STUDIES OF THE PHYSIOLOGICAL ACTION
OF FOLLISTATIN
IN THE PORCINE OVARY

A Thesis Submitted to the College of
Graduate Studies and Research
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in the Department of Animal and Poultry Science
University of Saskatchewan
Saskatoon

By
Colleen Rae Christensen
Spring 1999

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UNIVERSITY OF SASKATCHEWAN
College of Graduate Studies and Research

SUMMARY OF DISSERTATION
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of the requirements for the

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by
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University of Saskatchewan
Spring 1999

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Studies of the Physiological Action of Follistatin in the Porcine Ovary

To investigate the physiological action of follistatin in the swine ovary a recombinant porcine follistatin (rpFS) with apparent molecular weights of 39, 46, 48 and 50 kDa was expressed and characterized. The rpFS crossreacted with antibodies against native porcine follistatin and with activin A. One rpFS isotype was purified by monoclonal antibody affinity chromatography (rp-305 a.a. FS). In porcine granulosa cell cultures, rp-305 a.a. FS suppressed estradiol-17β accumulation (ED$_{50}$ = 0.9 μg/ml) independent of activin. Gilts were vaccinated against rpFS to determine the effect of follistatin immunoneutralization on litter size or ovulation rate. In Experiment 1, forty-seven gilts were vaccinated four times with rpFS (FS n=23) or with a control vaccine (CTL n=24). The naturally matured, cycling gilts were bred and piglet numbers were recorded at farrowing. FS vaccination did not increase total litter size. However, grouping by low ($\leq$1:400) or high anti-follistatin antibody titer (>1:400) responses showed an increased litter size in gilts with a high titer (total piglets: FS high titer = 13.0 ± 0.8; FS low titer = 10.8 ± 0.6; CTL 11.4 ± 0.5; p = 0.08). In Experiment 2, sixty-nine gilts were vaccinated three times with rpFS (n=35) or CTL (n=34) vaccines. The gilts were induced and synchronized into estrus using PG600, PGF$_{2\alpha}$ and boar exposure; cycling gilts received a fourth vaccination. Reproductive tracts were collected two weeks after the second observed estrus (FS n = 14, CTL n = 15). FS vaccination did not increase the number of corpora lutea (FS =13.2 ± 0.5, CTL = 14.5 ± 0.7) or corpora albicantia (FS = 12.1 ± 1.9, CTL = 12.3 ± 2.0), but appeared to effect normal ovarian morphology increasing the number of blood filled follicles and corpora lutea. In conclusion, rp-305 a.a. FS appears to negatively affect estradiol accumulation in
vitro. Vaccination against follistatin in naturally cycling gilts enhanced reproductive potential. Follistatin vaccination of pharmacologically induced gilts did not increase ovulation rate, but biological effects were apparent suggesting that pharmacological induction of estrus may have confounded the follistatin vaccination treatment.

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ABSTRACT

To investigate the physiological action of follistatin in the swine ovary a recombinant porcine follistatin (rpFS) with apparent molecular weights of 39, 46, 48, and 50 kDa was expressed and characterized. The rpFS crossreacted with antibodies against native porcine follistatin and with activin A. One rpFS isotype was purified by monoclonal antibody affinity chromatography (rp-305 a.a. FS). In porcine granulosa cell cultures, rp-305 a.a. FS suppressed estradiol-17β accumulation (ED₅₀ = 0.9 μg/ml) independent of activin. Gilts were vaccinated against rpFS to determine the effect of follistatin immunoneutralization on litter size or ovulation rate. In Experiment 1, forty-seven gilts were vaccinated four times with rpFS (FS n=23) or with a control vaccine (CTL n=24). The naturally matured, cycling gilts were bred and piglet numbers were recorded at farrowing. FS vaccination did not increase total litter size. However, grouping by low (≤1:400 n=16) or high anti-follistatin antibody titer (>1:400 n=7) responses showed an increased litter size in gilts with a high titer (total piglets: FS high titer = 13.0 ± 0.8; FS low titer = 10.8 ± 0.6; CTL 11.4 ± 0.5; p = 0.08). In Experiment 2, sixty-nine gilts were vaccinated three times with rpFS (n=35) or CTL (n=34) vaccines. The gilts were induced and synchronized into estrus using PG600, PGF₂α and boar exposure; cycling gilts received a fourth vaccination. Reproductive tracts were collected two weeks after the second observed estrus (FS n = 14, CTL n = 15). FS vaccination did not increase the number of corpora lutea (FS =13.2 ± 0.5, CTL = 14.5 ± 0.7) or corpora albicantia (FS = 12.1 ± 1.9, CTL = 12.3 ± 2.0), but appeared to
effect normal ovarian morphology increasing the number of blood-filled follicles and corpora lutea. In conclusion, rp-305 a.a. FS appears to negatively affect estradiol accumulation in vitro. Vaccination against follistatin in naturally cycling gilts enhanced litter size. Follistatin vaccination of pharmacologically induced gilts did not increase ovulation rate, but biological effects were apparent suggesting that pharmacological induction of estrus may have confounded the follistatin vaccination treatment.
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Finally, I would like to thank my brothers and all of my friends, especially Mike, who helped keep me sane and aware of the fact that there is life after graduate studies.
DEDICATION

This thesis is dedicated to my parents, Raymond and Sandra Christensen. Thank you for teaching me “do the best that you can” and “to finish whatever you start”.
# TABLE OF CONTENTS

 PERMISSION TO USE ............................................................................. i

 ABSTRACT ............................................................................................... ii

 ACKNOWLEDGEMENTS ......................................................................... iv

 DEDICATION ........................................................................................... v

 TABLE OF CONTENTS .......................................................................... vi

 LIST OF TABLES .................................................................................... ix

 LIST OF FIGURES .................................................................................. x

 LIST OF ABBREVIATIONS ..................................................................... xii

 1. INTRODUCTION ................................................................................ 1
     1.1 Objectives .................................................................................... 3

 2. LITERATURE REVIEW ..................................................................... 4
     2.1 Brief Overview of the Regulation of the Female Reproductive Cycle .... 4
         2.1.1 Production and Action of Gonadotropin Releasing Hormone ........ 4
         2.1.2 Production and Action of Gonadotropins .................................... 5
         2.1.3 Production and Action of Steroid Hormones ............................... 5
         2.1.4 Action of Intraovarian Regulatory Factors ................................... 7
     2.2 The Physical Structure, Location and Regulation of Inhibin, Activin,
         and follistatin .................................................................................. 7
         2.2.1 Overview of the Physical Structure of Inhibin, Activin, and Follistatin .... 7
         2.2.2 Inhibin ................................................................................... 8
             2.2.2.1 Inhibin Gene Regulation ..................................................... 8
             2.2.2.2 Inhibin Protein Structure and Protein Production ................. 8
                 2.2.2.2.1 Technical Problems Associated with Quantifying Inhibin .... 10
             2.2.2.3 Ovarian Expression of Inhibin .......................................... 11
             2.2.2.4 Extragonadal Expression of Inhibin .................................... 11
             2.2.2.5 Inhibin Receptor .............................................................. 11
             2.2.2.6 Beta subunit binding protein - Alpha2-macroglobulin .......... 12
         2.2.3 Activin .................................................................................. 13
             2.2.3.1 Activin Gene Regulation .................................................. 13
             2.2.3.2 Activin Protein Structure ................................................. 13
             2.2.3.3 Extragonadal Activin ....................................................... 14
             2.2.3.4 Activin Receptor ............................................................ 15
         2.2.4 Follistatin .............................................................................. 15
             2.2.4.1 Follistatin Gene Structure ................................................ 16
4. ACTIVE VACCINATION AGAINST FOLLISTATIN AFFECTS REPRODUCTIVE POTENTIAL IN CYCLING GILTS ................................................. 101
   4.1 Abstract .................................................................................. 101
   4.2 Introduction ............................................................................ 102
   4.3 Materials and Methods ............................................................ 104
   4.4 Results ................................................................................. 111
   4.5 Discussion ............................................................................. 129
   4.6 Implications ........................................................................... 137

5. GENERAL DISCUSSION .................................................................. 138

6. BIBLIOGRAPHY .......................................................................... 148

A. APPENDIX .................................................................................. A.1
B. APPENDIX .................................................................................. B.1
C. APPENDIX .................................................................................. C.1
LIST OF TABLES

Table 2.1 *In situ* hybridization of ovine ovaries indicating amount of expression of follistatin, α and β subunits just prior to ovulation (day -1) (Tisdall et al. 1994) ................................................................. 38

Table 4.1 The effect of active vaccination against follistatin on maturation and litter size (mean ± SEM) of gilts allowed to mature naturally. ....................... 121

Table 4.2 A chi-square analysis of the proportion of follistatin vaccinated animals which exhibited estrus compared to the proportion of control vaccinated animals which exhibited estrus at two estrus periods ........................................ 123

Table 4.3 The effect of active vaccination against follistatin in gilts on the number of ovarian structures. ................................................................. 124

Table A.1 Anti-follistatin antibody titer of blood samples taken 2 weeks after 3rd booster. ................................................................................................. A.2

Table B.1 Raw data collected for follistatin and control vaccinated gilts in Experiment 1. ................................................................................................. B.1

Table B.2 Raw data collected for follistatin and control vaccinated gilts in Experiment 2. ................................................................................................. B.3
LIST OF FIGURES

Figure 2.1 Physical structure and organization of inhibin and activin (Mather et al. 1992) ................................................................. 9

Figure 2.2 Genomic structure of the follistatin gene (Shimasaki et al. 1988) ................. 17

Figure 2.3 Physical structure of follistatins isolated from porcine follicular fluid (Sugino et al. 1993) ................................................................. 19

Figure 2.4 A proposed model for the physical interactions of activin, follistatin and their receptors (Halvorsen and DeCherney 1996) ......................... 36

Figure 2.5 Activin is critical for follicle differentiation in cattle and rats, as it is able to induce FSH receptors (Findlay 1993) ........................................ 39

Figure 2.6 The "activin tone" theory. Activin may be more important for stimulating folliculogenesis in immature follicles (Hillier and Miro 1993) .......... 40

Figure 3.1 Construction of the pFSHUneo plasmid. ........................................... 58

Figure 3.2 Yield of recombinant porcine follistatin upon repeated heat shock was quantitated to determine the yield over time ......................................... 69

Figure 3.3 A 10 % SDS-PAGE gel shows continuous production of recombinant follistatin ................................................................. 70

Figure 3.4 Western blot indicating the presence of heat shock proteins in FCC815 conditioned media ............................................................ 72

Figure 3.5 Purification of recombinant porcine follistatin using anionic exchange chromatography ................................................................. 75

Figure 3.6 A 10% SDS-PAGE gel of non purified FCC815 conditioned media and follistatin eluant obtained from a monoclonal affinity column ............. 76

Figure 3.7.1 A Western blot of FCC815 conditioned media electrophoresed in sample buffer containing β-mercaptoethanol ........................................ 78

Figure 3.7.2 A Western blot of FCC815 conditioned media electrophoresed in sample buffer containing dithiothreitol ........................................ 79

Figure 3.8 A radiolabelled activin-follistatin ligand blot assay was developed to characterize the binding of follistatin with activin ................................ 81

Figure 3.9 An activin-follistatin ligand blot assay was developed to characterize the binding of follistatin with activin ........................................ 82

x
Figure 3.10.1 Granulosa cells cultured from porcine antral follicles of 1-3 mm diameter were utilized to determine the biological activity of rp-305 a.a. FS .........................................................85

Figure 3.10.2 Granulosa cells cultured from porcine antral follicles of 1-3 mm diameter were utilized to determine the biological activity of rp-305 a.a. FS .........................................................86

Figure 3.11 Anti-follistatin monoclonal antibody 4-6D9 appears to enhance estradiol-17β accumulation without the addition of exogenous follistatin. ............88

Figure 3.12 Biological activity of human recombinant activin A in porcine granulosa cell culture bioassay system..................................................90

Figure 4.1 ELISA plates coated with FCC815 conditioned media were used to determine anti-follistatin antibody titer .................................................112

Figure 4.2 A scattergraph of the antibody titers induced in follistatin vaccinated gilts in Experiment 1 .................................................................114

Figure 4.3 Scattergraphs of the antibody titers induced in follistatin vaccinated gilts in Experiment 2 .................................................................115

Figure 4.4 A Western blot indicating that an antibody raised to recombinant porcine follistatin bound rp-305 a.a. FS of approximately 49 kDa molecular weight. Competitive binding of the anti-follistatin antibody to rh-288 a.a. FS decreased the binding of these anti-follistatin antibodies to recombinant porcine follistatin immobilized in the nitrocellulose ..........116

Figure 4.5 Biological activity of polyclonal anti-follistatin antibody on estradiol-17β accumulation in the porcine granulosa cell culture assay. ...118

Figure 4.6 Effect of polyclonal anti-follistatin antibody from high titer gilts plus rp-305 a.a. FS or rh-288 a.a. FS on estradiol-17β accumulation .............. 119

Figure 4.7.1 A photograph of formalin fixed ovaries of two follistatin vaccinated gilts......................................................................................... 126

Figure 4.7.2 H and E stain of the corpora hemorrhagica-like structure on the top of the right hand ovary of 4.7.1.........................................................127

Figure 4.8 A photograph of a frozen ovary from a follistatin vaccinated animal which contained a hemorrhaged follicle.........................................128

Figure A.1 Antigenic index of the 305 a.a. recombinant porcine follistatin. ..........A.3
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>a.a.</td>
<td>amino acid</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cells</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Bo</td>
<td>initial binding (no antigen only antibody)</td>
</tr>
<tr>
<td>cDNA</td>
<td>copy DNA</td>
</tr>
<tr>
<td>cv</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>CA</td>
<td>corpora albicantia</td>
</tr>
<tr>
<td>CH</td>
<td>corpora hemorrhagica</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary</td>
</tr>
<tr>
<td>CL</td>
<td>corpora lutea</td>
</tr>
<tr>
<td>CTL</td>
<td>control vaccinated</td>
</tr>
<tr>
<td>DES</td>
<td>diethylstilbestrol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's minimum essential media</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ED50</td>
<td>50% effective dose</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FS</td>
<td>follistatin vaccinated</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin releasing hormone</td>
</tr>
<tr>
<td>hCG</td>
<td>human chorionic gonadotropin</td>
</tr>
<tr>
<td>H and E stain</td>
<td>hemotoxylin and eosin stain</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N’’-2-ethane sulfonic acid</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>HSPG</td>
<td>heparan sulfate proteoglycans</td>
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<tr>
<td>ID</td>
<td>identification</td>
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<tr>
<td>IGF</td>
<td>insulin like growth factor</td>
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<td>immunoglobulin</td>
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<td>IL</td>
<td>interleukin</td>
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<td>interferon gamma</td>
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<td>immunoradiometric assay</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
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<tr>
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<td>disassociation constant</td>
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<td>luteinizing hormone</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>Mab</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MDBK</td>
<td>Madin-Darby bovine kidney cell line</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential media</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>-------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>NBCS</td>
<td>new born calf serum</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
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<td>probability (for tests of significance)</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PBST</td>
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<td>PG600</td>
<td>400 IU PMSG + 400 IU hCG</td>
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<td>PGF(_{2\alpha})</td>
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<td>protein kinase A</td>
</tr>
<tr>
<td>PK-C</td>
<td>protein kinase C</td>
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<tr>
<td>PMSF</td>
<td>phenylmethylsulfonylfluoride</td>
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<tr>
<td>PMSG</td>
<td>pregnant mare serum gonadotropin</td>
</tr>
<tr>
<td>rp-305 a.a. FS</td>
<td>recombinant porcine follistatin produced in section 3.</td>
</tr>
<tr>
<td>rh-288 a.a. FS</td>
<td>recombinant human follistatin available from NIH</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>stdev</td>
<td>standard deviation</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate - polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Sf9</td>
<td><em>Spodoptera frugiperda</em> ovarian cell line</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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1. INTRODUCTION

In female animals, numerous hormones interact to drive and facilitate an estrous cycle. Follicle growth is primarily driven by follicle stimulating hormone (FSH), yet a number of other hormones are also important for folliculogenesis, follicle recruitment, and follicle selection. Several intraovarian factors modify the action of FSH in the mammalian ovary including follistatin, activin, and inhibin. Follistatin interacts with the transforming growth factor β (TGFβ) family, binding the common beta subunit of activin and inhibin. Because of the integral interaction of follistatin with these hormones, any discussion of the physiological action of follistatin must also include discussion of the function of activin and inhibin.

The majority of physiological investigations of follistatin, activin, and inhibin have relied on in vitro experiments utilizing tissue from rats, cattle, sheep, and humans. These experiments have indicated that follistatin, activin, and inhibin may be involved in regulating ovarian follicle growth and atresia, and follicle differentiation. Follistatin neutralizes activin activity, yet it has not been shown to modify inhibin activity even though it is capable of binding inhibin. Follistatin also has steroidogenic activity in granulosa cell cultures independent of its interaction with activin. There is little information concerning the activity of follistatin in swine in vitro or in vivo.
Follistatin, activin, and inhibin are expressed in multiple forms. Follistatin is produced as several protein isotypes which differ in the degree of glycosylation and C-terminal truncation. These post-translational modifications may be species and tissue specific. In reproductive tissues, the beta subunit of activin and inhibin exists as two isotypes. Therefore, there are two mature inhibin protein isotypes and three mature activin isotypes. This variety of hormone expression has complicated the measurement of these hormones in biological fluids and has increased the necessity for experimental reagents of a high specificity and purity. Recombinant DNA technology and monoclonal antibody production are beginning to supply the reagents necessary to investigate follistatin, activin, and inhibin physiology. However, at the present time commercial supplies of the reagents are scarce.

Investigation of the physiological action of a hormone often involves the use of agonists and antagonists. Immunoneutralization is a technique where antibodies to an endogenous target hormone are induced or are supplied to an animal in order to neutralize the biological activity of the target hormone. Therefore, immunoneutralization of an endogenous hormone is a method of producing a hormone antagonist. Vaccination of animals against endogenous hormones also has been used as a practical technique to modify reproductive potential. Vaccination against androstenedione or inhibin has been investigated for usefulness as fecundity vaccines; vaccination against gonadotropin releasing hormone (GnRH) has been investigated as a contraceptive. Follistatin is produced in several reproductively relevant tissues.
Immunoneutralization of follistatin may be used to investigate the function of follistatin in reproduction and may be used to modify reproductive potential.

1.1 Objectives

The research described in this thesis was designed to investigate the following objectives:

1. To express and characterize a recombinant porcine follistatin.

2. To vaccinate cycling gilts against follistatin to determine the effect on litter size.

3. To vaccinate cycling gilts against follistatin to determine the effect on ovulation rate.
2. LITERATURE REVIEW

2.1 Brief Overview of the Regulation of the Female Reproductive Cycle

The hypothalamus coordinates all activities of the female reproductive cycle through signals which differ in chemical substance, quantity, and frequency of release. Through the oscillating pattern of negative and positive feedback of steroid, ovarian, and intrapituitary factors to the hypothalamus, a cyclic pattern is produced which allows a female mammal to physically prepare for coitus and pregnancy and to repeat the sequence if these events do not occur.

2.1.1 Production and Action of Gonadotropin Releasing Hormone

To begin an estrous cycle the GnRH pulse generator stimulates the pulsatile release of GnRH from the medial basal hypothalamus, preoptic area and suprachiasmatic nucleus (Kumar et al. 1991) into the hypophyseal portal system. The frequency and amplitude of GnRH pulses changes throughout the estrous cycle due to effects of progesterone and estrogen feedback. In swine, high GnRH pulse frequency is more effective in stimulating an acute release of luteinizing hormone (LH) than of follicle stimulating hormone (FSH). High GnRH pulse frequency inhibits FSH synthesis and release. Low GnRH pulse frequency supports FSH synthesis and release, but does not effectively increase LH concentrations (Lukin-Jayes et al. 1997). The response of FSH to experimental alterations of GnRH levels is much less pronounced and occurs more slowly than that of LH. This has led investigators to believe that although GnRH
pulsatile release is critical for both LH and FSH pulsatile release, other factors besides GnRH must regulate FSH synthesis and release (Esbenshade et al. 1986, Clark 1989, Lukin-Jayes et al. 1997).

2.1.2 Production and Action of Gonadotropins

Gonadotropins are dimeric glycoproteins consisting of a common α subunit and an unique β subunit. Upon GnRH stimulation they are produced in the anterior pituitary, released into the blood stream, and transported to the ovaries. In the ovary, LH and FSH are primarily responsible for stimulating follicle growth in follicles with greater than four layers of granulosa cells, stimulating antrum formation, steroidogenesis, and ovulation (Esbenshade et al. 1990). LH and FSH are produced at low levels throughout the luteal phase of the estrous cycle. Positive feedback of estrogen during the follicular phase allows a surge of LH and FSH to be released into the circulation stimulating ovulation. In swine, circulating levels of FSH decrease rather than increase just prior to the initial stages of preovulatory follicle maturation (Guthrie et al. 1992). A secondary surge of FSH occurs in swine approximately 27 hours after the peak LH secretion coincident with an increase in progesterone (van de Wiel et al. 1981).

2.1.3 Production and Action of Steroid Hormones

Estrogen and progesterone production in the ovaries is controlled by the gonadotropins and modified by intraovarian regulatory factors. Steroidal feedback to the hypothalamus and the pituitary modify the amount of gonadotropin production induced by GnRH.
The steroid hormones also have autocrine and paracrine actions within the ovary. Testosterone and dihydrotestosterone inhibit FSH-induced granulosa cell LH receptor induction and granulosa aromatase activity in porcine granulosa cell cultures (Hunter et al. 1992). Estrogen induces LH and FSH receptors and stimulates the LH surge required for ovulation. Estrogen also modulates its own production, and the estrogen concentration in follicular fluid has been correlated to oocyte maturation (Foxcroft and Hunter 1985; Hunter and Wiesak 1990). However, the effect of estrogen in vitro also appears to be variable and has been shown to depend on dose, cell maturity and length of exposure to estrogen (Hutz 1989). In swine, Hunter et al. (1992) showed that low doses of estrogen have stimulatory effects on steroidogenesis only in cells isolated from medium and large follicles, whereas high doses have inhibitory effects on granulosa cells recovered from all follicle sizes. The stimulatory effect of estrogen on porcine granulosa function in small follicles has lead to the theory that estrogen, produced by the largest follicles in the pool of follicles selected for ovulation, may stimulate development of smaller follicles of that selected pool (Hunter et al. 1992).

Production of estrogen by ovarian follicles in an environment containing low levels of FSH is critical for the selection of follicles for ovulation. The concentration of estrogen measured is indicative of a follicle’s health. Porcine atretic follicles have less estrogen, androstenedione and progesterone in follicular fluid, and less P-450C17, 3βHSD and aromatase mRNA and Ki-67 (a marker of cell proliferation) than non-atretic follicles (Garrett et al. 1997). Estrogenic follicles are defined as those that have > 100 ng/ml estrogen in follicular fluid (Foxcroft and Hunter 1985, Guthrie et al. 1993). Estrogen content in follicular fluid in the Meishan, a highly prolific breed, is greater than that in the Large White (Biggs et al. 1990 in Hunter et al. 1992).
2.1.4 Action of Intraovarian Regulatory Factors

As the hormone profiles throughout the estrous cycle were elucidated it became clear that there must be regulation of FSH independent of that for LH (Tonetta and di Zerega 1989). An intraovarian factor which would inhibit the release of FSH was proposed in 1932 and named “inhibin”.

Intraovarian factors modify the actions of the gonadotropins and are involved in each step of folliculogenesis. An increasingly large number of factors have been isolated from the ovary. Intraovarian factors are locally produced, their production is regulated locally by other hormones or factors, and finally, they display either autocrine or paracrine action (Findlay et al. 1993). Inhibin, activin, and follistatin are all produced in the ovary and have been identified as intraovarian regulators of the ovarian cycle.

2.2 The Physical Structure, Location, and Regulation of Inhibin, Activin, and Follistatin

2.2.1 Overview of the Physical Structure of Inhibin, Activin, and Follistatin

A unique, intricate relationship exists between three intraovarian factors, follistatin, activin, and inhibin. Activin and inhibin, members of the transforming growth factor beta (TGFβ) superfamily, are dimeric proteins which share a common β subunit. Follistatin is a monomeric protein which can bind either activin or inhibin through their common β subunit.
2.2.2 Inhibin

2.2.2.1 Inhibin Gene Regulation

The production of inhibin in granulosa cells and luteal cells (primate only) is stimulated principally by FSH (Vale et al. 1994). FSH-induced inhibin synthesis is enhanced in the presence of estrogen, testosterone, or androstenedione (Findlay et al. 1993). In the presence of FSH, low concentrations of LH stimulate inhibin synthesis and high concentrations of LH or human chorionic gonadotropin (hCG) inhibit inhibin synthesis. Several other factors appear to regulate inhibin synthesis and differences between species are apparent. Activin, insulin-like growth factor-I (IGF-I), and TGFβ stimulate basal and FSH induced inhibin production in cultured rat granulosa cells. However, TGFβ inhibits basal and FSH induced inhibin production in cultured porcine granulosa cells (Findlay et al. 1993). Recently, in rat granulosa cell cultures it has been shown that the stimulatory effect of activin on inhibin α subunit production requires IGF-I (Kubo et al. 1998). Epidermal growth factor (EGF), transforming growth factor α (TGFα), GnRH, and follistatin inhibit the synthesis of inhibin in rat granulosa cell cultures (Ling et al. 1990; Findlay 1993). In the presence of FSH, interferon gamma (INFγ) inhibits basal inhibin production; in the absence of FSH, INFγ stimulates basal inhibin production in rat granulosa cell cultures. In rat pituitary cell cultures, inhibin has been shown to down-regulate the expression of α subunit mRNA (Bilezikjian et al. 1996). In the same system follistatin did not affect inhibin α subunit mRNA.

