PROLACTIN AND ITS RECEPTOR IN THE FOLLICULAR HIERARCHY OF CHICKENS

By

SHENQIANG HU

Department of Animal Science

McGill University, Montreal, Canada

December 2016

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree

of

DOCTOR OF PHILOSOPHY

ABSTRACT

In galliformes, prolactin (PRL) is thought to have both pro- and anti-gonadal effects on steroidogenesis by ovarian follicles dependent on its concentration. However, the role of the major isoform (glycosylated PRL, G-PRL) is unknown. Therefore, a comparison between non-glycosylated (NG-) and G-PRL on steroidogenesis in chicken different size class follicles was carried out. Contrary to the observations in mammals where G-PRL generally shows lower bioactivity than NG-PRL, both isoforms affected both basal and gonadotrophin-stimulated steroidogenesis over a broad concentration range. In granulosa cells of either preovulatory F3-F1 or prehierarchical 6-8 mm follicles, lower levels of NG-PRL stimulated basal estradiol (E_2) and progesterone (P_4) secretion but at higher levels this effect was reduced. However, G-PRL inhibited basal E_2 and P_4 secretion in a dose-dependent manner in prehierarchical granulosa cells in contrast to an inducible effect in preovulatory granulosa cells. In accordance with the data for steroids, levels of *StAR*, *CYP11A1*, *CYP19A1* and *3β-HSD* transcripts were up- or down-regulated by both isoforms. Furthermore, G-PRL was less potent than NG-PRL in suppressing FSH- or LH-induced E_2 and P_4 secretion in preovulatory granulosa cells, whereas G-PRL reduced but NG-PRL enhanced FSH-induced P_4 secretion in prehierarchical granulosa cells. In the population of prehierarchical < 6 mm follicles, NG-PRL had a greater effect on suppressing FSH-induced E_2 secretion than G-PRL, whereas G-PRL was more effective than NG-PRL in suppressing FSH-induced P_4 production. In contrast, with the exception of the synergistic actions of LH and G-PRL on P_4 production in the 4-6 mm follicles, both isoforms reduced LH-stimulated E_2 and P_4 production in all < 6 mm follicles. Thus, glycosylation of PRL can differentially modify its actions on basal and gonadotropin-stimulated steroidogenesis depending on the concentration, the type of gonadotropin (FSH or LH) and the stage of follicle development. Consistent with the effects of PRL on steroidogenesis, the PRL receptor (PRLR) was widely distributed in the follicular hierarchy, with the highest expression in the < 2 mm follicles and a progressive decline as the follicles matured. FSH had the greatest stimulatory effect on expression of *PRLR* in prehierarchical granulosa cells but this effect declined with follicle maturation, whereas LH showed no effects in all follicular classes examined. NG- and G-PRL were observed to have differential effects on basal and FSH-mediated *PRLR* expression according to the concentration and the stage of follicle development. As a newly identified ligand for the PRLR in non-mammalian vertebrates including

chickens, PRL-like protein (PRL-L) had a similar expression pattern to PRLR during follicle development, with maximal expression in the < 2 mm follicles. Furthermore, *PRL-L* was more abundant in prehierarchical than preovulatory granulosa cells. FSH increased while LH did not affect expression of *PRL-L* in prehierarchical granulosa cells. Basal and FSH-induced *PRL-L* expression were differentially regulated by either isoform of PRL depending on the concentration. Therefore, it is suggested that the differential actions of PRL isoforms on follicular cell steroidogenesis are mediated through modulating the expression of *PRLR* and *PRL-L*. By using activators and/or inhibitors, we further demonstrated that in prehierarchical granulosa cells, multiple signaling pathways including PKA, PKC, PI3K-Akt-mTOR and AMPK were not only directly involved in but they could also converge to modulate ERK2 activity to regulate FSH-induced *PRLR* and *PRL-L* expression.

ABSTRAIT

Chez les galliformes, c'est pensé que la prolactine (PRL) a des effets pro- et anti-gonadiques sur la stéroïdogenèse par les follicules ovariens en fonction de sa concentration. Cependant, le rôle de l'isoforme majeure (PRL glycosylée, G-PRL) est inconnu. Par conséquent, une comparaison entre la prolactine non glycosylée (NG-) et G-PRL sur la stéroïdogenèse dans des follicules de différentes classes de taille de poulet a été effectuée. Contrairement aux observations chez les mammifères où la G-PRL présente généralement une bioactivité plus faible que la NG-PRL, les deux isoformes ont affecté la stéroïdogenèse basale et aussi stimulée par la gonadotrophine à nombreuses concentrations. Dans les cellules de la granulosa des follicules préovulatoires F3-F1 ou préhérarchiques de 6 à 8 mm, des niveaux inférieurs de NG-PRL ont stimulé la sécrétion basale d'estradiol (E_2) et de progestérone (P_4), mais à des niveaux plus élevés cet effet a été réduit. Cependant, la G-PRL a inhibé la sécrétion basale d'E_2 et de P_4 de manière dose-dépendante dans des cellules de granulosa préhérarchiques, contrairement à un effet inducible dans des cellules de granulosa préovulatoires. Conformément aux enregistrements sur les stéroïdes, les niveaux des transcrits *StAR, CYP11A1, CYP19A1* et *3ß-HSD* ont été régulés à la hausse ou à la baisse par les deux isoformes. En outre, la G-PRL était moins puissante que la NG-PRL dans la suppression de la sécrétion d'E_2 et P_4 induite par la FSH ou la LH dans les cellules de granulosa préovulatoires, tandis que la G-PRL réduit mais la NG-PRL augmentait la sécrétion de P_4 induite par la FSH dans les cellules granulosa préhérarchiques. Dans la population de follicules préhérarchiques < 6 mm, la NG-PRL a eu un effet plus important sur la suppression de la sécrétion d'E_2 induite par la FSH que la G-PRL, tandis que la G-PRL était plus efficace que la NG-PRL pour inhiber la production de P_4 induite par la FSH. Contrairement, à l'exception des actions synergiques de LH et de G-PRL sur la production de P_4 dans les follicules de 4-6 mm, les deux isoformes ont réduit la production d'E_2 et P_4 stimulée par LH dans tous les follicules < 6 mm. Ainsi, la glycosylation de la PRL peut modifier de manière différentielle ses actions sur la stéroïdogenèse basale et stimulée par la gonadotrophine en fonction de la concentration, du type de gonadotrophine (FSH ou LH) et du stade de développement des follicules. Conformément aux effets de la PRL sur la stéroïdogenèse, le récepteur PRL (PRLR) a été largement distribué dans la hiérarchie folliculaire, avec la plus haute expression dans les follicules de < 2 mm et un déclin progressif à mesure que les follicules se développaient. La FSH a eu le plus grand effet stimulant sur l'expression du *PRLR* dans les

cellules de la granulosa préhérarchique, mais cet effet a diminué avec la maturation des follicules, alors que la LH n'a montré aucun effet dans toutes les classes folliculaires examinées. On a observé que NG- et G-PRL ont des effets différentiels sur l'expression du *PRLR* basal et médiée par la FSH en fonction de la concentration et du stade de développement du follicule. En tant que ligand nouvellement identifié pour le PRLR chez des vertébrés non mammifères comprenant des poulets, la protéine PRL-like (PRL-L) présentait un profil d'expression similaire au *PRLR* pendant le développement du follicule, avec une expression maximale dans les follicules < 2 mm. De plus, le *PRL-L* était plus abondant dans les cellules préhérarchiques que dans les granulosa préovulatoires. La FSH a augmenté tandis que la LH n'a pas affecté l'expression de *PRL-L* dans les cellules granulosa préhérarchiques. L'expression de *PRL-L* basale et induite par la FSH était différentiellement régulée par l'isoforme de la PRL selon la concentration. Par conséquent, il est suggéré que les actions différentielles des isoformes de PRL sur la stéroïdogenèse des cellules folliculaires sont médiées par modulation de l'expression de PRLR et de PRL-L. En utilisant des activateurs et / ou des inhibiteurs, on a également démontré que dans des cellules de granulosa préhérarchiques, des voies de signalisation multiples comprenant PKA, PKC, PI3K-Akt-mTOR et AMPK étaient non seulement directement impliquées mais elles pourraient également converger pour moduler l'activité de ERK2 pour réguler l’expression de PRLR et PRL-L induite par FSH.

ACKNOWLEDGEMENTS

To begin with, I would like to express my most sincere gratitude and appreciation to my PhD supervisor, Dr. David Zadworny, for his financial support and scientific guidance. Without his supervision, encouragement and understanding, it would not have been possible to complete my doctoral program at this time period. Most importantly, I have learned a lot from his excellent mentorship in both science and life that will be beneficial for my whole life.

I would like to extend my heartfelt gratitude to the China Scholarship Council (CSC) and the Department of Animal Science, McGill University, for the financial support. I would also like to thank my advisory committee, Dr. Raj Duggavathi and Dr. Xin Zhao, for their constant support and constructive suggestions.

I am grateful to Dr. Vilceu Bordignon and Dr. Sarah Kimmins for permission to use their laboratory facilities. I really appreciate Dr. Roger I. Cue for his valuable suggestions on statistical analysis. My special thanks go to Dr. Dana Praslickova for her encouragement and constant care during my PhD. I am also thankful to Paul Meldrum and Robert McEwen for taking care of the birds in the Macdonald Campus Farm.

I am very thankful to all my friends of the Animal Science department, especially Siyuan Huang, Keith Siklenka, Karina Gutierrez, Werner Giehl Glanzner, Milena Taibi, Ejimedo Madogwe, Yasmin Schuermann and Luke Currin for their encouragement and help. I would like to offer my special thanks to Yimeng Shao and Agneev Mukherjee for their care and support.

Lastly, my deepest love and appreciation goes to my family for their unfailing love and support. Special thanks go to Weiwei Shi whose love, encouragement and support have helped me go through the most difficult time during this journey.

I dedicate this thesis to my deceased maternal grandfather during my PhD study, whom I will miss and love forever.

CONTRIBUTION TO KNOWLEDGE

Chapter II

This study represents the first to compare the effects of non-glycosylated (NG-) and glycosylated (G-) PRL on steroidogenesis by chicken ovarian follicles. In contrast to mammals where G-PRL is generally observed to have lower bioactivity than NG-PRL, both isoforms were biologically active in regulating basal and gonadotropin-stimulated E_2 and P_4 production. However, G-PRL had differing effects than NG-PRL depending on the concentration, the type of gonadotropin (FSH or LH) and the stage of follicle development. Specifically, basal E_2 and P_4 secretion in granulosa cells of either preovulatory F3-F1 or prehierarchical 6-8 mm follicles was induced by lower levels of NG-PRL but this effect was reduced as the concentration increased. However, despite a stimulatory effect in preovulatory granulosa cells, G-PRL inhibited basal E_2 and P_4 secretion in a dose-dependent manner in prehierarchical granulosa cells. Furthermore, G-PRL was less potent than NG-PRL in suppressing FSH- or LH-induced E_2 and P_4 secretion in preovulatory granulosa cells, whereas G-PRL reduced but NG-PRL enhanced FSH-induced P_4 secretion in prehierarchical granulosa cells. In follicular walls of each group of prehierarchical 4-6, 2-4 and < 2 mm follicles, NG-PRL had a greater effect on suppressing FSH-induced E_2 secretion than G-oPRL, whereas G-PRL was more effective than NG-PRL in suppressing FSH-induced P_4 production. In contrast, with the exception of the synergistic actions of LH and G-PRL on P_4 production in the 4-6 mm follicles, both isoforms reduced LH-stimulated E_2 and P_4 production in all < 6 mm follicles.

Chapter III

This chapter provides novel information about the expression pattern of the *PRLR* gene as well as its regulatory mechanisms in the follicular hierarchy. The mRNA levels of *PRLR* were highest in follicular walls of the smallest (< 2 mm) follicles following a progressive decline as the follicles matured. Furthermore, *PRLR* was more abundant in granulosa than theca layers in preovulatory follicles. Expression of *PRLR* was maximally stimulated by FSH in granulosa cells of prehierarchical 6-8 mm follicles but this effect declined as the follicles matured to F1, whereas, LH showed no effects in all size class follicles tested. The effects of NG- and G-PRL on basal and FSH-regulated *PRLR* expression in granulosa cells were variable dependent on the concentration

and the stage of follicle development. We further demonstrated that multiple signaling pathways, including PKA, PKC, PI3K, mTOR and AMPK, are not only directly involved in, but they can also converge to modulate ERK2 activity to regulate FSH-induced *PRLR* expression in prehierarchical 6-8 mm granulosa cells.

Chapter IV

As a recently identified ligand for the PRLR in non-mammalian vertebrates including chickens, the distribution and regulation of PRL-like protein (PRL-L) in the follicular hierarchy was described in this chapter. Highest levels of *PRL-L* transcript were observed in follicular walls of the < 2 mm follicles following a progressive decline during follicle maturation. Furthermore, *PRL-L* was expressed at higher levels in prehierarchical than preovulatory granulosa cells. In prehierarchical granulosa cells, FSH stimulated but LH did not affect basal *PRL-L* transcript levels, and dependent on the concentration NG- and G-PRL showed differential effects on both basal and FSH-induced *PRL-L* expression. Since PRL-L and PRLR displayed similar patterns of expression in the follicular hierarchy, these results suggest that PRL-L may act in a paracrine and/or autocrine fashion to activate the PRLR for some as yet unrecognized physiological function(s) in the ovary. In addition, results from activator and inhibitor studies also provided evidence for a positive role for PKA, PKC and PI3K signaling while a negative role for ERK2 in mediating FSH-induced *PRL-L* expression in prehierarchical granulosa cells.

CONTRIBUTION OF AUTHORS

In this thesis, three co-authored manuscripts are included.

Authors of manuscripts 1, 2 and 3 (Chapters II, III and IV)

Chapter II: S. Hu and D. Zadworny

S. Hu and D. Zadworny conceived and designed the study. S. Hu conducted the experiments, analyzed the data and wrote the manuscript. D. Zadworny reviewed and edited the manuscript. Submitted to *Domestic Animal Endocrinology*.

Chapter III: S. Hu, R. Duggavathi and D. Zadworny

S. Hu, R. Duggavathi and D. Zadworny conceived and designed the study. S. Hu conducted the experiments, analyzed the data and wrote the manuscript. R. Duggavathi and D. Zadworny reviewed and edited the manuscript. Published in *PLOS ONE*, 2017, 12(1): e0170409. doi:10.1371/journal.pone.0170409.

Chapter IV: S. Hu, R. Duggavathi and D. Zadworny

S. Hu, R. Duggavathi and D. Zadworny conceived and designed the study. S. Hu conducted the experiments, analyzed the data and wrote the manuscript. R. Duggavathi and D. Zadworny reviewed and edited the manuscript. Published in *General and Comparative Endocrinology*, 2017, 240: 191-197.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAs	Amino acids
AC	Adenylate cyclase
AFs	Atretic follicles
Akt	Protein kinase B
AMH	Anti-müllerian hormone
AMPK	AMP-activated protein kinase
ARRB	Beta-arrestin
AVT	Arginine vasotocin
BMP	Bone morphogenetic protein
3β-HSD	3β-hydroxysteroid dehydrogenase
17β-HSD	17β-hydroxysteroid dehydrogenase
cAMP	Cyclic adenosine monophosphate
CDS	Coding sequence
C/EBP	CCAAT/enhancer-binding protein
CRE	cAMP responsive element
CYP11A1	Cytochrome P450 side-chain cleavage enzyme
CYP17	Cytochrome P450 17α-hydroxysteroid dehydrogenase
CYP19A1	Cytochrome P450 aromatase
DA	Dopamine
DHEA	Dehydroepiandrosterone
DMEM/F12	Dulbecco's Modified Eagle's Medium/Nutrient Mixture F12
DMRT1	Doublesex and mab-3-related transcription factor 1
E_2	Estradiol
ECD	Extracellular domain
EGF	Epidermal growth factor
EIA	Enzyme immunoassay
ERα	Estrogen receptor α

ERK2	Extracellular signal-regulated protein kinase 2
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FOXL2	Forkhead box L2
FSH	Follicular-stimulating hormone
FSHR	FSH receptor
GDF9	Growth differentiation factor 9
GH	Growth hormone
GnIH	Gonadotropin-inhibiting hormone
GnRH	Gonadotropin-releasing hormone
G-oPRL	Glycosylated ovine PRL
GPCRs	G-protein-coupled receptors
5-HT	Serotonin
ICD	Intracellular domain
IGF	Insulin-like growth factor
JAK2	Janus kinase 2
LDL	Low density lipoprotein
LH	Luteinizing hormone
LHR	LH receptor
LPL	Lipoprotein lipase
LWFs	Large white follicles
LYFs	Large yellow follicles
MAPK	Mitogen-activated protein kinase
MBH	Mediobasal hypothalamus
MEK	MAP/ERK kinase
mTOR	Mammalian target of rapamycin
NG-oPRL	Non-glycosylated ovine PRL
P_4	Progesterone
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PGCs	Primordial germ cells

PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PL	Placental lactogen
POF	Postovulatory follicle
PRL	Prolactin
PRL-L	PRL-like protein
PRLR	PRL receptor
qPCR	Quantitative PCR
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOCS	Suppressor of cytokine signaling
SOX9	SRY (sex determining region Y)-box 9
StAR	Steroidogenic acute regulatory protein
STAT 5	Signal transducer and activator of transcription 5
SWFs	Small white follicles
SYFs	Small yellow follicles
T	Testosterone
TGF β1	Transforming growth factor β1
TM	Transmembrane domain
TNF-α	Tumor necrosis factor-α
TRH	Thyrotropin-releasing hormone
5’UTR	5’-untranslated regions
VIP	Vasoactive intestinal peptide
VLDL	Very low density lipoprotein
VPAC	VIP receptor

CHAPTER I
INTRODUCTION AND REVIEW OF LITERATURE

1.1 INTRODUCTION

Domestication and genetic selection of various galliformes have resulted in remarkable gains in reproductive performance and some White Leghorn hens have laid up to 365 eggs in one year. This remarkable feat is achieved because hens do not have a cyclic pattern of reproduction as in mammals but rather lay eggs in clutches which consist of eggs laid on consecutive days followed by 1 or more pause days. Clutch sizes of 50-100 eggs are not uncommon and the average domesticated hen produces more than 300 eggs each year. Thus, ovulation occurs on an almost daily basis. This requires that ovarian follicles be arranged in a distinct hierarchy with the largest destined for ovulation and the second largest maturing to become the largest within 25-26 hours of ovulation. The maintenance of this hierarchy requires a complex interplay within the hypothalamic-hypophyseal-ovarian axis but also within supporting structures such as the liver which synthesizes the large mass of yolk constituents required for follicular accretion.

Amongst the many endocrine factors affecting the maintenance of the follicular hierarchy in hens, prolactin (PRL) has been identified as a negative regulator. PRL was the first adenohypophyseal hormone to be isolated by Riddle and colleagues in the 1930's [1]. They named it after its ability to stimulate lactation in rabbits (*i.e.* pro-lactational) but also noted its ability to cause 'broodiness' in chickens. Broodiness is a requisite aspect of avian reproductive behaviour in feral hens that is associated with the incubation of eggs and the rearing of young after hatching. Subsequent measurement of blood levels of PRL indicated that in galliformes, PRL increased at sexual maturity, further increased during egg laying and in hens which exhibited incubation behaviour, the highest levels were associated with ovarian regression and the maintenance of incubation behaviour. Thus, hyperprolactinemia is associated with inhibition of the reproductive system in hens. Both indirect effects of PRL in the hypothalamic-hypophyseal axis on the release of gonadotrophins and direct effects on ovarian steroidogenesis have been shown [2-4] but the molecular mechanism is not well characterized. In addition, the circulating isoform of PRL significantly changes during egg laying in hens with the glycosylated isoform becoming dominant during ovarian regression and incubation behaviour [5, 6]. In mammals, the glycosylated isoform has lower binding affinity to the PRL receptor (PRLR) and thus, signal transduction is inhibited

[7]. However, the avian PRLR has a very different structure than its mammalian counterpart and has a duplicated extra-cellular domain (ECD) [8]. How avian PRL or its isoforms interact with its double antenna receptor is unknown.

In addition, a novel PRL-like protein (PRL-L) gene has been cloned in the chicken which is not present in mammals [9]. This gene is expressed in the ovary although the cellular location is unknown [9]. A specific receptor for PRL-L is not known but PRL-L can initiate signal transduction by binding to the chicken PRLR [10]. Because the PRLR is expressed in hen ovarian follicles [11] it is possible the PRL-L may have autocrine and/or paracrine effects within the follicular hierarchy. Since very little is known about the functions and regulation of PRL signaling within the follicular hierarchy, the objectives of the current study are: 1) to compare the effects of non-glycosylated (NG-) and glycosylated (G-) PRL on basal and gonadotropin-stimulated steroidogenesis by ovarian follicles at different developmental stages; 2) to determine the expression patterns and cellular distribution of PRL-L and PRLR during follicle maturation; 3) to identify the potential factors in regulation of expression of PRL-L and PRLR in granulosa cells and 4) to clarify the underlying intracellular mechanisms controlling expression of PRL-L and PRLR in granulosa cells.

1.2 REVIEW OF LITERATURE

1.2.1 Dynamics, characteristics and regulation of avian ovarian follicle development

1.2.1.1 Sexual development of the gonads and oogenesis

In most birds, the female is the heterogametic sex (ZW) and males are homogametic (ZZ). Sex determination and testes development during early embryogenesis is caused by a gene dosage effect linked to the Z-linked doublesex and mab-3-related transcription factor 1 (*DMRT1*) gene which in turn regulates several downstream genes involved in gonadal differentiation (*e.g. FOXL2*, *CYP19*, *SOX9*, etc.) [12]. Without this gene dosage effect, the default sex is female. In the chicken, both ovaries begin development after about 3 days of incubation but around the sixth day the right ovary and oviduct begins to regress. This regression is due to the inhibitory effects of anti-müllerian hormone (AMH) as well as lack of expression of estrogen receptor α (ERα) in the right tract [13].

Oogenesis starts with the formation of oogonia, which originate from the migration of primordial germ cells (PGCs) from the germinal crescent into the gonadal ridges following sexual differentiation. Thereafter, they continue to proliferate via mitosis and become oogonia [14, 15]. The oogonia further undergo rapid mitosis and then differentiate into primary oocytes in response to a variety of growth factors and early actions of steroids [16]. Similar to the situation in mammals, oogenesis in the ovary of most birds terminates before or around hatching, and all primary oocytes are arrested in the diplotene-stage of meiosis I which constitute the reserve for formation of future primordial follicles [17].

1.2.1.2 Folliculogenesis

After hatching, those diplotene-stage oocytes located in the ovarian cortex are temporarily arranged in groups or nests and are devoid of follicular covering. After a 4-5 day growth, nests of oocytes are broken down, and some pre-granulosa cells are stimulated to surround the oocytes by factors of intra-gonadal and extra-gonadal origin. Such pre-granulosa cells are reported to derive from the coelomic epithelium. In general, an oocyte surrounded with a single layer of flatted pre-granulosa cells is designated as the primordial follicle [17]. Most of these primordial follicles remain in a quiescent stage for years until they either undergo atresia or enter the developmental stage during the breeding season. Initiation of oocyte growth is tightly coupled with the morphological, histochemical and biochemical changes in the follicular cells [17].

Primordial follicles develop into primary follicles as the oocyte enlarges in size by developing more cytoplasmic components and the pre-granulosa cells multiply in number and go through the morphological change from flattened cells to cuboidal cells. Cells originated from mesenchymal elements begin to form theca layers which arrange outside of granulosa cells and are separated by a basement membrane. In addition, when the follicular diameter approaches around 2.5 mm, a perivitelline layer mainly consisting of carbohydrates and proteins appears between the oocyte and follicular cells. A number of microvilli projected from either the oocyte cytoplasm or the follicular cells develops in the inner side of the perivitelline layer, which facilitate communication between the oocyte and granulosa cells by forming gap junctions [18].

Thereafter, a small number of primary follicles develop into slow-growing prehierarchical follicles measuring 4-8 mm in diameter. During this period, the granulosa layer transforms from a single layer to become multilayered and the theca layer differentiates into a double-layered

structure including theca externa and interna. The former is made up of fibroblast-like cells, collagen fibers, blood vessels, nerves as well as a few steroid-producing cells, which can not only provide mechanical support for the follicle but it can also secrete estrogen. In contrast, the latter contains steroidogenic cells mainly responsible for the production of androgens. There is evidence that both granulosa and theca cells in this phase are gonadotropin-dependent due to the presence of the receptors (*i.e.* FSH and LH receptors) [19]. Meantime, the oocyte begins to initiate the uptake of yolk precursors and thus expands rapidly [17].

In the domestic hen, only one follicle among the 6-8 mm cohort will enter the preovulatory hierarchy per ovulation cycle, and then it undergoes a rapid growth phase by deposition of large amounts of yellow yolk. Specifically, the oocyte increases in size from about 9 mm to 40 mm and undergoes a 15-fold increase in its surface area. Also, the granulosa layer changes in its shape from multilayered to single-layered, and increases in both its cell number and surface area are observed. The preovulatory follicle consists of a primary oocyte in the center surrounded by six layers of tissues, including (from outside to inside) surface epithelium, connective tissue layer, theca externa and interna, basal lamina, granulosa layer, perivitelline layer and oolemma [20-22]. Large follicles of most female birds have a stigma, which is a visible nonvascular strip that can be ruptured when the oocyte is expelled into the oviduct [23]. Notably, the primary oocyte completes meiosis (*i.e.* from a diploid to a haploid number of chromosomes) just before ovulation, through which an ovum is produced.

1.2.1.3 Morphology of the reproductive system

In most adult birds, the female reproductive system consists of a single left ovary and its oviduct. The ovary is located just caudal to the lung and at the cranial end of the kidney. It is attached to dorsal abdominal wall by the mesovarian ligament and receives its blood supply from the ovarian artery mainly originating from the left renal artery. The ovarian artery can further divide into many branches that can pass through the surface of all follicles, thereby forming an arterial capillary network to allocate blood flow to each follicle according to their nutritional requirement [24, 25]. This blood-vascular system ensures rapid maturation and final ovulation of ovarian follicles by facilitating the uptake of yolk precursors synthesized by the liver [26]. In addition, the ovary is also surrounded by intricate connective, nerve, muscle and epithelial tissues,

which integrate external and internal signals into the ovary, thereby contributing to its normal physiological activity [27].

Ovarian follicles begin to develop during early post-hatch stages and exhibit a roughened and granular appearance [23]. With the initiation of puberty, follicles become more visible, and the ovary has a tapioca appearance which progresses to a "grape-like" appearance as the ovary sexually matures [23, 28]. In the laying hen, the functionally mature ovary contains follicles at different developmental stages (Figure 1.1). With the deposition of yolk precursors, the follicles are categorized as previtellogenic or vitellogenic. The previtellogenic follicles include hundreds of small white follicles (less than 2 mm, SWFs), 10 to 20 large white follicles (3 to 5 mm, LWFs) and 6 to 10 small yellow follicles (6 to 8 mm, SYFs), while the vitellogenic follicles comprise 5 to 8 large yellow follicles (9 to 40 mm, LYFs) which are arranged in a strict hierarchy. The largest vitellogenic follicle designated as F1 is the first to be ovulated, followed by the second largest (F2), etc. Once ovulation takes place, a single follicle from a cohort of SYFs is selected to enter the vitellogenic hierarchy [29]. A constant supply of new SYFs is provided by LWFs through rapid growth which are in turn replenished from cortical follicles embedded in the outer cortex of the ovary. Typically, prehierarchical follicles (*e.g.* SWFs, LWFs and SYFs) are more susceptible to atresia than hierarchical (preovulatory) follicles [30].

The oviduct is composed of five basic regions that are histologically distinguishable, including (from ovarian proximal to distal) infundibulum, magnum, isthmus, shell gland and vagina (Figure 1.1). At ovulation, the F1 follicle ruptures along the stigma and is released into the infundibulum where fertilization occurs if spermatozoa are present. During passage through the magnum, isthmus and shell gland albumen (egg white), shell membranes and shell are sequentially deposited. Finally, the completely formed egg is expulsed through the vagina and cloaca [23, 31].

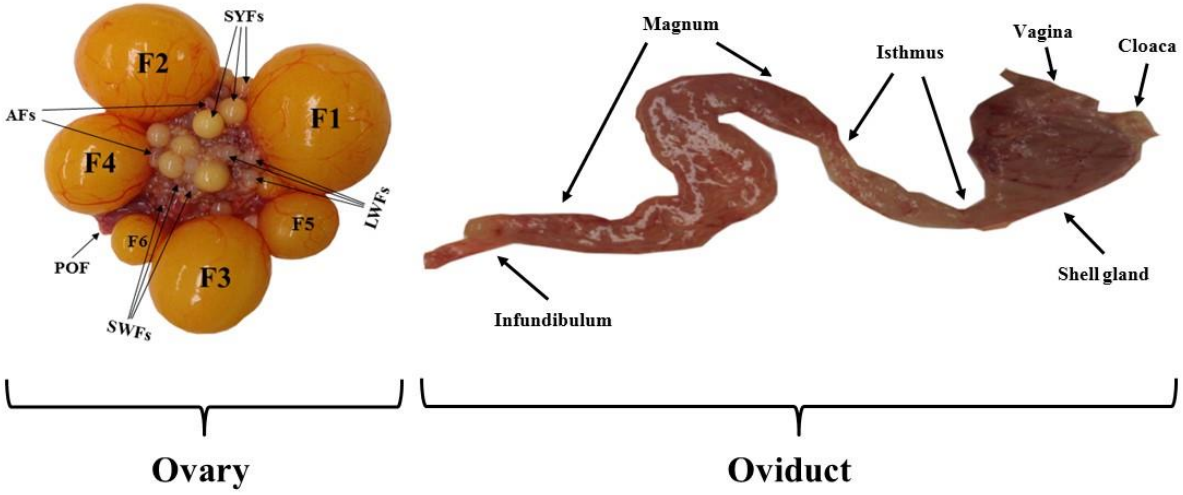


Figure 1.1 Morphology of the reproductive tract in female chickens.
SWFs, small white follicles; LWFs, large white follicles; SYFs, small yellow follicles; AFs, atretic follicles; POF, postovulatory follicle.

1.2.1.4 Ovulation and oviposition

During the breeding cycle, a limited number of follicles will be eventually ovulated, which represent the size of egg clutch and differ among different kinds of birds. Compared to most wild birds which normally lay two to ten eggs in a clutch and then stop laying to incubate the eggs, the domestic chicken can lay one egg each day throughout the year. The clutch size tends to be a reflection of the mother's nutrient reserve to ensure the survival of the nestlings, which obviously accounts for the interaction of genetic and environmental factors.

As the follicle advances in the vitellogenic hierarchy, the granulosa cells progressively secrete a substantial amount of progesterone (P_4) that reaches the maximal level in the largest follicle (F1). The increased secretion of P_4 subsequently acts through a feedback mechanism to induce the occurrence of a large preovulatory LH surge at six to eight hours before ovulation. Mechanisms controlling the timing of the LH surge have been related to the rhythmic expression of circadian clock genes in both the mediobasal hypothalamus (MBH) and the preovulatory follicles [32]. LH then stimulates the increasing production of prostaglandins and hydrolytic enzymes from the follicular walls, consequently resulting in an inflammatory response which disintegrates the stigma. Thereafter, smooth muscle contraction occurs and leads to the rupture of the F1 follicle. Unlike the situation in mammals, the postovulatory follicle in birds does not form the corpus luteum and

undergoes degeneration and absorption in a few days. Notably, there is evidence that the avian postovulatory follicle is an active structure during the first 24 h of its life, when it can produce a certain amount of P_4 and prostaglandin to promote next ovulation following oviposition, respectively [17].

After ovulation, the yolk-filled ovum spends about 24 h to pass through the oviduct before final oviposition. Oviposition is characterized by the expulsion of an egg from the oviduct to external environment through the uterine contraction, which is finely regulated by both neurohypophyseal hormones, such as arginine vasopressin and oxytocin, and ovarian steroids and prostaglandins [33]. Typically, the next ovulation takes place 15-60 mins after oviposition.

1.2.1.5 Steroidogenesis

Steroid hormones are indispensable for the establishment and maintenance of the ovarian follicular hierarchy in birds, and the main source of steroidogenesis is the ovary consisting of a stroma and numerous developing follicles which are responsible for the production of P_4, estradiol (E_2) and testosterone (T). As the precursor for steroid hormone synthesis, ovarian cholesterol mainly originates from low density lipoprotein (LDL). LDL is a product of the degradation of very low density lipoprotein (VLDL, one of yolk-forming components) synthesized by the liver, and this process is catalyzed by a key lipolytic enzyme namely lipoprotein lipase (LPL) [34].

The major steroidogenic pathways have been well established and are involved in a series of steroidogenic enzymes, including steroidogenic acute regulatory protein (StAR), cytochrome P450 side-chain cleavage enzyme (P450scc/CYP11A1), 3β-hydroxysteroid dehydrogenase (3β-HSD), cytochrome P450 17α-hydroxysteroid dehydrogenase (P450c17/CYP17), cytochrome P450 aromatase (P450 arom/CYP19) and 17β-hydroxysteroid dehydrogenase (17β-HSD). StAR plays a critical role in regulation of the rate-limiting step in steroid hormone synthesis, the transport of cholesterol from the outer to the inner mitochondrial membrane [35], where CYP11A1 initiates its conversion to pregnenolone, the precursor of all steroid hormones [36]. Two biosynthetic pathways are reported to direct the synthesis of steroid hormones from pregnenolone. In the Δ^4 pathway pregnenolone is converted to P_4 by the enzyme 3β-HSD, and subsequently P_4 is further converted to androstenedione by CYP17. In the Δ^5 pathway pregnenolone is converted to dehydroepiandrosterone (DHEA) by CYP17 and then to androstenedione by 3β-HSD. The

derivative androstenedione from both pathways is converted to T by 17β-HSD, and T is finally aromatized to E_2 catalyzed by CYP19 [13, 37-40].

In ovarian follicles of the laying hen, the biosynthesis of steroid hormones mainly occurs in the granulosa and theca layers. With follicular maturation, significant morphological changes are observed in both granulosa and theca layers as previously described, thereby contributing to variation in their steroidogenic capabilities. Specifically, non-hierarchical follicles are the major source of E_2 [41, 42], while P_4 is mainly produced by preovulatory follicles [43]. In follicles prior to selection, granulosa cells are arrested in an undifferentiated state and are considered to be steroidogenically incompetent [44] because of minimal expression of StAR [35] and CYP11A1 [36]. In contrast, subsequent to selection granulosa cells are differentiated and become steroidogenically active [44]. Thus, theca layers are the major site of steroidogenesis in non-hierarchical follicles while both granulosa and theca layers are steroidogenic in hierarchical follicles. The theca layers of non-hierarchical follicles predominantly produce androgens and estrogens via the Δ^5 pathway, independent of granulosa layers [45, 46]. During the transition of follicles from the prehierarchical to hierarchical stage, there is a shift in the steroidogenic pathway from Δ^5 to Δ^4 in theca layers, which is attributed to changes in the localization of 3β-HSD from the theca to the granulosa layer [47]. Nevertheless, another study reported that the Δ^5 and Δ^4 pathways function in both layers, with the preferred Δ^5 pathway in theca layers irrespective of the developmental stage and the preferred Δ^4 pathway in granulosa layers [37]. A three-cell model has been proposed for steroidogenesis in the hierarchical follicles. P_4 primarily synthesized by granulosa cells are transported to theca interna where they are required for the production of T. Subsequently, T is aromatized to E_2 in theca externa [44-46, 48]. It should be noted that the steroidogenic ability of granulosa layers increases while that of theca layers decreases through the preovulatory stage, consequently an elevation in P_4 and a reduction in E_2 secretion occur as follicles mature [43, 49]. Models summarizing the main steroidogenic pathways in chicken prehierarchical and hierarchical follicles are presented in Figure 1.2.

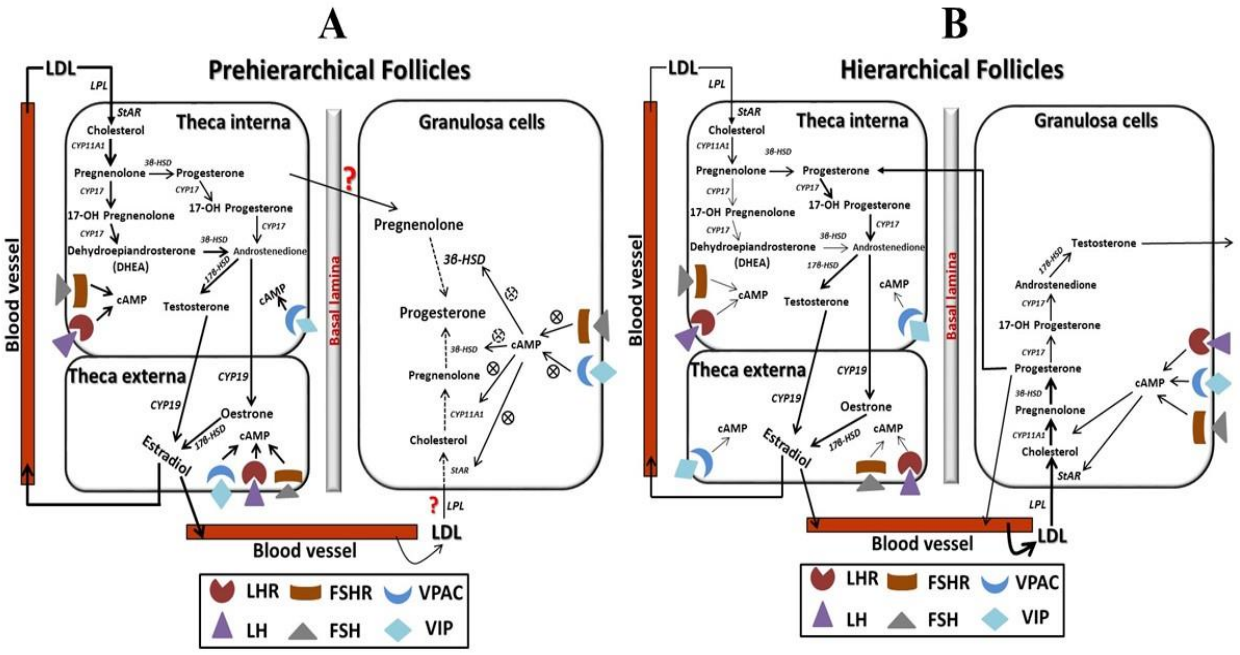


Figure 1.2 Schematic representation of steroidogenesis in both prehierarchical (A) and hierarchical follicles (B) of the chicken ovary.

In brief, the liver-synthesized VLDL circulates into the bloodstream and is transported into the follicular cells via receptor (*e.g.* VLDLR)-mediated endocytosis, where it undergoes lipolysis by LPL and is converted to LDL as the main precursor of steroid biosynthesis. In response to cAMP-induced signals via G-protein-coupled receptors (GPCRs; *e.g.* FSHR, LHR or VIP receptor, VPAC), cholesterol derived from LDL enters different types of cells where it undergoes the conversion to three main steroids, *i.e.* P_4, T and E_2, by a series of steroidogenic enzymes. The main site of steroidogenesis as well as its preferred steroidogenic pathways depends upon the stage of follicular development, which is mainly caused by lack of StAR and CYP11A1 activity in undifferentiated granulosa cells from prehierarchical follicles. Thereafter, the produced steroid hormones are released into the circulatory system to serve as a feedback mechanism to regulate steroidogenesis. Notably, interactions among granulosa cells, theca interna and externa occurs in the process of steroidogenesis. Solid lines indicate confirmed pathways for each cell type, and the thicker ones represent the preferred steroidogenic pathway at each step. Dotted lines indicate latent

minor pathways. '?' indicates unconfirmed cellular events. The symbol ⊗ signifies that this pathway remains quiescent or is suppressed at this stage, while the symbol ⛒ means that this pathway is absent or potentially inhibited. See text for further details.

1.2.1.6 Regulation of follicle development

Follicular development is a complex process controlled by a variety of endocrine, paracrine and autocrine factors. Various endocrine factors secreted by the hypothalamic-hypophyseal axis can act at the level of the ovary via the circulatory and/or nervous system in response to environmental and endocrine signals. The reduction in melatonin and gonadotropin-inhibiting hormone (GnIH) production leads to the onset of the breeding season because this decrease promotes gonadotropin-releasing hormone (GnRH) release followed by gonadotropin (FSH and LH) production, which can bind to the receptors within follicular cells, thereby regulating follicular basic functions [50, 51]. PRL may also play a role in follicular development, because levels of PRL increase during the breeding season. The mRNAs for either *PRL* or its receptor (*PRLR*) are present in the hypothalamic-hypophyseal axis and/or ovarian tissues, and PRL is involved in regulating gonadotropin secretion and ovarian steroidogenesis [7]. Furthermore, vasoactive intestinal peptide (VIP), a major PRL-releasing factor, is expressed in follicular cells and is considered as a key regulator of steroid hormone synthesis [52]. Additionally, several metabolic hormones also participate in regulation of follicular development, such as thyroid hormones [53], growth hormones (GHs) [54], adiponectin [55] and glucocorticoids [56].

In addition to endocrine hormones, many locally secreted factors from follicular cells (*e.g.* steroids, cytokines, growth factors, etc.) are also important for basal and gonadotropin-regulated basic functions of ovarian follicles. These factors include several members of the insulin-like growth factor family (IGFs), the epidermal growth factor family (EGFs), the transforming growth factor-β family (TGF-β), the fibroblast growth factor (FGFs) and the tumor necrosis factor-α (TNF-α) [57]. Recently, the crucial role of several oocyte-specific factors, such as growth differentiation factor 9 (GDF9) and bone morphogenetic factor 15 (BMP15), in regulating follicular development has been revealed. GDF9 can stimulate granulosa cell proliferation and BMP15 facilitates yolk deposition in growing follicles by decreasing the expression of the tight-junction protein occludin [58].

1.2.1.7 Follicle selection and intracellular mechanisms

In the laying hen, a cohort of follicles measuring 6-8 mm (about 6-10 per ovary) in diameter are called selectable follicles. Once ovulation occurs, a single follicle among these follicles will be selected into the preovulatory hierarchy in order to maintain the laying sequence. This process is termed follicle selection which is FSH-dependent since the follicle expressing the highest levels of FSH receptor (FSHR) within the 6-8 mm cohort is the next in line to enter the preovulatory hierarchy [59]. Activation of FSH signaling enhances cyclic adenosine monophosphate (cAMP) production, a second messenger essential for granulosa cell survival, thereby contributing to expression of several differentiation-inducing genes including *StAR*, *CYP11A1* and *LHR* [19]. Due to the expression of LHR these granulosa cells become responsive to LH and thus are able to secrete large amounts of P_4 in preparation for follicular rapid growth and ovulation [19]. In addition to the FSHR, other members of the GPCRs family, such as the VIP receptors (VPAC1and VPAC2), are also expressed in hen granulosa cells [60]. It has been suggested that lack of GPCR (*e.g.* FSHR and VPAC) signaling via cAMP in undifferentiated granulosa cells is caused by the desensitization of the receptors, which is mediated by GPCR kinases (GRKs) and the adaptor protein, beta-arrestin (ARRB) [29, 61, 62]. Interestingly, AMH is expressed at higher levels in hen prehierarchical than hierarchical granulosa cells [63] and is negatively involved in follicle selection by suppressing FSH responsiveness [63-65]. Additionally, two important regulators of follicle development (*i.e.* BMP6 [66] and vitamin D [67]) can regulate the expression of both FSHR and AMH in hen undifferentiated granulosa cells and consequently influence the process of follicle selection.

