EXPRESSION AND REGULATION OF SELECT INTERFERON STIMULATED GENES IN PORCINE ENDOMETRIUM DURING PREGNANCY

A Dissertation

by

MARGARET MARY JOYCE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2008

Major Subject: Biomedical Sciences

EXPRESSION AND REGULATION OF SELECT INTERFERON STIMULATED GENES IN PORCINE ENDOMETRIUM DURING PREGNANCY

A Dissertation

by

MARGARET MARY JOYCE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Approved by:

Chair of Committee,	Gregory A. Johnson
Committee Members,	Fuller W. Bazer
	Robert C. Burghardt
	Laurie A. Jaeger
	Gary R. Newton
	Thomas E. Spencer
Head of Department,	Evelyn Tiffany-Castiglioni

December 2008

Major Subject: Biomedical Sciences

ABSTRACT

Expression and Regulation of Select Interferon Stimulated Genes in Porcine Endometrium During Pregnancy. (December 2008)

Margaret Mary Joyce, B.S., Illinois State University

Chair of Advisory Committee: Dr. Gregory A. Johnson

Coordinated signals between the maternal endometrium and conceptus during the peri-implantation period are essential for the establishment and maintenance of pregnancy. In pigs, this involves estrogen secretion from conceptuses as the signal for maternal recognition of pregnancy. Pig conceptuses also secrete interferons (IFN) delta (IFND) and IFN gamma (IFNG). The uterine effects of pig IFNs are not known, although ruminant conceptuses secrete IFN tau (IFNT) for pregnancy recognition, and this increases the expression of IFN-stimulated genes (ISGs) in the endometrium. Therefore, studies were conducted to identify and characterize ISGs in the pig endometrium during pregnancy and to evaluate their regulation by estrogen and conceptus secretory proteins (CSPs) that contain IFNs.

In the first study, four classical ISGs, including interferon regulatory factor 1 (*IRF1*) and signal transducer and activator of transcription 2 (*STAT2*), were detected in the pig endometrium and increased after Day 12 of pregnancy, specifically in stroma. *IRF2*, a transcriptional repressor of ISGs, increased in luminal epithelium (LE) by Day 12 of pregnancy. The increase of *IRF2* was due to estrogen while the stromal increase of *IRF1* was due to IFN-containing CSP infusion.

In the second study, the ISG *STAT1* increased in LE after Day 12 of pregnancy and estrogen resulted in a similar increase. After Day 15 of pregnancy, *STAT1* increased in stroma. Infusion of IFN-containing CSPs resulted in a similar stromal increase.

In the third study, the ISGs swine leukocyte antigen (*SLA*) class I and beta-2microglobulin (*B2M)* increased in LE between Days 5 and 9 of the estrous cycle and pregnancy and decreased between Days 15 and 20 of pregnancy. By Day 15 of pregnancy, *SLAs* and *B2M* increased in stroma where they remained through Day 40. Progesterone increased *SLA* and *B2M* in LE, and a progesterone receptor antagonist ablated the upregulation while infusion of IFN-containing CSP increased *SLA* and *B2M* in stroma.

Collectively, these studies identify ISGs expressed in the pig endometrium during pregnancy. These genes may be involved in protecting the fetal semiallograft from immune rejection, limiting conceptus invasion through the uterine wall, and/or establishing a vascular supply to the conceptus. The interactions of estrogen, progesterone and IFNs to regulate cell-type specific expression of ISGs highlight the complex interplay between endometrium and conceptus for pregnancy recognition and implantation.

DEDICATION

To my Dad, Dudley.

ACKNOWLEDGEMENTS

I would like to thank my mentor and advisor, Dr. Greg Johnson for his guidance, patience and support throughout the course of this research. I would also like to thank my committee members, Drs. Fuller Bazer, Robert Burghardt, Laurie Jaeger, Gary Newton and Thomas Spencer for their assistance throughout my doctoral program. Thanks also to all the members of the collaborating laboratories for their assistance, patience and friendship.

Thanks also to my family and friends for all of their encouragement, support and love. This would not have been possible without all of you.

TABLE OF CONTENTS

		Page
ABSTRACT		iii
DEDICATION		v
ACKNOWLEDGEMENTS		vi
TABLE OF CONTENTS		vii
LIST OF FIGURES		ix
CHAPTER		
I	INTRODUCTION	1
II	LITERATURE REVIEW	3
	Blastocyst Development, Implantation, and Placentation	3
	Conceptus Estrogen as the Signal for Pregnancy Recognition	6
	Progesterone and Estrogen Receptors in the Uterine Endometrium	6
	Interferons and Interferon Signal Transduction Pathways	8
	Conceptus Interferons and Uterine Expression of Interferon Stimulated Genes	11
III	PIG CONCEPTUSES INCREASE UTERINE INTERFERON-REGULATORY FACTOR 1 (*IRF1*), BUT RESTRICT EXPRESSION TO STROMA THROUGH ESTROGEN-INDUCED *IRF2* IN LUMINAL EPITHELIUM	15
	Introduction	15
	Materials and Methods	17
	Results	25
	Discussion	34
IV	PIG CONCEPTUSES SECRETE ESTROGEN AND INTERFERONS TO DIFFERENTIALLY REGULATE UTERINE *STAT1* IN A TEMPORAL AND CELL-TYPE SPECIFIC MANNER	41
	Introduction	41
	Materials and Methods	43

CHAPTER Page

Results 49
Discussion 60

V UTERINE MAJOR HISTOCOMPATIBILITY CLASS I MOLECULES AND BETA 2 MICROGLOBULIN ARE REGULATED BY PROGESTERONE AND CONCEPTUS INTERFERONS DURING PIG PREGNANCY 68

Introduction 68
Materials and Methods 71
Results 77
Discussion 95

VI SUMMARY AND CONCLUSIONS 100

Summary 100
Conclusions 105

REFERENCES 106

VITA 128

LIST OF FIGURES

FIGURE Page

2.1 Pre-implantation development of the pig conceptus 4

2.2 Implantation phases 5

2.3 Maternal and conceptus signaling during early pregnancy 7

2.4 Type I and Type II IFN signaling pathways 10

3.1 Study 1 experimental design 18

3.2 Study 2 experimental design 19

3.3 Pig reproductive tract after ovariohysterectomy 21

3.4 Study 3 experimental design 22

3.5 Steady-state levels of mRNAs for the classical IFN-stimulated genes (**A**) *IRF1* (**a**), *STAT2* (**b**), *MIC* (**c**), and *B2M* (**d**), and the non-IFN stimulated gene (**B**) *IRF2* in pig endometria during the estrus cycle and pregnancy 27

3.6 *In situ* hybridization analysis of *IRF1* mRNA in pig uteri 28

3.7 *In situ* hybridization analysis of *STAT2* mRNA in pig uteri 29

3.8 *In situ* hybridization analysis of *IRF2* mRNA in cross-sections of pig uteri 30

3.9 Immunofluorescence localization of IRF1 protein in frozen cross-sections of pig endometria from day 15 of the estrus cycle (D15C) and D15 of pregnancy (P) 32

3.10 Localization of IRF1 protein and *IRF2* mRNA in cross-sections of pig uteri from study 2 33

3.11 Localization of IRF1 protein and *IRF2* mRNA in cross-sections of pig uteri from study 3 35

4.1 Study 4 experimental design 46

4.2 Study 1: *STAT1* increases in pregnant uterine endometrium in a cell-type-specific manner 51

4.3 Study 1: IFNδ and IFNγ are synthesized by pig conceptuses 53

4.4 Study 2: im estrogen injections increase *STAT1* in uterine luminal epithelium 55

FIGURE Page

4.5 Study 3: intrauterine infusion of pig CSPs that contain IFNδ and IFNγ increase *STAT1* in uterine stroma 58

4.6 Study 1: uterine *STAT1* increases in close proximity to paracrine release of IFNγ by implanting conceptuses 59

4.7 Study 4: inappropriate early estrogen results in demise of embryos and loss of IFN-induced *STAT1* 61

5.1 Experimental design for study 4 73

5.2 Steady-state levels of mRNA for *SLA-1, SLA-2, SLA-3*, *SLA-6, SLA-7, SLA-8* and β_2m genes in pig endometria during the estrous cycle and pregnancy 79

5.3 *In situ* hybridization analysis of *SLA-1, SLA-2,* and *SLA-3* mRNA in pig endometria 81

5.4 *In situ* hybridization analyses of *SLA-6, SLA-7,* and *SLA-8* mRNAs in pig endometria 82

5.5 *In situ* hybridization analysis of β_2m mRNA in pig endometria 84

5.6 *In situ* hybridization analysis of USP mRNA in pig endometria 85

5.7 Immunofluorescence localization of classical SLA class I molecules in frozen cross-sections of pig endometria during the estrous cycle (C) and pregnancy (P) 86

5.8 Treatment with CSP increased expression of classical SLA class I mRNAs 89

5.9 *In situ* hybridization analyses on expression of mRNAs 91

5.10 The effects of exogenous progesterone on endometrial expression of SLA class I and β_2m mRNAs and proteins 94

6.1 Proposed model of gene expression changes and regulation in pig endometrium 101

CHAPTER I

INTRODUCTION

Successful pregnancy requires coordinated maternal and conceptus signals. In pigs, one of the earliest signals is the secretion of estrogen on Day 11 by the developing conceptus [1]. This occurs as the pig conceptus transforms from a spherical to a tubular and then to a filamentous shape [2]. Conceptus estrogen acts on the maternal endometrium to redirect the luteolysin prostaglandin $F_{2\alpha}$ into the uterine lumen rather than the uterine vasculature, resulting in maintenance of the corpora lutea (CL), the source of progesterone required for pregnancy [1]. Exogenous estrogen injected intramuscularly on Days 11 through 15 of the estrous cycle results in CL maintenance, further indicating that estrogen is the signal for maternal recognition of pregnancy [1, 3, 4]. Premature exposure of the pregnant uterus to estrogen on Day 9 and Day 10 results in degeneration of the conceptuses by Day 15, demonstrating the delicate balance of maternal-fetal signaling essential for the establishment and maintenance of pregnancy [5].

The effects of ovarian progesterone and conceptus estrogen can be mediated by progesterone (PGR) and estrogen (ESR1) receptors. Expression of these receptors varies during early pregnancy. PGR is not detectable in the luminal epithelium (LE) after Day 10 or the glandular epithelium (GE) after Day 12 of pregnancy, but is continually present in the stroma [6]. Highest levels of ESR1 were detected in the LE and GE on Days 10 and 12 of pregnancy [7]. Levels decreased in both of these cell types by Day 15 and by Day 18, ESR1 is detectable in the LE by not the GE [7]. In the stroma, moderate levels of ESR1 are detected on Day 10 and Day 12, but after Day 15, ESR1 is no longer detectable [7]. These changes in PGR and ESR1 expression indicate that progesterone and estrogen may affect the uterine endometrium in a cell-specific manner.

This dissertation follows the style of Biology of Reproduction.

Pig conceptuses also secrete the Type I interferon delta (IFND) and the Type II IFN gamma (IFNG) on Days 12 through 20 [8]. These IFNs have not been shown to effect CL maintenance [9]. However, both Type I and Type II IFNs can induce expression of a number of genes termed interferon-stimulated genes (ISGs). The classical pathway by which ISGs are induced is the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway [10]. Activation of this pathway by Type I IFNs can result in the formation of ISG Factor 3 (ISGF3) which can bind to IFN-stimulated response elements (ISRE) and initiate transcription of a number of ISGs [11]. IFNG activation can result in the formation of a STAT1:1 homodimer, known as gamma activation factor (GAF) [12], which can bind to gamma activation sequences (GAS), resulting in transcription of ISGs [13].

Indeed, IFNs [14-21] and ISGs [16, 22-24] have been detected during early pregnancy in several species, most noteably in sheep [25-36], which utilize IFN tau (IFNT) for pregnancy recognition [4]. Studies in sheep have demonstrated that IFNT induces the expression of ISGs in the endometrium [28, 31, 33-35, 37-39]. Paracrine effects for porcine IFNs are suggested by localization of IFN receptors on endometrial epithelial cells [40], increased secretion of prostaglandin E_2 [41], and *MX1* expression in the stratum compactum stroma of pigs on Day 18 of pregnancy [42]. These studies suggest that induction or increases in uterine ISGs by conceptus IFNs is a phenomenon of early pregnancy in many, if not most, mammals and that these genes may be important for uterine receptivity and conceptus implantation and development.

CHAPTER II

LITERATURE REVIEW

Blastocyst Development, Implantation, and Placentation

In pigs, the 1-cell fertilized ovum or zygote undergoes cleavage to form a 2-cell embryo by 26 h after fertilization [2]. Embryos remain in the oviduct before entering the uterus at 48 to 56 h. Blastocyst formation is a key stage in early embryonic development when cells segregate into the embryonic disc, trophectoderm, extra-embryonic endoderm and blastocoel necessary for continued development and differentiation to a conceptus (embryo and associated extra-embryonic membranes) (Fig. 2.1) [2]. Blastocysts are 0.5 to 1 mm diameter spheres when they hatch from the zona pellucida and increase in size to Day 10 of pregnancy (2-6 mm) before undergoing a morphological transition to large spheres of 10 to 15 mm diameter and then tubular (15 mm by 50 mm) and filamentous (l mm by 100-200 mm) forms on Day 11. During the transition from tubular to filamentous forms, pig conceptuses elongate at 30 to 45 mm/h, primarily by cellular remodeling of trophectoderm [43]. However, hyperplasia is responsible for subsequent growth and elongation of the conceptus to 800-1000 mm length by Day 15 of pregnancy [2]. The period of rapid elongation of pig conceptuses is accompanied by estrogen production by trophectoderm [1], as well as interferons gamma (IFNG) and delta (IFND) [8, 40].

Implantation is highly synchronized, requiring reciprocal secretory and physical interactions between a developmentally competent conceptus and the uterine endometrium during a restricted period of the uterine cycle termed the “window of receptivity” [44-51]. In domestic animals, implantation follows a prolonged pre-attachment period, and does not result in embryo invasion past the basal lamina of the mucosal epithelium. As defined by Guillomot et al. [51] the initial phases of implantation are: (1) shedding of the zona pellucida; (2) pre-contact and blastocyst orientation; (3) apposition; and (4) adhesion, are common across species (Fig. 2.2).

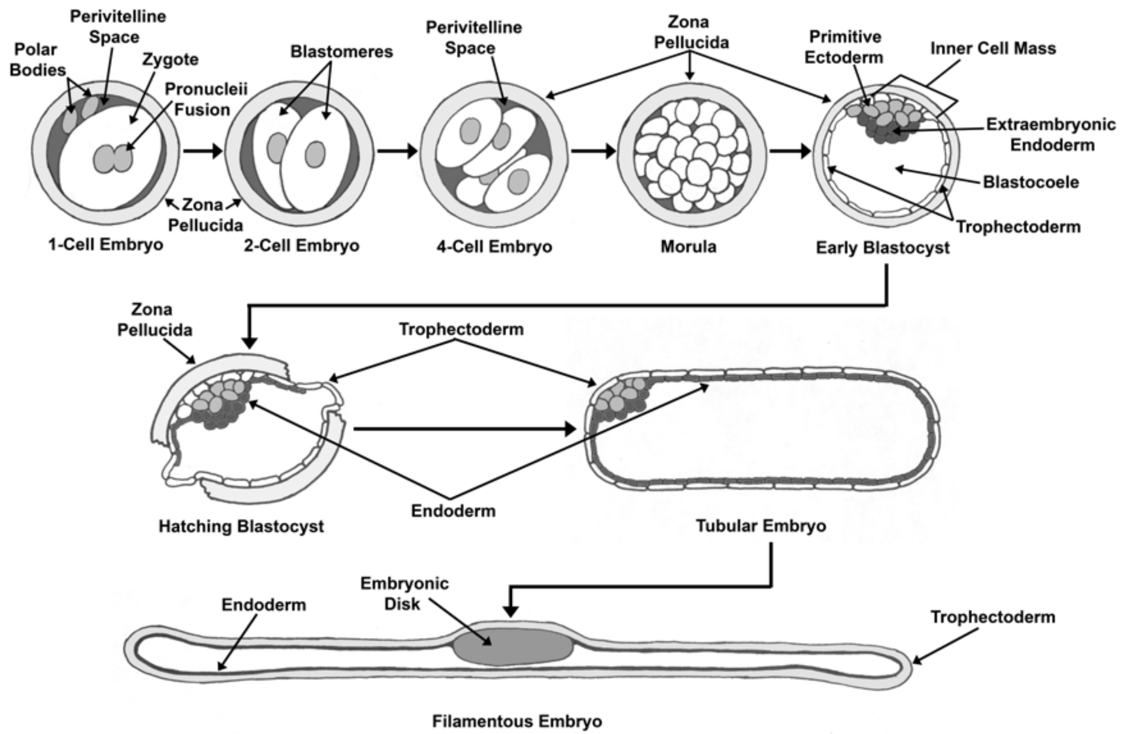


FIG. 2.1. Pre-implantation development of the pig conceptus. Embryos develop into blastocysts before hatching from the zona pellucida. They continue to increase in size, then on Day 10 transition from spherical to tubular to filamentous forms by Day 11 [2].*

* Reprinted with permission from Encyclopedia of animal science by Bazer FW, Johnson GA, Spencer TE, 2005, Taylor & Francis, London. Copyright 2005 by Taylor & Francis.

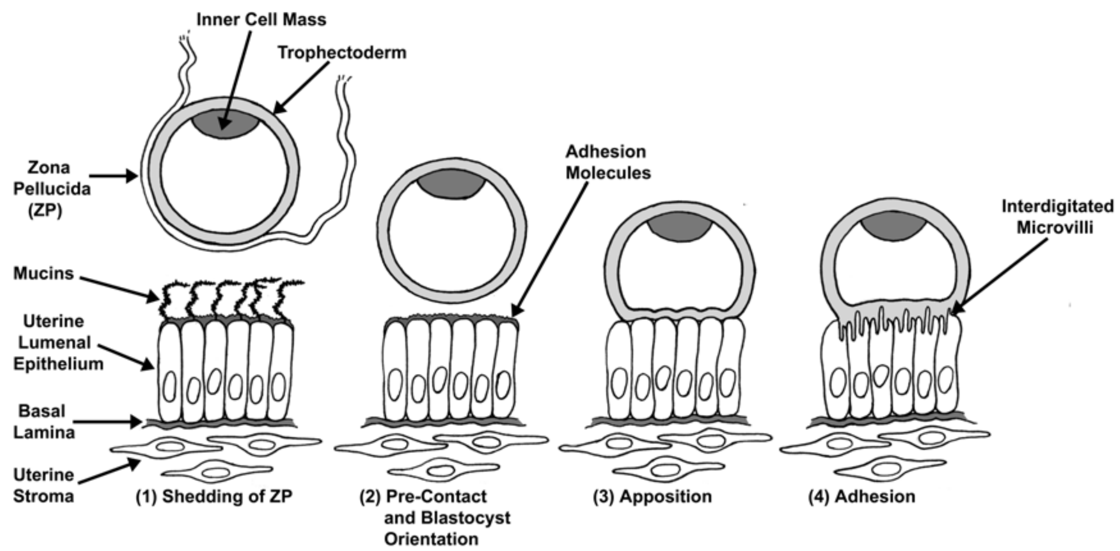


FIG. 2.2. Implantation phases. Potential phases of implantation involving interactions between the trophectoderm and the uterine luminal epithelium [52].*

* Reprinted with permission from Encyclopedia of animal science by Bazer FW, Johnson GA, Spencer TE, 2005, Taylor & Francis, London. Copyright 2005 by Taylor & Francis.

Conceptus attachment is thought to require loss of anti-adhesive molecules in the glycocalyx of LE, comprised largely of mucins that sterically inhibit attachment [53-55]. This results in "unmasking" of molecules, including selectins and galectins [56, 57], that contribute to initial attachment of conceptus to uterine luminal epithelium (LE). These low affinity contacts are then replaced by a more stable and extensive repertoire of adhesive interactions between integrins and maternal extracellular matrix (ECM) which appear to be the dominant contributors to stable adhesion at implantation [44, 45, 53, 58, 59].

Conceptus Estrogen as the Signal for Pregnancy Recognition

During the estrous cycle in pigs, the luteolysin prostaglandin $F_{2\alpha}$ (PGF) is secreted by the uterine LE and shallow GE toward the uterine vasculature (endocrine) and transported to the CL on the ovary, resulting in the destruction of the CL. However during pregnancy, maintenance of the CL requires that PGF be redirected into the uterine lumen (exocrine), where it is sequestered to exert its biological effect *in utero* and/or metabolized to prevent luteolysis [1]. This redirection of PGF secretion into the uterine lumen is associated with estrogen secretion by the conceptus, which occurs between Days 11 and 12 and Days 15 and 30 of pregnancy [1, 60]. Indeed, injection of 17β-estradiol on Days 11-15 of the estrous cycle results in PGF secretion into the uterine lumen and maintenance of the CL for a period equivalent to or slightly longer than pregnancy, a condition referred to as pseudopregnancy [1, 3, 4]. Collectively, these data strongly suggest that estrogen is at least in part the signal for maternal recognition of pregnancy in pigs (Fig. 2.3) and supports the endocrine-exocrine theory of pregnancy recognition, first described by Bazer and Thatcher [1].

Progesterone and Estrogen Receptors in the Uterine Endometrium

Effects of progesterone from the CL and estrogen from the conceptus on the uterine endometrium can be mediated through their respective receptors. However, expression of these receptors varies in a cell-type specific manner during the estrous

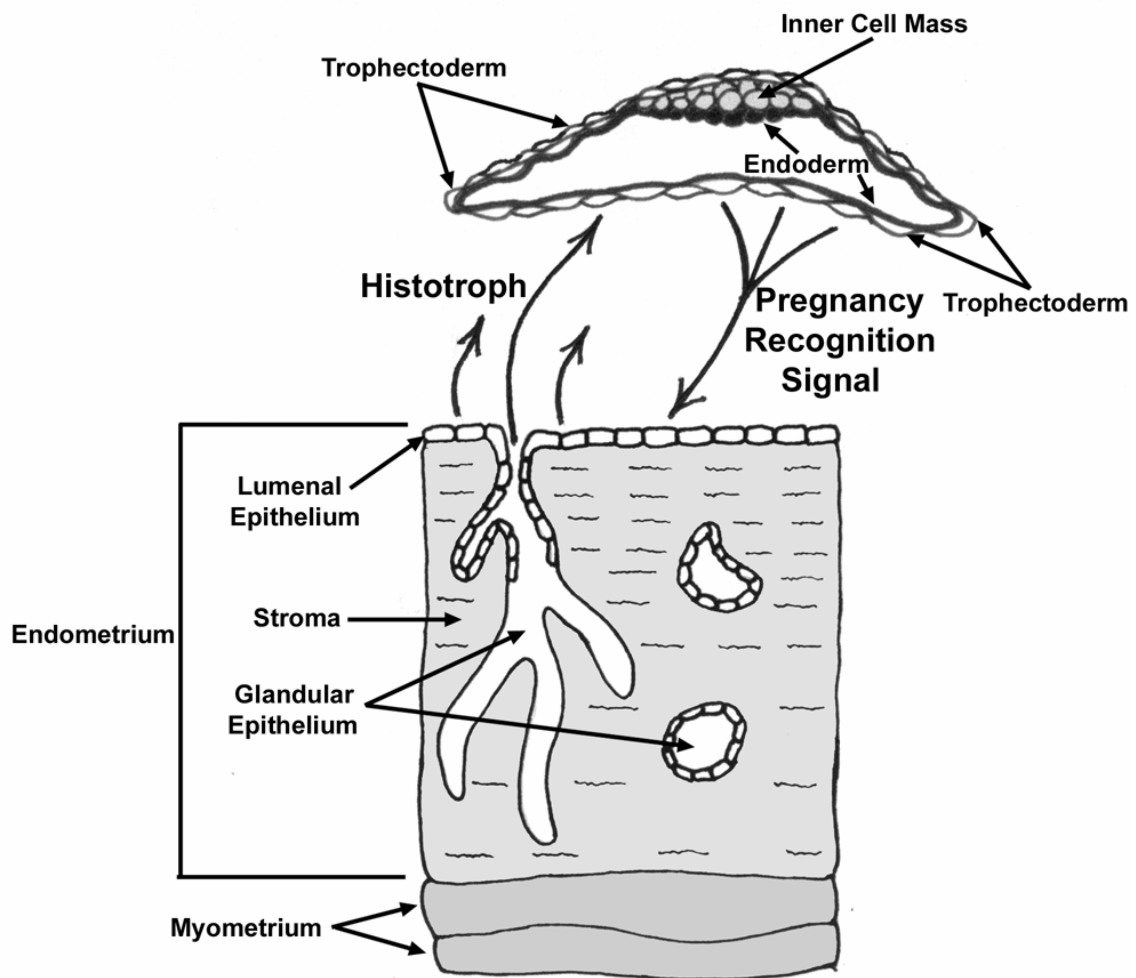


FIG. 2.3. Maternal and conceptus signaling during early pregnancy. Peri-implantation signaling between the conceptus and uterus for pregnancy recognition and production of histotroph [52].*

---

* Reprinted with permission from Encyclopedia of animal science by Bazer FW, Johnson GA, Spencer TE, 2005, Taylor & Francis, London. Copyright 2005 by Taylor & Francis.

cycle and early pregnancy. Progesterone receptor (PGR) is detectable in the LE, GE and stroma on Day 0 through Day 5 of the estrous cycle and early pregnancy [6]. However, prolonged progesterone exposure decreases PGR to undetectable levels in the LE by Day 10 and in the GE by Day 12 of the estrous cycle and pregnancy, but does not decrease stromal PGR [6].

Estrogen receptor (ESR1) expression differs from that of PGR. During the estrous cycle, ESR1 remains high in the LE and GE from Day 0 to 12, declines by Day 15 and then increases in the LE by Day 18 while remaining low in the GE [7]. Stromal expression is highest on Day 0, decreases by Day 5, is undetectable from Day 5 to Day 15, then increases by Day 18. Expression during early pregnancy is the same as during the estrous cycle except that ESR1 levels remain low or undetectable at Day 18 [7].

Interferons and Interferon Signal Transduction Pathways

Isaacs and Lindenmann discovered interferons (IFNs) in 1957 as a substance that could protect cells from viral infection [61]. IFNs are cytokines with many different biological functions including immunomodulatory, cell differentiative, anti-angiogenic and anti-proliferative effects [62, 63]. There are two types of IFNs, Type I or Type II IFNs. The Type I IFNs include IFNA, IFNB, IFND, IFNK, IFNT, and IFNW; however, there is only one known Type II IFN, IFNG [64].

The classical signaling pathway for IFNs is the Janus protein tyrosine kinase-signal transducers and activators of transcription (JAK-STAT) pathway [10]. Binding of a Type I IFN to its receptor induces dimerization of the receptor chains, IFNAR1 and IFNAR2, which activates the JAKs associated with each receptor chain, TYK2 and JAK1 respectively [10]. After activation, TYK2 phosphorylates IFNAR1, resulting in a docking site for signal transducer and activator of transcription (STAT) 2 [65], which then is phosphorylated by TYK2 and serves as a docking site for STAT1 [66, 67]. Subsequently, STAT1 is phosphorylated and the activated STAT1:2 heterodimer dissociates from the receptor complex. This complex can then associate with IFN regulatory factor (IRF) 9 to form the ISG Factor 3 (ISGF3) complex, translocate to the

nucleus and bind IFN-stimulated response elements (ISRE) in the promoters of several interferon stimulated genes (ISGs) to initiate transcription [11]. Type I IFNs can also induce formation of other STAT complexes, including STAT1:1, STAT3:3, STAT5:5 and STAT1:3 [68, 69], which can bind to gamma-activated sequences (GAS) in the promoters of another group of ISGs [10] (Fig. 2.4).

Biologically active IFN gamma forms a noncovalent dimer which binds to the two chains of its receptor complex, IFNGR1 and IFNGR2, in a 2:2 ratio [70, 71]. The two IFNGR1 chains are each associated with a JAK1 molecule and each IFNGR2 chain is associated with a JAK2 molecule [10]. After ligand binding, the receptor undergoes a conformational change that results in autophosphorylation and activation of JAK2, which then transphosphorylates JAK1 [72]. JAK1 subsequently phosphorylates the two IFNGR1 chains [73, 74], with which STAT1 proteins are associated. The STAT1 proteins are phosphorylated [75], dissociate from the receptor complex, form STAT1:1 homodimers, known as gamma activation factor (GAF) [12], and translocate to the nucleus where they can bind GAS elements and induce gene transcription [13] (Fig. 2.4).

