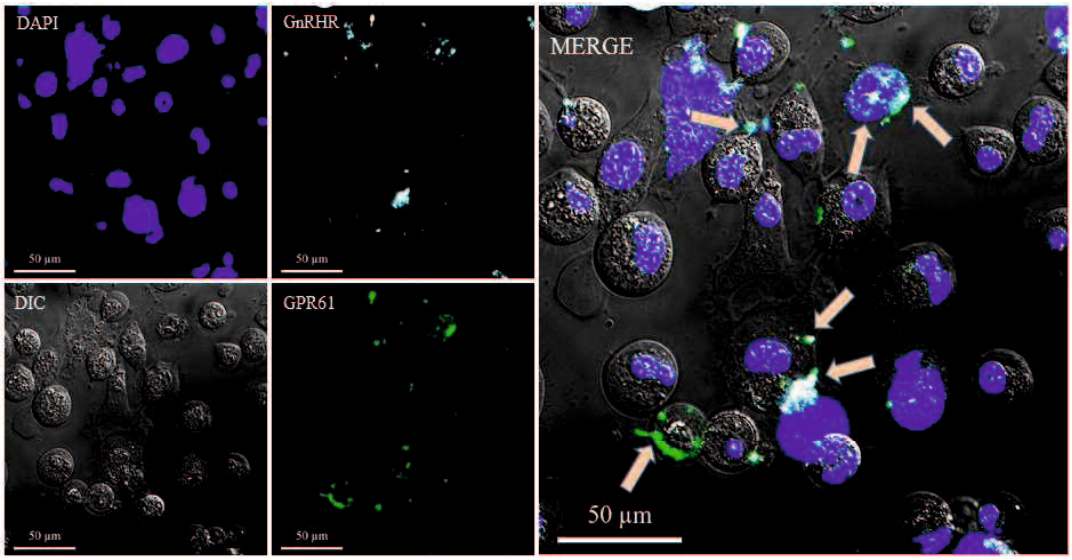


Fig. 4.6. Immunofluorescence analysis of GPR61 and GnRHR aggregation at the surface of cultured bovine AP cells without Triton X-100. Images of GnRHR (light blue) expression were captured by laser confocal microscopy; nuclei were counterstained with DAPI (dark blue). Scale bars, 50 µm.

4.4. Discussion

Previous studies have reported that gonadotrophs are a heterogeneous cell population comprising LH and FSH monohormonal and bihormonal subsets in rats, equines, and bovines (Townsend et al., 2004; Pals et al., 2008; Kadokawa et al., 2014). I speculated that LHβ-negative/GnRHR-positive and FSHβ-negative/GnRHR-positive cells are FSH and LH monohormonal gonadotrophs, respectively. Accordingly, I found that about 70% of gonadotrophs in bovine AP were GPR61-positive. I obtained a strong positive overlap coefficient between GPR61 and GnRHR on the cell-surface. The overlap coefficient was larger than a reported overlap coefficient between GnRHR and flotillin-1 in cultured LβT2 cells (Wehmeyer et al., 2014). GPR61 associates with Gs protein (Takeda et al., 2003; Toyooka et al., 2009) and stimulates ERK signaling in neurons (Hossain et al., 2016). In addition, GPR61 was found to inhibit cAMP-dependent reporter gene expression induced by forskolin in Chinese hamster ovary cells (Martin et al., 2015). The ERK and cAMP pathways inhibit and stimulate GnRH-induced LH secretion, respectively, in bovine gonadotrophs (Nakamura et al., 2015; Nakamura and Kadokawa, 2015; Rudolf and Kadokawa, 2016). These results suggest that the gonadotroph GPR61 has a very important role in inhibiting the GnRH-induced LH secretion by both decreasing cAMP and activating the ERK pathway. Moreover, these results suggest that the gonadotroph GPR61 must be decreased when pulsatile LH secretion is activated in heifers in the early luteal phase.

I found that GPR61 expression in bovine AP tissue was lower during the early luteal phase, when blood concentrations of estradiol and progesterone are low (Kadokawa and Yamada, 1999), as compared to other phases during the reproductive cycle. From study I, I found that GPR61 is expressed in the AP of heifers, and that the

level was higher during pre- as compared to post-ovulation. I also found half ERE and ERE- and PRE-like sequences in the 5'-flanking region of the bovine *GPR61* gene. A caveat here is that half ERE and PRE-like sequences are only five nucleotides long; such short sequence can appear at random every 1024 (= 4^5) nucleotides. Thus, some of the identified sequences may not be involved in the control gene expression. Whether estradiol or progesterone regulate *GPR61* expression remains to be determined. Gonadotropin secretion in ruminants is modulated by various factors including nutrition, estrous stage, season, and stress (Kadokawa et al., 1998; Phogat et al., 1999; Stackpole et al., 2003; Kadokawa, 2007). Therefore, further studies are required to clarify the relationship between these factors and GPR61 GnRHR in bovine gonadotrophs.

A recent study (Hossain et al., 2016) suggested that plasmalogens are ligands for GPR61. Plasmalogens are glycerophospholipids that activate ERK and Akt pathways in neuronal cells (Hossain et al., 2013), and are enriched in the lipid rafts of KB cells, a line of human epidermal carcinoma cells (Pike et al., 2002). In the present study, I demonstrated that GPR61 colocalizes with GnRHR on the surface of gonadotrophs. Therefore, further studies are required to clarify whether plasmalogen is the ligand for GPR61 expressed in gonadotrophs.

The GPR61 protein was approximately 23 kDa, as determined by western blotting; this was smaller than the size of 49 kDa predicted from the amino acid sequence. There are no previous reports of GPR61 protein size in any species. Band sizes in western blotting often differ from expected sizes in the case of membrane-bound proteins such as cell-surface receptors. This is due to a complex three-dimensional structure, which can include hydrophilic and lipophilic regions that form extracellular, transmembrane, and cytoplasmic domains (Larsoon, 2009; Rabillouid,

2009). For example, the molecular weight of GnRHR in cattle was previously estimated as 30 kDa (Hazum and Keinan, 1984), which was not consistent with the value of 37.6 kDa calculated based on the NCBI reference sequence (NP_803480). The molecular weight of GPR61 was also measured as 23 kDa under other reducing conditions using sample buffer containing either β-mercaptoethanol or dithiothreitol with incubation at either 65°C for 15 min or at 25°C for 16 h (data not shown). I also performed western blotting under non-reducing conditions using a sample buffer lacking either β-mercaptoethanol or dithiothreitol, and then, I measured the molecular weight of GPR61 as 18 kDa, smaller than 23 kDa (data not shown).

I found that about 25% of GPR61-positive cells were non-gonadotrophs. Although the precise function of GPR61 is unknown, GPR61-deficient mice exhibited obesity associated with hyperphagia (Nambu et al., 2011); moreover, GPR61 has been implicated in type 2 diabetes (Yuan et al., 2014). Our findings suggest that GPR61 in non-gonadotrophs may play an important role in regulating food intake and body weight, which is a possibility that bears further consideration in future investigations.

I could not detect FSH in immunocytochemistry assays on cultured AP cells. There are two possible reasons. The first possibility is that the used culture system may be appropriate only for detection of LH, GH, and prolactin, but not FSH because some factor (e.g. activin etc.) may be absent or present at insufficient level for continuous stimulation of FSH production. Another possibility is that FSH is a very difficult hormone to measure, unlike LH, because of its fragile 3-dimensional structure. For example, an radioimmunoassay kit for LH detection from the internationally renowned institute, NIDDK, contains an anti-LH polyclonal antibody and bovine LH, which can be used as both standard and labeling. However, NIDDK's radioimmunoassay kit for FSH estimation contains an anti-FSH polyclonal antibody,

bovine LH only as standard (high purity), and bovine FSH only for labeling (low purity). Therefore, it is possible that the anti-bovine FSH mouse monoclonal antibody cannot detect FSH in the cultured cells, although I have used the best anti-bovine FSH mouse monoclonal antibody in our study, and there is no other appropriate anti-bovine FSH antibody.

These data suggested that GPR61 colocalizes with GnRHR in the plasma membrane of gonadotrophs, and its expression changes stage-dependently in bovine anterior pituitary.

CHAPTER V

(Study III)

Heifers Express GPR153 in Gonadotrophs in Stage-Dependent Manner

Abstract

The study I discovered that GPR153 is expressed in the AP of heifers, leading us to speculate that GPR153 colocalizes with GnRHR in the plasma membrane of gonadotrophs and is expressed at specific times of the reproductive cycle. To test this hypothesis, I examined the coexpression of GnRHR, GPR153, and either LH or FSH in AP tissue and cultured AP cells by immunofluorescence microscopy. GPR153 was detected in gonadotrophs, with majority of them being colocalized with GnRHR and the remainder present at other parts of the cell surface or in the cytoplasm. I obtained a strong positive overlap coefficient (0.75 ± 0.02) between GPR61 and GnRHR on the cell-surface of cultured GnRHR-positive AP cells. Real-time PCR and western blot analyses found that expression was lower ($P < 0.05$) in AP tissues during early luteal phase as compared to pre-ovulation or mid- or late luteal phases. Additionally, the 5'-flanking region of the *GPR153* gene contained one ERE and several other sites with response elements similar to those of estrogen or progesterone. These data suggested that GPR153 colocalizes with GnRHR in the plasma membrane of gonadotrophs, and its expression changes stage-dependently in the bovine AP.

5.1. Introduction

GPR153 is an orphan receptor that is widely expressed in the brain, including in the hypothalamus arcuate nucleus and pituitary (Sreedharan et al., 2011). GPR153 gene is conserved in chimpanzee, Rhesus monkey, dog, cow, mouse, rat, chicken, zebrafish, and frog. The study I discovered that GPR153 is expressed in the AP of heifers and is significantly highly expressed in pre-ovulation APs. There have been very few studies on this receptor and hence limited references are available. In the present study, I tested the hypothesis that GPR153 colocalizes with GnRHR in plasma membrane of bovine gonadotrophs and that its expression is dependent on reproductive stage.

5.2. Materials and methods

5.2.1. Antibodies

I previously determined using the SOSUI v.1.11 algorithm that bovine GPR153 protein (610 amino acids; accession number XP_005217221 in NCBI reference bovine sequences) contains seven hydrophobic transmembrane domains linked by hydrophilic extracellular and intracellular loops (Hirokawa et al., 1998; http://harrier.nagahama-i-bio.ac.jp/sosui/). Bovine GPR153 has 89% homology to the human protein (609 amino acids; NCBI accession number AAH68275.1). I used a rabbit polyclonal anti-GPR153 antibody against the extracellular region of human GPR153 (ORB183887, Biorbyt) whose peptide antigen corresponds to amino acids 147–161 (VGWHDTSERFYTHGC) in the extracellular domain of human GPR153. This sequence has 100% homology to amino acids 147–161 in the extracellular domain of bovine GPR153 but not to other proteins in the bovine genome based on sequences retrieved from DNA Data Bank of Japan/GenBank/European Bioinformatics Institute Data Bank using the protein basic local alignment search tool. Details of other antibodies are similar to described in chapter IV.