The escape from ARRB-mediated desensitization of GPCRs as well as the resultant enhanced receptor signaling via cAMP in undifferentiated granulosa cells depend on the alleviation or removal of intracellular inhibitory signals mediated by various autocrine and/or paracrine factors originating from granulosa and theca cells as well as the oocyte [19, 29]. As one of the most-studied inhibitory signaling pathways, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling is responsible for blocking the initiation of FSH-responsiveness in undifferentiated granulosa cells [59, 68-70]. Please refer to [19, 29, 62] for more details about how MAPK/ERK precludes premature differentiation of prehierarchical granulosa cells and how this inhibitory signal is removed around the time of follicle selection. In addition to the MAPK/ERK, many other signaling pathways are also involved in the process of follicle

selection. The modulatory effects of protein kinase C (PKC) on granulosa cell steroidogenesis are developmental stage-dependent. In undifferentiated granulosa cells, inhibition of PKC enhances gonadotropin-induced StAR expression and promotes P_4 production, whereas, the opposite is seen in differentiated granulosa cells [69-72]. These data suggest an inhibitory role for PKC in follicles before selection but a stimulatory role in selected follicles. GPCR-induced cAMP signals are largely mediated by protein kinase A (PKA) to support granulosa cell survival and differentiation. Treatment with the PKA inhibitor, H89, causes a significant increase in granulosa cells apoptosis. PKA exerts its effects by either phosphorylating downstream transcription factors containing cAMP response elements or modulating other signaling pathways such as PKC and MAPK [73]. Phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) also participates in granulosa cell survival in hen preovulatory follicles [73, 74]. Besides, as a cellular sensor, AMP-activated protein kinase (AMPK) has been recently shown to regulate FSH- or IGF-mediated steroidogenesis (*i.e.* StAR expression and P_4 production) in chicken ovarian follicles [75, 76]. Nevertheless, potential cross-talk(s) between these signaling pathways and additional factors involved in granulosa cell survival and follicle selection are not fully understood and warrant further investigation.

1.2.2 PRL and its receptor in birds

1.2.2.1 Discovery of PRL and the PRL/GH/PL family

Almost a century ago, the hormone PRL was firstly discovered as a novel factor secreted from the anterior pituitary. The name was derived from its ability to stimulate the growth of the crop sac in pigeons and mammary gland development in rabbits (*i.e.* prolactin: pro-lactational) [1] but subsequently many other biological roles have been described. The more than 300 distinct biological actions of PRL have been broadly categorized into those involving water and electrolyte balance, growth and development, endocrinology and metabolism, brain and behaviour, reproduction and immune-regulation and protection [77]. Based on the similar structural characteristics and biological features among PRL, GH and placental lactogen (PL), they form a unique family of related proteins called the PRL/GH/PL family [77]. This family also includes other members such as somatolactin (SL), PRL-related proteins, proliferins, etc. [78]. The genes encoding PRL, GH and PL are predicted to derive from a common ancestral gene by gene

duplication about 500 million years ago [79]. Despite the structural homology, the different members of the PRL/GH/PL family differ in their physiological functions [80].

1.2.2.2 PRL structure

The gene encoding PRL has been cloned in many avian species including chickens [10, 81], turkeys [82], ducks [83], geese [84] and sparrows [85]. Similar to mammals, the chicken and turkey *PRL* genes are composed of 5 exons and 4 introns, with an overall length of about 9.4 kb and 6.7 kb, respectively. Moreover, the mature chicken and turkey PRLs also consist of 199 amino acids (AAs), with a molecular mass of about 23 kDa [8]. The avian PRLs contain 3 disulfide bonds composed of 6 cysteines and 5 highly conserved domains that can form 4 helix bundles, indicating a similar receptor binding domain and signal transduction pathway to mammalian PRLs [8].

There are two different promoter regions controlling the transcription of the mammalian *PRL* gene, including a proximal promoter region directing intra-pituitary *PRL* expression and a distal promoter region responsible for extra-pituitary *PRL* expression [77, 80, 86, 87]. However, the 5'-untranslated region (UTR) of the avian *PRL* gene is different from that in mammals. These sequence differences in the 5'-UTR of avian *PRL* lead to changes in its transcriptional and hormonal regulation [83, 88, 89]. A 24-bp insertion/deletion in the 5'-UTR of PRL is present in the domestic hen [89]. Homozygous insertion of the 24-bp nucleotide sequence leads to decreased PRL expression and consequently reduces broody behaviour [89] and increases egg production [90]. Other polymorphisms in the 5'-UTR and coding sequences (CDS) of the *PRL* gene have been identified in chickens which may affect transcriptional levels and functions of *PRL* [89-92]. Analysis of *PRL* promoters among different galliformes indicates several regulatory elements within this region, including the pituitary-specific transcription factor 1 (Pit-1) and VIP response element which are largely conserved [93, 94]. This suggests that the basic structure of the avian *PRL* promoter and the mechanisms involved in regulation of its transcription may be highly conserved.

Many factors from the hypothalamic-hypophyseal axis and other endocrine organs have been demonstrated to be involved in control of secretion of PRL from the anterior pituitary. In contrast to the predominantly inhibitory actions of the mammalian hypothalamus (principally secreting PRL inhibiting factors such as dopamine, DA, and y-aminobutyric acid, GABA) [7, 77, 80], PRL secretion in birds is mainly under the stimulatory control of several hypothalamic PRFs such as

VIP [95, 96], thyrotropin-releasing hormone (TRH) [97], arginine vasotocin (AVT) [98], serotonin (5-HT) [99]. Among them, VIP is considered to play a central role not only through stimulating PRL release but also through stimulating *PRL* gene expression by increasing transcription rate and enhancing mRNA stability [100, 101]. However, depending on the concentration DA may have different effects on PRL secretion in gallinaceous birds [102, 103]. In particular, DA can exert both stimulatory and inhibitory effects on PRL secretion at the hypothalamic level [102, 104], and inhibits PRL release by antagonizing VIP at the pituitary level via the DA D2 receptor (D2R) [105]. Please refer to [106-108] for more details about regulation of PRL secretion in birds.

1.2.2.3 Glycosylation of PRL

Although PRL mainly exists in the form of 23 kDa protein, multiple isoforms of PRL can result from alternative splicing, proteolytic cleavage and posttranslational modifications such as glycosylation in vertebrates [7]. In chickens and turkeys, the proportion of G- to NG-PRL changes during the reproductive cycle in hens. As egg laying proceeds, levels of G-PRL disproportionately increase in concert with increases in total blood levels of PRL to reach maxima if the hen shows incubation behaviour [5, 6]. G-PRL is N-linked despite the absence of the N-linked consensus sequence (N-X-S/T). However, 2 alternative sites (N-X-C) located at positions 56 and 197 are used in turkeys [109] but only one alternative site at position 56 is observed in chickens [81]. In most mammals, N-linked glycosylation occurs within helix 1 (at position 31) and in general, receptor binding and activation is greatly reduced by glycosylation. This may not be surprising since 3 of the 14 amino acids required for receptor binding are located in the proximity of this site hence, glycosylation may cause steric hindrance [7, 110]. The overall structure of avian PRL is very similar to mammals (conservation of helices, disulfide bridges, the same 14 residues etc.), however, glycosylation would result in a very different topology which may affect receptor binding and biological activity differently than in mammals. Shifts in the absolute concentration and the ratio of isoforms during the egg laying season may modulate the partitioning of biological effects of PRL in various target tissues [8]. In addition, the structure of the avian receptor is very different from mammals, therefore, many interactions between the receptor and the isoforms are possible to modify target cell responses [8]. In support of this, glyco-protein variants of gonadotrophins in mammals also change in level and have different effects on target tissues [111, 112].

1.2.2.4 Prolactin-like protein

In addition to PRL, a novel gene encoding PRL-like protein (PRL-L) was recently discovered and firstly cloned in chickens and zebrafish [9, 113]. Bioinformatic analysis indicates that the *PRL-L* gene is not present in all investigated mammals such as rat and human, whereas, it does exist in several other non-mammalian vertebrate species including tiger puffer, green puffer and zebra finch [9]. Moreover, it is predicted that this novel gene may evolve from an ancestral PRL/GH-like gene via two rounds of whole genome duplication events in non-mammalian vertebrate species, but has been lost in mammalian species during evolution [9]. Similar to most members of the PRL/GH/PL family, the *PRL-L* gene identified in chickens is also composed of 5 exons and 4 introns, encoding a 225 AA-PRL-like protein precursor containing a 25 AA-signal peptide, with a molecular mass of 22 kDa [9]. Its deduced AA sequence has a 30%-35% identity to that of vertebrate PRLs including chicken and zebrafish PRL. Nevertheless, the AA sequences within the regions that are critical for ligand-receptor binding are largely conserved between chicken PRL and PRL-L [9].

Different patterns of expression of *PRL* and *PRL-L* mRNAs are characterized in a variety of chicken tissues. In contrast to the predominant expression of *PRL* in pituitary, *PRL-L* is expressed in a broad spectrum of extra-pituitary tissues (*e.g.* brain, heart, ovary and testis) of both embryonic and adult chickens but has a minimal expression in pituitary. Notably, *PRL-L* mRNA is widely distributed in various regions of the chicken brain [9]. Different tissue distribution patterns between chicken *PRL* and *PRL-L* mRNA may be attributed to loss of a pituitary-specific transcriptional factor binding site essential for directing pituitary-specific expression of vertebrate *PRL*s within the promoter of *PRL-L* [9, 10, 114]. Accordingly, PRL-L may have distinct functions from PRL in non-mammalian vertebrate species.

Although a specific receptor for PRL-L has not been identified to date, the structural similarity of PRL-L to PRL [113, 114] suggested that PRL-L may interact with the PRLR. Indeed, chicken PRL-L does activate PRLR signal transduction albeit with lower affinity compared to PRL in a reporter construct expressed in HepG2 cells [10]. Furthermore, PRL-L is predicted to have a signal peptide and is detectable in extra-cellular locations [115]. Therefore, it is possible that PRL-L may serve as a ligand for the PRLR to act on target tissues mainly in an autocrine and/or paracrine fashion. It is also possible that interactions of PRL-L with the PRLR may be altered as

the ratio of PRL isoforms changes in circulation of the chicken during the reproductive season. However, other than the putative role of PRL-L in increasing cold-induced muscle growth in chicks [115], nothing is known about other physiological actions of PRL-L in tissues.

1.2.2.5 PRLR structure

The biological actions of PRL are known to be mediated by its receptor PRLR, which belongs to the class I cytokine receptor superfamily that is normally activated by ligand-induced homodimerization [116-118]. This superfamily also includes GHR, several interleukin receptors, granulocyte-colony stimulating factor receptors, leptin receptor and other cytokine receptors [77]. All members share a common WSXWS motif in the ECD and are normally located on the surface of cells in order to quickly recognize and respond to their ligands containing four α-helical strands [119-121]. In mammals, the *PRLR* gene has been characterized in a range of species [77] and are mapped to autosomal chromosomes (*e.g.* 2, 15, 16, 5 of rat [122], mouse [123], pig [124] and human [125], respectively). Multiple splice variants of *PRLR* have also been characterized, and these isoforms mainly vary in the length and composition of the cytoplasmic domain [77]. For example, one long and three short PRLR isoforms are identified in mice while three PRLR isoforms of 291(short), 393 (intermediate) and 591 (long) in rat [126, 127]. In addition, several soluble isoforms of PRLR without the cytoplasmic tail have also been discovered in mammals [118, 128].

In contrast, the genes encoding avian PRLRs are located on chromosome Z [129], and so far they have been cloned in chickens [10, 130], turkeys [11], pigeons [131], geese [132] and ducks [133]. The chicken *PRLR* gene spans about 94 kb and is comprised of at least 25 exons with 10 non-coding exons in the 5'-UTR and 15 exons within the CDS [10], whereas, its human and rat counterparts have multiple alternative first exons, a non-coding exon and 9 exons within the CDS [80]. In contrast to the mammals where alternative splicing of *PRLR* mRNA normally occurs between exons 9 and 11 [80], in chickens it is mainly found between either exons 2 and 4 or exons 7 and 9, thereby contributing to two novel transcript variants. One encodes an N-terminally truncated PRLR of 688 AAs, and the other encodes a short PRLR of 316 AAs devoid of the transmembrane and intracellular regions [10]. A similar *PRLR* variant lacking exon 3 is also reported in pigeons [131]. The physiological relevance (if any) of these variants is unknown. Interestingly, a novel partially duplicated *PRLR* gene was recently identified at the K locus in

Lohmann chickens [134] which is associated with the slow feathering trait utilized to type chicks by sex.

Like most cytokine receptors, the PRLR protein consists of an ECD, a short transmembrane domain (TM) and an intracellular domain (ICD). Although avian PRLR shares major structural properties with its mammalian orthologs, its AA length is extremely long (831 AAs versus 591 AAs) which results from a tandem duplication of the ECD [130]. Each unit of avian ECD (two units of 201 and 204 AAs) is similar to that in mammals (about 200 AAs) and contains the conserved four paired cysteine residues and a WSXWS motif. The TM of PRLR contains about 20 AAs and is important for dimerization. The ICD domain contains several tyrosine residues indispensable for phosphorylation after receptor dimerization and signal transduction. The membrane proximal region of the PRLR ICD contains a conserved proline-rich hydrophobic motif called box 1 and box 2. Box 1 is responsible for the folding of Src homology 2 (SH2)-binding domains, while box 2 is composed of hydrophobic residues that may participate in signal transduction [8, 77, 80].

In the mammalian model of signal transduction, interaction of PRL binding site 1 with a receptor molecule results in the formation of an inactive complex, whereas the interaction of binding site 2 with a second receptor results in structural reorientation and formation of an active complex. This dimerization of the receptor is required for signal transduction and 1:1 complexes which form when high levels of PRL are present are antagonistic [8]. Presumably, signal transduction occurs in a similar fashion in birds. However, the presence of a second potential receptor binding site on the ECD may allow for additional types of complexes to form. These complexes could be agonistic or antagonistic and could provide a mechanism for further partitioning the biological actions of PRL to different tissues. Moreover, since the relative proportion of the PRL isoforms significantly varies with the physiological status of the hen [5, 6], further interactions are likely possible. Furthermore, PRL-L is produced in many tissues and has been shown to activate the PRLR which could lead to additional interactions with the PRL isoforms and the PRLR.

1.2.2.6 Signal transduction

PRL-PRLR interactions initiate the activation of a cascade of intracellular signaling pathways. Similar to other cytokine receptors, PRLR lacks intrinsic enzymatic activity and thus transmits the

signal into the nucleus via multiple kinases that can induce the activation of many downstream effectors. Because of the presence of two relatively conserved regions (box 1 and box 2) containing specific tyrosine residues in the ICD of PRLR, ligand-mediated activation of PRLR can induce the phosphorylation of itself as well as numerous cellular kinases and then participate in the recruitment of associated cellular proteins to the receptor complex [7, 135].

One of the best known pathways associated with signal transduction of the PRLR is the JAK-STAT pathway, which has been described in detail in several recent reviews [7, 77, 80, 135-138]. Briefly, the interaction between one of PRL's binding sites with a PRLR molecule induces subsequent interaction of binding site 2 on the same PRL molecule with a second PRLR molecule. Following ligand binding and receptor homodimerization, the tyrosine kinase (*i.e.* Janus kinase 2, JAK2) that is constitutively associated with the ICD of PRLR is activated, which in turn phosphorylates the tyrosine residues (box 1 and box 2) of PRLR and themselves. After that, phosphorylated tyrosine residues of activated PRLR interact with STAT proteins (especially STAT1, STAT3, STAT5a and STAT5b) containing the SH2 domains, resulting in the phosphorylation of STAT docked at the receptor by linked JAK2. Then, phosphorylated STAT dissociates from the receptor and translocates into the nucleus by forming a transcriptional active dimer, eventually controlling expression of PRL-responsive genes involved in a range of cellular physiological activities [77, 80, 139]. In addition, other signal pathways and kinases linked to the signal transduction of PRLR are identified, including Ras/Raf/MAPK cascade, several members of the Src kinase family (*e.g.* c-src and Fyn) or insulin receptor substrates (IRS)-mediated PI3K cascade, the suppressor of cytokine signaling (SOCS) gene family associated with the JAK/STAT pathway [140], Akt Nek3-vav2-Rac1 pathway [141] and PI3K enhancer-A (PIKE-A) [142]. Very little is known about the avian PRL signaling pathways although it is assumed that signal transduction uses similar pathways.

1.2.3 Reproductive actions of PRL in birds

1.2.3.1 PRL during various reproductive states

Among the more than 300 separate functions of PRL examined to date, its reproductive actions represent the largest group and have been widely studied in vertebrates [77]. PRL secretion in pituitary lactotrophs is pulsatile and is subjected to seasonal and circadian regulation in the

majority of seasonally breeding animals [143]. Significantly, in both long- and short-day breeding birds, PRL has been demonstrated to act as a key mediator in control of the neuroendocrine photoperiodic reproductive response [144]. In Columbiformes such as pigeons and doves, the concentrations of PRL remain at low levels during courtship and early incubation but increase during the middle period of incubation in parallel with rapid growth of the crop sac [1, 145, 146]. PRL-stimulated proliferation of the crop-sac mucosal cells involves both direct actions through increasing the number of PRLRs within these cells and indirect actions on the secretion of additional factors from other organs [147, 148].

In galliformes, levels of circulating PRL have been associated with different reproductive states. In juvenile hens, levels of PRL are basal but increase after photostimulation, which may be a consequence of changing steroid secretion [106, 149-151]]. As eg laying proceeds, levels of PRL continue to increase over pre-photostimulated levels, and it seems that moderate PRL levels in good layers are related to optimum egg production [150, 152-154]. However, if a hen expresses incubation behaviour, a rapid rise (6-10 fold) in PRL levels occurs during the 3-7 days preceding the last oviposition. During this transition period, hens maintain their egg laying rate while simultaneously spending > 90 % of the day incubating the eggs. The high levels of PRL appears to be anti-gonadal and are strongly associated with the termination of egg production by broodiness [3, 153-156]. Following the termination of egg laying, in hens that exhibit photorefractoriness and moulting, PRL secretion declines markedly and is maintained at a relatively stable level [157-159]. In accordance with the changes in circulating PRL concentrations, pituitary levels of both PRL content and *PRL* transcript increase from the sexually immature to the actively laying turkey hens, and reach the maxima during incubation phase but decrease in moulting hens [82, 160, 161]. Increased pituitary *PRL* mRNA abundance and plasma PRL levels by photostimulation may account for increases in *PRL* transcription and its mRNA stability which decrease during the period from incubation to refractoriness [162]. In addition, levels of *PRLR* transcript also vary during the reproductive cycle in turkey hens, being the highest levels in the pituitary gland of incubating hens but lower levels during non-photostimulated stage [11]. It is notable that *PRLR* mRNA levels in the hypothalamus decline from the pre-laying to incubation phase in turkey and bantams hens [11, 163], suggesting that PRL may modulate its secretions in the pituitary gland via a feedback mechanism through actions in the hypothalamus. In addition, as the critical avian PRL-releasing factor, concentrations of VIP in hypophyseal portal blood are highest in incubating turkey hens,

followed by laying and photorefractory hens and lowest in non-photostimulated hens [164]. The number of VIP immunoreactive cells as well as levels of *VIP* mRNA in the hypothalamus are positively correlated with changes in pituitary levels of *PRL* and *PRLR* transcripts as well as plasma PRL concentrations [107, 165]. The varying levels of PRL, PRLR and VIP during the reproductive cycle suggest the important roles for PRL signaling in regulation of avian reproduction.

1.2.3.2 PRL and incubation behaviour

One of the main and most-studied roles of PRL in avian reproduction is involved in the initiation and maintenance of broodiness, specifically, *i.e.* incubation and broody behaviour. Broodiness can be characterized by persistent nesting, turning and retrieval of eggs, characteristic clucking and defense of the nest [166] and occurs during the periods of egg incubation and nurturing of offspring [167]. Broodiness can markedly reduce egg production by shortening the egg-laying period, and hens cease egg production during the incubation stage. Therefore, incubation behaviour is considered as a major hindrance to improve egg productivity in the domestic fowl.

Although the onset and maintenance of this behaviour is controlled by a variety of genetic and environmental factors, it is mainly associated with increasing circulating PRL levels. Firstly, as mentioned in the section 1.2.3.1, levels of plasma PRL reach the maxima during the incubation period in many species of birds showing that PRL may initiate the occurrence of incubation behaviour [3]. Secondly, physically preventing incubation or broody behaviour by nest-deprivation or the removal of eggs or young from breeding birds dramatically decrease plasma PRL levels but can be reversed by resumption of nesting or the return of eggs or young [156, 168-170]. Thirdly, active and/or passive immunizations against PRL, PRLR or its releasing factor, VIP, can significantly decrease circulating PRL levels and effectively inhibit incubation behaviour [171-175]. Lastly, some studies have shown that exogenous PRL injections accelerate the onset of incubation behaviour preceded by nesting behaviour, suggesting a major role of PRL in the transition from nesting behaviour to incubation behaviour [176, 177].

Nevertheless, PRL may not play a causal role in inducing avian incubation behaviour. In spite of some early studies showing that administrations of mammalian PRL are able to induce broodiness in domestic fowl [178, 179] and in laying turkey hens [180], some recent studies have

indicated the inability of PRL to induce incubation behaviour in a number of avian species including chickens, turkeys and ring doves [181, 182]. These controversial results are considered to be caused by differences in the source, purity and dosage of the PRL preparations examined. Please refer to recent reviews for more details [3, 167].

1.2.3.3 PRL and parental behaviour

PRL has also been identified as an essential regulator of parental care, including both maternal and paternal behaviour, in fish, birds and mammals such as primates [183]. There are two phases involved in parental care of birds, *i.e.* pre-hatching (incubation) and post-hatching (brooding and fostering the young) phases. The specific secretion patterns of PRL in both male and female birds during these two phases are associated with their caregiving behaviour in 25 species ranging from penguin to songbirds [183]. Moreover, a causal relationship was found between PRL and parental care in the ring dove, and the production of crop milk that is used to feed the young was also found to be PRL-dependent [184]. In addition, PRL secretion in the parents is stimulated when exposed to tactile and visual stimuli from the nest, the eggs and the chicks, and there are also strong connections among PRL, corticosterone and stress that is important for the maintenance of parental care [185].

1.2.3.4 PRL and clutch size

Clutch size is an important predictor of avian fitness. The physiological relevance of PRL in avian clutch-size determination has also been widely studied, and a negative relationship between plasma PRL and clutch size constitutes the PRL-based mechanism for clutch size determination [186]. Elevated levels of PRL exert an anti-gonadal effects via inhibition of LH to cause follicular degeneration and cessation of laying [187]. However, several opposite results have been recently reported, where no evidence for an inhibitory effect of PRL on LH secretion was found and the deterministic role of PRL in clutch size was uncoupled. It is also proposed that alternative control models of avian clutch size are likely involved in downstream regulation of PRL at the ovarian level and/or other unconsidered hormones [188, 189].

1.2.3.5 Pro- and anti-gonadal effects of PRL on ovarian functions

As previously described, in many avian species levels of PRL rise upon photostimulation, continue to increase as egg laying proceeds, reach the maxima during the incubation phase and decrease rapidly after removal of the eggs or hatching of the young. This temporal association leads to the hypothesis that depending on the concentration PRL may have both pro-gonadal and anti-gonadal roles. Low to moderate levels of PRL appears to be pro-gonadal for the establishment and maintenance of the ovarian follicular hierarchy and thereby egg production. However, when the concentration rises above a threshold value, the largest follicles are ovulated but no new prehierarchical follicles are recruited into the hierarchy and gonadal involution occurs. It has been established that both PRL and PRLR are widely expressed in the brain, hypothalamus and pituitary in birds [8, 10, 11, 93, 190]. Furthermore, *PRLR* mRNA is also abundant in the ovaries of chickens [10] and turkeys [11]. Therefore, PRL may have indirect effects on the hypothalamic-hypophyseal axis to regulate gonadotropins release as well as direct effects at the ovarian level. It is notable that in incubating hens which lose their clutch of eggs early in the reproductive season, PRL levels rapidly decline and egg production resumes within several weeks as new follicles are recruited into the hierarchy without the hen undergoing a moult or further photostimulation. Thus, the hypothalamic-hypophyseal-gonadal axis remains active and the hen has a mechanism to lay a new clutch of eggs should nesting fail during a reproductive season [3].

The pro-gonadal effects of PRL are supported by the rise in circulating PRL levels around the onset of sexual maturity. It has been previously reported that induction of gonadal development by photostimulation is associated with increases in PRL in both the pituitary gland and plasma in Japanese quail [191] and many other birds [3, 107, 192]. Photoperiodic stimulation of PRL secretion is also accompanied by changes in gonadotropins and ovarian steroid hormone levels [3, 107, 151], and furthermore injections of E_2 or P_4 alone, or in the combination, can increase PRL levels in ovariectomized turkeys but these increases are not comparable to normal PRL rises observed in intact laying turkeys [193]. However, whether or not elevated PRL levels are prerequisite for the onset of egg production have not been clearly defined in birds. Moreover, although immunizations against PRL have little or no effect on egg laying performance in broody birds such as turkeys [171] and bantam chickens [194], it depresses egg production in less- or non-broody chickens by reducing large white follicular growth and hence recruitment into the follicular

hierarchy [175]. Furthermore, immunization against recombinant chicken PRLR ECD also reduce hen egg laying rate compared to the control group under long photoperiods [175]. In addition, a recent study indicates that PRL has both stimulatory and inhibitory effects on steroid secretion by cultured chicken ovarian follicles, which is dependent on the type of follicular cell, the stage of follicle development and the stage of the ovulatory cycle [4]. Notably, although there are no mutagenic studies of the *PRL* and *PRLR* genes have been conducted in birds, both *PRL*- and *PRLR*-deficient mice are infertile due to impaired follicular and corpora luteal functions. Defects in pre-implantation and implantation ova, reduced fertilization rates as well as impaired mammary development and alveolar differentiation are observed in *PRLR*-null mice [195, 196]. Moreover, the PRLR is also expressed in the mouse oviduct and plays a role in the development of pre-implantation embryos [197].

Once the threshold level for PRL is exceeded, it appears to be anti-gonadal. Firstly, in incubating hens hyperprolactinemia has been strongly associated with reduced activity of the pituitary-gonadal axis [3, 107]. During the reproductive cycle in female birds, an inverse relationship between circulating LH and PRL levels has been demonstrated in a number of avian species [108, 144, 156, 198] which is also supported by the observation that nest-deprivation decreases plasma PRL but increases LH concentrations [155, 199]. Following ovariectomy of incubating hens, the higher levels of endogenous PRL prevent the expected rise in LH until PRL levels are suppressed [200, 201]. Compared to out-of-lay turkey hens, the higher levels of PRL in incubating hens may have stronger suppressive effects on pregnant mare serum gonadotropin (PMSG)-induced ovarian growth and steroidogenesis. It is thus concluded that increasing PRL concentrations during the incubation phase can inhibit the secretions of gonadotropins at the hypothalamic and/or pituitary level since circulating PRL concentrations, *PRL* and *PRLR* mRNAs levels within these tissues significantly change during the transition from the egg-laying to incubation state [82, 160, 163, 190, 202]. Secondly, in broody breeds of chickens and turkeys, intramuscular injections of PRL cause significant decreases in ovarian weight and the number of normal follicles but an increase in the number of atretic follicles [181]. Administrations of PRL into laying turkey hens reduce hypothalamic GnRH content followed by a dramatic decrease in circulating LH levels [203]. It also suppresses both basal and gonadotropin-induced E_2 and P_4 production. Basal levels of serum E_2 in laying turkey hens were significantly decreased after 30 min of injection of 1000 μg PRL. Moreover, pre-administrations of PRL at the doses of 250-1000

μg greatly reduced FSH- and/or LH-induced increases in serum E_2 and P_4 levels [204]. This suppressive effect of PRL may be mediated by its actions on expression of steroidogenic enzymes since *CYP19* mRNA levels in small white follicles were reduced by injections of PRL [205]. Also, regardless of the stages (early or later) of the reproductive cycle in domestic hen, subcutaneous administration of the dopamine antagonist 2-bromo-α-ergocriptine, resulted in fewer pause days and higher egg production which was correlated with lower PRL concentration and higher levels of E_2 and P_4 as well as LH [206, 207]. Thirdly, neutralization or inhibition of endogenous PRL via passive or active immunizations against PRL or VIP, attenuates the expression of incubation behaviour and improves egg production performance [171, 173, 194, 208, 209]. Lastly, physiological concentrations of PRL have been shown to exert inhibitory effects on both basal and LH-stimulated E_2 production in chicken small follicles [4, 210].

1.2.3.6 Actions of PRL in ovarian follicular cells

Although PRL has both pro- and anti-gonadal roles in regulation of avian ovarian activity, very little is known about its actions and regulation in theca and granulosa cells. In turkey hens, a previous study has shown that *PRLR* mRNA is expressed at higher levels in the stroma and small follicles than in large follicles [11]. However, the distribution of PRLR in cell types of turkey ovarian follicles as well as its expression pattern in different size class follicles in other avian species have not been investigated. Since the small follicles are the major source of estrogen (> 80%) in birds [11], PRL may play a role in regulation of estrogen synthesis. Indeed, *in vitro* studies by cultured intact follicles have suggested the suppressive effects of PRL on basal and LH-induced E_2 secretion in hen small follicles [4, 210]. In contrast, in chicken large follicles, dependent on the concentration, the stage of follicle development and the stage of the ovulatory cycle PRL can elicit either stimulatory or inhibitory effects on E_2 and P_4 secretion by cultured theca and granulosa layers, respectively [4]. The effects of PRL may be mediated through regulation of steroidogenic enzymes since 3β-HSD is reduced by PRL in granulosa cells [211]. Furthermore, increased phosphodiesterase (PDE) activity is induced by PRL in theca cells which would reduce the accumulation of cAMP associated with gonadotrophin stimulation and hence signal transduction [212, 213].

Currently, nothing is known about the effects of post-translational modifications of PRL on basal and gonadotropin-stimulated steroidogenesis as well as other basic functions (*e.g.*

proliferation and apoptosis) of follicular cells with the maturation of ovarian follicles. In addition, the expression patterns of the novel *PRL-L* gene and the *PRLR* gene in cell types and follicular size classes as well as their regulatory mechanisms during follicle development warrant further investigation.

1.3 RATIONALE, HYPOTHESES AND OBJECTIVES

Steroidogenesis by theca and granulosa cells is essential for the establishment and maintenance of the avian follicular hierarchy which requires complex interactions between pituitary hormones and locally produced factors in the ovary. Although accumulating evidence suggests that PRL has both pro- and anti-gonadal roles in the follicular hierarchy through both indirect actions on the hypothalamic-hypophyseal axis to regulate gonadotropins release and direct actions at the level of the ovary, very little is known about its precise actions in follicular cell steroidogenesis as well as the underlying regulatory mechanisms.

Since the relative and absolute amounts of G- to NG-PRL in both the pituitary gland and plasma significantly vary during various reproductive states in galliformes and it has been shown in mammals that glycosylation of PRL can influence the ligand binding affinity and consequently biological activity in selective target tissues, it is likely that glycosylation of PRL may modify its actions in the follicular hierarchy. Recently, a novel PRL-L gene has been identified in non-mammalian vertebrates including chickens and zebrafish. Given its ability to activate signaling transduction through the PRLR and high abundance in the chicken ovary, interaction(s) may occur between circulating PRL isoforms, intra-ovarian PRL-L and the PRLR to affect the follicular hierarchy. Since the steroidogenic competence of theca and granulosa cells is variable throughout follicle development, we hypothesize that 1) glycosylation may differentially modulate the actions of PRL in follicular cell steroidogenesis depending on the stage of follicle development and that 2) both PRLR and PRL-L are differentially distributed and regulated in the follicular hierarchy in chickens.

Therefore, the objectives of the present study are: 1) to compare the effects of G- and NG-PRL on basal and gonadotropin-stimulated steroidogenesis by ovarian follicles at different stages of development; 2) to determine the expression patterns and cellular distribution of PRL-L and PRLR during follicle development and 3) to investigate the independent and interactive effects of

gonadotropins and PRL isoforms on expression of PRL-L and PRLR in granulosa cells of different size class follicles as well as the underlying intracellular mechanisms.

1.4 REFERENCES

1. Riddle O, Bates RW, Dykshorn SW. The preparation, identification and assay of prolactin—a hormone of the anterior pituitary. American Journal of Physiology--Legacy Content 1933; 105:191-216.
2. Scanes CG. Pituitary gland. In: Avian Physiology: Springer; 1986: 383-402.
3. Goldsmith A. Prolactin in avian reproductive cycles. In: Hormones and behaviour in higher vertebrates: Springer; 1983: 375-387.
4. Hrabia A, Paczoska-Eliasiewicz H, Rząsa J. Effect of prolactin on estradiol and progesterone secretion by isolated chicken ovarian follicles. Folia biologica 2004; 52:197-203.
5. Bedecarrats G, Guemene D, Kühnlein U, Zadworny D. Changes in levels of immunoreactive prolactin isoforms during a reproductive cycle in turkey hens. General and comparative endocrinology 1999; 113:96-104.
6. Hiyama G, Kansaku N, Kinoshita M, Sasanami T, Nakamura A, Noda K, Tsukada A, Shimada K, Zadworny D. Changes in post-translational modifications of prolactin during development and reproductive cycles in the chicken. General and comparative endocrinology 2009; 161:238-245.
7. Freeman ME, Kanyicska B, Lerant A, Nagy G. Prolactin: structure, function, and regulation of secretion. Physiological reviews 2000; 80:1523-1631.
8. Zadworny D, Kansaku N, Bedecarrats G, Guemene D, Kuhnlein U. Prolactin and its receptor in galliformes. Avian and Poultry Biology Reviews 2002; 13:223.
9. Wang Y, Li J, Kwok AHY, Ge W, Leung FC. A novel prolactin-like protein (PRL-L) gene in chickens and zebrafish: cloning and characterization of its tissue expression. General and comparative endocrinology 2010; 166:200-210.
10. Bu G, Wang CY, Cai G, Leung FC, Xu M, Wang H, Huang G, Li J, Wang Y. Molecular characterization of prolactin receptor (cPRLR) gene in chickens: gene structure, tissue expression, promoter analysis, and its interaction with chicken prolactin (cPRL) and prolactin-like protein (cPRL-L). Molecular and cellular endocrinology 2013; 370:149-162.

11. Zhou JF, Zadworny D, Guemene D, Kuhnlein U. Molecular cloning, tissue distribution, and expression of the prolactin receptor during various reproductive states in Meleagris gallopavo. Biology of reproduction 1996; 55:1081-1090.
12. Smith CA, Roeszler KN, Ohnesorg T, Cummins DM, Farlie PG, Doran TJ, Sinclair AH. The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. Nature 2009; 461:267-271.
13. Bruggeman V, Van As P, Decuypere E. Developmental endocrinology of the reproductive axis in the chicken embryo. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 2002; 131:839-846.
14. Tsunekawa N, Naito M, Sakai Y, Nishida T, Noce T. Isolation of chicken vasa homolog gene and tracing the origin of primordial germ cells. Development 2000; 127:2741-2750.
15. Stebler J, Spieler D, Slanchev K, Molyneaux KA, Richter U, Cojocaru V, Tarabykin V, Wylie C, Kessel M, Raz E. Primordial germ cell migration in the chick and mouse embryo: the role of the chemokine SDF-1/CXCL12. Developmental biology 2004; 272:351-361.
16. Johnson A, Woods DC. Ovarian dynamics and follicle development. Reproductive Biology and Phylogeny of Aves 2007:243-277.
17. Guraya SS. Ovarian follicles in reptiles and birds. Springer Science & Business Media; 2013.
18. Wyburn G, Aitken R, Johnston H. The ultrastructure of the zona radiata of the ovarian follicle of the domestic fowl. Journal of Anatomy 1965; 99:469.
19. Johnson A, Woods DC. Dynamics of avian ovarian follicle development: cellular mechanisms of granulosa cell differentiation. General and comparative endocrinology 2009; 163:12-17.
20. Perry M, Gilbert A, Evans A. The structure of the germinal disc region of the hen's ovarian follicle during the rapid growth phase. Journal of anatomy 1978; 127:379.
21. Perry M, Gilbert A, Evans A. Electron microscope observations on the ovarian follicle of the domestic fowl during the rapid growth phase. Journal of anatomy 1978; 125:481.
22. Gilbert A, Evans A, Perry M, Davidson M. A method for separating the granulosa cells, the basal lamina and the theca of the preovulatory ovarian follicle of the domestic fowl (Gallus domesticus). Journal of Reproduction and Fertility 1977; 50:179-181.
23. Pollock CG, Orosz SE. Avian reproductive anatomy, physiology and endocrinology.

Veterinary Clinics of North America: Exotic Animal Practice 2002; 5:441-474.

24. Nalbandov A, James M. The blood-vascular system of the chicken ovary. American Journal of Anatomy 1949; 85:347-377.
25. Echols MS. Surgery of the avian reproductive tract. In: Seminars in Avian and Exotic Pet Medicine, vol. 11: Elsevier; 2002: 177-195.
26. Schjeide O, Wilkens M, McCANDLESS RG, Munn R, Peterson M, Carlsen E. Liver synthesis, plasma transport, and structural alterations accompanying passage of yolk proteins. American Zoologist 1963:167-184.
27. Brennan J, Capel B. One tissue, two fates: molecular genetic events that underlie testis versus ovary development. Nature Reviews Genetics 2004; 5:509-521.
28. Johnson A. Reproduction in the female. In: Avian physiology: Springer; 1986: 403-431.
29. Johnson A. Ovarian follicle selection and granulosa cell differentiation. Poultry science 2015; 94:781-785.
30. Johnson A, Bridgham J, Witty J, Tilly J. Susceptibility of avian ovarian granulosa cells to apoptosis is dependent upon stage of follicle development and is related to endogenous levels of bcl-xlong gene expression. Endocrinology 1996; 137:2059-2066.
31. Ritchie BW, Hsarrison GJ, Zantop D, Harrison L. Avian medicine: principles and application, abridged edition. Idaho Falls, ID: Wingers Publishing 1997.
32. Sellix MT, Menaker M. Circadian clocks in the ovary. Trends in Endocrinology & Metabolism 2010; 21:628-636.
33. Ubuka T, Bentley G. Neuroendocrine control of reproduction in birds. Hormones and Reproduction of Vertebrates 2011; 4:1-25.
34. Bujo H, Hermann M, Kaderli M, Jacobsen L, Sugawara S, Nimpf J, Yamamoto T, Schneider W. Chicken oocyte growth is mediated by an eight ligand binding repeat member of the LDL receptor family. The EMBO journal 1994; 13:5165.
35. Johnson A, Bridgham J. Regulation of Steroidogenic Acute Regulatory Protein and Luteinizing Hormone Receptor Messenger Ribonucleic Acid in Hen Granulosa Cells 1. Endocrinology 2001; 142:3116-3124.
36. Tilly J, Kowalski K, Johnson A. Cytochrome P450 side-chain cleavage (P450scc) in the hen ovary. II. P450scc messenger RNA, immunoreactive protein, and enzyme activity in developing granulosa cells. Biology of reproduction 1991; 45:967-974.

37. Lee K, Volentine K, Bahr J. Two Steroidogenic Pathways Present in the Chicken Ovary: Theca Layer Prefers Δ 5 Pathway and Granulosa Layer Prefers Δ 4 Pathway. Domestic Animal Endocrinology 1998; 15:1-8.
38. Hanukoglu I. Steroidogenic enzymes: structure, function, and role in regulation of steroid hormone biosynthesis. The Journal of steroid biochemistry and molecular biology 1992; 43:779-804.
39. LLEWELYN CA. Conversion of [4-14C] progesterone to androstenedione in vitro by thecal tissue from the ovary of the domestic fowl (Gallus domesticus). Journal of Endocrinology 1981; 89:283-288.
40. Etches R, Duke C. Progesterone, androstenedione and oestradiol content of theca and granulosa tissues of the four largest ovarian follicles during the ovulatory cycle of the hen (Gallus domesticus). Journal of Endocrinology 1984; 103:71-76.
41. Senior B, Furr B. A preliminary assessment of the source of oestrogen within the ovary of the domestic fowl, Gallus domesticus. Journal of reproduction and fertility 1975; 43:241-247.
42. Robinson F, Etches R. Ovarian steroidogenesis during follicular maturation in the domestic fowl (Gallus domesticus). Biology of Reproduction 1986; 35:1096-1105.
43. Bahr J, Wang S, Huang M, Calvo F. Steroid concentrations in isolated theca and granulosa layers of preovulatory follicles during the ovulatory cycle of the domestic hen. Biology of Reproduction 1983; 29:326-334.
44. Tilly J, Kowalski K, Johnson A. Stage of ovarian follicular development associated with the initiation of steroidogenic competence in avian granulosa cells. Biology of Reproduction 1991; 44:305-314.
45. PORTER TE, HARGIS BM, SILSBY JL, HALAWANI MEE. Differential steroid production between theca interna and theca externa cells: a three-cell model for follicular steroidogenesis in avian species. Endocrinology 1989; 125:109-116.
46. NITTA H, OSAWA Y, BAHR JM. Multiple steroidogenic cell populations in the thecal layer of preovulatory follicles of the chicken ovary. Endocrinology 1991; 129:2033-2040.
47. Nitta H, Mason J, Bahr JM. Localization of 3 beta-hydroxysteroid dehydrogenase in the chicken ovarian follicle shifts from the theca layer to granulosa layer with follicular maturation. Biology of reproduction 1993; 48:110-116.

48. HUANG ES-R, Nalbandov A. Steroidogenesis of chicken granulosa and theca cells: in vitro incubation system. Biology of reproduction 1979; 20:442-453.
49. Kato M, Shimada K, Saito N, Noda K, Ohta M. Expression of P450 17 alpha-hydroxylase and P450aromatase genes in isolated granulosa, theca interna, and theca externa layers of chicken ovarian follicles during follicular growth. Biology of reproduction 1995; 52:405-410.
50. Sharp P, Talbot R, Main G, Dunn I, Fraser H, Huskisson N. Physiological roles of chicken LHRH-I and-II in the control of gonadotrophin release in the domestic chicken. Journal of Endocrinology 1990; 124:291-299.
51. Wilson S, Chairil R, Cunningham F, Gladwell R. Changes in the hypothalamic contents of LHRH-I and-II and in pituitary responsiveness to synthetic chicken LHRH-I and-II during the progesterone-induced surge of LH in the laying hen. Journal of endocrinology 1990; 127:487-496.
52. Johnson A, Li Z, Gibney JA, Malamed S. Vasoactive intestinal peptide-induced expression of cytochrome P450 cholesterol side-chain cleavage and 17 alpha-hydroxylase enzyme activity in hen granulosa cells. Biology of reproduction 1994; 51:327-333.
53. Sechman A. The role of thyroid hormones in regulation of chicken ovarian steroidogenesis. General and comparative endocrinology 2013; 190:68-75.
54. Hrabia A. Growth hormone production and role in the reproductive system of female chicken. General and comparative endocrinology 2015; 220:112-118.
55. Chabrolle C, Tosca L, Crochet S, Tesseraud S, Dupont J. Expression of adiponectin and its receptors (AdipoR1 and AdipoR2) in chicken ovary: potential role in ovarian steroidogenesis. Domestic animal endocrinology 2007; 33:480-487.
56. Wang X-J, Li Y, Song Q-Q, Guo Y-Y, Jiao H-C, Song Z-G, Lin H. Corticosterone regulation of ovarian follicular development is dependent on the energy status of laying hens. Journal of lipid research 2013; 54:1860-1876.
57. Onagbesan O, Bruggeman V, Decuypere E. Intra-ovarian growth factors regulating ovarian function in avian species: a review. Animal reproduction science 2009; 111:121-140.
58. Johnson P, Stephens C, Giles J. The domestic chicken: Causes and consequences of an egg a day. Poultry science 2015; 94:816-820.
59. Woods DC, Johnson A. Regulation of follicle-stimulating hormone-receptor messenger

RNA in hen granulosa cells relative to follicle selection. Biology of reproduction 2005; 72:643-650.