Recent studies indicate that additional signaling pathways may be important for some of the biological functions of most IFNs and for the expression of ISGs. Full transcriptional activation of STAT1 requires phosphorylation on Ser727 [76]. Several kinases have been identified that regulate Ser727 phosphorylation, such as Ca^{2+}/calmodulin-dependent kinase II [77] and phosphatidylinositol 3'-kinase [78], indicating that they may play a role in ISG expression. Additionally, studies in which p38 MAPK was inhibited indicate that this kinase is required for induction of antiviral responses [79-81] and that inhibition of this kinase suppresses ISRE and GAS controlled gene expression [82, 83].

While the biological activity and expression of ISGs may involve many different signal transduction pathways, to date the only pathway that has been demonstrated to mediate the effects of the IFNs in the uterine endometrium is the JAK-STAT pathway [28, 84]. In ovine endometrial epithelial cells, IFN tau (IFNT) transiently phosphorylated STAT3, 5a/b, and 6, which briefly translocated to the nucleus, but did

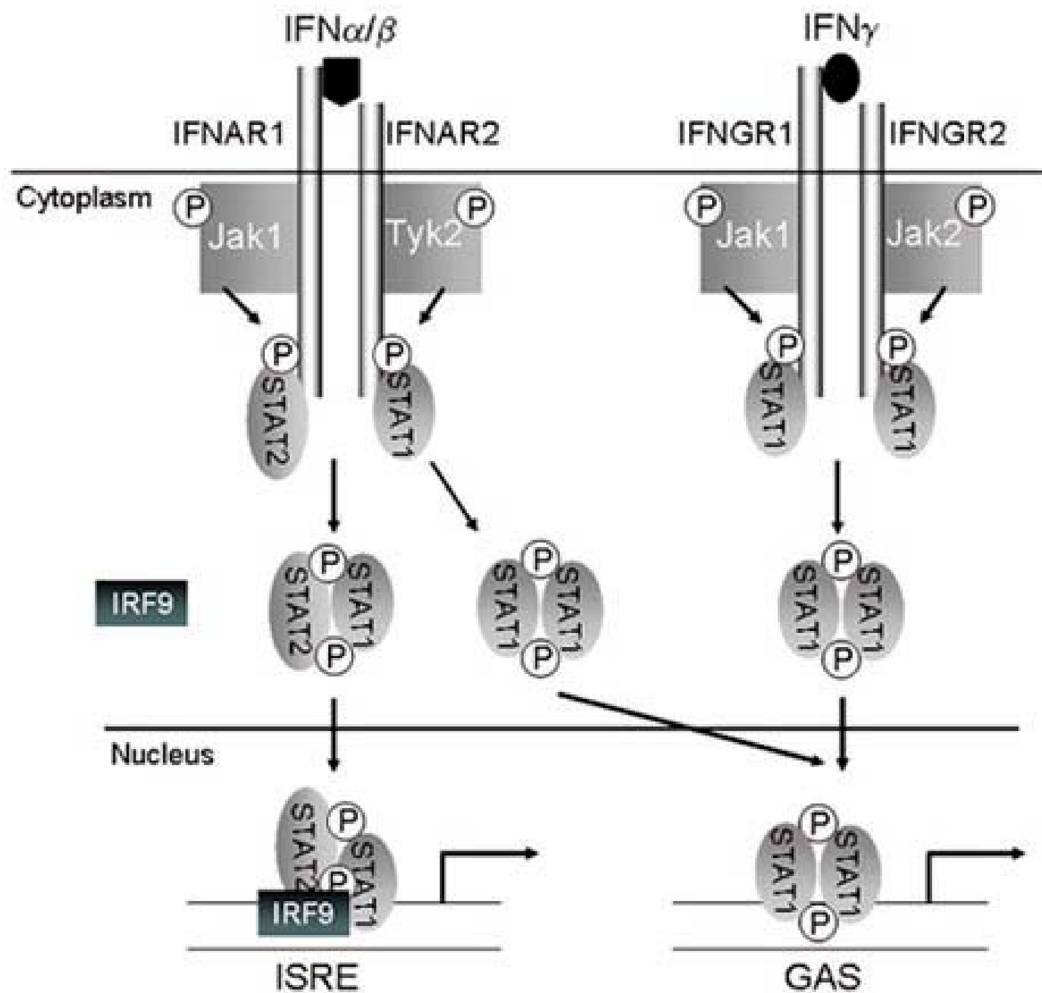


FIG. 2.4. Type I and Type II IFN signaling pathways. Schematic illustrating the JAK-STAT signaling pathways for Type I and Type II IFNs [85].*

---

* Reprinted with permission from STAT activation and differential complex formation dictate selectivity of interferon responses by Wesoly J, Szweykowska-Kulinska Z, Bluyssen HAR. Acta Biochimica Polonica 2007; 54:27-38. Copyright 2007 by Acta Biochimica Polonica.

not increase expression of these STATs [84]. In contrast, STAT1 and 2 were persistently phosphorylated and remained in the nucleus for a prolonged time. Expression of both of these STATs increased with IFNT treatment [84]. ISGF3 and STAT1 homodimers formed in these cells and bound to ISRE and GAS elements, respectively [84]. Time course analysis indicated that IFNT had short term effects on GAS-driven promoters (expression increased by 3 hours, but decreased by 24 hours), while IFNT had long-term effects on expression of genes with ISRE-containing promoters (increased expression at 3 and 24 hours) [84].

Conceptus Interferons and Uterine Expression of Interferon Stimulated Genes

Ruminants

IFNT is exclusively produced by ruminant conceptuses during early pregnancy [25-27]. In these species, IFNT is the antiluteolytic signal for maternal recognition of pregnancy [86]. It is synthesized and secreted from the mononuclear cells of the conceptus trophectoderm between Days 10 and 21-25 [87, 88]. IFNT acts on the luminal epithelium (LE) and superficial glandular epithelium (sGE) to suppress transcription of the *ESR1* gene, which prevents transcription of the oxytocin receptor gene [89-91] and production of luteolytic pulses of $PGF_{2\alpha}$ by the uterine endometrium [86], thereby maintaining progesterone secretion by the CL [90].

Characterization of the temporal and spatial pattern of expression for many of the ISGs in the uterus has been done in the sheep. Both chains of the Type I IFN receptor, IFNAR1 and IFNAR2, have been localized to the LE, GE and stroma of the ovine uterine endometrium [92], suggesting that IFNT from the conceptus should be able to increase expression of ISGs throughout the endometrium. However, ISGs induced by the classical IFN signal transduction pathway (JAK-STAT) are only expressed in the stroma and GE [28]. This may be due to expression of IRF2, a potent transcriptional repressor of ISGs, in the LE and sGE [28].

The temporal and spatial pattern for most of the classical ISGs in the sheep uterus is similar and can be illustrated by the expression pattern for *ISG15*. *ISG15*

mRNA is first detectable in the LE and stratum compactum stroma on Day 13 of pregnancy [29, 30]. By Day 15 of pregnancy, *ISG15* is no longer detectable in the LE [29, 30]. Instead, expression extends below the LE to the stratum spongiosum stroma [29, 30]. Expression is maintained through Day 25, then declines by Day 30 with expression limited to patches of the stratum compactum stroma along the maternal-conceptus interface where it remained throughout pregnancy [29, 30]. Other classical ISGs that have been characterized in the sheep uterus are *STAT1* [28], *STAT2* [28], *IRF1* [28], *IRF9* [28], *HLA* [31], *B2M* [31], *MX1* [32], *OAS1* [33], *CTSH* [34], *CTSK* [34], *IFITM1* [35, 93] and *IFITM3* [35, 93]. Transcription of *SOCS1*, *SOCS2*, and *SOCS3* increases during early pregnancy and in response to IFNT, but the spatial patterns of expression are currently unknown [36]. Additional potential ISGs have been identified by microarray analysis although the temporal and spatial pattern of expression is still to be determined [35].

Mice

In mouse placental tissue, antiviral activity consistent with Type I IFN has been detected [14], but may not be due to a classical Ifna or Ifnb [15]. However, trophoblast giant cells *in vitro* have been shown to secrete an Ifna-like factor [16]. Ifng is also present in mouse placentas from Days 10-18 [17]. On Days 10 and 12, Ifng is localized to the trophoblastic giant cells [17]. By Day 14, Ifng is detectable in the spongiotrophoblast and labyrinth region [17]. Ifng is no longer in the spongiotrophoblast by Day 16, but is exclusively expressed in the labyrinth region and remains through Day 18 [17]. Analysis of Ifng expression in the labyrinth region on Day 18 determined that it was primarily localized in endothelial cells immediately surrounding fetal blood vessels [17].

Isg15 mRNA has been detected within implantation sites during pregnancy in the mouse uterus [16, 22]. Interestingly, *Isg15* mRNA levels were much lower in artificially induced deciduomas, indicating that the conceptus may modulate expression [16, 22]. Additionally, endometrial stromal cells incubated with trophoblast giant cells media had

higher levels of *Isg15* mRNA compared to those incubated with control media, and this effect was blocked when the conditioned media was immunodepleted with Ifna antiserum [16].

Primates

First trimester human placental tissue also produces IFNG, with the most intense expression in villous syncytiotrophoblast and extravillous interstitial trophoblast [18]. Stimulation of first trimester human placental tissue with granulocyte-macrophage colony-stimulating factor (GM-CSF), platelet-derived growth factor (PDGF) or Sendai virus resulted in production and secretion of IFNA and IFNB [19-21]. Pre-treatment with both GM-CSF and PDGF followed by infection with Sendai virus resulted in higher levels of IFN then any of the treatments alone [19-21]. Regardless of treatment, the invasive extravillous trophoblasts produced the highest levels of IFN [19-21].

Recently, IFN-regulated genes have been shown to increase in decidualized endometrial fibroblasts in response to trophoblast conditioned media, although IFNA, IFNB, or IFNG was not detected in these conditioned stromal cells [23]. Additionally, *ISG15* has been localized to the decidua [24]. These results indicate that a Type I IFN may be secreted by human trophoblasts.

Pigs

As early as Day 11 of pregnancy, peri-implantation pig conceptuses produce acidic proteins that cross-react with human anti-IFNA antiserum [94] although peak antiviral activity was not measured in uterine flushings or conceptus culture media until Days 14 and 15 of pregnancy [95]. Both Type I IFN and Type II IFN are produced. The major species, which comprises 75% of the antiviral activity of pig conceptus secretory proteins (CSPs), is IFNG and the minor species (25%) is the novel Type I IFND [8, 40]. These pig conceptus IFNs are produced on approximately Days 12-20 of pregnancy, with maximal levels at about Days 15-16 [8, 96, 97].

Unlike IFNT, these IFNs do not appear to have an antiluteolytic effect on the CL, as intrauterine infusion of CSPs on Days 12 to 15 of the estrous cycle had no effect on the interestrus interval or temporal changes in plasma progesterone concentrations [9, 41], however, paracrine effects for IFNs are suggested by localization of IFN receptors on endometrial epithelial cells [40] and *MX1* expression in the stratum compactum stroma of pigs on Day 18 of pregnancy [42]. Although physiological roles for these IFNs in the pig uterus have not been determined, emerging evidence suggests that induction or increases in uterine ISGs by conceptus IFNs is a phenomenon of early pregnancy in many, if not most, mammals [16, 23, 98, 99]. Our working hypothesis is that pig conceptus IFNs increase uterine endometrial expression of ISGs during pregnancy, and that these genes have biological roles in uterine receptivity and conceptus implantation and development.

CHAPTER III

PIG CONCEPTUSES INCREASE UTERINE INTERFERON-REGULATORY FACTOR 1 (*IRF1*), BUT RESTRICT EXPRESSION TO STROMA THROUGH ESTROGEN-INDUCED *IRF2* IN LUMINAL EPITHELIUM*

Introduction

The successful establishment and maintenance of pregnancy requires orchestrated communication between the conceptus (embryo/fetus and associated extraembryonic membranes) and the uterus, which includes: (i) secretions from the conceptus to signal pregnancy recognition [4]; (ii) secretions from the uterine luminal epithelium (LE) and glandular epithelium (GE), i.e., the histotroph, to support the attachment, development and growth of the conceptus [100-102]; (iii) remodeling at the endometrial LE surface to allow intimate association between the conceptus trophectoderm and endometrium for implantation [45, 103, 104]; and (iv) remodeling of the endometrial stroma to generate a cytokine-rich environment that directly promotes angiogenesis, to provide hematotrophic support for the developing conceptus [105, 106].

In pigs, pregnancy recognition is the result of conceptus secretion of estrogens on Days 11 and 12 of pregnancy, which redirects PGF secretion from the uterine vasculature to the uterine lumen, where it is sequestered away from the CL [1, 3, 60, 107]. In contrast to pigs, sheep conceptuses secrete interferon tau (IFNT) to signal maternal recognition of pregnancy [4, 91, 99]. In addition to its antiluteolytic actions on the endometrium, IFNT increases the expression of a number of IFN-stimulated genes (ISGs) in the stroma of the ruminant uterus [28-32, 34, 35, 38, 39, 93, 108], including *MX1* [32], interferon regulatory factor 1 (*IRF1*) [28], signal transducer and activator of

---

* Reprinted with permission from Pig conceptuses increase uterine interferon regulatory factor 1 (*IRF1*), but restrict expression to stroma through estrogen-induced *IRF2* in luminal epithelium by Joyce MM, Burghardt JR, Burghardt RC, Hooper RN, Jaeger LA, Spencer TE, Bazer FW, Johnson GA. Biol Reprod 2007; 77:292-302. Copyright 2007 by Society for the Study of Reproduction.

transcription 2 (*STAT2*) [28], major histocompatibility complex (MHC) class I polypeptide-related alpha chain (*MIC*), and beta-2-microglobulin (*B2M*) [31].

Peri-implantation pig conceptuses also secrete IFNs. Cultured conceptuses from Day 11 of pregnancy have been shown to secrete proteins that cross-react with antiserum against IFN alpha [94], although peak antiviral activity was not measured in the uterine flushings or conceptus culture media until Days 14 and 15 of pregnancy [95]. Both type I IFN and type II IFN are produced. The major species, which comprises 75% of the antiviral activity of pig conceptus secretory proteins (CSPs), is the type II IFN gamma (IFNG) and the minor species (25%) is the novel type I IFN delta (IFND) [8, 40]. These IFNs do not appear to have antiluteolytic activities during pregnancy, as intrauterine infusion of CSPs on Days 12 to 15 of the estrus cycle had no effect on the interestrus interval or temporal changes in plasma progesterone concentrations [9, 41]. However, paracrine effects for IFNs are suggested by localization of IFN receptors on endometrial epithelial cells [40], increased secretion of prostaglandin E_2 [41], and *MX1* expression in the stratum compactum stroma of pigs on Day 18 of pregnancy [42]. The effects of these IFNs on pig endometrium have not been determined. Although it has been noted that high peri-implantation levels of IFNG coincide with the presence of uterine transforming growth factor β, interleukin 6 and MHC class II antigens in pigs [109, 110], increased uterine expression of classical ISGs has not been detected [111]. Indeed, treatment of Madin-Darby bovine kidney cells and bovine endometrial explant cultures with pig CSPs increased ISG expression, whereas a similar treatment had no effect on ISG expression in pig endometrial explants [111].

Our working hypothesis is that pig conceptus IFNs increase uterine endometrial expression of ISGs during pregnancy, and that these genes have biological roles in uterine receptivity and conceptus implantation and development. *IRF1* is a key intermediate in the induction cascade of many classical ISGs through its abilities to bind and transactivate IFN-stimulated response elements (ISRE) at their promoters [112-114]. Both type I and type II IFNs induce *IRF1* [112], which plays a role in placental development in the murine reproductive tract [113]. In sheep, *IRF1* expression increases

in the stroma and GE, but not in the LE, during early pregnancy, presumably due to the expression of *IRF2*, which is a potent transcriptional repressor of ISGs that is constitutively expressed in the LE and increases during early pregnancy [28]. Therefore, the objectives of the present studies were to determine whether *IRF1* and *IRF2* are expressed in the pig endometrium during pregnancy, and if so, whether the expression of these genes is regulated by conceptus estrogen and/or CSPs that contain IFNG and IFND.

Materials and Methods

Animals and Tissue Collection

All the experimental and surgical procedures complied with the Guide for Care and Use of Laboratory Animals and were approved by the Texas A&M University Laboratory Animal Care and Use Committee. Pigs were observed daily for estrus (Day 0) and exhibited at least two estrus cycles of normal duration (18-21 days) before use in these studies.

Study 1. To evaluate the effect of pregnancy status on endometrial gene expression, sexually mature pigs were assigned randomly at estrus to either cyclic or pregnant status. The pigs in the pregnant group were bred upon detection of estrus and 12 h and 24 h thereafter. Pigs were ovariohysterectomized on Day 5, 9, 12, or 15 of the estrus cycle or on Day 9, 10, 12, 13, 14, 15, 20, 25, 30, 35, 40, 60 or 85 of pregnancy (n = 3 pigs/day/status) (Fig. 3.1). Pregnancy was confirmed by the presence of normal conceptuses in the uterine flushings (Days 9-15) or at hysterectomy (Days 20-85).

Study 2. To evaluate the effect of estrogen-induced pseudopregnancy on uterine gene expression, pigs were assigned randomly at estrus to receive daily i.m. injections of either 5 ml corn oil (CO) vehicle or 5 mg 17β-estradiol benzoate (E_2; Sigma Chemical Company, St. Louis, MO) in 5 ml CO on Days 11, 12, 13, and 14 postestrus (n = 5 pigs/treatment). All pigs were ovariohysterectomized on Day 15 postestrus (Fig. 3.2).

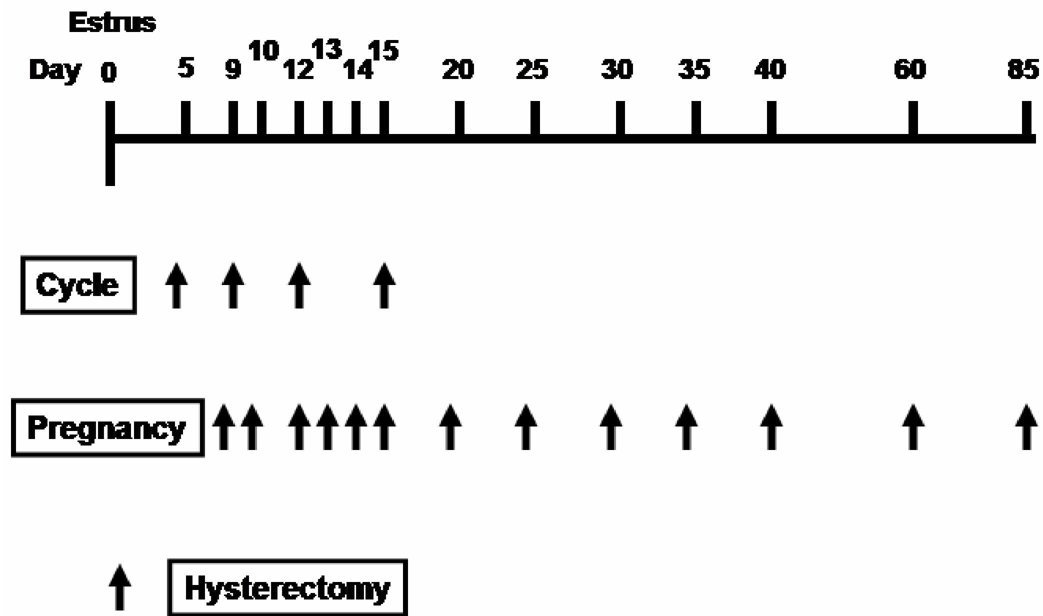


FIG. 3.1. Study 1 experimental design. Pigs were assigned randomly at estrus to either cyclic or pregnant status. Those in the pregnant group were bred at estrus and 12 h and 24 h thereafter. Pigs were ovariohysterectomized on the indicated days of the estrus cycle or pregnancy.

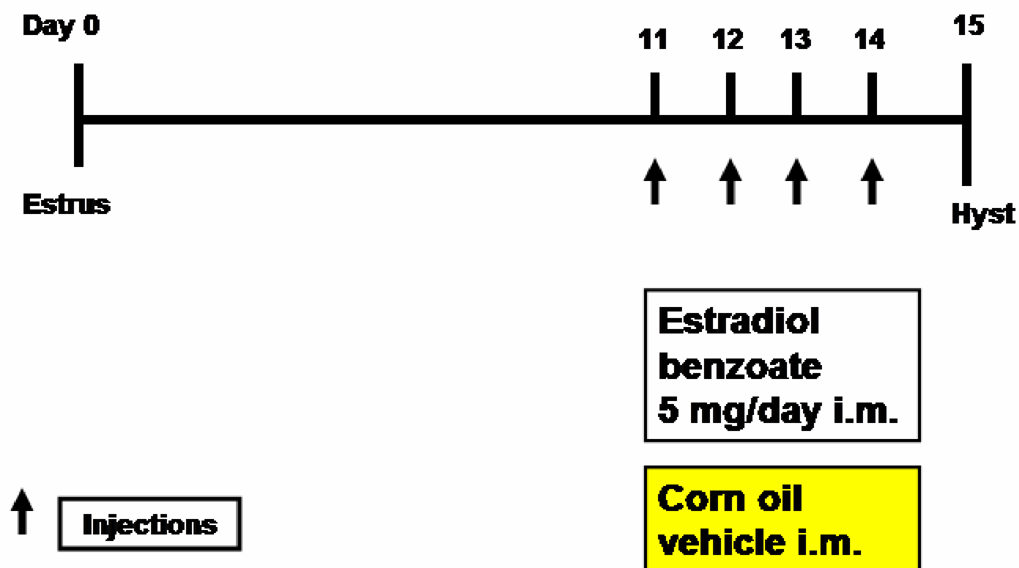


FIG. 3.2. Study 2 experimental design. Cyclic pigs received daily i.m. injections of either estradiol benzoate or corn oil vehicle on Days 11-15 postestrus. All pigs were ovariohysterectomized on Day 15 postestrus.

Study 3. To evaluate the effect of pig CSPs on uterine gene expression, pigs (n = 3) were injected i.m. with 5 mg E_2 in 5 ml of CO on Days 11, 12, 13, 14, and 15 postestrus, to induce pseudopregnancy. On Day 12 postestrus (coincident with the onset and prior to the peak of secretion of IFNs by the pig conceptuses [8, 94, 95]), each pig was surgically implanted with two indwelling ALZET osmotic pumps (Durect Corp., Cupertino, CA) with constant delivery rates of 10 µl/h. Briefly, each uterine horn was isolated via midline celiotomy, clamped, and severed from the uterine body at approximately 12.7 cm from the utero-tubal junction, while preserving the mesometrium and vascular supply to the uterine horn. The transected ends of each uterine horn and uterine body were closed using an inverting suture pattern of absorbable suture, and the serosa of the antimesometrial borders of each uterine horn and the uterine body were sutured together to prevent twisting of the horn. For each pump, a catheter was attached and inserted approximately 2 cm into the lumen of one uterine horn. Prior to surgery, the pumps were filled and equilibrated according to the manufacturer's instructions. For each pig, one uterine horn was infused by a pump that was filled with 35 mg of porcine serum albumin (Sigma), while the other uterine horn was infused by a pump that was filled with 35 mg of porcine CSPs. Thus, a 12.7-cm isolated section of the uterine horn, which retained full vascular supply, was completely exposed to the infusate; the uterine tissue samples were taken from these sections (Fig. 3.3). Pilot studies were conducted with infusion of India ink to confirm coverage of the uterus by the infusate. All gilts were ovariohysterectomized on Day 16 postestrus (coincident with maximal antiviral activity in the pig uterine flushings [95]) (Fig. 3.4).

At hysterectomy, several sections (~0.5 cm thickness) from the middle of each uterine horn or from the isolated pouch of an infused uterus were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2) and embedded in Paraplast-Plus (Oxford Laboaratory, St. Louis, MO). Several sections from each uterine horn were also embedded in Tissue-Tek Optimal Cutting Temperature (OCT) Compound (Miles, Oneonta, NY), snap frozen in liquid nitrogen, and stored at -80°C before sectioning.

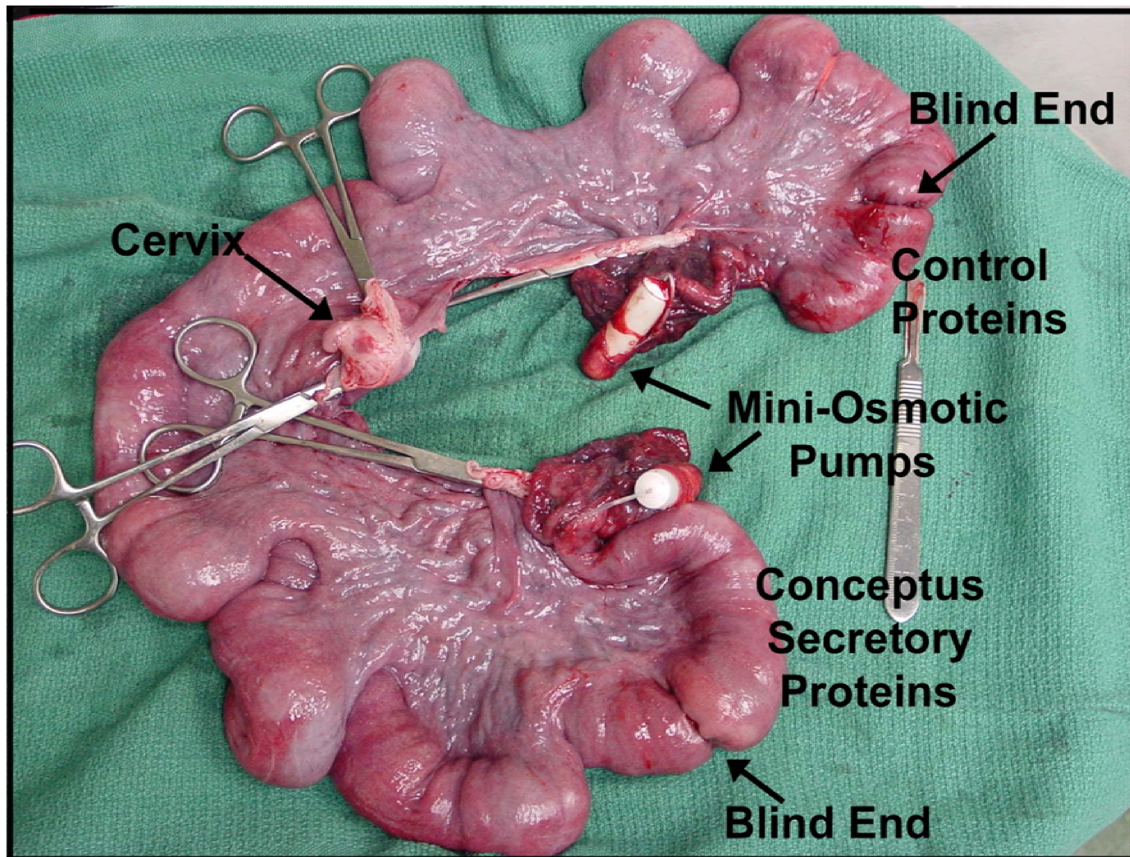


FIG. 3.3. Pig reproductive tract after ovariohysterectomy. On Day 12 postestrus, each uterine horn was surgically severed from the uterine body ~12.7 cm from the utero-tubal junction and two mini-osmotic pumps were surgically implanted. For each pump, a catheter was attached and inserted into the lumen of one uterine horn. For each pig, one pump was filled with control proteins and the other with porcine conceptus secretory proteins.