2.2.2.2 Inhibin Protein Structure and Protein Production

Mature inhibin is a 32 kDa heterodimer consisting of a 18 kDa α subunit and a 14 kDa β subunit. There are two known β subunit subtypes, βA and βB, therefore there
is an inhibin A and an inhibin B. In biological fluids, several molecular weight forms of inhibin are found which are composites of the α subunit precursor or the α subunit precursor and a β subunit (see Figure 2.1). Identification of inhibin isotypes in the circulation is complicated by the number of composite structures, the degree of glycosylation (Burger et al. 1995), and differences between species (Martens et al. 1997). Production of the β subunit may be the rate limiting step in the synthesis of inhibin. Bicsak et al. (1988) determined that although an α prosubunit form (38 kDa) could be located in rat granulosa cell lysate no βA subunit could be located. These investigators also illustrated that αβ dimers (inhibin) are preferentially arranged and secreted before ββ dimers (activin).

**ALTERNATE PROCESSING OF ALPHA AND BETA SUBUNITS**

![Diagram showing alternate processing of alpha and beta subunits]

- **Inhibins**
  - Inhibin A (58 kDa)
  - Inhibin A (32 kDa)
  - Inhibin B (32 kDa)

- **Activins**
  - Activin A (28 kDa)
  - Activin B (28 kDa)
  - Activin AB (28 kDa)

*Figure 2.1* Physical structure and organization of precursor and mature inhibin and activin isoforms (Mather et al. 1992). * indicates potential glycosylation sites
2.2.2.2.1 Technical Problems Associated with Quantifying Inhibin

While characterizing inhibin isoforms in biological fluids, it became clear that the levels of inhibin being measured included both biologically and non-biologically relevant forms. For example, the concentration of inhibin present during the swine estrous cycle was determined by radioimmunoassay (RIA), probing with a primary antibody specific for the N-terminal region of the α subunit. Hasegawa et al. (1988) found concentrations of 0.6-2.5 ng/ml α subunit in serum with the maximum concentration occurring four days before ovulation. Concentrations of inhibin were inversely related to FSH concentrations, but were not correlated with estrogen concentrations. Recently, inhibin concentrations were remeasured in swine serum using a two site immunoradiometric assay (IRMA) with a sensitivity of 21 pg/ml (Meyer and Wheaton 1996). The antibodies used were specific for inhibin A and could not measure inhibin B. The researchers could not detect dimeric inhibin A with this assay suggesting that the previous study had measured only the α subunit monomer form.

Two-site ELISAs and IRMAs (Knight and Muttukrishna 1994; Robertson et al. 1997) use monoclonal antibodies directed against two different epitopes, one on the α and the other on the β subunit, of the inhibin dimer. These assays have < 0.1% cross reaction with the free α subunit (Ying et al. 1997). This assay technique demonstrated that in human males inhibin B is the predominant circulating form. In females, inhibin A and B are both present, but peak expression of each occurs during different phases of the menstrual cycle (Robertson et al. 1997).

Comparisons between published literature from different laboratories using different inhibin quantification techniques is difficult. Attempts to standardize inhibin measurements were first made through allocation of a partially purified porcine follicular
fluid inhibin to the World Health Organization (WHO) for distribution and use as a positive control or antigen for labeling (Gaines Das et al. 1992). Subsequently, a standardized human recombinant inhibin was made available through the WHO (Rose and Gaines Das 1996).

2.2.2.3 Ovarian Expression of Inhibin

Guthrie et al. (1992) determined that porcine inhibin α subunit mRNA and protein production increased throughout preovulatory maturation and therefore greater amounts of inhibin were produced in large than in medium sized follicles. Size of the follicle may not indicate the absolute amount of inhibin production. During the porcine follicular phase, α subunit expression increased then decreased during late follicular phase in similarly sized follicles (Guthrie et al. 1992; Li et al. 1997a).

2.2.2.4 Extragonadal Expression of Inhibin

Inhibin α subunit RNA has also been identified in the placenta, pituitary, adrenal gland, spleen, kidney, brain, and spinal cord using the S1 nuclease assay (Meunier et al. 1988). The inhibin α subunit could not be isolated in swine pituitaries of either sex in two different breeds using RT-PCR technique (Li et al. 1997b).

2.2.2.5 Inhibin Receptor

Inhibin A binding sites have been identified in the granulosa, but not in the theca cell layer of ovarian follicles (Woodruff et al. 1990). It is generally believed that the "inhibin receptor" is the activin type II receptor. The β subunit of inhibin binds and
activates activin receptor II and IIb. Neither subunit of inhibin binds the activin type I receptor. The potency of inhibin activity in COS cells through the type II receptors is 6-30% of that of activin, depending on the type II receptor isotype (Martens et al. 1997). In some tissues inhibin antagonizes the actions of activin. In K562 cells, it has been shown that inhibin competes with activin for the activin type II receptor (Lebrun and Vale 1997). There is indication from two different experimental systems that an inhibin specific receptor also exists, however, it has not yet been identified.

2.2.2.6 Beta subunit binding protein - Alpha2-macroglobulin

Alpha2-macroglobulin weakly binds the β subunit of inhibin and activin. The binding capacity of the β subunit for α2-macroglobulin appears to be low affinity (K_D = 5 x 10^-7 M), but high capacity (DePaolo 1997). In the serum, the concentration of α2-macroglobulin is far greater than that of follistatin. Therefore, α2-macroglobulin is believed to be the major binding protein of activin and inhibin in the serum (Mather et al. 1997). Serum concentrations of α2-macroglobulin in humans and swine are 2-4 mg/ml (Meyer and Wheaton 1996). In mouse in vitro culture systems α2-macroglobulin does not appear to affect the bioactivity of activin or inhibin (Niemuller et al. 1995), but it may affect the half life of circulating activin and inhibin (DePaolo 1997). Binding by α2-macroglobulin may be a method of removing hormones from the circulation at different rates. Two forms of α2-macroglobulin exist which differ in the rate they are cleared from the circulation. Inhibin and follistatin preferentially bind to the α2-macroglobulin which is quickly cleared; activin binds the α2-macroglobulin which is slowly cleared from the circulation (Phillips et al. 1997). This is the first report to show that follistatin can be bound by α2-macroglobulin. It is unknown whether the activin-follistatin complex is bound by α2-macroglobulin. There is some indication that the
expression of $\alpha_2$-macroglobulin and follistatin binding proteins may be cell specific, allowing for another method for regulating activin activity. In rat decidual tissue $\alpha_2$-macroglobulin and follistatin are expressed in different cells during different periods of decidualization (Gu et al. 1995). The production of $\alpha_2$-macroglobulin may be regulated by the reproductive system. In rats, $\alpha_2$-macroglobulin is produced in antral ovarian follicles and corpus luteum, and its synthesis is enhanced by LH (Gaddy-Kurten et al. 1989).

2.2.3 Activin

2.2.3.1 Activin Gene Regulation

Very little is known about the regulation of activin synthesis or the temporal aspects of its synthesis within the ovary. In granulosa cell culture, activin production is stimulated by activation of the PK-C pathway \textit{in vitro} (Miyanaga et al. 1993). In rat anterior pituitary cultures, activin A stimulates the production of the $\beta_B$ subunit (Bilezikjian et al. 1996).

2.2.3.2 Activin Protein Structure

Activin is a 24 kDa homo or heterodimer with two $\beta$ subunits, $\beta_A$ and $\beta_B$ (Figure 2.1). In porcine granulosa cell layers more activin $\beta_A$ than $\beta_B$ mRNA is expressed. The expression of both $\beta_A$ and $\beta_B$ has been shown to decrease throughout the follicular phase (Guthrie et al. 1992; Li et al. 1997a). Hasegawa et al. (1994) isolated activin A, AB, and B with concentrations of 0.3, 0.24, 0.24 $\mu$g/ml from porcine follicular fluid. Three other beta subunit mRNA isoforms, $\beta_C$, $\beta_D$, $\beta_E$ have also been isolated, but their function is
not yet known (Hotton et al. 1995; Oda et al. 1995; Fang et al. 1996). The expression of βC and βE may be liver specific (Fang et al. 1997). The βD subunit has been detected only in the Xenopus.

The accurate and specific measurement of activin dimers is as problematic as measuring inhibin. For example, two-site ELISAs have measured the concentration of human activin A as approximately 128 pg/ml during the normal menstrual cycle, 500 pg/ml postmenopausal, and >4000 pg/ml during pregnancy (Knight et al. 1996). The concentration in estrogen active dominant follicles from cattle was approximately 2.5 ng/μl, in estrogen inactive follicles, 3.5 ng/μl, and below assay sensitivity in the serum of cycling heifers (Knight et al. 1996). An RIA with antibody specific for the βA subunit and with dissociation agents to remove bound follistatin, gave higher values than the two site ELISA; human female serum contained 4.17 ng/ml, and bovine follicular fluid 85.6 ng/ml (McFarlane et al. 1996).

2.2.3.3 Extragonadal Activin

Extragonadally, βA mRNA has been localized in spinal cord, adrenal cortex, cerebrum, bone marrow, liver, spleen, placenta, pancreatic A, B and D cells, skin, and vascular smooth muscle cells. Beta B mRNA has been localized in the pituitary, cerebrum, placenta, salivary glands, skin, and pancreatic B cells (Meunier et al. 1988; DePaolo et al. 1991; DePaolo 1997; Ying et al. 1997). In swine, both βA and βB mRNA have been identified in the pituitary (Li et al. 1997b).
2.2.3.4 Activin Receptor

Binding of activin to the plasma membrane and activation of internal signal transduction requires the interaction of two plasma membrane bound receptors: activin receptor types I and II. The type II activin receptor binds activin with a high affinity. The type I receptor requires coexpression of the type II receptor to interact with activin (Lebrun and Vale 1997). It is not clear whether activin directly binds the type II receptor if the activin-type II receptor complex holds activin and the type I receptor in close proximity (Gaddy-Kurten et al. 1995). Formation of the activin - type I - type II receptor complex allows auto and transphosphorylation of the type I receptor necessary for initiating the internal signal cascade (Willis et al. 1996). It is not known whether different isoforms of activin have different affinities to either activin receptor type (Mather et al. 1997). The binding affinity of activin A with receptor type IIA is \( K_D = 3.1 \times 10^{-10} \text{M} \), type IIB4 \( K_D 3.8 \times 10^{-10} \text{M} \), type IIB2 \( K_D 1 \times 10^{-10} \text{M} \) (de Winter et al. 1996). It has been shown that some activin receptors and TGFβ receptors are nonspecific and will bind other ligands besides activin or TGFβ (Derynck and Feng 1997). Little is known about the cellular specificity of activin receptor expression. Cameron et al. (1994) indicated that the predominant type II receptor expression was type IIA in rat granulosa cells, oocytes, and corpus luteum, although there was weak type IIB expression in corpus luteum, and granulosa during the diestrous period.

2.2.4 Follistatin

Follistatin is a monomeric glycoprotein which was first isolated from porcine follicular fluid (Esch et al. 1987). Follistatin is less potent than inhibin in suppressing the release of FSH, however, it is longer acting than inhibin in ovariectomized rats in
Follistatin has been identified as a specific high affinity, activin binding protein (Nakamura et al. 1990).

2.2.4.1 Follistatin Gene Structure

The follistatin gene is approximately six kilobases long and consists of six exons and five introns (Figure 2.2). Two mRNA variants are transcribed from the genomic DNA by alternative splicing (Shimasaki et al. 1988). One message encodes a precursor protein of 344 a.a.. The second message codes for an identical, but carboxyl terminal truncated protein of 317 a.a.. The first exon of the follistatin gene codes for the signal sequence; the following four exons are common to both mature follistatin isoforms. The sixth exon encodes for the extra 27 a.a. domain which is only found in the 344 a.a. precursor. Domains 2-4 show high homology to each other and are also similar to EGF and pancreatic trypsin inhibitor (Shimasaki et al. 1988). The primary structure of the follistatin gene is highly conserved (>98%) among the pig, human, and rat (Findlay et al. 1993). The follistatin promoter has three distinct cap sites each approximately 30 base pairs downstream of a distinct TATA-like sequence (Miyanaga and Shimasaki 1992). Both AP-1 and AP-2 binding sites, Sp-1 binding sites and a cAMP responsive element, as well as two 5’ flanking regions which inhibit transcription are present in the follistatin gene promoter (Miyanaga and Shimasaki 1992).
Figure 2.2 Genomic structure of the follistatin gene (Shimasaki et al. 1988).

2.2.4.2 Follistatin Gene Regulation

Follistatin mRNA production is regulated by FSH, but not by LH. FSH has a dose and time dependent stimulatory effect on steady state levels of follistatin mRNA. Activin has a biphasic action on basal and FSH induced follistatin production, being inhibitory at low doses and stimulatory at high doses (Findlay 1993). Bilezikjian et al. (1996) showed that follistatin can down-regulate its own production, and that inhibin inhibits and GnRH stimulates follistatin mRNA expression in rat pituitary cell cultures. Interestingly, in castrated male rams there is evidence that in vivo injection of IL-1β increases circulating concentrations of follistatin (Phillips et al. 1996). Both the PK-A and PK-C pathways are involved in stimulating follistatin synthesis (Miyanaga et al. 1993). Lindsell (1992) demonstrated that EGF and PK-C activators stimulate follistatin mRNA expression in cultured porcine granulosa cells in a dose and time dependent manner. In contrast, the synthesis of activin and inhibin is mediated through only the PK-C or PK-A pathways, respectively (Miyanaga et al. 1993).
2.2.4.3 Physical Structure of Follistatin

Follistatin is a glycosylated, monomeric protein. Follistatin is expressed as several different isotypes (Figure 2.3); the physiological function of these different isotypes has not yet been elucidated. There are two potential Asn-linked glycosylation sites at amino acids 95 and 259. It is not clear how glycosylation affects follistatin bioactivity, as mutagenesis of potential glycosylation sites of human 315 a.a. FS expressed in Chinese Hamster Ovary (CHO) cells does not interfere with activin binding or follistatin potency in a rat anterior pituitary bioassay (Inouye et al. 1991a). Differential glycosylation produces different molecular weight forms of follistatin. The dissociation constant of follistatin and activin has been estimated as approximately $6 \times 10^{-10}$ M (Nakamura et al. 1990; Sugino et al. 1993) which is greater than that of activin and its own receptor (see section 2.2.3.4). The binding of follistatin to activin is irreversible at physiological pH (Schneyer et al. 1994). Sugino et al. (1993) have shown that the most common form of follistatin in porcine follicular fluid is neither the 315 or 288 a.a. form, but a 303 a.a. form which may be derived from the 315 a.a. follistatin by proteolytic cleavage of the carboxyl terminus (Figure 2.3). The 288 a.a. follistatin comprises <1% (Findlay et al. 1993) or 10-20% of the total amount of follistatin in porcine follicular fluid (Sugino et al. 1993). The relative concentration of each of the follistatin isotypes may differ in serum and follicular fluid. In humans, the 315 a.a. follistatin is the major free follistatin isotype present in serum, and the 288 a.a. follistatin is the predominant free follistatin isotype in follicular fluid (Schneyer et al. 1996).
Figure 2.3 Physical structure of follistatins isolated from porcine follicular fluid (Sugino et al. 1993). * indicates sites of possible Asn-linked glycosylation.

Determination of follistatin concentration in biological fluids is complicated by inhibin or activin binding. To date no assays, other than chromatographical, have been developed which measure the different isoforms of follistatin. In swine follicular fluid, follistatin concentration have been identified as 5.6 mg/l (IRMA bound and unbound follistatin; Wakatsuki et al. 1996). It is not clear what role tissue origin contributes to circulating levels of follistatin. Long-term, the concentration of circulating follistatin does not decrease in post-menopausal women or ovariectomized in sheep. Following ovariectomy in sheep, circulating concentrations of follistatin return to preovariectomy levels within 48 hours indicating the regulation of circulating levels of follistatin is tightly controlled (Phillips et al. 1996). However, circulating concentrations of
follistatin do increase in women and rats following superovulatory treatment (Michel et al. 1990; Sugawara et al. 1990).

2.2.4.4 Ovarian Expression of Follistatin

Bovine and rat follistatin mRNA is first detected in the granulosa of secondary follicles and the amount of mRNA increases with follicle maturation. Peak expression of mRNA occurs in preovulatory follicles and newly formed corpora lutea. The amount of mRNA found in atretic follicles decreases with time (Findlay et al. 1993). In contrast, Tisdall et al. (1994) showed follistatin mRNA expression in granulosa of preantral, antral, and early atretic follicles, and, early and midphase luteal tissues in sheep. In sheep, follistatin expression in granulosa decreased in preovulatory follicles prior to ovulation.

During pubertal development, and the luteal phase of the estrous cycle in swine, the diameter of the follicle and follistatin expression are positively correlated (Li et al. 1997a). This work is similar to that by Lindsell (1992) who determined that follistatin mRNA expression increased with follicle size. However, during the follicular phase follistatin expression decreases regardless of the follicle diameter, with no change in the ratio of 288 a.a. to 315 a.a. mRNA. Decreased follistatin mRNA expression was accompanied by a decrease in follistatin protein concentrations in follicular fluid from 8 to 1 μg/ml (Li et al. 1997a).
2.2.4.5 Extragonadal Expression of Follistatin

The expression of follistatin in the pituitary is controversial. In the rat, follistatin is expressed by pituitary gonadotropes and folliculostellate cells during diestrus II (Kaiser et al. 1992) or during early proestrus (Halvorson et al. 1994). Follistatin mRNA has been localized in uterus, decidua, placenta, kidney, pancreas, muscle, lung, brain, thymus, adrenal gland, gut, heart, and bone (Michel et al. 1990; Petraglia et al. 1994). Michel et al. (1991) found similar ratios of mRNA expression for the 288 a.a. and 315 a.a. follistatin in all observed tissues. Follistatin is also expressed in tissues which do not express activin mRNA, for example, kidney, atherosclerotic plaques, and embryo implantation sites (Woodruff and Mather 1995).

Immunohistochemical techniques have not been able to localize follistatin protein in all tissues where mRNA has been characterized. Only the myometrium of the uterus, epithelium of the proximal and distal tubules of the kidney, the oviductal ampulla, the anterior pituitary, and the adrenal glands have been shown to contain follistatin protein (Kogawa et al. 1991; Michel et al. 1993). Interestingly, the cords of hepatic cells and the white bulbs of the spleen contain follistatin protein, but follistatin mRNA has not yet been identified (Michel et al. 1993). The dichotomy of mRNA and protein localization has several components. Transcription of a gene does not necessarily imply translation of the mRNA; other cellular signals may inhibit follistatin mRNA translation or decrease mRNA stability. Lack of follistatin specific antibodies has hindered the process of immunohistochemical analysis; to date follistatin antibodies are not available commercially nor have monoclonal antibodies specific for follistatin isotypes been developed. Finally, the immunohistochemical analyses cannot indicate the origin of the follistatin. Follistatin antigen identified in the assays may have originated
in the cells it was stained in or may have migrated from adjacent cells prior to tissue sampling.

2.2.4.6 Follistatin Receptor

The primary receptor identified for follistatin is the heparan sulfate proteoglycans (HSPG) on cell surfaces. The heparan binding site of follistatin is a basic amino acid rich region from amino acid 72-86 which also contains the activin:follistatin binding site (Sumitomo et al. 1995). There is an acidic amino acid rich region within the sixth exon, which may fold back onto the protein and interact with the basic amino acid rich, heparan binding site. The 315 a.a. follistatin is less able to interact with activin or heparan due to steric hindrance. The lower degree of interaction is mirrored in lower potency in pituitary cell culture (Sumitomo et al. 1995). The disociation constant of follistatin 288 a.a. for granulosa cell surface is (K_D=2 x 10^{-9} M); for 315 a.a. and granulosa cell surface there is no affinity (Sugino et al. 1993). Other studies have found that there is a small but significant association of follistatin 315 a.a. with cultured pituitary cells (Hashimoto et al. 1997).

Interaction of follistatin with HSPG on the cellular plasma membrane may not be exclusive. There is some indication that there may an independent follistatin receptor that is involved in follistatin modulation of progesterone synthesis (see section 2.3.3.1). As well, the effects of follistatin depletion in loss-of-function mutant mice are more widespread than the effect of activin depletion suggesting that follistatin may have independent activities or may interact with other members of the TGFβ family (see section 2.3.3.4).
2.3 Physiological Actions of Inhibin, Activin, and Follistatin

Inhibin and activin are biologically active primarily when they are in their dimerized form. The activin monomer, \( \beta_A \), is able to bind activin receptor type II with only 10-20% affinity of the dimer, however the monomer has < 1% the biological activity of the dimer (Husken-Hindi et al. 1994). There are indications that the \( \alpha \) subunit may have biological activity independent of and different to inhibin.

2.3.1 Inhibin

2.3.1.1 Physiological Actions of Inhibin in the Ovary

Inhibin has a potent effect in enhancing LH-induced androgen production in human theca cells (Hillier et al. 1991; Peng et al. 1996) and thecal cells from medium sized bovine follicles (Wrathall and Knight 1995), but does not enhance LH-induced androgen production in thecal cell cultures from small bovine follicles (Shukovski et al. 1993). In undifferentiated rat granulosa cell cultures, inhibin suppresses FSH induced aromatase activity, estrogen production, and progesterone production (Mather et al. 1992; Knight 1996). The \textit{in vivo} action of inhibin has yet to be elucidated. Injection of 1 \( \mu \)g of human recombinant inhibin into the rat ovarian bursa induced a small increase in the proportion of small follicles >350 \( \mu \)m and increased granulosa cellular proliferation (Woodruff et al. 1990). In contrast, vaccination against inhibin has increased ovulation rate in cattle (Glencross et al. 1994), sheep (O’Shea et al. 1993), and pigs (Brown et al. 1990; King et al. 1993).

The \( \alpha \) subunit may have inherent biological activity. In human granulosa-lutein cell culture the mature inhibin form has no effect on progesterone production; yet
antibodies specific to the α subunit decreased hCG stimulated progesterone production (Knight 1996). Schneyer et al. (1991) showed that a full length α precursor of inhibin can compete with FSH for the FSH receptor, decreasing FSH activity in granulosa cell culture.

2.3.1.2 Physiological Actions of Inhibin in the Pituitary

Inhibin produced in the ovary has an endocrine effect at the pituitary. In vivo inhibin suppresses serum FSH and FSHβ mRNA levels (Carroll et al. 1991; Vale et al. 1994). Inhibin also decreases FSH synthesis stimulated by GnRH and/or phorbol esters (Vale et al. 1994). Inhibin destabilizes FSHβ subunit mRNA (Vale et al. 1994) and may blunt gonadotropin stimulation of GnRH receptor synthesis (DePaolo 1997). In contrast, in perfused rat anterior pituitary cell cultures inhibin suppressed FSHβ mRNA expression, but was not able to inhibit FSH secretion (Weiss et al. 1993). The effect of inhibin on LH secretion is not clear. In some in vitro bioassay systems inhibin is able to inhibit GnRH stimulated LH secretion, however this has not been repeated in any in vivo studies (Vale et al. 1994). Antibodies specific to the α subunit of inhibin, obtained through active or passive immunization, increase the concentrations of circulating FSH in rats, sheep, cattle, and pigs (Finday et al. 1993; Knight 1996).

2.3.1.3 Loss of Inhibin Function Mutant Mice

Deletion of the α subunit gene creates a transgenic mouse which is unable to produce inhibin, but still is able to produce activin. Heterozygous mice were normal and fertile. Homozygous α subunit gene deficient mice were viable, but were infertile and susceptible to various gonadal tumors (Matzuk et al. 1992). The gross morphology of
the genitalia of the homozygous α subunit deficient mice was normal, suggesting that inhibin expression in the embryonic gonad was not essential for normal sexual differentiation and development.

It was not completely clear whether the tumor suppressing effect was due to inhibin, as the homozygous α subunit deficient mice had increased serum FSH levels, activin A and B levels (Trudeau et al. 1994). Cross breeding of inhibin α subunit deficient mice with GnRH deficient hypogonadal mice produced double knockout mice which did not exhibit gonadal or adrenal tumors (Kumar et al. 1996). Histological analysis of ovaries showed premalignant or malignant tumors similar to null α subunit animals; males had no tumor pathology. This suggests that tumor-suppressing activity of inhibin may be indirect and FSH concentrations are important in tumor development at least in the male mouse.

2.3.2 Activin

2.3.2.1 Physiological Actions of Activin in the Ovary

Activin enhances gonadotropin stimulated aromatase activity and estrogen production in rat granulosa cell cultures regardless of the follicle developmental stage, through stimulation of P-450SCC mRNA transcription (Mather et al. 1997). In the absence of FSH, activin does not have these effects. Activin has a biphasic effect on FSH receptor number in DES-primed immature rat granulosa cell culture, enhancing FSH-induced down-regulation of its receptor at concentrations of 3-30 ng/ml and inhibiting down-regulation at concentrations of approximately 100 ng/ml (Xiao et al. 1992). Furthermore, activin is able to induce FSH receptor number in the absence of FSH in DES-primed immature rat granulosa cell (Mather et al. 1997). In human
granulosa-lutein cell culture activin stimulates (Peng et al. 1996) or inhibits (Cataldo et al. 1994) estrogen production. The maturity of the granulosa-lutein cells used may have differed between these two studies.

Several investigations have been reported on the action of activin in progesterone synthesis. In undifferentiated rat granulosa cells, activin enhances FSH-induced progesterone synthesis and decreases the production of the progesterone metabolite, 20\alpha-hydroxyprogesterone (Findlay 1993). In partially differentiated rat granulosa cells, Miro et al. (1991) have shown that activin stimulates basal progesterone synthesis, but inhibits FSH-induced progesterone production. In bovine granulosa cell cultures of preovulatory follicles, activin A caused a time and dose-dependent inhibition of LH-induced progesterone and oxytocin synthesis (Shukovski and Findlay 1990), and prevented spontaneous luteinization in fully differentiated bovine cell cultures. In fully differentiated rat granulosa cell cultures, Miro et al. (1991) showed that both basal and FSH-induced progesterone production was inhibited by activin. In human granulosa lutein cell cultures, activin decreased both basal and hCG-induced progesterone synthesis (Knight 1996) or only the hCG-induced progesterone production (Peng et al. 1996). The inhibitory action of activin was shown to occur through down-regulation of 3\betaHSD, 84/85 isomerase and P-450_{SCC} mRNA (Mather et al. 1997). Surprisingly, the effect of activin in swine may be different from other species. In small (Ford and Howard 1997) and medium (Chang et al. 1996) porcine follicles, recombinant human activin A suppressed both basal and FSH-induced estrogen and progesterone production.