5.2.2. AP sample collection, protein and RNA analysis and immunofluorescence

Samples were collected in the same manner as detailed in chapter IV. Protein extraction and western blotting was done against GPR153 following the same procedure as described in chapter IV. RNA extraction, synthesis of cDNA and realtime PCR were carried out as described in chapter IV using the primer for GPR153. Immunofluorescence was similar to described in chapter IV except that rabbit polyclonal anti-GPR153 was used. Immunocytochemical analysis of cells was same as described in chapter IV using the rabbit polyclonal anti-GPR153. The obtained data were analyzed using the same methods as described in chapter IV (study

II).

5.2.3. Analysis of the GPR153 gene 5'-flanking region

The 5000-nucleotide sequence of the 5'-flanking region of the *GPR153* gene (chromosome 16: 47933012-47938011) was obtained using the online Ensembl and BLAT Search Genome program (http://genome.ucsc.edu) (Cow Jun. 2014, Bos_taurus_UMD_3.1.1/bosTau8). The sequence was analyzed using Genetyx software v.13 (Genetyx) for the presence of consensus response element sequences for estrogen—i.e., ERE (5'-GGTCANNNTGACC-3'), ERE-like sequence (5'-TGACCCCTGGGTCA-3') (Gruber et al., 2004), and half ERE (GGTCA, TGACC, or TGACT) (Liu et al., 1995), as well as for progesterone—i.e., PRE (5'-G/A G G/T AC A/G TGGTGTTCT-3') (Geserick et al., 2005) and half PRE (5'-TGTTCT-3') (Tsai et al., 1988).

5.3. Results

5.3.1. Relationship between GPR153 expression and estrous stage in bovine AP tissue

The ANOVA revealed a significant effect of estrous stage on GPR153 mRNA ($P < 0.05$; **Fig. 5.1**) and protein ($P < 0.05$; **Fig. 5.2**) expression: the level was lower in the AP of heifers in early luteal as compared to other phases of the reproductive cycle. The GPR153 band size was around 23 kDa by western blot analysis. Such band was not observed in the negative control membranes for which the primary antiserum had been omitted or pre-absorbed with antigen peptide or for which the normal guinea pig IgG had been used instead of the primary antibody.

5.3.2. Immunofluorescence analysis of GnRHR and GPR153 expression in bovine AP tissue

Coexpression of LH or FSH with GnRHR and GPR153 in bovine AP tissue was investigated by immunohistochemistry. LH and GPR153 showed colocalization, with the latter strongly expressed at the plasma membrane of LH-/GnRH-positive and LH-negative/GnRH-positive cells in each pituitary gland **(Fig. 5.3)**. FSH and GPR153 were also colocalized, with the latter strongly expressed at the plasma membrane of FSH-/ GnRH-positive and FSH-negative/GnRH-positive cells in each pituitary gland **(Fig. 5.4)**. GPR153 was detected in gonadotrophs, with majority of them being colocalized with GnRHR and the remainder present at other parts of the cell surface or in the cytoplasm. Percentages of single- and double-labeled GPR153- and GnRHR-positive cells were determined from 12 representative confocal images per pituitary gland. In each pituitary gland, there were an average of 52.3 ± 3.0 GnRHR-positive cells, 49.3 ± 2.2 GPR153-positive cells, and 43.3 ± 2.2 double-positive cells; 82.9% ±

2.7% of GnRHR-positive cells were GPR153-positive, whereas 87.8% ±0.6% of GPR153-positive cells were GnRHR-positive.

5.3.3. GPR153 and GnRHR aggregate on the surface of cultured AP cells

GPR153 and GnRHR aggregated on the surface of LH-/GnRH-positive and LH-negative/GnRH-positive cultured AP cells prepared by the PFA-Triton method **(Fig. 5.5)**. In the AP cells prepared by the CellCover method, GPR153 aggregated on the surface of GnRH-positive cells **(Fig. 5.6)**. The overlap coefficient between GPR153 and GnRHR was 0.75 ± 0.02 on the cell surface of cultured AP cells.

5.3.4. ERE, PRE, and similar sequences in the 5'-flanking region of bovine GPR153 gene

The 5'-flanking region of the bovine *GPR153* gene was analyzed for EREs, PREs, and similar sequences **(Table 5.1)**. One ERE, no PRE sequences, seven ERE-like, 23 half ERE, and three PRE-like sequences were identified.

Table 5.1. Analysis of ERE, ERE-like, half ERE, PRE, and PRE-like sequences in the 5000-nucleotide of 5'-flanking region of *GPR153* gene

Response	element	Number of sites	Position*
ERE	5'-GGTCANNNTGACC-3'	1	2826
ERE-like	5'-TGACCCCTGGGTCA-3'	7	1005, 1156, 1171, 1220, 2095, 2104, 3319
Half ERE	5'-GGTCA-3'	11	355, 957, 1005, 1064, 1171, 1220, 1934, 2104, 2322, 2826, 3951
Half ERE	5'-TGACC-3'	8	845, 900, 922, 1854, 2853, 3685, 3815, 4375
Half ERE	5'-TGACT-3'	4	1156, 2095, 3467, 3919
PRE	5'-G/A G G/T AC A/G TGGTGTTCT-3'	0	
PRE-like	5'- GGGTATAACCTGACATTCCG TCCTCTGGCTGAGTGTTCT-3'	1	627
PRE-like	5'-TGTTCT-3'	2	533, 660

*Nucleotide 5000 upstream of the start codon is defined as nucleotide 1.

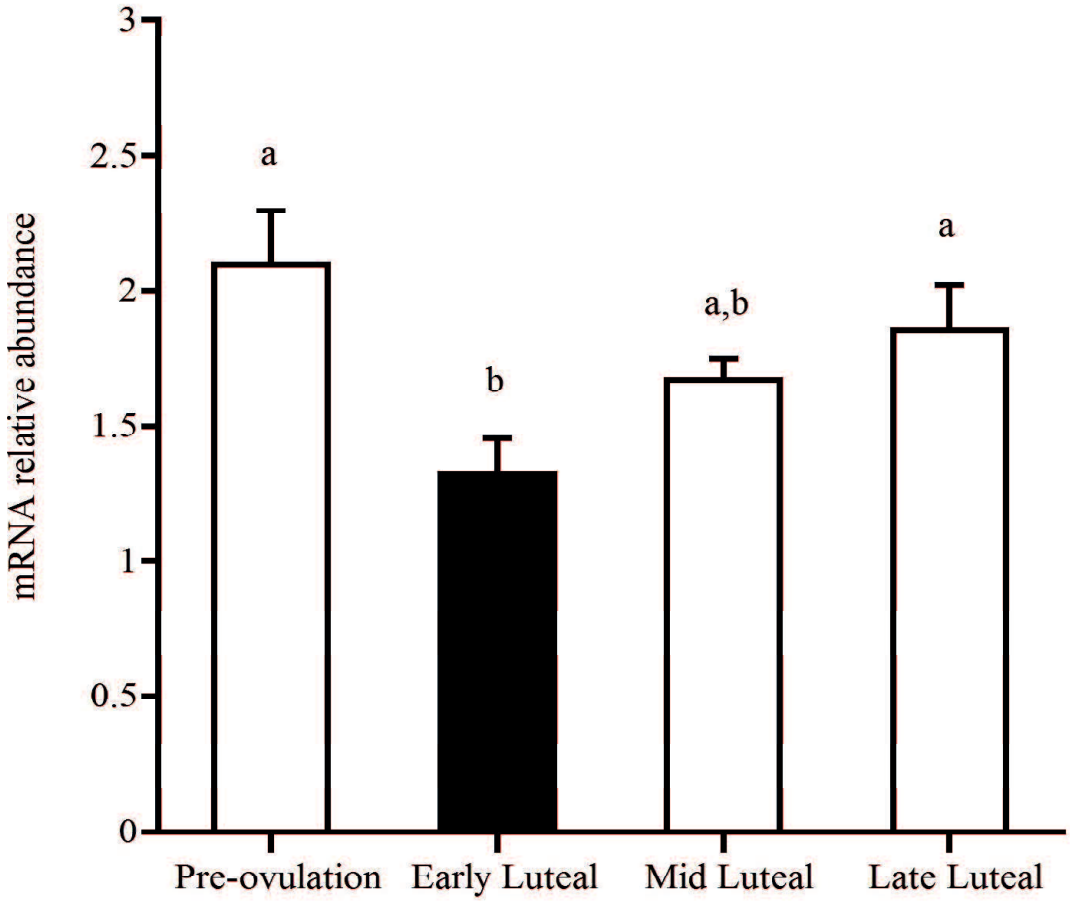


Fig 5.1. Relative *GPR153* mRNA levels (mean ± SEM) in bovine AP during pre-ovulation [day 19 to 21 (day 0 = day of estrus); n = 5], early luteal (day 2 to 5; n = 8), mid-luteal (day 8 to 12; n = 9), or late luteal (day 15 to 17; n = 7) phases, as determined by real-time PCR. Data were normalized to the geometric mean of *GAPDH* and *RAN-binding protein* (*RANBP10*) levels. Letters (a *vs.* b) indicate significant differences ($P < 0.05$) among phases.