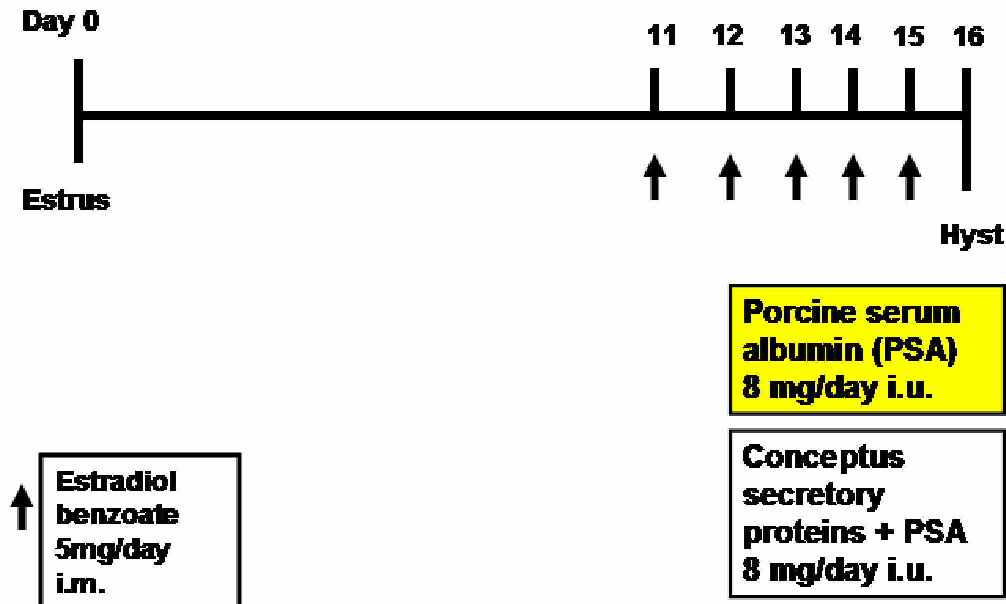


FIG. 3.4. Study 3 experimental design. Cyclic pigs received daily i.m. injection of estradiol benzoate on Days 11-15 postestrus. On Day 12 postestrus, each uterine horn was surgically severed from the uterine body ~12.7 cm from the utero-tubal junction and two mini-osmotic pumps were implanted. Prior to surgery, one pump was filled with porcine serum albumin (control protein) and the other was filled with porcine conceptus secretory proteins. For each pump, a catheter was attached and inserted into the lumen of one uterine horn.

The remaining endometrium was physically dissected from the myometrium, frozen in liquid nitrogen, and stored at -80°C for RNA extraction.

Preparation of Porcine CSPs

Using procedures previously described [41, 115], the conceptuses from Day 15-17 pregnant pigs (coincident with maximal production of IFNs by the conceptuses [8, 94, 95]) were recovered by flushing each uterine horn with 20 ml of minimal essential medium (MEM). The conceptuses were then cultured in MEM for 30 h at 37°C with rocking in a 50% O_2, 45% N_2, 5% CO_2 atmosphere. The culture medium was collected after centrifugation and protease inhibitors (Complete EDTA-free Protease Inhibitor Cocktail; Roche Diagnostics, Indianapolis, IN) were added. The culture supernatant was dialyzed (MWCO 3500; Spectrum Laboratories, Inc., Rancho Dominguez, CA) four times using 4 L of 10 mM Tris (pH 8.2) each time, and concentrated (MWCO 5000; Millipore Corp., Bedford, MA). The sample was then dialyzed (MWCO 1000, Spectrum Laboratories) against Dulbecco PBS (Sigma), protease inhibitors were added, and the sample was filter sterilized, assayed for protein concentration, and stored at 4°C until use.

RNA Isolation and Analyses

RNA isolation. Total cellular RNA was isolated from endometrial tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations.

Northern blot analysis. Total endometrial RNA (8 µg) was loaded onto a 1.2% agarose gel, electrophoresed, and transferred to a 0.2-µm nylon membrane, as described previously [116]. The blot was hybridized with radiolabeled antisense cRNA probes that were generated from linearized ovine *IRF1* [28], ovine *IRF2* [28], human *STAT2* [117], ovine *MIC* [31], or ovine *B2M* [31] plasmid templates. Radiolabeled riboprobes were generated by in vitro transcription with [α-^{32}P]uridine 5-triphosphate (Perkin-Elmer Life Sciences, Inc., Boston, MA) and the MAXIscript kit (Ambion, Austin, TX). After

washing, nonspecific hybridization was eliminated by RNase A digestion. Hybridization signals were detected by exposing the blot to a PhosphoImager screen and visualized using a Typhoon 8600 variable mode imager (Molecular Dynamics, Piscataway, NJ).

Slot blot analysis. The steady-state mRNA levels were assessed in endometrial total RNA samples (20 µg) by slot blot hybridization with radiolabeled antisense ovine *IRF1* [28], ovine *IRF2* [28], human *STAT2* [117], ovine *MIC* [31], or ovine *B2M* [31] cRNA probes using methods described previously [116]. To correct for variability in total RNA loading, a duplicate RNA slot membrane was hybridized with a radiolabeled antisense 18S rRNA (pT718S; Ambion) cRNA probe. The radiolabeled riboprobes were generated as described above. The membranes were washed, digested and hybridization signals were detected as descibed above.

In situ hybridization analysis. *IRF1*, *IRF2*, and *STAT2* mRNAs were localized in paraffin-embedded pig uterine tissues by *in situ* hybridization, as previously described [37]. Briefly, deparaffinized, rehydrated, and deproteinated uterine cross-sections (5-µm thickness) were hybridized with radiolabeled antisense or sense ovine *IRF1* [28], ovine *IRF2* [28], and human *STAT2* [117] cRNA probes, which were synthesized by in vitro transcription with [α-^{35}S]uridine 5-triphosphate (Perkin-Elmer Life Sciences). After hybridization, washing, and RNase A digestion, autoradiography was performed using NTB liquid photographic emulsion (Eastman Kodak, Rochester, NY). Slides were exposed at 4°C, developed in Kodak D-19 developer, counterstained with Harris modified hematoxylin (Fisher Scientific, Fairlawn, NJ), dehydrated, and protected with coverslips.

Immunofluorescence Analysis

Immunoreactive IRF1 protein was localized in frozen porcine uterine cross-sections (~8-10-µm thickness) by immunofluorescence staining using methods described previously [118]. Briefly, tissues were fixed in methanol at -20°C, washed in PBS that contained 0.3% (vol/vol) Tween-20, blocked in 10% normal goat serum, incubated overnight at 4°C with 1 µg/ml of rabbit antihuman IRF1 (sc-497; Santa Cruz

Biotechnology, Santa Cruz, CA) or rabbit IgG (negative control; Sigma), and detected with fluorescein-conjugated goat anti-rabbit IgG (Chemicon International, Temecula, CA). The slides were overlaid with Prolong antifade mounting reagent (Molecular Probes, Eugene, OR) and a coverslip.

Photomicrography

Digital photomicrographs of *in situ* hybridization and immunofluorescence staining were evaluated using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY), which was interfaced with an Axioplan HR digital camera and the Axiovision 4.3 software. Photographic plates were assembled using the Adobe Photoshop ver. 6.0 software (Adobe Systems Inc., San Jose, CA).

Statistical Analysis

The data were subjected to least-squares ANOVA using the general linear models procedures of the Statistical Analysis System (SAS, Cary, NC). The slot blot hybridization data were analyzed using 18S rRNA as a covariate to correct for differences in RNA loading. The data from study 1 were analyzed for the effect of day and status and their interaction where appropriate. For all other studies, the effects of treatment were determined by preplanned orthogonal contrasts. All tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error, and $P < 0.05$ was considered statistically significant. Data are presented as least-squares means with standard errors (SEMs). The SEM represents the pool of the mean derived using the root mean square error term generated by the SAS software.

Results

Effects of Pregnancy (Study 1)

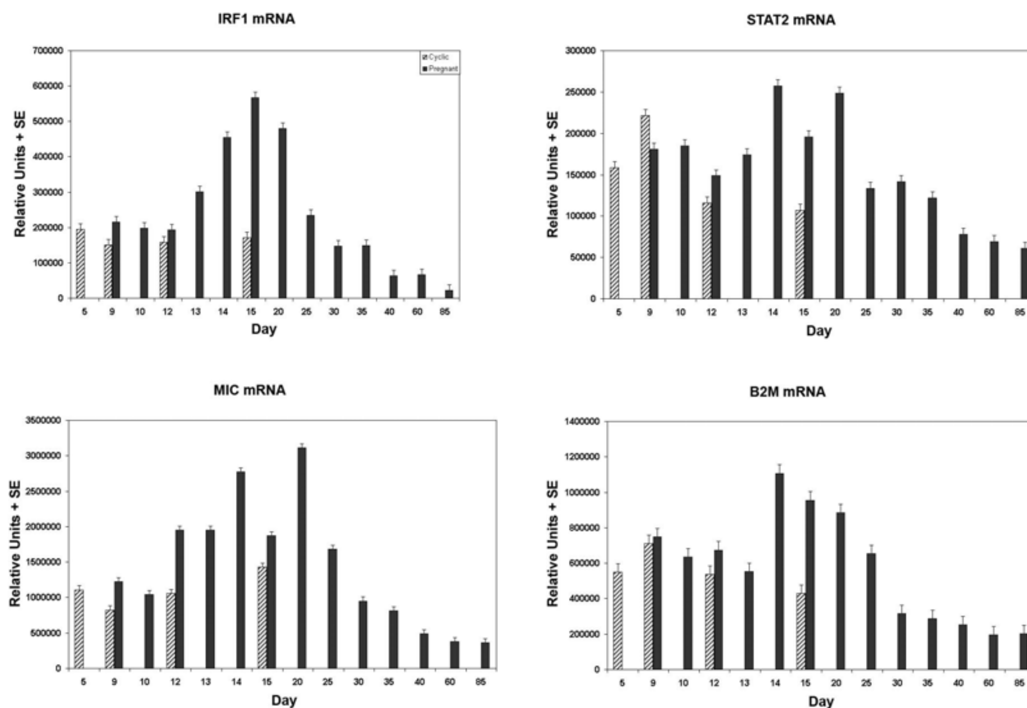
Steady-state levels of IRF1*,* STAT2*,* MIC*,* B2M*, and* IRF2 *mRNAs in the pig endometrium.* The ovine cDNAs for *IRF1*, *MIC*, and *B2M* revealed mRNAs of ~ 2.1

kb, ~1.7 kb, and ~1.0 kb, respectively, and the human cDNA for *STAT2* revealed mRNAs of ~4.5 kb and ~4.8 kb in Northern blot analysis of pig total endometrial RNA (data not shown). These mRNAs were similar in size to those detected using the same cDNAs with sheep total mRNA. The steady-state levels of *IRF1*, *MIC*, and *B2M* mRNAs in the porcine endometrium did not change ($P > 0.10$), whereas the *STAT2* mRNA levels increased between Day 5 and Day 9 during the estrus cycle, and decreased thereafter ($P < 0.005$, cubic effect of day) during the estrus cycle. During pregnancy, the *IRF1* mRNA levels were low on Day 9 to Day 12, increased almost 3-fold between Days 12 and 15, declined between Days 15 and 40, and remained low thereafter ($P < 0.001$, quartic effect of day) (Fig. 3.5A). The *STAT2* mRNA levels increased more than 2-fold between Days 12 and 14 ($P < 0.01$, linear effect of day) (Fig. 3.5A). The *MIC* mRNA levels increased nearly 3-fold between Days 10 and 14 of pregnancy, remained high through Day 20, declined between Days 20 and 40, and remained low thereafter ($P < 0.05$, cubic effect of day) (Fig. 3.5A). The *B2M* mRNA levels gradually decreased between Day 9 and Day 13, increased ~ 2-fold between Days 13 and 14, gradually declined through Day 25, and remained low thereafter ($P = 0.1$, quadratic effect of day) (Fig. 3.5A). Therefore, the levels of the mRNAs for the four classical ISGs, i.e., *IRF1*, *STAT2*, *MIC*, and *B2M*, are increased in the pig endometrium during the peri-implantation period.

The ovine cDNA for *IRF2* revealed a ~ 2.4 kb mRNA in both pig and sheep endometrial total RNA. The steady-state levels of *IRF2* mRNA did not change ($P > 0.10$) during the estrus cycle, whereas the *IRF2* mRNA levels increased from Day 9 to Day 13 in pregnant pigs, was maximal on Days 13-15, and decreased thereafter ($P < 0.001$, quadratic effect of day) (Fig. 3.5B).

In situ hybridization for IRF1*,* STAT2*, and* IRF2 *mRNAs in the pig endometrium*. The levels of *IRF1* (Fig. 3.6) and *STAT2* (Fig. 3.7) mRNAs were low in all endometrial cell types during the estrus cycle. During pregnancy, the *IRF1* and *STAT2* mRNAs were noticeably upregulated in the stratum compactum stroma of the endometrium between Day 12 and Day 15. The *IRF1* and *STAT2* mRNAs remained in the stratum

A) Classical Interferon-Stimulated Genes



B) Non-Interferon-Stimulated Gene

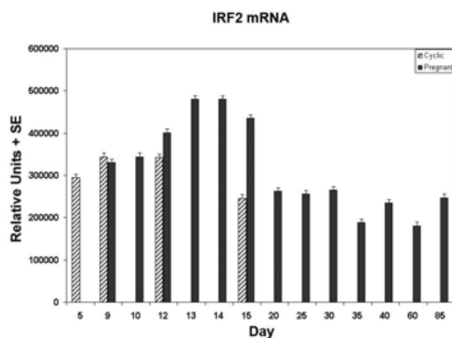


FIG. 3.5. Steady-state levels of mRNAs for the classical IFN-stimulated genes (**A**) *IRF1* (**a**), *STAT2* (**b**), *MIC* (**c**), and *B2M* (**d**), and the non-IFN stimulated gene (**B**) *IRF2* in pig endometria during the estrus cycle and pregnancy. The mRNA levels, expressed as least square means of the relative counts per minute with overall SEM, are normalized for differences in sample loading using 18S rRNA and represent 20 µg of total endometrial mRNA per sample.

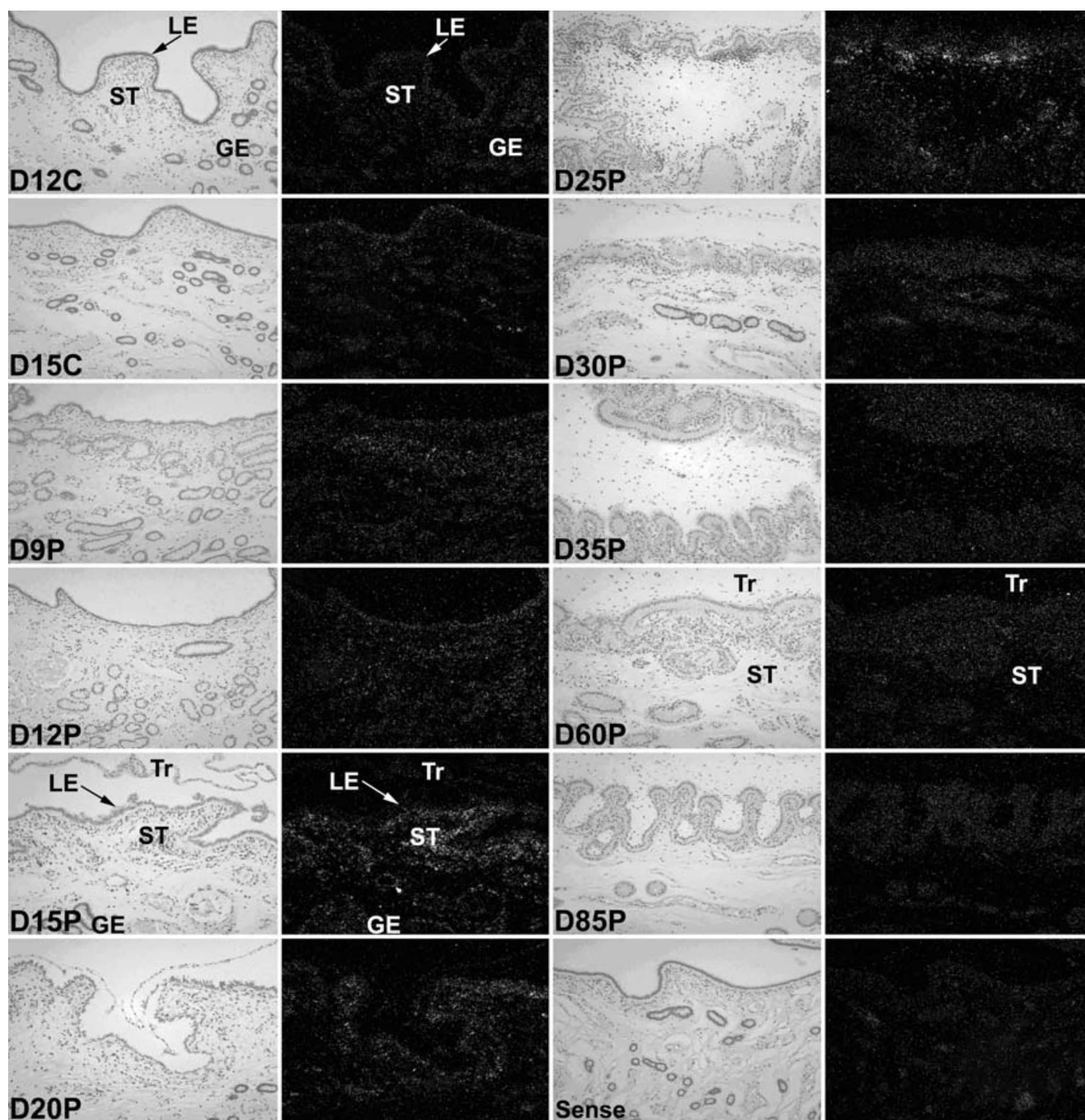


FIG. 3.6. *In situ* hybridization analysis of *IRF1* mRNA in pig uteri. Corresponding bright-field and dark-field images from different Days (D) of the estrus cycle (C) and pregnancy (P) are shown. A representative section from D12P hybridized with radiolabeled sense cRNA probe (Sense) serves as a negative control. LE, luminal epithelium; GE, glandular epithelium; ST, stratum compactum stroma; Tr, trophectoderm. The width of each field is 940 μm.

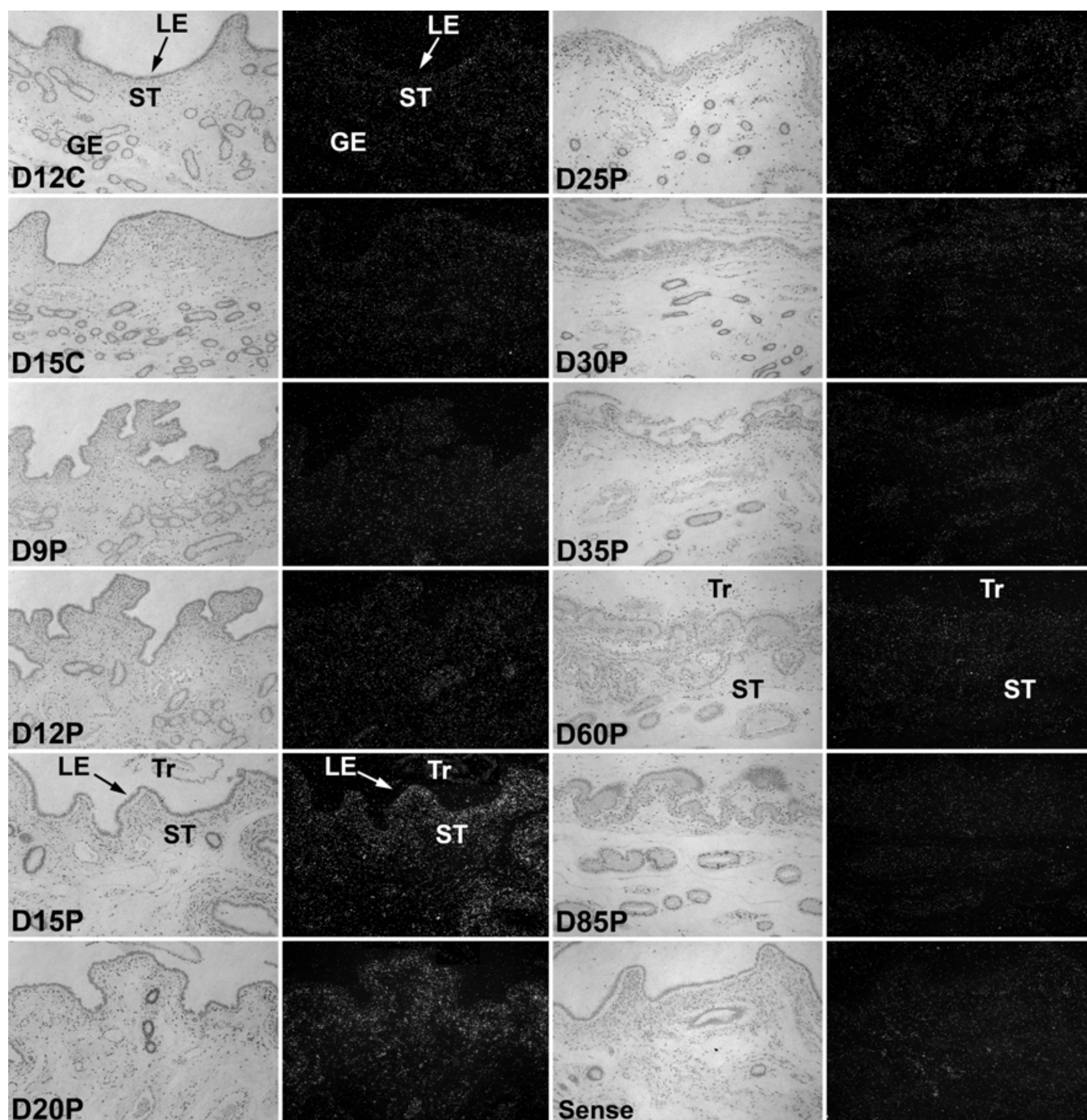


FIG. 3.7. *In situ* hybridization analysis of *STAT2* mRNA in pig uteri. Corresponding bright-field and dark-field images from different Days (D) of the estrus cycle (C) and pregnancy (P) are shown. A representative section from D15P hybridized with radiolabeled sense cRNA probe (Sense) serves as a negative control. LE, luminal epithelium; GE, glandular epithelium; ST, stratum compactum stroma; Tr, trophectoderm. The width of each field is 940 μm.

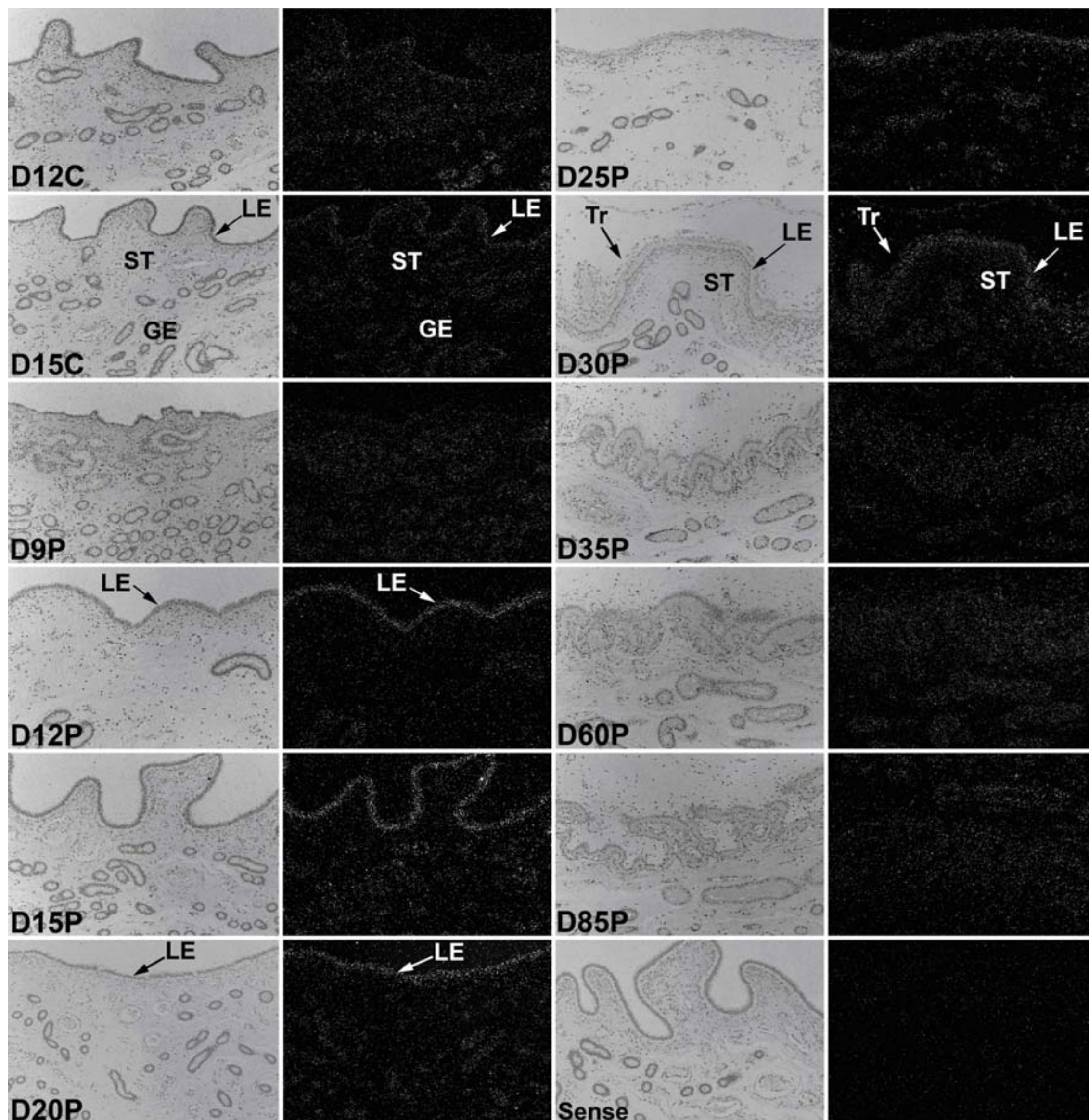


FIG. 3.8. *In situ* hybridization analysis of *IRF2* mRNA in cross-sections of pig uteri. Corresponding bright-field and dark-field images from different Days (D) of the estrus cycle (C) and pregnancy (P) are shown. A representative section from D12P hybridized with radiolabeled sense cRNA probe (Sense) serves as a negative control. LE, luminal epithelium; GE, glandular epithelium; ST, stratum compactum stroma; Tr, trophectoderm. The width of each field is 940 µm.

compactum stroma through Day 25 of pregnancy, and then decreased to very low levels through Day 85. The *IRF1* (Fig. 3.6) and *STAT2* (Fig. 3.7) mRNAs were not observed in the uterine LE of either the cyclic or pregnant pigs. Therefore, the mRNAs for two classical ISGs, i.e., *IRF1* and *STAT2*, are increased specifically in pig endometrial stroma during the peri-implantation period.

As illustrated in Figure 3.8, the level of *IRF2* mRNA was low during the estrus cycle. However, *IRF2* mRNA appeared in the LE of pregnant pigs on Day 12 and remained in the LE through Day 30. *IRF2* mRNA was not observed in the stroma or glands of the uteri from both cyclic and pregnant pigs.

Immunoreactive IRF1 protein. Consistent with the *in situ* hybridization results, the level of IRF1 protein was low in the endometrium on Day 15 of the estrus cycle, but was present in the endometrial stroma on Day 15 of pregnancy (Fig. 3.9). IRF1 protein was not observed in the LE of pregnant endometrium (Fig. 3.9).

Collectively, these data document two expression patterns during the peri-implantation period of pigs: 1) *IRF2* increased in LE cells on Day 12, at which time-point the elongated pig conceptuses secrete estrogen for pregnancy recognition [1]; and 2) the levels of classical ISGs increase in the endometrial stroma between Day 12 and Day 15, which temporally correlates with increased antiviral activity measured in uterine flushes exposed to conceptus secretion of IFNG and IFND [8, 94, 95].

Exogenous Estrogen Induces IRF2 *But Not* IRF1 *in Porcine Endometrium (Study 2)*

Intramuscular injections of E_2 did not alter the steady-state levels of *IRF1* mRNA in the pig endometrium compared to CO injection (for CO vs. E2, 146 634 vs. 115 756 ± 11 505 relative units of radioactivity; $P > 0.10$). Consistent with the slot blot hybridization results, the levels of immunoreactive IRF1 protein were similar in the endometria (Day 15) of cyclic pigs injected with E_2 and those injected with CO vehicle (Fig. 3.10A).