Activin appears to influence theca cell activity in vitro. Activin causes a dose-dependent decrease in LH and IGF-I-induced androstenedione production in rat and human theca cell cultures (Findlay, 1993; Hillier and Miro 1993). In bovine thecal cell cultures from medium sized follicles, activin did not directly affect basal
androstenedione production, but it decreased LH and estrogen stimulated androstenedione production (Wrathall and Knight 1995).

*In vivo* studies of activin activity have given variable results. Woodruff et al. (1990) showed that an injection of 1 μg of activin into the ovarian bursa of immature (25 day-old) rats decreased the proportion of follicles >350 μm and decreased cell proliferation relative to the uninjected control ovary. These results are opposite to those shown for inhibin injection into the ovarian bursa (see section 2.3.1.1). In contrast, daily injections of activin to intact immature female rats increased serum FSH, inhibin and estrogen concentrations, uterine weight, FSH receptor number, and the number of developing large follicles (Hasegawa et al. 1994). A second study in adult, cycling rats showed that daily administration of activin lead to premature superovulation of immature eggs and an increased incidence of atresia in large follicles (Erickson et al. 1995). Findlay et al. (1993) suggested that the discrepancy of activin activity between different biological assays may be due to the dose of activity utilized. Concentrations of >100 ng/ml activin decrease aromatase activity, which may have limited follicle growth in the study by Woodruff et al. (1990). There may be a gender difference in the action of activin. A single 50 μg bolus injection of activin had no effect on serum concentrations of FSH in adult male rats. In contrast, a 20 μg bolus injection of activin A given to diethylstilbestrol (DES)-primed immature female rats caused an increase in serum FSH concentrations (Findlay 1993).

Finally, separation of ovarian tissues in cell culture may not be appropriate to determine the activity of activin during folliculogenesis. During folliculogenesis, functional and morphological changes occur within a follicle. *In vitro* assay systems are distinct granulosa, theca, or granulosa cell-oocyte culture systems. Only in follicle culture systems are all three components of an *in vivo* follicle structure present. A recent
investigation in mice has shown the differentiated actions of activin, FSH, and the combination of activin and FSH on cultures of adult and immature mouse follicles. Yokota et al. (1997) cultured primary follicles from 11 day and 8 week-old mice. Follicle diameter, estrogen and inhibin production increased in immature mouse follicle cultures with increasing doses of activin or activin plus FSH, however in mature mouse follicle cultures only FSH increased the diameter of the follicles, and estrogen and inhibin production. In mature mouse follicle cultures, addition of activin to follicle cultures containing FSH decreased the diameter of the follicles, and estrogen and inhibin production. Addition of follistatin to the follicle cultures of mature rats partially reversed activin’s actions.

In conclusion, in vitro investigations of the action of activin have been more consistent than those conducted in vivo. In vitro activin appears to facilitate follicle growth through enhancing aromatase, FSH receptor number and estrogen production. Although the exact effect of activin on progesterone synthesis is still somewhat controversial the evidence suggests that activin can prevent luteinization of granulosa cells in vitro.

2.3.2.2 Physiological Action of Activin in the Pituitary

In vitro experiments utilizing pituitary cell cultures and recombinant activin A or B have shown that activin increased basal and GnRH-induced FSH release (Carroll et al. 1989; Corrigan et al. 1991). Carroll et al. (1991) showed that administration of 1ng/ml recombinant activin A increased FSHβ mRNA after four hours, increased FSH secretion after eight hours, and stabilized FSHβ mRNA with no effect on LH. In vitro bioassay of recombinant activin A and B stimulating FSH secretion showed that the
potency of each activin isoform was similar (ED$_{50}$ activin B = 2.1 +/- 0.6 ng/ml, activin A = 2.1 +/- 1.1 ng/ml) (Mason et al. 1989). However, native activin B may be significantly less potent than recombinant activin A or AB (Li et al. 1997a). Weiss et al. (1993) showed that, in a rat anterior pituitary perfusion system, activin was more effective than GnRH in stimulating FSHβ mRNA expression; addition of GnRH greatly enhanced activin's actions. Activin's actions were inhibited by addition of inhibin to the culture system. However, when FSH protein secretion was examined, GnRH, rather than activin, was more effective at increasing FSH secretion and activin augmented the action of GnRH.

Activin's actions on LH secretion in vivo is controversial and may be age, sex and species dependent. In immature and ovariectomized adult female rats activin A elevated FSHβ mRNA and serum FSH, but had no effect on adult male rats and only a modest effect on immature male rats (Carroll et al. 1991). In vivo administration of activin increased LH section during metestrus in rats (Vale et al. 1994) and during the follicular phase in monkeys (Woodruff and Mather 1995). Activin given to monkeys during the luteal phase caused a rapid decrease in circulating LH and progesterone concentrations and immediate menses (Woodruff and Mather 1995). Activin has been shown to induce GnRH receptors in rat pituitary cultures (Michel et al. 1993; DePaolo 1997). In addition, activin may affect the production of other pituitary hormones (Michel et al. 1993; Vale et al. 1994).

2.3.2.3 Other Actions of Activin

Activin has been identified as a growth factor and an apoptosis inducer in several tissues and cells of the immune system. Activin is involved in mesodermal and neural
tissue development in amphibians and may be involved also in mammalian tissue development (Woodruff and Mather 1995; Ying et al. 1997).

2.3.2.4 Loss of Activin Function Mutant Mice

Activin $\beta_A$, $\beta_B$ and activin receptor type II loss-of-function mutants have all been created to study the effects of removal of activin A, B, AB. Beta B deficient mouse mutants have impaired fertility and slightly higher concentrations of serum FSH (Vassalli et al. 1994). Beta A deficient mice developed to term, but died within 24 hours of birth due to palate and tooth defects which limited feeding (Matzuk et al. 1995a). Crossbreeding of heterozygotes deficient for $\beta_A$ and $\beta_B$, created offspring with an additive combination of defects seen in each deficient homozygote, but with no new deficiencies. This suggests that the lack of activin AB did not greatly affect normal physiology (Matzuk et al. 1995a). Activin receptor type II deficient mice were created which were expected to have similar phenotypic disruptions as activin deficient mice (Matzuk et al. 1995b). Some palate defects were noted, but these were not as severe as other $\beta_A$, $\beta_B$, and activin type II receptor deficient mutants and mice developed into adults. Serum FSH levels in activin type II receptor deficient mice were suppressed; puberty was delayed in male mice, and female mice were infertile. Females displayed thin uteri and small ovaries; oocyte and follicular development was normal, except for an increased rate of follicular atresia. The defects associated with type II deficient mice differed from those of $\beta_A$ or $\beta_B$ deficient mice, suggesting that $\beta_A$ and $\beta_B$ may signal through non-type II activin receptors.
2.3.3 Follistatin

2.3.3.1 Physiological Action of Follistatin in the Ovary

Since follistatin binds the β subunit of activin, the mature activin molecule has two binding sites for follistatin, whereas inhibin has only one follistatin binding site (Shimonaka et al. 1991). Follistatin will reverse the actions of activin if given in a 2:1 molar ratio of follistatin to activin (Kogawa et al. 1991). Follistatin (native porcine and rh-288 a.a.) has been reported to neutralize the action of activin in COS-1, K562, and P19 embryonic carcinoma cells by specifically inhibiting the binding of activin to activin type II receptors (de Winter et al. 1996). In contrast, Hashimoto et al. (1997) have shown that activin is able to associate with the type II receptor and follistatin in rat anterior pituitary cells and activin receptor transfected COS-7. As a result of these contradictory reports, the mechanism of follistatin neutralization of activin remains unclear. Follistatin does not appear to affect inhibin bioactivity.

In the ovary, the predominant effect of follistatin appears to be the inhibition of activin’s actions (Ling et al. 1990; Findlay et al. 1993). Follistatin causes a dose-dependent inhibition of activin stimulated aromatase activity, and also inhibits progesterone and inhibin synthesis. Follistatin increases FSH-induced progesterone synthesis, but has no effect on progesterone synthesis in the absence of FSH in DES-primed immature rat granulosa cell cultures (Xiao et al. 1990, 1992). Shukovsky et al. (1991) utilized bovine small and medium antral follicles incubated with LH (without activin) to study the effects of each molecular weight form of bovine native follistatin on progesterone and oxytocin synthesis. Their studies showed slight, but significant differences in the stimulation of steroid synthesis by each molecular weight species. A 35 kDa form of follistatin stimulated hormone synthesis more quickly and to a greater
extent than the 31 or 39 kDa forms. In partially and fully differentiated bovine granulosa cells, follistatin reversed the inhibitory effect of activin on progesterone and oxytocin synthesis in the presence of LH (Shukovski et al. 1991; Findlay 1993). In human granulosa-lutein cells, follistatin reversed activin inhibition of progesterone and estrogen production (Cataldo et al. 1994).

Follistatin reversed activin induced increases in FSH receptor number in DES-primed immature rat granulosa cell cultures (Nakamura et al. 1992; Xiao et al. 1992), but did not decrease FSH receptor numbers below basal levels. Follistatin prevented activin induced morphological changes in rat granulosa cells, but had no direct effect on morphology itself (Nakamura et al. 1992). The effects of follistatin in vitro suggest that it may enhance the process of luteinization by binding and inhibiting activin.

In bovine thecal cells from small follicles, a 31 kDa form of native bovine follistatin stimulated LH-induced progesterone secretion. The effects of follistatin on progesterone production were reversed by addition of activin to the culture medium (Shukovski et al. 1993). In a second study utilizing bovine thecal cells isolated from medium sized follicles, neither activin nor recombinant human 288 a.a. follistatin affected basal or induced progesterone production (Wrathall and Knight 1995). Follistatin alone did not affect basal or induced androstenedione production, but reversed the inhibitory effects of activin on androstenedione production (Wrathall and Knight 1995).

Follistatin may have activin independent effects in the granulosa cell layer. In undifferentiated granulosa cells, follistatin stimulated production of both progesterone and 20α-hydroxyprogesterone, whereas activin stimulates production of progesterone, but suppressed the production of 20 α-hydroxyprogesterone. As well, follistatin
enhanced forskolin stimulated progesterone synthesis, but had no effect on forskolin stimulation of aromatase or inhibin synthesis. Activin enhances all three parameters stimulated by forskolin (Findlay 1993). In human granulosa-lutein cell cultures, follistatin stimulated basal, but not hCG induced progesterone production without the requirement of activin (Li et al. 1993).

Follistatin functions as an activin binding protein, inhibiting the action of activin at several different sites. However, it may also have activin independent autocrine and paracrine actions. More investigations are necessary to determine if the follistatin isotype determines whether the particular action is activin independent or dependent and what cellular binding sites/receptors are involved.

2.3.3.2 Physiological Action of Follistatin in the Pituitary

*In vitro* and *in vivo* follistatin inhibited the activity of activin, suppressing the production of FSH. *In vivo* studies in ovariectomized rats have indicated that the action of follistatin may be through decreasing FSH pulse amplitude rather than pulse frequency (DePaolo et al. 1992). Different isoforms of follistatin have different potency in suppressing FSH secretion in pituitary culture systems. The potency of the 315 a.a. follistatin is approximately 5-33% that of inhibin; potency of 288 a.a. follistatin is similar to inhibin (Findlay et al. 1993) in rat anterior pituitary cell cultures. *In vivo*, the duration of FSH-suppression by follistatin is 10-21 hours, and that of inhibin is 4-9 hours in ovariectomized rats (DePaolo et al. 1991; Michel et al. 1993). The effect of follistatin on inhibiting activin activity in the pituitary has recently been shown to be via acceleration of activin A uptake into rat pituitary cells (Hashimoto et al. 1997). The exact role of follistatin and activin during the estrous cycle is unclear. Exogenous
follistatin can stimulate the secretion of activin B during late proestrus and early estrus in rats (DePaolo 1997), suggesting a role for follistatin facilitating, rather than inhibiting, the production of FSH. Furthermore, Halvorson et al. (1994) detected increased follistatin mRNA levels prior to increased FSHβ mRNA levels with no change in activin β subunit or activin receptor type II mRNA.

2.3.3.3 Other Actions of Follistatin

Follistatin reverses the actions of activin discussed in section 2.3.2.3. However, follistatin may not antagonize the action of activin in all systems. In a Xenopus ectodermal explant, heparinase mediated removal of the glycosaminoglycan chains of the proteoglycans inhibited mesodermal differentiation in response to activin (DePaolo 1997). This suggests, that in this system, the regular biological effect of follistatin would be to enhance the action of activin.

2.3.3.4 Loss of Follistatin Function Mutant Mice

Follistatin deficient mice were created using deletion gene targeting of all six follistatin exons (Matzuk et al. 1995c). Heterozygous animals developed normally and were fertile. Homozygous animals had severe muscle, skeletal, palate, and tooth defects and died within hours of birth. Some of these defects were not present in activin deficient mice (see section 2.3.2.4), including the skeletal defects and skin epidermis changes, suggesting that follistatin may have actions independent of activin or may act as a binding protein for other members of the TGFβ family.
2.3.3.5 Follistatin Overexpression Mutant Mice

Investigation of the effect of follistatin deficiency on fertility in adult mice was not possible in the loss-of-function mutants, therefore follistatin overexpression mice were created (Guo et al. 1998). The follistatin six exon transgene was identified in reproductive and non-reproductive tissue (liver, skin, brain, heart, lung, spleen, stomach, kidney, and small intestine). Not all transgenic animals expressed the transgene in all tissues or in the same quantity. Messenger RNA for the 315 a.a. follistatin was identified as the only follistatin mRNA expressed in all tissues examined. Most transgenic animals developed normally and were viable, although they had some minor skin defects. Animals with the greatest quantity and broadest tissue expression of the transgene were infertile. In males, testes were smaller, Leydig hyperplasia or tubular degeneration was present, and some animals had abnormal spermatogenesis. In females, ovaries were smaller, the uterus wall was thinner and in one line, folliculogenesis was blocked prior to follicle antrum formation. In the most severely affected animals, FSH and estrogen (females) were lowered. There was no change in serum follistatin or activin levels, suggesting the actions of follistatin were autocrine or paracrine.

2.4 Mechanisms of Action of Inhibin, Activin, and Follistatin

2.4.1 Proposed Physical Interactions

Follistatin may neutralize or enhance the actions of activin, depending on the tissue and the bioassay system utilized. A model of follistatin and activin interaction at the cell surface is depicted in Figure 2.4 (Halvorson and DeCherney 1996). In this model, follistatin associates with HSPG on the outer cell surface and also with activin;
activin associates with follistatin and the type II, but not type I receptor. However, the interaction of follistatin, activin, and the activin receptors may differ between cell types. de Winter et al. (1996) have shown that follistatin inhibits the interaction of activin and the type II receptor; Hashimoto et al. (1997) have shown that activin is able to bind both the type II receptor and follistatin at the cell surface. Moreover, it is not clear whether follistatin disassociates from the HSPG prior to association with activin. As discussed previously, different follistatin isoforms associate with HSPG with different affinities (see section 2.2.4.6). Follistatin isoforms with lower affinity to HSPG have less opportunity to bind activin at the cell surface, but may interact with activin in the interstitial area or in the circulation.

![Diagram](image)

**Figure 2.4** A proposed model for the physical interactions of activin, follistatin, and their receptors (Halvorsen and DeCherney 1996).

### 2.4.2 Sequential Expression of Follistatin and Inhibin $\alpha$ and $\beta$ Subunits

*In situ* hybridization techniques have been used to examine the expression of follistatin and $\alpha$ and $\beta$ subunits throughout the estrous cycle in pituitary and ovarian tissue of several species. The physical sites of expression have been discussed above (see sections 2.2.2, 2.2.3, 2.2.4). However, the expression of each of these hormones, in relation to the physiology of follicle recruitment and selection, is only beginning to be
examined. Guilbault et al. (1993) examined the concentration of $\alpha$ subunit, or dimeric inhibin, and the relationship of these proteins with the stage of the bovine estrous cycle. They found higher concentrations of $\alpha$ subunit during the growing phase relative to the regressing phase of the dominant follicle. The concentration of the $\alpha$ subunit was correlated positively with follicular fluid estrogen concentration. Interestingly, the concentration of dimeric inhibin was correlated inversely with the $\alpha$ subunit and estrogen concentration. In situ hybridization in sheep has shown that follistatin is expressed during different stages of follicle maturity than $\alpha$ or $\beta_A$ (Tisdall et al. 1994). Follistatin expression was detected in follicles with $>2$ layers of granulosa cells through to preovulatory follicles, whereas $\beta_A$ and $\alpha$ were detected only in medium to large antral follicles (Table 2.1). Singh (1997) determined the concentration of follistatin expressed in follicles and corpora lutea during the bovine estrous cycle. Follistatin protein expression was greater in dominant than in subordinate follicles and peaked during the mid growing phase of both waves examined. Follistatin concentration in corpora lutea peaked during day 11 of the estrous cycle and was not present during days 3 or 17. This data suggests that, in cattle, follistatin is produced in the dominant follicles and corpora lutea during the periods of their maximal function.
Table 2.1  *In situ* hybridization of ovine ovaries indicating amount of expression of follistatin, $\alpha$ and $\beta$ subunits just prior to ovulation (day -1). Follistatin mRNA expression is expressed during more stages of follicle maturity than the $\alpha$ or $\beta$ subunit (Tisdall et al. 1994).

<table>
<thead>
<tr>
<th>Follicle granulosa cells:</th>
<th>Follistatin</th>
<th>$\beta_\alpha$</th>
<th>$\alpha$</th>
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<tbody>
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<td>Primordial</td>
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<tr>
<td>Preantral</td>
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<tr>
<td>Antral</td>
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<tr>
<td>Preovulatory</td>
<td>(+)</td>
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<td>Atretic</td>
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<td>Corpus Lutea</td>
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<td>Oocytes</td>
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<td>Theca</td>
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<td>Stroma</td>
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<tr>
<td>Interstitial cells</td>
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<tr>
<td>Blood vessels</td>
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</table>

+$ = $ strong expression; (+) = weak expression; - = negative signal; +/- = signal intensity varied with level of atresia

The results of sequential expression and cell maturity dependent effects of activin and follistatin are combined in Figure 2.5 (Findlay 1993). Induction of FSH receptors by activin allows progression of granulosa cells from the “undifferentiated” to the “partially differentiated” state. Follistatin expression is induced by low levels of activin, therefore its activity succeeds activin activity. In “fully differentiated” cells both activin and follistatin affect the granulosa cell’s function. This model is based on studies in cattle and rat granulosa cell cultures and does not include the observations of Tisdall et al. (1994). There may be species differences in the sequence of intraovarian factor expression and cell maturity dependent effects.
Figure 2.5 Activin is critical for follicle differentiation in cattle and rats, as it is able to induce FSH receptors (Findlay 1993).

Detection of follistatin and inhibin subunits in ovarian tissue and the in vitro studies identifying the functions of these autocrine and paracrine hormones has led to the concept of "activin tone" (Figure 2.6; Hillier and Miro 1993). Activin is produced when the production of the \( \alpha \) subunit is low. During early follicle growth there is a period when activin is the predominant growth factor in a follicle’s microenvironment. With time, \( \alpha \) subunit and follistatin production may limit the available activin. Whether a follicle grows or becomes atretic depends on the concentration of available activin. Activin may be more important for stimulating folliculogenesis in immature follicles, and as a follicle matures, increasing production of follistatin decreases activin tone by binding and neutralizing activin’s action (Shintani et al. 1997).
Figure 2.6 The “activin tone” theory. Activin may be more important for stimulating folliculogenesis in immature follicles. As inhibin α subunit expression increases during the estrous cycle, activin is less able to exert its activity as αβ subunits are preferentially formed (Hillier and Miro 1993).

2.4.3 Limitations in the Current Knowledge of the Physiological Function of Follistatin, Activin, and Inhibin

In general, it is believed that follistatin’s predominant activity is as an activin binding protein. Activin stimulates estrogen production and granulosa cell differentiation favoring folliculogenesis; follistatin neutralizes this activity facilitating luteinization. Activin’s relative potency depends on the concentration of activin and follistatin, the follistatin isoform present, and the rate of α subunit production. What is
not clear is why: a) α subunit production exceeds β subunit synthesis by such a large amount, b) what the different functions of each of the isoforms of activin, follistatin, and inhibin are, or c) whether the actions of activin, follistatin, and inhibin are autocrine, paracrine, or endocrine. The shortest follistatin isoform binds HSPG with the highest affinity and has the highest probability of interaction with activin and the activin receptor. The function of the longer follistatin isoforms, which are more predominant than the 288 a.a. form and are not as closely associated with the cell surface, may differ from the 288 a.a. isoform, but the mechanism of action is unclear. Nor is it clear why follistatin does not affect inhibin activity. Finally, Chang et al. (1996) and Ford and Howard (1997) have indicated that, in swine, activin may stimulate atresia, which differs from all other mammal granulosa cell culture systems studied. How does activin physiology differ in swine from other species and why is activin activity different between species?

2.5 Immunoneutralization of Endogenous Hormones

Vaccination against reproductive hormones was first utilized to study hormonal actions, but commercial applications of the technology were subsequently pursued (D’Occhio 1993). Most hormone immunoneutralization experiments have focused on vaccination techniques which induce humoral immunity.

2.5.1 Immunoneutralization Technique Objectives

Vaccines stimulate the production of antibodies to a specific target antigen. The objective of immunoneutralization of an endogenous hormone is to stimulate the immune system to recognize an endogenous or “self” protein as a “foreign” protein.
Induction of neutralizing antibodies to a self-protein causes clearance of that protein through binding and removal of that protein from its site of action. Through induction of a large quantity of neutralizing antibodies, the normal endocrine regulatory feedback mechanisms of the animal are overridden, therefore the animal functions in an environment of altered physiology where the self-protein is no longer active (Reeves et al. 1989).

2.5.2 Components of a Vaccine

The efficacy of a vaccine depends on the antigenicity, and quantity of the vaccine antigen, and the use of adjuvants, carrier proteins, and immunostimulants (Povey and Carman 1997). A vaccine is composed of an antigen (+/- carrier protein) and an adjuvant. Peptide vaccines are often produced synthetically and utilize the most antigenic portion(s) of the target antigen. A peptide vaccine antigen is conjugated or crosslinked, to a larger carrier protein, which is foreign to the animal, in order to increase the antigenicity. Subunit vaccines are similar to peptide vaccines, but contain the entire amino acid sequence of the target hormone. Subunit vaccines are often produced by recombinant DNA technology. Fusion protein vaccines are a combination of the target hormone and a carrier protein linked in a DNA expression system to produce a chimeric protein.

An adjuvant is a substance which non-specifically enhances immune function through either stabilization of vaccine antigens, attraction of macrophages by microbial products in the adjuvant, or the physical properties of oil which allows it to form an emulsion with aqueous antigens (Redmond and Babiuk 1991). Emulsions regulate the release of the vaccine antigen to the immune system, prolonging stimulation. The type
of adjuvant used, the quantity of antigen, the age of the vaccinated animal, and the route
of vaccine entry into the animal can determine the amount and type of immune response
(Cryz 1996; Povey and Carman 1997). Examples of adjuvants include: metallic salts,
bacterial extracts, plant extracts, oils, surfactants, nucleic acid derivatives, polymers,
amines, and cytokines (Redmond and Babiuk 1991).

2.5.3 Antibody Production

T helper cells recognize antigen on the surface of antigen presenting cells (APC)
(Berzofsky 1991). Vaccine antigens are processed and presented by dendritic cells,
macrophages, or activated B cells, which recruit and activate T cells. A soluble vaccine
antigen is degraded by the APC lysosomes and associated with class II major
histocompatibility complex (MHC) on the APC cell surface (Cryz 1996). The proteases
involved in degrading the endocytosed antigen, the ability of the antigen peptides to bind
to the MHC class II molecules, and the MHC haplotype determine which vaccine
epitope(s) are presented (Berzofsky 1991). The type of APC involved in presenting the
vaccine antigen and the cytokine environment influence the type of T helper cell which
becomes activated. T helper subset 2 (Th2) is mainly responsible for B-lymphocyte
activation and formation of antibodies specific for the vaccine antigen.

Immature B cells, with Th2 cell assistance, proliferate and differentiate into IgG
producing plasma cells or memory B cells. During differentiation, B cell IgG genes
undergo somatic mutation so that the IgG produced is specific for the vaccine antigen
and has a high affinity for the antigen. With secondary vaccination, immune complexes
of antigen and antibody form. Complexes are engulfed by circulating macrophages or
dendritic cells which present the antigen to T cells in lymph node germinal centers.
Some immune complexes reach follicular dendritic cells of the draining lymph node and release antigen to memory B cells in the germinal center. Activated T cells provide growth and differentiation factors to plasma cells which produce IgG (Hentges 1994).

Vaccination against endogenous hormones stimulates a classic primary and secondary antibody formation response. With the primary vaccination immunoglobulin class M (IgM) antibodies are formed within days of vaccination. Isotypic switching occurs and IgG can be measured in the circulation within seven days. With subsequent vaccine administration only IgG antibody is detected at higher levels than during the primary vaccination; peak IgG formation occurs within 7-10 days following vaccination (Povey and Carman 1997).