60. Kim D, Johnson AL. Vasoactive intestinal peptide promotes differentiation and clock gene expression in granulosa cells from prehierarchal follicles. Molecular reproduction and development 2016; 83:455-463.
61. Kim D, Ocón-Grove OM, Johnson AL. Lack of FSH-Responsiveness in Granulosa Cells from Hen Prehierarchal Follicles Is Mediated by Beta-Arrestin. Biology of Reproduction 2012; 87:127-127.
62. Johnson AL. The avian ovary and follicle development: some comparative and practical insights. Turkish Journal of Veterinary and Animal Sciences 2014; 38:660-669.
63. Johnson P, Kent T, Urick M, Giles J. Expression and regulation of anti-mullerian hormone in an oviparous species, the hen. Biology of reproduction 2008; 78:13-19.
64. Johnson P, Kent T, Urick ME, Trevino L, Giles J. Expression of anti-Mullerian hormone in hens selected for different ovulation rates. Reproduction 2009; 137:857-863.
65. Johnson P. Follicle selection in the avian ovary. Reproduction in domestic animals 2012; 47:283-287.
66. Ocon-Grove OM, Poole D, Johnson A. Bone morphogenetic protein 6 promotes FSH receptor and anti-Müllerian hormone mRNA expression in granulosa cells from hen prehierarchal follicles. Reproduction 2012; 143:825-833.
67. Wojtusik J, Johnson PA. Vitamin D regulates anti-Mullerian hormone expression in granulosa cells of the hen. Biology of reproduction 2012; 86:91.
68. Woods DC, Haugen MJ, Johnson A. Opposing actions of TGFβ and MAP kinase signaling in undifferentiated hen granulosa cells. Biochemical and biophysical research communications 2005; 336:450-457.
69. Johnson A, Solovieva E, Bridgham J. Relationship Between Steroidogenic Acute Regulatory Protein Expression and Progesterone Production in Hen Granulosa Cells During Follicle Development 1. Biology of reproduction 2002; 67:1313-1320.
70. Johnson A, Lee J. Granulosa cell responsiveness to follicle stimulating hormone during early growth of hen ovarian follicles. Poultry science 2016; 95:108-114.
71. Woods DC, Haugen MJ, Johnson A. Actions of epidermal growth factor receptor/mitogen-activated protein kinase and protein kinase C signaling in granulosa cells from Gallus

gallus are dependent upon stage of differentiation. Biology of reproduction 2007; 77:61-70.

72. Woods DC, Johnson A. Protein kinase C activity mediates LH-induced ErbB/Erk signaling in differentiated hen granulosa cells. Reproduction 2007; 133:733-741.
73. Johnson A. Intracellular mechanisms regulating cell survival in ovarian follicles. Animal reproduction science 2003; 78:185-201.
74. Johnson A, Bridgham J, Swenson J. Activation of the Akt/Protein Kinase B Signaling Pathway Is Associated with Granulosa Cell Survival 1. Biology of reproduction 2001; 64:1566-1574.
75. Tosca L, Chabrolle C, Crochet S, Tesseraud S, Dupont J. IGF-1 receptor signaling pathways and effects of AMPK activation on IGF-1-induced progesterone secretion in hen granulosa cells. Domestic animal endocrinology 2008; 34:204-216.
76. Tosca L, Crochet S, Ferré P, Foufelle F, Tesseraud S, Dupont J. AMP-activated protein kinase activation modulates progesterone secretion in granulosa cells from hen preovulatory follicles. Journal of Endocrinology 2006; 190:85-97.
77. Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA. Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocrine reviews 1998; 19:225-268.
78. Soares MJ. The prolactin and growth hormone families: pregnancy-specific hormones/cytokines at the maternal-fetal interface. Reproductive Biology and Endocrinology 2004; 2:51.
79. Niall H, Hogan M, Sauer R, Rosenblum I, Greenwood F. Sequences of pituitary and placental lactogenic and growth hormones: evolution from a primordial peptide by gene reduplication. Proceedings of the National Academy of Sciences 1971; 68:866-869.
80. Ben-Jonathan N, LaPensee CR, LaPensee EW. What can we learn from rodents about prolactin in humans? Endocrine reviews 2008; 29:1-41.
81. Hanks M, Alonzi J, Sharp P, Sang H. Molecular cloning and sequence analysis of putative chicken prolactin cDNA. Journal of molecular endocrinology 1989; 2:21-30.
82. Wong EA, Ferrin NH, Silsby JL, El Halawani ME. Cloning of a turkey prolactin cDNA: expression of prolactin mRNA throughout the reproductive cycle of the domestic turkey (Meleagris gallopavo). General and comparative endocrinology 1991; 83:18-26.

83. Kansaku N, Ohkubo T, Okabayashi H, Guémené D, Kuhnlein U, Zadworny D, Shimada K. Cloning of duck PRL cDNA and genomic DNA. General and comparative endocrinology 2005; 141:39-47.
84. Liu Z, Shi Z, Liu Y, Li M, Huang Y, Yao B. Molecular cloning and characterisation of the Magang goose prolactin gene. General and comparative endocrinology 2008; 155:208-216.
85. Hiyama G, Sato T, Zadworny D, Kansaku N. Cloning of PRL and VIP cDNAs of the Java sparrow (Padda oryzivora). Animal Science Journal 2009; 80:176-186.
86. Berwaer M, Martial J, Davis J. Characterization of an up-stream promoter directing extrapituitary expression of the human prolactin gene. Molecular endocrinology 1994; 8:635-642.
87. Berwaer M, Monget P, Peers B, Mathy-Hartert M, Bellefroid E, Davis JR, Belayew A, Martial JA. Multihormonal regulation of the human prolactin gene expression from 5000 bp of its upstream sequence. Molecular and cellular endocrinology 1991; 80:53-64.
88. Ohkubo T, Tanaka M, Nakashima K. Molecular cloning of the chicken prolactin gene and activation by Pit-1 and cAMP-induced factor in GH3 cells. General and comparative endocrinology 2000; 119:208-216.
89. Jiang R-S, Xu G-Y, Zhang X-Q, Yang N. Association of polymorphisms for prolactin and prolactin receptor genes with broody traits in chickens. Poultry Science 2005; 84:839-845.
90. Cui J-X, Du H-L, Liang Y, Deng X-M, Li N, Zhang X-Q. Association of polymorphisms in the promoter region of chicken prolactin with egg production. Poultry science 2006; 85:26-31.
91. Liang Y, Cui J, Yang G, Leung FC, Zhang X. Polymorphisms of 5′ flanking region of chicken prolactin gene. Domestic animal endocrinology 2006; 30:1-16.
92. Bhattacharya T, Chatterjee R, Sharma R, Niranjan M, Rajkumar U. Associations between novel polymorphisms at the 5′-UTR region of the prolactin gene and egg production and quality in chickens. Theriogenology 2011; 75:655-661.
93. Kansaku N, Hiyama G, Sasanami T, Zadworny D. Prolactin and growth hormone in birds: Protein structure, gene structure and genetic variation. The journal of poultry science 2008; 45:1-6.
94. Hiyama G, Zadworny D, Kansaku N. Cloning and comparison of prolactin promoter in galliformes. The journal of poultry science 2009; 46:6-12.

95. Opel H, Proudman J. Stimulation of prolactin release in turkeys by vasoactive intestinal peptide. Proceedings of the Society for Experimental Biology and Medicine 1988; 187:455-460.
96. El Halawani M, Silsby J, Mauro L. Vasoactive intestinal peptide is a hypothalamic prolactin-releasing neuropeptide in the turkey (Meleagris gallopavo). General and comparative endocrinology 1990; 78:66-73.
97. Harvey S, Scanes C, Chadwick A, Bolton N. The effect of thyrotropin-releasing hormone (TRH) and somatostatin (GHRIH) on growth hormone and prolactin secretion in vitro and in vivo in the domestic fowl (Gallus domesticus). Neuroendocrinology 1978; 26:249-260.
98. Robinzon B, Koike T, Neldon H, Kinzler S, Hendry I, Halawani ME. Physiological effects of arginine vasotocin and mesotocin in cockerels. British poultry science 1988; 29:639-652.
99. Youngren OM, Chaiseha Y, El Halawani ME. Serotonergic stimulation of avian prolactin secretion requires an intact dopaminergic system. General and comparative endocrinology 1998; 112:63-68.
100. Tong Z, Pitts G, You S, Foster D, El Halawani M. Vasoactive intestinal peptide stimulates turkey prolactin gene expression by increasing transcription rate and enhancing mRNA stability. Journal of molecular endocrinology 1998; 21:259-266.
101. Xu M, Proudman J, Pitts G, Wong E, Foster D, Halawani ME. Vasoactive intestinal peptide stimulates prolactin mRNA expression in turkey pituitary cells: effects of dopaminergic drugs. Proceedings of the Society for Experimental Biology and Medicine 1996; 212:52-62.
102. Youngren OM, Pitts GR, Phillips RE, El Halawani ME. The stimulatory and inhibitor effects of dopamine on prolactin secretion in the turkey. General and comparative endocrinology 1995; 98:111-117.
103. Youngren OM, Pitts GR, Phillips RE, El Halawani ME. Dopaminergic control of prolactin secretion in the turkey. General and comparative endocrinology 1996; 104:225-230.
104. Youngren OM, Chaiseha Y, El Halawani ME. Regulation of prolactin secretion by dopamine and vasoactive intestinal peptide at the level of the pituitary in the turkey. Neuroendocrinology 1998; 68:319-325.
105. Al Kahtane A, Chaiseha Y, El Halawani M. Dopaminergic regulation of avian prolactin

gene transcription. Journal of molecular endocrinology 2003; 31:185-196.

106. Hall T, Harvey S, Chadwick A. Control of prolactin secretion in birds: a review. General and comparative endocrinology 1986; 62:171-184.

107. El Halawani M, Burke W, Millam J, Fehrer S, Hargis B. Regulation of prolactin and its role in gallinaceous bird reproduction. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 1984; 232:521-529.

108. Sharp PJ, Dawson A, Lea RW. Control of luteinizing hormone and prolactin secretion in birds. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology 1998; 119:275-282.

109. Corcoran D, Proudman J. Isoforms of turkey prolactin: evidence for differences in glycosylation and in tryptic peptide mapping. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry 1991; 99:563-570.

110. Sinha Y. Structural variants of prolactin: occurrence and physiological significance. Endocrine Reviews 1995; 16:354-369.

111. Wilson C, Leigh A, Chapman A. Gonadotrophin glycosylation and function. Journal of Endocrinology 1990; 125:3-14.

112. Perlman S, van den Hazel B, Christiansen J, Gram-Nielsen S, Jeppesen CB, Andersen KV, Halkier T, Okkels S, Schambye HT. Glycosylation of an N-terminal extension prolongs the half-life and increases the in vivo activity of follicle stimulating hormone. The Journal of Clinical Endocrinology & Metabolism 2003; 88:3227-3235.

113. Huang X, Hui MN, Liu Y, Yuen DS, Zhang Y, Chan WY, Lin HR, Cheng SH, Cheng CH. Discovery of a novel prolactin in non-mammalian vertebrates: evolutionary perspectives and its involvement in teleost retina development. PloS one 2009; 4:e6163.

114. Bu G, Liang X, Li J, Wang Y. Extra-pituitary prolactin (PRL) and prolactin-like protein (PRL-L) in chickens and zebrafish. General and comparative endocrinology 2015; 220:143-153.

115. Ijiri D, Higuchi A, Saegusa A, Miura M, Matsubara T, Kanai Y, Ohtsuka A, Hirabayashi M. Role of prolactin-like protein (PRL-L) in cold-induced increase of muscle mass in chicks. General and comparative endocrinology 2013; 186:94-100.

116. KELLY PA, DJIANE J, POSTEL-VINAY M-C, EDERY M. The prolactin/growth hormone receptor family. Endocrine reviews 1991; 12:235-251.

117. Goffin V, Kelly PA. The prolactin/growth hormone receptor family: structure/function relationships. Journal of mammary gland biology and neoplasia 1997; 2:7-17.
118. Goffin V, Kelly PA. Prolactin and growth hormone receptors. Clinical endocrinology 1996; 45:247-255.
119. Wells JA, de Vos AM. Hematopoietic receptor complexes. Annual review of biochemistry 1996; 65:609-634.
120. Mui AL-F, Miyajima A. Cytokine receptors and signal transduction. Progress in growth factor research 1994; 5:15-35.
121. Kishimoto T, Taga T, Akira S. Cytokine signal transduction. Cell 1994; 76:253-262.
122. Deng AY, Dene H, Rapp JP. Mapping of a quantitative trait locus for blood pressure on rat chromosome 2. Journal of Clinical Investigation 1994; 94:431.
123. Gearing D, Druck T, Huebner K, Overhauser J, Gilbert D, Copeland N, Jenkins N. The leukemia inhibitory factor receptor (LIFR) gene is located within a cluster of cytokine receptor loci on mouse chromosome 15 and human chromosome 5p12-p13. Genomics 1993; 18:148-150.
124. Vincent AL, Wang L, Tuggle CK, Robic A, Rothschild MF. Prolactin receptor maps to pig chromosome 16. Mammalian Genome 1997; 8:793-794.
125. Arden K, Boutin J-M, Djiane J, Kelly P, Cavenee W. The receptors for prolactin and growth hormone are localized in the same region of human chromosome 5. Cytogenetic and Genome Research 1990; 53:161-165.
126. Davis JA, Linzer DI. Expression of multiple forms of the prolactin receptor in mouse liver. Molecular Endocrinology 1989; 3:674-680.
127. Clarke DL, Linzer D. Changes in prolactin receptor expression during pregnancy in the mouse ovary. Endocrinology 1993; 133:224-232.
128. Postel-Vinay M. Growth hormone-and prolactin-binding proteins: soluble forms of receptors. Hormone Research in Paediatrics 1996; 45:178-181.
129. Dunn I. Genetic mapping of the chicken prolactin receptor gene: a candidate gene for the control of broodiness. British poultry science 1998; 39:23-24.
130. Tanaka M, Maeda K, Okubo T, Nakashima K. Double antenna structure of chicken prolactin receptor deduced from the cDNA sequence. Biochemical and biophysical research communications 1992; 188:490-496.

131. Chen X, Horseman ND. Cloning, expression, and mutational analysis of the pigeon prolactin receptor. Endocrinology 1994; 135:269-276.
132. Xing G, Zhao Q, Mao J, Liu T, Wang G. Identification and characterization of goose prolactin receptor. Poultry science 2011; 90:1050-1057.
133. Wang J, Hou S, Huang W, Yang X, Zhu X, Liu X. Molecular cloning of prolactin receptor of the Peking duck. Poultry science 2009; 88:1016-1022.
134. Bu G, Huang G, Fu H, Li J, Huang S, Wang Y. Characterization of the novel duplicated PRLR gene at the late-feathering K locus in Lohmann chickens. Journal of molecular endocrinology 2013; 51:261-276.
135. Goffin V, Binart N, Touraine P, Kelly PA. Prolactin: the new biology of an old hormone. Annual review of physiology 2002; 64:47-67.
136. Ben-Jonathan N, Mershon JL, Allen DL, Steinmetz RW. Extrapituitary prolactin: distribution, regulation, functions, and clinical aspects. Endocrine reviews 1996; 17:639-669.
137. Bouilly J, Sonigo C, Auffret J, Gibori G, Binart N. Prolactin signaling mechanisms in ovary. Molecular and cellular endocrinology 2012; 356:80-87.
138. Devi YS, Halperin J. Reproductive actions of prolactin mediated through short and long receptor isoforms. Molecular and cellular endocrinology 2014; 382:400-410.
139. Bachelot A, Binart N. Reproductive role of prolactin. Reproduction 2007; 133:361-369.
140. Pezet A, Favre H, Kelly PA, Edery M. Inhibition and restoration of prolactin signal transduction by suppressors of cytokine signaling. Journal of Biological Chemistry 1999; 274:24497-24502.
141. Clevenger CV. Roles and regulation of stat family transcription factors in human breast cancer. The American journal of pathology 2004; 165:1449-1460.
142. Chan CB, Liu X, Ensslin MA, Dillehay DL, Ormandy CJ, Sohn P, Serra R, Ye K. PIKE-A is required for prolactin-mediated STAT5a activation in mammary gland development. The EMBO journal 2010; 29:956-968.
143. Curlewis J. Seasonal prolactin secretion and its role in seasonal reproduction: a review. Reproduction, Fertility and Development 1992; 4:1-23.
144. Sharp PJ, Blache D. A neuroendocrine model for prolactin as the key mediator of seasonal breeding in birds under long-and short-day photoperiods. Canadian journal of physiology

and pharmacology 2003; 81:350-358.

145. Goldsmith A, Edwards C, Koprucu M, Silver R. Concentrations of prolactin and luteinizing hormone in plasma of doves in relation to incubation and development of the crop gland. Journal of Endocrinology 1981; 90:437-443.

146. Schooley JP, Riddle O, Bates RW. Effective Stimulation of Crop-Sacs by Prolactin in Hypophysecto-mized and in Adrenalectomized Pigeons. Proceedings of the Society for Experimental Biology and Medicine 1937; 36:408-411.

147. Horseman ND, Buntin JD. Regulation of pigeon cropmilk secretion and parental behaviors by prolactin. Annual review of nutrition 1995; 15:213-238.

148. Anderson TR, Pitts DS, Nicoll CS. Prolactin's mitogenic action on the pigeon crop-sac mucosal epithelium involves direct and indirect mechanisms. General and comparative endocrinology 1984; 54:236-246.

149. Sreekumar K, Sharp P. Effect of photostimulation on concentrations of plasma prolactin in castrated bantams (Gallus domesticus). Journal of neuroendocrinology 1998; 10:147-154.

150. Proudman J. Circulating prolactin levels at the end of the photophase and the end of the scotophase throughout the reproductive cycle of the turkey hen. Poultry science 1998; 77:303-308.

151. Khalil HA, Hanafy AM, Saleh SY, Medan MS. Comparative changes in the serum concentrations of inhibin-B, prolactin, gonadotropins and steroid hormones at different reproductive states in domestic turkey hens. Journal of Reproduction and Development 2009; 55:523-528.

152. Zadworny D, Walton J, Etches R. Effect of environment on the intake of food and water, body weight, egg production and plasma concentrations of corticosterone and prolactin in turkey hens. British poultry science 1986; 27:639-650.

153. Zadworny D, Walton J, Etches R. The relationship between plasma concentrations of prolactin and consumption of feed and water during the reproductive cycle of the domestic turkey. Poultry science 1985; 64:401-410.

154. Etches R, Cheng K. A homologous radioimmunoassay for turkey prolactin: Changes during the reproductive and ovulatory cycle. Poultry science 1982; 61:1354-1362.

155. Sharp P, Macnamee M, Sterling R, Lea R, Pedersen H. Relationships between prolactin, LH and broody behaviour in bantam hens. Journal of Endocrinology 1988; 118:279-286.

156. Goldsmith A, Burke S, Prosser JM. Inverse changes in plasma prolactin and LH concentrations in female canaries after disruption and reinitiation of incubation. Journal of Endocrinology 1984; 103:251-256.
157. Lien R, Siopes T. The relationship of plasma thyroid hormone and prolactin concentrations to egg laying, incubation behavior, and molting by female turkeys exposed to a one-year natural daylength cycle. General and comparative endocrinology 1993; 90:205-213.
158. Lien R, Siopes T. Turkey plasma thyroid hormone and prolactin concentrations throughout an egg laying cycle and in relation to photorefractoriness. Poultry science 1989; 68:1409-1417.
159. Proudman J, Siopes T. Relative and absolute photorefractoriness in turkey hens: profiles of prolactin, thyroxine, and triiodothyronine early in the reproductive cycle. Poultry science 2002; 81:1218-1223.
160. Karatzas C, Guemene D, Zadwomy D, Kuhnlein U. Changes in expression of the prolactin and growth hormone gene during different reproductive stages in the pituitary gland of turkeys. Reproduction Nutrition Development 1997; 37:69-79.
161. Cherms Jr F, Herrick R, McShan W, Hymer W. Prolactin Content of the Anterior Pituitary Gland of Turkey Hens in Diiferent Reproductive Stages. Endocrinology 1962; 71:288-292.
162. Tong Z, Pitts G, Foster D, El Halawani M. Transcriptional and post-transcriptional regulation of prolactin during the turkey reproductive cycle. Journal of molecular endocrinology 1997; 18:223-231.
163. Ohkubo T, Tanaka M, Nakashima K, Sharp P. Relationship between prolactin receptor mRNA in the anterior pituitary gland and hypothalamus and reproductive state in male and female bantams (Gallus domesticus). General and comparative endocrinology 1998; 111:167-176.
164. Youngren O, Chaiseha Y, Phillips R, El Halawani M. Vasoactive intestinal peptide concentrations in turkey hypophysial portal blood differ across the reproductive cycle. General and comparative endocrinology 1996; 103:323-330.
165. Chaiseha Y, El Halawani ME. Expression of vasoactive intestinal peptide/peptide histidine isoleucine in several hypothalamic areas during the turkey reproductive cycle: relationship to prolactin secretion. Neuroendocrinology 1999; 70:402-412.
166. Romanov MN, Talbot R, Wilson P, Sharp P. Genetic control of incubation behavior in the

domestic hen. Poultry science 2002; 81:928-931.

167. Buntin JD. Role of prolactin in avian incubation behavior and care of young: is there a causal relationship? Annals of the New York Academy of Sciences 1986; 474:252-267.

168. El Halawani M, Burke W, Dennison P. Effect of nest-deprivation on serum prolactin level in nesting female turkeys. Biology of Reproduction 1980; 23:118-123.

169. Kuwayama T, Shimada K, Saito N, Ohkubo T, Sato K, Wada M, Ichinoe K. Effects of removal of chicks from hens on concentrations of prolactin, luteinizing hormone and oestradiol in plasma of brooding Gifujidori hens. Journal of reproduction and fertility 1992; 95:617-622.

170. Richard-Yris M-A, Sharp P, Wauters A-M, Guémené D, Richard J-P, Forasté M. Influence of stimuli from chicks on behavior and concentrations of plasma prolactin and luteinizing hormone in incubating hens. Hormones and behavior 1998; 33:139-148.

171. Crisostomo S, Guemene D, Garreau-Mills M, Morvan C, Zadworny D. Prevention of incubation behavior expression in turkey hens by active immunization against prolactin. Theriogenology 1998; 50:675-690.

172. Crisóstomo S, Guémené D, Garreau-Mills M, Zadworny D. Prevention of the expression of incubation behaviour using passive immunisation against prolactin in turkey hens (Meleagris gallopavo). Reproduction Nutrition Development 1997; 37:253-266.

173. El Halawani M, Silsby J, Rozenboim I, Pitts G. Increased egg production by active immunization against vasoactive intestinal peptide in the turkey (Meleagris gallopavo). Biology of reproduction 1995; 52:179-183.

174. El Halawani M, Pitts G, Sun S, Silsby J, Sivanandan V. Active immunization against vasoactive intestinal peptide prevents photo-induced prolactin secretion in turkeys. General and comparative endocrinology 1996; 104:76-83.

175. Li W, Liu Y, Yu Y, Huang Y, Liang S, Shi Z. Prolactin plays a stimulatory role in ovarian follicular development and egg laying in chicken hens. Domestic animal endocrinology 2011; 41:57-66.

176. Buntin JD, Tesch D. Effects of intracranial prolactin administration on maintenance of incubation readiness, ingestive behavior, and gonadal condition in ring doves. Hormones and behavior 1985; 19:188-203.

177. El Halawani M, Rozenboim I. The ontogeny and control of incubation behavior in turkeys.

Poultry Science 1993; 72:906-911.
178. Riddle O, Bates RW, Lahr EL. Prolactin induces broodiness in fowl. American Journal of Physiology--Legacy Content 1935; 111:352-360.
179. Saeki Y, Tanabe Y. Changes in prolactin content of fowl pituitary during broody periods and some experiments on the induction of broodiness. Poultry Science 1955; 34:909-919.
180. Youngren O, El Halawani M, Silsby J, Phillips R. Intracranial prolactin perfusion induces incubation behavior in turkey hens. Biology of reproduction 1991; 44:425-431.
181. Opel H, Proudman J. Failure of mammalian prolactin to induce incubation behavior in chickens and turkeys. Poultry science 1980; 59:2550-2558.
182. Lehrman DS, Brody P. Does prolactin induce incubation behaviour in the ring dove? Journal of Endocrinology 1961; 22:269-275.
183. Schradin C, Anzenberger G. Prolactin, the hormone of paternity. Physiology 1999; 14:223-231.
184. Buntin JD. Neural and hormonal control of parental behavior in birds. Advances in the Study of Behavior 1996; 25:161-213.
185. Angelier F, Chastel O. Stress, prolactin and parental investment in birds: a review. General and comparative endocrinology 2009; 163:142-148.
186. Sockman KW, Schwabl H, Sharp PJ. The role of prolactin in the regulation of clutch size and onset of incubation behavior in the American kestrel. Hormones and Behavior 2000; 38:168-176.
187. Sockman KW, Sharp PJ, Schwabl H. Orchestration of avian reproductive effort: an integration of the ultimate and proximate bases for flexibility in clutch size, incubation behaviour, and yolk androgen deposition. Biological Reviews 2006; 81:629-666.
188. Ryan CP, Dawson A, Sharp PJ, Meddle SL, Williams TD. Circulating breeding and pre-breeding prolactin and LH are not associated with clutch size in the zebra finch (Taeniopygia guttata). General and comparative endocrinology 2014; 202:26-34.
189. Greives TJ, Kingma SA, Beltrami G, Hau M. Melatonin delays clutch initiation in a wild songbird. Biology letters 2012; 8:330-332.
190. Ohkubo T, Tanaka M, Nakashima K, Talbot R, Sharp P. Prolactin receptor gene expression in the brain and peripheral tissues in broody and nonbroody breeds of domestic hen. General and comparative endocrinology 1998; 109:60-68.

191. Goldsmith AR, Hall M. Prolactin concentrations in the pituitary gland and plasma of Japanese quail in relation to photoperiodically induced sexual maturation and egg laying. General and comparative endocrinology 1980; 42:449-454.
192. Sharp P, Klandorf H, McNeilly A. Plasma prolactin, thyroxine, triiodothyronine, testosterone, and luteinizing hormone during a photoinduced reproductive cycle in mallard drakes. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 1986; 238:409-413.
193. El Halawani M, Silsby J, Fehrer S, Behnke E. Effects of estrogen and progesterone on serum prolactin and luteinizing hormone levels in ovariectomized turkeys (Meleagris gallopavo). General and comparative endocrinology 1983; 52:67-78.
194. March J, Sharp P, Wilson P, Sang H. Effect of active immunization against recombinant-derived chicken prolactin fusion protein on the onset of broodiness and photoinduced egg laying in bantam hens. Journal of reproduction and fertility 1994; 101:227-233.
195. Ormandy CJ, Camus A, Barra J, Damotte D, Lucas B, Buteau H, Edery M, Brousse N, Babinet C, Binart N. Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. Genes & development 1997; 11:167-178.
196. Horseman ND, Zhao W, Montecino-Rodriguez E, Tanaka M, Nakashima K, Engle SJ, Smith F, Markoff E, Dorshkind K. Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene. The EMBO journal 1997; 16:6926-6935.
197. Shao R, Nutu M, Weijdegård B, Egecioglu E, Fernandez-Rodriguez J, Tallet E, Goffin V, Ling C, Billig H. Differences in prolactin receptor (PRLR) in mouse and human fallopian tubes: evidence for multiple regulatory mechanisms controlling PRLR isoform expression in mice. Biology of reproduction 2008; 79:748-757.
198. Goldsmith A, Williams D. Incubation in mallards (Anas platyrhynchos): changes in plasma levels of prolactin and luteinizing hormone. Journal of Endocrinology 1980; 86:371-379.
199. Wingfield JC, Goldsmith AR. Plasma levels of prolactin and gonadal steroids in relation to multiple-brooding and renesting in free-living populations of the song sparrow, Melospiza melodia. Hormones and Behavior 1990; 24:89-103.
200. Zadworny D, Etches R. Effects of ovariectomy or force feeding on the plasma concentrations of prolactin and luteinizing hormone in incubating turkey hens. Biology of

reproduction 1987; 36:81-88.

201. El Halawani M, Silsby J, Youngren O, Phillips R. Exogenous prolactin delays photo-induced sexual maturity and suppresses ovariectomy-induced luteinizing hormone secretion in the turkey (Meleagris gallopavo). Biology of reproduction 1991; 44:420-424.
202. Talbot RT, Hanks MC, Sterling RJ, Sang HM, Sharp PJ. Pituitary prolactin messenger ribonucleic acid levels in incubating and laying hens: effects of manipulating plasma levels of vasoactive intestinal polypeptide. Endocrinology 1991; 129:496-502.
203. Rozenboim I, Tabibzadeh C, Silsby J, El Halawani M. Effect of ovine prolactin administration on hypothalamic vasoactive intestinal peptide (VIP), gonadotropin releasing hormone I and II content, and anterior pituitary VIP receptors in laying turkey hens. Biology of reproduction 1993; 48:1246-1250.
204. Camper P, Burke W. The effects of prolactin on the gonadotropin induced rise in serum estradiol and progesterone of the laying turkey. General and comparative endocrinology 1977; 32:72-77.
205. Tabibzadeh C, Rozenboim I, Silsby JL, Pitts GR, Foster DN, El Halawani M. Modulation of ovarian cytochrome P450 17 alpha-hydroxylase and cytochrome aromatase messenger ribonucleic acid by prolactin in the domestic turkey. Biology of reproduction 1995; 52:600-608.
206. Reddy I, David C, Sarma P, Singh K. The possible role of prolactin in laying performance and steroid hormone secretion in domestic hen (Gallus domesticus). General and comparative endocrinology 2002; 127:249-255.
207. Reddy I, David C, Raju S. Effect of suppression of plasma prolactin on luteinizing hormone concentration, intersequence pause days and egg production in domestic hen. Domestic animal endocrinology 2007; 33:167-175.
208. Lea R, Dods A, Sharp P, Chadwick A. The possible role of prolactin in the regulation of nesting behaviour and the secretion of luteinizing hormone in broody bantams. Journal of Endocrinology 1981; 91:89-97.
209. Sharp P, Sterling R, Talbot R, Huskisson N. The role of hypothalamic vasoactive intestinal polypeptide in the maintenance of prolactin secretion in incubating bantam hens: observations using passive immunization, radioimmunoassay and immunohistochemistry. Journal of Endocrinology 1989; 122:5-NP.

210. Zadworny D, Shimada K, Ishida H, Sato K. Gonadotropin-stimulated estradiol production in small ovarian follicles of the hen is suppressed by physiological concentrations of prolactin in vitro. General and comparative endocrinology 1989; 74:468-473.
211. Taira H, Beck M. Activity of three-β-hydroxysteroid dehydrogenase in granulosa cells treated in vitro with luteinizing hormone, follicle-stimulating hormone, prolactin, or a combination. Poultry science 2006; 85:1769-1774.
212. Li Y, Yang C. Effect of prolactin on the steroidogenesis and proliferation of cultured theca cells from domestic hens. Poultry and Avian Biology Reviews 1995; 6:295.
213. Li C, Wang Y, Yang L. Effective mechanism of prolactin on the follicular theca cells from domestic fowl. Chinese Journal of Veterinary Science 1998; 1.

CHAPTER II

EFFECTS OF NON-GLYCOSYLATED AND GLYCOSYLATED PROLACTIN ON BASAL AND GONADOTROPIN-STIMULATED STEROIDOGENESIS IN CHICKEN OVARIAN FOLLICLES

Shenqiang Hu and David Zadworny*

Department of Animal Science, McGill University, Macdonald Campus, Ste. Anne de Bellevue, Quebec, H9X 3V9 Canada

* **Corresponding author.** E-mail: david.zadworny@mcgill.ca

Running title: Glycosylation of PRL on steroidogenesis in chickens

2.1 ABSTRACT

In galliformes, the circulating isoform of prolactin (PRL) significantly changes during different reproductive states. However, the role of the major isoform (glycosylated PRL, G-PRL) in ovarian steroidogenesis is unknown. The present study aimed to compare the effects of non-glycosylated (NG-) and G-PRL on basal and gonadotropin-stimulated estradiol (E_2) and progesterone (P_4) production in granulosa cells or follicular walls of chicken different size class follicles. In the initial experiment, granulosa cells of preovulatory F3-F1 and prehierarchical 6-8 mm follicles were incubated for 24 with different concentrations of NG- or G-PRL (0,1 ,10 ,100 or 1000 ng/ml). In the subsequent experiments, these categorized granulosa cells and follicular walls of prehierarchical 4-6, 2-4 and < 2 mm follicles were incubated for 24 h in the absence and presence of 10 ng/ml FSH or LH, or in combination with different concentrations of NG- or G-PRL (10, 100 or 1000 ng/ml). We observed that lower levels of NG-PRL induced E_2 and P_4 secretion in granulosa cells of either preovulatory or prehierarchical follicles but at higher levels this effect was reduced. In contrast, G-PRL promoted basal E_2 and P_4 secretion in preovulatory granulosa cells but was inhibitory in prehierarchical granulosa cells. Results obtained by real-time qPCR demonstrated that these effects were mediated through modulation the expression of *StAR*, *CYP11A1*, *CYP19A1* and *3β-HSD*. Furthermore, G-PRL was less potent than NG-PRL in inhibiting FSH- or LH-stimulated E_2 and P_4 production in granulosa cells of preovulatory follicles, whereas NG-PRL enhanced but G-PRL reduced FSH-induced P_4 production in those of prehierarchical follicles. In follicular walls from each group of prehierarchical 4-6, 2-4 and < 2 mm follicles, NG- and G-PRL had both stimulatory and inhibitory influences on the actions of FSH on E_2 and P_4 secretion, but both suppressed LH-induced E_2 and P_4 secretion except for the synergistic effects of LH and G-PRL on P_4 secretion by follicular walls of the 4-6 mm follicles. Taken together, these results suggest that both NG- and G-PRL are biologically active in regulating basal and gonadotropin-stimulated E_2 and P_4 production in chicken ovarian follicles. However, their effects are different depending on the concentration, the type of gonadotropin (FSH or LH) and the stage of follicle development.

Key words: Chicken; Prolactin; Glycosylation; Ovarian follicles; Estradiol; Progesterone

2.2 INTRODUCTION

In the laying hen, the main source of sex steroids are ovarian follicles, which consist of primary oocytes in the center surrounded by adjacent granulosa and distant theca layers which are the two major sites of steroidogenesis [1]. It has been established that both morphological structure and steroidogenic capability of granulosa and theca layers vary with follicular growth and development [2]. During the prehierarchical stage, theca cells of immature (< 8 mm) follicles mainly synthesize androgens and estrogens, whereas, granulosa cells are maintained in an undifferentiated state and are steroidogenically inactive [3]. However, once the follicles enter the preovulatory hierarchy, granulosa cells are differentiated and become steroidogenically active [3]. As follicles mature, P_4 secretion by granulosa cells increases while theca cells reduce E_2 biosynthesis [4]. The process of ovarian steroidogenesis is primarily under the control of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and many locally secreted factors [5, 6]. In addition, there is evidence that prolactin (PRL) is also involved in regulating vertebrate ovarian functions [7, 8].

In chickens and turkeys which display incubation behaviour, elevated levels of PRL are associated with ovarian involution as hens incubate their clutch of eggs. Suppression of PRL levels via active or passive immunization against PRL or its major releasing factor (vasoactive intestinal peptide, VIP) blocks incubation behaviour [9-12], suggesting that high levels of PRL are a requisite aspect of the behaviour. Evidence supports both indirect (inhibition of gonadotrophin release [13-16]) and direct (inhibition of steroidogenesis [17-20]) roles for high levels of PRL suppressing the hypothalamic-hypophyseal-ovarian axis to initiate ovarian regression. However, the effects of PRL are concentration dependent. At lower levels, PRL would appear to be pro-gonadal since passive immunization against PRL reduces large white follicular growth and hence recruitment into the follicular hierarchy in chickens [21]. In addition, photostimulation-induced increase in PRL secretion is associated with the onset of egg laying in parallel with increased ovarian steroid levels [22-24]. However, the effects of PRL on follicular cell steroidogenesis are variable according to the stage of the ovulation cycle, follicle size and concentration [25]. Consistent with the direct effects of PRL on ovarian functions, the receptor (PRLR) is widely expressed in the follicular hierarchy with higher levels in non-hierarchical follicles [26].

During the transition to incubation behaviour, hens simultaneously lay eggs while incubating eggs. At this time the concentration of PRL increases above a threshold value to change from pro- to anti-gonadal roles consistent with a shift in circulating PRL isoform. Both the absolute and relative amounts of glycosylated (G) to non-glycosylated (NG-) PRL change during this interval with the dominant form being G-PRL [27, 28]. The physiological role of this isoform is unknown although in mammals G-PRL has lower biological activity than NG-PRL and is postulated to down regulate PRL action in selective target tissues [29]. Since PRL glycosylation can influence the ligand binding affinity [30], it is possible that glycosylation may modify the biological actions of PRL in the ovary. Therefore, the present study aimed to examine the effects of NG- and G-PRL on both basal and gonadotropin-stimulated steroid production as well as the expression of steroidogenic enzyme genes in granulosa cells or follicular walls of follicles at different developmental stages in chickens.

2.3 MATERIALS AND METHODS

2.3.1 Hormones, chemicals and reagents

Recombinant human FSH (rhFSH, AFP8468A), ovine LH (oLH, AFP-5551B), non-glycosylated ovine PRL (NG-oPRL, AFP-10692C) and glycosylated ovine PRL (G-oPRL, AFP-5742B) were obtained from National Hormone & Peptide Program (Torrance, CA, USA). A stock solution of each hormone was correspondingly prepared with either PBS at appropriate pH or 0.01M $NaHCO_3$, and then stored in small aliquots at -80 ℃. The final concentration of each working solution used for corresponding treatment was prepared with culture medium. Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium/Nutrient Mixture F12 (DMEM/F12), 0.4% Trypan Blue Solution as well as penicillin and streptomycin mixture were purchased from Invitrogen Life technologies (Carlsbad, CA, USA). Type II collagenase was purchased from Sigma-Aldrich (Oakville, ON, Canada). Trizol reagent and high-capacity cDNA reverse transcription kit were purchased from Invitrogen Life technologies (Carlsbad, CA, USA). Power SYBR Green PCR Master Mix was purchased from D-Mark Bioscience (Toronto, ON, Canada). The enzyme immunoassay (EIA) kits for E_2 and P_4 were purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA). Protein Assay Kit and Bovine Serum Albumin (BSA) were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

2.3.2 Animals and tissue collection

All experimental procedures using chickens in this study were approved by the Faculty Animal Care Committee of McGill University. White Leghorn hens, 25-35 weeks of age and laying actively, were used in all studies described. Hens were fed *ad libitum* and kept in individual cages under standard conditions at the Poultry Complex of Macdonald Campus Farm, McGill University. The time of oviposition was monitored for each hen using surveillance camera (Lorex corporation, Maryland, USA), and ovulation was predicted to occur within 15-30 min after oviposition of the previous egg in the laying sequence. Hens, with an egg in the oviduct, were euthanized by cervical dislocation about 1-4 h before predicted time of a mid-sequence ovulation. After slaughter, the ovary from each hen was immediately removed and placed into ice-cold 0.9% NaCl solution. According to the diameter and the position in the follicular hierarchy, ovarian follicles were categorized into several groups, including the prehierarchical (< 2, 2-4, 4-6 and 6-8 mm) and the three largest preovulatory (namely F3-F1; 24-40 mm, F3 < F2 < F1) follicles. After being cleaned of surrounding vascular and connective tissues with fine tweezers, follicular walls containing theca and granulosa layers from the < 2, 2-4 and 4-6 mm follicles were obtained by dissecting follicles with sterile scalpel blades or needles following being washed with pre-heated culture media (DMEM/F12 supplemented with 1% penicillin-streptomycin mixture) to remove oocyte contents. Regarding the 6-8 mm and F3-F1 follicles, granulosa layers were separated from theca layers according to the method as previously described [1]. In brief, after being washed with ice-cold 0.9% NaCl solution, preovulatory follicles were cut with a scalpel blade along the line of the stigma, and intra-follicular contents including granulosa cell layers were immediately inverted into a dish containing culture medium while theca layers remained within the residual follicles. With respect to the 6-8 mm follicles, each follicle was slit and then inverted using fine forceps, granulosa cell layers were separated by gently shaking or peeling off from the inverted follicular tissue in culture medium.

2.3.3 Effects of PRL variants on basal steroidogenesis in granulosa cells of preovulatory F3-F1 and prehierarchical 6-8 mm follicles

Granulosa cell layers harvested from each group of the F3-F1 and 6-8 mm follicles were digested with 0.1% Type II collagenase, respectively. The number of each group of granulosa cells

were counted using a hemocytometer and cell viability was assessed by trypan blue exclusion test. The cells were then seeded onto 12-well culture plates at a density of ~3×10^5 cells/well in 1 ml DMEM/F12 medium containing 3% FBS and 1% penicillin-streptomycin mixture. Cells were cultured at 38.5 ℃ in a humidified atmosphere of 95% air and 5% CO_2. After 24 h of incubation with a medium change at 6 h, non-attached cells were removed by aspiration and adherent cells were washed three times with serum-free medium (DMEM/F12 supplemented with 1% penicillin-streptomycin mixture). Serum-free medium was used in all further incubations. The cells were subsequently treated with different concentrations of NG- or G-oPRL (0, 1, 10, 100 or 1000 ng/ml) for another 24 h. Finally, the culture media and cells were collected for steroid hormone determination and gene expression analyses, respectively. Each *in vitro* experiment was independently performed at least three times using tissues from different hens.

2.3.4 Effects of PRL variants on FSH- or LH-stimulated steroid secretion in granulosa cells or follicular walls of follicles at different developmental stages

Granulosa cells isolated from each group of the F3-F1 and 6-8 mm follicles were cultured as previously described. In addition, follicular walls harvested from each group of the 4-6, 2-4 and < 2 mm follicles were randomly allocated into 12-well culture plates in 1 ml DMEM/F12 medium containing 1% penicillin-streptomycin mixture and different treatments, respectively, with 1, 3 and 6 follicles per well corresponding to the 4-6, 2-4 and < 2 mm follicular class. Follicular walls were then incubated at 38.5 ℃ for 24 h in a humidified atmosphere of 95% air and 5% CO_2. Treatments herein include 0 or 10 ng/ml rhFSH or oLH alone, or in combination with different concentrations of NG- or G-oPRL (10, 100 or 1000 ng/ml). Finally, the culture media and follicular walls were collected for steroid hormone determination and protein concentration measurement, respectively. The secretion of steroid hormones by follicular walls was expressed per milligram of protein per 24 h, respectively. Each *in vitro* experiment was independently performed at least three times using tissues from different hens.

2.3.5 RNA isolation and real time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. The purity and concentration of RNA was determined using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the integrity of RNA was

assessed by visualization of the 28S/18S rRNA ratio after electrophoresis on 1.5% agarose gels. The cDNA was then synthesized from 1 µg RNA using high-capacity cDNA reverse transcription kit (Invitrogen, USA). Real time quantitative PCR (qPCR) reactions were performed on the CFX384^{TM} real-time PCR detection system (Bio-Rad, USA) using SYBR Green master mix (D-Mark Bioscience, Ontario, Canada). Reactions were conducted with the following conditions: pre-denaturation at 95 ℃ for 5 min, followed by 40 cycles of denaturation at 95 ℃ for 15 s and annealing/extension at corresponding temperature of each primer set for 30 s. The no-template controls and negative controls without reverse transcriptase were also included in all qPCR runs. Target specificity for each primer set was validated by melting curve analyses. In addition, the identity of all amplicons was verified by sequencing. Standard curves were generated by 5-fold serial dilutions of cDNA to determine the amplification efficiency of PCR reactions. The efficiencies were nearly 100% and had a coefficient of determination (R^2) > 0.98, allowing the use of the comparative Cq method (ΔΔCq) [31]. The *18S rRNA* gene was used as the internal control. All samples were amplified in triplicate and relative mRNA levels of target genes were normalized to *18S rRNA*. All qPCR results were shown as fold-differences in comparison to untreated control cells. The primers used for real time qPCR are listed in Table 2.7.1.