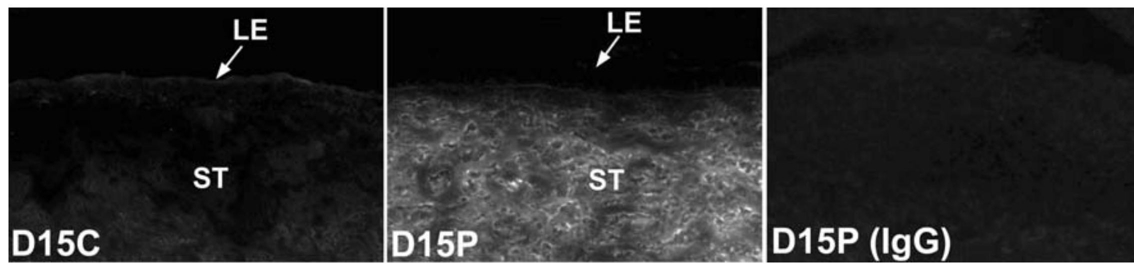
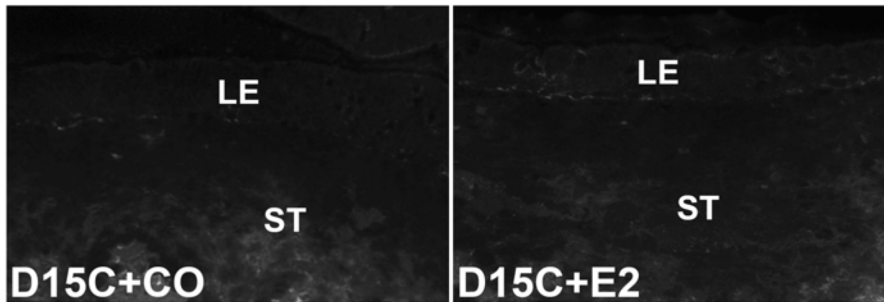


FIG. 3.9. Immunofluorescence localization of IRF1 protein in frozen cross-sections of pig endometria from day 15 of the estrus cycle (D15C) and D15 of pregnancy (P). Nonrelevant rabbit immunoglobulin (IgG) serves as a negative control. LE, luminal epithelium; ST, stratum compactum stroma. The width of each field is 540 µm.



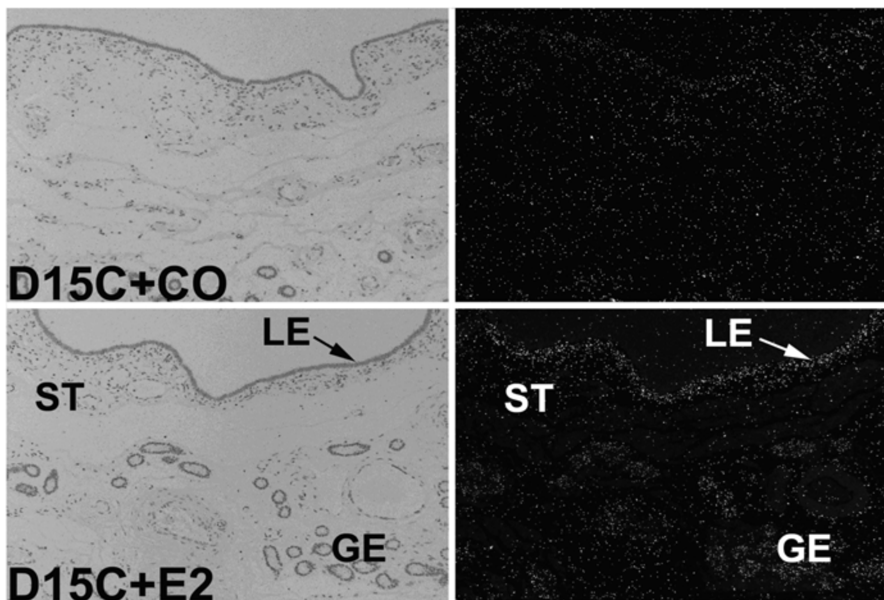


FIG. 3.10. Localization of IRF1 protein and *IRF2* mRNA in cross-sections of pig uteri from study 2. **A**) Immunofluorescence localization of IRF1 protein in frozen cross-sections of endometria and from Day 15 cyclic pigs injected i.m. with either the CO control (D15C+CO) or E_2 (D15C+E2). An example of nonrelevant rabbit immunoglobulin (IgG) is shown in Figure 3.9, and serves as a negative control. The width of each field is 540 μm. **B**) *In situ* hybridization analysis of *IRF2* mRNA in uterine cross-sections from pigs injected with either CO or E_2. Corresponding bright-field and dark-field images of the endometrium are shown. A representative section hybridized with radiolabeled sense cRNA probe (Sense) is shown in Figure 3.8, and serves as a negative control. The width of each field is 940 μm. LE, luminal epithelium; GE, glandular epithelium; ST, stratum compactum stroma.

In contrast, the levels of *IRF2* mRNA were increased ($P < 0.05$) in the endometria of pigs injected with E_2 as compared to those injected with CO vehicle (for CO vs. E_2, 245 844 vs. 343 684 ± 19 604 relative units of radioactivity). The *in situ* hybridization analyses revealed that *IRF2* mRNA was increased specifically by E_2 in the uterine LE (Fig. 3.10B).

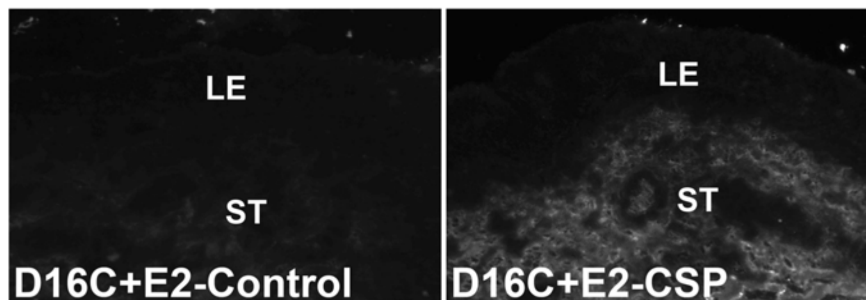
CSPs Regulate IRF1 *But Not* IRF2 *(Study 3)*

Steady-state levels of IRF1 *mRNA in the pig endometrium.* Intrauterine infusion of CSPs into the uterine horn of pigs treated with exogenous estrogen increased ($P < 0.1$) the steady-state levels of endometrial *IRF1* mRNA about 2-fold as compared to the uterine horns infused with control serum proteins. Consistent with the slot blot hybridization results, immunoreactive IRF1 protein abundance was noticeably greater in the stratum compactum stroma of the uterine horn infused with CSPs as compared to the uterine horn infused with control serum proteins (Fig. 3.11A).

In contrast, *in situ* hybridization for *IRF2* revealed that intrauterine infusion of CSP into Day-16 pigs treated with exogenous estrogen did not increase *IRF2* mRNA expression over the levels detected after intrauterine infusion of control serum proteins (Fig. 3.11B).

Discussion

Our results demonstrate that the levels of *IRF1*, *STAT2*, *MIC*, and *B2M* increase in endometria in the peri-implantation period, during which elongated pig conceptuses secrete IFND and IFNG [8, 40]. Furthermore, *IRF1* and *STAT2* are expressed in the endometrial stroma. It seems likely that IFND and/or IFNG pass through altered tight junctions between the uterine LE cells [119], to act in a paracrine manner to induce these genes in the endometrial stroma, since *IRF1*, *STAT2*, *MIC*, and *B2M* are known to be induced by both type I and type II IFNs [28, 112]. Indeed, the lower magnitude of increased expression of these genes in the endometria of pigs as compared to sheep correlates well with the differences noted between these species in the antiviral activities



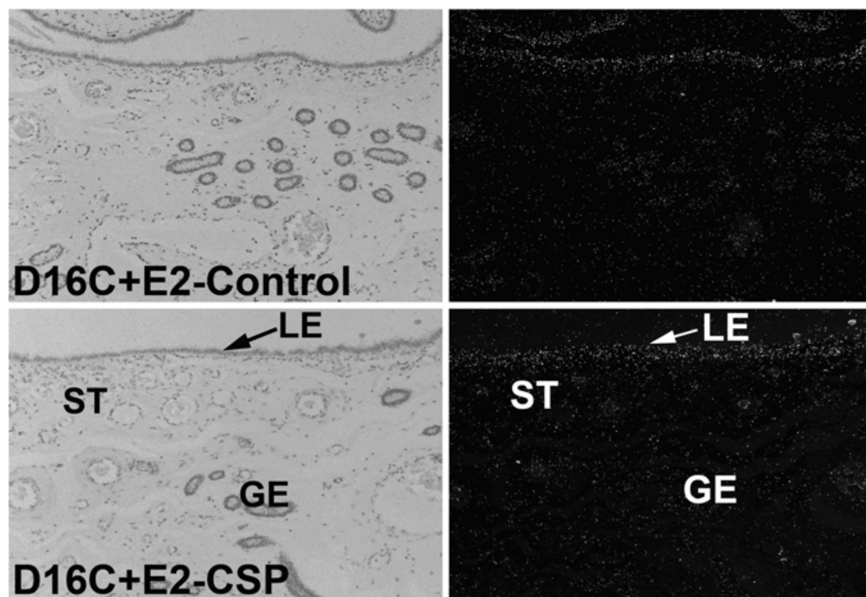


FIG. 3.11. Localization of IRF1 protein and *IRF2* mRNA in cross-sections of pig uteri from study 3. **A**) Immunofluorescence localization of IRF1 protein in frozen cross-sections of pig endometria from Day 16 cyclic pigs that received intrauterine infusion of control serum proteins (D16C+E2-Control) or CSPs (D16C+E2-CSP). An example of nonrelevant rabbit immunoglobulin (IgG) is shown in Figure 3.9, and serves as a negative control. The width of each field is 540 µm. **B**) *In situ* hybridization analysis of *IRF2* mRNA in uterine cross-sections from pigs injected with either CO or E_2. Corresponding bright-field and dark-field images of the endometrium are shown. A representative section hybridized with radiolabeled sense cRNA probe (Sense) is shown in Figure 3.8, and serves as a negative control. The width of each field is 940 µm. LE, luminal epithelium; GE, glandular epithelium; ST, stratum compactum stroma.

measured in the uterine flushes [8, 120]. Increases in *IRF1* and *STAT2* gene transcription in several cell lines require the activation of STAT1, formation of a homodimer (termed γ-activated factor or GAF), translocation to the nucleus, and transactivation through GAF binding to a γ-activated sequence (GAS) in the *IRF1* promoter [84, 112, 121, 122]. However, IRF1 can also coordinate with and maintain the induction cascade of other classical ISGs through binding and transactivating IFN-stimulated response elements (ISRE) at their promoters [112-114]. It is significant that the stromal distributions of *IRF1* and *STAT2* in the pig uterus are similar to the increases observed for *IRF1* and *STAT2* in pregnant sheep endometria exposed to IFNT [28], and this temporal/spatial pattern of expression is shared by several ISGs in sheep [28-32, 34, 35, 39, 123]. Cattle, mice, and primates express ISGs in the endometrial stroma or decidua of pregnancy [22, 24, 93, 108]. This is the first report of temporal changes in the gene expression levels of *IRF1*, *STAT2*, *MIC*, and *B2M* and in the spatial distributions of *IRF1* and *STAT2* in the pig uterus, and the first direct linkage of CSPs that contain IFNG and IFND to endometrial ISG expression in pigs.

Although it has been suggested that IRF1 induces and/or maintains the transcription of selected ISGs in the endometrial stroma of sheep [111], the pregnancy-specific roles of uterine ISGs remain conjectural. Hess and coworkers [23] treated decidualized human endometrial stromal cells with conditioned media from human trophoblasts, in studies similar to the in vivo intrauterine infusion experiments of the present study, and found that many ISGs, including *IRF1*, were upregulated. The upregulation of ISGs from the secretory products of human trophoblasts is likely due to the production of type I IFNs [21]. Mouse trophoblast giant cells have also been shown to produce a type I IFN-like molecule, which induces ISG expression in endometrial stromal cells [16]. Thus, emerging evidence suggests that the induction and increase in ISGs in the endometrium or decidua by conceptus IFNs are phenomena of early pregnancy in many mammals [23, 24, 124], including pigs (present study). Interestingly, a decidual-like transformation has been reported in the pregnant endometrial stroma of sheep, which suggests that the endometrium of noninvasive implanting species

undergoes remodeling that is somewhat similar to uterine decidua of species with invasive implantation [125]. Therefore, it is likely that ISGs facilitate remodeling within the stromal compartment of the uterus for uterine receptivity to conceptus implantation and placentation across disparate mammalian species.

As players in decidual/stromal remodeling, individual ISGs may be involved in protecting the fetal semiallograft from immune rejection, limiting conceptus invasion through the uterine wall, and/or establishing a vascular supply to the conceptus. Since IFNG, a protein secreted by pig conceptuses, is involved in endometrial vascular development in mice [126], it is reasonable to hypothesize that conceptus-derived IFNs upregulate ISGs, such as *IRF1*, to facilitate the vascular changes that are needed to provide hematotrophic support to the developing conceptus. Whether or not this is true, it is becoming increasingly clear that IFN induction of genes within the uterine stroma of mammals is a universal response to or component of mechanisms for the establishment and maintenance of pregnancy.

IRF2 is a potent repressor and attenuator of ISG expression and inhibits ISRE-containing genes through direct ISRE binding and coactivator repulsion [127, 128]. As such, IRF2 is an important regulator in gene networks of the IFN system [129, 130]. A previous study by Choi et al. [28] described the expression of *IRF2* in the endometrial LE of early pregnant sheep. In addition, the transcriptional activity of a promoter-reporter construct that contained five consensus ISRE-binding sites was strongly repressed by transient transfection of immortalized sheep stromal cells [131] with vectors that overexpressed ovine *IRF2* [28]. These data, along with the constitutive presence of IRF2 and lack of IRF1 and many other classical ISGs in the LE, have led to the hypothesis that IRF2 restricts the expression of ISGs in the LE by directly repressing their transcription and rendering IFNT unable to activate the classical JAK-STAT-IRF1 pathway [28, 99]. The present studies are the first to localize *IRF2* in the pig endometrium, and the similar temporal and spatial patterns of expression of *IRF2* and *IRF1* in pigs and sheep supports the idea that IRF2 represses the expression of ISGs in the LE of pigs, and perhaps in mammals in general.

In the present study, the conceptus and injections of estrogen induced *IRF2* expression, specifically in the endometrial LE. Estrogen receptor α (ESR1) is present in the LE on Day 12 of pregnancy [7], at which time-point the conceptuses secrete estrogens. Furthermore, estrogen is capable of regulating gene transcription through ESR1/Sp1 interactions [91] and the human *IRF2* promoter contains four Sp1 sites [130]. Estrogens are the maternal recognition signals that prevent CL regression [1]. In addition, conceptus estrogens modulate uterine gene expression to support the controlled inflammatory-like events that characterize changes in conceptus morphology and uterine remodeling for implantation in pigs [132]. Indeed, secreted phosphoprotein 1 (or osteopontin) is induced by estrogen in the LE [133]. Furthermore, conceptus secretion of estrogens correlates with conceptus secretion of interleukin 1β, which may in turn modulate the uterine response to this cytokine [134]. The importance of estrogen to early survival of pig conceptuses is underscored by the fact that premature exposure of the pregnant uterus to estrogen on Day 9 and Day 10 results in the degeneration of all pig conceptuses by Day 15 [5]. The present results strongly suggest that conceptus estrogens induce *IRF2* in the endometrial LE, thereby indirectly inhibiting conceptus IFNs from inducing *IRF1*, *STAT2*, and presumably other ISGs at the sites of conceptus attachment for implantation. The role that ISG repression plays in the establishment of pregnancy remains to be determined. However, in sheep, MHC class I and β2-microglobulin are silenced in the LE, presumably by IRF2 [31]. It has been hypothesized that the ablation of these key molecules, which are involved in host defense and immune histocompatibility of transplanted tissues at the maternal-placental interface, ensures acceptance of the conceptus semiallograft [31]. It is reasonable to predict similar mechanisms for the pig.

Interestingly, the placentas and offspring of *Irf1*$^{-/-}$ mice are smaller than their wild-type counterparts [110], a phenotype similar to that of several mouse strains that lack uterine natural killer (uNK) cells [135]. Uterine NK cells are associated with modification of the decidual spiral arteries that supply the conceptus with hematotrophic support [135]. In *Irf1*$^{-/-}$ mice, the uNK cells are fewer, smaller, and hypogranular [110].

Both Irf1 and Irf2 are involved in the development and/or function of peripheral NK cells. In *Irf1*$^{-/-}$ mice, the numbers of NK cells in the spleen and liver are reduced and cytolytic activity is absent [136, 137]. Irf1 also transcriptionally regulates interleukin 15 [138], which is involved in NK cell maturation [139]. In *Irf2*$^{-/-}$ mice, the number of NK cells and the NK cell cytotoxic activities of splenocytes are reduced [140]. While both Irf1 and Irf2 are important for peripheral NK cells, only Irf1 is involved in uNK cell development [110].

An attractive hypothesis for the pig is that conceptus secretion of IFNG and/or IFND increases IRF1 expression in the uterine stroma, which plays a role in increasing uNK cell cytolytic activity to expand maternal vascular support for developing conceptuses. Uterine NK cells are present and increase in the pig endometrium during early pregnancy due to the presence of the conceptus [141, 142]. These uNK cells may also transform into larger more granulated forms with increased cell cytolytic activity due to the uterine microenvironment [143]. This increase in functional activity of uNK cells in response to the conceptus does not occur in pseudopregnant pigs, which indicates an affect of the conceptus that is independent of conceptus estrogens [143]. Similar to the mouse, uNK cells may be involved in vascular changes that are important for embryo survival in pigs. Indeed, endometrial lymphocytes isolated near healthy conceptuses, but not those from sites of fetal arrest, are more numerous and express genes that are linked to angiogenesis [144]. These changes are associated with development of the subepithelial capillary bed, which is necessary for conceptus survival.

In conclusion, insights into the complex and overlapping events of pregnancy recognition and endometrial remodeling for implantation and placentation have been gained through examining the uterine expression levels of *IRF1*, *STAT2*, *MIC*, *B2M*, and *IRF2* in terms of stage of estrus cycle, day of pregnancy, treatment with E_2, and intrauterine infusion of CSP in pigs. The results suggest that pig conceptuses orchestrate precise temporal and spatial changes in uterine gene expression through initial secretion of estrogen, followed later by the expression of proteins, such as IFND and IFNG.

Estrogens from pig conceptuses or injected E_2 increase *IRF2* expression in the LE and limit the expression of selected ISGs, including IRF1, to the underlying stroma. It is likely that many other uterine genes critical for pregnancy success are regulated by a similar interplay between conceptus steroids and proteins. Since the trophoblasts of ruminants, rodents, primates, and pigs share the characteristic of secretion of multiple paracrine factors that profoundly affect uterine gene expression and uterine remodeling, insights from the present study advance our understanding of early pregnancy across mammalian species. While the key players at the uterine-placental interface require further definition, the interactions of estrogen, IFNs, and ISGs, including *IRF1* and *IRF2*, described here highlight the complex and precisely orchestrated interplay between the endometrium and conceptus that influences conceptus survival, implantation, and development.

CHAPTER IV

PIG CONCEPTUSES SECRETE ESTROGEN AND INTERFERONS TO DIFFERENTIALLY REGULATE UTERINE *STAT1* IN A TEMPORAL AND CELL-TYPE SPECIFIC MANNER*

Introduction

Implantation is the process by which the blastocyst attaches to the uterus for juxtaposition of embryonic and maternal circulations leading to the establishment of a functional placenta and successful pregnancy. During the peri-implantation period of pregnancy, uterine luminal epithelial (LE) cells and conceptus trophectoderm develop adhesion competence in synchrony to initiate an adhesion cascade within a restricted period of the uterine cycle termed the window of receptivity. These cells orchestrate bi-directional interactions between the blastocyst and uterine endometrium involving spatiotemporally regulated endocrine, paracrine and autocrine modulators that mediate cell-cell and cell-matrix interactions [46, 48, 104, 145-148]. The trophectoderm layer of the blastocyst produces a factor(s) that signals pregnancy recognition as well as forms the placental membranes that are ultimately responsible for ensuring pregnancy success.

Uterine endometrial responses to implantation are complex. In addition to remodeling of the uterine LE [51], both LE and glandular epithelia (GE) secrete histotroph to nourish and support development of the conceptus [102]. Uterine stroma transforms (i.e., decidualization) to control movement of the conceptus through the uterine wall during implantation while generating a cytokine-rich environment that directly promotes angiogenesis to ensure sufficient blood flow to the placenta for hematotrophic nourishment of fetal development [105, 149, 150].

---

* Reprinted with permission from Pig conceptuses secrete estrogen and interferons to differentially regulate uterine STAT1 in a temporal and cell-type specific manner by Joyce MM, Burghardt RC, Geisert RD, Burghardt JR, Hooper RN, Ross JW, Ashworth MD, Johnson GA. Endocrinology 2007; 148:4420-4431. Copyright 2007 by The Endocrine Society.

In pigs, pregnancy recognition is the result of conceptus secretion of estrogens on d 11 and 12 of pregnancy to redirect prostaglandin $F_{2\alpha}$ secretion from the uterine vasculature to the uterine lumen where it is sequestered away from the corpora lutea [1, 3, 86]. In addition, conceptus estrogens modulate uterine gene expression responsible for endometrial remodeling from d 13-25 of gestation required for implantation [132]. Secreted phosphoprotein 1 (*SPP1*, or osteopontin) is an extracellular matrix protein induced by estrogen in LE where it is hypothesized to influence trophectoderm and LE adhesion, signal transduction and cell migration [133]. Conceptus estrogen secretion also correlates with conceptus secretion of IL-1β, which may in turn modulate uterine response to this cytokine [134]. The importance of estrogen to implantation of pig conceptuses is underscored by the fact that premature exposure of the pregnant uterus to estrogen on d 9 and 10 results in degeneration of all pig conceptuses by d 15 [5].

Peri-implantation pig conceptuses also secrete interferons (IFNs) during the peri-implantation period. Cultured conceptuses from d 11 of pregnancy were first shown to secrete proteins that cross-reacted with antiserum against IFNα [94], but peak antiviral activity was not measured until d-14 and -15 conceptuses were cultured [95]. The major species (75% of antiviral activity in pig conceptus secretory proteins) is the type II IFNγ and the other (25%) is the type I IFNδ [8, 40]. However, in contrast to sheep conceptuses, in which a type I IFN (IFNτ) is the signal for maternal recognition of pregnancy [4], the IFNs produced by pig conceptuses do not appear to be antiluteolytic. Intrauterine infusion of conceptus secretory proteins on d 12 and 15 of the estrous cycle had no effect on interestrus interval or temporal changes in plasma progesterone concentrations [9].

IFNτ increases expression of a number of IFN-stimulated genes (ISGs) in the stroma of the ruminant uterus, including *MX1* and signal transducer and activator of transcription 1 (*STAT1*) [28, 32, 148]. It is noteworthy that paracrine actions of pig conceptus IFNs are also suggested by localization of IFN receptors on endometrial epithelial cells [40] and expression of *MX1* in the stroma of pigs on d 18 of pregnancy [42]. Although effects of these IFNs on pig endometrium have not been determined,

emerging evidence suggests that induction or increases in ISGs in the endometrium by conceptus IFNs is a phenomenon of early pregnancy in many mammals and may facilitate establishment of a uterine vascular supply to the conceptus [16, 21-24, 124].

Our working hypothesis is that pig conceptus IFNs increase uterine endometrial expression of the ISG *STAT1* during pregnancy and that *STAT1* has biological roles in uterine receptivity and conceptus implantation and development. Indeed, STAT1 activation generally results in transcription of genes that are antiproliferative, proapoptotic and proinflammatory that could profoundly influence endometrial remodeling for implantation and placentation [151]. However, in ruminants, endometrial ISG expression is a result of the signal for pregnancy recognition from the conceptus. Pigs utilize estrogens, not IFNs, for pregnancy recognition. Therefore, the objectives of the present studies were to determine whether *STAT1* is expressed in the pig endometrium during pregnancy, and if so, whether *STAT1* expression is regulated by conceptus estrogen and/or conceptus secretory proteins that contain IFNγ and IFNδ. Results provide compelling evidence that pig conceptus trophectoderm cells orchestrate precise temporal and cell-type-specific changes in uterine *STAT1* expression through initial secretion of estrogen, followed by IFNδ and IFNγ.

Materials and Methods

Animals and Tissue Collection

Experimental and surgical procedures complied with the Guide for Care and Use of Laboratory Animals and were approved by the Texas A&M University Laboratory Animal Care or the Oklahoma State Institutional Care and Use Committees. Pigs were observed daily for estrus (d 0) and exhibited at least two estrous cycles of normal duration before use in studies.

Study 1. To evaluate the effect of pregnancy on gene expression, pigs were assigned randomly to either cyclic or pregnant status. Those in the pregnant group were bred when detected in estrus and 12 and 24 h thereafter. Pigs were hysterectomized on

either d 5, 9, 12, or 15 of the estrous cycle or d 9, 10, 12, 13, 14, 15, 20, 25, 30, 35, 40, 60 or 85 of pregnancy (n = 3 pigs/d·status) (Fig. 3.1).

Study 2. To evaluate the effect of estrogen on uterine gene expression, pigs were assigned randomly to receive daily injections (im) of either 5 ml corn oil vehicle or 5 mg 17β-estradiol benzoate (Sigma Chemical Co., St. Louis, MO; 5 mg in 5 ml corn oil) on d 11, 12, 13, and 14 post estrus (n = 5 pigs per treatment). All pigs were hysterectomized on d 15 post estrus (Fig. 3.2).

Study 3. To evaluate the effect of pig conceptus secretory proteins on uterine gene expression, pigs (n = 3) were injected (im) with 5 mg 17β-estradiol benzoate (Sigma; 5 mg in 5 ml corn oil) on d 11, 12, 13, 14, and 15 post estrus. On d 12 post estrus (coincident with secretion of IFNs by pig conceptuses) [8, 94, 95], each pig was surgically implanted with two indwelling Alzet osmotic pumps (Durect Corp., Cupertino, CA) with a constant delivery rate of 10 µl/h. Each uterine horn was isolated via midline celiotomy, clamped, and severed from the uterine body at approximately 5 in. from the uterotubal junction while preserving the mesometrium and vascular supply to the uterine horn. The transected ends of each uterine horn and uterine body were sutured closed and the serosa of the antimesometrial borders of the horn and body sutured together to prevent twisting of the horn. For each pump, a catheter was attached and inserted approximately 2 cm into the lumen of one uterine horn. Before surgery, pumps were filled and equilibrated per manufacturer's instructions. For each pig, one uterine horn was infused by a pump filled with porcine serum albumin (35 mg; Sigma), whereas the other uterine horn was infused by a pump filled with porcine conceptus secretory proteins (CSPs) (35 mg) (Fig. 3.3). All pigs were hysterectomized on d 16 post estrus (coincident with maximal antiviral activity in pig uterine flushings) [95] (Fig. 3.4).

Preparation of porcine CSPs. As previously described [41, 115], conceptuses from d 15-17 pregnant pigs (coincident with maximal production of IFNs by conceptuses) [8, 94, 95] were recovered by flushing uterine horns, cultured for 30 h, dialyzed (MWCO 3500; Spectrum Laboratories, Inc., Rancho Dominguez, CA),

concentrated (MWCO 5000; Millipore Corp., Bedford, MA), filter sterilized, assayed for protein concentration, and stored at 4 C.

Study 4. To evaluate the effect of early estrogen administration on conceptus development and uterine gene expression, pigs were bred and assigned randomly to receive daily injections (im) of 2.5 ml corn oil or 5 mg estradiol cypionate (A. J. Legere, Scottsdale, AZ; 5 mg in 2.5 ml corn oil) on d 9 and 10 of gestation. Pigs were hysterectomized on d 10, 12, 13, 15, or 17 of pregnancy (n=4 pigs/day·treatment) (Fig. 4.1).

At hysterectomy, several sections (~0.5 cm) from the middle of each uterine horn were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2) and embedded in Paraplast-Plus (Oxford Laboratory, St. Louis, MO). Several sections from each uterine horn were also embedded in Tissue-Tek OCT Compound (Miles, Oneonta, NY), snap frozen in liquid nitrogen, and stored at -80 C. The remaining endometrium was physically dissected from the myometrium, frozen in liquid nitrogen, and stored at -80 C for RNA extraction.

Microarray Analysis

For study 4, microarray analysis was conducted using a spotted cDNA array representing mRNA transcripts from pig brain, oviduct, uterine endometrium, oocytes, early embryos, peri-implantation conceptuses, and fetal and ovarian tissues (developed at the University of Missouri using procedures previously described) [152].