2.5.4 Significance of Antibody Titer and Bioassay Techniques

In almost all reported experiments involving the induction of antibody toward reproductive hormones, antibody titer is the preferred reported measurement. Different assay techniques give differing amounts of antibody titer depending on the reference sample used. An example of how the significance of correlation between inhibin antibody titer and FSH concentrations is affected by the radiolabelled inhibin preparation used is discussed in section 2.5.5. The epitope(s) recognized by a binding assay may not be the same epitopes responsible for biological action. The amount of “neutralizing” antibody, that quantity of antibody which binds and inhibits the in vivo action of the target hormone, may be a more reliable indicator of vaccine efficacy. Unfortunately, the amount of neutralizing antibody present in a given experiment is rarely discussed and makes interpretation of the body of literature concerning immunoneutralization of reproductive hormones difficult.
2.5.5 Example of a Fecundity Protein Hormone Vaccine - Inhibin Vaccine

Researchers have actively and passively vaccinated swine (Brown et al. 1990; King et al. 1993; Wheaton et al. 1997), cattle (Morris et al. 1993; Glencross et al. 1994), sheep (Wheaton et al. 1992; O’Shea et al. 1993), mink (Ireland et al. 1992), rats, hamsters and marmosets (Sheth et al. 1992) against inhibin. Inhibin suppresses the release of FSH from the pituitary and suppresses FSH-induced aromatase activity and steroid production in granulosa cell cultures (see section 2.3.1.1).

Four types of inhibin vaccine antigens have been utilized in inhibin vaccination experiments: purified native inhibin from follicular fluid, fusion proteins, synthetic peptides (a partial fragment of \( \alpha \) subunit amino acid 1-32), and recombinant inhibins coupled to a carrier protein (O’Shea et al. 1994). Doses of inhibin protein used have ranged from 4 \( \mu \)g - 4 mg. Adjuvants utilized have included: Freund’s Complete, Incomplete, and non-ulcerative adjuvant or Montanide 888:Marcol 52. Vaccinations have been given at single and multiple sites, intramuscularly and subcutaneously, at various animal ages and with different numbers of booster vaccinations. The diversity of vaccine formulations and vaccination schedules has led to a wide range of antibody titers, but with little indication of antibody affinities and inhibin neutralizing abilities.

Vaccination of sheep has been shown to advance puberty and increase ovulation rate with an inhibin antibody titer of approximately 30% binding at 1:2000 serum dilution (O’Shea et al. 1993). Inhibin vaccination has been shown to increase FSH serum levels (Wheaton et al. 1992) or to have no effect on FSH levels (O’Shea et al. 1993). Bred ewes vaccinated against inhibin had a higher total number of lambs, although the number of lambs born alive did not differ between each group (\( B_0 = 30\% \) binding at 1:1000 serum dilution (Wrathall et al. 1992). The performance of these
animals was followed over a three year period (Fray et al. 1994). Antibody titer remained constant, with no difference in serum FSH levels. There was an significant increase in the number of lambs born per ewe over the three years with an overall mean of 2.77 +/- 0.2 versus a control vaccinated group with a lambing rate of 2.03 +/- 0.18. The authors did not discuss the number of stillborn animals in this study. There has only been one direct comparison of inhibin vaccination in different breeds of animals. Findlay et al. (1993) described an inhibin vaccination protocol in Booroolas, a prolific sheep breed, which showed a 2.3 fold increase in ovulation rate relative to a 3.2 fold increase in ovulation rate in Merinos.

Inhibin antibodies were shown to neutralize inhibin activity in a pituitary bioassay (Meyer et al. 1990). Ovine follicular fluid was shown to decrease FSH production in ovine pituitary cells in vitro. Preincubation of follicular fluid with serum from vaccinated sheep reversed the inhibition of FSH production. Although this bioassay has several significant faults, such as lack of purification of either follicular fluid or serum, it did display the predicted effects on FSH production.

In cattle, inhibin vaccination has been shown to increase ovulation rate and the incidence of twinning ($B_0 = 5-27\%$ at 1:40 serum dilution) (Morris et al. 1993). Inhibin vaccination has been shown to decrease circulating FSH levels ($B_0=15\%$ at 1:1000 serum dilution, Rhind et al. 1993), increase circulating FSH levels ($B_0=17\%$ at 1:2000 serum dilution, Glencross et al. 1992), or have no effect on serum FSH levels ($B_0= 50-126\%$ at 1:100 serum dilution, Morris et al. 1995; $B_0 = 4-50\%$ at serum dilution 1:2000, Scanlon et al. 1993).

In swine, inhibin vaccination increased ovulation rate ($B_0 = 5-7\%$ at 1:800 dilution Brown et al. 1990; $B_0= 9-22\%$ at 1:4000 serum dilution King et al. 1993) and
increased circulating FSH levels (King et al. 1993). Passive vaccination of sows with anti-inhibit antibodies blocked the drop in circulating FSH postweaning, however, blocking the FSH drop did not affect the subsequent litter size (Wheaton et al. 1997).

The predominant mechanism of action of inhibit vaccination is likely through increasing FSH concentrations. However, the most striking observation of these experiments is the variable effect of inhibit vaccination on FSH concentrations. Antibody titer and FSH concentration were correlated in some experiments and not in others. Circulating concentrations of FSH vary widely between animals of the same breed which makes it difficult to determine if FSH has increased over the control group average. As well, there are differences in the time period when blood samples were taken and/or the inhibit antigen used for vaccination or for antibody titer determination. The correlation between FSH concentrations and inhibit antibody titers may be greater in studies in which native inhibit or recombinant inhibit was used as an immunogen in the titer assay compared to an inhibit peptide vaccine (O’Shea et al. 1994). In summary, these observations cannot fully explain the difference in antibody titer and FSH concentrations and has led researchers to believe that there may be an intraovarian effect of inhibit vaccination, as well as neutralization of circulating inhibit (Findlay et al. 1993; O’Shea et al. 1994).

2.5.6 Example of a Fecundity Steroid Hormone Vaccine - Androstenedione Vaccine

Immunity to the steroid hormone, androstenedione, has been induced in sheep; a commercially available fecundity vaccine, Fecundin®, was produced as a result of this research (Scaramuzzi et al. 1993). Androstenedione was modified by conjugation to human serum albumin so that the hormone would be recognized as a foreign antigen.
Fecundin® has been reported to increase ovulation rate and number of offspring in sheep and cattle with optimal results with antibody titers between 1:500 - 1:3000. However, the efficacy of Fecundin® is variable and has also resulted in acyclicity, cystic follicles, and superovulation (Reeves et al. 1989).

The first proposed model of the mechanism of action of androstenedione vaccination postulated that androstenedione would have endocrine actions and suppress gonadotropin secretion from the pituitary. However, subsequent research showed that exogenous androstenedione did not affect circulating gonadotropin levels or ovulation rate, and that androstenedione vaccination increased LH pulsatility, but did not consistently affect FSH secretion (Scaramuzzi et al. 1993).

The second proposed model of androstenedione vaccination’s mechanism of action focused on the paracrine effects of androstenedione in the ovary. In different in vitro cell culture systems androgens have been shown to decrease estrogen receptor content and to augment FSH stimulated aromatase activity. These confounding effects have been combined in the proposal that high levels of androgen and low levels of FSH may lead to follicle atresia, whereas high levels of both androgen and FSH stimulate estrogen production. The researchers involved in androstenedione vaccination have not been able to clearly demonstrate the intraovarian effects of androstenedione vaccination, but have concluded that the antibodies to androstenedione may inhibit the transfer of androstenedione from the thecal cell layer to the granulosa cell layer and protect follicles from atresia associated with excess locally produced androgen (Scaramuzzi et al. 1993).
2.5.7 Example of a Contraceptive Protein Hormone Vaccine - GnRH Vaccine

Immunity to GnRH has been investigated as a means of controlling reproduction and carcass traits in livestock. One anti-GnRH vaccine, Vaxstrate®, is commercially available and other anti-GnRH vaccines for both livestock and domestic pets are under development. An anti-GnRH vaccine is also being investigated as a means to control reproduction in humans (Talwar et al. 1992).

There are management difficulties associated with raising gonad-intact livestock. Vaccination against GnRH has the potential to suppress reproduction and secondary sexual characteristics by reducing the amount of steroid hormone production. Intact bulls are aggressive; they can cause harm to producers and other animals, and have a higher incidence of bruised or dark-cutting meat at slaughter. Intact heifers/cows can result in unwanted pregnancies which slow weight gain and days to market. Intact boars have a tendency toward boar taint, an odor unpleasant to the consumer (Bonneau and Enright 1995). At present, the majority of male livestock is castrated by surgical methods. GnRH vaccination is an alternative which may appeal to people with ethical concerns about surgical castration.

GnRH vaccination in bulls has been shown to decrease circulating levels of LH and testosterone, scrotal circumference, testes weight, semen production and masculinity score, libido and aggressive behavior and increase marbling without affecting average daily gain (Robertson et al. 1982; Finnerty et al. 1994; Huxsoll et al. 1998). GnRH
vaccination in heifers has been shown to delay puberty, decrease ovulation rate, induce anestrus, arrest follicle growth, and decrease ovarian and uterine weight, the number of LH and GnRH receptors in ovarian tissue, circulating estrogen and progesterone (Adams and Adams 1990; Prendiville et al. 1995a, b), and to increase fat deposition and decrease protein deposition (Adams et al. 1990) without affecting growth rate (Prendiville et al. 1995a) or with a decreased growth rate (Adams and Adams 1990; Prendiville et al. 1995b).

GnRH vaccination in boars has decreased boar taint, decreased testes and accessory sex gland weight, and decreased circulating LH and testosterone levels (Falvo et al. 1986; Bonneau et al. 1994; Meloen et al. 1994), with a higher average daily gain, feed intake and feed efficiency compared to surgical castrates and with similar performance levels as intact boars.

As is the case with both inhibin and androstenedione vaccination there is individual animal variability in response to GnRH vaccination. The degree of the effect of GnRH vaccination in all experiments varied in magnitude and depended on the quantity of anti-GnRH antibodies induced.

2.6 Conclusion

The physiological evidence concerning the role of follistatin during the porcine estrous cycle is limited in comparison to that of cattle, humans, or rats. It is clear that
the porcine ovary produces follistatin, as well as activin and inhibin. Guthrie et al. (1992) and Li et al. (1997a) have shown that α and β subunits, and follistatin mRNA are dynamically regulated during the follicular phase of the porcine estrous cycle. It is difficult to determine whether the theories of the mechanism of hormone action developed in cattle, humans and rats can be transposed to swine. The role of activin and follistatin in porcine folliculogenesis is complex as shown by the studies of Chang et al. (1996) and Ford and Howard (1997) who observed that activin suppresses estrogen production, facilitating follicle atresia. Immunoneutralization of the inhibin α subunit in swine (Brown et al. 1990; King et al. 1993) has been shown to increase circulating FSH and ovulation rate, but as yet there have been no investigations of inhibin immunoneutralization on litter size. If “activin tone” is the major physiological mechanism influencing folliculogenesis in swine, enhancement of activin tone, through follistatin immunoneutralization, should be beneficial to increase ovulation rate.
3. CHARACTERIZATION OF A RECOMBINANT PORCINE FOLLISTATIN PRODUCED USING A HEAT SHOCK EXPRESSION SYSTEM

3.1 Abstract

Follistatin was initially identified through its ability to inhibit follicular stimulating hormone (FSH) secretion from the pituitary. But, because follistatin is present in low concentration in vivo, obtaining adequate quantities for characterizing the function of follistatin is difficult. We have cloned porcine follistatin cDNA in a novel expression system directed by the highly inducible bovine heat shock protein promoter, BoHSP70. The recombinant porcine follistatin with apparent molecular weights of 39, 46, 48, 50 kDa was expressed and secreted daily into the culture media at concentrations of 20-25 mg/l/4x10^7 cells, a rate which exceeds all previously reported values of recombinant follistatin production. The recombinant follistatin produced was a monomeric glycoprotein, immunologically indistinguishable from native follistatin. In a porcine granulosa cell culture, the recombinant porcine follistatin suppressed the accumulation of estradiol-17β without the requirement for activin stimulation, illustrating a novel action of follistatin.
3.2 Introduction

Follicular development in the ovary is a highly regulated process involving the gonadotropins and intrafollicular factors which modulate follicular response to gonadotropins. A unique, intricate relationship exists between three intraovarian factors, follistatin, activin, and inhibin. Activin and inhibin, members of the TGFβ superfamily, are dimeric proteins which share a common β subunit. Follistatin is a monomeric protein which can bind either activin or inhibin through the common β subunit (Shimonaka et al. 1991). Initially, each of these factors was purified and identified by its effect on FSH synthesis in pituitary cell cultures. Follistatin and inhibin both decrease FSH synthesis (Carroll et al. 1989; DePaolo et al. 1991), whereas activin increases FSH synthesis (Carroll et al. 1989). It appears that each of these three factors is expressed and produced sequentially in the ovary (Tisdall et al. 1994) and the relative concentration of each may determine the growth or development of a particular follicle (Hillier and Miro 1993).

In vitro studies using rat and bovine granulosa cells have shown that the actions of both activin and follistatin are similar in these species (Findlay 1993). Activin stimulates cellular proliferation, induces FSH receptors, and increases estrogen production during all stages of follicle growth. The effect of activin on progesterone production may differ with stage of follicle maturity. Activin enhances progesterone production in immature follicles and suppresses progesterone production in cell cultures from large or fully differentiated follicles (Findlay 1993). Nakamura et al. (1990)
reported that follistatin specifically bound activin with a high affinity and suggested that follistatin may act as an antagonist to activin, inhibiting its functions in vivo. Based on these investigations in granulosa cell cultures, activin is believed to promote folliculogenesis, and follistatin, to promote luteinization (Findlay 1993; Li et al. 1995).

Two follistatin mRNA have been identified, one coding for a 288 amino acid (a.a.) protein and the other for a 315 a.a. protein, which are produced by alternative splicing of a single follistatin gene (Shimasaki et al. 1988). Mature follistatin is expressed as both an unglycosylated and a glycosylated protein; the nature of this glycosylation may be species specific. To date, several glycosylated forms of follistatin have been identified (Findlay 1993). Shukovski et al. (1991) reported proteins of 31 kDa, 35 kDa, 39 kDa, and 45 kDa in bovine follicular fluid, and of 32 kDa and 35 kDa in porcine follicular fluid. Sugino et al. (1993) reported six molecular weight forms of follistatin from porcine follicular fluid; the major isoform was a 303 a.a. protein which may have been produced from the 315 a.a. follistatin during post-translation modification. The potency and prevalence of each molecular weight form of follistatin differs. The 288 a.a. form has an in vitro potency similar to that of inhibin in pituitary cell cultures, but this form comprises less than one percent of follistatin found in follicular fluid (Inouye et al. 1991b). The potency of the 315 a.a. follistatin is approximately 30% of the 288 a.a. follistatin (Findlay et al. 1993). All follistatin isoforms bind activin with similar affinity, therefore potency may be determined by heparan sulfate binding characteristics. The 288 a.a. follistatin isoform binds granulosa
cell surfaces with a high affinity ($K_D = 2 \times 10^{-9} \text{M}$), whereas the 315 a.a. follistatin has no affinity for heparan sulfate proteoglycans.

Elucidation of follistatin's physiological function requires large quantities of a biologically active hormone. In porcine follicular fluid, follistatin concentration is approximately 5.6 mg/l (Wakatsuki et al. 1996). Ueno et al. (1987) reported a method for extracting follistatin from porcine follicular fluid that yielded approximately 22 μg/l. Such low purification yields have limited the number and depth of studies to determine the nature of follistatin physiology. Recombinant DNA technology permits large scale production of recombinant proteins. Inouye et al. (1991b) produced recombinant forms of the 315 a.a. and 288 a.a. human follistatin in Chinese hamster ovary (CHO) cells at a production yield of approximately 3-5 mg/l of culture media. We have developed a stable transformed cell line, which produces a recombinant porcine follistatin of 305 amino acids, at approximately 20-25 mg/l/day.

3.3 Material and Methods

Definitions

"FCC815 conditioned media" refers to the culture media plus all proteins secreted into the media following the heat shock protocol. "Recombinant porcine follistatin" refers to the proteins recognized by the polyclonal antibody raised to native porcine follicular fluid (Figure 3.7.1). "rp-305 a.a. FS" refers to the recombinant porcine follistatin isotype which was purified by monoclonal antibody affinity chromatography.
Expression of Recombinant Porcine Follistatin

Isolation of the follistatin gene from ovarian granulosa cells

Isolation of the follistatin gene and cloning into the pGEM vector was carried out by Claire Lindsell during her Ph. D. thesis research (Lindsell 1992). Briefly, RNA was isolated from 2 g of ovarian tissue from an adult sow in luteal phase of the estrous cycle, using guanidium isothiocyanate/cesium chloride. Polymerase chain reaction primers were designed to amplify a region of the porcine cDNA sequence from the ATG initiation codon to nucleotide 1020 (Esch et al. 1987) and were synthesized on an Applied Biosystems 381A DNA synthesizer.

\[
5' \quad GGATCCATGGTCGGTCCCAAGCAC \quad 3' \\
5' \quad GGATCCTCAGTAGTCCTGGTCTTC \quad 3'
\]

The reverse transcriptase-polymerase chain reaction products were cloned into pGEM 3Zf(-) (Promega, Nepean, ON) and transformed into E. coli strain JM109 (Stratagene, La Jolla, CA) to create pGEM-FS. The identity of the cloned plasmid was confirmed by restriction endonuclease mapping and single strand DNA sequencing. This sequence was compared with the published genomic sequence of 2051 nucleotides (Esch et al. 1987) and was found to be identical except for four substitutions. The cDNA sequence contained three redundant substitutions and substitutions of cccgta for gggcta at nucleotides 31-36 of the signal peptide.

Construction of the follistatin expression vector (pFSHUno)

The expression plasmid pFSHUno (Figure 3.1), which contains the bovine HSP70 promoter, neomycin and ampicillin resistance genes, and follistatin cDNA, was produced
from the expression plasmid pG4HUno (Kowalski et al. 1993). pGEM-FS was
digested with NcoI and BamHI and a 1002 base pair fragment corresponding to
follistatin cDNA coding for signal sequence and the first 305 a.a. of mature follistatin,
was ligated into pG4HUno, replacing the bovine herpes virus glycoprotein gene (Figure
3.1).
Figure 3.1 Construction of the pFSHUneo plasmid.
Transfection and generation of a stable cell line, FCC815

Madin-Darby bovine kidney (MDBK) cells (ATCC # CCL22) were grown in Minimal Essential Medium (MEM) (Canadian Life Technologies Inc., Burlington, ON) supplemented with 10% fetal calf serum (FCS) (Hyclone Laboratories, Inc., Logan, UT). Transfection was carried out using 5 μg pFSHNeo DNA which had been purified three times by double cesium chloride ultracentrifugation and ethanol precipitation. pFSHNeo DNA and 40 μg of Lipofectin (Canadian Life Technologies Inc.) were incubated for 15 minutes at room temperature and then added dropwise to a tissue culture plate; 1x10^6 MDBK cells were plated in one 25 cm² well of a 4 well tissue culture plate. Cells were cultured at 37°C in a humidified, 5% CO₂ incubator in OptiMem media (Canadian Life Technologies Inc.). After 16 hours the cells were trypsinized and diluted 1:40 in MEM containing 10% FCS. The diluted cells were plated in ten 24-well Corning tissue culture plates. Neomycin resistant clones were selected by growth in MEM-10% FBS media supplemented with 666 μg/ml G418 (Canadian Life Technologies Inc.) and single-cell cloned in media containing 666 μg/ml G418.

Production of the recombinant porcine follistatin

FCC815 cells were cultured with MEM-10% FCS media in 150 cm² Corning tissue culture flasks at 37°C, in a 5% CO₂, humidified incubator. To induce the expression of recombinant follistatin, FCC815 cells were washed and incubated in MEM (without FCS) for 6 hours at 43°C in a second 5% CO₂, humidified incubator. The cells were returned to the 37°C incubator and incubated overnight. The next day the culture
supernatant was removed and centrifuged at 2000 g for 5 minutes to remove cellular debris. The same FCC815 flasks were cycled through additional daily heat shock cycles.

**Recombinant follistatin protein concentration determination**

The percentage of total protein in FCC815 conditioned media was determined by scanning a SDS-PAGE gel (Figure 3.3) using a ScanJet II system (Hewlett Packard) and analyzing percentage of total protein in each SDS-PAGE gel protein band using Biomed Instrument software (Advanced American Biotechnology, Fullerton, CA). The protein bands in the gel of the same molecular weight as those in a Western blot (Figure 3.7.1) were used to determine the percentage of recombinant porcine follistatin. FCC815 conditioned media protein concentrations were quantified by a protein assay (BioRad, Mississauga, ON). The percentage of recombinant follistatin was multiplied by the total protein measured by the BioRad protein assay to determine the concentration of recombinant follistatin produced and secreted into the culture supernatant.

**Purification of Recombinant Porcine Follistatin**

**Presence of heat shock proteins in FCC815 conditioned media**

The expression vector contained the HSP70 promoter, so the FCC815 conditioned media was tested for the presence of heat shock proteins. FCC815 conditioned media was resolved by SDS-PAGE electrophoresis under reducing conditions in sample buffer containing β-mercaptoethanol in a 10% mini-protean gel (BioRad). After SDS-PAGE electrophoresis the proteins were electroblotted onto a 0.45 μm nitrocellulose membrane
(BioRad). The blot was incubated with 0.5 % gelatin in 10 mM Tris buffered saline pH 8.0 for two hours. The primary antibody for this assay was: 1:500 dilution of SPA-820 purified monoclonal antibody raised to constitutive and induced HSP70 from HeLa cells (Stress Gen, Victoria, BC). The second antibody was: 1:1000 dilution of biotinylated goat anti-mouse IgG (1030-08, Southern Biotechnology Associates, Inc., Birmingham, AL), followed by 1:1000 dilution of streptavidin-horseradish peroxidase conjugate (Amersham Life Sciences, Oakville, ON). Dilutions were in 10mM Tris buffered saline, 0.5% Tween 20, pH 8.0; samples were incubated at room temperature with constant agitation for one hour unless otherwise specified. The specifically bound antibodies were visualized using a horseradish peroxidase substrate kit (BioRad).

**Follistatin purification by size exclusion**

The apparent molecular mass of recombinant porcine follistatin was first estimated as approximately 31-45 kDa. A combination of Centriprep centrifugation columns was used to purify the recombinant follistatin. A Centriprep 50 column (4310, Amicon, Beverly, MA) containing 10 ml of unconcentrated FCC815 conditioned media was centrifuged at 1800 g for 15 minutes. The eluant of this centrifugation (proteins < 50 kDa) was concentrated in a Centriprep 10 column (4304, Amicon) by centrifugation at 2500 g for 30 minutes. All concentration and eluant fractions were subjected to a BioRad protein analysis and electrophoresis in a 10 % SDS-PAGE gel.
**Follistatin purification by anionic exchange chromatography**

Fast Flow Q Sepharose (17-0510-10, Amersham Pharmacia Biotech, Baie d’Urfé, PQ) was used to separate recombinant porcine follistatin from other proteins in the FCC815 conditioned media. Several binding buffers were tested to determine the optimal conditions for binding recombinant porcine follistatin to the sepharose. A salt gradient was established to elute the bound proteins (Appendix C.1). All eluted fractions underwent electrophoresis in a 10% SDS-PAGE gel.

**Follistatin purification by affinity chromatography**

Attempts were made to purify recombinant porcine follistatin using sulfate ester coated cellulose beads (98175, Amicon) (Appendix C.2).

**Follistatin purification by monoclonal antibody affinity chromatography**

Recombinant follistatin was purified from FCC815 conditioned medium using a monoclonal antibody affinity column. The monoclonal antibody used (antibody 4-6D9) was raised against recombinant human 315 a.a. follistatin (monoclonal antibody generously supplied by Dr. Y. Shintani, Tokushima, Japan). The monoclonal antibody recognizes both recombinant human 288 and 315 a.a. follistatin (Dr. Shintani, personal communication). The antibody was crosslinked to protein A agarose (Pierce, Rockford, IL) using dimethyl pimelimidate (Pierce) (Appendix C.2). FCC815 conditioned media was added to the monoclonal antibody column and recombinant follistatin was eluted by addition of 100 mM glycine pH 2.8 (Appendix C.3). Purity of the eluted protein was determined in a 10% SDS-PAGE gel.
Characterization of Recombinant Porcine Follistatin

**Western blot analysis of recombinant porcine follistatin**

Media from heat shocked FCC815 cells was concentrated tenfold by centrifugation in a Centricon 10 (4205, Amicon). Electrophoresis and electroblotting procedures were the same as that for Western blot analysis of heat shock proteins. The primary antibody was rabbit anti-native porcine follistatin antibody (generously supplied by Dr. Ling, Whittier Institute, La Jolla, CA) diluted 1:500 in 0.5 % gelatin, 10mM Tris buffered saline pH 8.0, 0.5% Tween 20, and incubated with the nitrocellulose membrane for sixteen hours. This was followed by biotinylated goat anti-rabbit antibody (Canadian Life Technologies Inc.) and streptavidin-horseradish peroxidase conjugate (Amersham Life Sciences). Dilutions were 1:1000 in 10mM Tris buffered saline pH 8.0, 0.5% Tween 20; samples were incubated at room temperature with constant agitation for one hour unless otherwise specified.

**Follistatin-activin ligand blot - radiolabelled activin assay**

Recombinant human activin A (generous gift of Dr. Y. Eto, Ajinomoto Co. Inc., Kawasaki, Japan) was radiolabelled with iodine $^{125}$ (Appendix C.3). FCC815 conditioned media was electroblotted onto 0.45 μm nitrocellulose following electrophoresis in a 10 % SDS-PAGE gel. The nitrocellulose blots were blocked with 0.5 % gelatin in 10 mM Tris buffered saline pH 8.0, and incubated with radiolabelled activin (Appendix C.4). Attempts were made to neutralize the binding of radiolabelled activin with nitrocellulose blot bound recombinant porcine follistatin, by either
preincubation of the nitrocellulose blot with unlabelled activin A or by preincubation of radiolabelled activin A with a $\beta_A$ monoclonal antibody (MCA950S, Serotec, UK).

**Follistatin-activin ligand blot - colour visualization assay**

Recombinant human activin A (the same activin A as that used in the follistatin-activin radiolabelled ligand blot assay) was resolved in a 12% acrylamide mini-protean gel with either non-reducing sample buffer or sample buffer containing $\beta$-mercaptoethanol and electroblotted onto a 0.45 $\mu$m nitrocellulose membrane. The membrane was processed the same as the previous Western blot analyses, except that prior to addition of the anti-follistatin antibody, the membrane was incubated with FCC815 conditioned media (approximately 30 $\mu$g recombinant follistatin) for 16 hours.