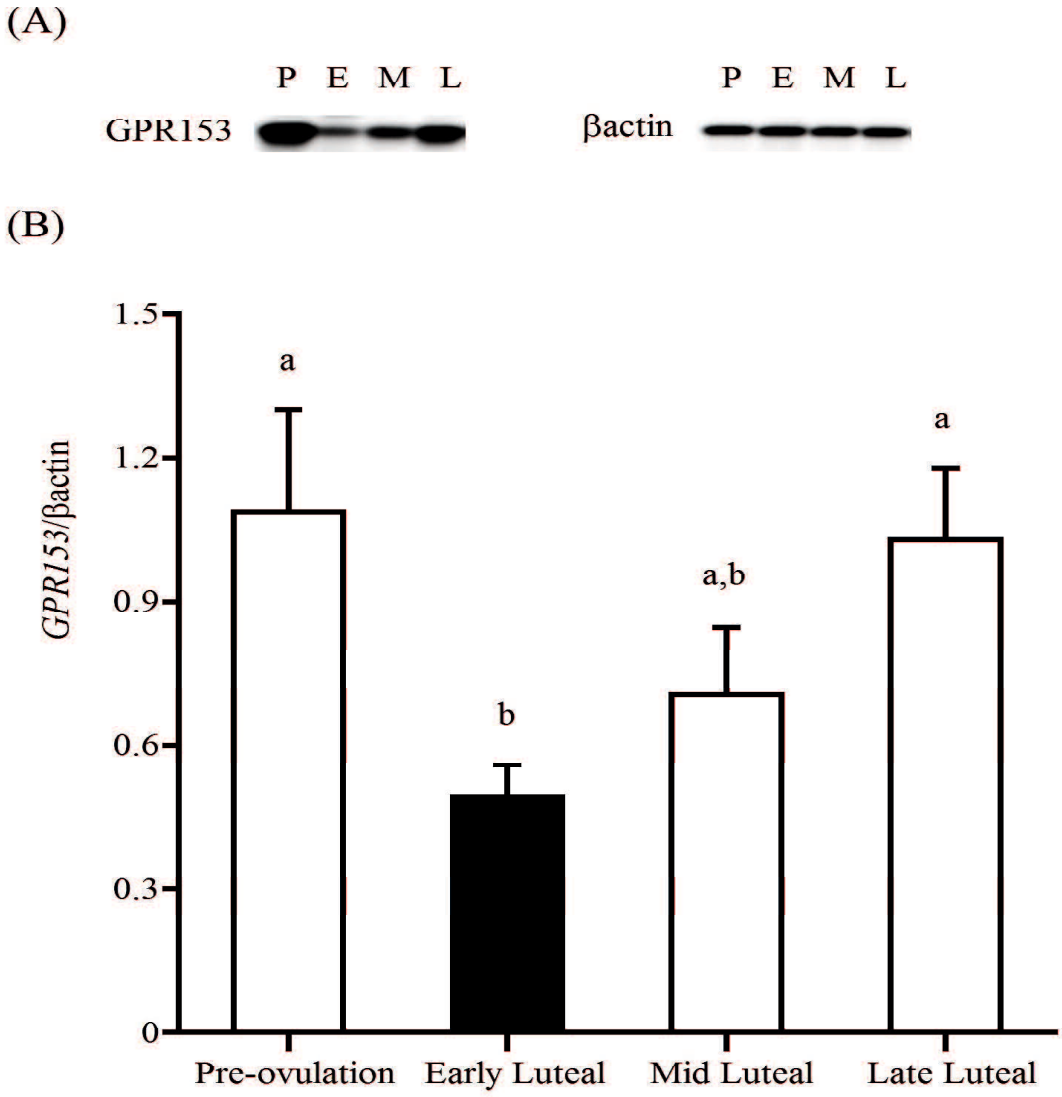


Fig 5.2. (A) Representative GPR153 and β-actin protein expression in bovine AP tissue obtained during the pre-ovulation (P), early luteal (E), mid-luteal (M), or late luteal (L) phases, as detected by western blotting. (B) GPR153 protein expression normalized to that of β-actin in bovine AP tissue obtained during pre-ovulation (n = 5), early luteal (n = 8), mid-luteal (n = 9), and late luteal (n = 7) phases. Letters (a *vs.* b) indicate significant differences ($P < 0.05$) among phases.

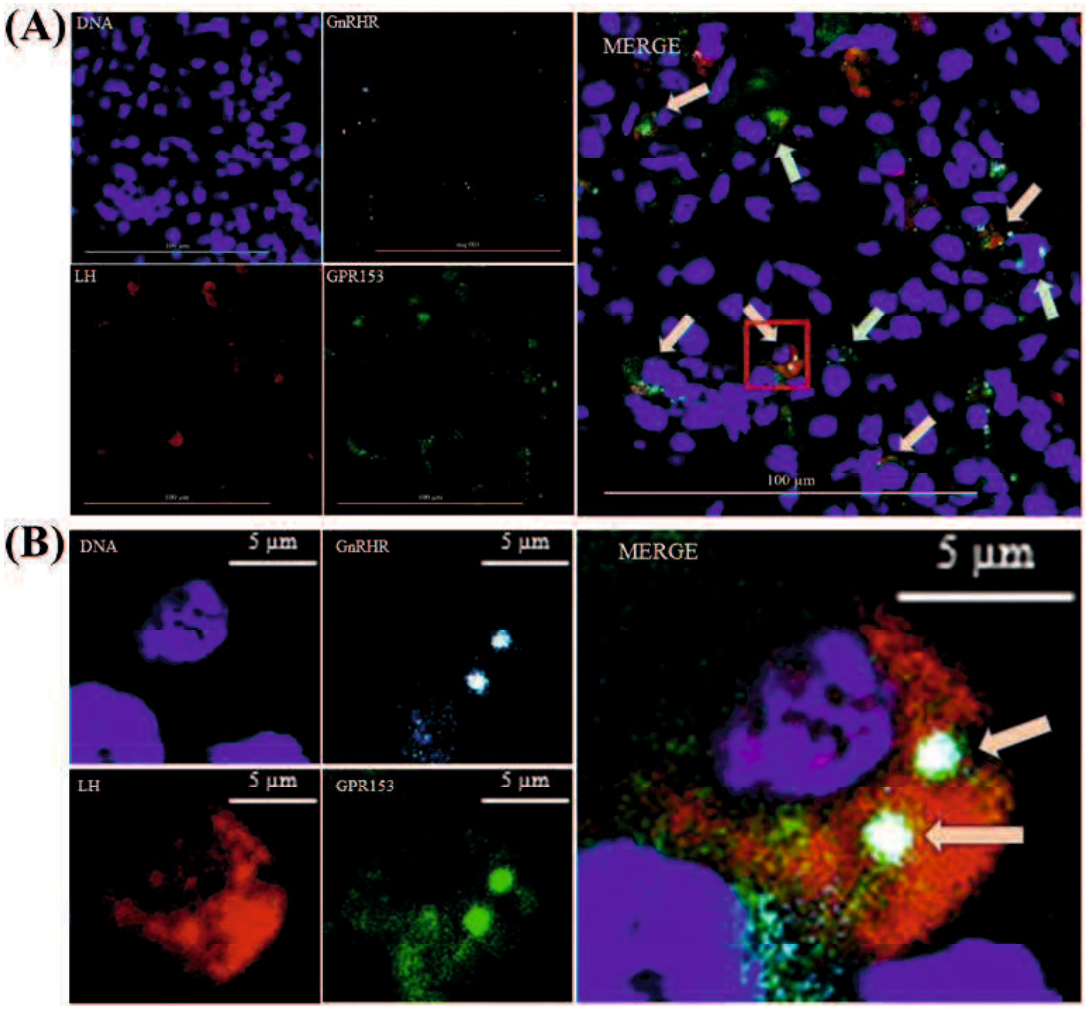


Fig 5.3. Immunofluorescence analysis of LH (red), GnRHR (light blue), and GPR153 (green) expression in bovine AP tissue by laser confocal microscopy (A; scale bars 100 µm). Higher magnification of the region of interest (shown with red square) is shown in (B; scale bars 5µm). Nuclei were counterstained with DAPI (dark blue). Yellow indicates colocalization of LH and GPR153. High GPR153 expression was observed at the plasma membrane of LH-/GnRH-positive cells (red arrows) and LH-negative/GnRH-positive cells (green arrows). Majority of the GPR153 colocalized with GnRHR in gonadotrophs.

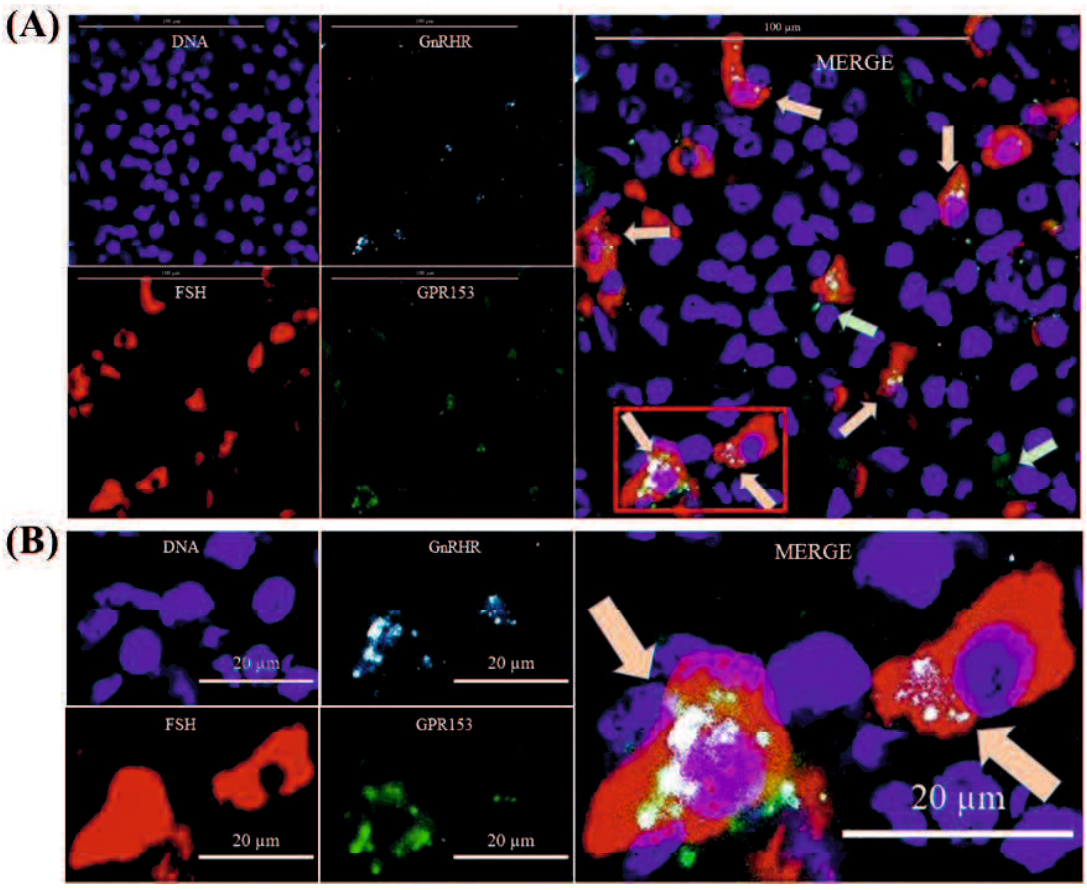


Fig 5.4. Immunofluorescence analysis of FSH (red), GnRHR (light blue), and GPR153 (green), in bovine AP tissue by laser confocal microscopy (A; scale bars 100 µm). Higher magnification of the region of interest (shown with red square) is shown in (B; scale bars 20 µm). Nuclei were counterstained with DAPI (dark blue) Yellow indicates colocalization of FSH and GPR153. High GPR153 expression was observed at the plasma membrane of FSH-/GnRH-positive cells (red arrows) and FSH-negative/GnRH-positive cells (green arrows). Majority of the GPR153 colocalized with GnRHR in gonadotrophs.