Total endometrial RNA (20 μg) was reverse transcribed and labeled using the 3DNA Array 50 Expression Array Detection Kit (Genisphere Inc., Hatfield, PA). Four hybridizations were conducted per the manufacturer's recommendations. For each replication, the total cDNA synthesis reaction volume for both treatments for each day was combined, cDNA volume concentrated to 3-10 μl (Microcon YM-30; Millipore), nuclease-free water added to a final volume of 10 μl, and slides hybridized (10 μl concentrated cDNA, 25 μl 2X formamide hybridization buffer, 2 μl LNA dT blocker, and 13 μl nuclease-free water) at 53 C for 16 h in a humidified hybridization cassette

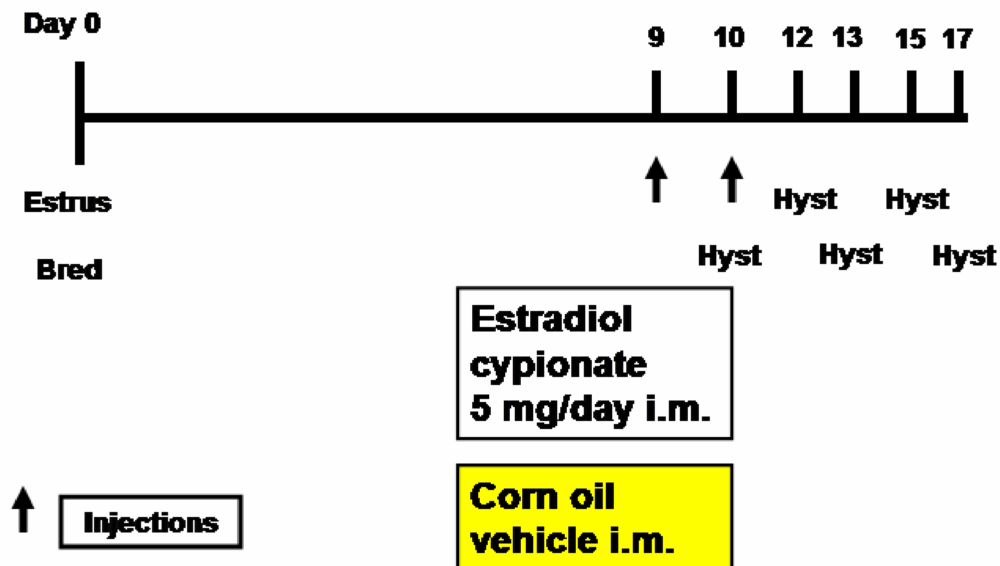


FIG. 4.1. Study 4 experimental design. Pregnant pigs received daily i.m. injections of estradiol cypionate or corn oil vehicle on Days 9 and 10 postestrus. Pigs from both treatments were hysterectomized on the indicated days of pregnancy.

using a 22 x 40 mm LifterSlip (Erie Scientific Co., Portsmouth, NH). After hybridization, slides were washed (2X SSC/0.2% SDS at 65 C for 15 min; 2X SSC, at room temperature for 15 min; and 0.2X SSC at room temperature for 15 min), rinsed in 95% ethanol for 2 min, and dried on a slide centrifuge. Secondary hybridizations were conducted at 50 C for 3 h, washed and dried as before. Each microarray slide was scanned with the Cy3 and Cy5 channels using the ScanArray Express (PerkinElmer Life Sciences, Inc., Wellesley, MA). Laser power and photomultiplier tube gain were adjusted for each slide to minimize variation between wavelengths. GenePix Auto Processor 3.0 software was used for data preprocessing, background correction, Local Loess pin-by-pin intensity normalization, and microarray statistical analysis (GPAP3.0, http://darwin.biochem.okstate.edu/gpap3; Weng, H., and P. Ayoubi, in preparation).

RNA Isolation and Analyses

RNA isolation. Total cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlesbad, CA) according to the manufacturer's recommendations.

RT-PCR analysis. Partial cDNAs for porcine *IFNδ* and porcine *IFNγ* were amplified by RT-PCR as previously described [30]. For *IFNδ*, conceptus total RNA from d 14 of pregnancy was reversed transcribed, and then gene-specific primers (GenBank accession no. Z22706; forward 5'-ATGGATTGTCCCCATGTAGG-3' and reverse 5'-CTGAGCTACCAGGGTTACCG-3') [153] were used. For *IFNγ*, porcine uterine endometrial RNA from d 15 of pregnancy was reverse transcribed, and then gene-specific primers (GenBank accession no. AY188090; forward 5'-CAGCTTTGCGTGACTTTGTG-3' and reverse 5'-TGAATGGCCTGGTTATCTTTG-3') were used. Both PCR products were cloned into a pCRII cloning vector using the TA cloning kit (Invitrogen) and confirmed by sequence analysis. A BLAST search for each was conducted to ensure that only target genes were evaluated.

Northern blot analysis. As previously described [116], 8 μg total RNA per lane was hybridized with a radiolabeled antisense human *STAT1* cRNA probe [154] generated by *in vitro* transcription with [α-^{32}P]uridine 5-triphosphate (PerkinElmer) and

a MAXIscript kit (Ambion, Austin, TX). Hybridization signals were detected by exposure to a PhosphoImager screen and visualized using a Typhoon 8600 variable mode imager (Molecular Dynamics, Piscataway, NJ).

Slot blot analysis. As previously described [116], 20 μg total RNA per slot was hybridized with a radiolabeled antisense human *STAT1* cRNA generated as above. To correct for variation in loading, a duplicate membrane was hybridized with a radiolabeled antisense 18S rRNA (pT718S; Ambion) cRNA probe. Hybridization signals were detected as above.

In situ hybridization analysis. As previously described [37], deparaffinized, rehydrated, and deproteinated uterine cross-sections (5 μm) were hybridized with radiolabeled antisense or sense human *STAT1* or pig *IFNγ* cRNA probes synthesized by *in vitro* transcription with [α-^{35}S]uridine 5-triphosphate (PerkinElmer). After hybridization, washes, and RNase A digestion, autoradiography was performed using NTB liquid photographic emulsion (Eastman Kodak, Rochester, NY). Slides were exposed at 4 C, developed in Kodak D-19 developer, counterstained with Harris' modified hematoxylin (Fisher Scientific, Fairlawn, NJ), dehydrated, and protected with coverslips.

Immunofluorescence Analysis

As previously described [118], frozen pig uterine cross-section (~8-10 μm) were fixed in -20 C methanol, washed in PBS containing 0.3% vol/vol Tween 20, blocked in 10% normal goat serum, incubated overnight at 4 C with 30 μg/ml mouse antihuman STAT1 (610185; BD Biosciences PharMingen, San Jose, CA), or 25 μg/ml mouse antiporcine IFNγ (I7662-18P; U.S. Biological, Swampscott, MA) or mouse IgG (negative control; Sigma), and detected with fluorescein-conjugated goat antimouse IgG (Chemicon International, Temecula, CA). Slides were overlaid with Prolong antifade mounting reagent (Molecular Probes, Eugene, OR) and a coverslip.

Photomicrography

Digital photomicrographs of *in situ* hybridization (autoradiographic film overviews as well as representative bright-field and dark-field images of liquid emulsion autoradiography) and immunofluorescence staining were evaluated using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) interfaced with an Axioplan HR digital camera and Axiovision 4.3 software. Photographic plates were assembled using Adobe Photoshop (version 6.0, Adobe Systems Inc., San Jose, CA).

Statistical Analysis

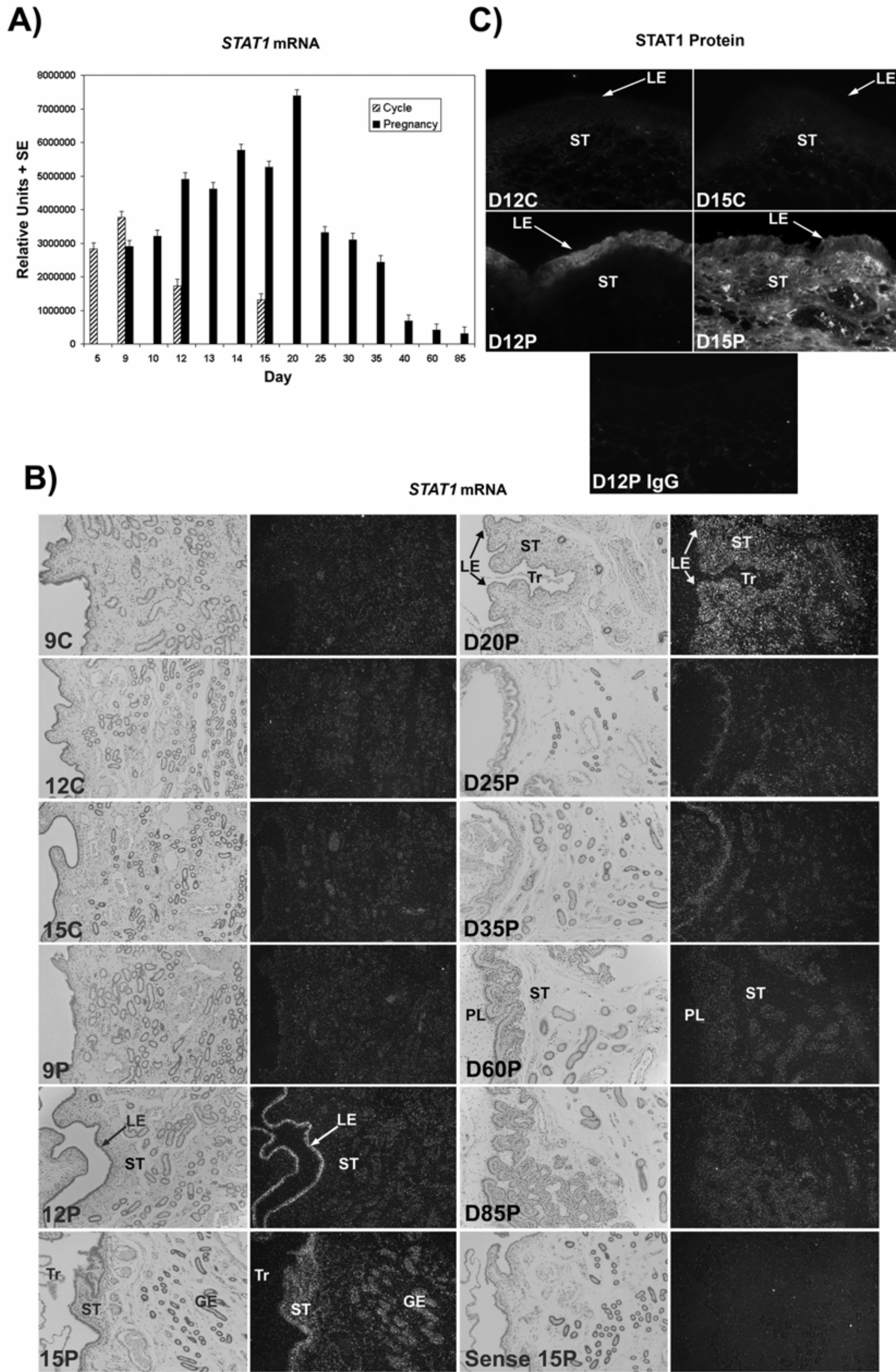
Data were subjected to least-squares ANOVA using the general linear models procedures of the Statistical Analysis System (SAS, Cary, NC). All slot blot hybridization data were analyzed using the 18S rRNA as a covariate to correct for differences in RNA loading. Data from study 1 were analyzed for effects of day and status and their interaction where appropriate. For all other studies, effects of treatment were determined by preplanned orthogonal contrasts. All tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error. Data are presented as least-squares means with SE.

Results

STAT1 Increases in Pregnant Uterine Endometrium in a Cell-Type-Specific Manner

The human cDNA for *STAT1* detected an approximately 4.2-kb mRNA by Northern blot analysis of pig total endometrial mRNA from d 15 of pregnancy (data not shown). This mRNA was similar in size to that detected using the same cDNA in sheep endometrial total mRNA. Pregnancy-specific up-regulation of endometrial *STAT1* mRNA was evaluated using slot blot hybridization (Fig. 4.2A). Steady-state levels of *STAT1* mRNA in pig endometrium decreased between d 9 and 12 of the estrous cycle ($P < 0.07$). However, during pregnancy, *STAT1* mRNA levels increased between d 10 and 20 ($P < 0.01$), decreased between d 20 and 25 ($P < 0.07$), decreased again between d 35 and 40 ($P < 0.07$), and remained low thereafter (Fig. 4.2A).

FIG. 4.2. Study 1: *STAT1* increases in pregnant uterine endometrium in a cell-type-specific manner. A, Steady-state levels of mRNA for *STAT1* in pig endometrium during the estrous cycle and pregnancy. The mRNA levels are expressed as least-square means of relative units of counts per minute with overall SEM, are normalized for differences in sample loading using 18S rRNA, and represent 20 µg total endometrial mRNA per sample. B, *In situ* hybridization analysis of *STAT1* mRNA in cross-sections of pig uterus. Corresponding bright-field and dark-field images from different days (D) of the estrous cycle (C) and pregnancy (P) are shown. A representative section from D15P hybridized with radiolabeled sense cRNA probe (Sense) serves as a negative control. Width of each field is 940 µm. C, Immunofluorescence localization of STAT1 protein in frozen cross-sections of pig endometrium from d 12 and 15 of the estrous cycle (C) and pregnancy (P). A representative section from D12P immunolocalized with nonrelevant mouse IgG serves as a negative control. Width of each field is 540 µm. PL, Placenta; ST, stratum compactum stroma; Tr, trophectoderm.


A)
STAT1 mRNA
Relative Units + SE
Cycle
Pregnancy
Day
C)
STAT1 Protein
LE
ST
D12C
D15C
D12P
D15P
D12P IgG
B)
STAT1 mRNA
9C
12C
15C
9P
12P
15P
Tr
GE
D20P
D25P
D35P
D60P
D85P
PL
Sense 15P

Temporal and spatial changes in *STAT1* mRNA (Fig. 4.2B) and protein (Fig. 4.2C) in the endometrium of cyclic and pregnant pigs were assessed by *in situ* hybridization and immunofluorescence analysis, respectively. *STAT1* mRNA was low in all endometrial cell types on d 9-15 of the estrous cycle. In contrast, *STAT1* mRNA increased in LE cells between d 9 and 12 of pregnancy. A second increase in *STAT1* mRNA was observed in endometrial stromal and GE cells between d 12 and 15 of pregnancy. *STAT1* mRNA remained high in LE, stroma, and GE cells through d 20 of pregnancy, after which levels decreased to those observed on d 9 (Fig. 4.2B). Consistent with *in situ* hybridization results, immunoreactive STAT1 protein was low in endometrium during the estrous cycle but was present in LE cells on d 12 of pregnancy and prominent in endometrial LE and stromal cells on d 15 (Fig. 4.2C).

Collectively, these data document two *STAT1* expression events in endometria during the peri-implantation period of pigs: 1) *STAT1* increases in LE cells on d 12 when elongated pig conceptuses secrete estrogen for pregnancy recognition [1, 3, 86], and 2) a second *STAT1* increase in endometrial stroma and GE cells between d 12 and 15 temporally correlates with increased antiviral activity measured in uterine flushes exposed to conceptus secretion of IFNγ and IFNδ [8, 94, 95]. Interestingly, expression of *STAT1* in pig stroma and GE is similar to that observed in sheep endometrium in response to conceptus IFNτ, but up-regulation of *STAT1* in LE is unique to the pig, which uses estrogen for pregnancy recognition.

IFNδ and IFNγ are Synthesized by Pig Conceptuses

Two major species of pig conceptus IFNs had previously been identified, the type II IFNγ and the type I IFNδ [8, 40]. Although these proteins were shown to be coexpressed in d-16 pig trophectoderm, no rigorous temporal and spatial localization of these genes was performed. Therefore in the present studies, pig-specific cRNA probes to *IFNγ* and *IFNδ* and antiserum to IFNγ were used to determine temporal and spatial localization of these IFNs in pig conceptus and uterine tissues (Fig. 4.3). RT-PCR

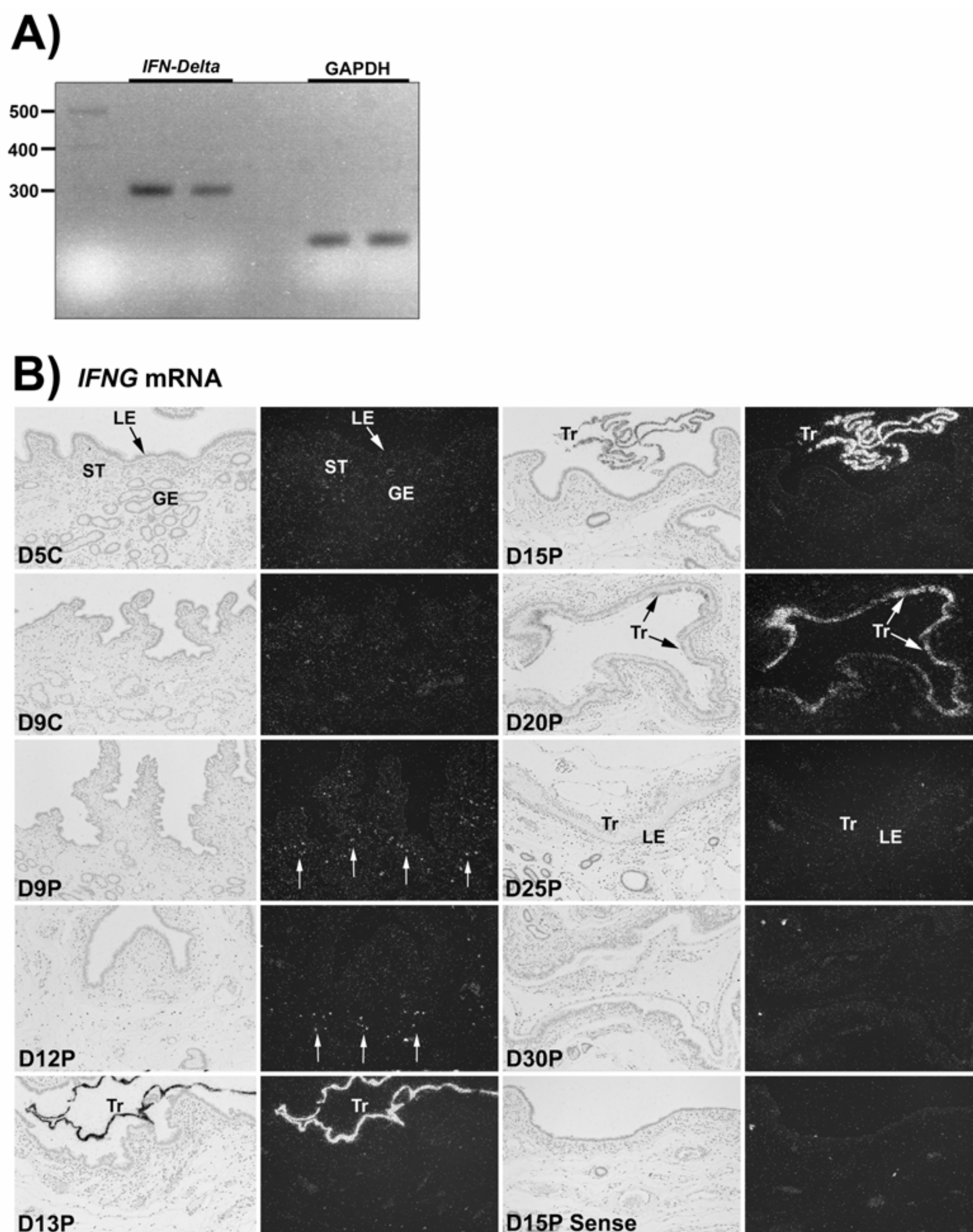


FIG. 4.3. Study 1: IFNδ and IFNγ are synthesized by pig conceptuses. A, RT-PCR analysis of *IFNδ* mRNA in two d-14 pig conceptus total RNA preparations. B, *In situ* hybridization analysis of *IFNγ* mRNA in cross-sections of the interface between pig uterus and conceptus. Corresponding bright-field and dark-field images from different days (D) of the estrous cycle (C) and pregnancy (P) are shown. A representative section from D15P hybridized with radiolabeled sense cRNA probe (Sense) serves as a negative control. On D9P and D12P, *arrows* indicate a population of cells within the endometrial stroma that express *IFNγ* mRNA. Width of each field is 940 µm. ST, stratum compactum stroma; Tr, trophectoderm.

analysis for *IFNδ* in d-14 conceptuses detected an approximately 296-kb mRNA (Fig. 4.3A). Sequence analysis identified that the PCR product was pig *IFNδ*; however, levels of *IFNδ* mRNA were not sufficiently high to be detectable in pig trophectoderm by our *in situ* hybridization procedure (data not shown). In contrast, robust hybridization was detected for *IFNγ* using *in situ* hybridization (Fig. 4.3B). *IFNγ* was not evident in endometrium during the estrous cycle but was expressed in a pregnancy-specific manner by a population of cells scattered within the endometrial stroma on d 9 and 12. It is likely these are endometrial lymphocytes that have been reported to express IFNγ in the pig [144]. Pig conceptus trophectoderm cells expressed high amounts of *IFNγ* mRNA by d 13 of pregnancy, and *IFNγ* remained readily detectable through d 20 (Fig. 4.3B). Similar to a previous report [40], immunoreactive IFNγ was localized to perinuclear membranes typically occupied by endoplasmic reticulum and Golgi appratus as well as cytoplasmic vesicles within trophectoderm cells, suggesting trafficking and secretion of IFNγ into the uterine lumen for access to endometrium (data not shown).

Estrogen Injections Given IM Increase STAT1 in Uterine Luminal Epithelium

The timing of *STAT1* expression in uterine LE coincides with the secretion of estrogen by pig conceptuses to signal pregnancy recognition. The potential involvement of estrogen in endometrial LE *STAT1* expression was evaluated by exogenous estrogen administration in postestrus cyclic pigs.

The im injection of estradiol benzoate did not alter steady-state levels of *STAT1* mRNA compared with corn oil injection in total pig endometrium, of which LE cells compose a small proportion ($P > 0.1$; Fig. 4.4A). However, *in situ* hybridization and immunofluorescence staining for *STAT1* revealed that estradiol benzoate increased *STAT1* mRNA and protein in the endometrial LE (Fig. 4.4, B and C). No increase in *STAT1* gene expression was observed in the LE of corn-oil-treated pigs. *STAT1* is therefore among the first genes shown to be regulated by estrogen in the pig uterine LE where it is temporally available to participate in pregnancy recognition and/or the adhesion cascade for implantation.

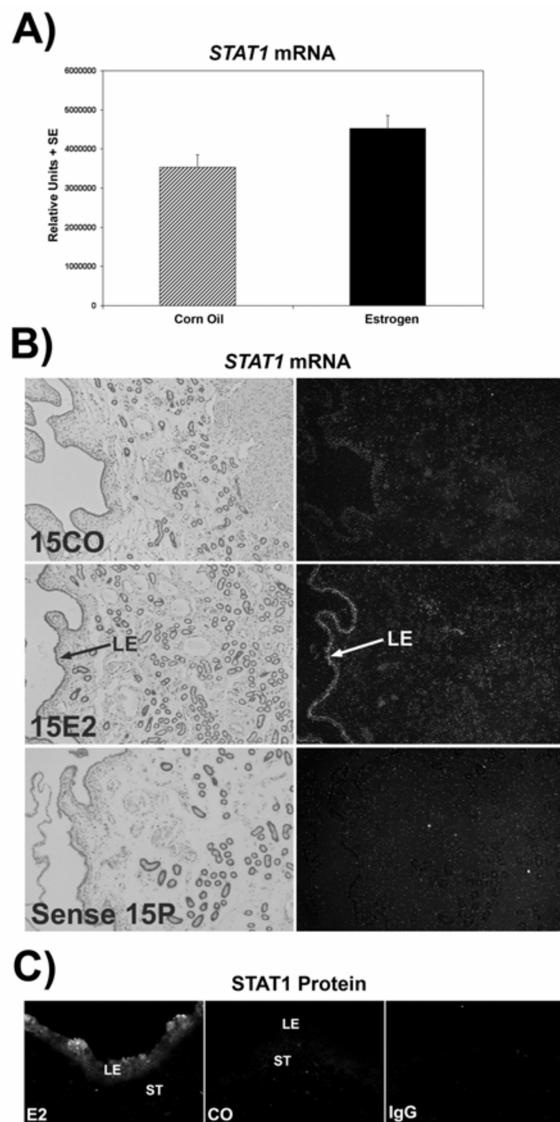


FIG. 4.4. Study 2: im estrogen injections increase *STAT1* in uterine luminal epithelium. A, Steady-state levels of *STAT1* mRNA in uterine endometrium of pigs injected im with corn oil or estradiol benzoate (estrogen). The mRNA levels are expressed as least-square means of relative units of counts per minute with overall SEM, normalized for differences in sample loading using 18S rRNA, and represent 20 µg total endometrial mRNA per sample. B, *In situ* hybridization analysis of *STAT1* mRNA in uterine cross-sections from corn oil control (CO) or estradiol benzoate (E2) injected pigs. Corresponding bright-field and dark-field images of endometrium are shown. A representative section from d 15 of pregnancy (P) hybridized with radiolabeled sense cRNA probe (Sense) serves as a negative control. Width of each field is 940 µm. C, Immunofluorescence localization of STAT1 protein in frozen cross-sections of pig endometrium from d-15 cyclic pigs injected im with corn oil (CO) or with estradiol benzoate (E2). A representative section from d 15 of pregnancy immunolocalized with nonrelevant mouse IgG serves as a negative control. Width of each field is 540 µm. ST, Stratum compactum stroma.

Intrauterine Infusion of Pig Conceptus Secretory Proteins That Contain IFNδ and IFNγ Increases STAT1 in Uterine Stroma

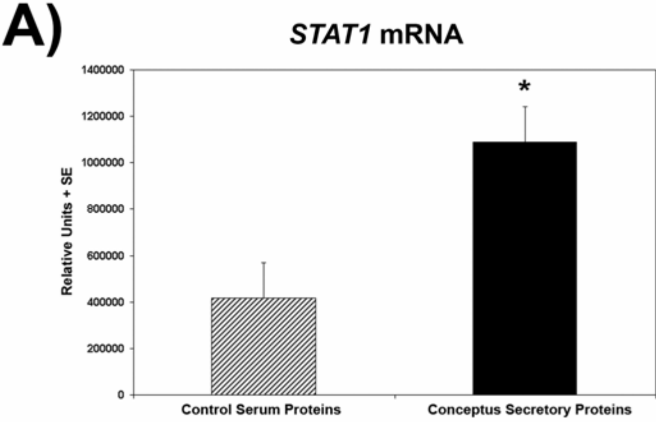
To investigate protein paracrine signals, including IFNδ and IFNγ, directed from the conceptus, CSPs were infused into the uterine lumen of estrogen-treated postestrus cyclic pigs.

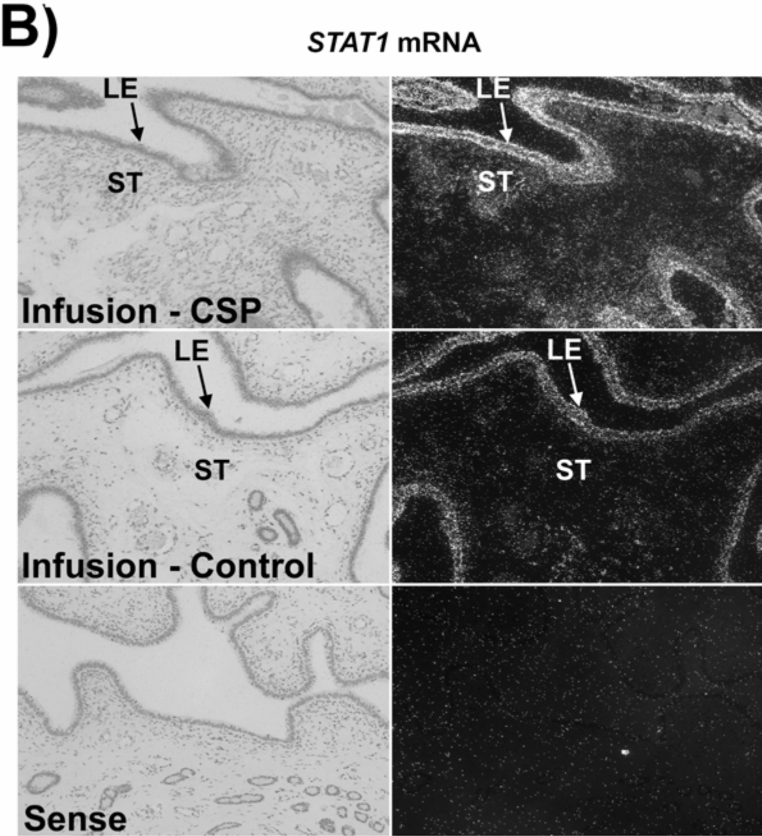
CSPs increased steady-state levels of endometrial *STAT1* mRNA expression over intrauterine infusion of control serum proteins (P = 0.08; Fig. 4.5A). Consistent with slot blot hybridization results, *in situ* hybridization and immunofluorescence analyses for *STAT1* demonstrated increased expression in the endometrial stratum compactum stroma of d-16 cyclic pigs intrauterine infused with CSP (Fig. 4.5, B and C). No increase in *STAT1* was observed when control serum proteins were infused into the uteri of d-16 cyclic pigs. Therefore, similar to sheep, *STAT1* increases in the sub-LE uterine wall, coincident with exposure to IFNs, where it is temporally available to facilitate remodeling within the stromal compartment of the uterus for implantation and placentation.