**Follistatin bioassay**

Granulosa cells from slaughterhouse porcine ovarian follicles with clear follicular fluid and a mean diameter of 1-3 mm were collected, dissected, and cultured similarly to previously published reports (Lindsell 1992, Xu et al. 1994) (Appendix C.6). Granulosa cells were plated at 3 x $10^5$ viable cells/well and cultured for 24 hours in a 37°C, 5% CO$_2$, humidified incubator in Dulbecco's Minimum Essential Media (DMEM) (Canadian Life Technologies Inc.) containing 5 $\mu$g/ml insulin (I1882, Sigma, St. Louis, MN), 100 IU penicillin (P7794, Sigma), 100 $\mu$g/ml streptomycin (S0890, Sigma), 1 $\mu$g/ml Fungizone® (15295-017, Canadian Life Technologies Inc.), and 10% new born calf serum (NBCS) (116010-142, Canadian Life Technologies Inc.). After 24 and 48 hours the media was changed to DMEM plus antibiotics, antimycotics and insulin, no NBCS.
The cultures were incubated for a further 48 hours. After four days in culture, the media was replaced with DMEM containing insulin, antibiotics, antifungal agents, 10^{-7} M 4-androstene 3-7-dione (A9630, Sigma), 100 ng/ml FSH (USDA-pFSH-I-1, USDA Animal Hormone Program, Beltsville, MD), and the culture treatments. Cultures were incubated with treatments for four days. At the end of the incubation, the conditioned media was removed and frozen at -20°C until radioimmunoassay. The cells were removed from the tissue culture plates by a 5 minute incubation with trypsin (25300-054, Canadian Life Technologies Inc.) at 37°C, pelleted in a microfuge, and frozen in 0.1% SDS (161-0302, BioRad) at -20°C until assayed for DNA content. The follistatin isotypes utilized in the bioassay included monoclonal affinity column purified recombinant porcine follistatin (rp-305 a.a. FS) and a 288 a.a. recombinant human follistatin (rh-288 a.a. FS) expressed in CHO cells (lot # B4384, National Hormone and Pituitary Program, the National Institute of Diabetes and Digestive and Kidney Diseases, the National Institute of Child Health and Human Development, and the U.S. Department of Agriculture). The activin A used in the bioassay was the same as that used in the follistatin-activin binding assays. The antibody used in the bioassay was the monoclonal antibody, 4-6D9.

**Estradiol-17β radioimmunoassay**

Estradiol-17β concentrations were determined by an established radioimmunoassay (Xu et al. 1995) (Appendix C.8). Intraassay coefficients of variation of two reference samples (0.2 ng/ml and 3.3 ng/ml) in 4 assays (n=6 replicates/assay) ranged from 5.8-15.1 and 16.3-18.3, respectively. Interassay coefficients of variation for the two reference samples were 10.5 and 18.4%, respectively.
DNA assay

DNA content was determined by fluorometry with a Hoeffer DNA Fluorometer TKO-100 (Hoefer Scientific Instruments, San Francisco, CA) according to the company’s recommended protocol. The intraassay coefficient of variation of a 50 ng/ml reference sample ranged from 4.2-10.5 % in 7 assays (n=5 replicates/assay); the interassay coefficient of variation was 8.3%.

Statistical analysis and follistatin potency determination

Significant effects of follistatin treatment were determined using the General Linear Model of analysis of variance in SAS (SAS Institute Inc. 1989, Cary, NC) testing the model: estradiol-17β = treatment. The contrast option in GLM was used to determine whether the effect of follistatin on estradiol-17β accumulation was linear. A Tukey’s studentized range test (SAS) was used to determine if treatment means were different. For each follistatin treatment the equation of a line was calculated from the relevant data points. The ED<sub>50</sub> value was extrapolated from this equation. The General Linear Model of analysis of variance was also used to test the significance of the monoclonal antibody treatment testing the model: estradiol-17β = follistatin or monoclonal antibody and the interaction between those two variables. A Tukey’s studentized range test was also used to determine if means between follistatin or follistatin monoclonal antibody treatments were different.
3.4 Results

Madin Darby bovine kidney (MDBK) cells were successfully transformed with an expression plasmid containing cDNA for a 305 a.a. porcine follistatin. This cell line is designated FCC815. Production of the recombinant porcine follistatin is induced by incubating the FCC815 cells at 43°C for six hours, with a yield of approximately 20-25 mg/liter/150cm² flask (4x10⁷ cells)/day (Figure 3.2). The recombinant porcine follistatin is secreted into the culture media so that FCC815 cells can be repeatedly heat shocked. This recombinant porcine follistatin has been identified by Western blot and activin ligand blot (Figures 3.7.1, 3.7.2, 3.8 and 3.9) and has biological activity in vitro (Figure 3.10.1). Some technical difficulties, related to follistatin purification and stability, were encountered while characterizing the recombinant porcine follistatin.

Production of Recombinant Porcine Follistatin

Production yield of recombinant porcine follistatin

To determine the yield of recombinant porcine follistatin and the number of cycles that the FCC815 cells would be viable, a confluent 150 cm² flask of FCC815 cells was repeatedly heat shocked and culture supernatants were collected daily and stored at -20°C. The FCC815 cells began to detach from the flask walls after 32 days. At day 38 the majority of the cells had detached and the heat shock cycles were discontinued. As Figure 3.3 indicates, SDS-PAGE analysis of media samples confirmed that the FCC815 cells continuously expressed the follistatin protein for a 38 day period. The system
appeared to have some variation with time, but production was relatively stable (Figure 3.2). Increased production on heat shock day 1 may be in part due to difficulty quantitating the percentage of recombinant porcine follistatin due to interference by the intensely staining 66 kDa protein band. Decreased production of recombinant follistatin on days 37 and 38 was associated with FCC815 cell death.
Figure 3.2 Yield of recombinant porcine follistatin upon repeated heat shock was quantitated to determine the yield over time. Production of recombinant follistatin was approximately 20-25 mg/liter/150cm² flask (4x10⁷ cells)/day and was fairly constant with time, decreasing after day 30 which coincided with the onset of FCC815 cell death.
Figure 3.3 A Coomassie stained 10% SDS-PAGE gel shows continuous production of recombinant porcine follistatin. The heat shock cycle number is displayed at the top of each lane. Each lane contains approximately 2 μg protein. The molecular weight of recombinant porcine follistatin ranged between 36.8 - 52 kDa, similar to the reported molecular weights of other follistatin isotypes. The protein band at approximately 66 kDa (heat shock lane 1) is most likely bovine serum albumin which is not apparent in the conditioned media by heat shock number 6 (data not shown).
Purification of Recombinant Porcine Follistatin

Purity of FCC815 conditioned media

FCC815 conditioned media contained proteins other than recombinant porcine follistatin. HSPs were identified by Western blotting (Figure 3.4). Two heat shock proteins of similar molecular mass were produced in heat shock cycle #1 and #2. With subsequent heat shocks, the concentration of heat shock proteins decreased below detectable Western blot limits. Bovine serum albumin was identified as one of the components of the conditioned media by observation of the molecular mass of the protein (Figure 3.3). FCC815 conditioned media may have contained other contaminates, such as other HSP induced by the heat shock protocol. Other HSP could not be confirmed since the HSP antibodies required for the assay, were not available.
Figure 3.4 Western blot indicating the presence of heat shock proteins in FCC815 conditioned media. Primary antibody was SPA-820 which recognizes both consitutive and induced heat shock 70 protein. Lane 1. Concentrated media from nontransformed, heat shocked Madin Darby Bovine Kidney cells; Lane 2. Eluant from concentrating media in Lane 1; Lane 3. Pool of heat shock cycles #1–4 of FCC815 conditioned media; Lane 4. Heat shock #1; Lane 5. Heat shock #2; Lane 6. Heat shock #3; Lane 7. Heat shock #4; Lane 8. Prestained Low Molecular Weight BioRad Protein Standards.
Follistatin purification by size exclusion

The protein analysis of the size exclusion centrifugation of FCC815 conditioned media indicated that 25.8% of the initial protein mass was contained in the eluant of the Centriprep 50 separation. Only 13.3% of the initial protein mass was contained in the Centriprep 10 concentrate. Electrophoresis in a 10% SDS-PAGE gel indicated that the Centriprep 50 separation did not exclusively separate protein by size. Proteins of approximately 60 kDa and lower were contained in the Centriprep 50 eluant and Centriprep 10 concentrate (gel not shown). Reanalysis of the FCC815 conditioned media in several different SDS-PAGE gels, and Western blots with several different molecular weight standards indicated that the apparent molecular weight of recombinant porcine follistatin was greater than that first recorded. Apparent molecular weight of recombinant porcine follistatin was subsequently estimated as 39, 46, 48, and 50 kDa (Figure 3.7.1).

Follistatin purification by anionic exchange

Approximately 1 mg (total protein) of FCC815 conditioned media was added to the column. Two protein peaks were eluted from the column with approximately 0.9071 and 0.9616 M NaCl. Analysis of protein content indicated that the peaks contained approximately 66 µg and 54 µg of protein, respectively. Silver staining of a 10% SDS-PAGE gel of the two spectrophotometric peaks indicated that neither peak contained a single protein species (Figure 3.5). Lane 1 clearly contained two protein bands. Lane 2 may have contained more that one protein band.
**Follistatin purification by affinity chromatography**

This protocol was attempted using several different ratios of FCC815 conditioned media to cellulose beads. All fractions were analyzed in a 10% SDS-PAGE gel; no single protein band was eluted using the sulfate ester coated cellulose beads.

**Follistatin purification by monoclonal antibody affinity chromatography**

A monoclonal antibody affinity column was used to purify follistatin from FCC815 conditioned media. Spectrophotometric analysis of the column eluant indicated the presence of a single protein peak. The eluant was separated in a 10% SDS-PAGE gel; the single protein recognized by the monoclonal antibody column was approximately 39 kDa molecular weight (Figure 3.6). This single isotype of recombinant porcine follistatin was named rp-305 a.a. FS and was used in the biological assays.

**Lability of the purified recombinant porcine follistatin**

Protein was retained on the filter of a Centricon 10 column (4205, Amicon) during concentration of rp-305 a.a. FS; only 52.4% of the protein was recovered. rp-305 a.a. FS was also lost during dialysis; recovery rates following dialysis ranged from 24-36%. rp-305 a.a. FS, frozen at -20°C in 100 mM Tris buffer pH 8.0, aggregated. In a silver stained SDS-PAGE gel, rp-305 a.a. FS samples taken following storage at -20°C contained more bands of a higher molecular weight than a sample taken prior to storage (gel not shown). The rp-305 a.a. FS used in biological assays was lyophilized as soon as possible following purification and stored at -80°C. The purified recombinant protein was dialyzed and stored in 0.01M PBS at -80°C one day prior to addition to the assay.
Figure 3.5 A silver stained, 10% SDS-PAGE gel of the anionic exchange chromatography purification of recombinant porcine follistatin. Two protein peaks were eluted by the column. Electrophoresis of the two peaks indicated that the protein peak eluted at 0.9071 M NaCl contained two protein species (Lane 1). The protein peak eluted at 0.9616 M NaCl may have contained more than one protein species (Lane 2). Bands at approximately 66 kDa are artifact associated with keratin contamination of buffers.
**Figure 3.6** A silver stained, 10% SDS-PAGE gel of the Lane 1 non-purified FCC815 conditioned media, and Lane 2 non-concentrated follistatin eluant obtained from a monoclonal affinity column. The monoclonal antibody used to obtain this purified follistatin fraction was raised against recombinant human 315 a.a. follistatin. Bands at approximately 66 kDa are artifact associated with keratin contamination of buffers.
Characterization of Recombinant Porcine Follistatin

Immunological analysis of recombinant porcine follistatin

Western blot analysis (Figure 3.7.1) of FCC815 conditioned media indicated four protein bands of approximately 39, 46, 48, 50 kDa which reacted specifically with rabbit anti-native porcine follistatin antibody. The antibody did not recognize a negative control lane containing bovine serum albumin (data not shown). The effect of post-translation modifications by the recombinant protein expression system on activin binding and biological activity of the recombinant porcine follistatin are unknown. The FCC815 conditioned media and recombinant Sf9 cell lysate of Sf9 cells transformed with the same cDNA sequence used to transform FCC815 cells (Lindsell 1992), were compared to determine if there were species differences. A Western blot, using the same procedure as Figure 3.7.1, indicated species differences in post-translation modifications (Figure 3.7.2).
Figure 3.7.1 A Western blot of FCC815 conditioned media (approximately 12 μg recombinant porcine follistatin) electrophoresed in sample buffer containing β-mercaptoethanol. A polyclonal antibody raised to native porcine follistatin recognized recombinant follistatin with apparent molecular weights of 39, 46, 48, 50 kDa.
Figure 3.7.2 Western blot of Lane 1 FCC815 conditioned media (approximately 6 μg recombinant porcine follistatin) reduced with DTT sample buffer, and Lane 2 recombinant porcine follistatin from approximately 60 μg transformed Sf9 insect cell lysate. First antibody was rabbit anti-porcine native follistatin. The blot indicates a species difference in post-translation glycosylation by two different expression systems.
**Follistatin-activin ligand blot - radiolabelled activin assay**

Autoradiographs of the incubation of radiolabelled activin A with nitrocellulose bound FCC815 conditioned media indicated a single band of approximately 39 kDa.

Preincubation of the nitrocellulose blot with 5 µg activin A prior to addition of radiolabelled activin A decreased the intensity of the 39 kDa band (Figure 3.8).

Preincubation of a βA subunit monoclonal antibody with the radiolabelled activin prior to incubation with the nitrocellulose bound follistatin did not appear to decrease the intensity of the 39 kDa band (data not shown).

**Follistatin-activin ligand blot - colour visualization assay**

When activin was electrophoresed under non-reducing conditions, the FCC815 conditioned media was able to bind specifically to the activin and a band of approximately 24 kDa was apparent (Figure 3.9, Lane 1). Reduction of activin with β-mercaptoethanol inhibited interaction of activin with the recombinant follistatin (Lane 2), indicating the importance of the dimeric structure of activin for follistatin-activin interaction.
Figure 3.8 A radiolabelled activin-follistatin ligand blot assay was developed to characterize the binding of follistatin with activin. Radiolabelled activin associated with nitrocellulose bound recombinant porcine follistatin of approximately 39 kDa (Lane 1 and 2). Preincubation of the nitrocellulose blot with unlabelled activin A decreased the binding of radiolabelled activin with the nitrocellulose bound follistatin (Lane 3 and 4). Two different lots of recombinant follistatin were used in these blots which may partially account for the difference in the 39 kDa band intensities of Lane 1 and 2.
**Figure 3.9** An activin-follistatin ligand blot assay was developed to characterize the binding of follistatin with activin. Activin was resolved in a 12% acrylamide gel under Lane 1 non-reducing and Lane 2 reducing conditions. The gel was transferred to a 0.45 μm nitrocellulose membrane and then incubated with recombinant porcine follistatin and rabbit anti-follistatin. Under non-reducing conditions a single band of approximately 24 kDa appears; reduction of the activin prevented follistatin recognition.
Biological activity of purified recombinant porcine follistatin.

Statistical analysis of the effect of recombinant follistatin on estradiol-17β accumulation on three separate sets of granulosa cell cultures experiments was conducted (n=3 replicates). The rp-305 a.a. FS significantly suppressed estradiol-17β accumulation in porcine granulosa cell cultures (p = 0.0005). Although the trend was to suppress estradiol-17β accumulation there also was considerable variation between experiments. For example, Figure 3.10.1 illustrates three replications of the recombinant follistatin dose response experiment carried out on different days. Two experiments showed a definite effect of rp-305 a.a. FS, however there was no response in the second experiment (middle graph). The estimated effective dose (ED$_{50}$) of these three combined assays was 0.9 µg/ml. A dose of 10 µg/ml of rp-305 a.a. FS was initially tested in this cell culture system. This dose appeared to detrimentally affect cell viability; cells detached from the cell culture surface at this concentration. The rh-288 a.a. FS was used to compare the biological activity of the rp-305 a.a. FS to another available follistatin isotype. Figure 3.10.2 indicates the biological activity of this 288 a.a. follistatin in the porcine granulosa cell bioassay and shows that it also significantly affected estradiol-17β accumulation (p = 0.0002); the estimated ED$_{50}$ of three assays was 14.7 µg/ml. The relationships between increasing doses of follistatin and estradiol-17β accumulation were not linear at the concentrations utilized in this experiment (rp-305 a.a. FS, p = 0.27; rh-288 a.a. FS, p = 0.45). A Tukey’s studentized range test indicated that only the 1 µg/ml rp-305 a.a. FS and the 10 µg/ml rh-288 a.a. FS treatment means were different from the other treatment means (p = 0.05). It should be noted that the ED$_{50}$ values represent potency values estimated from equations of theoretical lines.
Figure 3.10 Granulosa cells cultured from porcine antral follicles of 1-3 mm diameter were utilized to determine the biological activity of recombinant porcine follistatin. Increasing doses of rp-305 a.a. FS (0.1 ng/ml - 1 μg/ml, Figure 3.10.1) and rh-288 a.a. FS (0.1 ng/ml - 10 μg/ml, Figure 3.10.2) suppressed estradiol-17β accumulation. The six graphs represent three separate experiments for each recombinant follistatin and indicate the degree of variation between assays. Statistical analysis was carried out for each recombinant follistatin isotype (n = 3 replicates). Values on the graphs are the mean +/- standard deviation of n=3 wells/experiment. A Tukey's studentized range test for the combined effect of all follistatin assays on estradiol production indicated only the 1 μg/ml rp-305 a.a. FS and 10 μg/ml rh-288 a.a. FS treatment means were different from the other treatment means (p = 0.05).
Biological activity of an anti-follistatin monoclonal antibody

Preliminary investigations utilizing the 4-6D9 monoclonal anti-follistatin antibody indicated that the monoclonal antibody had significant biological activity in the porcine granulosa cell cultures ($p = 0.02$, Figure 3.11). When no exogenous follistatin was added to the culture treatment, increasing doses of 4-6D9 antibody stimulated estradiol-17β accumulation. A Tukey’s studentized range test indicated that the means between the 10 μg/ml and 0 μg/ml doses of follistatin monoclonal antibody were different at a significance value of $p = 0.1$. With addition of 1 μg/ml rh-288 a.a. FS to the culture treatment estradiol-17β accumulation was significantly suppressed ($p = 0.0001$) and the stimulatory effect of 4-6D9 was removed. The anti-follistatin monoclonal antibody appeared to have activity without addition of exogenous follistatin, which suggests that there may be endogenous production of follistatin in the porcine granulosa cell culture system. These conclusions are based on one experiment as the available amount of 4-6D9 monoclonal antibody was low (1 mg gift from Dr. Shintani). Further experiments should be carried out repeating and increasing the concentrations of 4-6D9 used, as well as including the rp-305 a.a. FS.
Figure 3.11 Anti-follistatin monoclonal antibody 4-6D9 (used to purify rp-305 a.a. FS) appears to enhance estradiol-17β accumulation without the addition of exogenous follistatin. Graph represents one experiment, n=3 wells/treatment, mean ± stdev. All antibody treatments, except 0 μg/ml Mab, are equalized to 10 μg/ml IgG.
Biological activity of recombinant human activin A

The biological activity of recombinant human activin A was tested in the porcinegranulosa cell assay so that the neutralizing effect of rp-305 a.a. FS on activin activitycould be investigated. However, a dose-dependent effect of human recombinant activinA on estradiol-17β accumulation was not apparent at concentrations which have beenpreviously reported to effect estradiol-17β accumulation in porcine granulosa cells (Fordand Howard 1997). Figure 3.12 shows the estradiol-17β accumulation response ofporcine granulosa cells following a 4 day incubation with activin A.
Figure 3.12

**Figure 3.12.1**

![Graph 1](image1)

**Figure 3.12.2**

![Graph 2](image2)

**Figure 3.12** Biological activity of human recombinant activin A in porcine granulosa cell culture bioassay system. A clear dose-dependent relationship between activin A treatment and estradiol-17β accumulation was not evident. Each graph represents one experiment (n=3 wells/treatment, mean ± stdev).
3.5 Discussion

Madin-Darby bovine kidney cells transformed with pFSHUneo were confirmed to produce recombinant porcine follistatin. The yield of recombinant follistatin in this system appears to be relatively consistent over time and the protein expression and/or presence in the conditioned media does not seem to detrimentally affect FCC815 cell viability. The expression system utilizes the bovine heat shock promoter region of the bovine HSP70A gene to drive transcription of the follistatin gene. The HSP70 promoter is one of the most strongly inducible promoters within mammalian cells and its induction is highly regulated. This expression system mimics the in vivo hsp70 activity and by linking the porcine follistatin gene to this promoter we have produced a highly inducible, high yield expression system.

The HSP expression system has several practical advantages. Recombinant follistatin can be synthesized in relatively large quantities (20-25 mg/l) with minimal manipulations. The promoter is induced simply by placing the cell culture at 43°C for six hours. The recombinant follistatin which is produced is secreted into the supernatant. Therefore, follistatin harvesting only requires the removal of the culture supernatant. As no cell lysis is necessary for harvesting the recombinant protein, the cells can be repeatedly induced. Furthermore, the Madin-Darby bovine kidney cell system post-translational modifications should be more similar to porcine than other available expression systems, as they have more closely related evolutionary pathways. Different species expression systems have unique post-translational events as illustrated.
in Figure 3.7.2. Other authors have noted differences in post-translational modifications between different species expression systems (Smith et al. 1985). Physiological differences between glycosylated recombinant porcine follistatin isotypes were not investigated in this thesis, since the means to differentiate between recombinant porcine follistatin isotypes were not available.

The FCC815 cells produce recombinant follistatin of four molecular masses which are recognized by a polyclonal antibody raised against native porcine follistatin. Non-reduced recombinant human activin A also recognized recombinant porcine follistatin in a radiolabelled activin A assay and a ligand blot. Chemical reduction of the activin A decreased the association of activin and follistatin below the ligand blot assay detection limits. Although non-purified FCC815 conditioned media was used in these assays, follistatin specificity was ensured by use of the polyclonal anti-follistatin antibody in the follistatin-activin ligand blot assay. Antibodies to follistatin were not used in the radiolabelled activin-follistatin binding assay. The follistatin isotype recognized was of a similar molecular weight as that recognized by the anti-follistatin monoclonal antibody used to purify rp-305 a.a. FS. Only one follistatin isotype, of approximately 39 kDa, was recognized by activin A. It is unclear why higher molecular weight forms of recombinant porcine follistatin did not interact with activin A. Glycosylation or the tertiary structure of the higher molecular weight recombinant porcine follistatin isotypes may have obstructed the activin binding site. However, de Winter et al. (1996) noted that all follistatin isoforms bound activin with similar dissociation constants. Several protocols were used to purify recombinant porcine
follistatin from the FCC815 conditioned media. The method ultimately chosen, monoclonal antibody affinity column purification, produced large amounts of a single species of purified follistatin. The monoclonal antibody used to purify FCC815 conditioned media was raised to recombinant human 315 a.a. follistatin. The monoclonal antibody recognized only the lowest molecular weight recombinant porcine follistatin expressed by FCC815 cells.

Follistatin is known to act as an activin binding protein which neutralizes the effects of activin in rat, bovine, and human granulosa cell cultures (Findlay 1993, Peng et al. 1996). The activin neutralizing ability of recombinant porcine follistatin was not examined in our biological assay system. In contrast to Ford and Howard (1997), we were not able to establish a dose-dependent effect of recombinant human activin A on estradiol-17β accumulation in granulosa cell cultures from small porcine ovarian follicles (Figure 3.12). The lack of a clear, repeatable effect of activin rendered examination of the biological neutralization of activin by recombinant porcine follistatin difficult to measure.

Chang et al. (1996), and Ford and Howard (1997), have reported that recombinant human activin A suppressed basal and FSH induced estrogen and progesterone production in porcine granulosa cell cultures, contradicting results obtained from bovine and rat granulosa cell cultures. Cataldo et al. (1994) have also indicated that activin suppressed estradiol accumulation, but only in 3/7 experiments. The results of this thesis (Figure 3.15) contrast those of Chang et al. (1996), and Ford and Howard
(1997). There may be species differences in the effect of activin, however, experimental conditions may also greatly affect the observed *in vitro* response. Growth factors, such as insulin or IGF-I, may be induced by the gonadotropin (Hammond et al. 1991) used in the bioassay which may affect steroid synthesis (Gooneraatne et al. 1990; Xu et al. 1995) confounding the effect of activin treatment. Activin itself may interact with endogenous or exogenous growth factors, further complicating the experimental system. Interactions between activin and IGF-I which affect steroid production have not yet been described. However, a recent study has shown an intricate relationship between activin and the IGF-I system (Kubo et al. 1998). In rat granulosa cell cultures, activin induced inhibin α subunit production requires IGF-I. The activin induced production of the α subunit was blocked by the addition of anti-IGF-I antibody or IGF-I binding proteins -4 or -5. The inhibition of α subunit production produced by these agents was reversed by the addition of IGF-I to the cell culture. Although all studies using porcine granulosa cells utilized substrate concentrations similar to experiments in other species, the exact experimental conditions required to simulate *in vivo* events in porcine granulosa cell cultures may be more critical.

Although effects of activin in this granulosa cell culture system were ambiguous, the effect of rp-305 a.a. FS on estradiol-17β in three replications was significant (Figure 3.10.1). rp-305 a.a. FS suppressed estradiol-17β accumulation at the 1 μg/ml dose without the requirement for exogenous activin. This activin independent effect of recombinant porcine follistatin on estradiol-17β accumulation is a novel finding. This trend was repeated in assays containing human recombinant 288 a.a. follistatin (Figure
3.10.2). Doses of 10 μg/ml rh-288 a.a. FS suppressed estradiol-17β accumulation. The effect of follistatin on estradiol-17β accumulation was reversed in the presence of the monoclonal antibody to recombinant human follistatin (Figure 3.11). Increasing doses of the monoclonal antibody enhanced estradiol-17β accumulation, confirming the activity of recombinant follistatin in the porcine granulosa cell bioassay. Addition of both the monoclonal antibody and exogenous follistatin inhibited the positive effect of the monoclonal antibody on estradiol-17β accumulation and suppressed estradiol-17β accumulation below control levels. The monoclonal anti-follistatin antibody had biological activity without addition of exogenous follistatin, suggesting that follistatin was produced by the granulosa cell cultures.