2.3.6 Measurement of E_2 and P_4

Concentrations of E_2 and P_4 in medium samples were measured according to the manufacturer's instructions using E_2 and P_4 EIA kits (Cayman Chemical Co., USA). The detection limits of E_2 and P_4 assay are approximately 6 pg/ml and 10 pg/ml, respectively. The cross-reactivity of E_2 antiserum with P_4, testosterone, 17α-E_2, aldosterone, cortisol and hydrocortisone is < 0.01%. The P_4 antiserum is reported to cross-react with testosterone (< 0.05%), pregnenolone (< 2.5%) and < 0.01% with 17α-E_2, estrone and estriol. Protein concentration in follicular walls was determined using Protein Assay Kit (Bio-Rad, USA) with the BSA as the standard.

2.3.7 Statistical analyses

All results are expressed as mean ± SEM of three independent experiments. The data from replicate experiments were analyzed by analysis of variance (ANOVA) with a randomized complete block design (each experimental run being a block). When a significant difference was observed, post hoc comparisons between groups were performed using Tukey's test. All statistical

analyses were performed using the Mixed procedure of SAS 9.4 (SAS Institute, Cary, USA). A p-value less than 0.05 was considered statistically significant.

2.4 RESULTS

2.4.1 Effects of PRL variants on basal steroidogenesis in granulosa cells of preovulatory F3-F1 follicles

We first examined the effects of NG- and G-oPRL on basal E_2 and P_4 secretion as well as the expression of several key steroidogenic enzymes (*i.e. StAR*, *CYP11A1*, *CYP19A1* and *3β-HSD*) in cultured preovulatory follicle granulosa cells. As shown in Fig. 2.7.1A-B, low levels of either NG- or G-oPRL tended to stimulate E_2 and P_4 production, whereas this stimulatory effect was reduced as the concentration increased. Compared to untreated cells, maximal stimulation ($P < 0.05$) of NG-oPRL on E_2 and P_4 production was achieved at 10 and 1 ng/ml, respectively. In contrast, 1 and 10 ng/ml G-oPRL exerted the greatest stimulatory effects ($P < 0.05$) on E_2 and P_4 production, respectively.

Consistent with the data for the steroid hormone, levels of *StAR*, *CYP11A1*, *CYP19A1* and *3β-HSD* transcripts were also induced by 1 or 10 ng/ml of either of NG- and G-oPRL (Fig. 2.7.1C-F). In comparison with the control group, 1 ng/ml NG-oPRL increased *StAR*, *CYP11A1*, *CYP19A1* and *3β-HSD* transcript levels by 1.9, 1.8, 2.0, and 2.1 fold ($P < 0.05$), respectively, while a 1.9-, 1.8- and 2.0-fold increase ($P < 0.05$) on the expression of *StAR*, *CYP11A1* and *CYP19A1* was stimulated by 1 ng/ml G-oPRL, respectively and 10 ng/ml G-oPRL induced a 1.9-fold increase ($P < 0.05$) on *3β-HSD* transcript levels. However, higher levels (100 or 1000 ng/ml) of NG and G-oPRL showed no effect ($P > 0.05$) on steroidogenic enzyme gene expression.

2.4.2 Effects of PRL variants on basal steroidogenesis in granulosa cells of prehierarchical 6-8 mm follicles

The effects of NG- and G-oPRL on basal E_2 and P_4 secretion and levels of *StAR*, *CYP11A1*, *CYP19A1* and *3β-HSD* transcripts in cultured prehierarchical follicle granulosa cells were shown in Fig. 2.7.2. Similar to that in preovulatory follicle granulosa cells, 1 ng/ml NG-oPRL increased ($P < 0.05$) but NG-oPRL at higher levels (10-1000 ng/ml) had no effect ($P > 0.05$) on E_2 and P_4 production. In contrast, low levels (1 or 10 ng/ml) of G-oPRL did not alter ($P > 0.05$) but both 100

and 1000 ng/ml G-oPRL suppressed ($P < 0.05$) the secretion of E_2 and P_4 (Fig. 2.7.2A-B). Furthermore, 1 ng/ml NG-oPRL upregulated ($P < 0.05$) expression of *StAR* and *CYP11A1*, and levels of *CYP19A1* and *3β-HSD* transcripts were also induced by the inclusion of 1 or 10 ng/ml NG-oPRL. However, although increasing concentrations of G-oPRL showed no effect ($P > 0.05$) on *StAR* and *CYP11A1* transcript levels, a decreasing tendency ($P < 0.05$) was observed in the expression of *CYP19A1* and *3β-HSD* (Fig. 2.7.2C-F).

2.4.3 Effects of PRL variants on FSH- or LH-stimulated E_2 and P_4 secretion in granulosa cells of preovulatory F3-F1 and prehierarchical 6-8 mm follicles

Since both PRL isoforms and gonadotropins are involved in regulating steroidogenesis in chicken ovarian follicles, the effects of their combination on E_2 and P_4 production were further investigated in cultured granulosa cells of both preovulatory F3-F1 and prehierarchical 6-8 mm follicles. As shown in Fig. 2.7.3 and 4, higher basal levels of E_2 and P_4 were observed in granulosa cells of preovulatory F3-F1 follicles than those of prehierarchical 6-8 mm follicles. Compared to the control group, stimulation with 10 ng/ml FSH or LH increased ($P < 0.05$) basal E_2 and P_4 secretion in both cell types.

In preovulatory granulosa cells, addition of NG-oPRL reduced FSH-stimulated E_2 and P_4 secretion maximally by 57% and 34% ($P < 0.05$), respectively. However, 10 ng/ml G-oPRL did not affect ($P > 0.05$) FSH-induced E_2 secretion but enhanced ($P < 0.05$) FSH-induced P_4 secretion, while at the doses of 100 and 1000 ng/ml G-oPRL suppressed ($P < 0.05$) FSH-stimulated E_2 production but had no effect ($P > 0.05$) on FSH-induced P_4 production (Fig. 2.7.3A-B). In contrast, NG-oPRL reduced ($P < 0.05$) LH-stimulated E_2 and P_4 production, while G-oPRL suppressed the stimulatory actions of LH on E_2 production in a dose-dependent manner but had no effect on ($P > 0.05$) LH-stimulated P_4 secretion (Fig. 2.7.4A-B).

In prehierarchical granulosa cells, an acute decrease ($P < 0.05$) in FSH-induced E_2 secretion occurred after the inclusion of 10-1000 ng/ml NG-oPRL, whereas, NG-oPRL tended to potentiate FSH-stimulated P_4 production with a maximal effect at 100 ng/ml ($P < 0.05$). However, G-oPRL reduced the stimulatory effects of FSH on E_2 and P_4 secretion in a dose-dependent manner (Fig. 2.7.3C-D). In contrast, LH-induced E_2 and P_4 secretion was abrogated ($P < 0.05$) by the addition of either 10-1000 ng/ml NG- or G-oPRL (Fig. 2.7.4C-D).

2.4.4 Effects of PRL variants on FSH- or LH-stimulated E_2 and P_4 secretion in follicular walls of prehierarchical < 6 mm follicles

Next, we evaluated the interactive effects of PRL isoforms and gonadotropins on steroid production by cultured follicular walls of the 4-6, 2-4 and < 2 mm follicles. As expected, basal production of E_2 was highest (about 2 ng/mg protein) in the < 2 mm follicles but decreased as the follicles matured, whereas basal P_4 was maintained at very low concentrations (from about 30 to 70 pg/mg protein) among the < 6 mm follicles. Stimulation with 10 ng/ml FSH increased ($P < 0.05$) basal E_2 secretion by all follicular walls tested. With regard to P_4, FSH increased ($P < 0.05$) its secretion by follicular walls of the 4-6 mm follicles but showed no effect ($P > 0.05$) in the < 4 mm follicles. In contrast to FSH, 10 ng/ml LH promoted ($P < 0.05$) basal E_2 and P_4 secretion in all follicular walls tested (Fig. 2.7.5 and 6).

Furthermore, an inhibitory effect of NG-oPRL on FSH-induced E_2 secretion was consistently observed in follicular walls of the < 6 mm follicles but the magnitude was dependent on its concentration and stage of follicle development. However, a combination of 10 ng/ml FSH and NG-oPRL at the dose of 10 or 100 ng/ml stimulated ($P < 0.05$) basal P_4 production by follicular walls of the < 4 mm follicles. In contrast, in the 4-6 mm follicles, 10 ng/ml G-oPRL augmented ($P < 0.05$) FSH-induced E_2 secretion but did not alter ($P > 0.05$) FSH-induced P_4 secretion, but at higher doses G-oPRL suppressed ($P < 0.05$) FSH-induced E_2 and P_4 production. FSH-induced E_2 secretion by follicular walls of the 2-4 mm follicles was enhanced ($P < 0.05$) by the addition of 10 or 100 ng/ml G-oPRL but was suppressed ($P < 0.05$) by 1000 ng/ml G-oPRL. Combined treatment with FSH and G-oPRL at all doses had no effect ($P >0.05$) on basal P_4 secretion. In addition, G-oPRL suppressed FSH-induced E_2 and P_4 production in a dose-dependent manner in the < 2 mm follicles (Fig. 2.7.5).

On the other hand, the inclusion of NG-oPRL inhibited LH-induced E_2 and P_4 production by follicular walls of all < 6 mm follicles in a dose-dependent manner. Similarly, G-oPRL exerted dose-dependent inhibitory effects on LH-stimulated E_2 secretion in all < 6 mm follicles. In contrast, 10 ng/ml G-oPRL enhanced but at the dose of 100 or 1000 ng/ml G-oPRL did not affect LH-induced P_4 secretion in the 4-6 mm follicles. In follicular walls of the < 4 mm follicles, G-oPRL suppressed LH-stimulated P_4 production in a dose-dependent manner (Fig. 2.7.6).

2.5 DISCUSSION

Basal secretion of E_2 by follicular walls was highest in the smallest (< 2 mm) follicles but declined with the maturation of follicles, while the concentration of P_4 was maintained at relatively low level in follicular walls of all prehierarchical < 6 mm follicles. In contrast, much lower levels of E_2 but higher P_4 production were observed in granulosa cells from preovulatory F3-F1 follicles. These data confirm previous observations [6] that non-hierarchical follicles are the major source of E_2 while P_4 is primarily produced by hierarchical follicles. We also observed that lower content of basal E_2 and P_4 was present in prehierarchical 6-8 mm than preovulatory F3-F1 granulosa cells, which could be due to reduced expression of key steroidogenic enzymes, StAR and CYP11A1 [6].

Comparison of the effects of NG- and G-PRL on ovarian steroidogenesis is important since the ratio of G- to NG-PRL significantly varies during different reproductive states (from about 30% during egg laying to 80% during the incubation period) in galliformes [27, 28]. Results from the present study revealed for the first time that both NG- and G-oPRL were biologically active in regulating basal and gonadotropin-stimulated steroidogenesis in either granulosa cells or follicular walls of chicken different size class follicles. However, their effects were variable according to the concentration, glycosylation, the type of gonadotropin (FSH or LH) and the stage of follicle development. These results are partially supported by the observations that NG-oPRL were effective in suppressing basal and LH-induced E_2 secretion by hen small ovarian follicles [19, 25]. In mammals, glycosylation may lower the receptor binding activity and consequently downregulates the actions of PRL in selective target tissues [29]. Nevertheless, in contrast to its mammalian counterpart, the avian PRLRs have a duplicated extracellular domain (ECD) [32, 33] which may allow for additional interactions with either of PRL isoforms, it is thus likely that the activity ratio of G- to NG-oPRL may change between mammals and birds. Further investigation of the interactions between NG-, G-PRL and repeated ECD of PRLR is required to elucidate how glycosylation modulates the physiological actions of PRL in birds.

Both NG- and G-oPRL affected basal steroidogenesis in granulosa cells. Lower doses (1 or 10 ng/ml) of NG- and G-oPRL stimulated E_2 and P_4 secretion in preovulatory granulosa cells but this effect diminished as the doses increased. Notably, under moderate concentrations (10-100 ng/ml), it appeared that NG-oPRL was more effective in inducing E_2 production while G-oPRL had a stronger effect on P_4 production. These results differ from those reported in an earlier study

which showed that PRL had no effect on basal P_4 secretion by granulosa cell layers isolated from each category of chicken F3, F2 and F1 follicles after a 3 or 5 h incubation [34]. This discrepancy may be due to differences in the stages of the ovulatory cycle (before or after ovulation), the source of PRL examined and the intensity of egg production of experimental hens. Indeed, there is evidence that both basal [35, 36] and PRL-regulated granulosa cell steroidogenesis [25] depend on the stage of the ovulatory cycle of the hen. Additionally, a recent study showed that NG-oPRL promoted basal E_2 and P_4 secretion by theca and granulosa layers of chicken preovulatory follicles, respectively [25]. However, divergent effects of NG- and G-oPRL on steroid production were found in granulosa cells of prehierarchical 6-8 mm follicles. Specifically, NG-oPRL stimulated while G-oPRL exerted dose-dependent inhibitory effects on basal E_2 and P_4 production. In accordance with the data for steroids, levels of *StAR*, *CYP11A1*, *CYP19A1* and *3β-HSD* transcripts were up- or down-regulated by both PRL isoforms dependent on the concentration and the stage of follicle maturation. In support of this, the ability of PRL in modulating ovarian steroidogenic gene expression was also shown by *in vivo* injections of oPRL into turkeys [37].

In agreement with previous studies in chickens [38-41], FSH or LH stimulated basal E_2 and P_4 secretion by granulosa cells from both prehierarchical and preovulatory follicles. However, the PRL isoforms had different effects on FSH- or LH-induced steroidogenesis dependent on the concentration, follicle type and steroid produced. Both NG- and G-oPRL suppressed gonadotropin-stimulated E_2 production in preovulatory granulosa cells, but G-oPRL was much less effective than NG-oPRL. Conversely, gonadotropin-stimulated P_4 secretion by preovulatory granulosa cells was reduced in the presence of NG-oPRL but was induced or not affected by the addition of G-oPRL. In contrast, in granulosa cells of prehierarchical follicles, NG-oPRL enhanced while G-oPRL reduced FSH-induced P_4 production. These results combined with the effects of PRL on basal steroidogenesis suggest that G-PRL below a threshold concentration may play a positive role in preovulatory follicles not only through facilitating basal steroidogenesis but also through attenuating the suppressive effects of NG-PRL on gonadotropin-stimulated steroid production. However, this effect diminishes with increasing concentration. Conversely, in prehierarchical granulosa cells, G-PRL, especially at higher concentrations, exhibits consistent negative effects on both basal and gonadotropin-stimulated steroidogenesis notwithstanding the stimulatory actions of NG-PRL on FSH-induced P_4 production. In galliformes that express incubation behaviour, hens typically lay a few eggs during the early incubation stage while

simultaneously incubating eggs. This stage is characterized by low to moderate absolute and relative amounts of G- to NG-PRL, whereas, egg laying terminates when the amounts reach maxima [27, 28, 42]. Thus, it would appear that hens oviposit the dominant follicles without recruiting replacements during this transitional phase. This collapse of the follicular hierarchy may, at least in part, be explained by the differential effects of G-PRL on basal and gonadotropin-induced steroidogenesis, as well as the actions of NG-PRL on gonadotropin-stimulated steroidogenesis between prehierarchical and preovulatory granulosa cells.

Consistent with the effects of PRL on follicular cell steroidogenesis, PRLR is present in all follicular classes with higher levels in the smaller follicles [26]. Thus, the smaller follicles are also potentially affected by PRL. The suppressive effects of NG-oPRL on basal E_2 secretion have been previously described in the < 6 mm follicle by cultured hen intact follicles [25]. The present study extended the effects of NG- and G-oPRL on FSH- and LH-stimulated steroid secretion by follicular walls of each class of the 4-6, 2-4 and < 2 mm follicles. Similar to the larger follicles, NG-oPRL had a greater effect on suppressing FSH-induced E_2 secretion than G-oPRL in all < 6 mm follicles. However, G-oPRL was more effective than NG-oPRL in suppressing FSH-induced P_4 production. This is in accord with the study of Porter *et al* who showed that in incubating turkey hens with elevated levels of endogenous PRL (predominantly G-PRL [27]) E_2 production by the 2-7 mm follicles was not stimulated over basal levels by FSH [43]. In addition, the latter study [43] reported that P_4 synthesis was higher in these follicles after FSH stimulation than in laying hens which have much lower levels of PRL (predominantly NG-PRL [27]). Similarly, we observed that the combination of FSH and NG-oPRL had a synergistic effect on P_4 secretion by follicular walls of the < 4 mm follicles. In contrast, with the exception of the synergistic actions of LH and G-PRL on P_4 production by follicular walls of the 4-6 mm follicles, both NG- and G-PRL reduced LH-stimulated E_2 and P_4 production in all < 6 mm follicles. These results were in agreement with a previous study in which PRL exerted inhibitory effects on LH-stimulated E_2 production by cultured intact follicles from each class of the < 1, 1-2 and 3-5 mm follicles in chickens [19]. Moreover, it was also supported by the observation that the small white follicular (SWF; 2-7 mm) cells from incubating turkey hens secreted less E_2 in response to LH than did those from laying hens [43]. In addition, in the presence of LH, P_4 secretions by the SWF cells from incubating hens were greater than those from laying hens [43], which may be partially caused by the synergistic actions of G-PRL and LH in stimulating P_4 secretion in the 4-6 mm follicles. These data clearly

indicate that PRL has the ability to influence the steroidogenic responsiveness of the < 6 mm follicles to gonadotropins (FSH or LH) which is associated with the degree of PRL glycosylation.

In conclusion, both isoforms of PRL were biologically active across a wide concentration range in modulating ovarian steroidogenesis in chickens. This contrasts with the observations in mammals where glycosylation of PRL is generally associated with reduced ligand binding activity and signal transduction. Furthermore, glycosylation differentially modulates the actions of PRL in basal and gonadotropin-stimulated follicular cell steroidogenesis depending on the concentration, the type of gonadotropin (FSH or LH) and the stage of follicle development, which appears to be involved in regulation of the follicular hierarchy during the reproductive cycle in galliformes.

2.6 ACKNOWLEDGEMENTS

We thank Dr.AF Parlow (National Hormone & Peptide Program) for providing us with oLH, rhFSH, NG- and G-oPRL. We are grateful to Profs. Raj Duggavathi, Sarah Kimmins and Vilceu Bordignon (McGill University) for their laboratory facilities. SH was supported by the Chinese Scholarship Council (CSC), Hector & Ada Ma Fellowship and Graduate Excellence Award of McGill University. This research was funded by the Discovery Grants of Natural Sciences and Engineering Council of Canada.

2.7 TABLES AND FIGURES

Table 2.7.1 Primer pairs for real-time quantitative PCR

Gene		Sequence (5' to 3')	Tm (℃)	GenBank Accession No.
StAR	F	GTCCCTCGCAGACCAAGTT	59.8	NM_204686
	R	GGTGCTTGGCGAAGTCCA		
CYP11A1	F	GCTTTGCCTTGGAGTCTGTG	60.4	NM_001001756
	R	GGTGACGGCGTCGATGAA		
CYP19A1	F	GAAAGCAATTCTGGTGACTCT	57.8	NM_001001761
	R	TGGGTGCATGGATAAGTCATT		
3β-HSD	F	GCTGGGCTTTGGAATTGAG	61.8	NM_205118
	R	CAGGTGGCGGTTGGTTGAG		
18S rRNA	F	TTAAGTCCCTGCCCTTTGTACAC	60	AF173612
	R	CGATCCGAGGAACCTCACTAAAC		

F, forward primer; R, reverse primer

Figure 2.7.1 Effects of non-glycosylated (NG-) and glycosylated (G-) ovine PRL (oPRL) on basal steroidogenesis in granulosa cells of preovulatory F3-F1 follicles.

(A-B) Effects of NG- and G-oPRL on E_2 and P_4 secretion. (C-F) Effects of NG- and G-oPRL on the mRNA levels of *StAR*, *CYP11A1*, *CYP19A1* and *3β-HSD*. Cells were incubated for 24 h with different concentrations of NG- or G-oPRL (0, 1, 10, 100 or 1000 ng/ml). Values are expressed as the mean ± SEM of three independent experiments. Relative mRNA level was normalized to *18S rRNA* and was compared to the control group. Different letters indicate significant differences at $P < 0.05$.

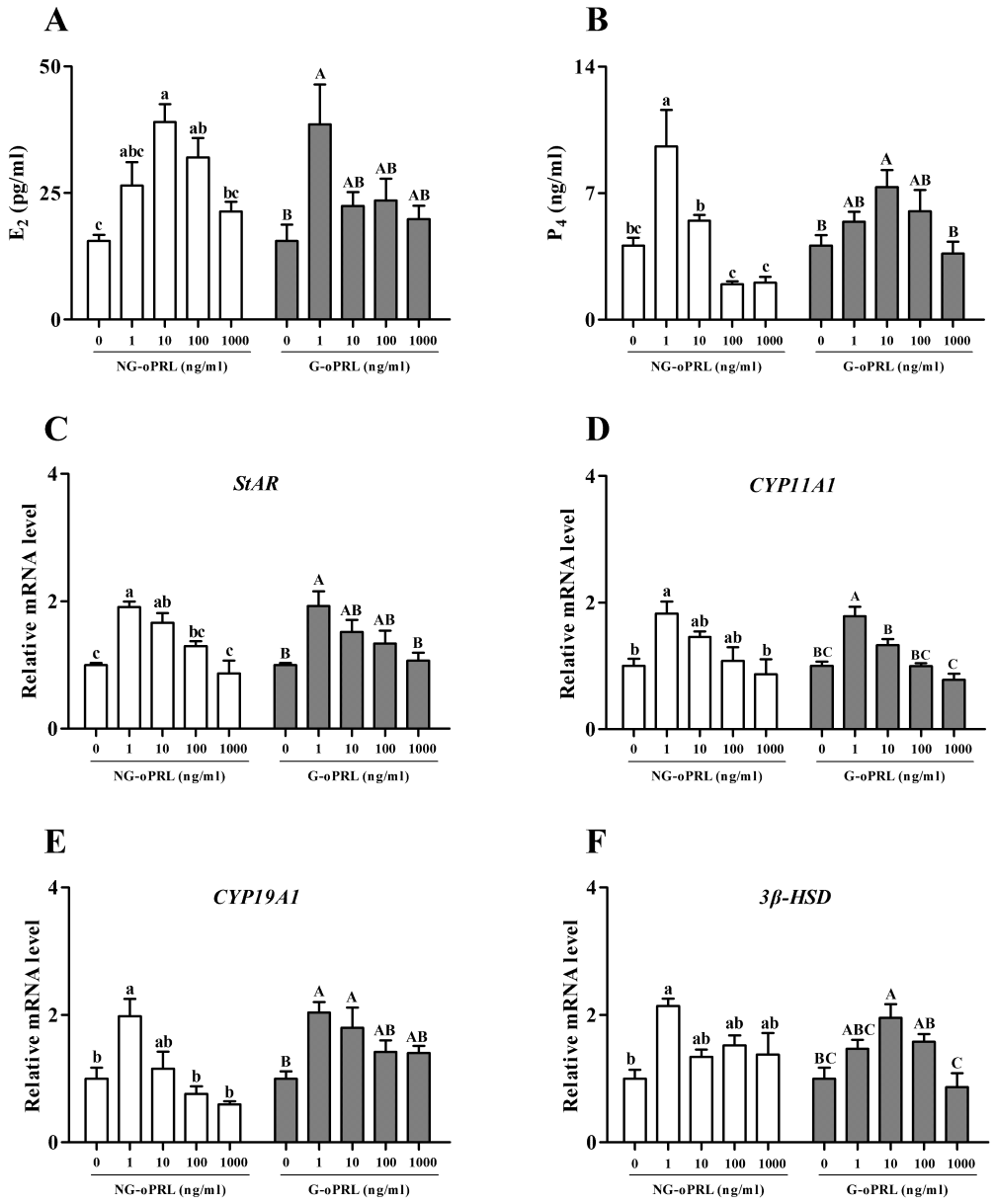


Figure 2.7.2 Effects of non-glycosylated (NG-) and glycosylated (G-) ovine PRL (oPRL) on basal steroidogenesis in granulosa cells of prehierarchical 6-8 mm follicles.

(A-B) Effects of NG- and G-oPRL on E_2 and P_4 secretion. (C-F) Effects of NG- and G-oPRL on the mRNA levels of *StAR*, *CYP11A1*, *CYP19A1* and 3*β-HSD*. Cells were incubated for 24 h with different concentrations of NG- or G-oPRL (0, 1, 10, 100 or 1000 ng/ml). Values are expressed as the mean ± SEM of three independent experiments. Relative mRNA level was normalized to *18S rRNA* and was compared to the control group. Different letters indicate significant differences at $P < 0.05$.

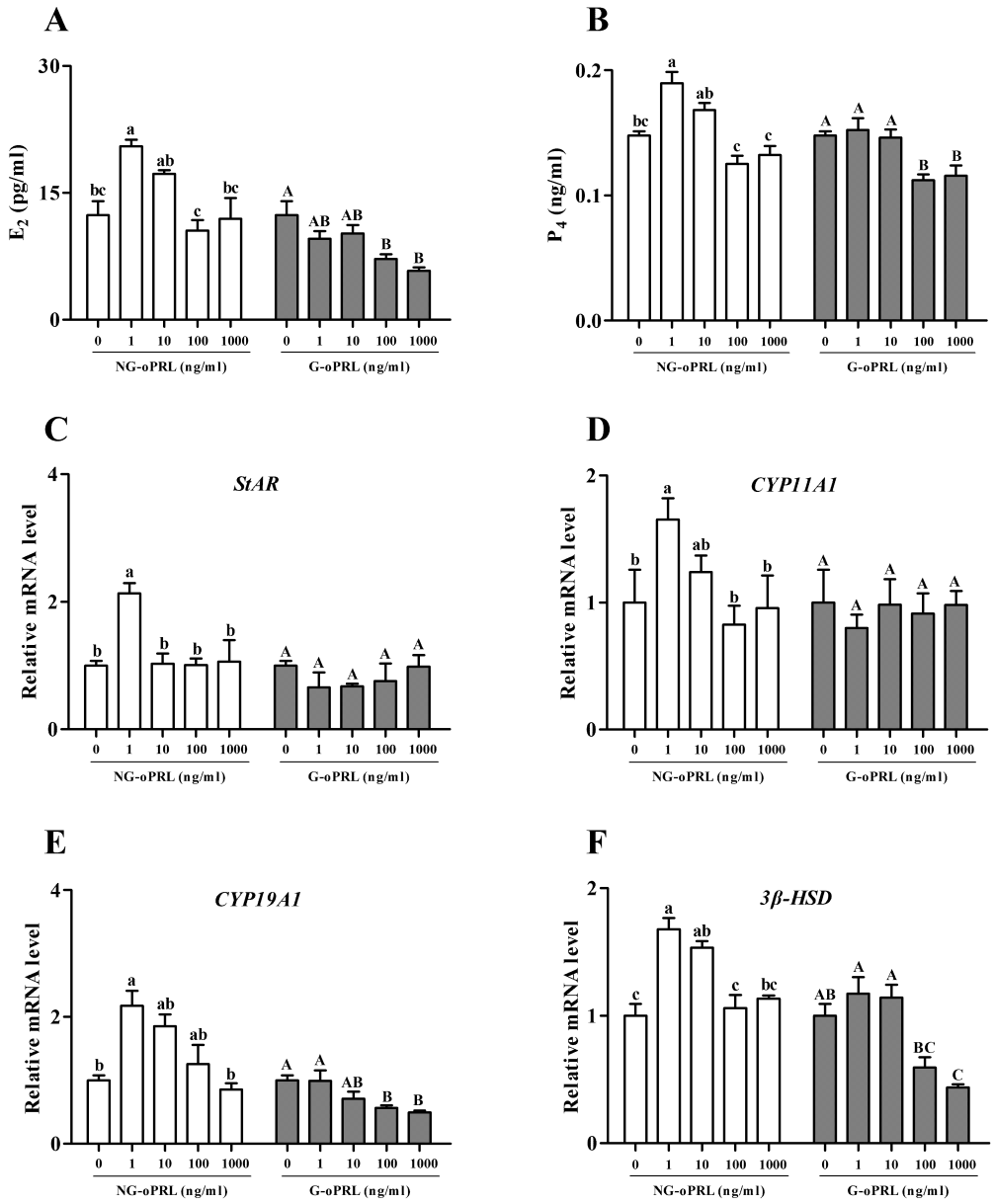


Figure 2.7.3 Effects of non-glycosylated (NG-) and glycosylated (G-) ovine PRL (oPRL) on FSH-stimulated E_2 and P_4 production in preovulatory F3-F1 (A-B) and prehierarchical 6-8 mm (C-D) granulosa cells.

Granulosa cells were incubated for 24 h without or with 10 ng/ml FSH in combination with different concentrations of NG- or G-oPRL (0, 10, 100 or 1000 ng/ml). Values are expressed as the mean ± SEM of three independent experiments. Different letters indicate significant differences at $P < 0.05$.

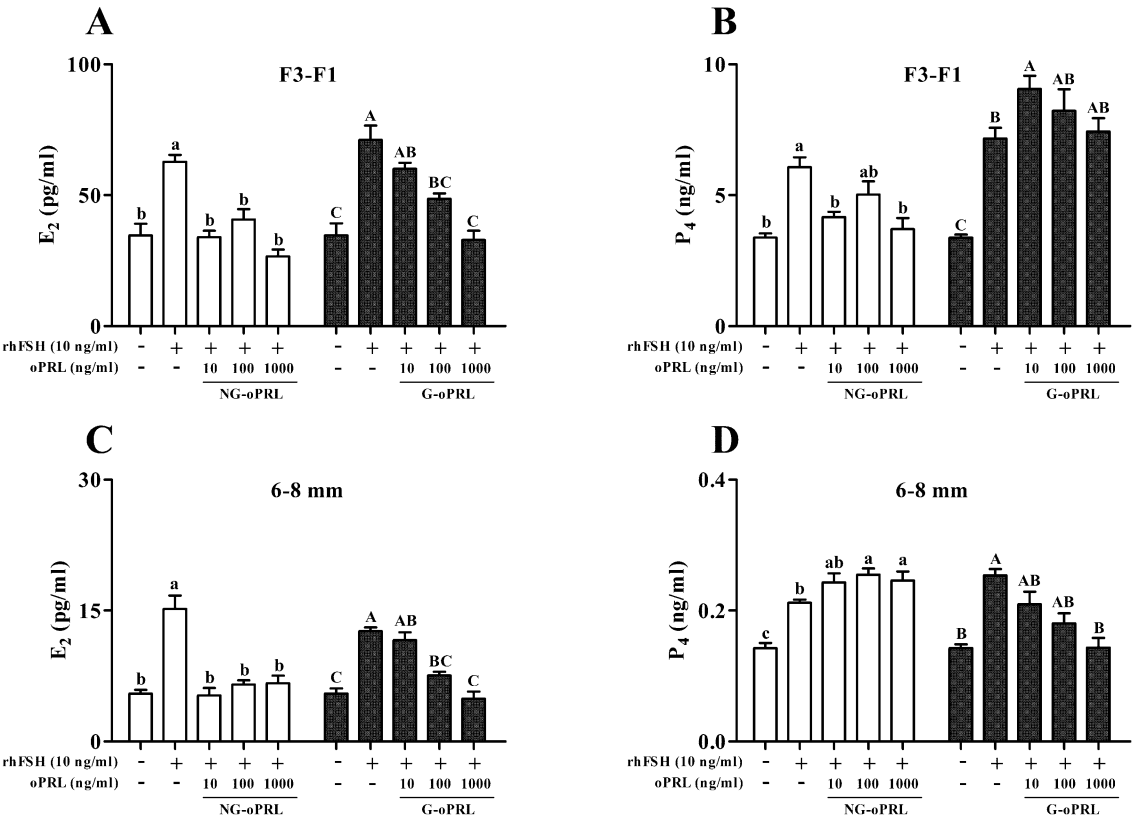


Figure 2.7.4 Effects of non-glycosylated (NG-) and glycosylated (G-) ovine PRL (oPRL) on LH-stimulated E_2 and P_4 production in preovulatory F3-F1 (A-B) and prehierarchical 6-8 mm (C-D) granulosa cells.

Granulosa cells were incubated for 24 h without or with 10 ng/ml LH in combination with different concentrations of NG- or G-oPRL (0, 10, 100 or 1000 ng/ml). Values are expressed as the mean ± SEM of three independent experiments. Different letters indicate significant differences at $P < 0.05$.

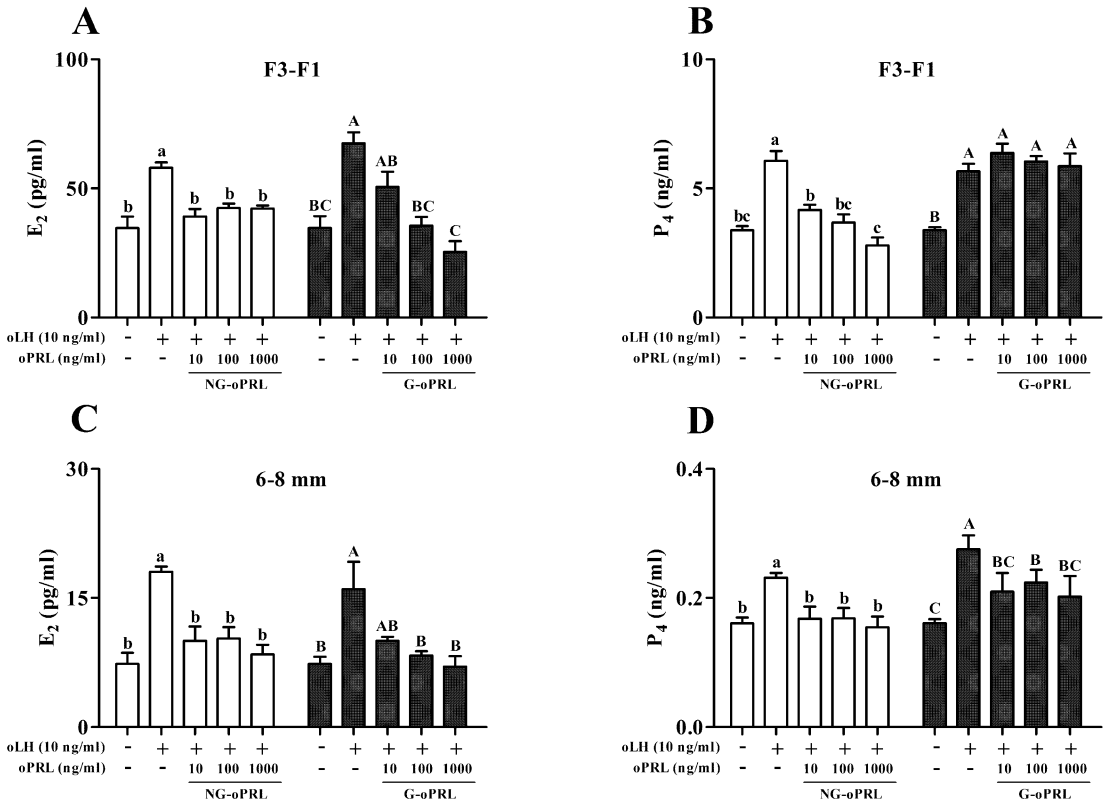


Figure 2.7.5 Effects of non-glycosylated (NG-) and glycosylated (G-) ovine PRL (oPRL) on FSH-stimulated E_2 and P_4 production in follicular walls of prehierarchical 4-6 (A-B), 2-4 (C-D) and < 2 mm (E-F) follicles.

Follicular walls were incubated for 24 h without or with 10 ng/ml FSH in combination with different concentrations of NG- or G-oPRL (0, 10, 100 or 1000 ng/ml). Values are expressed as the mean ± SEM of three independent experiments. Different letters indicate significant differences at $P < 0.05$.

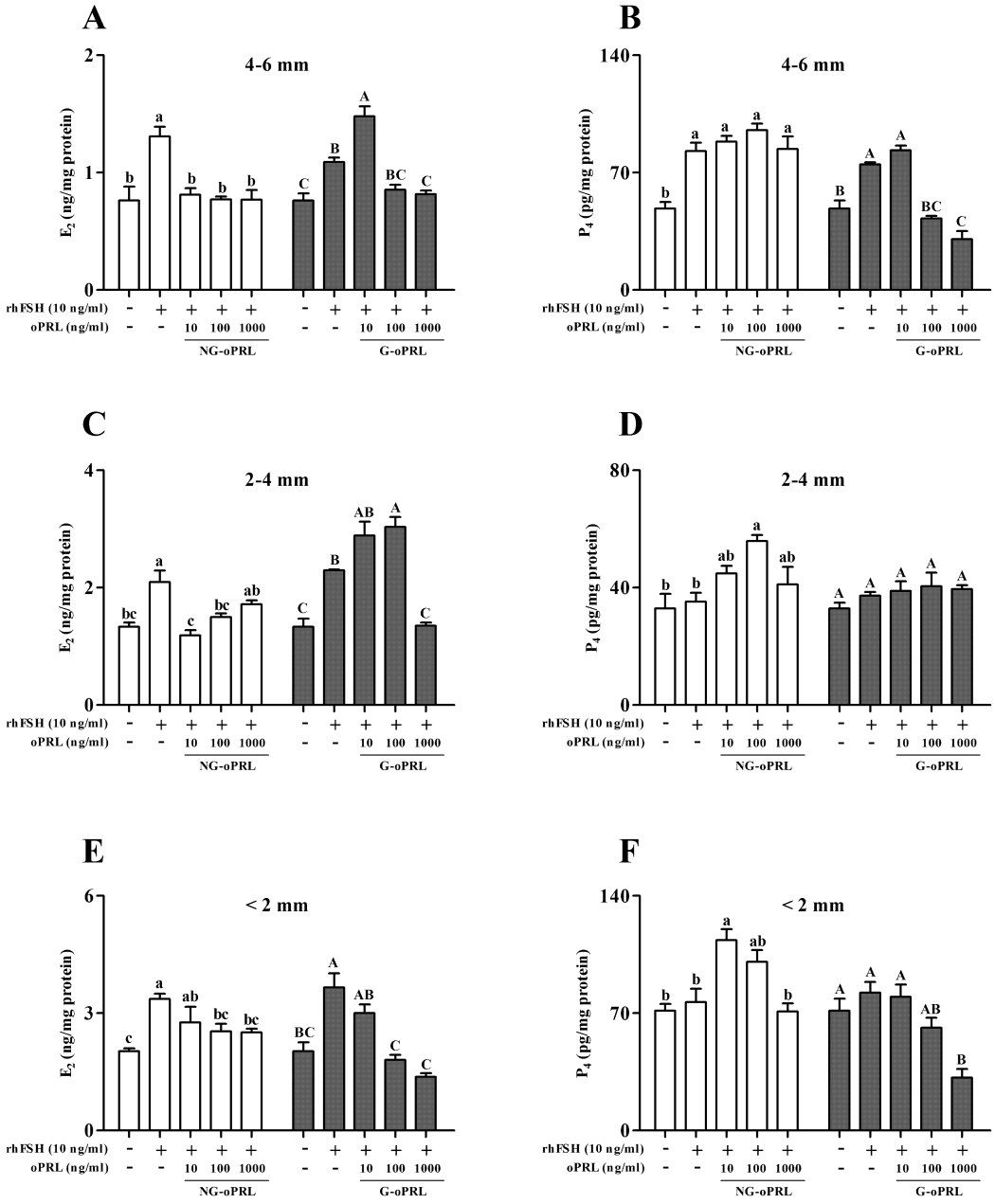
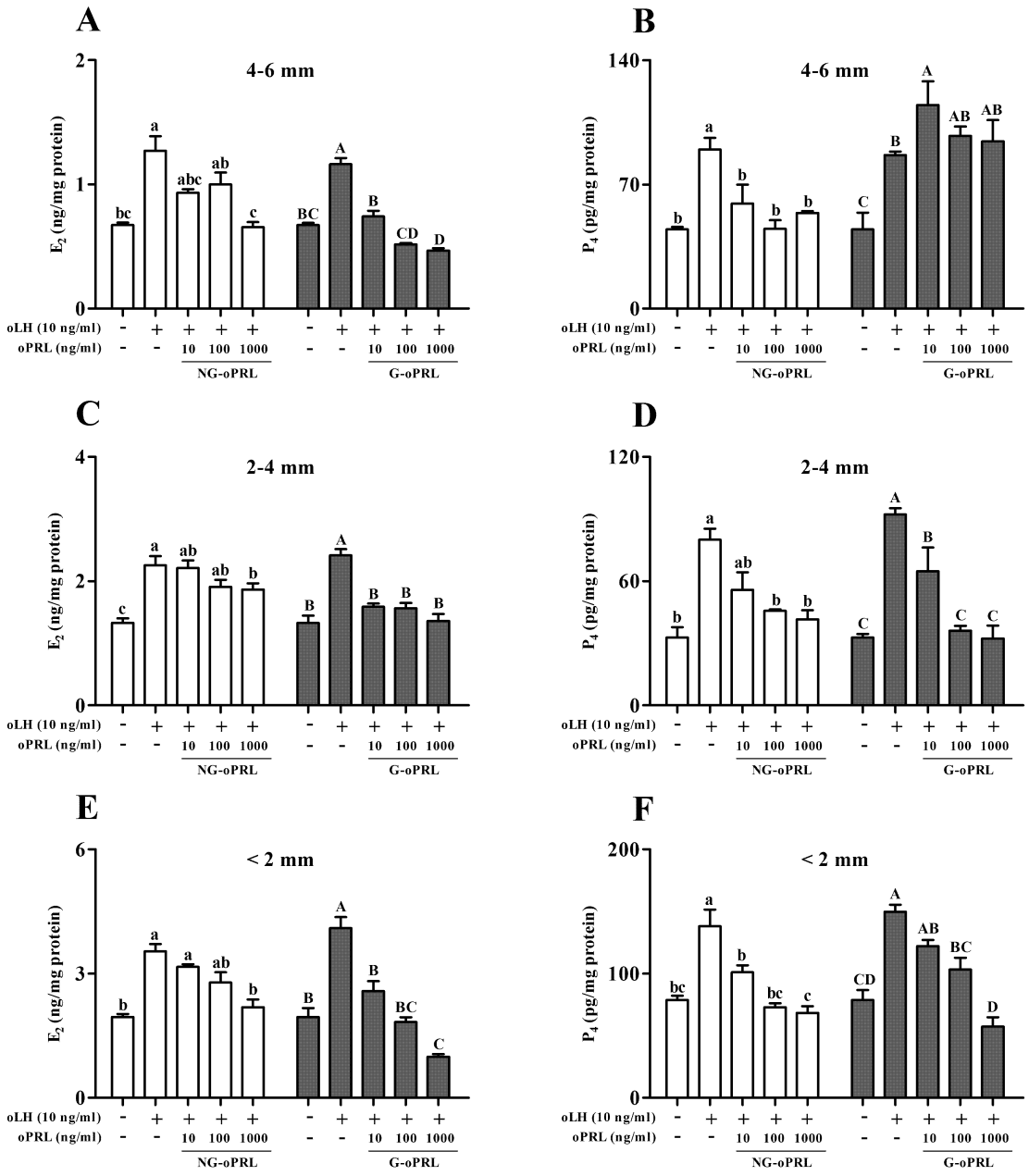


Figure 2.7.6 Effects of non-glycosylated (NG-) and glycosylated (G-) ovine PRL (oPRL) on LH-stimulated E_2 and P_4 production in follicular walls of prehierarchical 4-6 (A-B), 2-4 (C-D) and < 2 mm (E-F) follicles.

Follicular walls were incubated for 24 h without or with 10 ng/ml LH in combination with different concentrations of NG- or G-oPRL (0, 10, 100 or 1000 ng/ml). Values are expressed as the mean ± SEM of three independent experiments. Different letters indicate significant differences at $P < 0.05$.