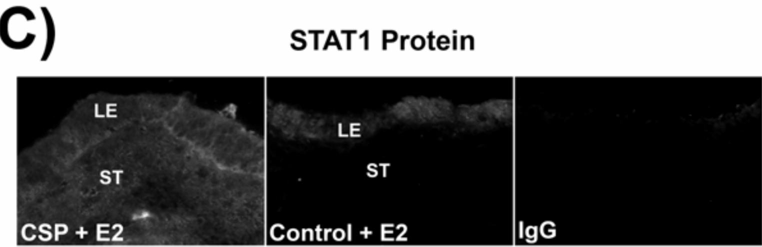
Uterine STAT1 Increases in Close Proximity to Paracrine Release of IFNγ by Implanting Conceptuses

Figure 4.6 illustrates the spatial distribution of *STAT1* mRNA in relation to location of the conceptus within the uterine lumen using autoradiographs of serial uterine cross-sections probed with *IFNγ* and *STAT1* cRNAs respectively. Significantly, *STAT1* increases in uterine endometrial LE, stroma, and GE cells in close proximity to the implanting conceptus that expresses estrogens, IFNδ, and IFNγ, with the amount of *STAT1* decreasing as distance from the point of contact between conceptus and uterus increases. These data strongly suggest that *STAT1* expression is regulated by paracrine secretions i.e., estrogens and/or IFNδ and IFNγ, from the implanting pig conceptus.

FIG. 4.5. Study 3: intrauterine infusion of pig CSPs that contain IFNδ and IFNγ increase *STAT1* in uterine stroma. A, Steady-state levels of *STAT1* mRNA in endometrium of pigs intrauterine infused with control serum proteins or with CSPs. The mRNA levels are expressed as least-square means of relative units of counts per minute with overall SEM, normalized for differences in sample loading using 18S rRNA, and represent 20 μg total endometrial mRNA per sample. *, Statistically different from control at $P < 0.08$. B, *In situ* hybridization analysis of *STAT1* mRNA in uterine cross-sections from control serum protein- and CSP-infused pigs. Corresponding bright-field and dark-field images of uterine endometrium are shown. A representative section from d 15 of pregnancy hybridized with radiolabeled sense cRNA probe (Sense) serves as a negative control. Width of each field is 940 μm. C, Immunofluorescence localization of STAT1 protein in frozen cross-sections of pig endometrium from d-16 cyclic pigs intrauterine infused with CSP or with control proteins. A representative section from d 15 of pregnancy immunolocalized with non-relevant mouse IgG serves as a negative control. Width of each field is 540 μm. ST, Stratum compactum stroma.


A)
STAT1 mRNA
Relative Units + SE
1400000
1200000
1000000
800000
600000
400000
200000
0
*
Control Serum Proteins
Conceptus Secretory Proteins


B)
STAT1 mRNA
LE
ST
Infusion - CSP
Infusion - Control
Sense


C)
STAT1 Protein
LE
ST
CSP + E2
Control + E2
IgG

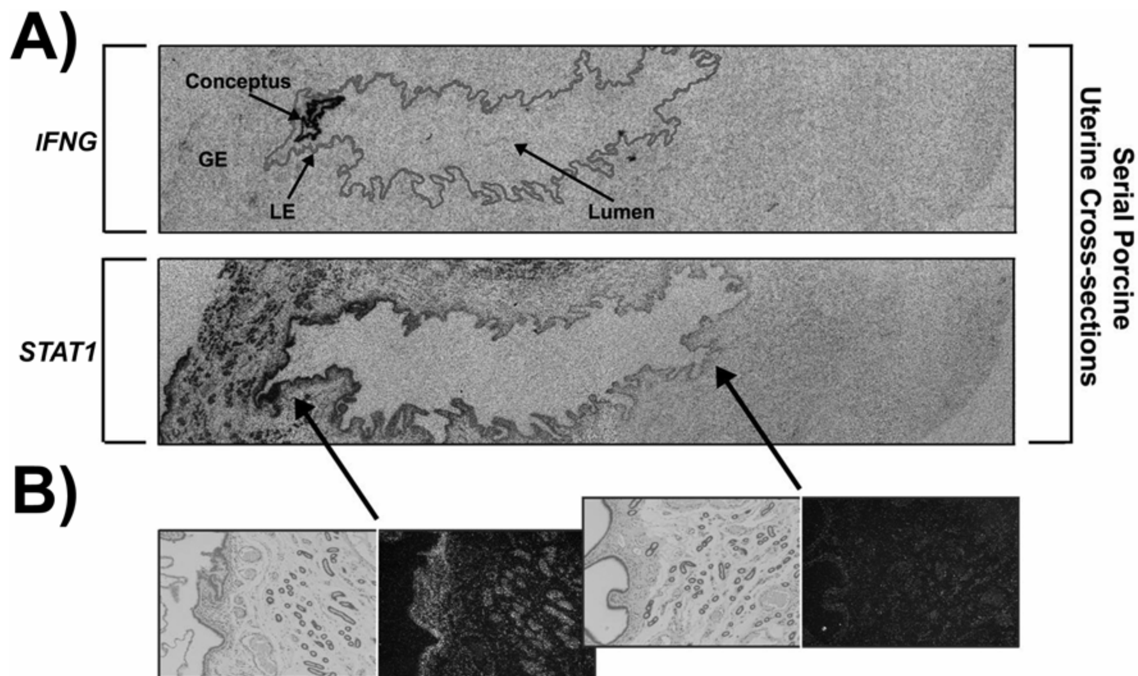


FIG. 4.6. Study 1: uterine *STAT1* increases in close proximity to paracrine release of IFNγ by implanting conceptuses. A, Representative autoradiographic images (Biomax-MR; Kodak) showing entire cross-sections of the uterine walls from d 15 of pregnancy probed with radiolabeled antisense pig *IFNγ* cRNA (top) or *STAT1* cRNA (bottom). The luminal epithelium of the *IFNγ*-probed tissue has been artificially outlined in *gray* for histological reference. Width of each field is 20 mm. B, Corresponding bright-field and dark-field images from the same sectioned uterus probed with *STAT1* in A. Width of each field is 940 µm.

Inappropriate Early Estrogen Results in Demise of Embryos and Loss of IFN-Induced STAT1

Early uterine exposure to estrogen on d 9 and 10 of pregnancy results in total embryo loss by d 15-17 of pregnancy [155]. Analysis of uterine *STAT1* mRNA expression using this experimental model system provides an opportunity to investigate the interrelationship between paracrine release of estrogens and IFNs by pig conceptuses.

Total endometrial *STAT1* mRNA was 2.1-fold lower on d 15 of early estrogen-treated than control pregnant pigs (P = 0.06; Fig. 4.7A). Although *STAT1* mRNA increased in LE of all animals in the study, stromal expression of *STAT1* was observed only in control pregnant pigs. No stromal expression of *STAT1* was detected in pigs exposed to early estrogen (Fig. 4.7B). These results indicate that inappropriate estrogen, leading to progressive conceptus degeneration, compromises paracrine release of IFNδ and IFNγ, which are required for stromal expression of *STAT1*.

Discussion

Our results demonstrate that cell-type-specific induction of *STAT1* in the pig uterus is differentially regulated by conceptus signals. Estrogen secretion by the conceptus on d 12, which is the signal for maternal recognition of pregnancy, temporally correlates with *STAT1* expression in the LE, and treatment of cyclic pigs with exogenous estrogen increased *STAT1* in the LE. Stromal induction of *STAT1* correlates with IFNδ and IFNγ secretion by the conceptus, and intrauterine infusion of CSPs, which contain IFNδ and IFNγ, into cyclic pigs treated with exogenous estrogen increased *STAT1* compared with intrauterine infusion of control proteins, similar to that observed on d 15 of pregnancy.

Up-regulation of *STAT1* within uterine LE, stroma, and GE in close proximity to the implanting conceptus implies paracrine regulation of *STAT1* by conceptus estrogens and IFNs. A similar conceptus-associated pattern of LE gene expression has previously been observed for *SPP1*, a gene that increases in the pig uterine LE in response to

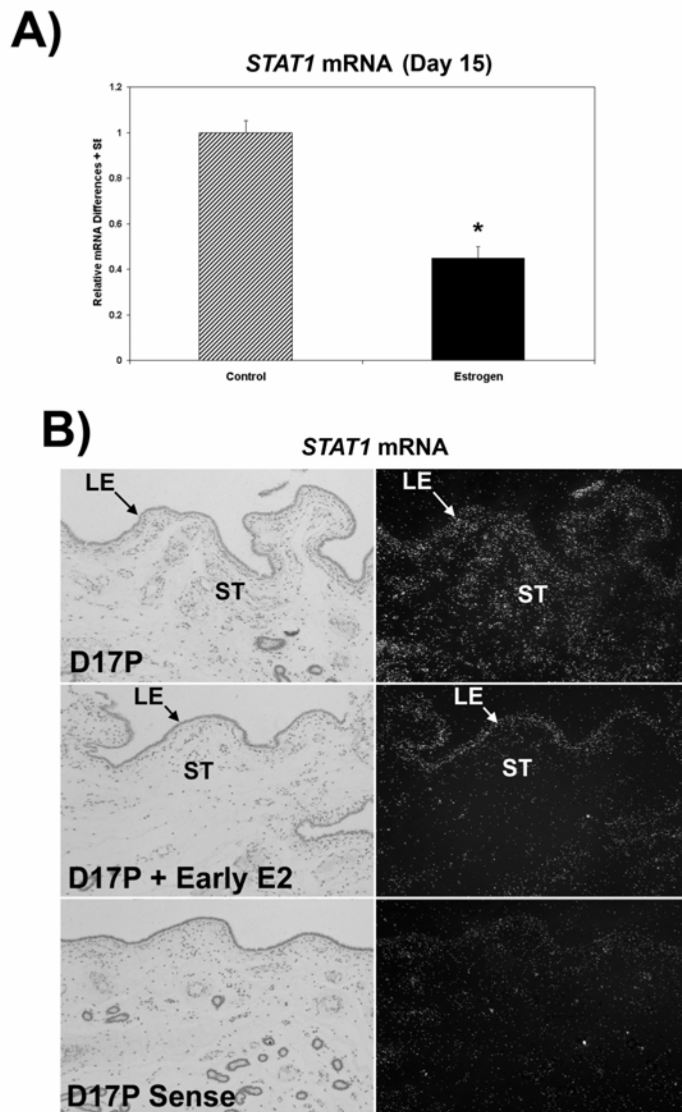


FIG. 4.7. Study 4: inappropriate early estrogen results in demise of embryos and loss of IFN-induced *STAT1*. A, Microarray analysis of total *STAT1* mRNA in endometrium of untreated (control) and early estrogen-treated pregnant pigs. The mRNA levels are expressed as relative mRNA differences between signals measured in the Cy3 and Cy5 channels using the ScanArray Express system with overall SEM and represent 20 µg total endometrial mRNA per sample. *, Statistically different from control at $P < 0.06$. B, *In situ* hybridization analysis of *STAT1* mRNA in uterine cross-sections from d-17 control pregnant (D17P) and early estrogen-treated pregnant pigs (D17 + Early E2). Corresponding bright-field and dark-field images of uterine endometrium are shown. A representative section from d 17 of pregnancy hybridized with radiolabeled sense cRNA probe (Sense) serves as a negative control. Width of each field is 940 µm. ST, Stratum compactum stroma.

conceptus estrogen [133]. It is likely that estrogen effects on the endometrium are restricted to regions near the conceptus due to sulfatase activity of trophectoderm. During pregnancy, pig endometrium rapidly converts estradiol to the biologically inactive estrone sulfate, and concentrations of estrone sulfate are high within the uterine lumen of pregnant pigs [156]. Trophectoderm has sulfatase activity that restores the biological activity of estrogen, allowing for a localized effect of estrogen to up-regulate *STAT1* and *SPP1* in LE.

In contrast, it is somewhat surprising that initial increases in stromal *STAT1* are restricted to sites of intimate association between the conceptus and uterus, given that IFNγ synthesis and secretion by pig conceptuses appears to be similar in magnitude to IFNτ production by sheep conceptuses (Fig. 4.3, B and C) [9]. Indeed, *STAT1* increases universally in the stroma and GE of pregnant sheep without regard to conceptus location within the lumen, presumably due to the high levels of secretion of IFNτ by conceptuses [30, 120]. One explanation for the spatial pattern of *STAT1* expression observed in the pig uterus is that IFNδ and IFNγ act synergistically to up-regulate ISGs. Interaction between type I and type II IFNs has been previously demonstrated [157]. It is plausible that high levels of IFNγ act on uterine stromal and GE cells to increase intracellular stores of ISGF3 so that the much lower levels of IFNδ can maximally up-regulate *STAT1* in close proximity to the implanting pig conceptus

To the best of our knowledge, this is the first report demonstrating estrogen regulation of *STAT1* gene expression. Induction of *STAT1* in LE may be the result of direct transcriptional activation. The protein kinase regulated by RNA (PKR) is an ISG, yet in the absence of IFN, induction can be mediated by Sp1 [158]. Interestingly, estrogen regulates oxytocin receptor expression in the uterine LE of sheep through GC-rich Sp1 promotor elements [91]. It is plausible that in a similar manner, estrogen up-regulates *STAT1* gene expression in pig LE through binding of Sp1 sites. Alternatively, induction of *STAT1* in LE may be indirect through the induction of a putative estromedin. This estromedin would be released from uterine cells, because exogenous estrogen, in the absence of a conceptus, induced *STAT1* in uterine LE. Stromal

estromedins that regulate gene expression in adjacent epithelia have been reported in the murine uterus [159]. However, estrogen receptor α (ESR1) is nearly undetectable in pig endometrial stroma from d 5-15 of pregnancy [7], and only low levels of estrogen receptor β (ESR2) have been detected in total pig endometrium [160, 161]. Because ESR1 is prominent in pig LE through d 12 of pregnancy [7], it is possible that estrogen binds ESR1 in LE to induce release of an unknown autocrine factor that up-regulates *STAT1* in LE.

Although estrogen regulation of *STAT1* gene expression has not previously been reported, estrogen has been shown to induce transcriptional activation of STATs [162]. This activation required cytoplasmic localization of ESR1 and ESR2 [162]. Additionally, MAPK, Src-kinase, and phosphatidylinositol-3-kinase activity were involved in this activation [162]. Because ESR1 is present in the pig uterine LE at the time of estrogen release by conceptuses [7], and ESR2 is barely detectable in the pig uterus [161], it is likely that *STAT1* regulation would be mediated via ESR1. However, estrogen receptor involvement may not be essential. Kennedy and co-workers [163] used estrogen receptor-negative osteoblast and breast cancer cells to demonstrate estrogen-dependent *STAT1* activation.

Although estrogens, secreted by pig conceptuses on d 12 of gestation, function to establish pregnancy [1, 3, 86], secretion of estrogen also overlaps with initiation of attachment of the conceptus to the uterine luminal surface for implantation on d 13 of pregnancy [164]. Indeed, both pregnancy recognition and implantation require rapid morphological elongation of trophectoderm that coincides with elevated conceptus estrogen synthesis and release [132]. The timing and extent of estrogen exposure can have dramatic effects on conceptus development and survival. Insufficient distribution of estrogen, as seen in litters with fewer than two piglets per uterine horn at the time of trophectoderm elongation, results in failure to prevent luteolysis and subsequent termination of pregnancy [165]. On the contrary, adverse timing of estrogen exposure in the form of naturally occurring alfatoxins in moldy corn on d 9 and 10 of gestation results in conceptus degeneration during the period of placental attachment to the uterine

surface [155, 166]. Indeed, conceptus estrogens are believed to regulate implantation success by altering gene expression, including *SPP1* [133] and IL-1β [134], in uterine LE to initiate a cascade of molecular events that modifies the luminal glycocalyx for conceptus implantation [164, 167]. It is reasonable to propose that elongating pig conceptuses secrete estrogen to increase expression of *STAT1* in LE and that this gene has a role in pregnancy recognition and/or the adhesion cascade for implantation.

Pig conceptuses also secrete IFNs during the peri-implantation period [8], a phenomenon shared by humans, rodents, and ruminants [4, 16, 21]. However the secretion of both type I and type II IFNs, IFNδ and IFNγ, respectively, is unique to the pig. In general, both type I and II IFNs can induce *STAT1* through the classical Janus kinase-STAT signaling pathway leading to γ-activation factor binding of γ-activated sequence (GAS) elements and induction of gene transcription [13]. In addition, IFNδ signals through a similar, yet distinct, pathway leading to ISGF3 complex binding of IFN-stimulated response elements in the promoters of several ISGs to initiate transcription [11].

Although the type I IFNα and type II IFNγ each induce expression of largely nonoverlapping sets of genes, they can also act in concert to produce synergistic interactions leading to mutual reinforcement of physiological responses [168]. This synergy has been demonstrated for cooperative induction of ISGs such as *STAT1*. Normally relatively nonresponsive to IFNγ, combined treatment of cells with IFNγ followed by IFNα results in higher-magnitude ISG induction [168]. In addition, cotreatment with IFNγ and IFNα extends the period of ISG expression over IFNα alone [157]. For typical ISGs, type I IFNs induce rapid expression, independent of protein synthesis, followed by a protein synthesis-dependent suppression of transcription within 6 h. IFNγ overrides IFNα-induced ISG repression, allowing continuous expression of ISGs for greater than 24 h [157]. Clearly, IFNδ and IFNγ may profoundly influence uterine physiology through cooperative induction of cytokine-specific transcription factors, such as *STAT1*, that allow reinforcement of effects of distinct cell-surface ligands while maintaining the specificities of the individual inducing IFNs.

It is estimated that the IFNs control the transcription of several hundred genes to influence cell functions. STAT1 activation mediates transcriptional responses to many cytokines and growth factors that are generally antiproliferative, proapoptotic, and proinflammatory [151]. Interestingly, recent work by Hartman *et al.* [169] has shown that gene transcription that results from STAT1 activation depends on the type of interferon that activates STAT1. Because STAT1 homodimers form after both IFNγ and IFNα stimulation, it was expected that IFNγ-induced STAT1 binding sites would predominately overlap with those of the IFNα-induced STAT1 sites. However, IFNγ and IFNα treatments result in dramatic differences in target sites for STAT1 binding. Sixty-six percent of the STAT1 binding sites observed with IFNγ treatment were absent in IFNα-treated cells, and 75% of IFNα-induced STAT1 binding sites were not present in IFNγ-treated cells [169]. Indeed, IFNγ-induced STAT1 homodimers bind sites not occupied by STAT1 upon IFNα induction, and visa versa, indicating that many STAT1 IFNγ sites are not used in the IFNα response, and many STAT1 IFNα sites are not used in the IFNγ response [169]. Therefore, pig conceptuses provide the potential for highly complex and differential cell-type-specific gene expression in the mesenchymal component of the pregnant uterus through conceptus cosecretion of IFNδ and IFNγ to induce *STAT1*.

Although it is well established that conceptus IFNτ induces expression of numerous ISGs in the stroma and GE of ruminants [98], the pregnancy-specific roles of uterine ISGs in general remain conjectural. Recent analysis of human endometrium by Hess *et al.* [23] may shed light on ISG function(s) within the endometrium. In these studies, culture medium from human trophoblasts was incubated with decidualizing endometrial stromal cells, and global gene expression was assessed in the decidualized cells. IFN-induced or related genes constituted a significant percentage of those that were up-regulated in decidualizing cells in response to paracrine signals from the trophoblast [23]. Human trophoblasts have previously been reported to produce IFN [21], and clearly, results from Hess *et al.* [23] and others [16, 22, 24, 124] indicate decidualizing stroma of mice and humans undergoes an IFN-like response similar to that

observed in the endometrial stroma of ruminants, and now pigs. Interestingly, a decidual-like transformation has previously been reported in the pregnant endometrial stroma of sheep, suggesting that the endometrium of noninvasive implanting species undergoes remodeling somewhat similar to that observed in the decidua of invasive implanting species [125]. Therefore it is likely that ISGs facilitate remodeling within the stromal compartment of the uterus for implantation and placentation across disparate mammalian species. As players in decidual/stromal remodeling, individual ISGs could be involved in protecting the fetal semi-allograft from immune rejection, in limiting conceptus invasion through the uterine wall, and/or in establishing a vascular supply to the conceptus. Because IFNγ, a protein secreted by pig conceptuses, is believed to initiate endometrial vascular development [126], it is reasonable to hypothesize that conceptus-derived IFNs up-regulate ISGs such as *STAT1* to facilitate vascular changes necessary to provide hematotrophic support to the developing conceptus. Whether this is the case or not, it is becoming increasingly clear that IFN induction of genes within the uterine stroma of mammals is a universal response to, or component of, a progressing pregnancy.

It is particularly intriguing that *IRF1*, a prototypic IFNγ/STAT1-responsive gene [109], is not detectable in the uterine LE of peri-implantation sheep and pigs [28, 170]. However, the concommittant expression of interferon regulatory factor 2 (IRF2), a potent transcriptional repressor of ISGs [127, 129], in LE has led to the hypothesis that IRF2 prevents ISG expression in LE, whereas ISG expression in the underlying stroma continues unabated [28, 170]. Indeed, most ISGs, including *STAT1* are not expressed in the LE of sheep [148]. Results of the present study indicate that conceptus estrogen secretion on d 12 alters this physiology by inducing *STAT1* in pig LE without the subsequent induction of *IRF1*. Given that the human *IRF1* promoter contains seven Sp1 sites [130] and that estrogen is capable of regulating gene transcription through ESR1/Sp1 interactions [91], estrogen may also regulate *IRF1* expression. Indeed, 17β-estradiol can downregulate *IRF1* expression [171, 172] without effecting *STAT1* expression [171], and this effect appears to be mediated by ESR1 [172, 173]. The

temporal/spatial pattern of uterine ESR1 correlates with this idea [7]. ESR1 is detectable in pig LE on d 15 where *STAT1,* but not *IRF1,* is expressed [170]. ESR1 is not present in d-15 stroma where both *STAT1* and *IRF1* are prominently expressed [170].

Collectively, *in vivo* results support the conclusion that conceptus estrogen and IFNs regulate uterine ISGs in a complex cell-type-specific manner. In the case of *STAT1*, conceptus estrogen increases *STAT1* in LE as part of the pregnancy recognition signal that may also serve to remodel the apical surface of LE to allow attachment of the conceptus, whereas conceptus IFNδ and IFNγ increase *STAT1* in the underlying uterine stroma that may play a role in the remodeling of the uterine wall for maximal blood flow to the developing fetus. It is likely that many other uterine genes considered critical for pregnancy success are regulated by similar interplay between conceptus steroid and protein secretion. Because the trophoblasts of ruminants, rodents, and primates share with pigs the secretion of multiple paracrine factors that profoundly affect uterine gene expression and uterine remodeling, insights from the present studies impact our understanding of early pregnancy across mammalian species. Although the key players at the uterine-placental interface require further definition, the interactions of estrogen, IFNs, and *STAT1* described here highlight the complex, precisely orchestrated interplay between endometrium and conceptus that influences conceptus survival, implantation, and development.

CHAPTER V

UTERINE MAJOR HISTOCOMPATIBILITY CLASS I MOLECULES AND BETA 2 MICROGLOBULIN ARE REGULATED BY PROGESTERONE AND CONCEPTUS INTERFERONS DURING PIG PREGNANCY*

Introduction

Implantation is the process by which the blastocyst attaches to the uterus for juxtaposition of embryonic membranes with maternal uterine endometrium to establish histotrophic and hematotrophic exchange of nutrients and gases leading to the establishment of a functional placenta. During placentation, intimate physical contact between uterine and placental cells facilitates bi-directional interactions involving spatiotemporally regulated endocrine, paracrine and autocrine modulators that mediate cell-cell and cell-matrix interactions essential for successful establishment and maintenance of pregnancy [46, 145, 148]. These interactions may also, at least in part, prevent rejection of the conceptus (embryo/fetus and associated extraembryonic membranes), which is a semi-allograft within the uterine environment.

Medawar recognized that the laws of transplantation biology dictate rejection of the conceptus as a semiallogeneic tissue with paternal as well as maternal histocompatibility Ags [174, 175], however many details of how the conceptus is protected from a potentially hostile immune environment remain unclear. Nevertheless, the conceptus *in utero* secures its own position, establishes an immunological truce with its mother, and obstructs or directs her immune system to contribute to the immunologic privileged state of the trophoblast [176].

The bulk of immune response to tissue grafts is directed to the MHC Ags. MHC

---

* Reprinted with permission from Uterine MHC class I molecules and β2-microglobulin are regulated by progesterone and conceptus interferons during pig pregnancy by Joyce MM, Burghardt JR, Burghardt RC, Hooper RN, Bazer FW, Johnson GA. J Immunol 2008; 181:2494-2505. Copyright 2008 by The American Association of Immunologists, Inc.

molecules are polymorphic cell surface glycoproteins that present peptide Ags to TCRs, and bind to inhibitory and activating receptors on NK cells and other leukocytes. MHC class I molecules are categorized as either classical or nonclassical and contain a transmembrane α-chain associated noncovalently with an extracellular β-chain called β_2-microglobulin (β_2m). The classical MHC class I molecules are expressed on most somatic cells and present peptides derived from self proteins or from proteins of intracellular pathogens to CTL; therefore, they are involved in immune recognition of foreign pathogens and transplanted tissue. Additional class I MHC molecules termed nonclassical are less polymorphic and are restricted in cell-type specific expression [177]. Protection of the conceptus from the maternal immune system involves down-regulation of MHC class I by the trophoblast [177-181]. Additionally, in some species such as humans, nonclassical monomorphic MHC class I molecules are expressed by the trophoblast, which may protect the trophoblast cells from NK cell attack and T lymphocytes [177, 178]. However, when placentation is noninvasive, trophectoderm cells do not express any MHC class I during the first trimester of pregnancy. Notable examples of noninvasive placentation include the entire placenta of pigs, all regions of placentation excluding the invasive chorionic girdle that develops into the endometrial cups of horses, and the interplacentomal uterine-placental interface of ruminants [177, 180, 182].

Cytokines and hormones regulate expression of MHC molecules during conceptus development, as well as the tissue differentiation and remodeling that occurs at the uterine-placental interface [102, 103, 105, 183-188]. Mattsson et al. [189] reported in mice increased uterine, but not placental, expression of MHC class I and class II molecules in response to IFN. Choi and co-workers [31] reported complex regulation of MHC class I and β_2m in uterine and placental tissue of sheep. In these studies, MHC class I and β_2m were inhibited in endometrial luminal epithelium (LE), but paradoxically stimulated by IFN-τ, the pregnancy recognition signal in sheep [148], in both endometrial stromal cells, and in glandular epithelium (GE) [31].

In pigs, conceptuses secrete estrogens on days 11 and 12 of pregnancy as the

signal for pregnancy recognition [1]. In addition, conceptus estrogens modulate uterine gene expression responsible for uterine remodeling for implantation and placentation from days 13 to 25 of gestation [132]. The importance of estrogen is underscored by the fact that premature exposure of the pregnant uterus to estrogen on days 9 and 10 results in degeneration of all pig conceptuses by day 15 [5].