The potency of the follistatin effect on estradiol-17β accumulation varied between assays. The between assay variability may have been due partially to the nature of primary, static granulosa cell cultures. Variability in primary cell cultures can be due to cell biochemical heterogeneity, the degree of cell to cell communication or confluency, and the viability of the cultured cells. There are some studies which indicate these variables may affect estradiol-17β accumulation in porcine granulosa cell cultures. Biochemical heterogeneity of the ovulating population of follicles has been identified in swine (Hunter et al. 1992); there is also evidence that within a porcine follicle, granulosa cells have variable estrogen production potential (Ford and Howard 1997). Confluent ovine pituitary cell cultures have been shown to produce less follistatin than less confluent pituitary cell cultures (Farnworth et al. 1995). In contrast, Saito et al. (1991) demonstrated that there is a logarithmic acceleration in follistatin secretion from rat
granulosa cell cultures following an increase in the initial density of plated cells. The regulation of follistatin, activin, and inhibin is dynamic. Follicle stimulating hormone was added to the cultures to induce estrogen production, however FSH also stimulates the production of each of these growth factors in porcine granulosa cell cultures (Lindsell 1992, Demura et al. 1993). In addition, activin has been shown to modulate both follistatin and inhibin production (Findlay 1993). Therefore, the assay of biological effects of recombinant porcine follistatin overlaid endogenous, dynamic regulation of the same growth factors which may have also been affecting estradiol-17β accumulation in the cell culture system.

In order to decrease experimental error the same batches of FSH and follistatin were used throughout the experiments. To decrease the effects of granulosa cell heterogeneity, granulosa cells from a large number of follicles were pooled. All culture wells were initially plated at the same seeding density; viability of the granulosa cells was determined by Trypan blue dye-exclusion assay prior to plating. At the end of each experiment DNA content of the granulosa attached to the tissue culture wells was determined. Further understanding of the time course and complexity of the endogenous feedback systems and experimental conditions affecting the estrogen producing ability of the porcine granulosa cell system would be required before the assay variability could be decreased. To fully investigate the in vitro actions of follistatin in the porcine granulosa system a larger quantity of highly purified, highly specific reagents would be required, including each of the follistatin isoforms, activin A, AB and B and monoclonal
antibodies to each hormone isotype. Moreover, a stable granulosa cell line may be necessary to decrease assay variation.

The rp-305 a.a. FS suppressed estradiol accumulation with an estimated ED$_{50}$ of 0.9 $\mu$g/ml compared to an estimated ED$_{50}$ of 14.7 $\mu$g/ml of the rh-288 a.a. follistatin. The rp-305 a.a. FS was approximately tenfold more potent than the rh-288 a.a. follistatin. These results differ from other follistatin isoform potency results in rat pituitary cell cultures in which the 288 a.a. follistatin isoform is approximately threefold more potent than the 315 a.a. isoform (Sugino et al. 1993). The potency difference between the recombinant follistatin proteins in the porcine granulosa bioassay system may be partly artifactual, as the ED$_{50}$ values were calculated from an equation for a theoretical line. Post-translational modifications of follistatin may have differed between the expression systems used (rp-305 a.a. FS expressed in Madin Darby bovine kidney cells and rh-288 a.a. FS expressed in CHO cells). However, differences in follistatin glycosylation have not yet been shown to alter activin binding ability or potency in a rat pituitary cell culture system (Inouye et al. 1991a). The difference in potencies may be due partially to the type of assay system. The rh-288 a.a. FS was tested in a heterologous species system. Perhaps the affinity of rh-288 a.a. FS for HSPG in a human cell culture system would be greater than in the porcine cell culture system. As well, the potency of each follistatin isoform may be species or tissue culture specific. Native follistatin isoforms have been purified and investigated in bovine granulosa cell cultures. In immature bovine granulosa cell cultures the 35 kDa follistatin isoform has a
greater potency and stimulates progesterone and oxytocin production more quickly than the 31 or 39 kDa follistatin isoforms (Shukovski et al. 1991).

It appears that the suppressive effect of follistatin was through an activin independent mechanism since exogenous activin was not added to the experimental system. However, exogenous follistatin may have interacted with endogenous activin, decreasing the amount of "activin tone" in the assay wells (section 2.4.2). A Western blot demonstrated the activin binding ability of recombinant porcine follistatin (Figure 3.9). The quantity of some activin isotypes has been measured in porcine follicular fluid: 1.1 μg/ml activin A (Shintani et al. 1991) and 0.6 μg/ml activin AB (Evans et al. 1997). It is possible that in this granulosa cell assay system, only the highest concentrations of follistatin utilized were able to overwhelm the endogenous production of activin. To determine whether follistatin is acting as a binding protein in this granulosa cell assay system, activin neutralization experiments utilizing α2-macroglobulin or anti-activin antibody would be required.

This is the first report of an activin independent effect of follistatin on estradiol levels in granulosa cell culture. The results of the bioassay suggest interesting implications for the in vivo effect of follistatin as the concentrations of rp-305 a.a. FS used in the bioassay are physiologically relevant. Follistatin concentrations of 5.6 μg/ml (Wakatsuki et al. 1996) and 1-8 μg/ml follistatin (Li et al. 1997a) have been recorded in porcine follicular fluid. The 303 a.a. follistatin isotypes constitute approximately 76 % of the total follistatin (Figure 2.3, Sugino et al. 1993) and may have a comparable
potency to the 305 a.a. recombinant porcine follistatin. The estimated ED$_{50}$ for recombinant 305 a.a. follistatin in our bioassay system was 0.9 μg/ml. It is possible that similar amounts of 303 a.a. follistatin exist in the follicular fluid of porcine follicles. However, it is not clear how much follistatin might be bound to activin or HSPG. The net activity of follistatin in a particular follicle may depend on the relative concentration of all follistatin isotypes. A follicle may respond to external factors and change the degree of post-translational modification or mRNA splicing of follistatin to create a “fine tuning” system which may or may not interact with activin. To date, in-depth studies of the biological activity of different follistatin isotypes have not yet been conducted.

The recombinant porcine follistatin expressed in the heat shock expression system was recognized clearly by activin A and by anti-follistatin antibody. In a porcine granulosa cell assay the purified recombinant porcine follistatin isoform, rp-305 a.a. FS, demonstrated biological activity. This biological activity of the follistatin isoform is unique, because it suppresses the accumulation of estradiol-17β without the requirement of activin. These biological assay results suggest a need for further experiments to elucidate the physiological action of follistatin in the porcine ovary. Future experiments using porcine granulosa cell cultures will be necessary to identify the interaction of follistatin and activin in the bioassay system, the amount of free follistatin and activin present in the system, the relative proportions of the follistatin isotypes present, and the potency of follistatin isoforms in cell cultures of more differentiated granulosa. The study of Kubo et al. (1998) indicates that there may be interactions with follistatin, activin and the IGF-I system which may potentially affect estrogen production in
granulosa cells. Further experiments are also needed to elucidate the interaction of these families of intraovarian regulators.
4. ACTIVE VACCINATION AGAINST FOLLISTATIN AFFECTS REPRODUCTIVE POTENTIAL IN CYCLING GILTS

4.1 Abstract

Pork Improvement Canada primiparous sows (gilts) were vaccinated against follistatin with the objective to increase litter size (Experiment 1) and ovulation rate (Experiment 2). In Experiment 1, 47 gilts were vaccinated four times between 4 weeks of age and breeding against a recombinant porcine follistatin (FS) or a sham vaccine (CTL) and were allowed to mature naturally prior to breeding on the second or third estrus. At breeding, FS antibody titers ranged from 0 - 1:6400 in the FS vaccinated gilts, and were not detectable in the CTL gilts. Overall, follistatin vaccination did not affect the total number of piglets born. However, separation of the FS vaccinated gilts into low (<1:400, n=16) and high (>1:400, n=7) titer groups revealed differences in the total number of piglets born (FS high titer = 13.0 ± 0.8, FS low titer = 10.8 ± 0.6, CTL = 10.9 ± 0.4: p=0.08) and the number of piglets born alive (FS high titer = 12.9 ± 0.9, FS low titer = 10.0 ± 0.5, CTL = 10.3 ± 0.4: p=0.01). In Experiment 2, 69 gilts were vaccinated similarly to Experiment 1. At 85 kg, gilts were induced into estrus with a combination of PG600 and hCG, and synchronized in estrous using PGF<sub>2α</sub>. At the second estrus, antibody titers ranged from 0 - 1:6400 in the FS vaccinated treatment group and no antibodies to FS were detected in the CTL group. Later in the second luteal phase, gilts
were sent to the abattoir and reproductive tracts were collected. There was no significant difference in the number of corpora lutea (FS = 13.2 ± 0.5, CTL = 14.5 ± 0.7) or corpora albicantia (FS = 12.1 ± 1.9, CTL = 12.3 ± 2.0) between treatments. FS vaccinated gilts showed an increased number of luteal structures which resembled corpora hemorrhagica (p = 0.04) and follicles which contained blood clots. Separation of the FS vaccinated gilts into low (≤ 1:400, n=10) and high (> 1:400, n=3) titer groups suggested a negative association between titer and corpora lutea number (p=0.05). The first study showed that vaccination of gilts against follistatin increases litter size. In the second study ovarian changes occurred, but an effect of follistatin vaccination on ovulation rate was not apparent, possibly due to confounding effects of pharmacological estrus induction.

4.2 Introduction

Immunoneutralization, through either active or passive vaccination of endogenous hormones, has been investigated as a tool to increase fecundity in livestock. Active vaccination of an endogenous hormone is a highly specific and reversible process. However, the individual animal’s reaction to the immunogen can be highly variable and it is difficult to control the dynamic regulation of an endogenous hormone. Active vaccination against an endogenous hormone can induce antibodies which inhibit, enhance, or have no effect on the biological activity of the target hormone. Fecundin®, an androstenedione vaccine, is the only commercially available fecundity vaccine. It has been reported to induce a 20% increase in lamb crop (Reeves et al. 1989). Attempts to vaccinate against inhibin have increased the total number of lambs born (Wrathall et al.
1992), twinning rate in cattle (Morris et al. 1993), and ovulation rate in swine (Brown et al. 1990; King et al. 1993). To date, only one report has indicated that vaccination of an endogenous hormone, somatostatin, can increase litter size in swine (Kirkwood et al. 1990).

Follistatin, an activin binding protein, inhibits the folliculogenic actions of activin and promotes follicle luteinization (Findlay 1993). It also inhibits activin induced FSH release (DePaolo et al. 1991). As well as being expressed in the pituitary and ovarian tissues, follistatin has also been identified in other reproductive and non-reproductive tissues including placenta, decidua, uterus, kidney, pancreas, muscle, lung, brain, thymus, adrenal gland, gut, heart, and bone (Michel et al. 1993; Petraglia et al. 1994). In human placental cell culture, follistatin may inhibit progesterone synthesis through binding and neutralizing activin (Petraglia et al. 1994). The majority of investigations have focused on the physiological actions of follistatin in humans, rats, and cattle. Swine were chosen as the target species for this investigation to further elucidate the physiological function of follistatin in this species.

Follistatin immunoneutralization could potentially affect all tissues where follistatin is expressed. The objective of Experiment 1 was to determine whether vaccination against follistatin would affect the litter size of bred gilts. Vaccination against follistatin increased litter size in gilts, therefore the objective of Experiment 2 was to determine if the effect of follistatin vaccination in Experiment 1 was through increased ovulation rate.
4.3 Materials and Methods

Experiment 1

**Animals and animal care**  Camborough 15 (Pork Improvement Canada Inc., Acme, AB) gilts were housed by the Prairie Swine Center Inc., Saskatoon, SK, Canada. Experimental protocols were approved by the University of Saskatchewan Animal Care Protocol Committee and animal care was given in compliance with the standards provided by the Canadian Council on Animal Care.

**Experimental design, breeding and vaccination schedule**  Forty seven animals were assigned to two treatment groups: follistatin vaccination (FS n=23) or control vaccination (CTL n=24). All animals were allowed to mature naturally. At approximately 5 months of age the gilts were observed twice daily for estrus in the presence of a mature boar, and were bred on the second or third estrus to accommodate sow rotations. The gilts were bred by natural service, once per day for two consecutive days. The age and weight of the gilts at each estrus were recorded. Gilts received their primary vaccination at 4 weeks of age. Booster vaccinations were given at 7 weeks of age and at approximately 80 kg. A final booster vaccination was given 7-10 days prior to breeding.

**Vaccine formulation and delivery**  **Follistatin Vaccine:** Recombinant porcine follistatin was produced as described in section 3. FCC815 conditioned medium was
used in the follistatin vaccine. The conditioned medium primarily contained
recombinant follistatin (Figure 3.4), but may have contained trace amounts of heat shock
proteins (Figure 3.5). Each vaccine dose contained 200 μg of unconjugated, non-
modified FS; follistatin protein concentration was determined by the same protocol used
in section 3.3. **Control Vaccine:** The control vaccine did not contain protein. Saline
was emulsified in adjuvant and given as a sham vaccination.

Emulsification of the vaccines was achieved using a Branson 450 Sonifier (VWR
Scientific Inc., Edmonton, AB). The adjuvant used for the primary vaccination of
follistatin gilts was Freund's Complete Adjuvant (0638, Difco Laboratories, Detroit, MI).
The adjuvant used for the primary vaccination of control gilts, and for the booster
vaccinations for both treatments was Freund's Incomplete Adjuvant (0639, Difco
Laboratories). All vaccines were given in a 2 ml volume, intramuscularly at one site in
the neck region. Blood samples were taken prior to and 14 days after each vaccination.
Jugular venous blood samples were collected into plain Vacutainer tubes (Fisher
Scientific Inc., Edmonton, AB), stored at room temperature for 6 hours, at 4°C for 16
hours, and centrifuged at 2000 g for 20 minutes. Serum was stored at -20°C until
assayed for anti-follistatin antibodies.

**Experiment 2**

**Animals, animal care, vaccine formulation and delivery** Animal care, vaccine
formulation and delivery were the same as in Experiment 1 with a few exceptions.
Camborough 15 x Cana boar (Pork Improvement Canada Inc.) gilts were used. The adjuvant used for the primary vaccination against follistatin was Freund's Complete Adjuvant (15721-02, Canadian Life Technologies Inc., Burlington, ON). The adjuvant used for the primary vaccination of control gilts, and follistatin and control booster vaccinations was Freund's Incomplete Adjuvant (15720-014, Canadian Life Technologies Inc.).

**Experimental design** Sixty-nine gilts were assigned to two treatment groups: follistatin vaccination (FS n=35) and control vaccination (CTL n=34). Those gilts which did not display behavioral estrus in response to estrus induction were excluded from the experiment. The number of gilts which responded to estrus induction and continued to cycle are listed in Table 4.2.

**Vaccination schedule and estrus synchronization** Gilts received their primary vaccination at 5 weeks of age. Booster vaccinations were given at 8 weeks of age and at approximately 85 kg and 95 kg. At 114 days of age, the gilts were assigned to two blocks based on weight. At a mean weight of 85 kg, before puberty was evident, all gilts were synchronized into estrus using a pharmacological regime (Bolamba and Sirard 1996). On day 0 of synchronization each animal received a booster vaccination (booster #2), 400 IU PMSG and 200 IU hCG (PG600, Intervet, Boxmeer, Netherlands); on day 3 each gilt received 750 IU hCG (Chorulon, Intervet). From a few days prior to and through each estrus, gilts were allowed rail contact with a mature boar twice daily and observed for signs of physical and behavioral estrus. Those gilts which displayed estrus
following PG600 and hCG injection (FS n=24, CTL n=20) received a final booster vaccination (booster #3) on day 16 and 350 μg/day PGF₂α (Estrumate, Coopers Agropharm Inc., Ajax, ON) from days 16-19 to increase estrus synchrony between the gilts by lysing their corpora lutea. At day 14-17 of the second estrous cycle gilts were sent to slaughter and the reproductive tracts were collected. Sampling and serum extraction procedures were identical to those in Experiment 1.

**Ovarian morphology** Reproductive tracts were collected between days 14-17 following the second observed estrus and the number of ovulation sites was counted. During collection it became apparent that some ovaries contained potentially unusual luteal or follicular structures. Some corpora lutea structures contained a large, central blood clot similar to the appearance of a corpora hemorrhagica even though 14-17 days had passed since the last observed estrus. In addition, there appeared to be a number of follicles with dark red/black follicular fluid. All potentially unusual ovarian structures were recorded and submitted to the Veterinary Pathology Laboratory of the Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada for hematoxylin and eosin staining. Five animals (3 FS vaccinated and 2 CTL vaccinated) had fully formed corpora lutea indicating they had displayed “silent” estrus. The serum sample taken 14 days after the second booster was used for antibody titer determination and statistical analysis of treatment effects for these animals.

**Development of antibody titer assay** Two methods were used to develop an assay to quantitate the amount of antibody raised during follistatin vaccination a liquid phase titer
assay and an enzyme-linked immunosorbent assay (ELISA). **Liquid Phase Titer Assay:** Iodine\textsuperscript{125} was incorporated into rh-288 a.a. FS using chloramine T reduction (Appendix C.6). Several different methods were investigated to precipitate the antibody bound radiolabelled follistatin. The agents tested included: charcoal, protein A agarose and polyethylene glycol in various buffers, using test tubes of different material (Appendix C.6). **ELISA:** Only two follistatin isotypes were available for use as a coating antigen, rp-305 a.a. FS and rh-288 a.a. FS. Non-purified FCC815 conditioned media was used as a coating antigen. Serum was preincubated with conditioned media from non-transformed heat shocked MDBK cells or from heat shocked MDBK cells transformed with a follistatin negative expression cassette (pSVD2neo) to bind and remove antibody which recognized heat shock proteins (Appendix C.7). Purified rh-288 a.a. FS was also used as a coating antigen; this antigen was used in the final anti-follistatin antibody ELISA protocol.

**Determination of antibody titers**  **Follistatin Antibody Titers:** Follistatin antibody titer was measured by ELISA. rh-288 a.a. FS was coated at 100 ng/well in 0.01 M phosphate buffered saline (PBS) on Immulon 4 plates (011-010-3855, Dynatech Laboratories Inc., Chantilly, VA), and incubated at 4°C overnight. The next day the plates were blocked with 1% bovine serum albumin (A-4503, Sigma, St. Louis, MO) in 0.01 M PBS + 0.05% Tween 20 + 0.1mM phenylmethylsulfonylfluoride, pH 7.4 (PMSF, P-7626, Sigma) (PBST) and incubated for one hour. Serum samples diluted in PBST were added to the plate, followed by incubations with biotinylated rabbit anti-swine IgG antibody diluted 1:1000 (B-7015 Sigma), and streptavidin-horseradish peroxidase conjugate diluted
1:1000 (1231, Amersham Life Sciences Inc., Arlington Heights, IL). All incubations were for one hour, at room temperature, with constant agitation. Between incubations the plates were washed three times with PBST (no PMSF). Prior to colour development plates were also washed with ddH₂O. Antibody titer was visualized with 3,3′,5,5′-tetramethyl-benzidine dihydrochloride (T-3405, Sigma) in phosphate-citrate buffer (P-4922, Sigma). After 10 minutes the optical density at 650 nm was determined using a 96 well plate reader (Molecular Devices, Fisher Scientific, Edmonton, AB). Inter assay coefficient of variation (cv) was 5.9 % and intra assay cvs varied between 2.1 - 9.8 %. Titer was defined as one dilution of (sample - pooled CTL vaccinated serum) greater than 3 standard deviations of the blank (no serum) wells. Control Gilts: Sera from all control vaccinated gilts were tested in the follistatin antibody titer assay.

**Western blotting** Approximately 2.5 µg rp-305 a.a. FS was resolved by SDS-PAGE electrophoresis under reducing conditions in a 10% mini-protean gel (BioRad, Mississauga, ON). Electrophoresis, electroblotting and nitrocellulose blocking were the same as Western blots in section 3.3. 20 µg of anti-follistatin antibody was diluted in TTBS and incubated with the nitrocellulose membrane for sixteen hours. This was followed by biotinylated goat anti-porcine antibody (B-7015 Sigma) and streptavidin-horseradish peroxidase conjugate (1231 Amersham Life Sciences). Dilutions were 1:1000 in TTBS, and incubated at room temperature with constant agitation for one hour unless otherwise specified. The specifically bound antibodies were visualized using a horseradish peroxidase substrate kit (BioRad). 20 µg of anti-follistatin antibody was also preincubated with 80 µg rh-288 a.a. follistatin for six hours, and incubated with a
nitrocellulose blot for 16 hours to determine whether anti-follistatin antibodies could be bound competitively by a different follistatin isotype.

**Follistatin antibody bioassay, estradiol-17β radioimmunoassay, DNA assay**

All protocols were carried out as described in section 3.3. The polyclonal anti-follistatin antibody tested was from immunized gilts in Experiment 2.

**Immunoglobulin Purification with Protein A Agarose**

Protein A agarose slurry (0020334, Pierce) was washed twice with 10 mM Tris pH 7.5. One ml of gilt serum, 1 ml 10 mM Tris pH 7.5 and 1 ml protein A agarose were incubated for 1 hour at room temperature with constant agitation. The slurry was poured into a disposable column and the eluant discarded. The protein A agarose was washed with 10 bed volumes of 100 mM Tris pH 7.5 and 10 bed volumes of 10 mM Tris pH 7.5. The immunoglobulins were eluted with 100 mM glycine pH 3.0 and 1.0 M Tris pH 7.5 was immediately added to eluant to neutralize pH. Immunoglobulin concentration was determined by O.D. at 280 nm.

**Statistics** The effects of vaccine treatment and anti-follistatin antibody titer group were determined using the General Linear Model of analysis of variance in SAS (SAS Institute Inc. 1989) testing the model: parameter = treatment or antibody titer group. Means comparisons were done using LSMEANS in SAS. Each data population was examined for distribution skewness. A chi-square analysis was done to determine if the proportion of gilts entering estrus differed between the CTL and FS vaccinated groups.
The effects of follistatin antibody and follistatin on estradiol-17β accumulation were determined using the General Linear Model of analysis of variance in SAS testing the model: estradiol-17β accumulation = follistatin antibody or follistatin dose.

4.4 Results

Anti-follistatin antibody titer assay  Liquid Phase Titer Assay  Incorporation of $^{125}$I into rh-288 a.a. follistatin ranged from 23.8 - 69.1 μCi/μg. Separation on a Sephadex G100 column revealed only one radioactive compound. Each permutation of precipitation agent, buffer, and test tube precipitated approximately 60% of the radioactive label. Incubation with serum from follistatin vaccinated animals did not change the total precipitated counts indicating there was not a good separation of antibody bound radiolabelled follistatin from radiolabelled follistatin. The optimal separation protocol may not have been utilized, or radiolabelling follistatin may have altered (damaged) the follistatin antigen so that it was not recognized by anti-follistatin antibody. Subsequent attempts focused on developing an ELISA based assay system.

ELISA: Preincubation of serum from follistatin vaccinated animals with conditioned media from heat shocked, transformed MDBK cells (pSVD2neo) gave an ELISA with a typical antibody dilution profile (Figure 4.1). False positives, those gilts with an antibody titer to HSP (or other contaminants of the FCC815 conditioned media not contained in heat shocked pSVD2neo conditioned media), could not be identified. As well, the between day coefficient of variation using this assay system was 22%.

Therefore the rh-288 a.a. FS was used as a coating antigen to determine the anti-follistatin antibody titers in Experiment 1 and 2 as rh-288 a.a. FS is a purified protein.
Figure 4.1 ELISA plates coated with FCC815 conditioned media were used to determine anti-follistatin antibody titer. Preincubation of serum samples from FS vaccinated gilts with conditioned media from heat shocked pSVD2neo cells indicates that diluted samples followed a typical ELISA dilution curve.
Anti-follistatin antibody responses and characterization

**Antibody titer**  In both experiments, not all of the gilts vaccinated with recombinant porcine follistatin developed a measurable titer. The range of antibody titers was from not detectable to 1:6400 in both Experiment 1 and 2 (Figures 4.2 and 4.3). Gilts vaccinated with the control vaccine had no measurable titer to follistatin.

**Antibody specificity**  The *in vitro* binding ability of the induced anti-follistatin antibodies was measured in a Western blot (Figure 4.4). Anti-follistatin antibody recognized recombinant porcine follistatin (Lane 1) of approximately 49 kDa molecular weight. Preincubation of the anti-follistatin antibody with 80 µg rh-288 a.a. FS decreased the binding of the antibody to the recombinant porcine follistatin (Lane 2).
Figure 4.2 A scattergraph of the antibody titers induced in follistatin vaccinated gilts in Experiment 1. Blood samples were taken during the first day of breeding.

* Animals which had undetectable antibody titers are listed in the scattergraph as "0" antibody titer.
Figure 4.3 Scattergraphs of the antibody titers induced in follistatin vaccinated gilts in Experiment 2. Blood samples were taken during the first day of the second observed estrus.