2.8 REFERENCES

1. Gilbert AB, Evans AJ, Perry MM, Davidson MH. A method for separating the granulosa cells, the basal lamina and the theca of the preovulatory ovarian follicle of the domestic fowl (Gallus domesticus). J Reprod Fertil 1977; 50:179-181.
2. Huang ES, Nalbandov AV. Steroidogenesis of chicken granulosa and theca cells: in vitro incubation system. Biol Reprod 1979; 20:442-453.
3. Tilly JL, Kowalski KI, Johnson AL. Stage of ovarian follicular development associated with the initiation of steroidogenic competence in avian granulosa cells. Biol Reprod 1991; 44:305-314.
4. Bahr JM, Wang SC, Huang MY, Calvo FO. Steroid concentrations in isolated theca and granulosa layers of preovulatory follicles during the ovulatory cycle of the domestic hen. Biol Reprod 1983; 29:326-334.
5. Onagbesan O, Bruggeman V, Decuypere E. Intra-ovarian growth factors regulating ovarian function in avian species: a review. Anim Reprod Sci 2009; 111:121-140.
6. Johnson AL, Woods DC. Dynamics of avian ovarian follicle development: cellular mechanisms of granulosa cell differentiation. Gen Comp Endocrinol 2009; 163:12-17.
7. Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA. Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocr Rev 1998; 19:225-268.
8. Bouilly J, Sonigo C, Auffret J, Gibori G, Binart N. Prolactin signaling mechanisms in ovary. Mol Cell Endocrinol 2012; 356:80-87.
9. Crisostomo S, Guemene D, Garreau-Mills M, Morvan C, Zadworny D. Prevention of incubation behaviour expression in turkey hens by active immunization against prolactin. Theriogenology 1998; 50:675-690.
10. Crisóstomo S, Guemene D, Garreau-Mills M, Zadworny D. Prevention of the expression of incubation behaviour using passive immunisation against prolactin in turkey hens (Meleagris gallopavo). Reproduction Nutrition Development 1997; 37:253-266.
11. El Halawani M, Silsby J, Rozenboim I, Pitts G. Increased egg production by active immunization against vasoactive intestinal peptide in the turkey (Meleagris gallopavo). Biology of reproduction 1995; 52:179-183.

12. El Halawani M, Pitts G, Sun S, Silsby J, Sivanandan V. Active immunization against vasoactive intestinal peptide prevents photo-induced prolactin secretion in turkeys. General and comparative endocrinology 1996; 104:76-83.
13. El Halawani M, Silsby J, Youngren O, Phillips R. Exogenous prolactin delays photo-induced sexual maturity and suppresses ovariectomy-induced luteinizing hormone secretion in the turkey (Meleagris gallopavo). Biology of reproduction 1991; 44:420-424.
14. Reddy I, David C, Sarma P, Singh K. The possible role of prolactin in laying performance and steroid hormone secretion in domestic hen (Gallus domesticus). General and comparative endocrinology 2002; 127:249-255.
15. Sharp PJ, Blache D. A neuroendocrine model for prolactin as the key mediator of seasonal breeding in birds under long- and short-day photoperiods. Can J Physiol Pharmacol 2003; 81:350-358.
16. Rozenboim I, Tabibzadeh C, Silsby J, El Halawani M. Effect of ovine prolactin administration on hypothalamic vasoactive intestinal peptide (VIP), gonadotropin releasing hormone I and II content, and anterior pituitary VIP receptors in laying turkey hens. Biology of reproduction 1993; 48:1246-1250.
17. Camper P, Burke W. The effects of prolactin on the gonadotropin induced rise in serum estradiol and progesterone of the laying turkey. General and comparative endocrinology 1977; 32:72-77.
18. Tabibzadeh C, Rozenboim I, Silsby JL, Pitts GR, Foster DN, El Halawani M. Modulation of ovarian cytochrome P450 17 alpha-hydroxylase and cytochrome aromatase messenger ribonucleic acid by prolactin in the domestic turkey. Biology of reproduction 1995; 52:600-608.
19. Zadworny D, Shimada K, Ishida H, Sato K. Gonadotropin-stimulated estradiol production in small ovarian follicles of the hen is suppressed by physiological concentrations of prolactin in vitro. Gen Comp Endocrinol 1989; 74:468-473.
20. Zadworny D, Etches RJ. Effect of pregnant mare serum gonadotropin on plasma prolactin, luteinizing hormone, estradiol, and ovarian growth in incubating and out-of-lay turkeys. Poult Sci 1988; 67:319-326.
21. Li WL, Liu Y, Yu YC, Huang YM, Liang SD, Shi ZD. Prolactin plays a stimulatory role in ovarian follicular development and egg laying in chicken hens. Domest Anim Endocrinol

2011; 41:57-66.

22. Goldsmith AR, Hall M. Prolactin concentrations in the pituitary gland and plasma of Japanese quail in relation to photoperiodically induced sexual maturation and egg laying. General and comparative endocrinology 1980; 42:449-454.
23. El Halawani M, Silsby J, Fehrer S, Behnke E. Effects of estrogen and progesterone on serum prolactin and luteinizing hormone levels in ovariectomized turkeys (Meleagris gallopavo). General and comparative endocrinology 1983; 52:67-78.
24. Hall T, Harvey S, Chadwick A. Control of prolactin secretion in birds: a review. General and comparative endocrinology 1986; 62:171-184.
25. Hrabia A, Paczoska-Eliasiewicz H, Rzasa J. Effect of prolactin on estradiol and progesterone secretion by isolated chicken ovarian follicles. Folia Biol (Krakow) 2004; 52:197-203.
26. Zhou JF, Zadworny D, Guemene D, Kuhnlein U. Molecular cloning, tissue distribution, and expression of the prolactin receptor during various reproductive states in Meleagris gallopavo. Biology of Reproduction 1996; 55:1081-1090.
27. Bedecarrats G, Guemene D, Kuhnlein U, Zadworny D. Changes in levels of immunoreactive prolactin isoforms during a reproductive cycle in turkey hens. Gen Comp Endocrinol 1999; 113:96-104.
28. Hiyama G, Kansaku N, Kinoshita M, Sasanami T, Nakamura A, Noda K, Tsukada A, Shimada K, Zadworny D. Changes in post-translational modifications of prolactin during development and reproductive cycles in the chicken. Gen Comp Endocrinol 2009; 161:238-245.
29. Sinha Y. Structural variants of prolactin: occurrence and physiological significance. Endocrine Reviews 1995; 16:354-369.
30. Ben-Jonathan N, Mershon JL, Allen DL, Steinmetz RW. Extrapituitary prolactin: distribution, regulation, functions, and clinical aspects. Endocr Rev 1996; 17:639-669.
31. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25:402-408.
32. Zhou JF, Zadworny D, Guemene D, Kuhnlein U. Molecular cloning, tissue distribution, and expression of the prolactin receptor during various reproductive states in Meleagris gallopavo. Biol Reprod 1996; 55:1081-1090.

33. Tanaka M, Maeda K, Okubo T, Nakashima K. Double antenna structure of chicken prolactin receptor deduced from the cDNA sequence. Biochemical and biophysical research communications 1992; 188:490-496.
34. Hammond RW, Burke WH, Hertelendy F. Prolactin does not affect steroidogenesis in isolated chicken granulosa cells. Gen Comp Endocrinol 1982; 48:285-287.
35. Etches R, Duke C. Progesterone, androstenedione and oestradiol content of theca and granulosa tissues of the four largest ovarian follicles during the ovulatory cycle of the hen (Gallus domesticus). Journal of Endocrinology 1984; 103:71-76.
36. Bahr J, Wang S, Huang M, Calvo F. Steroid concentrations in isolated theca and granulosa layers of preovulatory follicles during the ovulatory cycle of the domestic hen. Biology of Reproduction 1983; 29:326-334.
37. Tabibzadeh C, Rozenboim I, Silsby JL, Pitts GR, Foster DN, El Halawani M. Modulation of ovarian cytochrome P450 17 alpha-hydroxylase and cytochrome aromatase messenger ribonucleic acid by prolactin in the domestic turkey. Biol Reprod 1995; 52:600-608.
38. Johnson AL, Bridgham JT. Regulation of steroidogenic acute regulatory protein and luteinizing hormone receptor messenger ribonucleic acid in hen granulosa cells. Endocrinology 2001; 142:3116-3124.
39. Johnson AL, Solovieva EV, Bridgham JT. Relationship between steroidogenic acute regulatory protein expression and progesterone production in hen granulosa cells during follicle development. Biol Reprod 2002; 67:1313-1320.
40. Sechman A, Antos P, Katarzyńska D, Grzegorzewska A, Wojtysiak D, Hrabia A. Effects of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin on secretion of steroids and STAR, HSD3B and CYP19A1 mRNA expression in chicken ovarian follicles. Toxicology letters 2014; 225:264-274.
41. Johnson A, Lee J. Granulosa cell responsiveness to follicle stimulating hormone during early growth of hen ovarian follicles. Poultry science 2016; 95:108-114.
42. Zadworny D, Walton JS, Etches RJ. Effect of environment on the intake of food and water, body weight, egg production and plasma concentrations of corticosterone and prolactin in turkey hens. Br Poult Sci 1986; 27:639-650.
43. Porter T, Hargis B, Silsby J, El Halawani M. Enhanced progesterone and testosterone secretion and depressed estradiol secretion in vitro from small white follicle cells of

incubating turkey hens. General and comparative endocrinology 1989; 74:400-405.

CONNECTING STATEMENT 1

In Chapter II we evaluated the roles of NG- and G-PRL in basal and gonadotropin-stimulated steroidogenesis in the follicular hierarchy. We observed that both NG- and G-PRL were functionally active in regulating basal and gonadotropin-stimulated E_2 and P_4 secretion in either granulosa cells or follicular walls of ovarian developing follicles. However, their effects were variable dependent on the concentration of PRL, the degree of PRL glycosylation, the type of gonadotropin (FSH or LH) and the stage of follicle development.

Since PRL exerts its effects through interaction with the receptor PRLR and glycosylation of PRL may influence the ligand binding affinity, it is likely that the differential effects of NG- and G-PRL on follicular cell steroidogenesis during follicle maturation can be mediated through modulation of PRLR expression. Therefore, in the following chapter (Chapter III) we firstly determined the expression profile and cellular distribution of PRLR in the follicular hierarchy and then investigated the independent and interactive effects of gonadotropins (FSH or LH) and PRL isoforms (NG- or G-PRL) on PRLR expression as well as the underlying intracellular mechanisms in granulosa cells of different size class follicles.

CHAPTER III

REGULATORY MECHANISMS UNDERLYING THE EXPRESSION OF PROLACTIN RECEPTOR IN CHICKEN GRANULOSA CELLS

Shenqiang Hu, Raj Duggavathi and David Zadworny*

Department of Animal Science, McGill University, Macdonald Campus, Ste. Anne de Bellevue, Quebec, H9X 3V9 Canada

* **Corresponding author**. E-mail: david.zadworny@mcgill.ca

Running title: Mechanisms of PRLR expression in chickens

3.1 ABSTRACT

Prolactin (PRL) has both pro- and anti-gonadal roles in the regulation of avian ovarian functions through its interaction with the receptor (PRLR). However, neither the pattern of expression of PRLR nor its regulatory mechanisms during follicle development have been clearly defined. The objective of the present study was to investigate mechanisms of *PRLR* expression in chicken granulosa cells. Levels of *PRLR* transcript were highest in the stroma and walls of follicles < 2 mm in diameter and progressively declined with the maturation of follicles. In preovulatory follicles, *PRLR* was expressed at higher levels in granulosa than theca layers. FSH exerted the greatest stimulatory effect on *PRLR* and *StAR* expression in cultured granulosa cells of the 6-8 mm follicles but this effect declined as follicles matured to F1. In contrast, LH did not alter the expression of *PRLR* in granulosa cells of all follicular classes but increased levels of *StAR* in F2 and F1 granulosa cells. Both non-glycosylated- (NG-) and glycosylated- (G-) PRL upregulated basal *PRLR* expression in granulosa cells of the 6-8 mm, F3 or F1 follicles but had little effect in F2 follicles. Furthermore, FSH-stimulated *PRLR* expression was reduced by the addition of either isoform of PRL especially in F2 granulosa cells. These results indicate that *PRLR* is differentially distributed and regulated by FSH or PRL variants independently or in combination in the follicular hierarchy. By using activators and inhibitors, we further demonstrated that multiple signaling pathways, including PKA, PKC, PI3K, mTOR and AMPK, are not only directly involved in, but they can also converge to modulate ERK2 activity to regulate FSH-mediated *PRLR* and *StAR* expression in undifferentiated granulosa cells. These data provide new insights into the regulatory mechanisms controlling the expression of *PRLR* in granulosa cells.

Key words: Granulosa cells; PRLR; Regulatory mechanism; ERK2; Chicken

3.2 INTRODUCTION

In chickens, ovarian follicles go through initial (activation of cortical follicles) and cyclic (follicle selection) recruitment before ovulation. These events are tightly coupled with the morphological and functional changes in granulosa cells [1]. In follicles prior to selection, granulosa cells are undifferentiated and steroidogenically inactive [2] due to low levels of expression of the two key genes required for steroidogenesis, steroidogenic acute regulatory protein (StAR) [3] and cytochrome P450 side chain cleavage ($P450_{scc}$) enzyme [4]. Subsequent to selection, granulosa cells are differentiated and become steroidogenically active [5]. The process of follicle selection is mainly under the control of follicle stimulating hormone (FSH) [5, 6]. Within the cohort of prehierarchical 6-8 mm follicles, a single follicle showing the highest expression of FSH receptor (FSHR) in the granulosa layer is likely to be next in line to enter the preovulatory hierarchy [7]. FSH signaling leads to the differentiation of granulosa cells by controlling the expression of several steroidogenic genes such as *StAR*, $P450_{scc}$ and luteinizing hormone receptor (*LHR*), which is achieved via modulation of multiple intracellular signaling cascades, including protein kinase A (PKA), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinases (ERKs) and AMP-activated protein kinase (AMPK) [1, 5, 8, 9]. In differentiated granulosa cells, LHR substitutes for the dominant role of FSHR in further promoting LH-induced steroidogenesis which is largely mediated by the PKA pathway [5].

Evidence is accumulating that in addition to gonadotropins, prolactin (PRL) may also play a critical role in the follicular hierarchy in birds. The anti-gonadal effects of PRL are substantiated by the strong association of high PRL secretion with degeneration and disappearance of the follicular hierarchy during incubation in many species of birds. In broody breeds of chickens and turkeys, intramuscular injections of 10-160 IU of PRL cause significant decreases in ovarian weight and the number of normal follicles but an increase in the number of atretic follicles [10]. These effects are indirect via inhibition of the hypothalamo-adenohypophyseal axis and the release of LH [11, 12] and direct since PRL suppresses both basal and gonadotropin-induced levels of progesterone and estradiol production by the ovary [13]. In contrast, neutralization or inhibition of endogenous PRL via passive or active immunizations against PRL or its releasing factor, vasoactive intestinal peptide (VIP), attenuated the expression of incubation behaviour and improved egg production performance [14-18]. Nevertheless, the observations that plasma PRL

levels gradually increase before the onset of sexual maturity and egg laying still occurs during early stages of incubation in birds also imply that PRL below a threshold concentration appears to be pro-gonadal. Indeed, in less- or non-broody chickens, immunizations against PRL or its receptor (PRLR) depressed egg production by reducing large white follicular growth and hence recruitment into the follicular hierarchy [19]. Furthermore, *in vitro* effects of PRL on steroid secretion by cultured ovarian follicles are stimulatory or inhibitory dependant on the concentration of PRL, the type of follicular cells and the stages of follicle development as well as the stage of the ovulatory process [20]. Nevertheless, so far little is known about the involvement of the PRL-PRLR system in the process of follicle selection as well as how it is regulated in birds.

It is well known that PRL exerts its effects through interaction with the receptor, PRLR [21]. Despite extremely low or even undetectable levels of *PRL* transcript in the chicken ovary [22-24], *PRLR* mRNA is abundant in the ovaries of chickens [25] and turkeys [26]. In particular, *PRLR* transcript is expressed at higher levels in walls of small follicles than those of large follicles in turkeys [26]. Therefore, it is likely that PRL may affect the follicular hierarchy mainly in an endocrine manner. However, the expression pattern of *PRLR* in cell type or follicular size classes during follicle development in chickens has not been investigated. In addition, post-translational modification contributes to different forms of circulating PRL in birds and glycosylated (G-) PRL is a major isoform dependent on the stage of the reproductive cycle. Since glycosylation is able to modulate the biological activity of PRL by influencing its receptor-binding efficiency [21] and the ratio of G- to non-glycosylated (NG-) PRL varies during various reproductive stages in chickens [27] and turkeys [28], interactions between G-, NG-PRL and PRLR may occur to partition the effects of PRL on ovarian follicles. Thus, it is of importance to investigate the effect of PRL glycosylation on PRLR expression during follicle development. The objectives of the present study were: 1) to determine the expression profile and cellular distribution of *PRLR* during chicken follicle development; 2) to investigate the effects of gonadotropins on *PRLR* expression; 3) to examine the role of NG- and G-PRL in basal and gonadotropin-regulated *PRLR* expression; 4) to elucidate the cellular mechanisms controlling the modulatory effects of FSH on *PRLR* expression by cultured chicken granulosa cells.

3.3 MATERIALS AND METHODS

3.3.1 Hormones, chemicals and reagents

Recombinant human FSH (rhFSH, AFP8468A), ovine LH (oLH, AFP-5551B), non-glycosylated ovine PRL (NG-oPRL, AFP-10692C) and glycosylated ovine PRL (G-oPRL, AFP-5742B) were obtained from National Hormone & Peptide Program (Torrance, CA, USA). A stock solution of each hormone was correspondingly prepared with either PBS at appropriate pH or 0.01M $NaHCO_3$, and then stored in small aliquots at -80 ℃. The final concentration of each working solution used for corresponding treatment was prepared with culture medium. Protein kinase A (PKA) activator (Forskolin) and inhibitor (H89), protein kinase C (PKC) activator (Phorbol 12-myristate 13-acetate, PMA) and inhibitor (GF109203X), phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002), mammalian target of rapamycin (mTOR) inhibitor (Rapamycin), extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitor (PD0325901) and AMP-activated protein kinase (AMPK) activator (AICAR) were purchased from Selleck Chemicals (Houston, TX, USA). Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium/Nutrient Mixture F12 (DMEM/F12), 0.4% Trypan Blue Solution as well as penicillin and streptomycin mixture were purchased from Invitrogen Life technologies (Carlsbad, CA, USA). Type II collagenase as well as phosphatase and protease inhibitor cocktail were purchased from Sigma-Aldrich (Oakville, ON, Canada). Trizol reagent and high-capacity cDNA reverse transcription kit were purchased from Invitrogen Life technologies (Carlsbad, CA, USA). Power SYBR Green PCR Master Mix was purchased from D-Mark Bioscience (Toronto, ON, Canada). Protein Assay Kit, Bovine Serum Albumin (BSA), 4 × Laemmli buffer, 2-mercaptoethanol and Immun-Star Western C Chemiluminescent Kit were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Rabbit monoclonal anti-phospho- and anti-total-ERK1/2 primary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). A rabbit polyclonal anti-β-actin primary antibody and a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody were purchased from Abcam (Cambridge, UK).

3.3.2 Animals and tissue collection

All experimental procedures using chickens in this study were approved by the Faculty Animal Care Committee of McGill University. White Leghorn hens, 25-35 weeks of age and

laying actively, were used in all studies described. Hens were fed *ad libitum* and kept in individual cages under standard conditions at the Poultry Complex of Macdonald Campus Farm, McGill University. The time of oviposition was monitored for each hen using surveillance camera (Lorex corporation, Maryland, USA), and ovulation was predicted to occur within 15-30 min after oviposition of the previous egg in the laying sequence. Four hens were randomly selected for ovarian tissue collection and were killed approximately 1-4 h before predicted time of a mid-sequence ovulation by cervical dislocation. After slaughter, the ovary from each hen was immediately removed and placed into ice-cold 0.9% NaCl solution. According to the diameter and the position in the follicular hierarchy, ovarian follicles were categorized into several groups, including the stroma, prehierarchical (< 2, 2-4, 4-6 and 6-8 mm) and preovulatory (9-12 mm and F5-F1, 13-40 mm; F5 < F4 < F3 < F2 < F1) follicles. Follicular walls consisting of a mixture of cells including theca and granulosa cells were collected from the < 2 mm to F4 follicles, and theca and granulosa cell layers were isolated from F3-F1 follicles, respectively, according to the method as previously described [29]. To further determine changes in the expression of proteins of interest between undifferentiated and differentiated follicles, another 4 hens were sacrificed to collect the stroma, follicular walls from the < 2 to 4-6 mm follicles as well as the theca and granulosa cell layers isolated from each category of the 6-8 mm, F3, F2 and F1 follicles, respectively. All samples were snap frozen in liquid nitrogen and then stored at -80 ℃ until analysis.

3.3.3 Granulosa cell culture and treatments

Granulosa cell layers harvested from each category of prehierarchical (6-8 mm) follicles as well as the three largest preovulatory follicles (F3, F2 and F1) were digested with 0.1% Type II collagenase, respectively. The number of each category of granulosa cells were counted using a hemocytometer and cell viability was assessed by trypan blue exclusion test. The cells were then seeded onto 12-well culture plates at a density of ~3×10^5 cells/well in 1 ml DMEM/F12 medium containing 3% FBS and 1% penicillin-streptomycin mixture. Cells were cultured at 38.5 ℃ in a humidified atmosphere of 95% air and 5% CO_2. After 24 h of incubation with a medium change at 6 h, non-attached cells were removed by aspiration and adherent cells were washed three times with serum-free medium (DMEM/F12 supplemented with 1% penicillin-streptomycin mixture). Serum-free medium was used in all further incubations. The cells were subsequently treated with 10 ng/ml rhFSH, or 10 ng/ml oLH, or different concentrations of NG- and G-oPRL (0, 1, 10, 100

or 1000 ng/ml), or in combination for another 24 h. To further explore the involvement of signaling pathways, the cells were cultured in the absence or presence of 10 ng/ml rhFSH, or together with corresponding activator or inhibitor of intracellular signaling cascades for another 4 h. Finally, the culture media were removed and the cells were collected for either RNA isolation or protein extraction. Each *in vitro* experiment was independently performed at least three times using tissues from different hens.

3.3.4 RNA isolation and real time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. The purity and concentration of RNA was determined using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the integrity of RNA was assessed by visualization of the 28S/18S rRNA ratio after electrophoresis on 1.5% agarose gels. The cDNA was then synthesized from 1 μg RNA using high-capacity cDNA reverse transcription kit (Invitrogen, USA). Real time quantitative PCR (qPCR) reactions were performed on the CFX384^{TM} real-time PCR detection system (Bio-Rad, USA) using SYBR Green master mix (D-Mark Bioscience, Ontario, Canada). Reactions were conducted with the following conditions: pre-denaturation at 95 ℃ for 5 min, followed by 40 cycles of denaturation at 95 ℃ for 15 s and annealing/extension at corresponding temperature of each primer set for 30 s. The no-template controls and negative controls without reverse transcriptase were also included in all qPCR runs. Target specificity for each primer set was validated by melting curve analyses. In addition, the identity of all amplicons was verified by sequencing. Standard curves were generated by 5-fold serial dilutions of cDNA to determine the amplification efficiency of PCR reactions. The efficiencies were nearly 100% and had a coefficient of determination (R^2) > 0.98, allowing the use of the comparative Cq method (ΔΔCq) [30]. The *18S rRNA* gene was used as the internal control. All samples were amplified in triplicate and relative mRNA levels of target genes were normalized to *18S rRNA*. All qPCR results were shown as fold-differences in comparison to an appropriate reference tissue or untreated control. The primers used for real time qPCR are listed in Table 3.7.1.

3.3.5 Protein extraction and western blot analysis

Tissue and cell lysates were prepared in Triton X-100 lysis buffer containing 10 mM Tris-Hcl (pH 7.5), 5 mM EDTA (pH 8.0), 150 mM NaCl, 30 mM sodium pyrophosphate, 30 mM sodium

fluoride, 1 mM sodium orthovanadate activated and 0.5% Triton X-100 as well as phosphatase and protease inhibitor cocktail. Protein concentration was determined by using Protein Assay Kit with Bovine Serum Albumin as the standard (Bio-Rad, USA). Equal amounts of protein lysates (20-40 μg) mixed with appropriate 4 × Laemmli buffer were boiled at 95 ℃ for 5 mins and then resolved by 10 % sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). After being transferred to nitrocellulose membranes, the membranes were subsequently blocked with 5% skim milk and then incubated overnight at 4 ℃ with corresponding primary antibodies. Following being washed 3 times with TBST (10 mins each time), the membranes were further incubated with the secondary antibody for 1.5 h at room temperature and then washed 3 times with TBST. The immunoblotted proteins were finally detected with Immun-Star Western C Chemiluminescent Kit (Bio-Rad, USA) by using Chemidoc Analyzer (Bio-Rad, USA). With regard to proteins of similar size, the membranes were stripped using stripping buffer (20 ml 20% SDS; 12.5 ml 0.5 M Tris-Hcl, pH 6.8; 67.5 ml DEPC H_2O and 0.8 ml 2-mercaptoethanol) and then washed with TBST, followed by being blocked with 5% skim milk before re-incubation with another primary antibody. The rabbit monoclonal anti-phospho- and anti-total-ERK1/2 primary antibodies were used at 1:1,000 dilution. The rabbit polyclonal anti-β-actin primary antibody was used at 1:5,000 dilution, while the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody was used at 1:10,000. Densitometric analysis was performed using Image Lab software (Version 4.1, Bio-Rad), and the relative abundance of phospho-ERK2 was normalized to total ERK2 and then was expressed as fold changes compared to an appropriate tissue or untreated control.

3.3.6 Statistical analyses

All results are expressed as mean ± SEM of a minimum of three independent experiments. The *in vivo* data of *PRLR* mRNA levels in follicular walls and ERK protein abundance in follicular walls, granulosa and theca layers, as well as the *in vitro* data from replicate experiments were analyzed by analysis of variance (ANOVA) with a randomized complete block design (each subject in the former or each experimental run in the latter being a block). The *in vivo* data of *PRLR* expression between preovulatory F3-F1 granulosa and theca layers were subjected to an ANOVA with two-way split-plot design (the subject by follicle being the plot and the type of cell layer being the sub-plot). When a significant difference was observed, post hoc comparisons

between groups were performed using Tukey's test. All statistical analyses were performed using the Mixed procedure of SAS 9.4 (SAS Institute, Cary, USA). A p-value less than 0.05 was considered statistically significant.

3.4 RESULTS

3.4.1 Expression pattern of *PRLR* mRNA in chicken ovarian follicles

Changes in levels of *PRLR* mRNA in the stroma, prehierarchical (< 2, 2-4, 4-6 and 6-8 mm) and preovulatory (9-12 mm and F5-F1; F5 < F4 < F3 < F2 < F1) follicles are presented in Fig. 3.7.1. The highest levels of *PRLR* mRNA were observed in the stroma and follicles < 2 mm in diameter. Subsequently, their levels progressively declined as the follicles developed (Fig. 3.7.1A). In preovulatory follicles (F3-F1), *PRLR* transcript was more abundant in granulosa than theca layers and their levels decreased in granulosa layers as the follicles approached to the largest diameter (Fig. 3.7.1B).

3.4.2 Gonadotropin regulation of *PRLR* expression in chicken granulosa cells

Since *PRLR* was minimally expressed in theca layers compared to granulosa layers in the hierarchical follicles (Fig. 3.7.1B), we further investigated its regulation by gonadotropins in granulosa cells from the 6-8 mm (undifferentiated) follicles and F3-F1 (differentiated) follicles. We also evaluated the abundance of *StAR* transcript, which is regulated by gonadotropins, as a validation of our cell culture model. The effects of FSH and LH on expression of *StAR* and *PRLR* were different depending on the degree of granulosa cell differentiation. In agreement with data previously reported [3, 31], FSH stimulated a 3.5-fold increase ($P < 0.05$) in the mRNA levels of *StAR* in granulosa cells of the 6-8 mm follicles, whereas, this stimulatory effect was progressively reduced as the follicles matured to F1. As expected, LH did not alter *StAR* transcript levels ($P > 0.05$) in cells of either the 6-8 mm or F3 follicles, but increased its levels by 5.7- and 3.1-fold ($P < 0.05$) in F2 and F1 follicles, respectively (Fig. 3.7.2A). In contrast, a similar pattern of FSH stimulation on *PRLR* mRNA expression was also observed in granulosa cells from the 6-8 mm to F1 follicles with the most inducible effect (a 4.5-fold increase, $P < 0.05$) in the 6-8 mm follicles, whereas, LH had no effect on *PRLR* transcription in cells from all follicular classes examined ($P > 0.05$; Fig. 3.7.2B).

3.4.3 Effects of PRL variants on basal and FSH-stimulated *PRLR* mRNA levels in chicken granulosa cells

In galliformes, levels of circulating PRL and the degree of glycosylation of PRL significantly vary during different reproductive stages. Hence, we investigated the effects of non-glycosylated (NG-) and glycosylated (G-) ovine PRL (oPRL) on *PRLR* expression in granulosa cells of follicles at different developmental stages. Granulosa cells of the 6-8 mm follicles were most sensitive to increasing concentrations of either isoform of PRL and a similar dose-response of *PRLR* mRNA levels was observed with maximal stimulatory effects of NG- and G-oPRL achieved at doses of 10 and 100 ng/ml ($P < 0.05$), respectively (Fig. 3.7.3A). A similar pattern was observed in granulosa cells from F1 follicles (Fig. 3.7.3D). In granulosa cells of F3 and F2 follicles, NG-oPRL had minimal effect on *PRLR* mRNA levels, whereas G-oPRL was stimulatory in F3 but tended to be inhibitory in F2 granulosa cells (Fig. 3.7.3B and C).

Since either FSH or PRL isoforms were involved in modulating levels of the *PRLR* transcript, we further examined the effects of their interaction in undifferentiated (6-8 mm) and differentiated (F2) granulosa cells. Both PRL isoforms inhibited FSH-induced expression of *PRLR* in granulosa cells of F2 follicles ($P < 0.05$), whereas, these effects were not significant except for 10 ng/ml G-oPRL in the 6-8 mm follicles (Fig. 3.7.4).

3.4.4 Role of PKA and PKC pathways in FSH-induced *PRLR* and *StAR* expression in undifferentiated granulosa cells

Since FSH is essential for the selection of follicles within the 6-8 mm cohort into the preovulatory hierarchy [5] and stimulated the greatest increase in *PRLR* and *StAR* transcript levels in granulosa cells of the 6-8 mm follicles, we sought to elucidate the cellular mechanisms underlying FSH-induced *PRLR* and *StAR* expression in such size class follicles. The role of PKA and PKC signaling pathways was firstly evaluated using pharmacological activators and inhibitors. The doses of PKA activator and inhibitor (*i.e.* Forskolin and H89, respectively) were derived from earlier studies in chickens [2, 32-34]. Consistent with previous results, FSH treatment increased *PRLR* and *StAR* mRNA levels in all cultured granulosa cells of the 6-8 mm follicles ($P < 0.05$; Fig. 3.7.5). Furthermore, FSH-induced increase in levels of *PRLR* and *StAR* transcripts was mimicked by 10 μM Forskolin but was suppressed by 20 μM H89 ($P < 0.05$; Fig. 3.7.5A and C).

Since different doses of PKC activator and inhibitor (*i.e.* PMA and GF109203X, respectively) have been used in chicken granulosa cells [31, 35, 36], the dose-response effect of either PMA or GF109203X on *PRLR* expression was first determined in the present study (Suppl. Fig. 3.7.9). Compared to untreated cells, 20 nM PMA and 10 μM GF109203X effectively stimulated and inhibited *PRLR* mRNA levels ($P < 0.05$), respectively. These concentrations were used in subsequent experiments. Treatment with PMA enhanced basal levels of *PRLR* transcript similar to FSH while GF109203X had no effect on FSH-induced *PRLR* expression (Fig. 3.7.5B). In contrast, PMA reduced basal *StAR* levels and the FSH-stimulated increase in *StAR* transcript was further enhanced by addition of GF109203X ($P < 0.05$; Fig. 3.7.5D).

3.4.5 Role of PI3K-Akt-mTOR and AMPK pathways in FSH-induced *PRLR* and *StAR* expression in undifferentiated granulosa cells

Next, we determined the role of PI3K-Akt-mTOR and AMPK signaling pathways in FSH-induced increase in levels of *PRLR* and *StAR* transcripts. The doses of the PI3K inhibitor (LY294002) as well as the mTOR inhibitor (Rapamycin) were used according to recent studies in geese [37] and chickens [38], and the dose of the AMPK activator AICAR was based on a study in chicken granulosa cells [9]. Neither 20 μM LY294002 nor 10 μM Rapamycin altered FSH-induced *PRLR* expression ($P > 0.05$; Fig. 3.7.6A), whereas it was suppressed by treatment with 1 mM AICAR ($P < 0.05$; Fig. 3.7.6B). In contrast, either of LY294002 and Rapamycin further enhanced FSH-induced *StAR* transcript levels which were also raised by addition of AICAR ($P < 0.05$; Fig. 3.7.6C and D).

3.4.6 Abundance of ERK2 in chicken developing follicles and its role in FSH-induced expression of *PRLR* and *StAR* in undifferentiated granulosa cells

Because ERK2 plays a crucial role in preventing premature differentiation in granulosa cells from chicken prehierarchical follicles [5], we then investigated its expression pattern in ovarian follicles during development as well as its role in FSH-induced *PRLR* and *StAR* expression in 6-8 mm granulosa cells. By using rabbit anti-mouse total- or phospho-ERK1/2 monoclonal antibodies, it was observed that only ERK2 protein with the size of ~ 42 kDa was detected. In the stroma and walls of < 6 mm follicles, levels of either total- or phospho-ERK2 remained unchanged ($P > 0.05$), except for a non-significant increase in ERK2 phosphorylation in < 4 mm follicles. Furthermore,

the abundance of ERK2 in theca and granulosa cell layers from prehierarchical (6-8 mm) and preovulatory (F3-F1) follicles was also determined. ERK2 phosphorylation in theca layers did not alter as follicles matured ($P > 0.05$), but much higher levels of ERK phosphorylation were found in granulosa layers of the 6-8 mm follicles than those of F3-F1 follicles ($P < 0.05$; Fig. 3.7.7A).

The dose of the MEK inhibitor PD0325901 was used according to its effective inhibition on ERK2 phosphorylation in granulosa cells of the 6-8 mm follicles (Suppl. Fig. 3.7.10). Treatment with 1 μM PD0325901 further enhanced FSH-induced increase in levels of both *PRLR* and *StAR* transcripts ($P < 0.05$; Fig. 3.7.7B and C).

3.4.7 Effects of manipulation of several intracellular signaling pathways on basal and FSH-stimulated ERK2 phosphorylation in undifferentiated granulosa cells

Since there is a difference in levels of ERK2 phosphorylation between undifferentiated and differentiated granulosa cells and crosstalk of ERK signaling with other signaling pathways commonly occurs in regulating ovarian steroidogenesis [39], we further investigated the effects of manipulation of other signaling pathways on basal and FSH-mediated ERK2 phosphorylation in granulosa cells of the 6-8 mm follicles. Compared to untreated cells, stimulation with 10 ng/ml FSH for 4 h stimulated phosphorylation of ERK2 ($P < 0.05$), which was mimicked by addition of 10 μM Forskolin. Inhibition of PKA by 20 μM H89 did not alter ($P > 0.05$) basal but suppressed ($P < 0.05$) FSH-induced ERK2 phosphorylation. In contrast, treatment with the PKC inhibitor GF109203X had no effects on both basal and FSH-stimulated ERK phosphorylation ($P > 0.05$). However, activation of PKC by 20 nM PMA increased basal levels of ERK phosphorylation ($P < 0.05$; Fig. 3.7.8).

Furthermore, blockage of PI3K or mTOR signaling using 20 μM LY294402 or 10 μM Rapamycin, respectively, did not impair the activity of ERK2 when compared to control cells ($P > 0.05$). However, in the presence of FSH, Rapamycin further increased ($P < 0.05$) while LY294002 showed no effect on FSH-induced ERK2 activation ($P > 0.05$). Activation of AMPK signaling by 1mM AICAR stimulated basal ($P < 0.05$) but failed to further enhance FSH-induced ERK2 phosphorylation ($P > 0.05$; Fig. 3.7.8).

3.5 DISCUSSION

In chickens, PRL has been shown to have direct effects on the ovary to modulate steroidogenesis yet the distribution of its receptor PRLR within the follicular hierarchy has not been quantified. In the current study we show that maximal expression of *PRLR* transcript was observed in the stroma and walls of follicles < 2 mm in diameter before a progressive decline with follicle maturation (Fig. 3.7.1A). Since the smaller (< 2 mm) follicles represent the largest follicular class in the ovary and are thought to be the major source of estrogen [40] and exogenous PRL could suppress both basal and gonadotropin-stimulated estrogen production by cultured hen these small follicles [20, 41], it is likely that PRL may have a dominantly negative influence on steroidogenesis in the smaller follicles. However, such inhibitory effects by PRL may be attenuated or even become stimulatory dependent on its concentration and the stage of follicle development. Divergent effects of PRL upon steroidogenesis in porcine granulosa cells was demonstrated to be associated with the degree of cell differentiation [42]. Furthermore, in chickens, a stimulatory role for PRL in recruiting large white follicles into small yellow ones was suggested through immunizations against PRL or PRLR [19]. Dependent on the dose of PRL, the stage of follicle development and the stage of the ovulatory cycle, PRL could be either stimulatory or inhibitory on estradiol secretion by the theca layers or progesterone production by the granulosa layers of F3-F1 follicles [20]. Notably, in the preovulatory hierarchy, the *PRLR* transcript was more abundant in granulosa than theca layers (Fig. 3.7.1B), implying a relatively more important role of PRL signaling in regulating progesterone production and progesterone-induced ovulation. Indeed, activity of 3β-hydroxysteroid dehydrogenase (3βHSD), a key enzyme in the progesterone biosynthetic pathway, was shown to be regulated by gonadotropins and PRL in granulosa cells of chicken F3-F1 follicles [43], and PRL tended to suppress LH-induced premature ovulation in chickens [44]. Accordingly, we speculated that varying levels of *PRLR* transcript during follicle development are related to changes in the process of follicular cell steroidogenesis as a result of their different responsiveness to gonadotropins and PRL.

Although there is a close relationship between gonadotropins and PRL in control of ovarian functions in many species of birds, to our knowledge, information about their actions in modulating PRL signaling through the PRLR at the cellular level remains scarce. The present study provides the first evidence for the independent and interactive effects of gonadotropins and PRL

variants on expression of the *PRLR* gene by cultured chicken granulosa cells. These effects were different dependent on the stage of follicle development (*i.e.* the degree of granulosa cell differentiation). In undifferentiated granulosa cells, FSH induced the greatest increase in the mRNA levels of *PRLR* in parallel with that in the *StAR* transcript, whereas, this stimulatory effect gradually declined in F3 and F2 granulosa cells and eventually became inhibitory in F1 cells. In contrast, LH had no effect on *PRLR* mRNA expression in granulosa cells from all size class follicles examined although it did increase levels of *StAR* transcript in F2 and F1 granulosa cells (Fig. 3.7.2). The magnitude of gonadotropin (FSH or LH) stimulation on expression of *StAR* unequivocally supported the observation that there is a shift from FSHR dominance in undifferentiated follicles to LHR dominance in the preovulatory hierarchy [5]. In addition to the *StAR* gene, elevated levels of P450scc and LHR as well as progesterone production induced by FSH are also recognized as markers of granulosa cell differentiation [5]. Although the role for PRL signaling in modulating granulosa cell differentiation in galliformes is not known, the greatest stimulatory effect of FSH on *PRLR* transcription in chicken undifferentiated granulosa cells may suggest a positive effect of PRL by potentiating the actions of FSH during follicle selection. These novel data are supported by the observations in rat granulosa cells where FSH promoted the expression and formation of functional PRLRs *in vivo* and *in vitro* and PRL enhanced FSH-induced progesterone production by amplifying the expression of StAR, P450scc and 3βHSD [45, 46].

In galliformes, the ratio of circulating G- to NG-PRL significantly varies during different stages of the reproduction cycle [27, 28]. Stimulation of undifferentiated granulosa cells with either variant produced a similar effect in inducing upregulation of *PRLR* transcript, whereas, G-PRL had a dominant effect in the hierarchal follicles (Fig. 3.7.3). In galliformes that express incubation behaviour, hyperprolactinemia is associated with large shift in absolute and relative levels of G-PRL (from about 30 % during egg laying to about 70 % during incubation behaviour). Hens typically lay 3-5 eggs from the time the behaviour is first expressed until termination of lay and involution of the ovary. Thus, it would appear that hens oviposit the dominant follicles without recruiting replacements during this transitional phase. It is possible that these increasing levels of G-PRL induce increased PRLR and hence sensitivity to the inhibitory effects of PRL. In many species, G-PRL is reported to have decreased binding activity and consequently lower biological

activity. However, avian receptors have a duplicated extracellular domain [26, 47]. NG- and G-PRL may interact differently within these ligand binding domains to affect signal transduction.

Furthermore, FSH alone up-regulated the expression of *PRLR* but this effect was reduced by the inclusion of either PRL isoform especially in the F2 granulosa cells (Fig. 3.7.4). These data indicated an antagonistic effect of PRL on the actions of FSH in regulating *PRLR* expression in chicken granulosa cells and the magnitude of such inhibition is probably dependent on the concentration of PRL and the degree of granulosa cell differentiation. A role for PRL in regulating the actions of FSH was previously reported in porcine granulosa cells where lower levels of PRL enhanced FSH-induced FSHR binding but higher levels of PRL led to a decrease in the number of FSHR [48]. Furthermore, locally secreted growth factors such as the BMP system was involved in modifying the actions of PRL in FSH-induced steroid production in rat granulosa cells [45]. Thus, the inhibitory effect of PRL on FSH-induced *PRLR* expression in chicken granulosa cells may be related to its interaction with FSH signaling via modifying the FSHR binding as well as a potential negative feedback by other unknown autocrine/paracrine growth factors such as the BMP system. Taken together, these results indicated that expression of *PRLR* in granulosa cells is cooperatively regulated by gonadotropins and circulating PRLs in dose- and follicular size-dependent manners. However, the relationship between FSH and PRL signaling on regulation of PRLR expression during follicle development remains unclear, hence any potential networking between these pathways warrants further investigation.

Thereafter, we further investigated the role of several signaling pathways in FSH-induced PRLR and StAR expression in chicken undifferentiated granulosa cells. As in mammals, the conserved binding sites for several transcription factors such as CCAAT/enhancer-binding protein (C/EBP) and SP1 are also characterized in the promoter region of chicken *PRLR* [25]. FSH is able to modulate the expression and activity of both C/EBP and SP1 via multiple signaling pathways in a variety of cell types [49, 50], thereby regulating the transcription of *PRLR*. Use of activators to activate the PKA or PKC pathways both increased basal levels of *PRLR* transcript, whereas, only the inhibition of PKA abolished FSH-induced *PRLR* expression (Fig. 3.7.5A and B), indicating that FSH regulates PRLR expression in a PKA-dependent manner. Activation of PKA increased *StAR* transcript levels while its inhibition blocked FSH-induced *StAR* expression. In contrast, addition of the PKC activator (PMA) reduced *StAR* levels but inhibition of PKC by GF109203X augmented the stimulatory effects of FSH on *StAR* expression (Fig. 3.7.5C and D).