Peri-implantation pig conceptuses also secrete IFNs. The major species is type II IFN-γ and the other is type I IFN-δ [8, 40]. In contrast to sheep conceptuses, in which a type I IFN-τ is the signal for maternal recognition of pregnancy [148], the IFNs produced by pig conceptuses do not appear to be antiluteolytic [9]. However, both sheep and pig conceptus IFNs increase expression of a number of IFN-stimulated genes (ISGs) in uterine stroma [148, 170, 190, 191]. Although physiological roles for these IFNs in the pig uterus have not been determined, emerging evidence suggests that induction or increases in uterine ISGs by conceptus IFNs is a phenomenon of early pregnancy in many, if not most, mammals [16, 23, 124, 170, 190, 191].

Our working hypothesis is that pig conceptus IFNs increase uterine endometrial expression of the classical and nonclassical MHC class I molecules. In pigs these molecules are known as classical swine leukocyte Ag (SLA) class I genes (*SLA-1, SLA-2, SLA-3*) and nonclassical SLA class I (*SLA-6, SLA-7, SLA-8*) genes [192], and β_2m during pregnancy. The temporal cell type-specific expression of these genes plays a role in preventing immune disruption of pregnancy. Therefore, the objective of the present study is to determine 1) the temporal and spatial expression of SLA class I and β_2m genes in pig endometrium during pregnancy, and 2) whether their expression is regulated by estrogen, progesterone, or conceptus secretory proteins (CSPs) that contain IFN-γ and IFN-δ. Results provide compelling evidence that pig conceptus trophectoderm cells induce uterine stromal expression of SLA class I and β_2m genes through secretion of IFN-δ or IFN-γ, but expression is silenced in LE as a possible means of preventing immune rejection at the uterine-placental interface.

Materials and Methods

Animals and Tissue Collection

Experimental and surgical procedures complied with the Guide for Care and Use of Laboratory Animals and were approved by the Texas A&M University Laboratory Animal Care and Use Committee. Pigs were observed daily for estrus (day = 0) and exhibited at least two estrous cycles of normal duration before use in these studies.

Study one. To evaluate the effect of pregnancy on endometrial gene expression, pigs were assigned randomly to either cyclic or pregnant status. Those in the pregnant group were bred when detected in estrus and 12 and 24 h thereafter. Pigs were hysterectomized on either day 5, 9, 12, or 15 of the estrous cycle or day 9, 10, 12, 13, 14, 15, 20, 25, 30, 35, 40, 60 or 85 of pregnancy (n = 3 pigs/day/status) (Fig. 3.1).

Study two. To evaluate the effect of estrogen on endometrial gene expression, pigs were assigned randomly to receive daily injections (i.m.) of either 5 ml corn oil vehicle or 5 mg of 17β-estradiol benzoate (5 mg in 5 ml of corn oil; Sigma-Aldrich) on days 11-14 postestrus (n = 5 pigs/treatment). This dose of 17β-estradiol is used to induce pseudopregnancy in pigs [132]. All pigs were hysterectomized on day 15 postestrus (Fig. 3.2).

Study three. To evaluate the effect of pig CSPs on endometrial gene expression, pigs (n = 3) were injected (i.m.) with 5 mg 17β-estradiol benzoate (5 mg in 5 ml of corn oil; Sigma-Aldrich) on days 11-15 postestrus. On day 12 postestrus (coincident with secretion of IFNs by pig conceptuses [8, 95]), each pig was surgically implanted with two indwelling ALZET osmotic pumps (Durect Corporation) with a constant delivery rate of 10 µl/h. Each uterine horn was isolated via midline celiotomy, clamped, and severed from the uterine body at ~5 inches from the utero-tubal junction while preserving the mesometrium and vascular supply to the uterine horn. The transected ends of each uterine horn and uterine body were sutured closed and the serosa of the antimesometrial borders of the horn and body sutured together to prevent twisting of the uterine horn. For each pump, a catheter was attached and inserted ~2 cm into the lumen of one uterine horn. Before surgery, pumps were filled and equilibrated per the

manufacturer's instructions. For each pig, one uterine horn was infused from a pump filled with porcine serum albumin (35 mg; Sigma-Aldrich), whereas the other uterine horn was infused from a pump filled with porcine CSP (35 mg) (Fig. 3.3). All pigs were hysterectomized on day 16 postestrus (coincident with maximal antiviral activity in pig uterine flushings [95]) (Fig. 3.4).

Preparation of porcine CSP. As previously described [41], conceptuses from day-15 to day-17 pregnant pigs (coincident with maximal production of IFNs by conceptuses [8, 95]) were recovered by flushing uterine horns and cultured for 30 h. After recovery, medium was dialyzed (m.w. cutoff, 3500; Spectrum Laboratories), concentrated (m.w. cutoff, 5000; Millipore), filter sterilized, assayed for protein concentration (Bio-Rad) and stored at 4°C because IFN-γ is unstable to freezing and thawing.

Study four. To evaluate the effects of progesterone on endometrial gene expression, pigs were ovariectomized on day 4 postestrus and assigned randomly to receive daily injections (i.m.) of either progesterone (200 mg; Sigma-Aldrich) or progesterone plus ZK137,316 (75 mg), a progesterone receptor antagonist generously provided by Dr. K. Chwalisz (Shering AG, Berlin, Germany), on days 4-12 postestrus. All pigs were hysterectomized on day 12 postestrus (n = 5 pigs/treatment) (Fig. 5.1).

At hysterectomy, several sections (~0.5 cm) from the middle of each uterine horn were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2) and embedded in Paraplast-Plus (Oxford Laboratory). Several sections from each uterine horn were also embedded in Tissue-Tek OCT Compound (Miles), snap frozen in liquid nitrogen, and stored at -80°C. The remaining endometrium was physically dissected from the myometrium, frozen in liquid nitrogen, and stored at -80°C for RNA extraction.

RNA Isolation and Analyses

RNA isolation. Total cellular RNA was isolated using TRIzol reagent (Invitrogen), according to the manufacturer's recommendations.

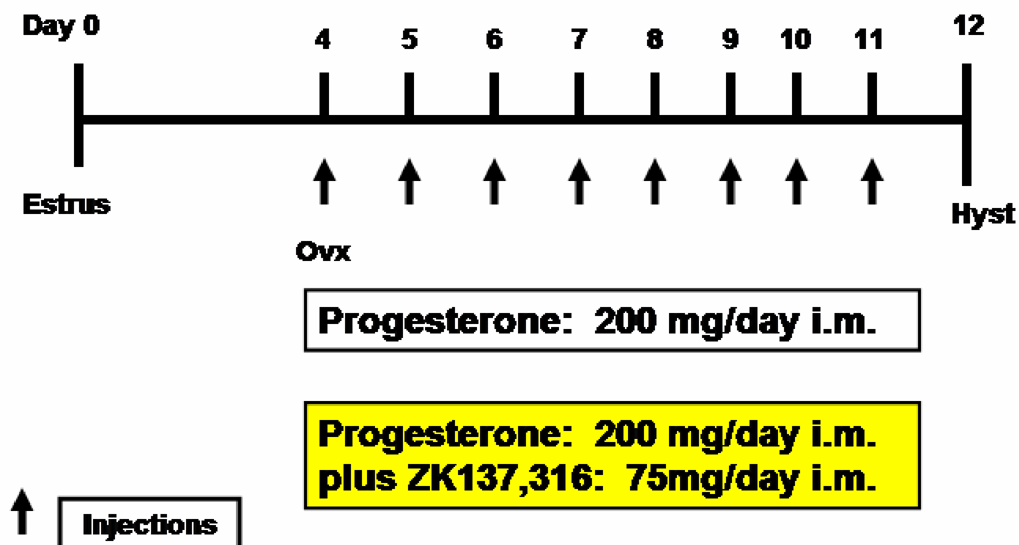


FIG. 5.1. Experimental design for study 4. Cyclic pigs were ovariectomized on Day 4 postestrus. They were then assigned randomly to receive daily i.m. injections of either progesterone or progesterone plus ZK137,316 on Days 4-12 postestrus. All pigs were hysterectomized on Day 12 postestrus.

RT-PCR analyses. A partial cDNA that cross-hybridizes with the classical SLA class I genes (*SLA-1*, *SLA-2*, and *SLA-3*), as well as partial cDNAs for *SLA-6, SLA-7, SLA-8*, β_2m gene, and ubiquitin-specific protease (USP) gene were amplified by RT-PCR as previously described [30]. For all genes, porcine uterine endometrial RNA from day 15 of pregnancy was reverse transcribed, then the following gene-specific primers were used to amplify the PCR products: classical SLA class I (forward) 5'-ATACCTGGAGATGGGGAAGG-3', (reverse) 5'-CCTTGGTAAGGGACACATCG-3', 500-bp product; *SLA-6* (forward) 5'-TTGGTATCCCGGCCCGGCCACGGTAGTG-3', (reverse) 5'-TGCCCGATACTGTTCAGCTACTCCC-3', 443-bp product [193]; *SLA-7* (forward) 5'-TATTGCGATCGGAACACACGCATC-3', (reverse) 5'-GCATGCCACTTCCAGGTAGGCTCTGC-3', 309-bp product [193]; *SLA-8* (forward) 5'-GCCACGGGGAGCCCCGGTACCTTGAG-3', (reverse) 5'-GAAGCGCTCATGAGCACGGGACTTG-3', 422-bp product [193]; β_2m (forward) 5'-ATGATATCCCACTTTTCACACCGCTCCAGTAGC-3', (reverse) 5'-ATAGATCTGGATTCATCCAACCCAGATGCAGC-3', 439-bp product [193]; and USP (forward) 5'-AGAGGATGACAGTGCCAAGG-3', (reverse) 5'-CTGCTTCCAACAGGTCTTCC-3', 473-bp product (GenBank accession no. AF134195). All PCR products were cloned into a pCRII cloning vector using the TA Cloning kit (Invitrogen) and confirmed by sequence analysis. A BLAST search for each was conducted to ensure that only target genes were evaluated.

Slot blot analyses. As previously described [116], duplicate membranes with 20 µg of total RNA per slot were hybridized with radio-labeled antisense porcine classical SLA class I genes, *SLA-6, SLA-7,* and *SLA-8* and β_2m gene RNA probes generated by *in vitro* transcription with [α-^{32}P]UTP (Perkin-Elmer Life Sciences) and a MAXIscript kit (Ambion). To correct for variation in loading, a duplicate membrane was hybridized with a radio-labeled antisense 18 S rRNA (pT718S; Ambion) RNA probe. Hybridization signals were detected by exposure to a PhosphoImager screen and visualized using a Typhoon 8600 variable mode imager (Molecular Dynamics).

In situ hybridization analyses. As previously described [29], deparaffinized,

rehydrated, and deproteinated uterine cross-sections (5 µm; 1 cross-section each from three separate blocks from each animal) were hybridized with radio-labeled antisense or sense classical SLA class I genes*, SLA-6, SLA-7,* and *SLA-8* and β_2m or USP gene RNA probes synthesized by *in vitro* transcription with [α-^{35}S]UTP (Perkin-Elmer). After hybridization, washes and RNase A digestion, autoradiography was performed using NTB liquid photographic emulsion (Eastman Kodak). Slides were exposed at 4°C, developed in Kodak D-19 developer, counterstained with Harris' modified hematoxylin (Fisher Scientific), dehydrated, and protected with coverslips.

Immunofluorescence Analyses

As previously described [118], frozen uterine cross-sections (~8-10 µm; 1 cross-section each from three separate blocks from each animal) were fixed in -20°C methanol, washed in PBS containing 0.3% v/v Tween 20 (rinse solution), blocked in 10% normal goat serum, incubated overnight at 4°C with 10 µg/ml mouse anti-porcine MHC class I (PT85A; VMRD), or 10 µg/ml mouse IgG (negative control; Sigma-Aldrich) and detected with 2 µg/ml fluorescein-conjugated goat anti-mouse IgG (Chemicon International). Slides were overlaid with Prolong anti-fade mounting reagent (Molecular Probes) and a coverslip.

SLA class I protein was colocalized with either β_2m protein or von Willibrand factor in frozen uterine cross-sections by immunofluorescence staining as previously described [194]. Briefly, sections were cut, fixed, washed, and blocked as we described. After dipping in rinse solution at room temperature, sections were incubated overnight at 4°C with the initial primary Ab (10 µg/ml mouse anti-porcine MHC class I or mouse IgG). Following washes, sections were incubated with 2 µg/ml initial secondary Ab (fluorescein-conjugated goat anti-mouse IgG), washed, and incubated overnight at 4°C with the second primary Ab (20 µg/ml rabbit anti-human β_2m, RDI-CBL307; Research Diagnostics, 10 µg/ml rabbit anti-human von Willebrand factor, AB7356; Chemicon International, or 10 µg/ml rabbit IgG, negative control; Sigma-Aldrich). Following washes, sections were incubated with 2 µg/ml secondary Ab (Texas Red-conjugated goat

anti-rabbit IgG; Molecular Probes), washed, dipped in distilled-deionized water, and overlaid with anti-fade mounting reagent as described.

Photomicrography

Digital photomicrographs of *in situ* hybridization (brightfield and darkfield images) and immunofluorescence staining were evaluated using an Axioplan 2 microscope (Carl Zeiss) interfaced with an Axioplan HR digital camera and Axiovision 4.3 software. For immunofluorescence colocalization of proteins, digital camera settings were evaluated to confirm that no "spectral bleed through" FITC signal was detectable in the Texas Red filter set and vice versa. In these studies, once the distribution of individual Ags was established, the codistribution of two Ags was investigated simultaneously in individual sections using compatible primary and FITC or Texas Red secondary Ab combinations with appropriate filter sets. Individual fluorophore images were recorded sequentially with AxioVision 4.3 software and evaluated in multiple fluorophore overlay images recorded in Zeiss Vision Image (.zvi) file format, which were subsequently converted to Tagged Image File (.tif) format. Photographic plates were assembled using Adobe Photoshop (version 6.0, Adobe Systems). All sections from each day per treatment were assessed as a group, and sections exhibiting the most representative hybridization or immunostaining pattern for each day per treatment were selected for inclusion in photographic plates.

Statistical Analyses

All slot blot hybridization data were subjected to least-squares ANOVA using the general linear models procedures of the Statistical Analysis System. Data were analyzed using the 18 S rRNA as a covariate to correct for differences in RNA loading and for effects of day and status and their interaction where appropriate. All tests of significance were performed using the appropriate error terms according to the expectation of the mean square for error. Slot blot hybridization data are presented as least squares means with SE.

Results

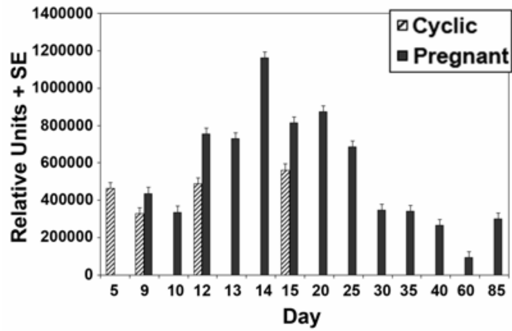
Effects of Pregnancy From Study One

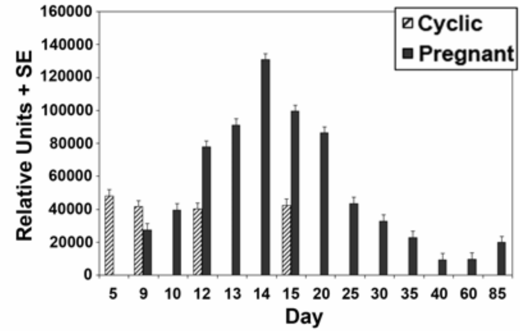
Partial cDNAs for classical SLA class I genes *SLA-1*, *SLA-2* and *SLA-3*, the nonclassical class I genes *SLA-6, SLA-7, SLA-8*, as well as β_2m gene were detected in day-15 pregnant pig endometria by RT-PCR analyses. Steady-state mRNAs in endometrium from the estrous cycle and pregnancy were then examined using antisense RNA probes and slot blot hybridization (Fig. 5.2). There was no significant change in total steady-state mRNA during the estrous cycle for any of these mRNAs ($p > 0.1$). During the ~115 days of pig pregnancy, steady-state mRNAs increased between day 9 and 14, decreased between day 14 and 60 and remained low through day 85 (classical and nonclassical SLA class I mRNAs, $p < 0.005$, quartic effect of day; β_2m mRNA, $p = 0.06$, quartic effect of day) (Fig. 5.2).

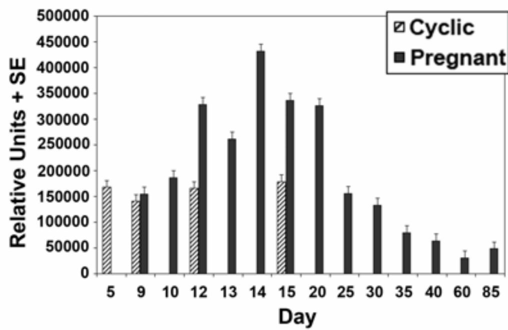
In situ hybridization was used to localize classical and nonclassical SLA class I mRNAs as well as β_2m mRNAs to specific cell types within the endometria of cyclic and pregnant pigs. Messenger RNA for *SLA-1*, *SLA-2* and *SLA-3* increased in the LE, GE, and blood vessels between days 5 and 9 of the estrous cycle and remained in these cell types through day 15 (Fig. 5.3). During pregnancy, the pattern of expression for *SLA-1*, *SLA-2,* and *SLA-3* mRNAs was the same as observed for the estrous cycle through day 12. However by day 15, expression increased in the stratum compactum stroma (note the difference in the spatial distribution of mRNA in endometrium from day 15 of the estrous cycle vs day 15 of pregnancy, Fig. 5.3), where it remained detectable through day 40 of pregnancy. Significantly, *SLA-1*, *SLA-2*, and *SLA-3* mRNAs were no longer detectable in the LE by day 20 of pregnancy (expanded view of day 25 shown in Fig. 5.3).

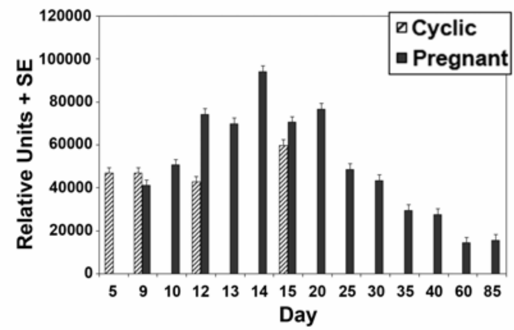
The three nonclassical SLA class I mRNAs exhibited similar patterns of expression in pig endometria by *in situ* hybridization. *SLA-6, SLA-7,* and *SLA-8* mRNAs increased in LE after day 9 of the estrous cycle and early pregnancy (Fig. 5.4). These mRNAs then decreased in LE after day 15 of pregnancy and were not detectable in LE on day 25 (Fig. 5.4). However, similar to the classical SLA mRNAs, *SLA-6*, *SLA-7*, and

FIG. 5.2. Steady-state levels of mRNA for *SLA-1, SLA-2, SLA-3*, *SLA-6*, *SLA-7*, *SLA-8* and β_2m genes in pig endometria during the estrous cycle and pregnancy. Steady-state levels of mRNA for the classical *SLA-1, SLA-2, SLA-3* genes (A), the nonclassical *SLA-6*, *SLA-7,* and *SLA-8* genes (B-D), and β_2m gene (E) in pig endometria during the estrous cycle and pregnancy were determined by slot blot hybridization. The mRNA levels, expressed as least square mean of relative units of cpm with overall SEM, are normalized for differences in sample loading using 18 S rRNA. The mRNA levels represent 20 μg of total endometrial mRNA per sample. Each of these mRNAs was increased during pregnancy over the estrous cycle.


A) SLA (1,2,3)
Cyclic
Pregnant
Relative Units + SE
1400000
1200000
1000000
800000
600000
400000
200000
0
5 9 10 12 13 14 15 20 25 30 35 40 60 85
Day


B) SLA 6
Cyclic
Pregnant
Relative Units + SE
160000
140000
120000
100000
80000
60000
40000
20000
0
5 9 10 12 13 14 15 20 25 30 35 40 60 85
Day


C) SLA 7
Cyclic
Pregnant
Relative Units + SE
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
5 9 10 12 13 14 15 20 25 30 35 40 60 85
Day


D) SLA 8
Cyclic
Pregnant
Relative Units + SE
120000
100000
80000
60000
40000
20000
0
5 9 10 12 13 14 15 20 25 30 35 40 60 85
Day

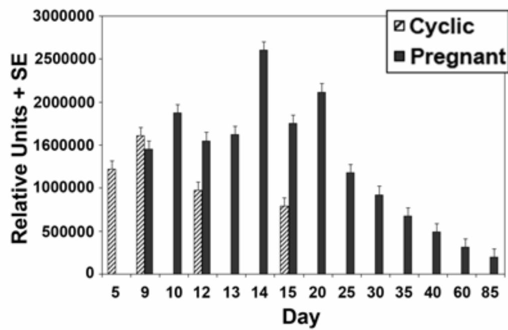
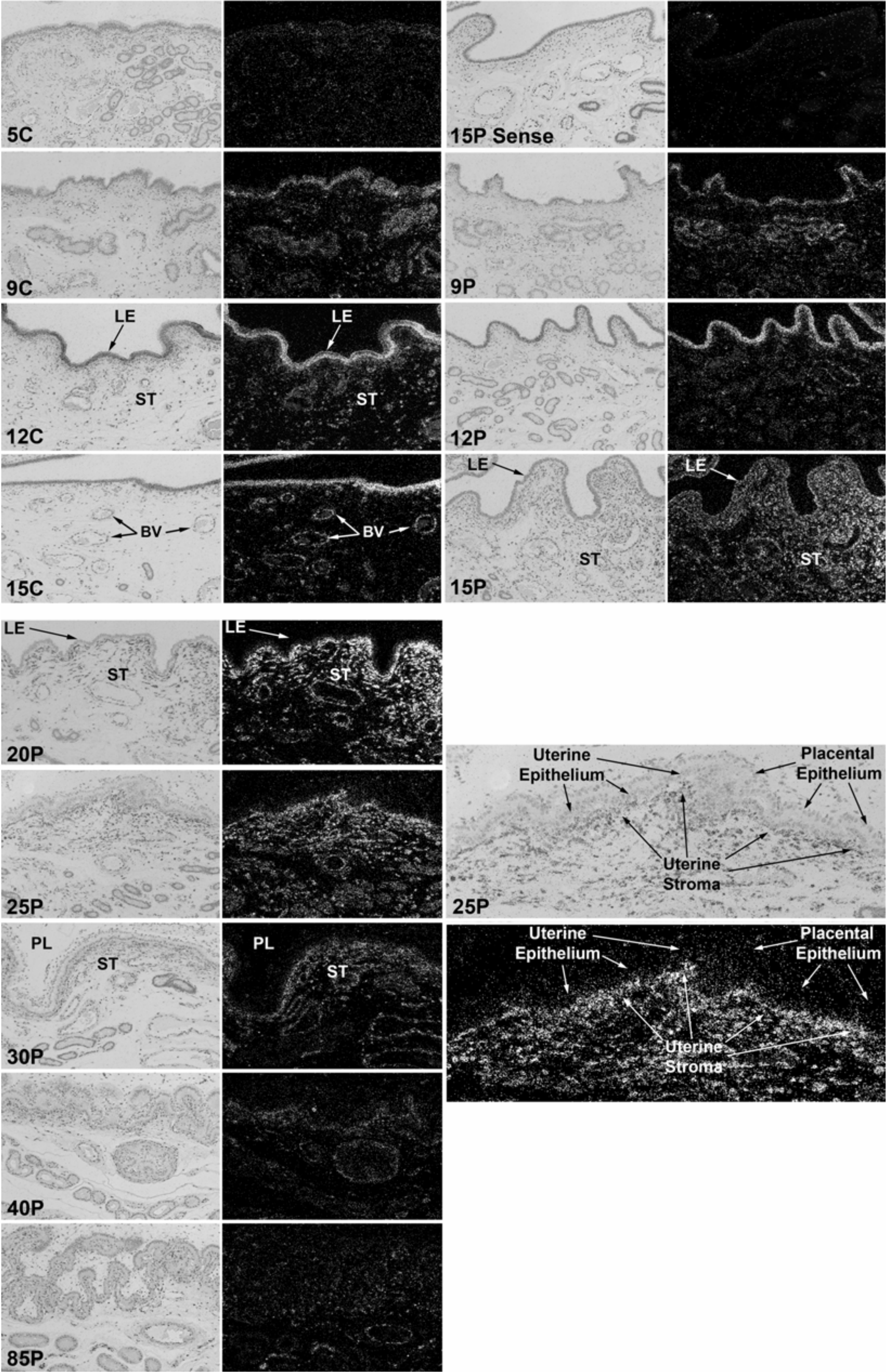

E) B2M
Cyclic
Pregnant
Relative Units + SE
3000000
2500000
2000000
1600000
1000000
500000
0
5 9 10 12 13 14 15 20 25 30 35 40 60 85
Day

FIG. 5.3. *In situ* hybridization analysis of *SLA-1, SLA-2,* and *SLA-3* mRNA in pig endometria. Corresponding brightfield and darkfield images from different days of the estrous cycle (C) and pregnancy (P) are shown. A representative section from day 15 of pregnancy hybridized with radio-labeled sense RNA probe (Sense) serves as a negative control. An expanded view of the photomicrographs of the uterine-conceptus interface on day 25 of pregnancy indicating the lack of hybridization for *SLA-1, SLA-2,* and *SLA-3* mRNAs in epithelia at this interface is shown. LE, stratum compactum stroma (ST), placenta (PL), and blood vessels (BV) are indicated. The width of each field is 870 μm.


5C
15P Sense
9C
9P
LE
LE
ST
ST
12C
12P
BV
BV
15C
LE
LE
ST
ST
15P
LE
LE
ST
ST
20P
25P
Uterine
Epithelium
Placental
Epithelium
Uterine
Stroma
25P
PL
ST
PL
ST
30P
Uterine
Epithelium
Placental
Epithelium
Uterine
Stroma
40P
85P

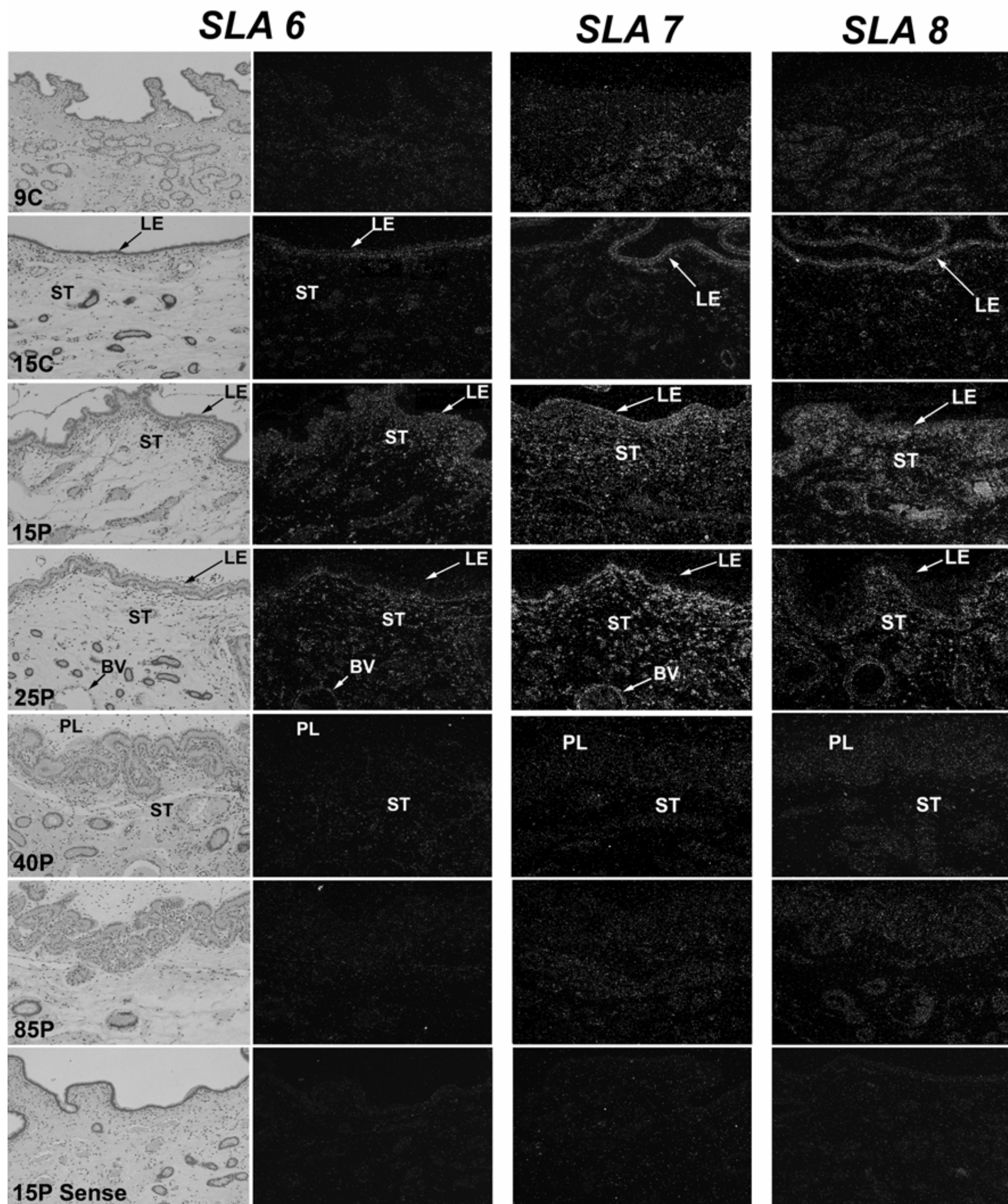


FIG. 5.4. *In situ* hybridization analyses of *SLA-6*, *SLA-7,* and *SLA-8* mRNAs in pig endometria. Corresponding brightfield and darkfield images from different days of the estrous cycle (C) and pregnancy (P) are shown for *SLA-6*, whereas only darkfield images are shown for *SLA-7* and *SLA-8*. Representative sections from day 15 of pregnancy hybridized with radio-labeled sense RNA probes (Sense) served as negative controls. LE, stratum compactum stroma (ST), placenta (PL), and blood vessels (BV) are indicated. The width of each field is 870 μm.