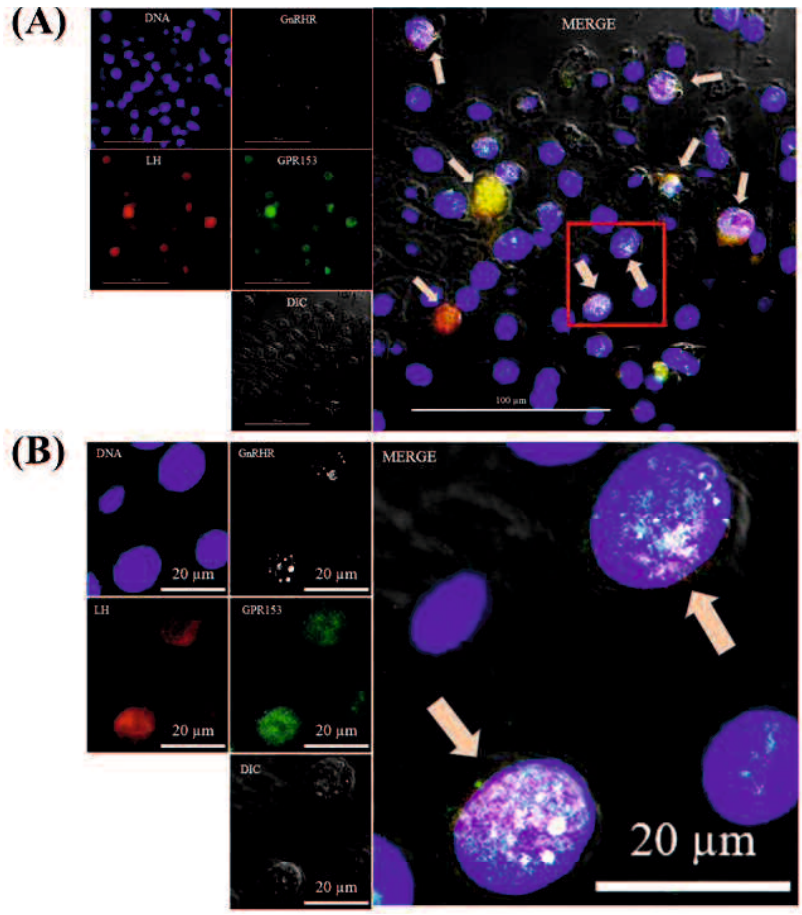


Fig 5.5. Immunofluorescence analysis of LH (red), GnRHR (light blue), and GPR153 (green) expression, in cultured bovine AP cells treated with Triton X-100, by laser confocal microscopy (A; scale bars 100 µm). Higher magnification of the region of interest (shown with red square) is shown in (B; scale bars 20 µm). Nuclei were counterstained with DAPI (dark blue). Cell contours can be seen by differential interference contrast (DIC). Anti-GPR153 and -GnRHR antibodies bound GPR153 and GnRHR, respectively, on the gonadotroph surface and cytoplasm. High GPR153 expression was observed at the plasma membrane of LH-/GnRH-positive cells (red arrows) and LH-negative/GnRH-positive cells (green arrows). Majority of the GPR153 colocalized with GnRHR in gonadotrophs.

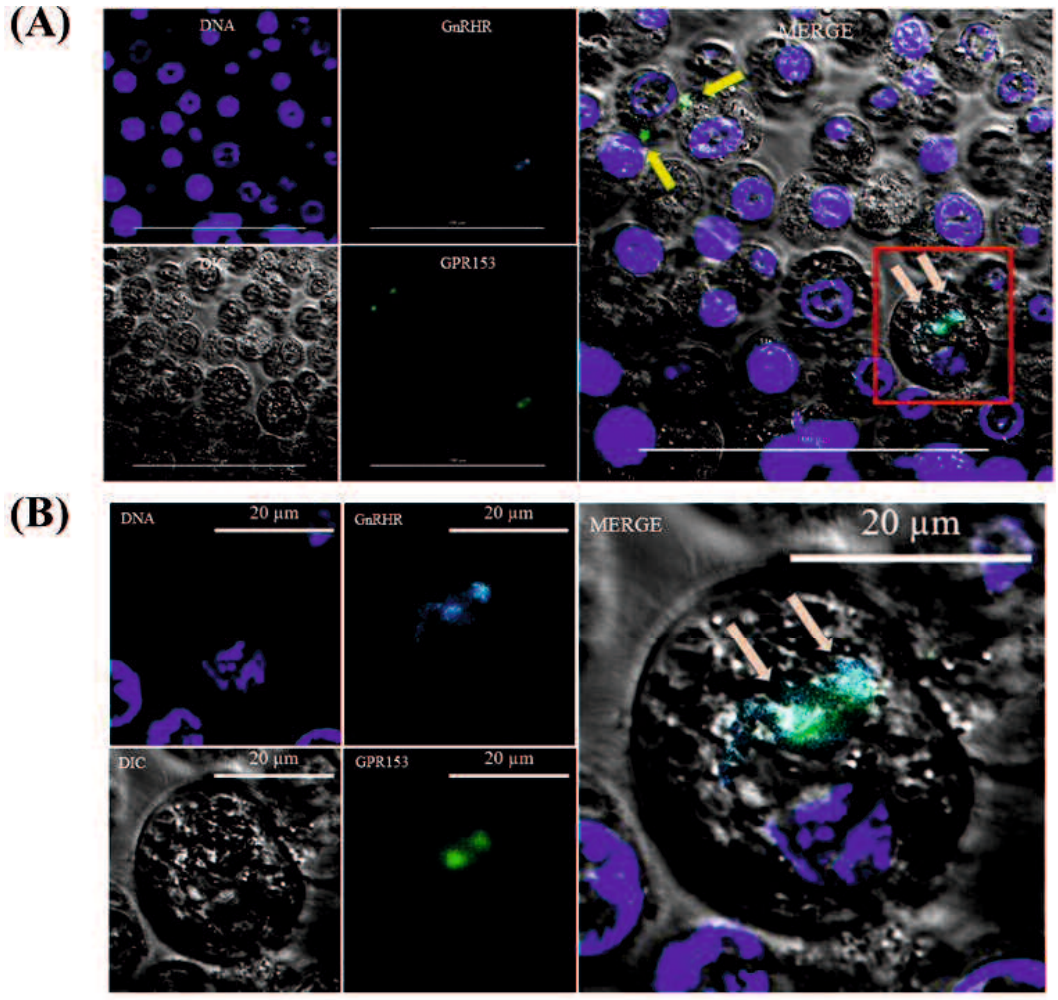


Fig 5.6. Immunofluorescence analysis of GPR153 and GnRHR aggregation at the surface of cultured bovine AP cells without Triton X-100 treatment (A; scale bars 100 µm). Higher magnification of the region of interest (shown with red square) is shown in (B; scale bars 20 µm). Images of GnRHR (light blue) expression were captured by laser confocal microscopy; nuclei were counterstained with DAPI (dark blue). Cell contours can be seen by differential interference contrast (DIC). Anti-GPR153 and -GnRHR antibodies bound GPR153 and GnRHR, respectively, on the gonadotroph surface. GPR153 and GnRHR were highly expressed by appeared aggregated. Majority of the GPR153 colocalized with GnRHR in gonadotrophs (green arrows).

5.4. Discussion

Gonadotrophs are a heterogeneous cell population comprising LH and FSH monohormonal and bihormonal subsets in rats, equines, and bovines (Townsend et al., 2004; Pals et al., 2008; Kadokawa et al., 2014). I speculated that LH-negative/GnRHR-positive and FSH-negative/GnRHR-positive cells are FSH and LH monohormonal gonadotrophs, respectively. Accordingly, I found that about 83% of gonadotrophs in bovine AP were GPR153-positive. I obtained a strong positive overlap coefficient between GPR153 and GnRHR on the cell-surface. The overlap coefficient was larger than a reported overlap coefficient between GnRHR and flotillin-1 in cultured LβT2 cells (Wehmeyer et al., 2014). Lipid rafts containing GnRHR also harbor glucocorticoid receptor (Wehmeyer et al., 2014) in the LβT2 cells. Glucocorticoid receptor increases GPR153 gene expression in A549 human lung adenocarcinoma cells (Wang et al., 2004). Although GPR153's function and ligand(s) are unknown, these results suggest that GPR153 may affect GnRHR downstream signaling, although additional studies are required to identify its ligand.

Pulsatile LH secretion is activated in heifers in early luteal phase when blood concentrations of estradiol and progesterone are low (Kadokawa & Yamada, 1999). I found that GPR153 expression in bovine AP tissue was lower during this phase as compared to others during the reproductive cycle. The study I found that GPR153 is expressed in the AP of heifers, and that the level was higher during pre- as compared to post-ovulation. I also found ERE, half ERE and ERE- and PRE-like sequences in the 5'-flanking region of the bovine *GPR153* gene in this study. The 5'-flanking region of the *GPR153* gene contained only one site of ERE. Koohi et al. (2007) reported that one site of ERE is enough to receive estrogen-activated nuclear estrogen receptor α for bovine oxytocin promoter to show estrogen response. However, Derecka et al.

(2006) reported that same ERE can show estrogen response in the presence of thymidine kinase promoter, but not cytomegalovirus promoter in a reporter plasmid (Derecka et al., 2006). Therefore, further studies are required to clarify whether the discovered ERE receive estrogen-activated nuclear estrogen receptor α to show estrogen response. Also we need to be careful that bovine gonadotroph express GPR30, the membrane estrogen receptor (Rudolf & Kadokawa, 2013; Rudolf & Kadokawa, 2016). Therefore, further studies are required to clarify whether estradiol or progesterone regulate *GPR153* expression.

The GPR153 protein was approximately 23 kDa, as determined by western blotting; this was smaller than the size of 66 kDa predicted from the amino acid sequence. There are no previous reports of GPR153 protein size in any species. The molecular weight of GPR153 was also measured as 23 kDa under other reducing conditions using sample buffer containing β-mercaptoethanol with incubation at either 65°C for 15 min or at 25°C for 16 h (data not shown).