These results confirm previous findings showing that PKA is positively involved in but PKC is negatively involved in granulosa cell differentiation in chickens [31, 51]. In addition, neither LY294002 nor Rapamycin suppressed FSH-induced *PRLR* levels but both did potentiate the stimulation of FSH on *StAR* expression (Fig. 3.7.6A and C). Since both pro-survival and proliferative effects of the PI3K-Akt-mTOR signaling have been demonstrated in chicken or goose granulosa cells [37, 52, 53], it is hypothesized that in hen undifferentiated follicles, suppression of this pathway may depress proliferation and promote steroidogenesis to initiate granulosa cell differentiation. As a cellular energy sensor, AMPK is widely expressed in chicken preovulatory granulosa cells and is involved in FSH- or IGF-regulated steroidogenesis via modulation of several steroidogenic enzymes expression such as StAR and 3βHSD [9, 54]. It was observed that activation of AMPK by AICAR reduced FSH-induced *PRLR* mRNA levels but enhanced FSH-induced *StAR* expression in chicken undifferentiated granulosa cells (Fig. 3.7.6B and D), suggesting that AMPK also participates in chicken granulosa cell differentiation through regulation of PRLR and StAR.

The actions of these signaling pathways in FSH-induced PRLR and StAR expression may be implicated in the regulation of ERK2 phosphorylation. As reported by other researchers [3, 38], only ERK2 was present in chicken ovarian tissues examined (Fig. 3.7.7A). The widespread expression of ERK2 in walls of < 6 mm follicles as well as in theca and granulosa cell layers from > 6 mm follicles imply that it plays a universal role in the hierarchy. However, greater levels of ERK2 phosphorylation were detected in undifferentiated (6-8 mm) than differentiated (F3-F1) granulosa cells, suggesting different actions of ERK2 between prehierarchical and preovulatory follicles. Indeed, ERK2 precludes premature differentiation in granulosa cells from prehierarchical follicles through inhibition of steroidogenesis, whereas, in preovulatory follicles ERK2 enhanced LH-induced progesterone production [3, 31, 35]. In agreement with the effects of the two MEK inhibitors (*i.e.* U0126 and PD98059) on FSH-induced StAR levels in chicken granulosa cells [3, 51], inhibition of ERK2 by PD0325901 enhanced FSH-stimulated *PRLR* and *StAR* expression in undifferentiated granulosa cells (Fig. 3.7.7B and C), further confirming a predominantly negative role of ERK2 in follicles prior to selection. Similar to the observations in mammalian immature granulosa cells [55], in chicken undifferentiated granulosa cells FSH treatment induced an increase in ERK2 phosphorylation which was abolished by addition of PD0325901 (Suppl. Fig. 3.7.10). Although FSH-induced ERK2 phosphorylation appeared paradoxical with the inhibitory effect of

ERK2 on FSH-stimulated steroidogenesis during the prehierarchical phase, there is evidence that transient activation and subsequent termination of ERK2 is required for the differentiation of granulosa cells in chickens [56]. In chicken undifferentiated granulosa cells, stimulation of TGFβ1 on *FSHR* transcription is abrogated via activation of ERK2 by the EGF receptor ligands (*e.g.* TGFα, betacellulin) [5], whereas, temporal activation of ERK2 is required for TGFα or betacellulin-induced *TGFβ1* expression which implicates ERK2-mediated expression of MAPK phosphatases [56, 57]. Likewise, a feedback mechanism whereby ERK2 is initially activated and subsequently terminated by FSH via modulation of phosphatase activity may exist in chicken prehierarchical follicles, thereby contributing to granulosa cell differentiation by controlling the expression of several differentiation-inducing genes such as *FSHR*, *TGFβ1* and *PRLR*.

In accordance with the effects of 8-bromo-cAMP [55], Forskolin increased basal levels of ERK2 phosphorylation but H89 reduced FSH-induced ERK2 activation (Fig. 3.7.8), indicating that in chicken undifferentiated granulosa cells FSH stimulates ERK2 activation in a cAMP/PKA-dependent manner. Activation of PKC by PMA increased basal levels of ERK2 phosphorylation but inhibition of PKC by GF109203X did not alter FSH-induced ERK2 phosphorylation, which corresponded with their effects on PRLR expression, suggesting that PMA mediates *PRLR* transcription through the PKC/ERK2 pathway. Furthermore, the PI3K inhibitor, LY294002, had no effect but the mTOR inhibitor, Rapamycin, enhanced FSH-induced ERK phosphorylation (Fig. 3.7.8). In chicken myoblasts 50 μM LY294002 reduced basal and insulin-induced ERK phosphorylation [38]. This discrepancy may be due to differences in the cell type, the dose of LY294002 and the hormone examined. Our results indicate that blockade of the mTOR signaling may to some extent promote granulosa cell differentiation by inhibiting proliferation and inducing differentiation via transient activation of ERK. Addition of the AMPK activator (AICAR) increased basal levels of ERK phosphorylation and resulted in a nonsignificant decrease in FSH-induced ERK phosphorylation in undifferentiated granulosa cells (Fig. 3.7.8). The stimulatory effect of AICAR on basal ERK phosphorylation as well as its divergent effects on FSH-induced ERK phosphorylation have been reported in hen F1 and F3 plus F4 follicles [9]. These observations suggest that differential effects of the AMPK signaling on FSH-induced steroidogenesis in granulosa cells are dependent on the stage of follicle maturation and are involved in regulation of ERK activity.

In conclusion, our data suggest that *PRLR* was differentially expressed and regulated by FSH or PRL variants independently, or in combination in the follicular hierarchy. In undifferentiated granulosa cells, FSH-induced *PRLR* and *StAR* expression was mediated by multiple signaling pathways including PKA, PKC, PI3K, mTOR and AMPK. All of these pathways may also converge to modulate ERK activity to regulate granulosa cell differentiation.

3.6 ACKNOWLEDGEMENTS

We thank Dr.AF Parlow (National Hormone & Peptide Program) for providing us with oLH, rhFSH, NG- and G-oPRL. We are grateful to Profs. Sarah Kimmins and Vilceu Bordignon (McGill University) for their laboratory facilities. SH was supported by the Chinese Scholarship Council (CSC), Hector & Ada Ma Fellowship and Graduate Excellence Award of McGill University. This research was funded by the Discovery Grants of Natural Sciences and Engineering Research Council of Canada (RD and DZ).

3.7 TABLES AND FIGURES

Table 3.7.1 Primer pairs for real-time quantitative PCR

Gene		Sequence (5' to 3')	Tm (℃)	GenBank Accession No.
PRLR	F	CCTTCCACCAGTGCTTCAA	56.4	NM_204854
	R	AGGAGGCTGACTGTTAGGT		
StAR	F	GTCCCTCGCAGACCAAGTT	59.8	NM_204686
	R	GGTGCTTGGCGAAGTCCA		
18S rRNA	F	TTAAGTCCCTGCCCTTTGTACAC	60	AF173612
	R	CGATCCGAGGAACCTCACTAAAC		

F, forward primer; R, reverse primer

Figure 3.7.1 Expression pattern of *PRLR* mRNA in chicken developing follicles.

(A) Abundance of *PRLR* mRNA in the stroma and walls of prehierarchical (< 2, 2-4, 4-6 and 6-8 mm) and preovulatory (9-12 mm, F5 and F4) follicles. (B) Relative *PRLR* mRNA levels in theca and granulosa cell layers isolated from the three largest preovulatory follicles (namely F3-F1; F3 < F2 < F1). Relative expression level was normalized to *18S rRNA*. Data are expressed as fold differences ± SEM compared to either the stroma or F3 granulosa cells (n = 4 hens). Different lowercase letters indicate a significant effect of follicular developmental stage, whilst different uppercase letters indicate a significant effect of cell type (granulosa versus theca cell layer). $P < 0.05$ was accepted as statistically significant.

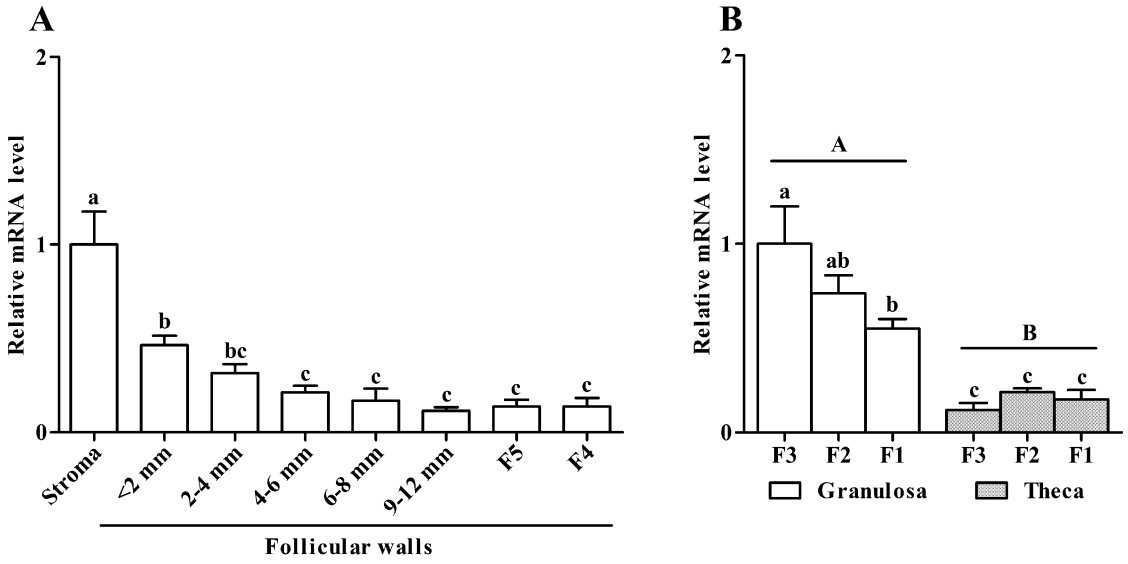


Figure 3.7.2 Effects of gonadotropins on levels of *StAR* (A) and *PRLR* (B) transcripts in chicken granulosa cells of different size class follicles after a 24-h culture.

Relative expression level was normalized to *18S rRNA*. Data are expressed as fold differences ± SEM of six independent experiments using tissues from different hens and are compared to control cells of each follicular class. *, $P < 0.05$ compared to control cells within each follicular class.

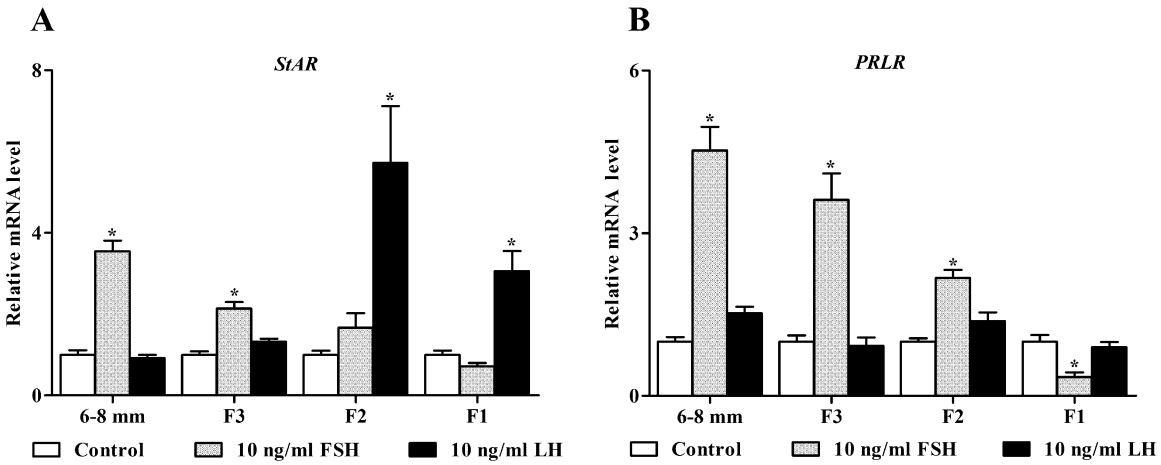


Figure 3.7.3 Regulation of *PRLR* transcript by non-glycosylated (NG-) and glycosylated (G-) ovine PRL (oPRL) (0, 1, 10, 100 or 1000 ng/ml) in cultured granulosa cells of the 6-8 mm (A), F3 (B), F2 (C) and F1 (D) follicles after a 24-h culture.

Relative expression level was normalized to *18S rRNA*. Data are expressed as fold differences ± SEM of three independent experiments using tissues from different hens and are compared to control cells of each follicular class. Different letters indicate significant differences at $P < 0.05$.

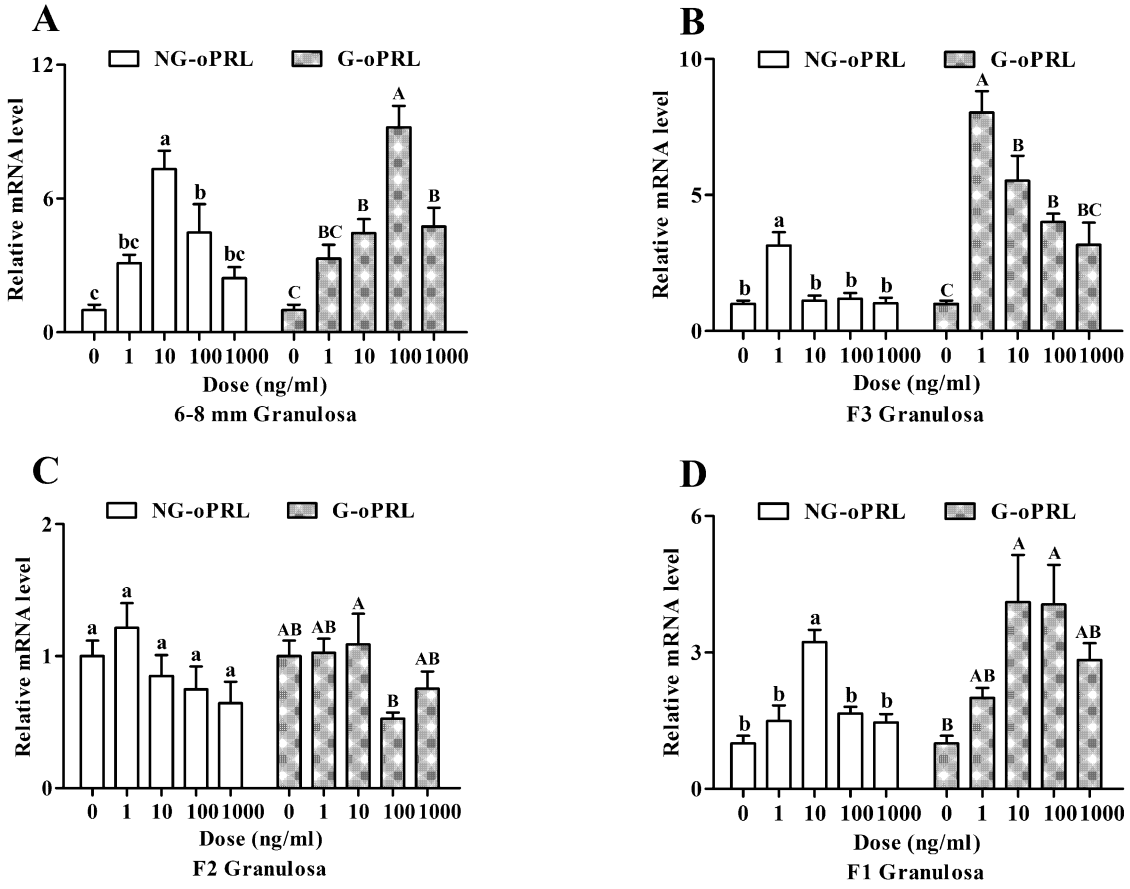


Figure 3.7.4 Effects of non-glycosylated (NG-) and glycosylated (G-) ovine PRL (oPRL) (0, 10 or 100 ng/ml) on FSH-mediated *PRLR* mRNA expression in cultured granulosa cells of the 6-8 mm (A) and F2 (B) follicles after a 24-h culture.

Relative expression level was normalized to *18S rRNA*. Data are expressed as fold differences ± SEM of three independent experiments using tissues from different hens and are compared to control cells of each follicular class. *, $P < 0.05$ compared to control cells; $^{\#}$, $P < 0.05$ compared to cells only stimulated by 10 ng/ml FSH.

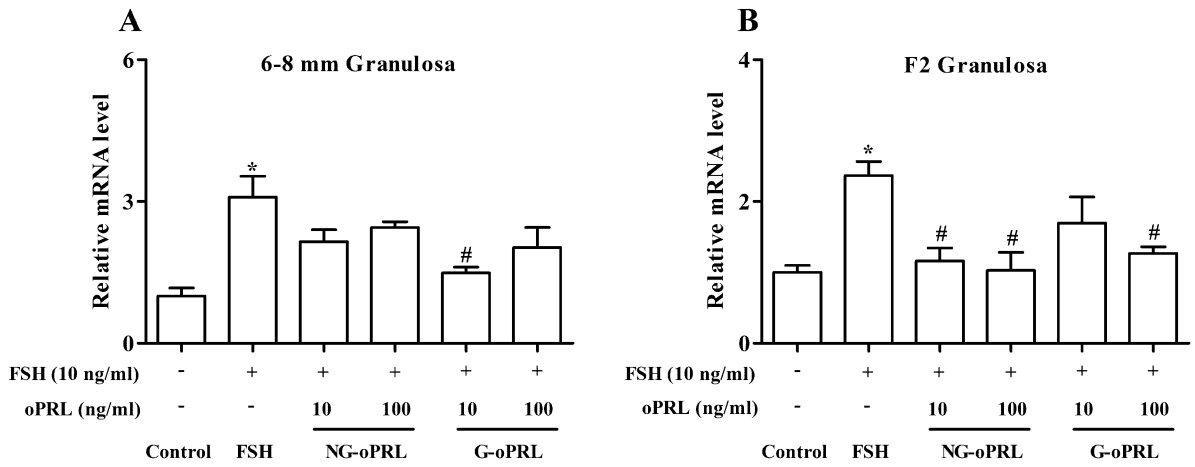


Figure 3.7.5 Role of PKA and PKC signaling pathways in FSH-induced *PRLR* and *StAR* mRNAs expression in cultured granulosa cells of prehierarchical (6-8 mm) follicles.

(A and C) Changes in relative mRNA levels of *PRLR* (A) and *StAR* (C) after culture of granulosa cells for 4 h in the absence or presence of 10 ng/ml FSH in combination with PKA activator (10 µM Forskolin) or inhibitor (20 µM H89). (B and D) Changes in relative mRNA levels of *PRLR* (B) and *StAR* (D) in granulosa cells treated without or with 10 ng/ml FSH in combination with PKC activator (20 nM PMA) or inhibitor (10 µM GF109203X). Relative expression level was normalized to *18S rRNA*. Data are expressed as fold differences ± SEM of three independent experiments using tissues from different hens and are compared to control cells. *, $P < 0.05$ compared to control cells; $^{\#}$, $P < 0.05$ compared to cells only stimulated by 10 ng/ml FSH.

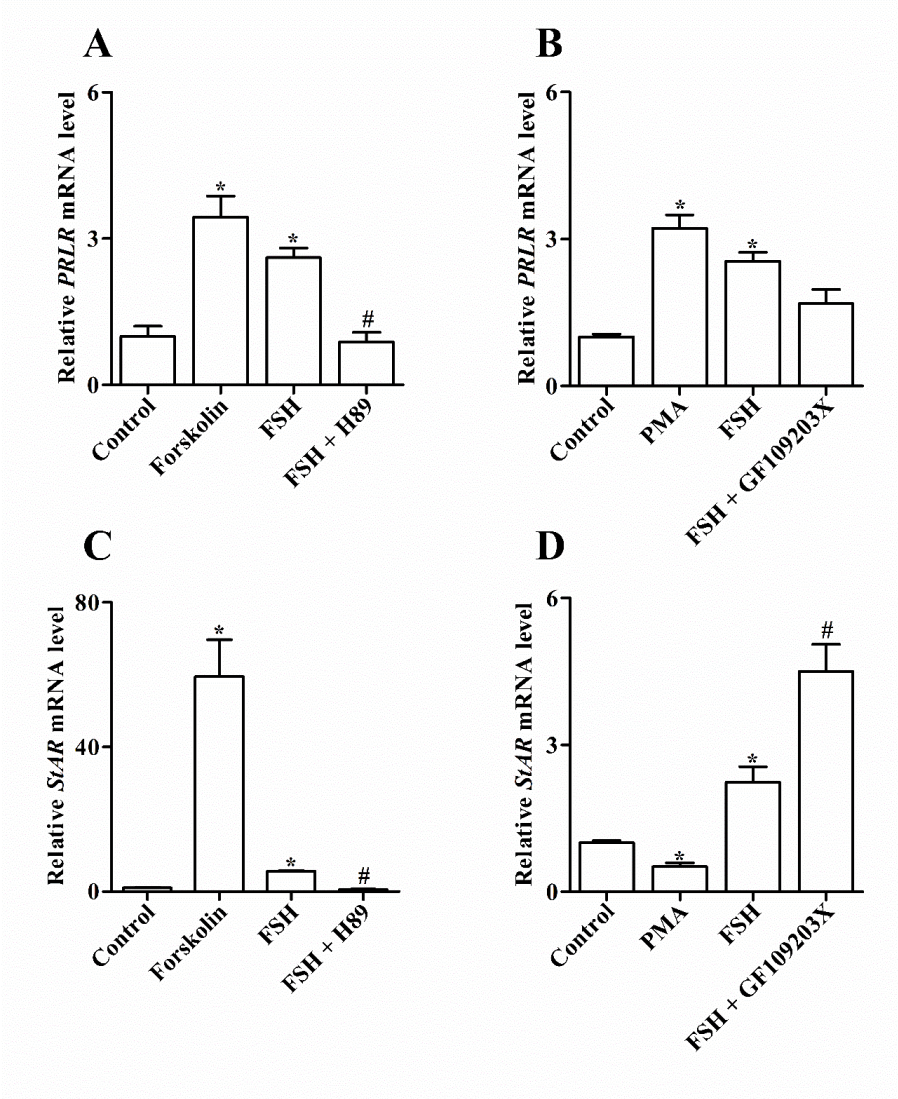


Figure 3.7.6 Role of PI3K-Akt-mTOR and AMPK signaling pathways in FSH-induced *PRLR* and *StAR* mRNAs expression in cultured granulosa cells of prehierarchical (6-8 mm) follicles. (A and C) Changes in relative mRNA levels of *PRLR* (A) and *StAR* (C) after culture of granulosa cells for 4 h in the absence or presence of 10 ng/ml FSH together with PI3K inhibitor (20 µM LY294002) or mTOR inhibitor (10 µM Rapamycin). (B and D) Relative *PRLR* (B) and *StAR* (D) mRNAs levels in granulosa cells cultured for 4 h in the absence or presence of 10 ng/ml FSH, or together with AMPK activator (1 mM AICAR). Relative expression level was normalized to *18S rRNA*. Data are expressed as fold differences ± SEM of three independent experiments using tissues from different hens and are compared to control cells. *, $P < 0.05$ compared to control cells; $^{\#}$, $P < 0.05$ compared to cells only stimulated by 10 ng/ml FSH.

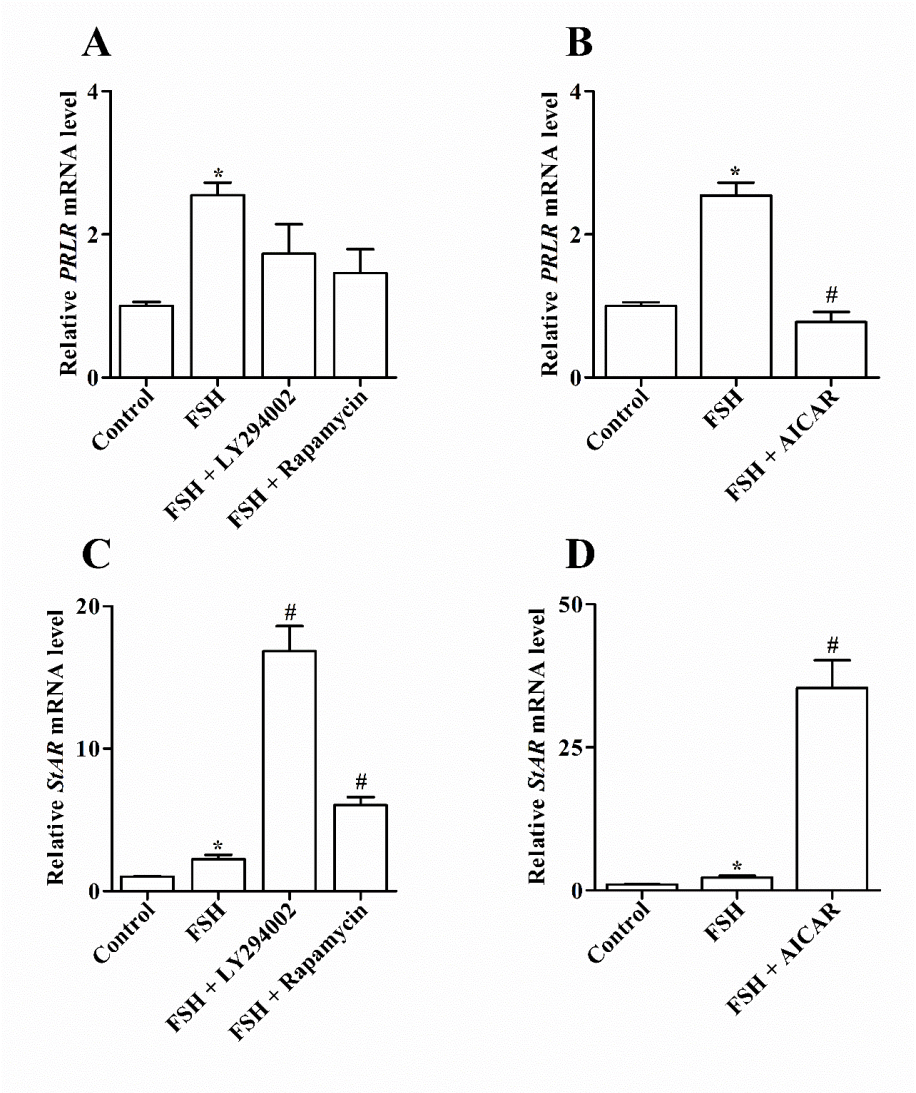


Figure 3.7.7 Abundance of ERK2 protein in chicken developing follicles and its role in FSH-induced *PRLR* and *StAR* expression in cultured granulosa cells of 6-8 mm follicles.

(A) Top panel: Representative western blot analysis of phosphorylated and total ERK2 in the stroma and walls of prehierarchical (< 2, 2-4 and 4-6 mm) follicles as well as in theca and granulosa cell layers separated from the 6-8 mm and F3-F1 follicles. β-actin was used as loading control. Bottom panel: Quantitative analysis of relative protein abundance of phosphorylated ERK2 by densitometry using Image Lab software (Version 4.1, Bio-Rad laboratories). Relative protein abundance was normalized to total ERK2. Data are expressed as fold differences ± SEM compared to an appropriate tissue (n = 4 hens). Bars with different letters are significantly different at $P < 0.05$. (B and C) Changes in relative mRNAs levels of *PRLR* (B) and *StAR* (C) in granulosa cells treated without or with 10 ng/ml FSH, or together with the MEK inhibitor (1 μM PD0325901) after a 4-h culture. Relative mRNA expression level was normalized to *18S rRNA*. Data are expressed as fold differences ± SEM of three independent experiments using tissues from different hens and are compared to control cells. *, $P < 0.05$ compared to control cells; $^{\#}$, $P < 0.05$ compared to cells only stimulated by 10 ng/ml FSH.

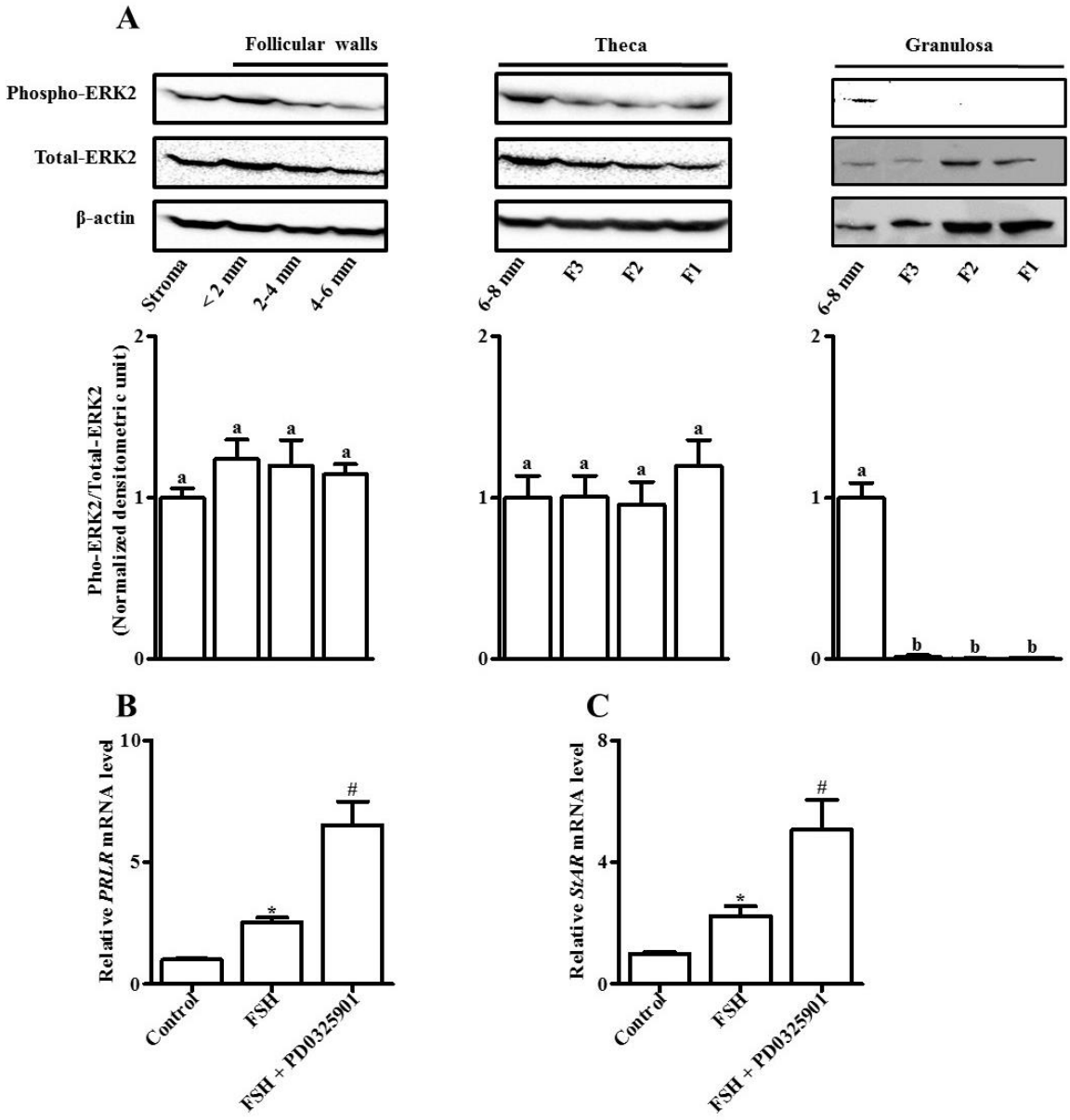
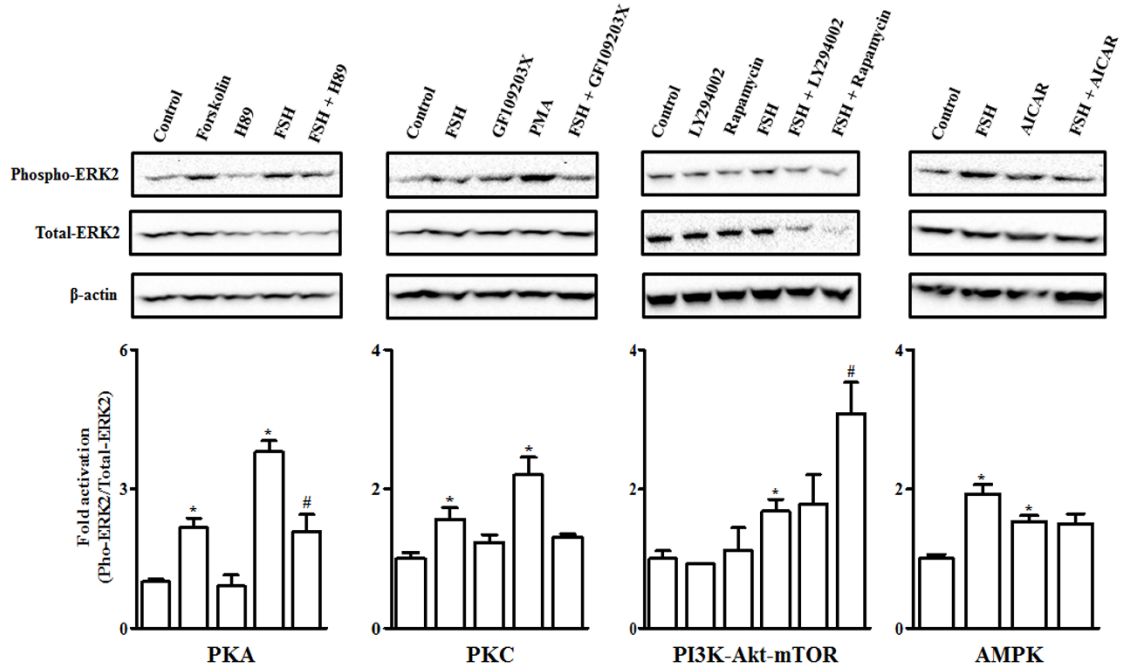

A
Follicular walls
Theca
Granulosa
Phospho-ERK2
Total-ERK2
β-actin
Stroma
< 2 mm
2-4 mm
4-6 mm
6-8 mm
F3
F2
F1
Pho-ERK2/Total-ERK2 (Normalized densitometric unit)
B
Relative PRLR mRNA level
Control
FSH
FSH + PD0325901
C
Relative StAR mRNA level

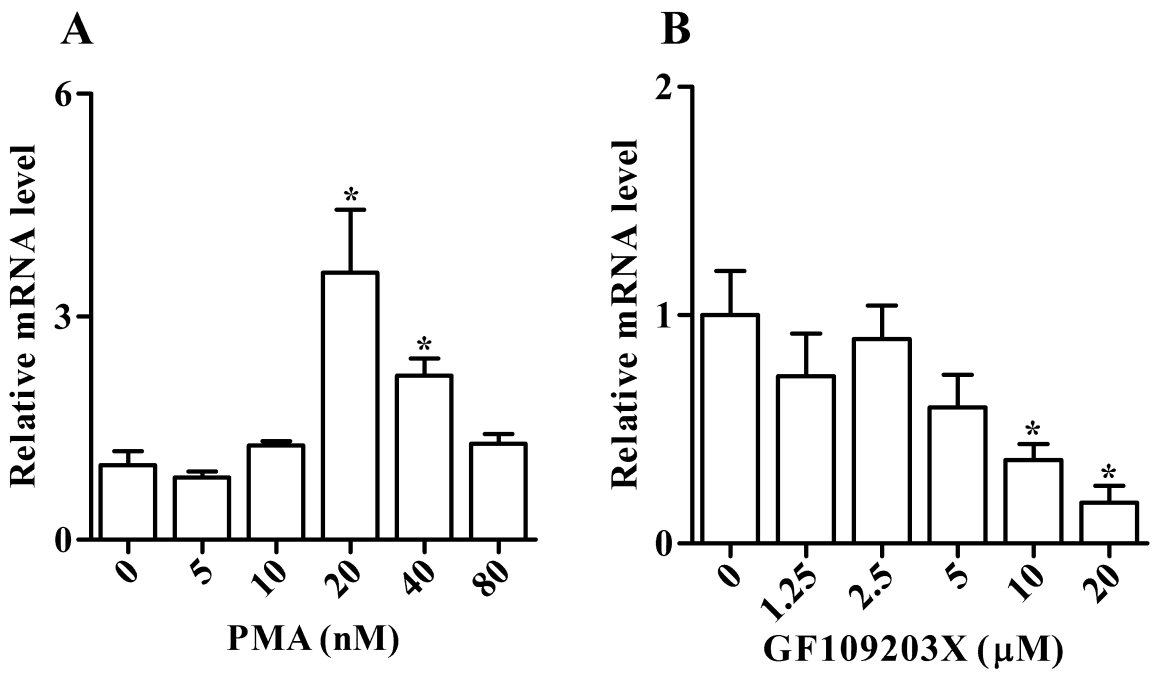
Figure 3.7.8 Effects of manipulation of several intracellular signaling pathways on basal and FSH-induced ERK2 phosphorylation in cultured granulosa cells of prehierarchical (6-8 mm) follicles.

Top panel: Representative western blot analysis of phosphorylated and total ERK2 in granulosa cells treated without or with 10 ng/ml FSH in combination with PKA activator or inhibitor (10 µM Forskolin or 20 µM H89, respectively), or PKC activator or inhibitor (20 nM PMA or 10 µM GF109203X, respectively), or PI3K inhibitor (20 µM LY294002) or mTOR inhibitor (10 µM Rapamycin), or AMPK activator (1 mM AICAR). β-actin was used as loading control. Bottom panel: Quantitative analysis of relative protein abundance of phosphorylated ERK2 by densitometry using Image Lab software (Version 4.1, Bio-Rad laboratories). Relative protein abundance was normalized to total ERK2. Data are expressed as fold differences ± SEM of three independent experiments using tissues from different hens and are compared to control cells. *, $P < 0.05$ compared to control cells; $^{\#}$, $P < 0.05$ compared to cells only stimulated by 10 ng/ml FSH.

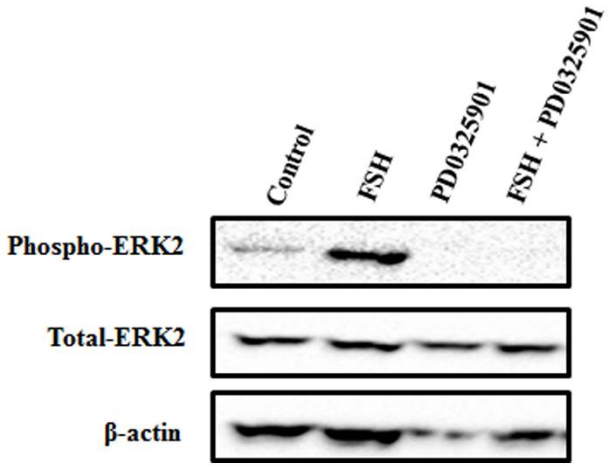


Supplemental figure 3.7.9 Dose optimization of the activator (PMA) and inhibitor (GF109203X) of PKC in regulation of *PRLR* expression in cultured granulosa cells of prehierarchical (6-8 mm) follicles.

(A and B) Changes in relative *PRLR* mRNA levels in granulosa cells responding to different doses of either PMA (0, 5, 10, 20, 40 or 80 nM) (A) or GF109203X (0, 1.25, 2.5, 5, 10 or 20 μM) (B) after a 4-h culture. Relative expression level was normalized to *18S rRNA*. Data are expressed as mean ± SEM of three independent experiments using tissues from different hens and are compared to control cells. *, $P < 0.05$; compared to control cells.



Supplemental figure 3.7.10 Representative western blot analysis of phosphorylated and total ERK2 in cultured granulosa cells of prehierarchical (6-8 mm) follicles treated without or with 10 ng/ml FSH, or in combination with the MEK inhibitor (1 μM PD0325901). β-actin was used as loading control.



3.8 REFERENCES

1. Johnson AL. Ovarian follicle selection and granulosa cell differentiation. Poultry science. 2015;94(4):781-5. doi: 10.3382/ps/peu008. PubMed PMID: 25535403.
2. Tilly JL, Kowalski KI, Johnson AL. Stage of ovarian follicular development associated with the initiation of steroidogenic competence in avian granulosa cells. Biology of reproduction. 1991;44(2):305-14. PubMed PMID: 1849025.
3. Johnson AL, Bridgham JT. Regulation of steroidogenic acute regulatory protein and luteinizing hormone receptor messenger ribonucleic acid in hen granulosa cells. Endocrinology. 2001;142(7):3116-24. doi: 10.1210/endo.142.7.8240. PubMed PMID: 11416034.
4. Tilly JL, Kowalski KI, Johnson AL. Cytochrome P450 side-chain cleavage (P450scc) in the hen ovary. II. P450scc messenger RNA, immunoreactive protein, and enzyme activity in developing granulosa cells. Biology of reproduction. 1991;45(6):967-74. PubMed PMID: 1666853.
5. Johnson AL, Woods DC. Dynamics of avian ovarian follicle development: cellular mechanisms of granulosa cell differentiation. General and comparative endocrinology. 2009;163(1-2):12-7. doi: 10.1016/j.ygcen.2008.11.012. PubMed PMID: 19059411.
6. Johnson PA. Follicle selection in the avian ovary. Reproduction in domestic animals = Zuchthygiene. 2012;47 Suppl 4:283-7. doi: 10.1111/j.1439-0531.2012.02087.x. PubMed PMID: 22827382.
7. Woods DC, Johnson AL. Regulation of follicle-stimulating hormone-receptor messenger RNA in hen granulosa cells relative to follicle selection. Biology of reproduction. 2005;72(3):643-50. doi: 10.1095/biolreprod.104.033902. PubMed PMID: 15537865.
8. Johnson AL. Intracellular mechanisms regulating cell survival in ovarian follicles. Animal reproduction science. 2003;78(3-4):185-201. doi: 10.1016/S0378-4320(03)00090-3. PubMed PMID: WOS:000185268900004.
9. Tosca L, Crochet S, Ferre P, Foufelle F, Tesseraud S, Dupont J. AMP-activated protein kinase activation modulates progesterone secretion in granulosa cells from hen preovulatory follicles. The Journal of endocrinology. 2006;190(1):85-97. doi: 10.1677/joe.1.06828. PubMed PMID: 16837613.
10. Opel H, Proudman JA. Failure of mammalian prolactin to induce incubation behaviour in chickens and turkeys. Poultry science. 1980;59(11):2550-8. PubMed PMID: 7465521.

11. Rozenboim I, Tabibzadeh C, Silsby JL, El Halawani ME. Effect of ovine prolactin administration on hypothalamic vasoactive intestinal peptide (VIP), gonadotropin releasing hormone I and II content, and anterior pituitary VIP receptors in laying turkey hens. Biology of reproduction. 1993;48(6):1246-50. PubMed PMID: 8391330.

12. Sharp PJ, Macnamee MC, Sterling RJ, Lea RW, Pedersen HC. Relationships between prolactin, LH and broody behaviour in bantam hens. The Journal of endocrinology. 1988;118(2):279-86. PubMed PMID: 3171469.

13. Camper PM, Burke WH. The effects of prolactin on the gonadotropin induced rise in serum estradiol and progesterone of the laying turkey. General and comparative endocrinology. 1977;32(1):72-7. PubMed PMID: 863224.

14. Lea RW, Dods AS, Sharp PJ, Chadwick A. The possible role of prolactin in the regulation of nesting behaviour and the secretion of luteinizing hormone in broody bantams. The Journal of endocrinology. 1981;91(1):89-97. PubMed PMID: 7299322.

15. El Halawani ME, Silsby JL, Rozenboim I, Pitts GR. Increased egg production by active immunization against vasoactive intestinal peptide in the turkey (Meleagris gallopavo). Biology of reproduction. 1995;52(1):179-83. PubMed PMID: 7711177.

16. Sharp PJ, Sterling RJ, Talbot RT, Huskisson NS. The role of hypothalamic vasoactive intestinal polypeptide in the maintenance of prolactin secretion in incubating bantam hens: observations using passive immunization, radioimmunoassay and immunohistochemistry. The Journal of endocrinology. 1989;122(1):5-13. PubMed PMID: 2769162.

17. Crisostomo S, Guemene D, Garreau-Mills M, Morvan C, Zadworny D. Prevention of incubation behaviour expression in turkey hens by active immunization against prolactin. Theriogenology. 1998;50(4):675-90. PubMed PMID: 10732158.

18. March JB, Sharp PJ, Wilson PW, Sang HM. Effect of active immunization against recombinant-derived chicken prolactin fusion protein on the onset of broodiness and photoinduced egg laying in bantam hens. Journal of reproduction and fertility. 1994;101(1):227-33. PubMed PMID: 8064686.