* Animals which had undetectable antibody titers are listed in the scattergraph as "0" antibody titer.
Figure 4.4 A Western blot indicating that 20 μg of an antibody raised to recombinant porcine follistatin bound recombinant porcine follistatin (Lane 1) of approximately 49 kDa molecular weight. Competitive binding of 20 μg of anti-follistatin antibody to 80 μg rh-288 a.a. FS decreased the binding of these anti-follistatin antibodies to recombinant porcine follistatin immobilized in the nitrocellulose (Lane 2).
Biological activity of polyclonal anti-follistatin antibodies

The porcine granulosa cell assay was used to determine whether immunoglobulin from follistatin vaccinated gilts (Experiment 2) neutralized the activity of exogenous follistatin \textit{in vitro}. The immunoglobulin used was affinity purified from serum samples; anti-follistatin antibody was not separated from the total immunoglobulin population. A range of results was observed (Figures 4.5 and 4.6). The immunoglobulin from FS vaccinated gilts appeared to suppress estradiol-17β accumulation in porcine granulosa cell cultures (Figure 4.5.1, antibody group: \( p = 0.01 \), antibody dose: \( p = 0.02 \)), contradicting results with an anti-follistatin monoclonal antibody (Figure 3.11). In experiment 4.5.2, 100 \( \mu \text{g/ml} \) IgG from FS vaccinated gilts appeared to suppress estradiol-17β accumulation relative to CTL IgG, however, in this experiment the CTL immunoglobulin gave high estradiol-17β concentrations relative to other antibody experiments (Figure 4.5.1, 4.6.1, 4.6.2). In experiment 4.5.2, the total amount of immunoglobulin added into the culture wells was not equalized, making interpretation of the biological activity of the immunoglobulin from FS vaccinated gilts difficult; statistical analysis was not carried out on this experiment. Two more experiments were carried out on the same day in which all incubations contained equal amounts of immunoglobulin. In either experiment, follistatin did not significantly suppress estradiol-17β accumulation and the swine anti-follistatin antibodies did not significantly enhance estradiol-17β accumulation (Figures 4.6.1 and 4.6.2).
Figure 4.5.1

Effect of Antibody on Estradiol-17β Accumulation in Granulosa Cell Culture

- CTL IgG
- IgG from non detectable anti-FS Ab titer gilts
- IgG from low anti-FS Ab titer gilts
- IgG from high anti-FS Ab titer gilts

Figure 4.5.2

Effect of Antibody and rp-305 a.a, FS on Estradiol-17β Accumulation in Granulosa Cell Culture

- 100 ug/ml CTL IgG
- 0 ug/ml IgG
- 1 ug/ml IgG from FS gilts
- 10 ug/ml IgG from FS gilts
- 100 ug/ml IgG from FS gilts

Figure 4.5 The effect of affinity purified IgG from FS vaccinated gilts on estradiol-17β accumulation in the porcine granulosa cell culture assay. The IgG from the FS immunized gilts appears to have biological activity, without the addition of exogenous follistatin, suppressing estradiol-17β accumulation at the highest concentration of antibody tested (Figures 4.5.1 and 4.5.2). The anti-follistatin antibody titer groups refer to: those which did not develop an anti-follistatin antibody titer, or developed a low (≤ 1:400) or high (> 1:400) anti-follistatin antibody titer (Figure 4.5.1). In Figure 4.5.2, "IgG from FS gilts" refers to the amount of IgG purified from the serum of high anti-follistatin antibody titer gilts. The control IgG was purified from CTL vaccinated gilts. Each graph represents one experiment (n=3 wells/treatment, mean ± stdev).
Figure 4.6.1

Effect of Antibody and Follistatin on Estradiol-17β Accumulation in Granulosa Cell Culture

![Graph showing the effect of antibody and follistatin on estradiol-17β accumulation in granulosa cell culture.](image)

Figure 4.6.2

Effect of Antibody and Follistatin on Estradiol-17β Accumulation in Granulosa Cell Culture

![Graph showing the effect of antibody and follistatin on estradiol-17β accumulation in granulosa cell culture.](image)

**Figure 4.6** Effect of anti-follistatin antibody from high titer gilts plus rp-305 a.a. FS or rh-288 a.a. FS on estradiol-17β accumulation. Each graph represents one experiment (n=3 wells/treatment, mean ± stdev). All antibody treatments are equalized to 100 μg/ml IgG/well.
Effect of vaccination on maturation and litter size  Table 4.1 lists the effect of follistatin vaccination on litter characteristics in Experiment 1; Table B.1 contains the raw animal data. Vaccination against follistatin did not affect the timing of puberty; the gilts first displayed estrus at approximately 105 kg and 176 days of age. The gilts were bred by natural service at their second or third estrus. As a group, vaccination against follistatin did not affect litter size statistically (total piglets born p = 0.5, piglets born alive p = 0.4), however, separation of FS vaccinated gilts into low and high titer groups demonstrated a difference between titer groups and between high titer and control groups (total number of piglets born, FS high titer = 13.0 ± 0.8, FS low titer = 10.8 ± 0.6, CTL = 10.9 ± 0.4; p=0.08: number of piglets born alive, FS high titer = 12.9 ± 0.9, FS low titer = 10.0 ± 0.5, CTL = 10.3 ± 0.4; p=0.01: number of stillborn piglets, FS high titer = 0.1 ± 0.1, FS low titer = 0.6 ± 0.2, CTL = 0.4 ± 0.2; p=0.6: number of mummified piglets, FS high titer = 0, FS low titer = 0.2 ± 0.1, CTL = 0.3 ± 0.1; p=0.5). Although there was no significant difference in the number of mummified piglets between antibody titer groups, the lower numbers of mummified piglets in the FS high titer group significantly increased the significance of the follistatin antibody effect on the "number of piglets born alive" relative to the "total piglets born" comparison. The effect of both the boar and the estrus in which the gilts were bred was analyzed using the General Linear Model of SAS and was found to not affect litter size (statistical data not shown).
Table 4.1 The effect of active vaccination against follistatin on maturation and litter size (mean ± SEM) of gilts allowed to mature naturally.

<table>
<thead>
<tr>
<th></th>
<th>Vaccine Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Follistatin Vaccinated</td>
</tr>
<tr>
<td></td>
<td>Low Titer (≤1:400)</td>
</tr>
<tr>
<td>n</td>
<td>16</td>
</tr>
<tr>
<td>Piglets Born Alive</td>
<td>10.0 ± 0.5x</td>
</tr>
<tr>
<td>Stillborn Piglets</td>
<td>0.6 ± 0.2x</td>
</tr>
<tr>
<td>Mummified Piglets</td>
<td>0.2 ± 0.1x</td>
</tr>
<tr>
<td>Total Piglets</td>
<td>10.8 ± 0.6z</td>
</tr>
<tr>
<td>First Estrus Age (Days)</td>
<td>175.2 ± 3.9x</td>
</tr>
<tr>
<td>First Estrus Weight (kg)</td>
<td>105.0 ± 2.7x</td>
</tr>
</tbody>
</table>

ab values with different superscripts indicate a difference p < 0.05 between FS and CTL groups

ux values with different superscripts indicate a difference p < 0.05 between means in low titer, high titer, and CTL gilts

yz values with different superscripts indicate a difference p < 0.1 between means in low titer, high titer, and CTL gilts
**Estrus synchronization** In Experiment 2, gilts came into estrus 3-8 days after injection with PG600. The interval between first and second estrus ranged from 18-28.5 days. There was no significant difference between treatments for the duration to the onset of estrus at either the first (p = 0.9) or second estrus period (p = 0.2). A chi-square analysis indicated that FS vaccination did not significantly affect the number of animals cycling at either estrus (Figure 4.2).

**Effect of vaccination on ovulation rate** The ovaries of one follistatin vaccinated gilt in Experiment 2 contained 60 corpora albicantia. The test of skewness indicated this gilt was an outlier and it was removed from further statistical analysis (final analysis: FS n=13; CTL n=15). There was no significant difference in the number of corpora lutea or corpora albicantia between vaccination treatments. Grouping the gilts by antibody titer indicated some differences between titer groups (Table 4.3). Those gilts with an antibody titer > 1:400 had fewer corpora lutea than low titer gilts or control vaccinated gilts (p=0.05).
Table 4.2 A chi-square analysis of the proportion of follistatin vaccinated animals which exhibited estrus compared to the proportion of control vaccinated animals which exhibited estrus at two estrus periods.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Induced Estrus</th>
<th></th>
<th>Second Estrus</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Observed</td>
<td>$\chi^2$ and</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td>Number of</td>
<td>in Estrus</td>
<td>p-values</td>
<td>Number of</td>
</tr>
<tr>
<td>Gilts</td>
<td></td>
<td></td>
<td></td>
<td>Gilts</td>
</tr>
<tr>
<td>Follistatin Vaccination</td>
<td>35</td>
<td>24</td>
<td>0.71, p=0.40</td>
<td>24</td>
</tr>
<tr>
<td>Control Vaccination</td>
<td>34</td>
<td>20</td>
<td></td>
<td>20</td>
</tr>
</tbody>
</table>
Table 4.3 The effect of active vaccination against follistatin in gilts on the number of ovarian structures (mean ± SEM).

<table>
<thead>
<tr>
<th>Vaccine Treatment</th>
<th>Follistatin Vaccinated</th>
<th>Control Vaccinated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low Titer (≤1:400)</td>
<td>High Titer (&gt;1:400)</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Corpora Lutea</td>
<td>13.9 ± 0.5\textsuperscript{a}\textsuperscript{u}</td>
<td>11.0 ± 0.6\textsuperscript{x}</td>
</tr>
<tr>
<td>Corpora Albicantia</td>
<td>13.1 ± 2.2\textsuperscript{x}</td>
<td>8.7 ± 4.2\textsuperscript{x}</td>
</tr>
<tr>
<td>Corpora Hemorrhagica</td>
<td>1.4 ± 0.7\textsuperscript{a}</td>
<td>0.3 ± 0.3\textsuperscript{u}</td>
</tr>
<tr>
<td>Hemorrhaged Follicles</td>
<td>0.3 ± 0.2\textsuperscript{x}</td>
<td>0.3 ± 0.3\textsuperscript{x}</td>
</tr>
</tbody>
</table>

\textsuperscript{a,b} values with different superscripts indicate a difference \( p < 0.05 \) between FS and CTL groups.

\textsuperscript{u,x} values with different superscripts indicate a difference \( p < 0.05 \) between means in low titer, high titer, and CTL gilts.
**Ovarian tissue characteristics**  In Experiment 2, more luteal structures ($p = 0.04$) containing a central blood clot, resembling corpora hemorrhagica, were observed in follistatin vaccinated gilts (Figures 4.7.1 and 4.7.2). Fully developed luteal cells had formed around the periphery of these structures, yet along the septa and the center of the luteal structures, cells were in an intermediary state. These intermediary cells had a fibroblastic-like shape, a less darkly stained nucleus, and an increased proportion of cytoplasm to nucleus relative to normal, healthy granulosa cells. In some of the ovaries containing corpora hemorrhagica deposits of hemosiderin, neutrophils, basophils and leukocytes were found within the intermediary area of the luteal structure. The follistatin vaccinated gilts had a greater incidence of follicles containing a blood clot (Table 4.3 and Figure 4.8), although this trend was not statistically significant ($p = 0.4$). A total of 8 hemorrhaged medium follicles were observed in 4/14 follistatin vaccinated animals. Only 2 control vaccinated pigs had 2 small hemorrhaged follicles (Table B.2). Hematoxylin and eosin staining of the hemorrhaged follicles showed all follicles to be in a state of atresia.
Figure 4.7.1 A photograph of formalin fixed ovaries of two follistatin vaccinated gilts. The ovary on the left contained four sites of incomplete luteinization.
Figure 4.7.2 H and E stain of the corpora hemorrhagica-like structure on the top of the right hand ovary of Figure 4.7.1. 20X magnification. Luteal cells around the perimeter of the luteal structure had a normal morphology. The cells surrounding the blood clot appeared to be in an intermediate stage between granulosa and luteal cells. Cells had a fibroblastic shape, a smaller nucleus:cytoplasm ratio and a less dark staining nuclei than granulosa cells. Subsequent staining with Prussian Blue showed that areas which did not stain during hematoxylin and eosin staining were deposits of hemosiderin.
Figure 4.8 A photograph of a frozen ovary from a follistatin vaccinated animal which contained a hemorrhaged follicle.
4.5 Discussion

Immunoneutralization of follistatin requires the induction of specific anti-follistatin antibodies which are able to bind and neutralize the biological activity of follistatin. *In vitro*, we have demonstrated that the antibodies produced against recombinant porcine follistatin bind recombinant porcine follistatin (Figure 4.4). Binding of the anti-follistatin antibodies to recombinant porcine follistatin was competitively displaced by preincubation of anti-follistatin antibodies with rh-288 a.a. FS illustrating the specificity of the interaction.

The results of the biological activity of the polyclonal anti-follistatin antibodies *in vitro* were disappointing. The objective of this area of investigation was to determine whether the biological activity of the anti-follistatin antibodies was opposite that of the follistatin antigen itself. This would indicate that the anti-follistatin antibodies would be able to neutralize the *in vivo* activity of endogenous follistatin. However, the polyclonal anti-follistatin antibodies suppressed (Figure 4.5) or had no effect on estradiol-17β accumulation (Figure 4.6). In contrast, a monoclonal anti-follistatin antibody specifically enhanced the accumulation of estradiol-17β in porcine granulosa cell cultures (Figure 3.11). The biological activity of the monoclonal and polyclonal anti-follistatin antibodies may have differed due to the increased concentration and specificity of the monoclonal antibody relative to the serum purified polyclonal antibody. The results of the polyclonal antibodies experiments indicate the difficulty investigating the inhibitory effect of a substance in a dynamic biological system. Biological assays
examining the neutralizing effects of antibodies directed against pathogens have been successful in obtaining a quantitative value for the biological activity of the antibody. In these assays, de novo synthesis of the target antigen is more easily controlled. Neutralizing assays for antibodies directed against endogenous hormones must examine the activity of the antibody in a system where the antigen is also a dynamically regulated hormone. Possible feedback regulation of follistatin (or other factors which affect estradiol-17β accumulation) may have masked the effects of anti-follistatin polyclonal antibodies in the biological assay.

In both experiments, the anti-follistatin antibody response was variable and included a number of animals which did not produce measurable titers to the follistatin vaccine. The lack of anti-follistatin antibody response may have been the result of a poor immune response or limitations in the follistatin antibody assay system. Those animals which did not recognize follistatin as “foreign” may not have had effective vaccine antigen processing and presentation within the MHC molecule of the APC. As well, there may have been a lack of antigenic epitopes in the recombinant porcine follistatin vaccine. The vaccine contained FCC815 conditioned media in which the recombinant porcine follistatin was not modified or conjugated. Unmodified, unconjugated porcine follistatin would not normally be expected to be antigenic. However, the vaccine pretrial experiment (Appendix A) indicated that unmodified, unconjugated follistatin in Freund’s adjuvant was more antigenic than biotinylated, avidin associated follistatin in Freund’s adjuvant. The molar ratio of follistatin to biotin utilized in the pretrial experiment may not have been optimal; biotinylation may have
altered or covered important antigenic determinants. Further studies of antigen carriers and coupling ratios will be necessary to increase the immunogenicity of the follistatin vaccine so that a more consistent and adequate immune response is induced.

There were several limitations to the antibody titer assay. The antibody titer to follistatin may have been below the limits of the assay. A serum titer assay measures the unbound antibody in the circulation. In those animals in which no antibody titer was detected, a small antibody response may have been induced, but binding by endogenous follistatin may have prevented measurement in the assay system. It would have been more appropriate to measure the antibody titer in follicular fluid during estrus, but this was impossible to measure due to technical limitations. The heterologous assay system using rh-288 a.a. follistatin as a coating antigen to capture antibodies raised against recombinant porcine follistatin may have provided a lower estimate of the antibody response. Glycosylation of the rh-288 a.a. FS and recombinant porcine follistatin may have been sufficiently different to affect antibody recognition of the rh-288 a.a. follistatin. Furthermore, some epitopes recognized by the follistatin antibodies may have been directed to the C-terminal end of the recombinant porcine follistatin which was not expressed in the rh-288 a.a. follistatin isoform. Although these assay limitations were recognized by the investigators before the experiments began, rh-288 a.a. follistatin was chosen as the ELISA coating antigen as it was the most readily available, purified follistatin. Also, false positives could not be eliminated and coefficients of variation were higher in assays using recombinant porcine follistatin than in those obtained using
rh-288 a.a. FS. Difficulties in heterologous assay systems for follistatin have been noted by others (Gilfillan and Robertson 1994).

The FCC815 conditioned media contained heat shock proteins, as well as recombinant porcine follistatin, the major protein secreted by heat shocked FCC815 cells (Figures 3.3 and 3.4). There is some indication that heat shock proteins are antigenic (Newport 1991) and may chaperone antigenic peptides during MHC class I restricted antigen presentation (Blachere et al. 1993; Suō and Srivastava 1995). However, conjugation, rather than association, of a vaccine antigen with the heat shock protein may be necessary to illicit an immune response (Barrios et al. 1994). Almost all investigations involving heat shock proteins utilize the heat shock proteins isolated from the particular tumor, virus, bacterium or parasite that is being investigated. One investigation utilizing heat shock proteins from normal mammalian tissue suggests that the peptides associated with the heat shock proteins from normal tissues are not antigenic (Udono and Srivastava 1993). These combined studies suggest that the heat shock proteins in the FCC815 conditioned media may not have contributed to the immune response following active follistatin vaccination.

The titer of anti-follistatin antibody raised was variable (Figures 4.2 and 4.3). Only 7/23 of the FS vaccinated gilts in Experiment 1 achieved an antibody titer of >1:400. As a group, FS vaccination did not increase litter size, however active vaccination against endogenous follistatin increased litter size in gilts with titers >1:400 (Table 4.1). The increase in number of piglets born alive in the high anti-follistatin
antibody gilts was greater than the increase in number of piglets born alive following somatostatin vaccination (Kirkwood et al. 1990). However, there was less individual variability in animal antibody titer in response to the somatostatin vaccine.

Ovulation rate can be modulated by affecting FSH secretion, follicle recruitment, selection, or atresia. Vaccination against follistatin may have affected follistatin physiology both in the pituitary or the ovary. However, only one variable was examined due to experimental limitations for example, inability to daily observe and track folliculogenesis by ultrasound in swine, the cost limitations of housing and technician labor, and biosecurity constriction restricting time of entering swine housing after entering the abbatoir. This variable was the number of corpora lutea following the second estrus after pharmacological estrus induction.

Follistatin vaccination did not significantly increase ovulation rate following pharmacological estrus induction or the subsequent estrous cycle (Table 4.3). Subdivision of the follistatin vaccinated group into similar titer groups, as in Experiment 1, gave contradictory results. Those gilts with an antibody titer > 1:400 had fewer corpora lutea than low titer or control vaccinated gilts. However, these observations must be interpreted with caution as the number of animals in the high titer group was small (n=3).

Vaccination against somatostatin (Kirkwood et al. 1990) and inhibin (Brown et al. 1990; King et al. 1993) have increased ovulation rates in swine. It is interesting that
follistatin vaccination did not induce an increase in ovulation rate in Experiment 2. However, a stimulatory effect of follistatin vaccination on ovulation rate cannot be dismissed at present. The differences in animal management between Experiment 1 and 2 may have affected follistatin or activin regulation. The pharmacological agents used to induce estrus have been shown to affect follistatin, inhibin α subunit, and activin receptor type II expression (Shimasaki et al. 1989; Shinozaki et al. 1992; Tuuri et al. 1994; Eramaa et al. 1995; Fraser et al. 1995). The gilts in Experiment 2 also underwent the physical stress as a result of more frequent blood sampling and injections, as well as psychological stress of frequent animal handling and pen mixing. The effect of stress on ovarian follistatin regulation is unknown, yet there is evidence suggesting that stress affects non-gonadal sources of follistatin. RU28362, a synthetic glucocorticoid, decreases follistatin production in porcine endothelial cells (Michel et al. 1996). Surgery has been shown to stimulate follistatin synthesis in male and female gonadoectomized or sham gonadoectomized sheep (Klein et al. 1993; Phillips et al. 1996). These experiments illustrate the dynamic regulation of a non-gonadal source of follistatin in response to stress, although the exact mechanism leading to increased follistatin concentrations is not clear. It is conceivable that a non-gonadal source of follistatin was stimulated by stress in gilts in Experiment 2. This non-gonadal source of follistatin may have bound up circulating anti-follistatin antibody so that less antibody was present in gilt ovaries. The range of antibody titers in both experiments is similar, however, the proportion of animals with a titer > 1:400 was smaller in Experiment 2.
Although ovulation rate was not altered in Experiment 2, the ovaries of follistatin vaccinated gilts in Experiment 2 contained unusual luteal and follicle structures (Figures 4.7.1, 4.7.2 and 4.8). The follistatin vaccination group contained ovarian luteal structures which appeared to be in an intermediary stage between corpora hemorrhagica and corpora lutea. It is difficult to determine whether these structures represent secondary ovulation sites or delayed luteinization of normal or abnormal ovulation sites since the gilt’s ovaries were not scanned daily using ultrasonography. However, no gilts displayed a prolonged estrus or two periods of estrus within three weeks. The periphery of these corpora hemorrhagica-like sites contained what appeared to be fully formed luteal cells. Therefore, these corpora hemorrhagica-like sites are probably delayed luteinization sites. The follistatin vaccinated group contained a greater number of follicles with blood clots and these follicles appeared to be atretic. It is unclear how follistatin vaccination may have delayed luteal formation or facilitated follicle atresia. The hemosiderin and blood clots may be indicative of wound formation. Anti-follistatin:follistatin complexes could have precipitated, bound and activated complement which could initiate an inflammatory and a blood coagulation reaction. Follistatin has been localized throughout the granulosa layer in follicles and proximal to capillaries in luteal cells (Singh 1997). A large amount of antibody:antigen at these sites, especially in the luteal structures, potentially could disrupt blood circulation. However, this theory is difficult to examine, because specific antibodies directed to components of swine complement are not available. Staining for swine immunoglobulin would be difficult. General staining would be high and a method for quantifying the amount of stained immunoglobulin at the wound site relative to a non-wound site would
need to be developed. The tissues containing these potential wound sites would need to be doubly stained for follistatin and immunoglobulin, or an antibody specific for follistatin bound immunoglobulin would need to be developed.

The effect of follistatin vaccination and genotype was not fully explored in this thesis. Two different swine lines were utilized in Experiment 1 and 2, due to limited numbers of the Camborough 15 gilts. The gilts used in Experiment 2 are raised commonly as market hogs due to lean growth characteristics of the Cana sire. Metabolic differences between the two lines may have affected the reproductive characteristics of the gilts. A small study carried out by the PSCI indicated that litter numbers of Camborough 15 x Cana gilts bred by artificial insemination were similar to those of Camborough 15 bred by artificial insemination.

Active vaccination against follistatin induced biological effects. Litter size in bred gilts with an anti-follistatin antibody titer of >1:400 was increased significantly. The presence of antibodies against recombinant porcine follistatin during pharmacologically induced estrus may have disrupted the normal process of luteinization and follicle development. This may have resulted in an increased number of corpora hemorrhagica-like structures and hemorrhaged follicles in midluteal phase gilts. Further studies in naturally maturing and cycling Camborough 15 gilts with an improved follistatin vaccine may be necessary to detail the effect of follistatin immunoneutralization on ovulation rate and ovarian characteristics.
4.6 Implications

This is the first experiment which has shown that vaccination against follistatin increases litter size in naturally cycling swine. In this experiment gilts received four vaccinations against follistatin, but only 7/23 animals were shown to have an increased litter size which was associated with an increased anti-follistatin antibody titer. An improved immune response would be necessary to confirm the increase in swine fecundity before commercial applications could be considered.
5. GENERAL DISCUSSION

The physiological actions of follistatin, activin, and inhibin have been investigated in rat, mouse, human, bovine, and sheep experimental systems. In these systems follistatin has been demonstrated to facilitate follicle luteinization by reversing activin induced FSH receptor induction, estrogen production, granulosa cell proliferation, and morphological changes (Findlay 1993). There is some indication that in bovine granulosa and thecal cell cultures, and human granulosa-lutein cell cultures, follistatin has activin independent effects (Shukovski et al. 1991; Li et al. 1993; Shukovski et al. 1993). Contrary to its effects in other mammalian cell cultures, activin suppresses estradiol accumulation in porcine cultured granulosa cell cultures (Chang et al. 1996; Ford and Howard 1997). The in vivo physiological actions of follistatin in swine have not yet been determined. To investigate follistatin activity in the porcine ovary, a recombinant porcine follistatin was cloned and expressed. The biological action of the recombinant porcine follistatin in porcine granulosa cell culture was investigated and gilts were vaccinated with the recombinant porcine follistatin to determine effects of follistatin immunoneutralization on reproductive parameters.

Prior to initiating investigations into the biological activity of the recombinant porcine follistatin, several technical problems had to be resolved. Recombinant porcine
follistatin had to be purified from FCC815 conditioned media (section 3). Purification of a single, biologically active recombinant porcine follistatin was difficult. The recombinant follistatin was labile and a variety of specific, high purity reagents (anti-follistatin antibody, other follistatin isoforms, activin isoforms), which would be useful for purifying and characterizing the recombinant porcine follistatin, were not available. Due to these technical difficulties only one recombinant porcine follistatin isoform, rp-305 a.a. FS, was purified and characterized in this thesis. The biological activity of rp-305 a.a. FS in vitro was compared to the only other readily available follistatin isoform, rh-288 a.a. FS. Both the rp-305 a.a. and rh-288 a.a. FS suppressed the accumulation of estradiol-17β in porcine granulosa cell cultures. The potency of both follistatins varied between experiment replications, possibly due to daily variations inherent to the granulosa cell culture system.