I found that about 17% of GPR153-positive cells were non-gonadotrophs. Although the precise function of GPR153 is unknown, in rats, the antisense oligodeoxynucleotide knockdown of GPR153 caused a reduction in food intake (Sreedharan et al., 2011). Our findings suggest that GPR153 in non-gonadotrophs may play an important role in regulating food intake, which is a possibility that bears further consideration in future investigations.

These data suggested that GPR153 colocalizes with GnRHR in the plasma membrane of gonadotrophs, and its expression changes stage-dependently in bovine anterior pituitary.

CHAPTER VI

(Study IV)

Method for Isolating Pure Bovine Gonadotrophs from AP using Magnetic Nanoparticles and Anti-GnRHR Antibody

Abstract

No methods are currently available for rapidly isolating gonadotrophs from AP in any species. We developed a method for preparing pure bovine gonadotrophs from a heterogeneous AP cell mixture by magnetic separation and our original antibody against the N terminus of bovine GnRHR. A bovine AP cell mixture was incubated with the anti-GnRHR antibody, anti-dextran antibody-conjugated secondary antibody, and dextran-coated magnetic nanoparticles for magnetic isolation. Approximately 5.2 $\times 10^6$ cells were isolated per AP of Japanese Black heifers (26 months of age) and cultured, and confocal microscopy confirmed to be GnRHR- and LH-positive, corresponding to a purity of 100%. Approximately 44.5 μg of total protein was extracted from the pure gonadotrophs per AP.

6.1. Introduction

Gonadotrophs in the AP are important cells; however, the AP has a heterogeneous cell population and gonadotrophs constitute only 10 to 15% of all cells in the AP and are scattered among other cell types in rats and humans (Ben-Shlomo et al., 2011). To clarify the mechanisms of action of hormone-secreting AP cells, previous studies have tried to purify specific cell types from heterogeneous AP cell mixtures; however, these attempts have resulted in failure to obtain gonadotrophs in rats (Wynick et al., 1990) or in a low purity of gonadotrophs in sheep (Chen et al., 1994).

We recently developed a highly specific antibody that recognizes the extracellular region at the N terminus of bovine GnRHR (Kadokawa et al., 2014). We could obtain pure bovine gonadotrophs utilizing the antibody for fluorescence-activated cell sorting analysis of bovine AP cell mixture (Kadokawa et al., 2014). However, the slow speed of this method was unsatisfactory, since the system could only process one sample at a time.

The EasySep magnetic separation system (Stemcell Technologies) is a simple method for the simultaneous isolation and purification of cells using a strong magnet. Recently, microglia were purified from murine brain using the EasySep magnetic separation method (Jose et al., 2015).

In the present study, we developed a new method to obtain pure bovine gonadotrophs from AP using EasySep and the anti-GnRHR antibody.

6.2. Materials and methods

6.2.1. Labeling of anti-GnRHR antibody

FITC was conjugated to anti-GnRHR antibody (Kadokawa et al., 2014) using the Surelink FITC Labeling kit (Kirkegaard & Perry Laboratories) according to the manufacturer's protocol. Briefly, 250 μg antibodies (1.67 nM) were incubated with 99.6 nM Surelink FITC for 60 min at room temperature. The mixture was loaded onto a spin ultrafilter included in the kit. Labeled antibody on the ultrafilter was separated from free FITC by centrifugation at 14,000 × g for 10 min at room temperature. Purified FITC-labeled antibody was stored in a light-shielded 1.5-mL microtube containing 0.5 mL of 0.1% BSA in PBS at 4°C.

Using an EZ-Link Sulfo-NHS-LC-Biotinylation kit (Pierce Biotechnology) according to the manufacturer's protocol, 100 μg of anti-GnRHR was labeled. A reaction mixture was prepared at a 1:20 of molar ratio of anti-GnRHR to biotin and incubated at room temperature for 30 min. The reaction mixture was then loaded onto a NAP-5 gel filtration column (GE Healthcare) that had been washed twice with PBS (pH 7.4) and once with 1% BSA dissolved in PBS to minimize the loss of labeled antibody inside the column. Labeled anti-GnRHR was separated from free biotin on the column by elution with PBS, and the 6th to 25th elution drops were collected in 1.5-mL microtubes containing 0.5 mL of 0.1% BSA PBS.

6.2.2. AP collection and cell preparation

APs were obtained from post-pubertal Japanese Black heifers (n = 6, 26 months of age, various stages of the estrous cycle) and cells prepared as discussed in chapter IV. After washing five times in 10mL of HEPES, the tissues were then incubated at 37°C in 10 mL of HEPES buffer containing 11,200 U collagenase (032–

22364; Wako) and 1% BSA (Wako) for 45 min while pipetting every 5 min. After washing with 2% FBS in HEPES buffer (2% FBS), the cells were resuspended in 1 mL of the same solution. We did not use pancreatin to avoid damaging GnRHR on the surface of gonadotrophs. Cell suspensions were passed through a 200-μm filter (Filcons cup; As One Corporation), followed by a 30-μm filter (CellTrics, Partec GmbH) to obtain a single-cell preparation. The yield was $1.9 \times 10^7 \pm 0.9 \times 10^7$ cells per AP, as determined using a cell counter (Model TC20; Bio-Rad). Cell viability was > 95%, as confirmed by Trypan Blue exclusion and the same cell counter.

6.2.3. Magnetic nanoparticle separation using the FITC Positive Selection kit

We used the EasySep Other Cells FITC Positive Selection kit (18558, Stemcell Technologies) according to the manufacturer's instructions. We compared non-fixed cells and cells fixed with CellCover and assessed whether there were any differences in the LH, FSH, and β-actin bands in the western blot. Dissociated AP cells were resuspended in 1 mL of 2% FBS dissolved in Dulbecco's PBS containing 1 mM EDTA (2% FBS). Half of the cell suspension (0.5 mL) was transferred to a 12 × 75 mm polystyrene tube (M. Watanabe & Co.), then, cells were fixed for 2 min at room temperature with CellCover, washed three times with 2% FBS, and incubated for 15 min at room temperature in the dark with 1.5 μg of FITC-labeled anti-GnRHR antibody dissolved in 0.5 mL of 2% FBS. The remaining 0.5 mL of cell suspension was transferred to a separate tube and incubated for 15 min at room temperature with 1.5 μg of FITC-labeled anti-GnRHR antibody without fixation. After the primary antibody reaction, fixed or non-fixed cells were incubated for 15 min at room temperature with 50 μl of the anti-dextran antibody-conjugated anti-FITC antibody. The reaction mixture was incubated for 10 min with 25 μl of dextran-coated magnetic

nanoparticles; the reaction mixture volume was made up to 2.5 mL with 2% FBS with gentle mixing by pipetting. The tube was placed on the EasySep magnet for 5 min at room temperature, and the cell suspension was decanted in a continuous motion to pour off the supernatant fraction (discarded solution) into another separate polystyrene tube, allowing the magnetically labeled cells (i.e., isolated cells) to be retained within the magnetic field. The discarded solution was transferred to a protein low binding microtube (Proteosave SS; Sumitomo Bakelite), which was centrifuged at 400 × g for 5 min at room temperature to obtain the non-isolated cell pellet; this was resuspended in 500 µl of 2% FBS, and 40 µl of the cell suspension were loaded into a µ-Slide VI microscopy chamber (Ibidi) for confocal microscopy observation. The polystyrene tube containing isolated cells was removed from the magnet, and 2.5 mL of 2% FBS were added to the tube. After mixing by pipetting, the tube was replaced on the magnet for 5 min, and inverted to pour off the supernatant fraction. This washing step was repeated, and isolated cells were resuspended in 500 µl of 2% FBS and transferred to another protein low binding microtube; 40 µl were then loaded into another lane of the same µ-Slide VI microscopy chamber for confocal microscopy observation, while 20 µl were used for both cell counts with the same cell counter and Trypan Blue exclusion. The remaining 440 µl of the cell suspension were centrifuged at 400 × g for 5 min at room temperature and the pellet was stored at −80°C until western blot analysis.

6.2.4. Single-fluorescence immunocytochemistry

Isolated and non-isolated fixed cells in the µ-Slide VI microscopy chamber were visualized by the confocal microscopy. Fluorescence and differential

interference contrast images were acquired with CCD camera to prepare single-plane confocal images.

6.2.5. Magnetic nanoparticle separation using the Biotin Positive Selection kit for confocal microscopy observation

We could culture the isolated cells by the above-mentioned FITC Positive Selection method. However, confocal microscopy detected the FITC signal on the surface of approximately 5 % of cells cultured for 3 days; thus, the isolated cells were inappropriate for further confocal microscopic evaluation. Therefore, we additionally used the EasySep Other Cells Biotin Positive Selection kit (18559, Stemcell Technologies) for confocal microscopic observation of the isolated cells after culture.

Dissociated AP cells from AP tissue of post-pubertal Japanese Black heifers (n = 4, 26 months of age) was prepared by the protocol described above. The dissociated AP cells were resuspended in 1 mL of 2% FBS dissolved in DMEM. The cell suspension was incubated for 15 min at room temperature with 3 μg of biotin-labeled anti-GnRHR antibody. After the primary antibody reaction, cells were incubated for 15 min at room temperature with 100 μl of the anti-dextran antibody-conjugated streptavidin. The reaction mixture was incubated for 10 min with 50 μl of dextran-coated magnetic nanoparticles, and then, the cells attached by anti-GnRHR were isolated by the magnet by the protocol described above. The isolated cells were resuspended in 1 mL of DMEM containing 1% nonessential amino acids (100×), 100 IU/mL penicillin, 50 μg/mL streptomycin, 10% horse serum, and 2.5% FBS. The cell suspension (150 μl per lane) was loaded into six lanes of a μ-Slide VI microscopy chamber and cultured at 37°C in a humidified atmosphere of 5% CO_2 for 84 h, while 20 μl of the cell suspension were used for both cell counts with the same cell counter and Trypan Blue exclusion.

The cultured cells in the μ-Slide VI microscopy chamber were treated with 4% PFA in PBS for 3 min, then treated with 0.1% Triton X-100 in PBS for 3 min. After blocking with PBS containing 10% normal goat serum (Wako Pure Chemicals) for 30 min, the treated cells were incubated with the same antibodies for GnRHR, LH, and FSH dissolved in 0.5% normal goat serum PBS (final concentration of each antibody was 1:1,000) for 2 h at room temperature, followed by incubation with a cocktail of fluorochrome-conjugated secondary antibodies (Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 546 goat anti-mouse IgG, and Alexa Fluor 647 goat anti-guinea pig IgG; all 4 μg/mL from Thermo Fisher Scientific) and 1 μg/mL DAPI (Wako Pure Chemicals) for 2 h at room temperature. To verify the specificity of the signals, we included several negative controls in which the primary antibodies had been omitted or pre-absorbed with the antigen peptides or in which normal animal IgGs were used instead of the primary antibodies. Then, the stained cells in the μ-Slide VI microscopy chamber were visualized by the same confocal microscope to prepare single-plane confocal images in order to confirm presence of LH, FSH, and GnRHR.