19. Li WL, Liu Y, Yu YC, Huang YM, Liang SD, Shi ZD. Prolactin plays a stimulatory role in ovarian follicular development and egg laying in chicken hens. Domestic animal endocrinology. 2011;41(2):57-66. doi: 10.1016/j.domaniend.2011.03.002. PubMed PMID: 21600726.

20. Hrabia A, Paczoska-Eliasiewicz H, Rzasa J. Effect of prolactin on estradiol and

progesterone secretion by isolated chicken ovarian follicles. Folia biologica. 2004;52(3-4):197-203. PubMed PMID: 19058560.

21. Freeman ME, Kanyicska B, Lerant A, Nagy G. Prolactin: structure, function, and regulation of secretion. Physiological reviews. 2000;80(4):1523-631. PubMed PMID: 11015620.

22. Hanks MC, Alonzi JA, Sharp PJ, Sang HM. Molecular cloning and sequence analysis of putative chicken prolactin cDNA. Journal of molecular endocrinology. 1989;2(1):21-30. PubMed PMID: 2765112.

23. Wang Y, Li J, Yan Kwok AH, Ge W, Leung FC. A novel prolactin-like protein (PRL-L) gene in chickens and zebrafish: cloning and characterization of its tissue expression. General and comparative endocrinology. 2010;166(1):200-10. doi: 10.1016/j.ygcen.2009.10.007. PubMed PMID: 19854191.

24. Wilkanowska A, Mazurowski A, Mroczkowski S, Kokoszynski D. Prolactin (PRL) and prolactin receptor (PRLR) genes and their role in poultry production traits. Folia biologica. 2014;62(1):1-8. PubMed PMID: 24745142.

25. Bu G, Ying Wang C, Cai G, Leung FC, Xu M, Wang H, et al. Molecular characterization of prolactin receptor (cPRLR) gene in chickens: gene structure, tissue expression, promoter analysis, and its interaction with chicken prolactin (cPRL) and prolactin-like protein (cPRL-L). Molecular and cellular endocrinology. 2013;370(1-2):149-62. doi: 10.1016/j.mce.2013.03.001. PubMed PMID: 23499864.

26. Zhou JF, Zadworny D, Guemene D, Kuhnlein U. Molecular cloning, tissue distribution, and expression of the prolactin receptor during various reproductive states in Meleagris gallopavo. Biology of reproduction. 1996;55(5):1081-90. PubMed PMID: 8902221.

27. Hiyama G, Kansaku N, Kinoshita M, Sasanami T, Nakamura A, Noda K, et al. Changes in post-translational modifications of prolactin during development and reproductive cycles in the chicken. General and comparative endocrinology. 2009;161(2):238-45. doi: 10.1016/j.ygcen.2009.01.007. PubMed PMID: 19523395.

28. Bedecarrats G, Guemene D, Kuhnlein U, Zadworny D. Changes in levels of immunoreactive prolactin isoforms during a reproductive cycle in turkey hens. General and comparative endocrinology. 1999;113(1):96-104. doi: 10.1006/gcen.1998.7187. PubMed PMID: 9882548.

29. Gilbert AB, Evans AJ, Perry MM, Davidson MH. A method for separating the granulosa

cells, the basal lamina and the theca of the preovulatory ovarian follicle of the domestic fowl (Gallus domesticus). Journal of reproduction and fertility. 1977;50(1):179-81. PubMed PMID: 864645.

30. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8. doi: 10.1006/meth.2001.1262. PubMed PMID: 11846609.

31. Johnson AL, Solovieva EV, Bridgham JT. Relationship between steroidogenic acute regulatory protein expression and progesterone production in hen granulosa cells during follicle development. Biology of reproduction. 2002;67(4):1313-20. PubMed PMID: 12297550.

32. Sechman A, Pawlowska K, Hrabia A. Effect of 3,3',5-triiodothyronine and 3,5-diiodothyronine on progesterone production, cAMP synthesis, and mRNA expression of STAR, CYP11A1, and HSD3B genes in granulosa layer of chicken preovulatory follicles. Domestic animal endocrinology. 2011;41(3):137-49. doi: 10.1016/j.domaniend.2011.05.007. PubMed PMID: 21798688.

33. Zakany R, Szucs K, Bako E, Felszeghy S, Czifra G, Biro T, et al. Protein phosphatase 2A is involved in the regulation of protein kinase A signaling pathway during in vitro chondrogenesis. Experimental cell research. 2002;275(1):1-8. doi: 10.1006/excr.2002.5487. PubMed PMID: 11925100.

34. Kansaku N, Shimada K, Saito N, Hidaka H. Effects of protein kinase A inhibitor (H-89) on VIP- and GRF-induced release and mRNA expression of prolactin and growth hormone in the chicken pituitary gland. Comparative biochemistry and physiology Part C, Pharmacology, toxicology & endocrinology. 1998;119(1):89-95. PubMed PMID: 9568378.

35. Woods DC, Johnson AL. Protein kinase C activity mediates LH-induced ErbB/Erk signaling in differentiated hen granulosa cells. Reproduction. 2007;133(4):733-41. doi: 10.1530/REP-06-0261. PubMed PMID: 17504917.

36. Jin Y, Zhang C, Lin X, Zeng W. Prostaglandin involvement in follicle-stimulating hormone-induced proliferation of granulosa cells from chicken prehierarchical follicles. Prostaglandins & other lipid mediators. 2006;81(1-2):45-54. doi: 10.1016/j.prostaglandins.2006.07.002. PubMed PMID: 16997131.

37. Wen R, Hu S, Xiao Q, Han C, Gan C, Gou H, et al. Leptin exerts proliferative and anti-apoptotic effects on goose granulosa cells through the PI3K/Akt/mTOR signaling pathway. The

Journal of steroid biochemistry and molecular biology. 2015;149:70-9. doi: 10.1016/j.jsbmb.2015.01.001. PubMed PMID: 25576904.

38. Duchene S, Audouin E, Crochet S, Duclos MJ, Dupont J, Tesseraud S. Involvement of the ERK1/2 MAPK pathway in insulin-induced S6K1 activation in avian cells. Domestic animal endocrinology. 2008;34(1):63-73. doi: 10.1016/j.domaniend.2006.11.001. PubMed PMID: 17166687.

39. Stocco DM, Wang X, Jo Y, Manna PR. Multiple signaling pathways regulating steroidogenesis and steroidogenic acute regulatory protein expression: more complicated than we thought. Molecular endocrinology. 2005;19(11):2647-59. doi: 10.1210/me.2004-0532. PubMed PMID: 15831519.

40. Lee KA, Bahr JM. Utilization of Substrates for Testosterone and Estradiol-17-Beta Production by Small Follicles of the Chicken Ovary. Domestic animal endocrinology. 1994;11(3):307-14. doi: Doi 10.1016/0739-7240(94)90022-1. PubMed PMID: WOS:A1994NX93900007.

41. Zadworny D, Shimada K, Ishida H, Sato K. Gonadotropin-stimulated estradiol production in small ovarian follicles of the hen is suppressed by physiological concentrations of prolactin in vitro. General and comparative endocrinology. 1989;74(3):468-73. PubMed PMID: 2744414.

42. Veldhuis JD, KLASE P, HAMMOND JM. Divergent Effects of Prolactin upon Steroidogenesis by Porcine Granulosa Cells in Vitro: Influence of Cytodifferentiation*. Endocrinology. 1980;107(1):42-6.

43. Taira H, Beck MM. Activity of three-beta-hydroxysteroid dehydrogenase in granulosa cells treated in vitro with luteinizing hormone, follicle-stimulating hormone, prolactin, or a combination. Poultry science. 2006;85(10):1769-74. PubMed PMID: 17012167.

44. Tanaka K, Kamiyoshi M, Tanabe Y. Inhibition of premature ovulation by prolactin in the hen. Poultry science. 1971;50(1):63-6. PubMed PMID: 5550480.

45. Nakamura E, Otsuka F, Inagaki K, Miyoshi T, Yamanaka R, Tsukamoto N, et al. A novel antagonistic effect of the bone morphogenetic protein system on prolactin actions in regulating steroidogenesis by granulosa cells. Endocrinology. 2010;151(11):5506-18. doi: 10.1210/en.2010-0265. PubMed PMID: 20810564.

46. Wang C, Hsueh A, Erickson GF. Induction of functional prolactin receptors by follicle-stimulating hormone in rat granulosa cells in vivo and in vitro. Journal of Biological Chemistry.

1979;254(22):11330-6.

47. Tanaka M, Maeda K, Okubo T, Nakashima K. Double antenna structure of chicken prolactin receptor deduced from the cDNA sequence. Biochemical and biophysical research communications. 1992;188(2):490-6.

48. Porter MB, Brumsted JR, Sites CK. Effect of prolactin on follicle-stimulating hormone receptor binding and progesterone production in cultured porcine granulosa cells. Fertility and sterility. 2000;73(1):99-105.

49. Ramji DP, Pelagia F. CCAAT/enhancer-binding proteins: structure, function and regulation. Biochemical Journal. 2002;365(3):561-75.

50. Alliston TN, Maiyar AC, Buse P, Firestone GL, Richards JS. Follicle stimulating hormone-regulated expression of serum/glucocorticoid-inducible kinase in rat ovarian granulosa cells: a functional role for the Sp1 family in promoter activity. Molecular endocrinology. 1997;11(13):1934-49.

51. Woods DC, Haugen MJ, Johnson AL. Actions of epidermal growth factor receptor/mitogen-activated protein kinase and protein kinase C signaling in granulosa cells from Gallus gallus are dependent upon stage of differentiation. Biology of reproduction. 2007;77(1):61-70. doi: 10.1095/biolreprod.106.059394. PubMed PMID: 17409374.

52. Eresheim C, Leeb C, Buchegger P, Nimpf J. Signaling by the extracellular matrix protein Reelin promotes granulosa cell proliferation in the chicken follicle. The Journal of biological chemistry. 2014;289(14):10182-91. doi: 10.1074/jbc.M113.533489. PubMed PMID: 24573679; PubMed Central PMCID: PMC3974987.

53. Johnson AL, Bridgham JT, Swenson JA. Activation of the Akt/protein kinase B signaling pathway is associated with granulosa cell survival. Biology of reproduction. 2001;64(5):1566-74. PubMed PMID: 11319165.

54. Tosca L, Chabrolle C, Crochet S, Tesseraud S, Dupont J. IGF-1 receptor signaling pathways and effects of AMPK activation on IGF-1-induced progesterone secretion in hen granulosa cells. Domestic animal endocrinology. 2008;34(2):204-16. doi: 10.1016/j.domaniend.2007.03.001. PubMed PMID: 17478073.

55. Hunzicker-Dunn M, Maizels ET. FSH signaling pathways in immature granulosa cells that regulate target gene expression: Branching out from protein kinase A. Cellular signalling. 2006;18(9):1351-9. doi: 10.1016/j.cellsig.2006.02.011. PubMed PMID: WOS:000238868200001.

56. Woods DC, Johnson AL. Phosphatase activation by epidermal growth factor family ligands regulates extracellular regulated kinase signaling in undifferentiated hen granulosa cells. Endocrinology. 2006;147(10):4931-40. doi: 10.1210/en.2006-0194. PubMed PMID: 16840544.

57. Woods DC, Haugen MJ, Johnson AL. Opposing actions of TGFbeta and MAP kinase signaling in undifferentiated hen granulosa cells. Biochemical and biophysical research communications. 2005;336(2):450-7. doi: 10.1016/j.bbrc.2005.08.107. PubMed PMID: 16139244.

CONNECTING STATEMENT 2

In Chapters II and III, we investigated the single and combined actions of gonadotropins and PRL isoforms in regulation of steroidogenesis and expression of PRLR as well as intracellular regulatory mechanisms in ovarian follicles. We found that basal E_2 and P_4 secretion was differentially regulated by gonadotropins or PRL isoforms independently or in combination possibly through modulating PRLR expression in the follicular hierarchy. Additionally, we also observed that PRLR was widely expressed throughout follicle development.

PRL-like protein (PRL-L), a homolog of PRL, has been recently identified in non-mammalian vertebrates including chickens. Because of its ability to bind to the PRLR which is widely distributed in the follicular hierarchy, interactions may occur among circulating PRL isoforms, intra-ovarian PRL-L and PRLR to regulate follicular cell steroidogenesis. Therefore, in the next chapter (Chapter IV) we investigated the expression pattern of PRL-L in the follicular hierarchy and examined the roles of gonadotropins and PRL isoforms in regulation of PRL-L expression as well as the underlying intracellular mechanisms in granulosa cells.

CHAPTER IV

EXPRESSION AND REGULATION OF PROLACTIN-LIKE PROTEIN MESSENGER RNA IN UNDIFFERENTIATED CHICKEN GRANULOSA CELLS

Shenqiang Hu, Raj Duggavathi and David Zadworny*

Department of Animal Science, McGill University, Macdonald Campus, Ste. Anne de Bellevue, Quebec, H9X 3V9 Canada

* **Corresponding author.** E-mail: david.zadworny@mcgill.ca

Running title: Regulatory mechanisms of PRL-L in chickens

4.1 ABSTRACT

Prolactin-like protein (PRL-L; LOC417800) is a homolog of PRL in non-mammalian vertebrates and can act as a functional ligand of PRL receptor (PRLR). Despite its widespread expression in extrapituitary tissues, mechanisms of regulation of PRL-L in the chicken ovary remain unknown. In this study, we first examined *PRL-L* expression in chicken ovarian developing follicles. *PRL-L* transcript levels were highest ($P < 0.05$) in follicular walls of < 2 mm follicles and progressively declined during follicle maturation. Undifferentiated granulosa cells of 6-8 mm follicles had higher ($P < 0.05$) *PRL-L* mRNA levels than differentiated granulosa cells of F3, F2 or F1 follicles. In cultured undifferentiated granulosa cells, levels of *PRL-L* transcript were increased ($P < 0.05$) by follicle stimulating hormone (FSH) treatment while were not altered by the addition of luteinizing hormone (LH). In addition, 10 ng/ml non-glycosylated (NG-) and 1 ng/ml glycosylated (G-) PRL increased ($P < 0.05$) but at higher levels (100 or 1000 ng/ml) both showed no effects on *PRL-L* expression. Furthermore, 100 ng/ml NG-PRL enhanced ($P < 0.05$) FSH-induced *PRL-L* expression, whereas the effects of G-PRL were not significant. These results suggest that *PRL-L* mRNA is differentially expressed in the follicular hierarchy and its high abundance in undifferentiated granulosa cells is under the regulation of FSH or PRL variants independently or in combination. Moreover, in undifferentiated granulosa cells we also provide evidence for a positive role for PKA, PKC and PI3K signaling while a negative role for ERK2 in mediating FSH stimulation of *PRL-L* transcription.

Key words: Chicken; Ovary; Follicle development; Granulosa cells; Prolactin-like protein; Signaling pathway

4.2 INTRODUCTION

Recently, a novel prolactin-like protein (PRL-L; LOC417800) has been discovered in several non-mammalian vertebrate species including chicken, zebra finch, tiger puffer, green puffer and zebrafish [1]. It is evolved from an ancestral PRL/GH-like gene, but has been lost in mammals during evolution [2, 3]. A specific receptor for PRL-L has not been discovered, however, structural similarity of PRL-L to PRL [2, 3] suggests that PRL-L may interact with the PRL receptor (PRLR). Indeed, chicken PRL-L does activate PRLR signal transduction albeit with lower affinity compared to PRL in a reporter construct expressed in HepG2 cells [4]. It has been shown that PRL-L may be involved in increasing cold-induced muscle growth in chicks [5]. Apart from that, nothing is known about other physiological actions in non-mammalian species despite its widespread expression in multiple tissues [1]. In contrast to PRL that is predominantly expressed in pituitary, PRL-L is expressed in extra-pituitary tissues including the ovary in both embryonic and adult chickens [1]. However, the distribution to cell type or follicular size class in the chicken ovary is unknown.

The establishment and maintenance of ovarian follicular hierarchy is essential for egg production in birds and occurs as a result of synergistic interactions between pituitary gonadotropins and locally secreted factors within the ovary [6, 7]. In chickens and turkeys, PRL has both pro- and anti-gonadal roles on ovarian function. Plasma levels of PRL increase after photostimulation and are maintained at higher levels during the period of egg laying [8-10], and active immunization against PRL suppresses chicken egg production by reducing follicular recruitment into the hierarchy [11]. However, at high concentrations PRL is anti-gonadal and is associated with ovarian involution [8-10] and inhibition of steroidogenesis [12-16]. Consistent with the direct effects of PRL on ovarian functions, PRLR is expressed in all follicle size classes with the highest levels found in the small follicles (< 2 mm) [17]. Because of its ability to bind to the PRLR, interaction(s) may occur among circulating PRL, intra-ovarian PRL-L and PRLR to regulate the follicular hierarchy.

Since there is a paucity of information about the involvement of the *PRL-L* gene in the follicular hierarchy, it is important to investigate the expression and regulation of PRL-L in order to understand its potential role in modulating PRL signaling and ovarian function. The objectives of the present study were: 1) to determine the expression pattern of *PRL-L* in chicken ovarian

follicles at different developmental stages; 2) to investigate the effects of FSH and LH in regulation of *PRL-L* mRNA levels; 3) to investigate the effects of non-glycosylated (NG-) and glycosylated (G-) PRL on basal and gonadotropin-stimulated expression of PRL-L; 4) to explore the role of intracellular signaling pathways in gonadotropin regulation of *PRL-L* expression in undifferentiated chicken granulosa cells.

4.3 MATERIALS AND METHODS

4.3.1 Hormones, chemicals and reagents

Recombinant human FSH (rhFSH, AFP8468A), ovine LH (oLH, AFP-5551B), non-glycosylated ovine PRL (NG-oPRL, AFP-10692C) and glycosylated ovine PRL (G-oPRL, AFP-5742B) were obtained from National Hormone & Peptide Program (Torrance, CA, USA). A stock solution of each hormone was correspondingly prepared with either PBS at appropriate pH or 0.01M $NaHCO_3$, and then stored in small aliquots at -80 ℃. The final concentration of each working solution used for corresponding treatment was prepared with culture medium. Protein kinase A (PKA) activator (Forskolin) and inhibitor (H89), protein kinase C (PKC) activator (Phorbol 12-myristate 13-acetate, PMA) and inhibitors (GF109203X and RO318220), phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002), mammalian target of rapamycin (mTOR) inhibitor (Rapamycin) and extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitor (PD0325901) were purchased from Selleck Chemicals (Houston, TX, USA). Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium/Nutrient Mixture F12 (DMEM/F12), 0.4% Trypan Blue Solution as well as penicillin and streptomycin mixture were purchased from Invitrogen Life technologies (Carlsbad, CA, USA). Type II collagenase was purchased from Sigma-Aldrich (Oakville, ON, Canada). Trizol reagent and high-capacity cDNA reverse transcription kit were purchased from Invitrogen Life technologies (Carlsbad, CA, USA). Power SYBR Green PCR Master Mix was purchased from D-Mark Bioscience (Toronto, ON, Canada).

4.3.2 Animals and tissue collection

All experimental procedures using chickens in this study were approved by the Faculty Animal Care Committee of McGill University. White Leghorn hens, 25-35 weeks of age and laying

actively, were used in all studies described. Hens were fed *ad libitum* and kept in individual cages under standard conditions at the Poultry Complex of Macdonald Campus Farm, McGill University. The time of oviposition was monitored for each hen using surveillance camera (Lorex corporation, Maryland, USA), and ovulation was predicted to occur within 15-30 min after oviposition of the previous egg in the laying sequence. Four hens were randomly selected for ovarian tissue collection and were killed approximately 1-4 h before predicted time of a mid-sequence ovulation by cervical dislocation. After slaughter, the ovary from each hen was immediately removed and placed into ice-cold 0.9% NaCl solution. According to the diameter and the position in the follicular hierarchy, ovarian follicles were categorized into several groups, including the stroma, prehierarchical (< 2, 2-4, 4-6 and 6-8 mm) and preovulatory (9-12 mm and F5-F1, 13-40 mm; F5 < F4 < F3 < F2 < F1) follicles. Follicular walls consisting of a mixture of cells including theca and granulosa cells were collected from < 2 mm to F4 follicles, and theca and granulosa cell layers were isolated from F3-F1 follicles, respectively, according to the method as previously described [18]. To further determine the difference in the expression of PRL-L between undifferentiated and differentiated granulosa cell layers, another 6 chickens were sacrificed to collect granulosa cell layers from each category of the 6-8 mm, F3, F2 and F1 follicles, respectively. All samples were snap frozen in liquid nitrogen and then stored at -80 ℃ until analysis.

4.3.3 Granulosa cell culture and treatments

Ovarian follicles were grouped by the diameter and stage of maturation, and granulosa cell layers were harvested and digested with 0.1% Type II collagenase. The number of granulosa cells were subsequently counted using a hemocytometer and cell viability was estimated by trypan blue exclusion test. The cells were then seeded into 12-well culture plates at a density of ~3×10^5 cells/well in 1ml DMEM/F12 medium containing 3% FBS and 1% penicillin-streptomycin mixture. Cells were cultured at 38.5 ℃ in a humidified atmosphere of 95% air and 5% CO_2. After 24 h of incubation with a medium change at 6 h, non-attached cells were removed by aspiration and adherent cells were washed three times with serum-free medium supplemented with 1% penicillin-streptomycin mixture. Cells were subsequently incubated in serum-free medium without or with corresponding treatment for another 24 h. These treatments include 10 ng/ml rhFSH or oLH, or different concentrations of NG- and G-oPRL (0, 1, 10, 100 or 1000 ng/ml), or in combination. To further explore the involvement of signaling pathways, cells were cultured in the

absence or presence of 10 ng/ml rhFSH, or together with corresponding activator or inhibitor(s) of several intracellular signaling cascades for another 4 h. Finally, the culture media were removed and the cells were collected for RNA isolation. Each *in vitro* experiment was independently performed at least three times on different cultures.

4.3.4 RNA isolation and real time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. The purity and concentration of RNA was determined using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA, and the integrity of RNA was assessed by visualization of the 28S/18S rRNA ratio after electrophoresis on 1.5% agarose gels. The cDNA was then synthesized from 1 µg RNA using a high-capacity cDNA reverse transcription kit (Invitrogen, USA). Chicken-specific primers for *PRL-L* (GenBank No. NM_001165912) (forward: 5´AGCCACTTTCCCGCTTTA3´; reverse: 5´TGCCCATTGACTGCATCT3´) and *18S rRNA* (GenBank No. AF173612) (forward: 5´TTAAGTCCCTGCCCTTTGTACAC 3´; reverse: 5´CGATCCGAGGAACCTCACTAAAC3´) were designed using Primer Premier Version 6 software (Premier Biosoft International, CA, USA) and synthesized by Invitrogen Life technologies. Real time quantitative PCR (qPCR) reactions were performed on the CFX384^{TM} real-time PCR detection system (Bio-Rad) using SYBR Green master mix (D-Mark Bioscience, ON, Canada). Reactions were conducted with the following conditions: pre-denaturation at 95℃ for 5 min, followed by 40 cycles of denaturation at 95℃ for 15 s and annealing/extension at 60℃ for 30 s. The no-template controls and negative controls without reverse transcriptase were also included in all qPCR runs. Target specificity for both primer pairs was validated by melting curve analyses. In addition, the identity of both amplicons was verified by sequencing. A standard curve was generated by 5-fold serial dilutions of cDNA to determine the amplification efficiency of PCR reactions. The efficiencies for both prime pairs were nearly 100% and had a coefficient of determination (R^2) > 0.98, allowing the use of the comparative Cq method (ΔΔCq) [19]. The *18S rRNA* gene was used as the internal control. All samples were amplified in triplicate and relative mRNA levels of *PRL-L* were normalized to *18S rRNA*. All qPCR results were shown as fold-differences in comparison to an appropriate reference tissue or control treatment.

4.3.5 Statistical analyses

All results were presented as mean ± SEM of data from at least three separate experiments. The data from replicate experiments were analyzed using one-way randomized block ANOVA with each experimental run being a block. When a significant difference was observed, post hoc comparisons between the groups were performed using Duncan's Multiple Range Test. All statistical analyses were performed using the General Linear Model procedure of SAS 9.4 (SAS Institute, Cary, USA). $P < 0.05$ was considered statistically significant.

4.4 RESULTS

4.4.1 Distribution of *PRL-L* mRNA during chicken ovarian follicle development

Levels of *PRL-L* mRNA were determined in hen ovarian stroma and follicular walls of prehierarchical and preovulatory follicles. Among developmental stages examined, *PRL-L* mRNA levels were higher in follicular walls of slow-growing (1-8 mm) prehierarchical follicles than those of rapid-growing (9-12 mm) hierarchical follicles. Highest abundance of *PRL-L* mRNA was detected in follicular walls of < 2 mm follicles, and minimal expression was observed in those of the F5-F4 follicles (Fig. 4.7.1).

4.4.2 Effects of gonadotropins on *PRL-L* expression in undifferentiated chicken granulosa cells

Since theca cell layers of hierarchical follicles (F3-F1) did not show detectable levels of *PRL-L* mRNA (data not shown), we focused on the expression and regulation of *PRL-L* mRNA abundance in granulosa cells from undifferentiated (6-8 mm) and differentiated (F3-F1) follicles. As shown in Fig. 4.7.2A, *PRL-L* transcript levels were much higher in freshly isolated granulosa cell layers from undifferentiated (6-8 mm) follicles than those from differentiated (F3-F1) follicles. Thus, the effects of gonadotropins on *PRL-L* mRNA expression were further determined in undifferentiated granulosa cells. FSH treatment enhanced the mRNA levels of *PRL-L* by 2.5-fold when compared to untreated cells ($P < 0.05$), while stimulation with LH showed no significant effects on *PRL-L* mRNA levels ($P > 0.05$, Fig. 4.7.2B).

4.4.3 Effects of PRL variants on *PRL-L* expression in undifferentiated chicken granulosa cells

The degree of glycosylation of PRL is known to vary with reproductive state in the chicken. Therefore, we examined the direct effects of non-glycosylated (NG-) and glycosylated (G-) ovine PRL on *PRL-L* mRNA levels in undifferentiated granulosa cells (Fig. 4.7.3). Either NG- or G-oPRL at relatively low doses appeared to have stimulatory effects on *PRL-L* mRNA expression, while at higher doses the response was minimal. Specifically, 10 ng/ml NG-oPRL stimulated a 3.8-fold increase in levels of *PRL-L* transcript ($P < 0.05$), and 1 ng/ml G-oPRL increased levels of *PRL-L* mRNA by 3.7 fold ($P < 0.05$).

4.4.4 Effects of PRL variants on FSH-induced *PRL-L* expression in undifferentiated chicken granulosa cells

Next, we tested the modulatory effects of PRL variants on FSH-induced *PRL-L* expression in undifferentiated granulosa cells. The inclusion of NG-oPRL at a low level (10 ng/ml) showed no effects on ($P > 0.05$), but at a relatively higher level (100 ng/ml) enhanced ($P < 0.05$) FSH-induced *PRL-L* mRNA levels. However, neither 10 ng/ml nor 100 ng/ml G-oPRL altered FSH-stimulated *PRL-L* levels ($P > 0.05$, Fig. 4.7.4).

4.4.5 Role of PKA and PKC pathways in FSH-induced *PRL-L* expression in undifferentiated chicken granulosa cells

To determine whether PKA and PKC signaling pathways are involved in FSH-stimulated *PRL-L* expression in granulosa cells of 6-8 mm follicles, the activators and inhibitors of PKA (Forskolin and H89, respectively) and PKC (PMA and RO318220 or GF109203X, respectively) were used. The doses of Forskolin and H89 were derived from earlier studies in chickens [20-23]. As shown in Fig. 4.7.5A, treatment with 10 μM Forskolin or 10 ng/ml FSH induced a significant increase in the levels of *PRL-L* mRNA ($P < 0.05$). This FSH-induced effect on *PRL-L* mRNA abundance was blocked by H89 treatment ($P < 0.05$).

Since different doses of PMA and GF109203X have been used in chicken granulosa cells [24-26], we first determined dose-response effects of either PMA or GF109203X on *PRL-L* expression (Suppl. Fig. 4.7.7). Compared to untreated cells, 10 and 20 nM PMA enhanced ($P < 0.05$, Suppl. Fig. 4.7.7A), while 10 and 20 μM GF109203X suppressed *PRL-L* mRNA levels

($P < 0.05$, Suppl. Fig. 4.7.7B). Then, 20 nM PMA and 10 μM GF109203X were selected to be used in subsequent experiments. While 20 nM PMA increased basal *PRL-L* mRNA abundance to levels similar to that of FSH, inhibition of PKC with 2.5 μM RO318220 or 10 μM GF109203X decreased FSH-induced *PRL-L* expression ($P < 0.05$, Fig. 4.7.5B).

4.4.6 Role of PI3K-Akt-mTOR pathway in FSH-induced *PRL-L* expression in undifferentiated chicken granulosa cells

To further explore other signaling pathways by which FSH can enhance *PRL-L* mRNA levels, granulosa cells from 6-8 mm follicles were cultured for 4 h in the absence or presence of 10 ng/ml FSH and/or PI3K inhibitor (20 μM LY294002) or mTOR inhibitor (10 μM Rapamycin). Treatment with the doses of the inhibitors has been previously demonstrated to be effectively in blocking either PI3K or mTOR signal in goose granulosa cells [27] and chicken muscle cells [28]. As shown in Fig. 4.7.6A, levels of *PRL-L* mRNA induced by FSH declined when treated in combination with LY294002 ($P < 0.05$) but were not altered by addition of Rapamycin ($P > 0.05$).

4.4.7 Role of ERK2 pathway in FSH-induced *PRL-L* expression in undifferentiated chicken granulosa cells

We then explored the role of ERK2 in FSH-induced *PRL-L* expression. Granulosa cells from 6-8 mm follicles were treated without or with 10 ng/ml FSH and/or the MEK inhibitor (1 μM PD0325901). The dose for PD0325901 effectively suppressed phosphorylation of ERK2 in undifferentiated chicken granulosa cells (data not shown). Stimulation with PD0325901 resulted in an increase in FSH-stimulated *PRL-L* expression ($P < 0.05$, Fig. 4.7.6B).

4.5 DISCUSSION

The non-mammalian vertebrate gene encoding PRL-L has been shown to be expressed in a wide variety of extra-pituitary tissues including the ovary of embryonic and adult chickens [1, 2]. However, neither the cell type nor the tissue location within the ovary has been characterized. In this study, we observed that *PRL-L* mRNA was present in the ovary with the highest levels observed in follicular walls of the smallest (< 2 mm) follicles. As follicles grow and mature, however, levels of *PRL-L* gradually declined. In the smaller (< 8 mm) follicles the cell type expressing *PRL-L* remains unknown as expression was determined in follicular walls in the present

study. However, in preovulatory (F3-F1) follicles expression of *PRL-L* was restricted to the granulosa cells. In light of these results, it is plausible that PRL-L is mainly expressed in granulosa cells of prehierarchical (< 8 mm) follicles.

The function of PRL-L in the follicular hierarchy is not known. However, it is notable that levels of its transcripts mirror those of the PRLR, which are also expressed at higher levels in the pre-hierarchical rather than the hierarchical follicles [17]. PRL-L has been shown to bind to and activate signal transduction through the chicken PRLR using a reporter construct expressed in HepG2 cells [4]. In addition, PRL-L was predicted to have a signal peptide [1] and has been shown to be secreted by chick muscle cells [5]. Therefore, it is possible that PRL-L acts in an autocrine and/or paracrine fashion in the follicular hierarchy by activating the PRLR. However, the physiological roles in modulating follicular recruitment and accretion remain to be investigated.

It is well known that the initiation, progression and maturation of avian ovarian follicles are tightly coupled with morphological and functional changes in all elements of follicular walls. In particular, granulosa cells from prehierarchical cohorts are arrested in an undifferentiated state (steroidogenically inactive), while those from preovulatory follicles are maintained in a differentiated state (steroidogenically active) [29]. This transition is facilitated by the change from FSH receptor (FSHR) dominance during the prehierarchical phase to LH receptor (LHR) dominance during the preovulatory stage [29]. FSH signaling activates a series of cAMP-dependent genes such as steroidogenic enzymes and LHR, thereby leading to the initiation of granulosa cell differentiation [29, 30]. In contrast, subsequent to follicle selection LH-induced production of large amounts of progesterone promote rapid maturation and ovulation of hierarchical follicles [30]. Thus, the declining expression of *PRL-L* in preovulatory granulosa cells may, at least in part, be due to the acquisition of LH-responsiveness. Whereas the relatively higher expression in 6-8 mm prehierarchical granulosa cells may be sustained by FSH-stimulated signals. Additionally, the small follicles are thought to be the major source of estrogen [31], supporting possible paracrine and/or autocrine actions of PRL-L in regulation of estrogen production.

Very little is known about the factors that regulate the expression of PRL-L in any cell type. To our knowledge, only cold exposure of neonatal chicks for 24 h is known to induce *PRL-L* expression in the skeletal muscle [5] and no studies have addressed mechanisms of regulation of its expression in the ovary. In the present study, we showed that both gonadotrophins and PRL

variants had significant effects on the *PRL-L* gene expression. In undifferentiated granulosa cells of prehierarchical (6-8 mm) follicles, addition of FSH significantly promoted while stimulation with LH did not alter basal *PRL-L* mRNA levels. In addition, a stimulatory effect on *PRL-L* mRNA levels was observed in granulosa cells treated with either NG- or G-oPRL at a lower level, whereas, this effect became minimal when the level of either NG- or G-PRL was higher. Nevertheless, G-oPRL at very low levels (1 ng/ml) seemed to be more effective than NG-oPRL in inducing *PRL-L* mRNA expression in undifferentiated granulosa cells. The role of PRL-L in the follicular hierarchy is unknown. However, PRL has been shown to have both pro- and anti-gonadal effects in chickens dependent on its concentration. At low levels, PRL has been shown to enhance steroidogenesis dependent on the stage of the ovulatory cycle and follicle size class [13], whereas, at higher concentrations PRL inhibits steroidogenesis and causes ovarian involution [8-10]. Moreover, the proportion of G- to NG-PRL increases from about 30% to 80 % associated with hyperprolactinemia and ovarian involution in hens [32, 33]. How PRL-L may be involved in this process is unclear but interactions among isoforms of PRL, its receptor and PRL-L warrants further investigation. Furthermore, FSH and NG-oPRL appeared to have an additive effect on *PRL-L* mRNA levels in granulosa cells derived from 6-8 mm follicles, while G-oPRL treatment had no significant effects on FSH-stimulated *PRL-L* expression. In mammals, it has been shown in granulosa cells that PRL may differentially modulate FSH signaling by altering the number of FSH-induced FSHRs [34] and that locally secreted growth factors such as the bone morphogenetic proteins (BMPs) system are involved in mediating the actions of PRL in FSH-stimulated steroid production [35]. Thus, the differential effects of NG- and G-oPRL on FSH-induced *PRL-L* expression may be associated with their different roles in influencing FSH signaling and/or potential feedback by autocrine/paracrine growth factors such as the BMP system. Taken together, these results imply that crosstalk occurs between the FSH and PRL signaling pathways in regulation of PRL-L expression in undifferentiated granulosa cells that varies dependent on the concentration and the degree of glycosylation of PRL. Any potential networking between the pathways requires further investigation.

Since FSH treatment increased *PRL-L* mRNA levels in undifferentiated (6-8 mm) granulosa cells, we further explored the role of intracellular signaling pathways. Activation of PKA using Forskolin induced *PRL-L* expression similar to FSH. Its inhibition using H89 abolished FSH-driven increase in *PRL-L* mRNA levels. As a cAMP-dependent protein kinase, PKA feeds into

multiple signaling pathways that are involved in mediating the complex actions of FSH in mammalian [36] and avian [37] granulosa cells. Since a cAMP-responsive element (CRE) was predicted in chicken *PRL-L* promoter region [1], it is plausible that PKA mediates FSH regulation of *PRL-L* expression through the CRE-binding protein. In addition, the stimulatory effect of FSH on *PRL-L* expression was also mimicked by the PKC activator PMA, and inhibition of PKC using RO318220 or GF109203X decreased FSH-induced *PRL-L* transcript abundance, indicating that PKC signaling cascade may be positively involved in FSH-induced *PRL-L* expression in undifferentiated granulosa cells.

We also observed that the PI3K inhibitor (LY294002) abrogated, while the mTOR inhibitor (Rapamycin) had no effect on FSH-induced *PRL-L* expression in undifferentiated granulosa cells. These results indicate that PI3K pathway contributes to FSH-induced *PRL-L* expression independent of mTOR signaling. In addition, inhibition of ERK2 signaling by the MEK inhibitor (PD0325901) upregulated *PRL-L* mRNA levels in FSH-stimulated granulosa cells. While the mammalian genome has ERK1 ($p44^{MAPK}$) and ERK2 ($p42^{MAPK}$) proteins as two key members of mitogen-activated protein kinases [38], ERK2 is demonstrated to be the only isoform in avian species [28]. It was shown to be a negative regulator in FSH-induced differentiation and StAR expression in undifferentiated chicken granulosa cells [24]. In light of these observations, ERK2 may play negative roles in regulation of FSH-induced *PRL-L* expression in undifferentiated granulosa cells. Taken together, all these data derived from inhibitor studies suggested that in undifferentiated chicken granulosa cells, the stimulation of FSH on transcription of *PRL-L* is regulated by multiple simultaneous pathways that have both positive and negative impact.

In conclusion, *PRL-L* transcript was widely expressed in all developing follicles and had higher levels in undifferentiated than differentiated granulosa cells. The transcription of *PRL-L* in undifferentiated granulosa cells was regulated by FSH or PRL variants independently, or in combination. Furthermore, the stimulatory actions of FSH on *PRL-L* expression was simultaneously mediated by multiple signaling pathways including the classic AC/cAMP/PKA and the inositol phospholipid signaling pathways. Activation of these pathways in concert with the activation of the PRL pathway resulted in crosstalk in regulation of *PRL-L* transcription, which could be variable dependent on the concentration of PRL and the degree of PRL glycosylation. Additional studies to determine the protein levels of PRL-L

in the follicular hierarchy, to clarify the regulatory mechanisms controlling its expression, as well as further unveiling its physiological actions in the reproductive system are required.

4.6 ACKNOWLEDGEMENTS

We thank Dr.AF Parlow (National Hormone & Peptide Program) for providing us with oLH, rhFSH, NG- and G-oPRL. We are grateful to Profs. Sarah Kimmins and Vilceu Bordignon (McGill University) for their laboratory facilities. SH appreciates the financial support from the Chinese Scholarship Council (CSC), Hector & Ada Ma Fellowship and Graduate Excellence Award of McGill University. This research was funded by the Discovery Grants of Natural Sciences and Engineering Research Council of Canada (RD and DZ).

4.7 FIGURES

Figure 4.7.1 Characterization of *PRL-L* mRNA abundance in chicken ovarian follicles.
Relative *PRL-L* mRNA levels in ovarian stroma and follicular walls of prehierarchical (< 2, 2-4, 4-6 and 6-8 mm) and preovulatory (9-12 mm, F5 and F4) follicles. Relative mRNA abundance of *PRL-L* was normalized to *18S rRNA*. Bars (mean ± SEM) with different letters are significantly different ($P < 0.05$).

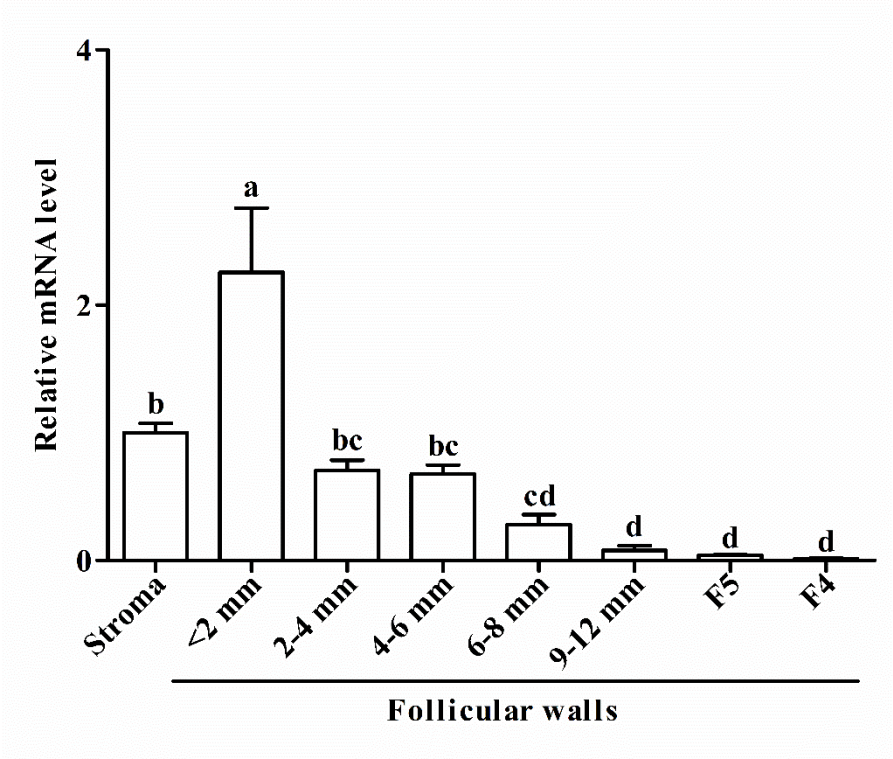


Figure 4.7.2 Expression and gonadotropin regulation of *PRL-L* mRNA in chicken granulosa cells.

(A) Abundance of *PRL-L* mRNA in granulosa cell layers separated from 6-8 mm and F3-F1 follicles. (B) Effects of FSH or LH (10 ng/ml) on relative *PRL-L* mRNA levels in granulosa cells of 6-8 mm follicles after a 24-h culture. Relative mRNA abundance of *PRL-L* was normalized to *18S rRNA*. Bars (mean ± SEM) with different letters are significantly different ($P < 0.05$).

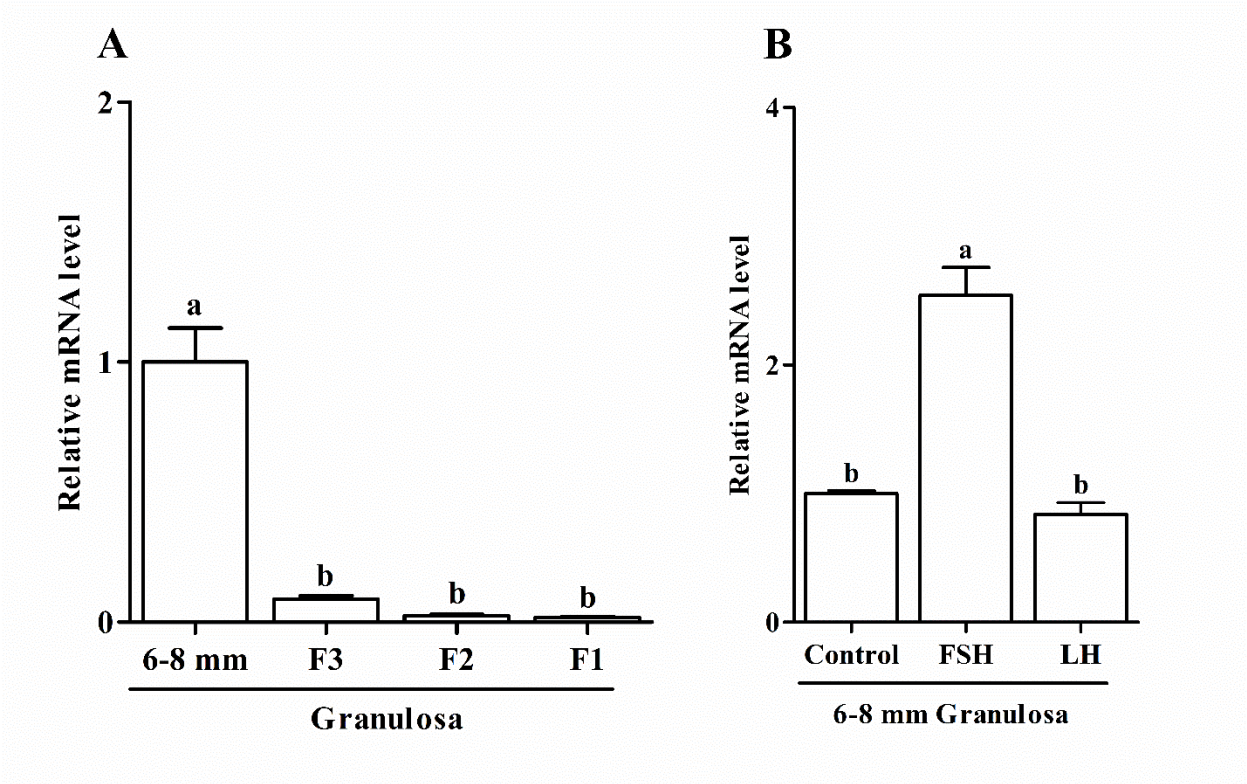


Figure 4.7.3 Regulation of *PRL-L* mRNA abundance by non-glycosylated (NG-) and glycosylated (G-) ovine PRL (oPRL) (0, 1, 10, 100 or 1000 ng/ml) in cultured granulosa cells of chicken 6-8 mm follicles after a 24-h culture.