Both rp-305 a.a. FS and rh-288 a.a. FS confirm that follistatin suppressed estradiol-17β accumulation in porcine granulosa cells cultured from small follicles, but there were potency differences between the two recombinant follistatin isoforms tested. The ED₅₀ of rp-305 a.a. FS was 0.9 µg/ml and the ED₅₀ of rh-288 a.a. FS was 14.7 µg/ml. The HSPG disassociation constant, which is affected by the length of the follistatin amino acid sequence, may be the most influential variable affecting potency. In a rat anterior pituitary cell culture bioassay, the potency of the 288 a.a. form exceeds that of both the 303 a.a. and the 315 a.a. forms. The 288 a.a. follistatin form has been shown to associate with proteoglycans on the cell surface with the greatest affinity (Findlay et al. 1993; Sugino et al. 1993). It is thought that the highly acidic amino acid
region at the C-terminal end of the 315 a.a. and 303 a.a. follistatin associates with the basic amino acid, heparan sulfate binding region and decreases the affinity of the longer follistatin isoforms for the HSPG on cell surfaces. The disassociation constant of follistatin isoforms associated with HSPG has been shown to be positively correlated with the rate of endocytic degradation in rat anterior pituitary cell cultures (Hashimoto et al. 1997). The recombinant follistatin produced in this thesis research contained 305 a.a.; the highly acidic amino acid stretch finishes at amino acid number 304. There was an additional tyrosine molecule in this recombinant protein, which may have interfered with the interaction of the acidic tail with the main body of the follistatin protein. As a result, this recombinant follistatin may have been able to bind HSPG more tightly than the 288 a.a. recombinant human follistatin. Other authors have indicated the physical interaction of follistatin, HSPG, activin, and activin receptors may differ between cell systems (de Winter et al. 1996).

The association of follistatin, HSPG, and activin has been the most investigated mechanism of follistatin action model. However, this model does not explain adequately all experimental observations or allow for the possibility that follistatin exerts biological effects other than through binding to activin. There is evidence showing different effects of follistatin and activin on progesterone metabolism, which suggests a follistatin mode of action independent of activin (section 2.3.3.1). As well, comparison of the loss-of-function mutant mice indicates that follistatin may bind to other members of the TGFβ family (section 2.3.3.4). When examining the role of follistatin in porcine granulosa cell cultures it was observed that specific inhibitory actions of follistatin may not necessarily
be dependent on the presence of exogenous activin. This does not rule out that follistatin may have neutralized endogenous activin production by the granulosa cells, as this effect could not be identified. Alternative mechanisms of follistatin action include: a) binding to an independent follistatin receptor or b) follistatin interaction and modulation of inhibin, TGFβ, or other members of the TGFβ family. Further experiments with specific activin, inhibin, and TGFβ inhibitors are necessary to further elucidate the various actions of follistatin in the porcine ovary.

In the ovary, follicular health is determined by its estrogenic activity. The ovulating follicle population has higher estrogen:progesterone ratios in follicular fluid. Yet the relative proportions and interactions of intrafollicular factors controlling the synthesis of estrogen, which leads to ovulating or non-ovulating follicles, are not understood. Follistatin may be one of the factors that affects follicle selection for ovulation. Production and secretion of follistatin by follicles selected for ovulation may decrease estrogen synthesis in those follicles not selected for ovulation. For example, several different follistatin isoforms exist and the isoforms appear to have different potencies. Differential expression of the various follistatin isoforms may be a method to regulate estrogen production selectively. Follicles may constantly produce follistatin throughout folliculogenesis, yet the particular follistatin isoform produced, or the amount of production of an isoform, may change depending on the biochemical activity of the follicle or of its neighbors. For example, once a follicle has been selected for ovulation the follicle may increase production of both the 288 a.a. and 303 a.a. follistatin (or 315 a.a. follistatin). The 288 a.a. follistatin may bind HSPG of the follicle in which it was
produced and the longer isoforms may exert their biological effect on neighboring follicles. In this manner, the follicle protects its own estrogen production (as the shorter isoform does not potently suppress estrogen production, section 3), while decreasing estrogen production in neighboring follicles.

In this thesis, immunoneutralization of follistatin was used to determine the physiological role of follistatin in fecundity. One of the variables affecting fecundity, ovulation rate, was further investigated to elucidate a potential mechanism for the increase in litter size observed following vaccination against follistatin. Immunoneutralization experiments have inherent limitations. They exert limited control over the mechanism and the magnitude of the desired physiological response. For instance, it is known that follistatin is expressed in non-reproductive tissues, although its role is not understood. It is difficult to determine if potential "non-reproductive" follistatin immunoneutralization effects, and the specificity and degree of anti-follistatin antibody response, may represent confounding effects in these experiments.

As a group, vaccination against follistatin resulted in a slight, but non-significant increase in the number of piglets born alive (Tables 4.1 and B.1). The range of antibody titers measured in follistatin vaccinated gilts at breeding was quite variable (Figure 4.2). Those gilts that achieved a titer >1:400 farrowed more live piglets than control vaccinated gilts. Therefore, vaccination against follistatin caused an antibody dose-dependent effect on litter size. The effect of follistatin vaccination on ovulation rate was also examined. Follistatin vaccination did not affect ovulation rate; subdivision of the
follistatin vaccinated gilts indicated a potentially negative relationship between high anti-follistatin titer and corpora lutea number. However, the animal numbers in this high titer anti-follistatin antibody group were very low. In addition, the experimental design, specifically the pharmacological induction of estrus, may have confounded the effect of anti-follistatin antibody on ovarian folliculogenesis. A second experiment would need to be conducted utilizing a different experimental design addressing these confounding factors before a relationship could be established between anti-follistatin antibody titer and ovulation rate (section 4.5).

In the follistatin vaccinated gilts of Experiment 2, there were more ovaries that contained hemorrhaged follicles than in the control vaccinated group. Luteal structures resembling corpora hemorrhagica were found only in the follistatin vaccinated group. Histological analysis of the hemorrhaged follicles indicated the follicles were atretic and the corpora hemorrhagica-like luteal structures contained the blood breakdown product, hemosiderin. It is unclear whether the presence of these unusual ovarian structures would affect reproductive potential of the gilts negatively. The presence of hemorrhaged, atretic follicles could potentially decrease ovulation rate. Furthermore, progesterone production by luteal structures with fewer luteal cells may have been lower. The presence of these unusual ovarian structures may be a specific effect of follistatin immunoneutralization itself, or the result of a confounding interaction between the vaccine treatment and pharmacological estrus induction. Although the ovaries of the gilts in Experiment 1 were not observed, the follistatin vaccination protocol in Experiment 1 did not appear to impair gilt fertility. In contrast, there was a significant
increase in litter size, following follistatin vaccination. This lack of a negative effect of follistatin vaccination in Experiment 1 suggests that the ovarian effects observed in Experiment 2 are probably the result of a confounding interaction between follistatin vaccination and pharmacological estrus induction. The unusual morphological observations in Experiment 2 point to an important role for follistatin in folliculogenesis, which may be more evident during pharmacological estrus induction in gilts. Further studies on the role of follistatin in folliculogenesis could lead to improved fecundity in estrus induced gilts. There are a limited number of observations of the effect of the reagents used in estrus induction and follistatin vaccination. The number of animals in the follistatin vaccinated group that were observed in estrus following estrus induction was greater (although not statistically significant) than in the control group (Table 4.2 “Induced Estrus”). In addition, there is some limited evidence for an effect of follistatin in pharmacological estrus management in cattle. Singh (1997) vaccinated adult cattle against follistatin using the same immunogen as that used in this thesis. He followed the pattern of ovarian follicle growth in the cattle’s ovaries using daily transvaginal ultrasound. The proportion of small follicles increased during the estrous cycle in follistatin vaccinated cattle, yet only one dominant follicle developed in each follicle wave. In a second experiment, a suboptimal superovulatory regime administered to follistatin vaccinated cattle gave similar numbers of viable oocytes as those collected from non-follistatin vaccinated cattle, which received the full superovulatory regime.

Increased fecundity following vaccination against follistatin may not be achieved solely through an increased ovulation rate. Swine litter size is also dependent on embryo
viability, uterine capacity (Gama and Johnson 1992), and the presence of an allelic variant of the estrogen receptor (Rothschild et al. 1996). Follistatin is expressed in the uterus, placenta, and decidua (Michel et al. 1990; Petraglia et al. 1994). A few observations concerning follistatin during human gestation have been noted. Serum levels of follistatin increase throughout pregnancy in humans (Wakatsuki et al. 1996). In human placental cell culture, follistatin neutralizes activin induced stimulation of hCG and progesterone production (Petraglia et al. 1994). However, the activity of follistatin during gestation in swine, a species in which the major site of progesterone production is not the placenta, is not clear. Limitations in uterine capacity, especially during late gestation, influence embryonic survival (Gama and Johnson 1993). Follistatin vaccination may have affected uterine capacity as there were half as many mummified piglets in the follistatin vaccination group as in the control vaccination group. However, these numbers were not statistically significant (Tables 4.1 and B.1). The improvements in fecundity observed as the result of follistatin vaccination may have been the result of a number of different effects, of which some may have been additive.

The results of the experiments involving follistatin vaccination are similar to other endogenous hormone vaccination experiments. In general, the results of the experiments are variable. This is due partially to technical difficulties associated with vaccination against endogenous hormones, for example, achieving a neutralizing antibody response, which epitopes the antibody recognizes, when samples are taken, and what type of assay is used (section 4.5). However, the variability of the biological response also indicates the complex physiology and the redundancy of intraovarian
hormones. The production of FSH and estrogen are both modulated by inhibin and follistatin. Inhibin vaccination has been shown to increase ovulation rate in swine (Brown et al. 1990; King et al. 1993). The effect of inhibin vaccination on FSH levels and ovulation rate in other livestock species is more variable (section 2.5.5). Reduction of one hormone through immunoneutralization may cause a compensatory increase in receptor number or in activity of other hormones. Vaccination against multiple target hormones, such as both follistatin and inhibin, may be a more effective method to modulate ovulation rate in swine.

In conclusion, recombinant porcine 305 a.a. follistatin was expressed in a bovine heat shock system and its identity was confirmed through immunological and activin binding determinants. In porcine granulosa cell culture, both recombinant porcine 305 a.a. and recombinant human 288 a.a. follistatin suppressed estradiol-17β accumulation at the highest doses utilized. The suppressive effect of follistatin on estradiol-17β accumulation appears to be activin independent. The rp-305 a.a. concentrations necessary to suppress estradiol accumulation were in the same range as the concentration of 303 a.a. follistatin in porcine follicular fluid. Thus, the follicle luteinizing action of follistatin in porcine granulosa cell culture is similar to that in other species (section 2.3.3.1), but the role of activin in this mechanism requires further clarification. Induced estrogen suppression may be a mechanism of negative follicle selection by autocrine or paracrine mechanisms. Further investigations of the relevance of these in vitro observations to physiological events in folliculogenesis is necessary.
Follistatin production by reproductive tissues is important for regulation of reproduction in swine. However, the physiological mechanisms, the follistatin isotypes and the tissues involved are not clear. Active vaccination against follistatin increased litter size in gilts. This was opposite to the increased impaired fertility observed with follistatin overexpression in transgenic mice (section 2.3.3.5). Attempts to examine the effect of active vaccination against follistatin on ovulation rate gave unexpected results. Follistatin vaccination did not increase ovulation rate, however, this experiment may have been confounded by pharmacological estrus induction or stress. This experiment emphasizes the complicated multisite regulation and activity of follistatin in gilts.

The physiological role of follistatin in the porcine ovary has not been elucidated fully. The mechanism of follistatin action and the relevance of endogenous follistatin isotypes is still unclear. Yet the data in this thesis suggest that follistatin is an active and important factor in reproduction. Recombinant porcine follistatin suppresses estradiol-17β accumulation, and immunoneutralization of endogenous follistatin affects litter size. The broad distribution of follistatin and its potential interaction with activin or other members of the TGFβ family may indicate that follistatin acts as a compensatory factor in many tissues, fine-tuning a system driven primarily by other factors.
6. BIBLIOGRAPHY


A. APPENDIX

FOLLISTATIN VACCINE FORMULATION TRIAL

To determine an optimal follistatin vaccine formulation follistatin was either not modified or was biotinylated and emulsed in two different adjuvants. The vaccine test groups were:

- Vaccine 1: Control vaccine - saline : Quil A adjuvant
- Vaccine 2: Biotinylated follistatin + avidin : Freund’s adjuvant
- Vaccine 3: Unmodified follistatin : Freund’s adjuvant
- Vaccine 4: Biotinylated follistatin + avidin : Quil A adjuvant
- Vaccine 5: Unmodified follistatin : Quil A adjuvant

At 28 days of age, six gilts/vaccine group were given one primary vaccination (day 0) and three booster vaccinations (days 22, 40, 85). Complete Freund’s adjuvant was given with the primary vaccination in groups 2 and 3 and incomplete Freund’s adjuvant was given for all subsequent booster vaccinations. Vaccine groups 2-5 contained 200 μg recombinant porcine follistatin per vaccine dose. All vaccines were given as a 2 ml volume, intramuscularly in the neck region. Blood samples were taken by jugular venipuncture, two weeks after all vaccinations. Blood samples were handled the same as section 4. The ELISA antibody titer protocol used was developed prior to the ELISA used in section 4.

A.1 Biotinylation

1. Prepare 10 mg/ml biotin-amidocaproate N-hydroxysuccinimide ester (B2643, Sigma) solution in DMSO and add to FCC815 conditioned media in a 5:1 molar ratio.
2. Incubate at room temperature for one hour with constant agitation.
3. Centrifuge solution.
4. Add avidin (20339, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) solution to biotin:follistatin solution in 1:0.5 solution.
5. Incubate at room temperature for one hour with constant agitation.
A.2 ELISA Protocol

Materials:

Immunolon™ 1 plates (011-010-3355, Dyeatech Laboratories Inc., Chantilly, VA)
Coating buffer: 30 mM Na₂CO₃ + 70 mM NaHCO₃, pH 9.5
Dilution buffer: TBS
Blocking buffer: 0.5 % BSA in dilution buffer
2nd antibody: phosphatase labeled goat anti-porcine IgG (H+L) (15-14-06 Kirkegaard & Perry Laboratories, Gaitherburg, MD) diluted 1:1000
Substrate solution: Sigma FAST™ (B-5655, Sigma) 1mg/ml

One aliquoted FCC815 conditioned media was used for this assay. The stock was diluted 1:100, added to the plates and stored at 4°C for 16 hours before the assay began. Serum samples were diluted on the day of the assay and serially diluted on the assay plate. All incubations were for one hour at room temperature with constant agitation. Titer was defined as 2X an individual animal's prebleed sera diluted to 1:400 (a dilution which was similar to a blank (nc serum) value).

Table A.1 Anti-follistatin antibody titer of blood samples taken from gilts 2 weeks after 3rd booster.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Vaccine</th>
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<td>5</td>
<td>nd</td>
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<td>6</td>
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</table>

nd = no detectable antibody titer

cv = 11.5%
Figure A.1 Antigenic index of the 305 a.a. recombinant porcine follistatin. The antigenic index is composed of the hydrophilicity and surface probability plots, chain flexibility and protein secondary structure. Graphs were created using the Protean, Version 3.14 software of DNASTAR Inc.
B. APPENDIX

DATA

Table B.1  Raw data collected for follistatin and control vaccinated gilts in Experiment 1.

<table>
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<tr>
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<th>Bred Wt</th>
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<th>Stillborn</th>
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nd = no detectable antibody titer in the anti-follistatin antibody titer assay
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Table B.2  Raw data collected for follistatin and control vaccinated gilts in Experiment 2.

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nd = no detectable antibody titer in the anti-follistatin antibody titer assay
silent = animal was not observed in physical or behavioral estrus yet ovaries contained corpora lutea
s = hemorrhaged follicle was 1-3 mm in diameter
m = hemorrhaged follicle was >3-6mm in diameter
C. APPENDIX

PROTOCOLS

Standard SDS-PAGE conditions: Constant voltage 125V; running time ~ 40 minutes.

Standard Electroblotting "Wet Transfer": Constant current 300 mA; running time ~ 60 minutes.

C.1 Follistatin purification by anionic exchange chromatography

Equipment:
- EconoColumn 2.5 x 30 cm (737-2532, BioRad)
- Peristaltic pump P-1 (18-1110-91, Amersham Pharmacia Biotech)
- Single path UV-1 monitor (18-1110-91, Amersham Pharmacia Biotech)
- Spectrophotometer (BD-41, Kipp and Zonen)

Buffers:
- Binding Buffer: 0.05M PO₄ pH 5.5
- Initial Column Buffer: 0.05M PO₄ pH 7.5
- Mixing Column Buffer: 0.05M PO₄ pH 7.5 + 1M NaCl

Resin: Fast Flow Q sepharose beads (17-0510-10, Amersham Pharmacia Biotech)

Salt Gradient: This was established by creating a siphon between reservoirs containing the initial and mixing column buffers. The mixing buffer was added to the initial buffer in a reservoir which was being constantly mixed by a magnetic stir bar. Velocity of the reaction was controlled by the speed of the peristaltic pump (6 ml/min)

Chromatography: Resin was prepared by washing with 3 bed volumes ddH₂O, 1.5 bed volumes 0.5 M NaOH, 1.5 bed volumes 2M NaCl and 2 bed volumes ddH₂O. Washed column with 0.05M PO₄ pH 5.5 until spectrograph reached baseline. Add FCC815 conditioned media and continue washing with 0.05M PO₄ pH 5.5 until spectrograph reached baseline. Begin the salt gradient. Collect all protein peaks.
C.2 Follistatin purification by affinity chromatography

Buffers:

Binding Buffer: 10 mM HEPES, 0.1M NaCl, pH 7.0
Elution buffer: 10 mM HEPES, 3.0 M NaCl, pH 7.0

Resin: Matrix Cellulose® (1-351, Amicon)

Procedure:
1. Incubate washed resin in a 10 ml conical tube with FCC815 conditioned media at 4°C for 16 hours with constant agitation.
2. Centrifuge at 2000g for 10 minutes.
3. Wash the pellet with binding buffer twice.
4. Release the cellulose sulfate bound follistatin by resuspending the pellet in 10 ml Elution buffer and incubate 1-2 hours at room temperature with constant agitation.
5. Dialyze the eluant of #4.

C.3 Monoclonal affinity column preparation

1. Wash protein A or G (20398, Pierce) agarose slurry with 50 mM sodium borate pH 8.2, twice.
2. Incubate 1 ml of washed beads with 5 mg antibody for one hour at room temperature with constant agitation.
3. Centrifuge at 2000 g for 5 minutes.
4. Wash beads twice with 50 mM sodium borate pH 8.2.
5. Resuspend in 10 ml 0.1M triethanolamine pH 8.2.
6. Add dimethyl pimelimidate:2HCl (21667, Pierce, Rockford, IL) at a molar ratio of IgG:DMP of ~1:30.
7. Incubate for one hour at room temperature with constant agitation.
8. Centrifuge at 2000 g for 5 minutes.
9. Incubate for 10 minutes in 0.1M ethanolamine pH 8.2 at room temperature with constant agitation.
10. Neutralize the reaction by incubating beads with 50 mM sodium borate pH 8.2.
C.4 Purifying FCC815 conditioned media using an IgG-Protein A or G agarose column

Purifications were carried out in disposable chromatography columns.

1. Wash the IgG linked agarose slurry with ~10 bed volumes 10 mM Tris pH 7.5.
2. Add sample to the column.
3. Wash sample with ~10 bed volumes 100 mM Tris pH 7.5.
4. Wash sample with ~10 bed volumes 10 mM Tris pH 7.5.
5. Elute purified sample with 100 mM glycine pH 2.8.
6. Collect 2 ml fractions, add 100 ml 1M Tris pH 7.5 so that the pH in the fractions is immediately neutralized.
7. Lyophilize all glycine fractions at < -20°C and store at -80°C until use.
8. Dialyse lyophilized fractions against 0.05M PBS using SpectraPor 6000-8000 MWCO tubing, prior to use in biological assays. Recovery rates for dialysis range from 24-36%.

C.5 Radiolabeling activin A

Materials: 30 μg chloramine T (10 μl of 3.0 mg/ml)
10% BSA
3.0 μg activin A
1 mCi iodine I¹²⁵
Buffer: 0.05M PO₄
Reaction Time: 45 seconds

Incorporation: 73-212 μCi/μg activin A

Purification of Iodination Reaction: PD10 prepared column (17-0851-01, Amersham Pharmacia Biotech)

Radiolabelled activin A was used in activin:follistatin binding assays without further purification.
C.6 Follistatin-activin binding assay

1. Electrophoresise ~6 μg recombinant porcine follistatin, reduced in sample buffer containing β-mercaptoethanol in a 10% SDS-PAGE gel.
2. Electroblot onto 0.45 μm nitrocellulose paper.
3. 30 minute wash of nitrocellulose in 3% IGEPAL CA-630 (I-3021 Sigma) in physiological saline containing 100 KIU aprotinin (817123, Trasylol, Miles Canada Inc, Etobicoke, Ontario) and 1mM PMSF (P-7626, Sigma) at 4°C with constant agitation.
4. 2 hour incubation with 0.5% gelatin, 10mM Tris buffered saline, 0.5% Tween 20 (RD 6435, BDH Inc), pH 8.0 containing 1mM PMSF at 4°C with constant agitation.
5. 16 hour incubation with 50,000 counts of radiolabelled activin in 0.5% gelatin, 10mM Tris buffered saline, 0.5% Tween 20, pH 8.0 containing 1mM PMSF at 4°C with constant agitation.
6. 3 washes with saline containing 0.1% Tween 20 at 4°C with constant agitation.
7. Allow the nitrocellulose blots to dry at room temperature and then place in autoradiograph cassette with X-ray film at -80°C until the film is ready to develop.

C.7 Estradiol 17β radioimmunoassay

Materials:
- Estradiol-6-carboxymethyl oximino-(2-[125I]jodo histamine) (IM135 Amersham Pharmacia Biotech)
- Estradiol antiserum (NCR rabbit A11, obtained from Dr. N. Rawlings, University of Saskatchewan)
- Estradiol 17β (E8875, Sigma)

Buffer: 0.1 M PBS containing 0.1% gelatin

Day 1: Add 100 μl radiolabelled estradiol, 100 μl estradiol antiserum, buffer (100 μl or 200 μl) and 100 μl estradiol-17β standard or sample (100 μl or 200 μl) for a total volume of 500μl.

Day 2: Add 100 μl 1% bovine gamma globulin (G7516, Sigma). Vortex.
Add 1 ml 30% PEG 8000 (B80016, BDH, Inc.). Vortex.
Centrifuge tubes at 3000 rpm for 30 min.
Decant and allow tubes to drain for > 1 hour.
Count in a γ counter.
C.8 Porcine ovary collection and granulosa cell isolation

1. Ovaries were collected from prepubertal gilts slaughtered at Intercontinental Packers Ltd., Saskatoon, SK.

2. All ovaries were collected and kept on ice in saline containing antibiotics.

3. Upon returning to the laboratory ovaries were sorted by follicle size. Only those ovaries which contained follicles which were < 3mm were used.

4. Granulosa cells were dissected from ovarian follicles as follows:
   - a follicle was punctured and follicular fluid was removed
   - with forceps the granulosa layer was dissected from the follicle
   - tissue was kept in media containing 10% NBCS on ice until washing

5. Granulosa cells were washed by pelleting at 800 rpm, media was removed and ~ 20 ml fresh media was added. Cells were gently stirred in the media, then pelleted and washed once more.

6. Cells were counted in a hemocytometer and cell viability was determined using 0.4% trypsin blue (15250-012, Canadian Life Technologies) and 1% nigrosin (N4763, Sigma).

7. Cells were plated at 300,000 viable cells per well in a Corning 24 well (62408-350, VWR Canlab, Mississauga, ON). (day 0)

8. Media was changed on days 1 and 2.

9. Media was changed and treatments were added on day 4.

10. On day 8, media was harvested and frozen at -20°C until the estradiol 17β assay was conducted. The cells were removed from the tissue culture plates by a 5 minute incubation with trypsin at 37°C, pelleted in a high speed microfuge and frozen at -20°C until the DNA assay was completed. Cell viability at the end of the incubations was not conducted with trypsin blue as it interfered with the DNA assay. Cells attached to the cell culture wells were assumed to be viable.

Media day 0 - 1:
Dulbecco's MEM
5 µg/ml insulin
100 µg/ml streptomycin
100 IU pencillin
1 µg/ml Fungizone®
10% NBCS

Media day 1-8: Day 0 media without 10% NBCS.
C.9 Radiolabeling of rh-288 a.a. follistatin

Materials: 30 μg chloramine T (10 μl of 3.0 mg/ml)
100 μl sodium metabisulfite (100 μl of 1.0 mg/ml)
3.0 μg rh-288 a.a. FS
1 mCi iodine I\(^{125}\)

Buffer: 0.05M PO\(_4\)

Reaction Time: 45 seconds

Incorporation: 23.8 - 69.1 μCi μCi/μg rh-288 a.a. FS

Purification of Iodination Reaction: PD10 prepared column (17-0851-01, Amersham Pharmacia Biotech)

C.10 Liquid-phase anti-follistatin antibody titer assay

Day 1: 100 μl radiolabelled follistatin
100 μl antibody
200 μl buffer (tried 0.5% BSA, 1% BSA, and 0.1% gelatin in 0.05M phosphate buffer)
Vortex, incubate 16 hours at 4°C

Day 2: Add 400 μl protein A, 1% charcoal or 6% polyethylene glycol, vortex.
Centrifuge at 3000 rpm for 30 minutes.
Decant and count on a γ counter.

Tried glass and plastic (polypropylene and polystyrene) test tubes.
C.11 ELISA - FCC815 conditioned media as coating antigen

Materials: Immunon 4 plates (011-010-3855, Dynatech)
2nd antibody - biotinylated anti-swine IgG (B7015, Sigma)
Strep-avidin HRP (RPN1231 Amersham Pharmacia Biotech)
Substrate development: tetramethylbenzidine dihydrochloride tablets (T3405, Sigma)

Dilution buffer: 0.01M PBS + 1 mM PMSF (added fresh from stock before each dilution)

Coating: 100 µl, FCC815 stock, 4°C, 16 hours

Preincubation: serum samples incubated 1:1 with heat shock protein stock,
total volume 100 µl, 4°C, 16 hours
- heat shock protein stock was conditioned media from MDBK cells transformed with a negative expression cassette (pSVD2neo)

All other steps were done at room temperature with constant agitation for 1 hour.

Blocking: 200 µl dilution buffer + 1% BSA
Serum samples: 100 µl
2nd antibody: 100 µl of 1:2000 dilution
streptavidin HRP: 100 µl
TMB substrate: 100 µl
OD reading: 650 nm