6.2.6. Data analysis

Differences in total cell or total protein yield per AP between fixed isolated cells and non-fixed isolated cells were compared with the non-paired t test. The level of significance was set at $P < 0.05$. Data are expressed as mean ± SEM.

6.3. Results

6.3.1. Cell and protein yield per pituitary gland

The total cell yields were $5.2 \times 10^6 \pm 2.0 \times 10^6$ and $7.6 \times 10^6 \pm 1.1 \times 10^6$ cells per AP using the protocols for preparing fixed and non-fixed gonadotrophs, respectively; the total protein yields were 44.5 ± 7.0 and 39.1 ± 9.2 µg per AP, respectively. There were no significant differences in the values obtained for fixed and non-fixed cells. Viability of non-fixed cells was > 90%.

6.3.2. Immunocytochemical detection of GnRHR-expressing AP cells

We observed more than 10^3 cells for each AP utilizing confocal microscope. All isolated cells were positive for the FITC signal resulting from the binding of FITC-labeled anti-GnRHR antibody expressed on the surface of gonadotrophs **(Fig. 6.1A)**. In contrast, most non-isolated cells were negative for the FITC signal **(Fig. 6.1B)**, although a few immunoreactive cells were detected.

6.3.3. Confocal microscopy observation of cultured isolated cells

The total cell yields were $7.5 \times 10^6 \pm 1.2 \times 10^6$ cells per AP after the isolation by the Biotin Positive Selection. Cell viability was > 90%. We observed more than 10^3 cells for each AP utilizing confocal microscopy **(Fig. 6.2)**, and confirmed that all of the cells expressed GnRHR and LH, after 84 h.

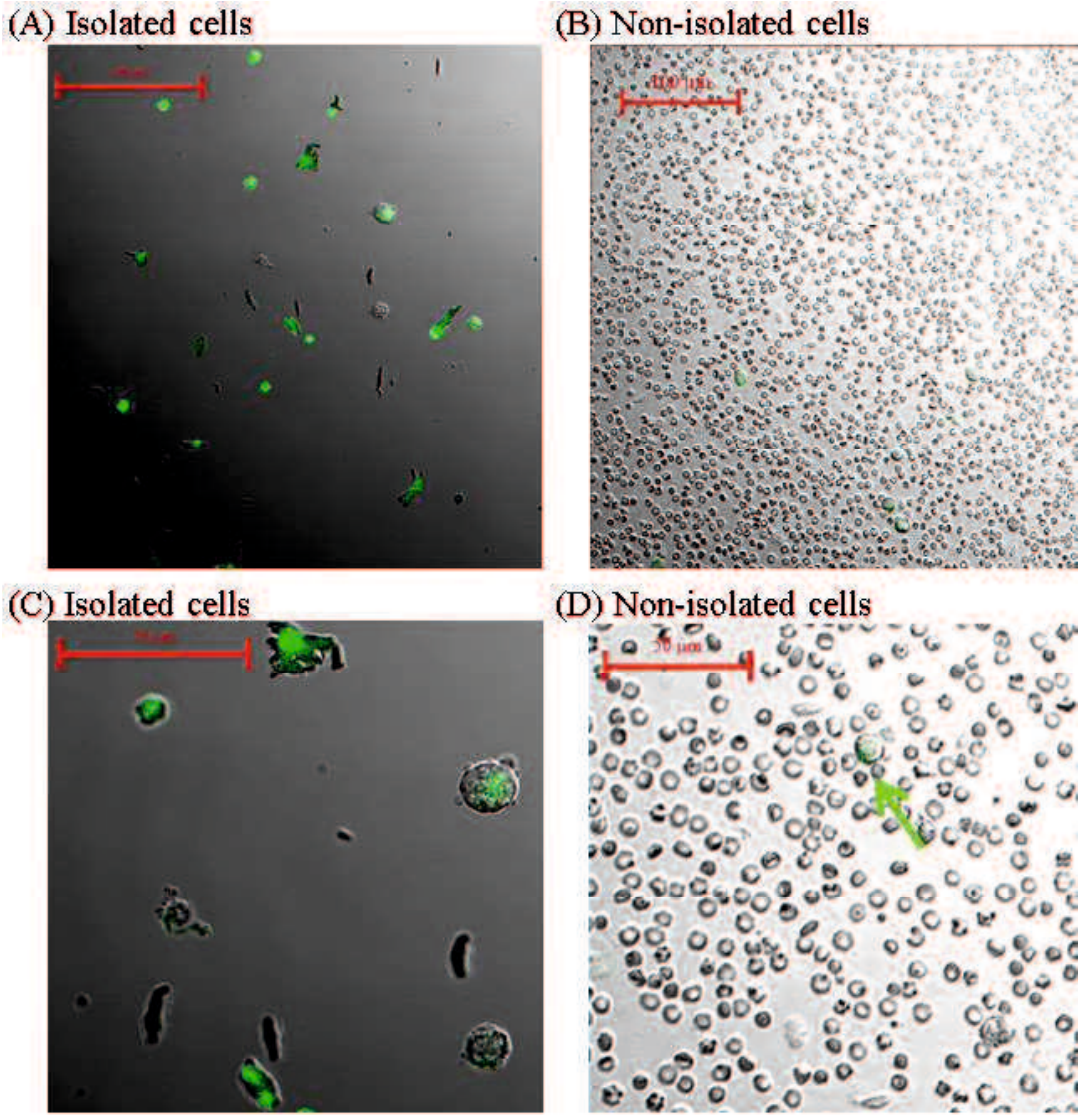


Fig. 6.1. Representative merged FITC and differential interference contrast images of isolated (A, C) and non-isolated (B, D) cells at low (A, B; scale bars=100 μm) and high (C, D; scale bars=50 μm) magnification, obtained by confocal microscopy. FITC-labeled anti-GnRHR antibody was detected on the surface of all isolated cells that were not treated with Triton X-100. Note that all cells were floating and moving inside the microscopy chamber (17 mm long, 3.8 mm wide and 0.4 mm thickness). The green arrow in panel D indicates FITC-positive cells that were not isolated.

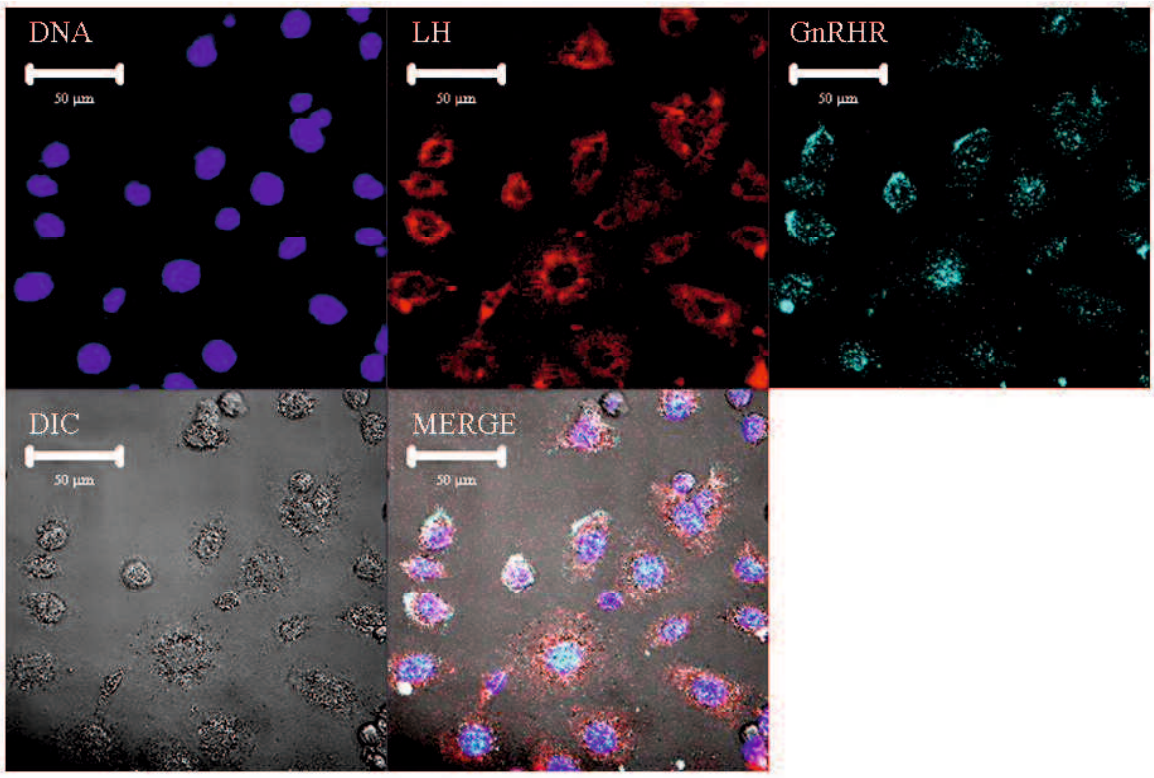


Fig. 6.2. Immunocytochemical analysis of gonadotrophs cultured for 84 h after isolation from heterogeneous bovine AP cells in the microscopy chamber, as visualized by laser confocal microscopy. Cells were labeled with antibodies against LH (red) and GnRHR (light blue); Nuclear DNA was counterstained with DAPI (dark blue), and cell morphology was visualized by differential interference contrast (DIC) imaging. The MERGE is an overlay of the four panels. Cells were treated with Triton X-100, and anti-GnRHR antibody was detected in both the surface and cytoplasm of all isolated cells. Note that all cells were attached to the microscopy chamber. Scale bars=50 µm.

6.4. Discussion

Gonadotrophs are larger than other secretory cells in rat AP (Wynick et al., 1990). The FITC-positive cells observed in this study were larger than those that were negative for FITC, and their shape and size were very similar to bovine gonadotrophs that were immunoreactive to anti-LH and anti-GnRHR antibodies in previous studies (Kadokawa et al., 2014; Bastings et al., 1991). The results of our western blot analysis indicated that the isolated cells contained LH. The results of our confocal microscopic observations indicated that the cells cultured after isolation contained GnRHR, and LH. We therefore conclude that the FITC-positive cells are bovine gonadotrophs, which were purified in the present study by a novel method.