Relative mRNA abundance of *PRL-L* was normalized to *18S rRNA*. Bars (mean ± SEM) with different letters are significantly different ($P < 0.05$).

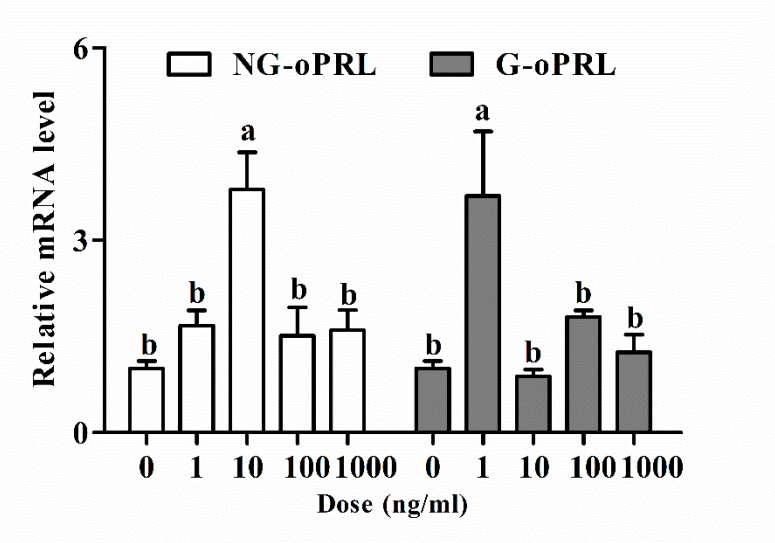


Figure 4.7.4 Effects of non-glycosylated (NG-) and glycosylated (G-) ovine PRL (oPRL) (0, 10 or 100 ng/ml) on FSH-induced *PRL-L* expression in cultured granulosa cells isolated from chicken 6-8 mm follicles after a 24-h culture.

Relative mRNA abundance of *PRL-L* was normalized to *18S rRNA*. Bars (mean ± SEM) with different letters are significantly different ($P < 0.05$).

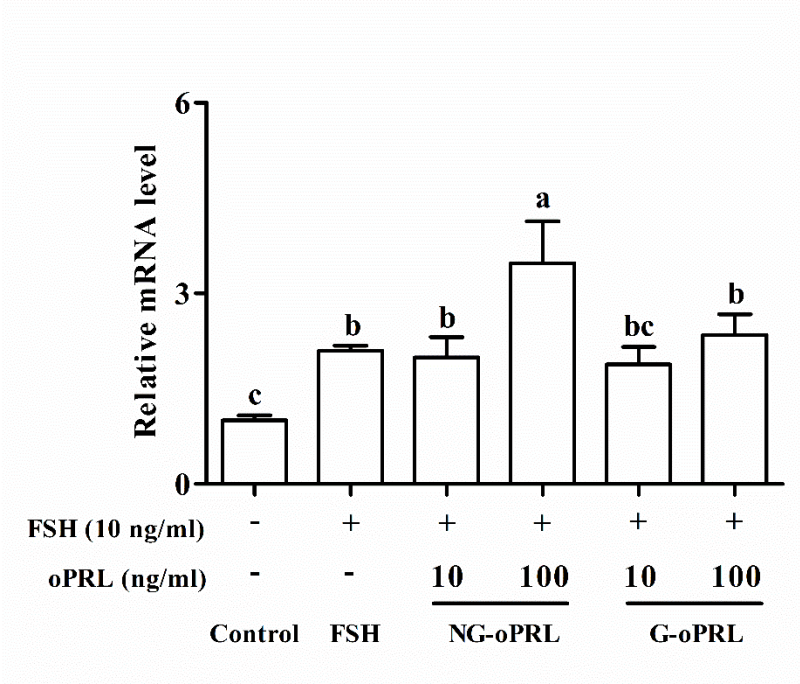


Figure 4.7.5 Role of PKA and PKC signaling pathways in FSH-induced *PRL-L* expression in granulosa cells isolated from chicken 6-8 mm follicles.

(A) Changes in relative *PRL-L* mRNA levels after culture of granulosa cells for 4 h in the absence or presence of 10 ng/ml FSH and/or PKA activator (10 µM Forskolin) or PKA inhibitor (20 µM H89). (B) Changes in *PRL-L* mRNA levels in granulosa cells treated without or with 10 ng/ml FSH and/or PKC activator (20 nM PMA) or PKC inhibitors (2.5 µM RO318220 or 10 µM GF109203X) after a 4-h culture. Relative mRNA abundance of *PRL-L* was normalized to *18S rRNA*. Bars (mean ± SEM) with different letters are significantly different ($P < 0.05$).

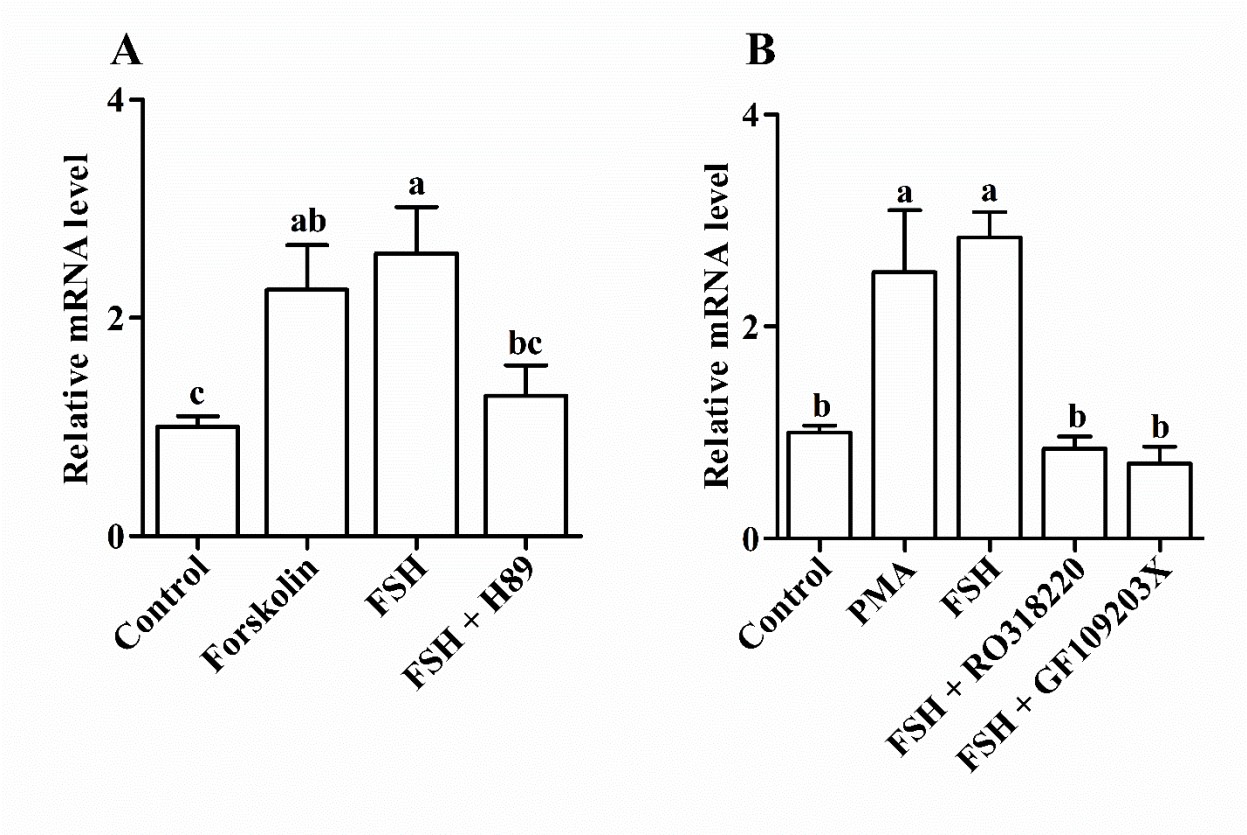
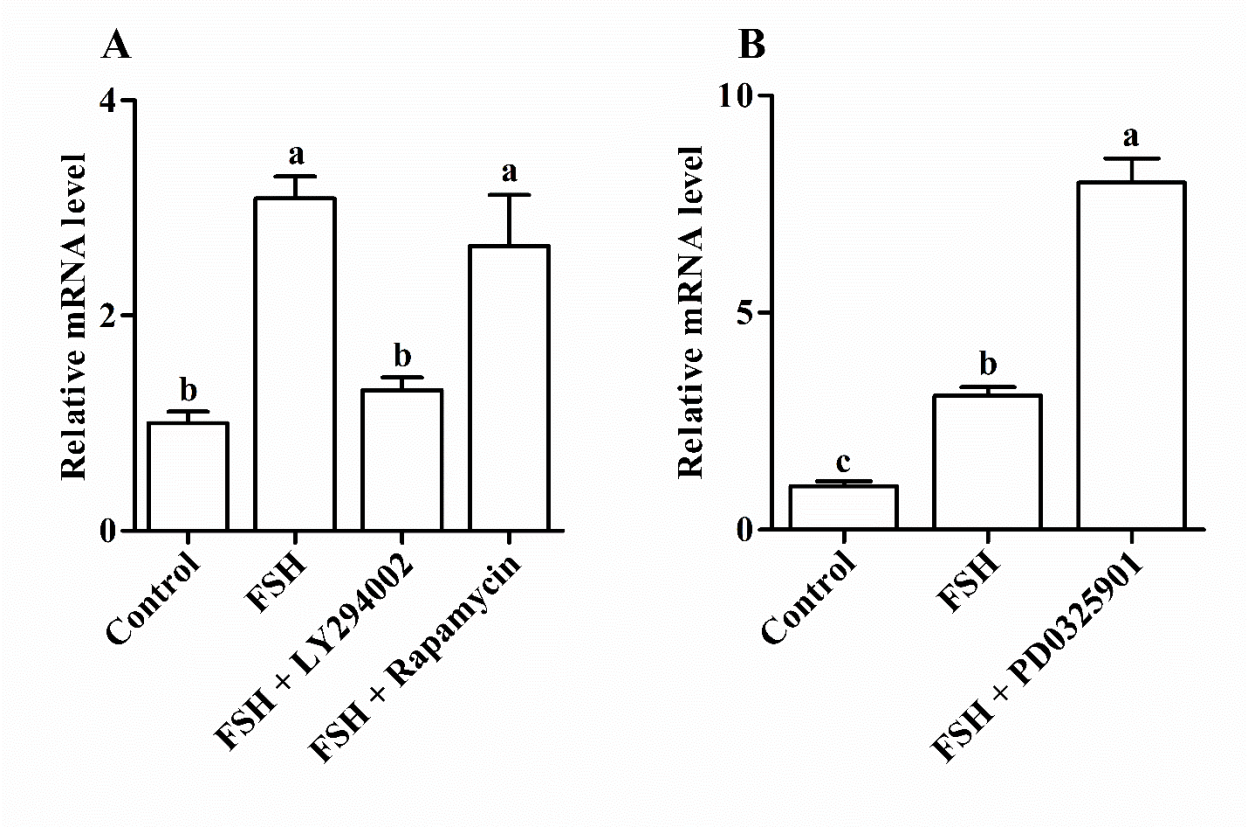


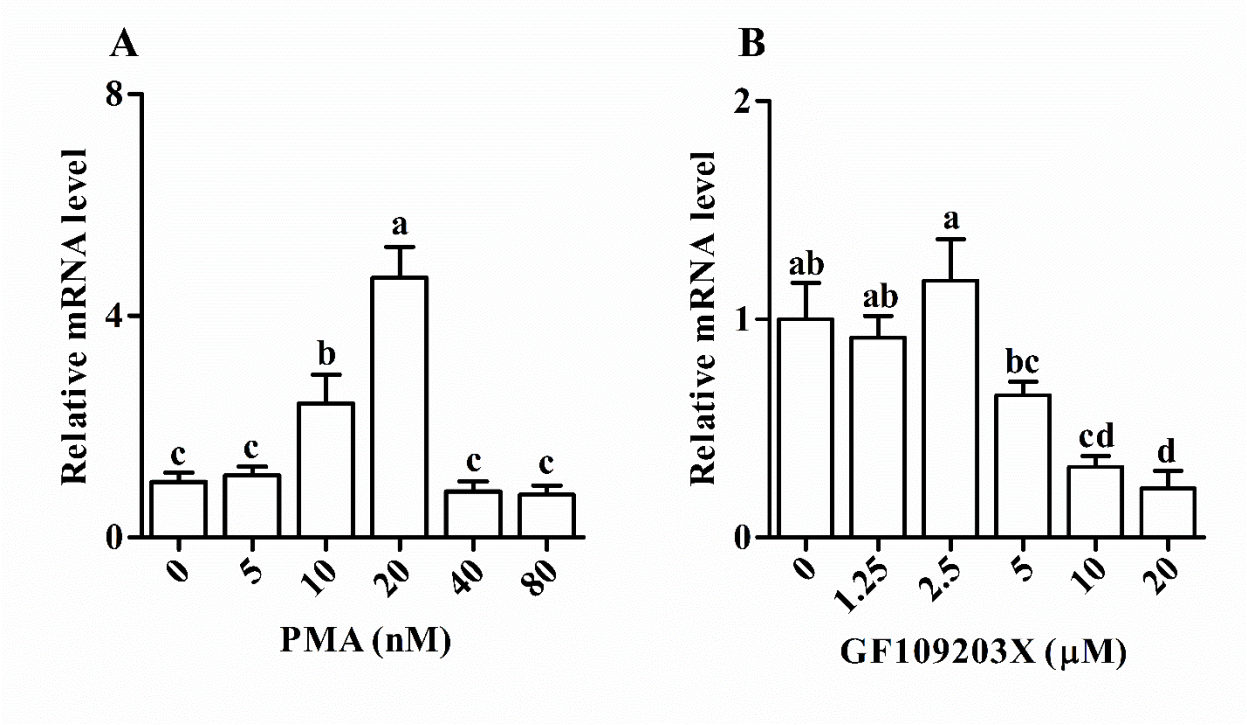
Figure 4.7.6 Role of PI3K-Akt-mTOR and ERK2 signaling pathways in FSH-induced *PRL-L* expression in granulosa cells isolated from chicken 6-8 mm follicles.

(A) Changes in relative *PRL-L* mRNA levels after culture of granulosa cells for 4 h in the absence or presence of 10 ng/ml FSH and/or PI3K inhibitor (20 μM LY294002) or mTOR inhibitor (10 μM Rapamycin). (B) Relative *PRL-L* mRNA levels in granulosa cells treated without or with 10 ng/ml FSH and/or the MEK inhibitor (1 μM PD0325901). Relative mRNA abundance of *PRL-L* was normalized to *18S rRNA*. Bars (mean ± SEM) with different letters are significantly different ($P < 0.05$).



Supplemental figure 4.7.7 Dose optimization of the activator (PMA) and inhibitor (GF109203X) of PKC in regulation of *PRL-L* expression in granulosa cells isolated from chicken 6-8 mm follicles.

(A and B) Changes in relative *PRL-L* mRNA levels in granulosa cells responding to different doses of either PMA (0, 5, 10, 20, 40 or 80 nM) (A) or GF109203X (0, 1.25, 2.5, 5, 10 or 20 μM) (B) after a 4-h culture. Relative mRNA abundance of *PRL-L* was normalized to *18S rRNA*. Bars (mean ± SEM) with different letters are significantly different ($P < 0.05$).



4.8 REFERENCES

1. Wang Y, Li J, Yan Kwok AH, Ge W, Leung FC. A novel prolactin-like protein (PRL-L) gene in chickens and zebrafish: cloning and characterization of its tissue expression. Gen Comp Endocrinol 2010; 166:200-210.
2. Bu G, Liang X, Li J, Wang Y. Extra-pituitary prolactin (PRL) and prolactin-like protein (PRL-L) in chickens and zebrafish. Gen Comp Endocrinol 2015; 220:143-153.
3. Huang X, Hui MN, Liu Y, Yuen DS, Zhang Y, Chan WY, Lin HR, Cheng SH, Cheng CH. Discovery of a novel prolactin in non-mammalian vertebrates: evolutionary perspectives and its involvement in teleost retina development. PLoS One 2009; 4:e6163.
4. Bu G, Ying Wang C, Cai G, Leung FC, Xu M, Wang H, Huang G, Li J, Wang Y. Molecular characterization of prolactin receptor (cPRLR) gene in chickens: gene structure, tissue expression, promoter analysis, and its interaction with chicken prolactin (cPRL) and prolactin-like protein (cPRL-L). Mol Cell Endocrinol 2013; 370:149-162.
5. Ijiri D, Higuchi A, Saegusa A, Miura M, Matsubara T, Kanai Y, Ohtsuka A, Hirabayashi M. Role of prolactin-like protein (PRL-L) in cold-induced increase of muscle mass in chicks. Gen Comp Endocrinol 2013; 186:94-100.
6. Johnson PA, Stephens CS, Giles JR. The domestic chicken: Causes and consequences of an egg a day. Poult Sci 2015; 94:816-820.
7. Onagbesan O, Bruggeman V, Decuypere E. Intra-ovarian growth factors regulating ovarian function in avian species: a review. Anim Reprod Sci 2009; 111:121-140.
8. Sharp PJ, Blache D. A neuroendocrine model for prolactin as the key mediator of seasonal breeding in birds under long- and short-day photoperiods. Can J Physiol Pharmacol 2003; 81:350-358.
9. Zadworny D, Walton JS, Etches RJ. The relationship between plasma concentrations of prolactin and consumption of feed and water during the reproductive cycle of the domestic turkey. Poult Sci 1985; 64:401-410.
10. Zadworny D, Walton JS, Etches RJ. Effect of environment on the intake of food and water, body weight, egg production and plasma concentrations of corticosterone and prolactin in turkey hens. Br Poult Sci 1986; 27:639-650.

11. Li WL, Liu Y, Yu YC, Huang YM, Liang SD, Shi ZD. Prolactin plays a stimulatory role in ovarian follicular development and egg laying in chicken hens. Domest Anim Endocrinol 2011; 41:57-66.
12. Zadworny D, Shimada K, Ishida H, Sato K. Gonadotropin-stimulated estradiol production in small ovarian follicles of the hen is suppressed by physiological concentrations of prolactin in vitro. Gen Comp Endocrinol 1989; 74:468-473.
13. Hrabia A, Paczoska-Eliasiewicz H, Rzasa J. Effect of prolactin on estradiol and progesterone secretion by isolated chicken ovarian follicles. Folia Biol (Krakow) 2004; 52:197-203.
14. Reddy IJ, David CG, Sarma PV, Singh K. The possible role of prolactin in laying performance and steroid hormone secretion in domestic hen (Gallus domesticus). Gen Comp Endocrinol 2002; 127:249-255.
15. Porter TE, Hargis BM, Silsby JL, el Halawani ME. Enhanced progesterone and testosterone secretion and depressed estradiol secretion in vitro from small white follicle cells of incubating turkey hens. Gen Comp Endocrinol 1989; 74:400-405.
16. Tabibzadeh C, Rozenboim I, Silsby JL, Pitts GR, Foster DN, el Halawani M. Modulation of ovarian cytochrome P450 17 alpha-hydroxylase and cytochrome aromatase messenger ribonucleic acid by prolactin in the domestic turkey. Biol Reprod 1995; 52:600-608.
17. Zhou JF, Zadworny D, Guemene D, Kuhnlein U. Molecular cloning, tissue distribution, and expression of the prolactin receptor during various reproductive states in Meleagris gallopavo. Biol Reprod 1996; 55:1081-1090.
18. Gilbert AB, Evans AJ, Perry MM, Davidson MH. A method for separating the granulosa cells, the basal lamina and the theca of the preovulatory ovarian follicle of the domestic fowl (Gallus domesticus). J Reprod Fertil 1977; 50:179-181.
19. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25:402-408.
20. Tilly JL, Kowalski KI, Johnson AL. Stage of ovarian follicular development associated with the initiation of steroidogenic competence in avian granulosa cells. Biol Reprod 1991; 44:305-314.
21. Sechman A, Pawlowska K, Hrabia A. Effect of 3,3',5-triiodothyronine and 3,5-diiodothyronine on progesterone production, cAMP synthesis, and mRNA expression of

STAR, CYP11A1, and HSD3B genes in granulosa layer of chicken preovulatory follicles. Domest Anim Endocrinol 2011; 41:137-149.

22. Zakany R, Szucs K, Bako E, Felszeghy S, Czifra G, Biro T, Modis L, Gergely P. Protein phosphatase 2A is involved in the regulation of protein kinase A signaling pathway during in vitro chondrogenesis. Exp Cell Res 2002; 275:1-8.
23. Kansaku N, Shimada K, Saito N, Hidaka H. Effects of protein kinase A inhibitor (H-89) on VIP- and GRF-induced release and mRNA expression of prolactin and growth hormone in the chicken pituitary gland. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 1998; 119:89-95.
24. Johnson AL, Solovieva EV, Bridgham JT. Relationship between steroidogenic acute regulatory protein expression and progesterone production in hen granulosa cells during follicle development. Biol Reprod 2002; 67:1313-1320.
25. Woods DC, Johnson AL. Protein kinase C activity mediates LH-induced ErbB/Erk signaling in differentiated hen granulosa cells. Reproduction 2007; 133:733-741.
26. Jin Y, Zhang C, Lin X, Zeng W. Prostaglandin involvement in follicle-stimulating hormone-induced proliferation of granulosa cells from chicken prehierarchical follicles. Prostaglandins Other Lipid Mediat 2006; 81:45-54.
27. Wen R, Hu S, Xiao Q, Han C, Gan C, Gou H, Liu H, Li L, Xu H, He H, Wang J. Leptin exerts proliferative and anti-apoptotic effects on goose granulosa cells through the PI3K/Akt/mTOR signaling pathway. J Steroid Biochem Mol Biol 2015; 149:70-79.
28. Duchene S, Audouin E, Crochet S, Duclos MJ, Dupont J, Tesseraud S. Involvement of the ERK1/2 MAPK pathway in insulin-induced S6K1 activation in avian cells. Domest Anim Endocrinol 2008; 34:63-73.
29. Johnson AL, Woods DC. Dynamics of avian ovarian follicle development: cellular mechanisms of granulosa cell differentiation. Gen Comp Endocrinol 2009; 163:12-17.
30. Johnson PA. Follicle selection in the avian ovary. Reprod Domest Anim 2012; 47 Suppl 4:283-287.
31. Lee KA, Bahr JM. Utilization of Substrates for Testosterone and Estradiol-17-Beta Production by Small Follicles of the Chicken Ovary. Domestic Animal Endocrinology 1994; 11:307-314.
32. Bedecarrats G, Guemene D, Kuhnlein U, Zadworny D. Changes in levels of

immunoreactive prolactin isoforms during a reproductive cycle in turkey hens. Gen Comp Endocrinol 1999; 113:96-104.

33. Hiyama G, Kansaku N, Kinoshita M, Sasanami T, Nakamura A, Noda K, Tsukada A, Shimada K, Zadworny D. Changes in post-translational modifications of prolactin during development and reproductive cycles in the chicken. Gen Comp Endocrinol 2009; 161:238-245.
34. Porter MB, Brumsted JR, Sites CK. Effect of prolactin on follicle-stimulating hormone receptor binding and progesterone production in cultured porcine granulosa cells. Fertility and sterility 2000; 73:99-105.
35. Nakamura E, Otsuka F, Inagaki K, Miyoshi T, Yamanaka R, Tsukamoto N, Suzuki J, Ogura T, Makino H. A novel antagonistic effect of the bone morphogenetic protein system on prolactin actions in regulating steroidogenesis by granulosa cells. Endocrinology 2010; 151:5506-5518.
36. Hunzicker-Dunn M, Maizels ET. FSH signaling pathways in immature granulosa cells that regulate target gene expression: Branching out from protein kinase A. Cellular Signalling 2006; 18:1351-1359.
37. Johnson AL. Intracellular mechanisms regulating cell survival in ovarian follicles. Animal Reproduction Science 2003; 78:185-201.
38. Zassadowski F, Rochette-Egly C, Chomienne C, Cassinat B. Regulation of the transcriptional activity of nuclear receptors by the MEK/ERK1/2 pathway. Cell Signal 2012; 24:2369-2377.

CHAPTER V
GENERAL DISCUSSION AND CONCLUSIONS

5.1 GENERAL DISCUSSION AND CONCLUSIONS

In galliformes, ovarian follicles committed to ovulate are normally arranged in a well-defined follicular hierarchy which is indispensable for egg production. Disrupted or abnormal follicle development are associated with reduced reproductive efficiency and may even cause infertility and ovarian cancer in domestic hens. For instance, in *ad libitum*-fed broiler breeder hens, excessive follicle development due to more than one follicle being selected into the preovulatory hierarchy results in reduced high-quality egg production in parallel with some reproductive disorders. Therefore, it is of great value to elucidate the factors involved in regulating the establishment and maintenance of the avian follicular hierarchy. This may not only expand our knowledge of the characteristics and regulation of vertebrate follicular growth and development but may also contribute to the optimization and development of conventional breeding and management strategies and/or new technologies including molecular and transgenic techniques that can improve reproductive efficiency in commercial (*e.g.* broiler breeder hens), threatened and endangered avian species. Furthermore, the chicken ovary has recently been exploited for the study of human ovarian cancer, since the laying hen is the only animal other than humans that can spontaneously develop ovarian cancer, with approximately 30-35% incidence by 3.5 years of age [1]. A series of morphological, histochemical and biochemical, as well as molecular and cellular studies have shown the similarity in the phenotype and etiology of the disease between the domestic hen and human [1]. Thus, such work may to some extent be useful for the establishment of early diagnosis and therapeutic strategies of human ovarian cancer.

The maintenance of the follicular hierarchy is orchestrated by complex interactions between endocrine hormones mainly released from the hypothalamic-hypophyseal axis and many locally secreted factors in the ovary. In contrast to gonadotropins, much less is known about the involvement of PRL signaling in the follicular hierarchy although its role in incubation behavior has been well recognized over the last half-century. In hens that express incubation behavior,

hyperprolactinemia is typically related to follicular disruption and ovarian regression. Evidence supports both indirect (inhibition of gonadotrophin release [2-5]) and direct (inhibition of steroidogenesis [6-9]) roles for high levels of PRL suppressing the hypothalamic-hypophyseal-ovarian axis to initiate ovarian regression. Nonetheless, PRL at lower levels would appear to be pro-gonadal since photostimulation induces increased levels of circulating PRL associated with the initiation of egg laying accompanied by elevated concentrations of ovarian steroids. In addition, immunization against PRL has been shown to retard the development of white follicle growth and their recruitment into the preovulatory hierarchy in chicken hens [10]. Moreover, *in vitro* studies of cultured hen ovarian follicles suggest that the effects of PRL on basal E_2 and P_4 production were variable according to the concentration, the type of follicular cells, the stage of follicle development and the stage of the ovulation cycle [11]. Thus, it is assumed that PRL may have both pro- and anti-gonadal roles in the follicular hierarchy depending on the concentration. However, the molecular mechanism has not yet been clearly defined.

Multiple isoforms of PRL resulting from post-translational modifications have been identified in vertebrates, and among them G-PRL represents the major variant in birds [12]. Significantly, the relative and absolute amounts of G- to NG-PRL changes during various reproductive states in turkeys [13] and chickens [14]. In mammals, glycosylation of PRL may reduce its binding affinity with the receptor PRLR and consequently the biological activity in target tissues since 3 of the 14 amino acids required for receptor binding are located in the proximity of this site hence, glycosylation may cause steric hindrance [12, 15]. Nevertheless, glycosylation of avian PRL may result in a different topology which may interact differently with the receptor to exert its biological actions than its mammalian counterpart. Notably, compared to mammals, avian PRLR has a duplicated ligand binding domain, which allows for more complex interactions with PRL isoforms. Thus, it is likely that glycosylation may modulate the actions of PRL in hen ovarian follicles differently than in mammals. However, so far there is a paucity of information about the role of G-PRL in the follicular hierarchy.

Therefore, our first study was conducted to compare the effects of NG- and G-PRL on basal and gonadotropin-stimulated steroidogenesis by cultured hen different size class follicles. In granulosa cells of preovulatory F3-F1 follicles, both isoforms at lower levels induced basal E_2 and P_4 secretion but these effects declined as the concentrations increased. However, in contrast to an inducible effect of NG-PRL, G-PRL suppressed basal E_2 and P_4 secretion in a dose-dependent

manner in granulosa cells of prehierarchical 6-8 mm follicles. The effects of both isoforms on basal steroid production were further demonstrated to be mediated through modulating expression of several key steroidogenic enzymes (*i.e.* StAR, CYP11A1, CYP19A1 and 3β-HSD). In addition, G-PRL was shown to be less potent than NG-PRL in suppressing FSH- or LH-stimulated E_2 and P_4 production in preovulatory granulosa cells, whereas, in prehierarchical granulosa cells, G-PRL reduced but NG-PRL enhanced FSH-stimulated P_4 production. These results indicated that G-PRL below a threshold concentration may play a positive role in preovulatory follicles not only through facilitating basal steroidogenesis but also through attenuating the suppressive effects of NG-PRL on gonadotropin-stimulated steroid production. This effect was gradually reduced as the concentration increased. However, in prehierarchical 6-8 mm follicles G-PRL was more effective than NG-PRL in suppressing both basal and gonadotropin-stimulated steroid production. Thus, the different effects of NG- and G-PRL on basal and gonadotropin-stimulated steroidogenesis between prehierarchical and preovulatory follicles may contribute, at least in part, to the collapse of the follicular hierarchy in galliformes that express incubation behavior. This may result from the ovulation of the dominant follicles without recruiting replacements during the transition from egg laying to incubation phase. In support of this hypothesis there is a 2-3 fold increase in the proportion of G- to NG-PRL during this transition [13, 14].

In follicular walls of each class of prehierarchical 4-6, 2-4 and < 2 mm follicles, NG-PRL had a greater effect on suppressing FSH-induced E_2 secretion than G-oPRL, whereas G-PRL was more effective than NG-PRL in suppressing FSH-induced P_4 production. In contrast, with the exception of the synergistic actions of LH and G-PRL on P_4 production in the 4-6 mm follicles, both isoforms reduced LH-stimulated E_2 and P_4 production in all < 6 mm follicles. Taken together, in contrast to the observations in mammals where G-PRL has a reduced bioactivity than NG-PRL, both isoforms are biologically active in regulating basal and gonadotropin-stimulated steroidogenesis by chicken ovarian follicles. Nevertheless, G-PRL has differing effects than NG-PRL depending on the concentration, the type of gonadotropin (FSH or LH) and the stage of follicle development. In terms of practical implications, results from the first study may open a new window on improving female reproductive efficiency by shortening incubation time and therefore extending the egg laying period in incubating hens, which may be achieved by genetically and/or non-genetically controlling the relative and absolute amounts of circulating G-PRL. Even so, further studies in

broody birds such as turkeys and bantams should also be carried out regarding the genetic differences between non-broody and broody hens.

Since PRL exerts its effects through interaction with the receptor PRLR, in the second study we further investigated the expression pattern of PRLR and its regulatory mechanisms in the follicular hierarchy. Levels of *PRLR* transcript were highest in follicular walls of prehierarchical < 2 mm follicles but declined as the follicles matured. Varying levels of *PRLR* mRNA in the follicular hierarchy may be related to differences in the steroidogenic response of different size class follicles to gonadotropins or PRL isoforms alone, or combination. Indeed, it was observed that the single and combined effects of FSH and PRL isoforms on *PRLR* expression in granulosa cells were variable dependent on the stage of follicle development (*i.e.* the degree of granulosa cell differentiation). FSH had the greatest stimulatory effect on *PRLR* expression in granulosa cells of prehierarchical 6-8 mm follicles but this effect declined as the follicles matured to F1. Either PRL isoform induced basal *PRLR* expression in granulosa cells of the 6-8 mm, F3 and F1 follicles but had little effects in F2 follicles. Furthermore, FSH-stimulated *PRLR* expression was inhibited by the inclusion of either isoform of PRL especially in F2 granulosa cells. Thus, depending on the concentration and follicular size, PRL isoforms elicit differential effects on basal and gonadotropin-stimulated steroidogenesis through modulating *PRLR* expression during follicle development.

By using activators and/or inhibitors, several signaling pathways including PKA, PKC and AMPK were demonstrated to be directly involved in the stimulatory effect of FSH on *PRLR* expression in prehierarchical granulosa cells. In addition, ERK2 was the only isoform identified in hen ovarian follicles. Its widespread expression throughout follicle development suggested a universal role in the follicular hierarchy. However, there were higher levels of ERK2 phosphorylation in prehierarchical than preovulatory granulosa cells. Moreover, inhibition of ERK2 enhanced FSH-induced *PRLR* expression in prehierarchical granulosa cells, suggesting that ERK2 negatively participated in FSH-induced *PRLR* expression. Nevertheless, we found that FSH induced ERK2 phosphorylation possibly through modulating activity of PKA, PKC, mTOR and AMPK pathways. This may support the notion that transient activation and subsequent termination of ERK2 is required for granulosa cell differentiation [16]. Therefore, it is concluded that multiple signaling pathways including PKA, PKC, mTOR and AMPK are not only directly involved in, but

they can also converge to modulate ERK2 activity to regulate FSH-induced *PRLR* expression in undifferentiated granulosa cells.

PRL-L, a homolog of PRL, has been recently identified in non-mammalian vertebrates including chickens [17]. Because of its ability to bind to the PRLR that is widely distributed in the follicular hierarchy [18, 19], interactions may occur among circulating PRL isoforms, intra-ovarian PRL-L and PRLR to affect follicular cell steroidogenesis. In the third study, we investigated the expression and regulation of PRL-L in the follicular hierarchy. Maximal expression of *PRL-L* was observed in follicular walls of the < 2 mm follicles following a progressive decline with the maturation of follicles. Since the expression pattern of *PRL-L* mirrored that of the *PRLR* gene during follicle development and PRL-L was predicted to have a signal peptide [17] and has been shown to be secreted by chick muscle cells [20], it is possible that PRL-L may act in an autocrine and/or paracrine fashion in the follicular hierarchy by activating the PRLR. In prehierarchical granulosa cells, FSH increased but LH did not alter basal *PRL-L* transcript levels, while either NG- or G-PRL at a low level stimulated *PRL-L* expression but at higher levels this effect was minimal. Furthermore, FSH and NG-PRL appeared to have a synergistic effect on *PRL-L* mRNA levels, whereas the inclusion of G-oPRL did not alter FSH-stimulated *PRL-L* expression. These data imply that crosstalk may occur between the FSH and PRL signaling in regulating *PRL-L* expression in granulosa cells depending on the concentration and the degree of PRL glycosylation. In addition, we further demonstrated that FSH-induced *PRL-L* expression in prehierarchical granulosa cells was positively regulated by PKA, PKC and PI3K but was negatively regulated by ERK2.

In conclusion, widespread expression of PRLR and PRL-L during follicle development supports the importance of the PRL/PRL-L signaling in the follicular hierarchy. The observations that NG- and G-PRL exerted differential effects on basal and gonadotropin-stimulated steroidogenesis by different size class follicles demonstrate that glycosylation of PRL can modify its actions in follicular cell steroidogenesis, which are mediated through modulating expression of *PRLR* and *PRL-L*. In addition, regulation of *PRLR* and *PRL-L* transcription by FSH are mediated by multiple signaling pathways that have positive and negative impacts, including PKA, PKC, PI3K-Akt-mTOR, AMPK and ERK2.

5.2 FUTURE DIRECTION

- In the present study, we demonstrated that both PRL isoforms are biologically active across a broad concentration range in regulating steroidogenesis during follicle development in chickens, which contrasts with the observations in mammals where G-PRL generally has a lower bioactivity than NG-PRL. Considering the double antenna structure of avian PRLR, further studies are required to clarify the interactions between both isoforms and each repeated ligand binding unit of PRLR as well as respective downstream signaling cascades.
- Since glycosylation of PRL is able to modulate its effects on steroidogenesis in developing follicles which can thereby be involved in the establishment and maintenance of the follicular hierarchy, it is of importance to determine the factors inducing and the mechanisms controlling the glycosylation of PRL during the reproductive cycle so as to improve reproductive performance in incubating hens.
- Only transcripts of *PRL-L* and *PRLR* in the follicular hierarchy were examined in the present study. Specific antibodies against chicken PRL-L and PRLR are needed to be produced in order to further determine the protein expression using other techniques such as western blotting and immunohistochemistry. Highest levels of both *PRL-L* and *PRLR* mRNAs in the smallest (< 2 mm) follicles may suggest an important role for the PRL/PRL-L signaling in primordial follicle recruitment and estrogen biosynthesis. However, their exact roles as well as the underlying regulatory mechanisms are not clear and await further investigation.
- FSH signaling plays a pivotal role in the process of follicle selection that ensures the occurrence of almost daily ovulation in commercial laying hens. Our results showed that FSH induces the greatest increase in both *PRL-L* and *PRLR* mRNAs expression in granulosa cells of prehierarchical 6-8 mm follicles, indicating that both are downstream targets of FSH and may have a role in follicle selection. In addition, both PRL isoforms are found to be able to affect FSH-induced *PRLR* and *PRL-L* expression in these follicles. Therefore, future work on the role of the PRL/PRL-L signaling in the process of follicle selection is required. Crosstalk between the FSH and PRL/PRL-L signaling pathways during follicle development is also needed to be further elucidated.

- Since PRL-L is widely distributed in the follicular hierarchy and can be coordinately regulated by FSH and PRL isoforms, it is thought to be functional during follicular growth and development. Its exact actions on the functions of ovarian follicles (*e.g.* steroidogenesis, proliferation, differentiation and apoptosis) as well as interactions among circulating PRL isoforms, intra-ovarian PRL-L and repeated ligand binding units of PRLR remain unknown and need to be further studied. Additional studies on the actions of PRL isoforms on follicular cell proliferation, differentiation and apoptosis together with the physiological functions of PRL-L in other extra-pituitary tissues are also required.

5.3 REFERENCES

1. Johnson PA, Giles JR. The hen as a model of ovarian cancer. Nature Reviews Cancer 2013; 13:432-436.
2. El Halawani M, Silsby J, Youngren O, Phillips R. Exogenous prolactin delays photo-induced sexual maturity and suppresses ovariectomy-induced luteinizing hormone secretion in the turkey (Meleagris gallopavo). Biology of reproduction 1991; 44:420-424.
3. Reddy I, David C, Sarma P, Singh K. The possible role of prolactin in laying performance and steroid hormone secretion in domestic hen (Gallus domesticus). General and comparative endocrinology 2002; 127:249-255.
4. Sharp PJ, Blache D. A neuroendocrine model for prolactin as the key mediator of seasonal breeding in birds under long-and short-day photoperiods. Canadian journal of physiology and pharmacology 2003; 81:350-358.
5. Rozenboim I, Tabibzadeh C, Silsby J, El Halawani M. Effect of ovine prolactin administration on hypothalamic vasoactive intestinal peptide (VIP), gonadotropin releasing hormone I and II content, and anterior pituitary VIP receptors in laying turkey hens. Biology of reproduction 1993; 48:1246-1250.
6. Camper P, Burke W. The effects of prolactin on the gonadotropin induced rise in serum estradiol and progesterone of the laying turkey. General and comparative endocrinology 1977; 32:72-77.
7. Tabibzadeh C, Rozenboim I, Silsby JL, Pitts GR, Foster DN, El Halawani M. Modulation of ovarian cytochrome P450 17 alpha-hydroxylase and cytochrome aromatase messenger ribonucleic acid by prolactin in the domestic turkey. Biology of reproduction 1995; 52:600-

608.

8. Zadworny D, Shimada K, Ishida H, Sato K. Gonadotropin-stimulated estradiol production in small ovarian follicles of the hen is suppressed by physiological concentrations of prolactin in vitro. General and comparative endocrinology 1989; 74:468-473.
9. Zadworny D, Etches R. Effect of pregnant mare serum gonadotropin on plasma prolactin, luteinizing hormone, estradiol, and ovarian growth in incubating and out-of-lay turkeys. Poultry science 1988; 67:319-326.
10. Li W, Liu Y, Yu Y, Huang Y, Liang S, Shi Z. Prolactin plays a stimulatory role in ovarian follicular development and egg laying in chicken hens. Domestic animal endocrinology 2011; 41:57-66.
11. Hrabia A, Paczoska-Eliasiewicz H, Rząsa J. Effect of prolactin on estradiol and progesterone secretion by isolated chicken ovarian follicles. Folia biologica 2004; 52:197-203.
12. Freeman ME, Kanyicska B, Lerant A, Nagy G. Prolactin: structure, function, and regulation of secretion. Physiological reviews 2000; 80:1523-1631.
13. Bedecarrats G, Guemene D, Kühnlein U, Zadworny D. Changes in levels of immunoreactive prolactin isoforms during a reproductive cycle in turkey hens. General and comparative endocrinology 1999; 113:96-104.
14. Hiyama G, Kansaku N, Kinoshita M, Sasanami T, Nakamura A, Noda K, Tsukada A, Shimada K, Zadworny D. Changes in post-translational modifications of prolactin during development and reproductive cycles in the chicken. General and comparative endocrinology 2009; 161:238-245.
15. Sinha Y. Structural variants of prolactin: occurrence and physiological significance. Endocrine Reviews 1995; 16:354-369.
16. Woods DC, Johnson A. Phosphatase activation by epidermal growth factor family ligands regulates extracellular regulated kinase signaling in undifferentiated hen granulosa cells. Endocrinology 2006; 147:4931-4940.
17. Wang Y, Li J, Kwok AHY, Ge W, Leung FC. A novel prolactin-like protein (PRL-L) gene in chickens and zebrafish: cloning and characterization of its tissue expression. General and comparative endocrinology 2010; 166:200-210.
18. Bu G, Wang CY, Cai G, Leung FC, Xu M, Wang H, Huang G, Li J, Wang Y. Molecular

characterization of prolactin receptor (cPRLR) gene in chickens: gene structure, tissue expression, promoter analysis, and its interaction with chicken prolactin (cPRL) and prolactin-like protein (cPRL-L). Molecular and cellular endocrinology 2013; 370:149-162.

19. Zhou JF, Zadworny D, Guemene D, Kuhnlein U. Molecular cloning, tissue distribution, and expression of the prolactin receptor during various reproductive states in Meleagris gallopavo. Biology of reproduction 1996; 55:1081-1090.

20. Ijiri D, Higuchi A, Saegusa A, Miura M, Matsubara T, Kanai Y, Ohtsuka A, Hirabayashi M. Role of prolactin-like protein (PRL-L) in cold-induced increase of muscle mass in chicks. General and comparative endocrinology 2013; 186:94-100.