We obtained 10^7 heterogeneous cells per AP after the protocol without pancreatin and isolated 10^6 gonadotrophs per AP from the heterogeneous cells. Gonadotrophs constitute only 10%–15% of all AP cells (Ben-Shlomo et al., 2011); thus, our yield was consistent with estimated values. We obtained about 40 μg total protein per AP, which is sufficient for mass spectrometry-based quantitative proteomics (Chahrour et al., 2015) and proteomics for new protein discovery (Beck et al., 2011).

Nonetheless, our method had certain challenges. Firstly, rat gonadotrophs are not a homogeneous population, and include a wide range of subtypes (Yoshimura et al., 1981), while ovine gonadotrophs can be non-polarized, polarized, and semi-polarized (Crawford et al., 1981). Our method cannot distinguish between different gonadotroph populations. Secondly, GnRHR can move between the cell surface and the cytoplasm (Kadokawa et al., 2014), but our method is only effective for AP cells with cell surface GnRHR expression. Therefore, additional studies are required to identify other cell surface proteins that can distinguish gonadotroph subpopulations.

Dairy cows can secrete LH from AP only weakly in the postpartum period (Kaodkawa et al., 1998) however, the precise mechanisms remain to be clarified. This method may be useful for preparing pure gonadotroph samples from dairy cows.

In conclusion, the method described in this study provides a novel and simple method for the rapid simultaneous purification of bovine gonadotrophs from heterogeneous AP cell mixture using the EasySep magnetic separation method and our original anti-GnRHR antibody.

CHAPTER VII

(Study V)

GPR61 and GPR153 are Expressed in Purified Bovine Gonadotrophs

Abstract

We used the purified gonadotrophs to study the expression of GPR61 and GPR153. Western blotting for the protein extracted from the purified gonadotrophs confirmed the expression of GPR61 and GPR153.

7.1. Introduction

The study I discovered two GPRs, GPR61 and GPR153, which showed significantly higher expression during the pre-ovulatory stage of estrus cycle in bovine AP.

This study aimed to evaluate that GPR61 and GPR153 are expressed in the purified gonadotrophs.

7.2. Materials and methods

APs were collected, and gonadotrophs were isolated using the method described in chapter VI, then stored in deep freezer.

The isolated cell pellets were thawed and homogenized by pipetting and vortexing in 18 µl Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Rockford, IL, USA) containing protease inhibitors (Halt protease inhibitor cocktail; Thermo Fisher Scientific). The total amount of protein in 3 µl of each sample was measured with a bicinchoninic acid kit (Thermo Fisher Scientific), and 10 µg total protein were analyzed alongside 10 µg protein extracted from the AP tissue of two heifers (26 months of age, in the middle of the luteal phase and estrus phase) as control. AP tissue collected from heifers was flash-frozen in liquid nitrogen after collection in the same slaughterhouse on the previous day. Samples were boiled in sample buffer solution with reducing reagent, 4x Laemmli sample buffer (Bio-Rad) containing 10% (v/v) β-mercaptoethanol at 100°C for 3 min. Western blotting were done as same as described in chapter IV.

7.3. Results and discussion

Western blots showed an immune reactive protein band with an apparent molecular weight of 23kDa for GPR61 **(Fig. 7.1A)** and 22kDa for GPR153 **(Fig. 7.1B)** in the protein extracted from the isolated cells.

This study confirmed the expression of GPR61 and GPR153 in gonadotrophs. Further studies are required to explore their role in gonadotroph.

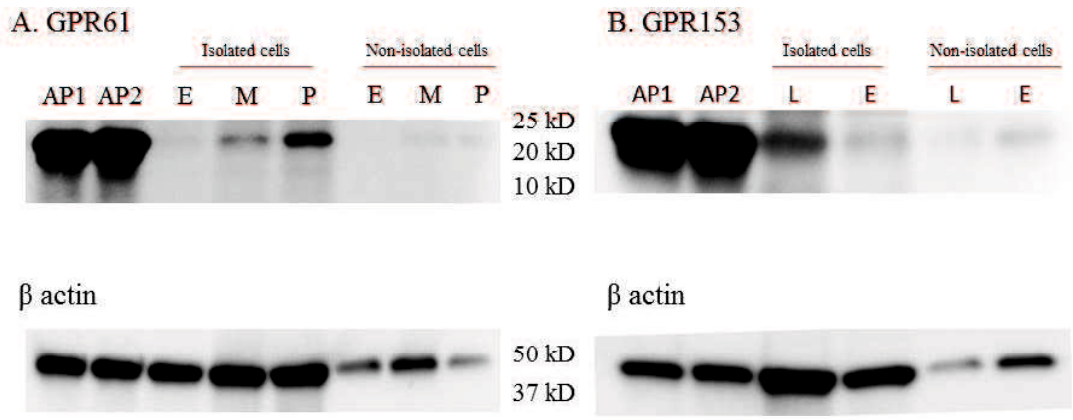


Fig. 7.1. Western blotting of isolated gonadotropes, non-isolated AP cells and AP tissues (positive control) using antibodies against (A) GPR61 and (B) GPR153 along with β actin. The symbol represent as: anterior pituitary luteal (AP1), anterior pituitary estrus (AP2), pre-ovulation (P), early luteal (E), mid-luteal (M), or late luteal (L) phases.

CHAPTER VIII

(Study VI)

Estimation of Importance of GPR173

Utilizing Phoenixin for LH Secretion

Abstract

The study I discovered the expression of GPR173 in bovine AP. Phoenixin may be a ligand of GPR173. Therefore, this chapter evaluated importance of GPR173 for LH secretion from cultured bovine AP cells utilizing phoenixin. However, phoenixin (both phoenixin 14 and phoenixin 20) did not show any significant effect on LH secretion. Therefore, the results did not support any important role of GPR173 for LH secretion form bovine AP.

8.1. Introduction

The study I discovered the expression of GPR173 in bovine AP. Phoenixin is a newly identified, endogenous peptide abundantly produced in the hypothalamus and is reported to be highly conserved across non-mammalian (fish, amphibians, chicken) and mammalian species (rodents, human, bovines) (Treen et al., 2016). Its expression in hypothalamus, pituitary gland and ovary suggests its possible role at different level of HPG axis (Stein et al., 2016). Phoenixin is also expressed in the arcuate neurons that express kisspeptin and contact GnRH neurons in the medial preoptic area, suggesting that phoenixin may stimulate GnRH neurons (Treen et al., 2016). Phoenixin may be the ligand of GPR173 in the brain and the gonads (Treen et al., 2016). Therefore, I evaluated importance of GPR173 for LH secretion from cultured bovine AP cells utilizing phoenixin.

8.2. Materials and methods

APs were collected from post-pubertal Japanese Black heifers in the middle of the luteal phase (n = 8, 26 months old) and cultured. After 58 h culture, the culture medium was replaced by new medium containing either phoenixin 14 or phoenixin 20 (from 1nM to 10000 nM) for further 24 h. Then, medium was replaced with 280ul of 0.1% BSA DMEM containing the same concentration of phoenixin 14 or phoenixin 20. The cells were incubated while gently shaking for 5 min, and then, cells were treated with 20 µL of 15 nM GnRH (Peptide Institute Inc.) dissolved in DMEM for 2 h in order to stimulate LH secretion. The pretreatment plus the GnRH treatment yielded a final concentration of 1 nM, 10 nM, 100 nM, 1000nM or 10,000 nM phoenixin and a final concentration of 1 nM of GnRH. The "control" wells received 0.1% BSA DMEM only. The "GnRH" wells 0.1% BSA DMEM and 20ul of 15nM GnRH. After incubation with GnRH, the medium was collected for immunoassay of LH (Rudolf and Kadokawa, 2014).

LH concentrations in the treated samples for each pituitary were averaged, and the mean LH values were expressed as a percentage of the control value. The mean for the control wells was set at 100%. The statistical significance of differences in LH concentration was analyzed by one-factor ANOVA followed by Fisher's PLSD test. The level of significance was set at $P < 0.05$. Data are expressed as mean ± SEM.

8.3. Results and discussion

The mean LH concentration in the medium of both GnRH wells and the wells treated with either phoenixin 14 **(Fig. 8.1)** or phoenixin 20 **(Fig. 8.2)** were significantly greater ($P < 0.01$) than that of the control wells. None of the wells containing either phoenixin 14 or phoenixin 20 displayed significant differences in mean LH concentrations compared to GnRH wells.

An intracerebroventricular injection of phoenixin stimulated LH secretion in diestrus rats (Stein et al., 2016). The loss of endogenous phoenixin in hypothalamus results in decreased GnRH receptor expression in the AP in rats (Treen et al., 2016). Also phoenixin knockdown by small interfering RNA delayed estrus cycle in rats (Treen et al., 2016). However, neither phoenixin 14 nor phoenixin 20 showed any effect on LH secretion from cultured bovine AP cells. Therefore, phoenixin may have role only in hypothalamus. Phoenixin exert an anxiolytic effect in mice (Jiang et al., 2015) and in obese men (Hofmanna et al., 2017), therefore, phoenixin might have any non-reproductive function in bovines.

Therefore, the results did not support any important role of GPR173 for LH secretion from bovine AP.

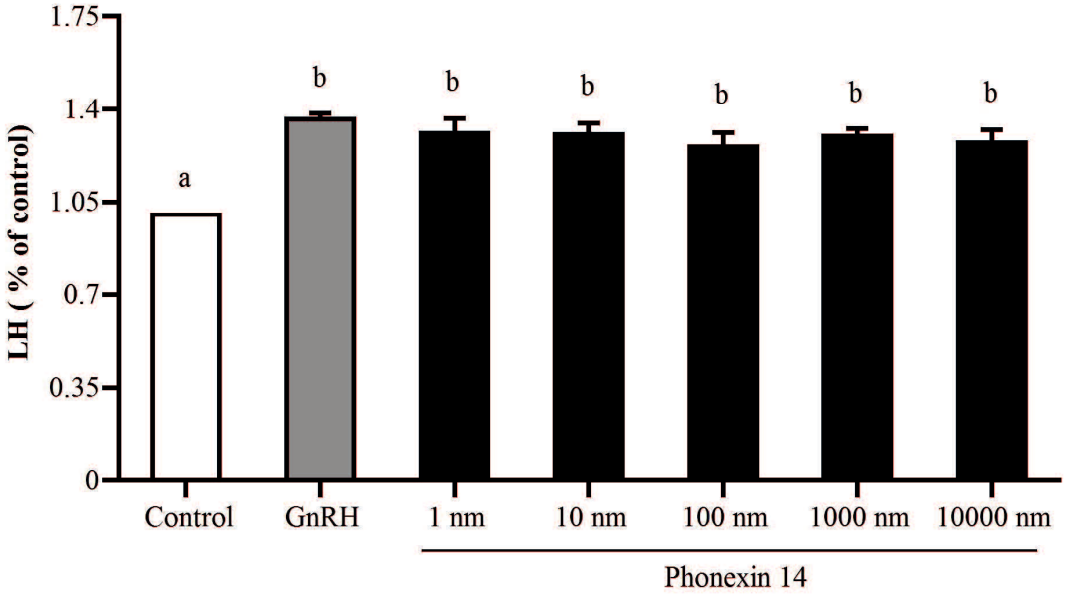


Fig. 8.1. Effect of various concentrations of phoenixin 14 on GnRH induced LH secretion from cultured bovine AP cells. LH concentrations in control cells (cultured in medium alone) were averaged and the mean value set at 100%. LH concentrations of the treated groups are expressed as a percentage of the control. Each value represents the mean concentration ± SEM. Letters (a *vs*. b) indicate significant differences ($P < 0.01$) between control and other treatment.

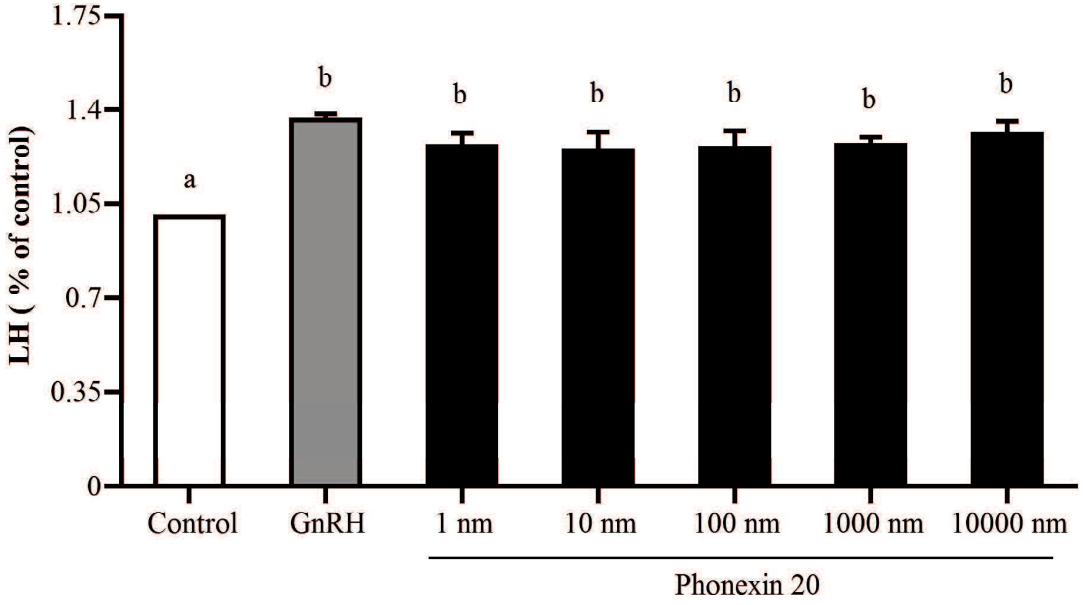


Fig. 8.2. Effect of various concentrations of phoenixin 20 on GnRH induced LH secretion from cultured bovine AP cells. LH concentrations in control cells (cultured in medium alone) were averaged and the mean value set at 100%. LH concentrations of the treated groups are expressed as a percentage of the control. Each value represents the mean concentration ± SEM. Letters (a *vs.* b) indicate significant differences ($P < 0.01$) between control and other treatment.

CHAPTER IX

(Study VII)

Estimation of Importance of GPR101 and GPR173

Utilizing GnRH-(1-5) for LH Secretion

Abstract

The metabolite of GnRH, GnRH-(1-5), may be a ligand of GPR101 and GPR173. Therefore, this chapter evaluated the effect of GnRH-(1-5) on LH secretion from the cultured bovine AP cells. However, GnRH-(1-5) did not show any significant effect on LH secretion. Therefore, the results did not support any important role of GPR101 and GPR173 for LH secretion from bovine AP.

9.1. Introduction

GnRH-(1-5) is a metabolite of GnRH produced by thimet oligopeptidase (EP24.15) (Larco et al., 2015). GnRH-(1-5) may be a ligand of GPR101 and GPR173 (Cho-Clark et al., 2014; Larco et al., 2013). GnRH-(1-5) increases GnRH expression in GT1-7 cells, and GnRH-(1-5) may stimulate GnRH secretion in sheep (Larco et al., 2015). The study I discovered GPR101 expression in the pre-ovulation AP (RPKM was 0.80±0.33) and the post-ovalution AP (RPKM was 1.68±0.53) although there was no significant difference between pre- and post-ovulation AP (pre/post ratio was 0.48, $P > 0.1$). Therefore, I evaluated importance of GPR101 and GPR173 for LH secretion from cultured bovine AP cells utilizing GnRH-(1-5).

9.2. Materials and methods

APs were collected from post-pubertal Japanese Black heifers in the middle of the luteal phase (n = 8, 26 months old) and cultured. After 58 h cultures, the culture medium was replaced by new medium containing GnRH-(1-5) (from 1nM to 1000 nM) for further 24 h. Then, medium was replaced with 280ul of 0.1% BSA DMEM containing the various concentration of with GnRH-(1-5) (from 1nM to 1000 nM). The cells were incubated while gently shaking for 5 min, and then, cells were treated with 20 μL of 15 nM GnRH (Peptide Institute Inc., Osaka, Japan) dissolved in DMEM for 2 h in order to stimulate LH secretion. The pretreatment plus the GnRH treatment yielded a final concentration of 1 nM, 10 nM, 100 nM or 1000nM of GnRH-(1-5), and 1 nM of GnRH. The "control" wells received 0.1% BSA DMEM only. The "GnRH" wells 0.1% BSA DMEM and 20ul of 15nM GnRH. After incubation with GnRH, the medium was collected for immunoassay of LH.

The mean for the control wells was set at 100%. The statistical significance of differences in LH concentration was analyzed by one-factor ANOVA followed by Fisher's PLSD test. The level of significance was set at $P < 0.05$. Data are expressed as mean ±SEM.

9.3. Results and discussion

The mean LH concentration in the medium of both GnRH wells and the wells treated with GnRH-(1-5) **(Fig. 9)** were significantly greater ($P < 0.01$) than that of the control wells. None of the wells containing GnRH-(1-5) displayed significant differences in mean LH concentrations compared to GnRH wells.

Therefore, the results did not support any important role of GPR101 and GPR173 for LH secretion from bovine AP.

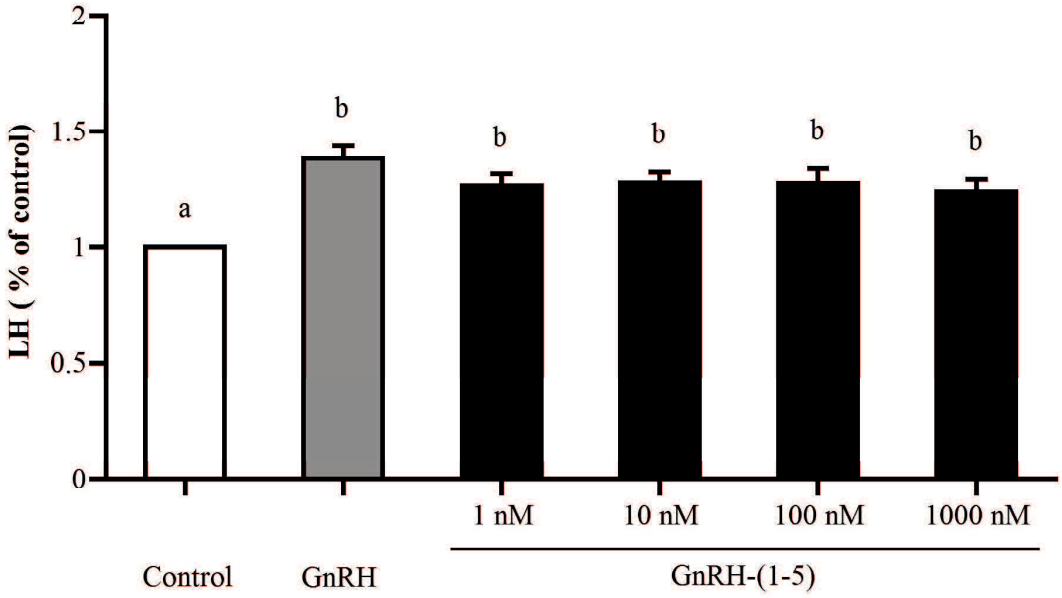


Fig. 9. Effect of various concentrations of GnRH-(1-5) on GnRH induced LH secretion from cultured bovine AP cells. LH concentrations in control cells (cultured in medium alone) were averaged and the mean value set at 100%. LH concentrations of the treated groups are expressed as a percentage of the control. Each value represents the mean concentration ± SEM. Letters (a *vs* b) indicate significant differences ($P < 0.01$) between control and other treatment.

CHAPTER X

General Discussion and Conclusion

10.1. General discussion

The study I discovered that bovine APs expressed 12,769 annotated genes which is about 58% of the 22,000 genes of cattle genome (Bovine Genome Sequencing and Analysis Consortium, 2009). Especially, the study I discovered 259 receptors and 364 binding protein genes. Among 259 receptor genes, 18 are the orphan receptors, including the two GPRs, *GPR 61* and *GPR153,* differently expressed in the pre- and post-ovulation APs. Furthermore, the studies II, III and V clarified that both GPR 61 and GPR153 colocalize with GnRHR on the surface of gonadotrophs. The study I also discovered 13 binding protein genes differently expressed in the pre- and post-ovulation APs. Therefore, AP collects various information from both brain and periphery.

The study I also confirmed that primary roles of AP are endocrine. *PRL* was the highest expressed AP hormonal genes, suggesting many roles of prolactin. Only 31 genes showed higher expression in the post-ovulation AP than the pre-ovulation AP, whereas about 12 times that number (365) of genes was expressed more in the pre-ovulation AP than the post-ovulation AP. Therefore, APs are likely to play important roles for various physiological functions especially before ovulation, the first step toward pregnancy.

This thesis did not support importance of GPR101 and GPR173 for LH secretion in the VI and VII utilizing the ligand candidates recently reported. We need to wait to find importance of other orphan GPCRs for LH secretion from bovine AP, especially for GPR61 and GPR153.

10.2. Conclusion

In conclusion, this thesis clarified the global gene expression in bovine AP before and after ovulation, and also this thesis discovered the new important receptors GPR61 and GPR153 expressed in gonadotrophs in stage-dependent manner.

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