SLA-8 mRNAs increased in the stratum compactum stroma by day 15 of pregnancy, where they remained through day 25, and then decreased to undetectable levels by day 40 (Fig. 5.4). Expression was also detected in endothelia cells in endometrial stroma on all days of the estrous cycle and pregnancy (Fig. 5.4).

In situ hybridization for β_2m mRNA was generally more intense than was observed for SLA mRNAs; however, the general patterns of expression for these mRNAs were similar. The β_2m mRNA increased in the LE and GE between days 5 and 9 of the estrous cycle and pregnancy, and remained in these cell types through day 15 (Fig. 5.5). During pregnancy, the pattern of expression for β_2m mRNA was the same as observed for the estrous cycle through day 12. However by day 15, expression increased in the stratum compactum stroma (note the difference in the spatial distribution of mRNA in endometrium from day 15 of the estrous cycle vs day 15 of pregnancy) (Fig. 5.5). The β_2m remained detectable in the stratum compactum stroma through day 30 and became undetectable in LE by day 20 of pregnancy. Expression of β_2m mRNA by GE was more robust than the SLA mRNAs on all days examined, and diverged from the general pattern of SLA mRNA expression by further increasing in GE between days 60 and 85 of pregnancy (Fig. 5.5). Additionally, β_2m mRNA was detectable in endothelial cells in the endometrial stroma on all days of the estrous cycle and pregnancy (Fig. 5.5).

Because mRNAs for *SLA-1, SLA-2, SLA-3, SLA-6, SLA-7,* and *SLA-8* and β_2m gene decreased in uterine LE between days 15 and 20 of pregnancy, endometrial expression of the USP gene, a negative regulator of type I IFN signaling [195], was examined. *In situ* hybridization revealed that USP mRNA increased between days 15 and 20 of pregnancy in the LE, where it continued to be expressed through day 40 (Fig. 5.6).

Immunofluorescence analysis of pig endometria indicated that immunoreactive classical SLA class I molecules increased in LE between days 9 and 12 of the estrous cycle and pregnancy (Fig. 5.7). Consistent with *in situ* hybridization results, SLA class I molecules were maintained in the LE of cyclic endometrium. In pregnant endometrium, SLA class I molecules were not detectable in LE by day 15 and remained absent from

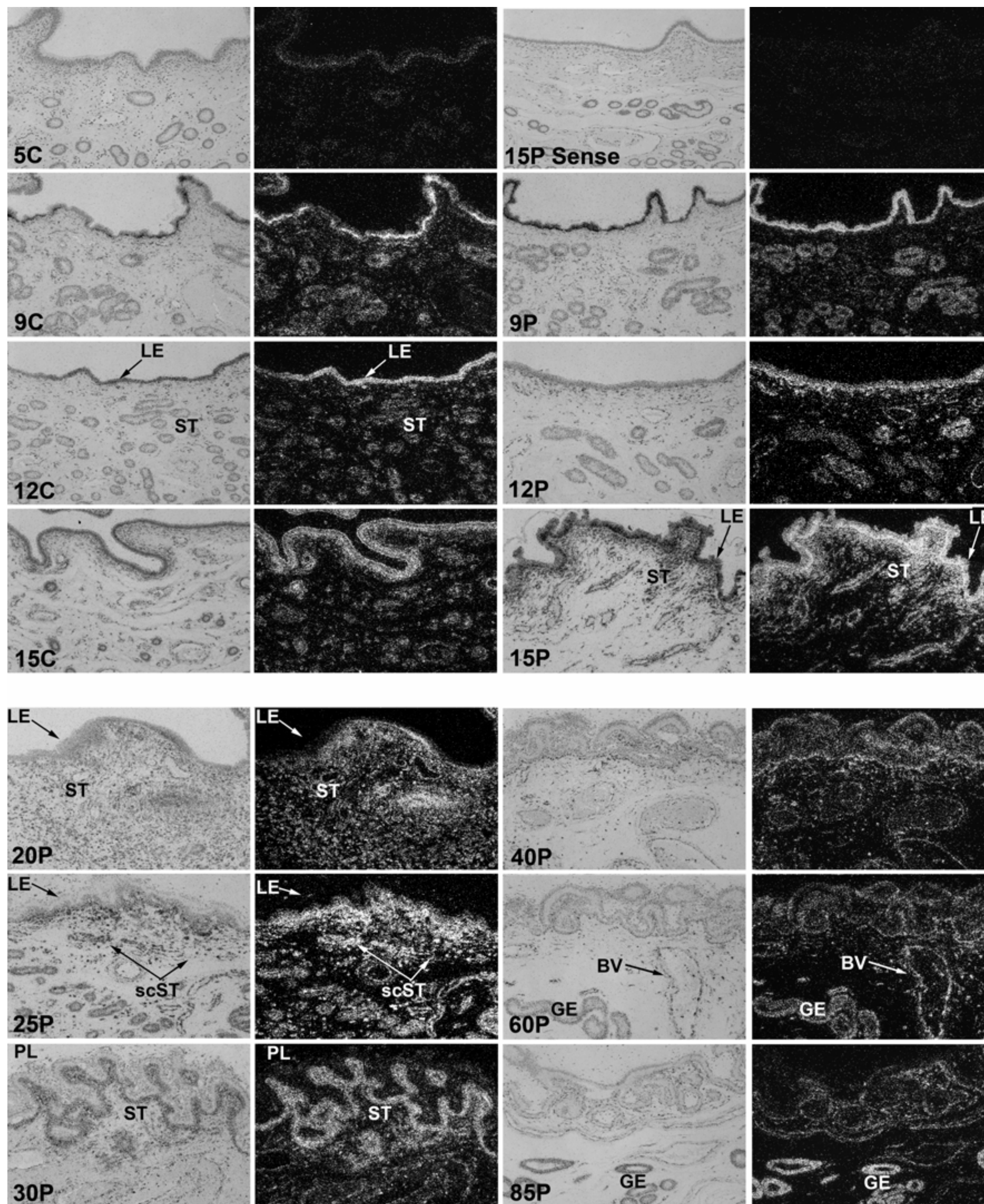


FIG. 5.5. *In situ* hybridization analysis of β_2m mRNA in pig endometria. Corresponding brightfield and darkfield images from different days of the estrous cycle (C) and pregnancy (P) are shown. A representative section from day 15 of pregnancy hybridized with radio-labeled sense RNA probe (Sense) served as a negative control. LE, GE, stratum compactum stroma (ST), placenta (PL), and blood vessels (BV) are indicated. The width of each field is 870 μm.

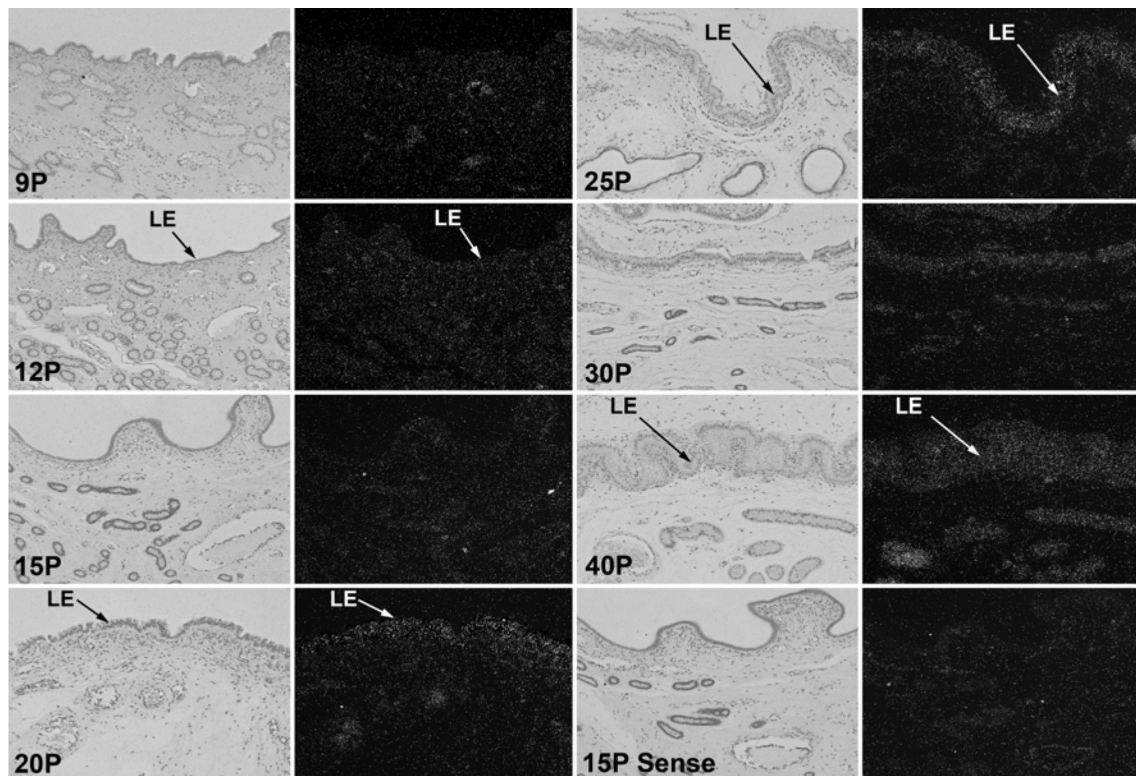


FIG. 5.6. *In situ* hybridization analysis of USP mRNA in pig endometria. Corresponding brightfield and darkfield images in LE from different days of pregnancy (P) are shown. A representative section from day 15 of pregnancy hybridized with radio-labeled sense RNA probe (Sense) served as a negative control. The width of each field is 870 μm.

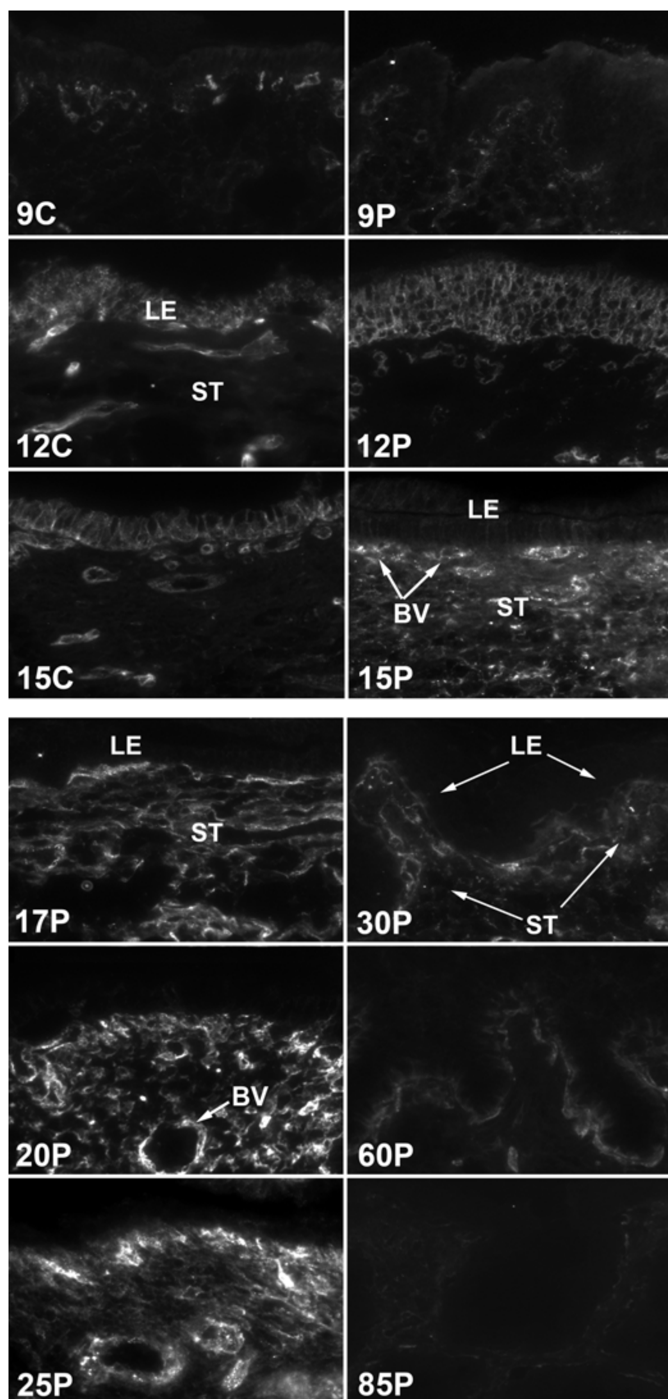


FIG. 5.7. Immunofluorescence localization of classical SLA class I molecules in frozen cross-sections of pig endometria during the estrous cycle (C) and pregnancy (P). A nonrelevant mouse Ig (mIgG) served as a negative control as illustrated in Fig. 5.10. LE, stratum compactum stroma (ST), and blood vessels (BV) are indicated. The width of each field is 540 µm.

LE through day 85 (Fig. 5.7). Similar to the temporal and spatial expression of SLA mRNAs, immunoreactive SLA class I molecules increased in the stratum compactum stroma by day 15 of pregnancy, remained abundant through day 25, and then progressively decreased through day 85 (Fig. 5.7). An increase of SLA class I molecules in endometrial stroma was not observed during the estrous cycle (Fig. 5.7). In addition, SLA class I protein was present in endothelial cells of the endometrium throughout the estrous cycle and pregnancy (Fig. 5.7).

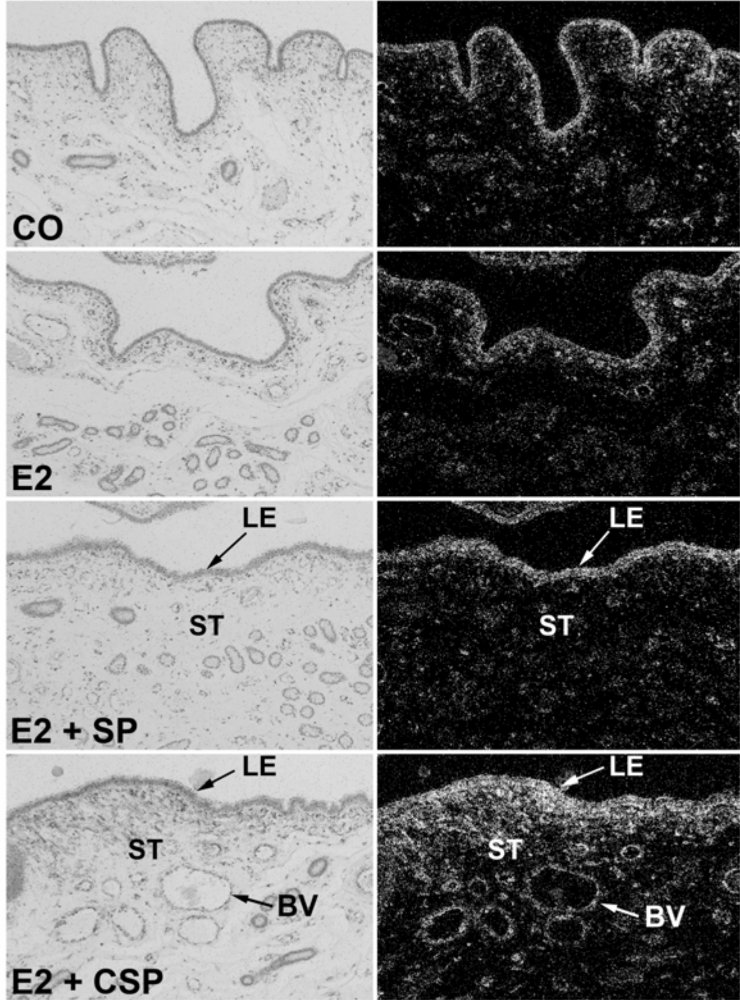
Collectively, results from study one indicate that classical and nonclassical SLA, as well as β_2m, 1) increases in uterine LE before implantation; 2) increases in uterine stratum compactum stroma during the peri-implantation period (days 10-25); and 3) decreases in uterine LE immediately after initial attachment of trophectoderm to uterine LE during early placentation. Therefore, although expression prominently increases within the uterine-placental environment, classical SLA class I mRNA and molecules and nonclassical SLA class I and β_2m mRNAs are conspicuously absent at the immediate interface between uterine and placental epithelia of pigs after day 15, perhaps due to expression of USP in uterine LE.

Effects of Exogenous Estrogen and CSPs for Studies Two and Three

The i.m. injections of estradiol benzoate did not alter expression of classical SLA class I mRNA in endometrial LE of day-15 cyclic-treated pigs compared with corn oil vehicle treatment (Fig. 5.8A). In contrast, intrauterine infusion of CSPs into the uterine horns of day-16 cyclic pigs treated with exogenous estrogen increased expression of classical SLA class I mRNAs in the stratum compactum stroma compared with intrauterine infusion of control serum proteins (Fig. 5.8A). In agreement with mRNA results, immunofluorescence analysis indicated the intrauterine infusion of CSP increased SLA class I molecules in the stratum compactum stroma (Fig. 5.8B).

Similar to SLA class I mRNA, *in situ* hybridization for *SLA-6* (Fig. 5.9A), *SLA-7* (Fig. 5.9B), *SLA-8* (Fig. 5.9C), and β_2m gene (Fig. 5.9D) indicated no effect of exogenous estrogen on expression of these mRNAs in day 15 cyclic pig endometria.

FIG. 5.8. Treatment with CSP increased expression of classical SLA class I mRNAs. A, *In situ* hybridization analysis of *SLA-1, SLA-2,* and *SLA-3* mRNA. B, Immunofluorescence localization of classical SLA molecules. Cross-sections of pig endometria from day-15 cyclic pigs injected i.m. with either corn oil control (CO) or estradiol benzoate (E2), or from estrogen-treated day-16 cyclic pigs in which either control serum proteins (E2 + SP) or CSPs (E2 + CSP) were infused into the uterine lumen are shown. Corresponding brightfield and darkfield images of *in situ* hybridization from different treatments are shown. A representative section from day 15 of pregnancy hybridized with radio-labeled sense RNA probe (15P Sense) served as a negative control for *in situ* hybridization as illustrated in Fig. 5.3. A nonrelevant mouse Ig (mIgG) served as a negative control as illustrated in Fig. 5.10. LE, luminal epithelium, stratum compactum stroma (ST), and blood vessels (BV) are indicated. The width of each *in situ* hybridization field is 870 µm and the width of each immunofluorescence localization field is 540 µm.


A)
CO
E2
LE
ST
E2 + SP
LE
ST
BV
E2 + CSP

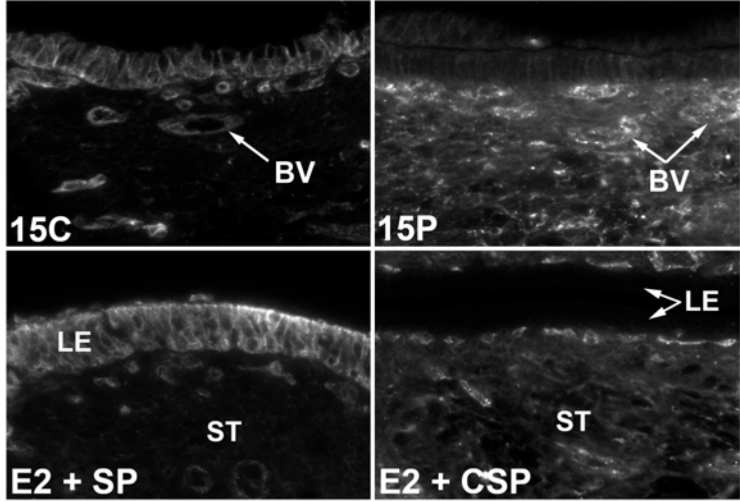
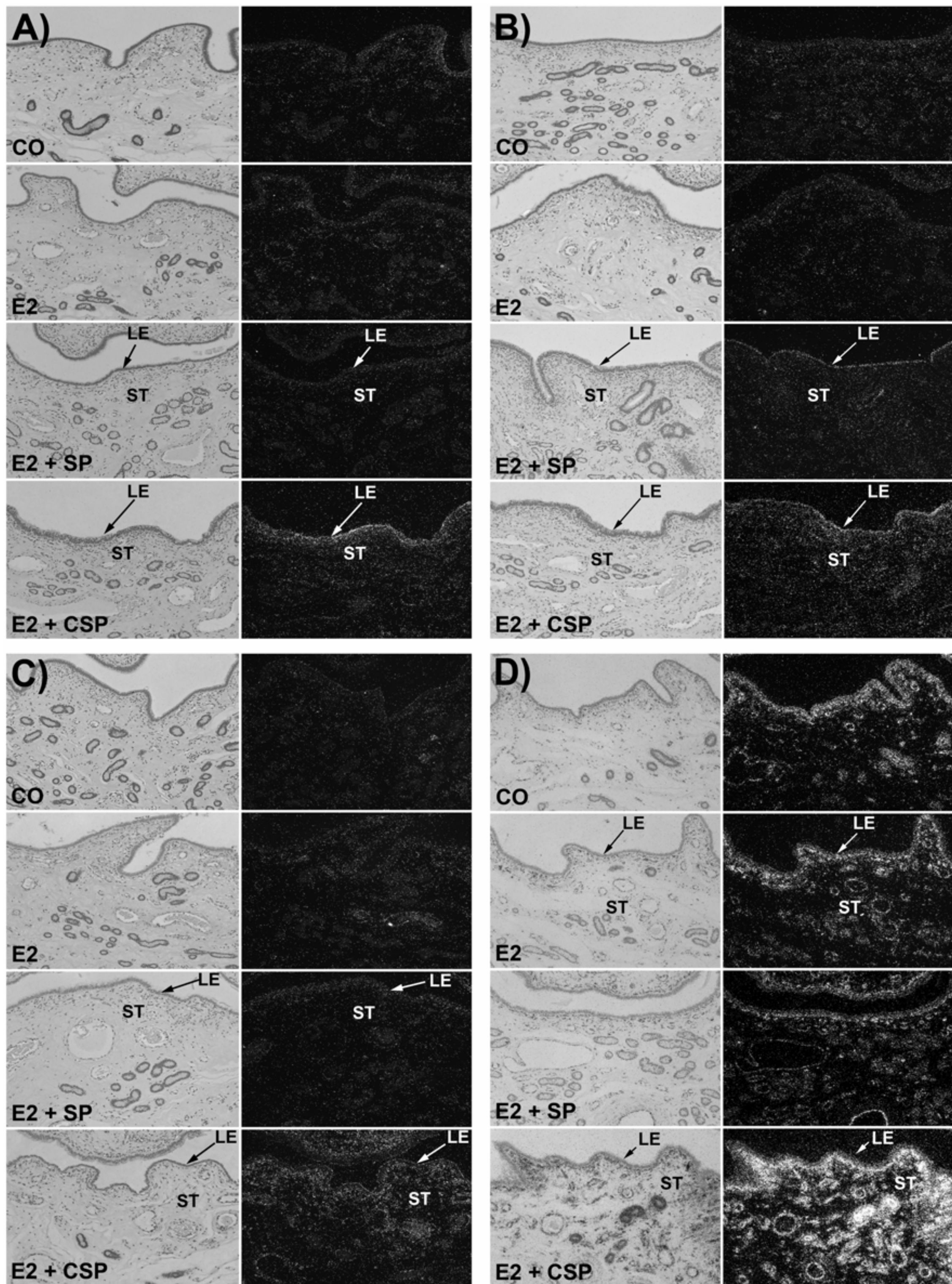

B)
BV
15C
BV
15P
LE
ST
E2 + SP
LE
ST
E2 + CSP

FIG. 5.9. *In situ* hybridization analyses on expression of mRNAs. *SLA-6* (A), *SLA-7* (B), *SLA-8* (C) and β_2m (D) mRNAs in cross-sections of pig endometria from day-15 cyclic pigs injected i.m. with either corn oil control (CO) or estradiol benzoate (E2), or from estrogen-treated day-16 cyclic pigs, which received intrauterine infusions of either control serum proteins (E2 + SP) or CSPs (E2 + CSP). Corresponding brightfield and darkfield images from different treatments are shown. Representative sections from day 15 of pregnancy hybridized with radio-labeled sense RNA probes (15P Sense) served as negative controls as illustrated for *SLA-6, SLA-7,* and *SLA-8* in Fig. 5.4 and for β_2m in Fig. 5.5. LE and stratum compactum stroma (ST) are indicated. The width of each field is 870 µm.


A)
CO
E2
LE
ST
E2 + SP
E2 + CSP
B)
C)
D)

However, intrauterine infusion of CSP into estradiol benzoate-treated day-16 cyclic pigs increased mRNA levels for *SLA-6*, *SLA-7*, *SLA-8,* and β_2m gene in the stratum compactum stroma compared with infusion of serum proteins (Fig. 5.9A-D).

Collectively, results from study two and study three strongly suggest that classical and nonclassical SLA, as well as β_2m increase in endometrial stroma in response to IFN-γ or IFN-δ present in CSP of the peri-implantation period, but expression of these mRNAs and molecules in the endometrial LE is not induced by conceptus estrogens alone, although these estrogens are accepted to be the pregnancy recognition signal.

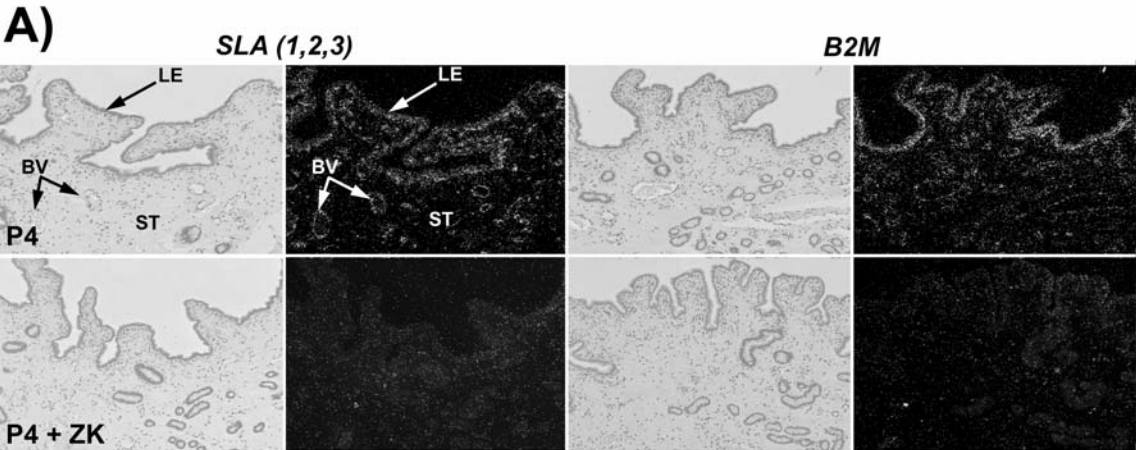
Effects of Exogenous Progesterone for Study Four

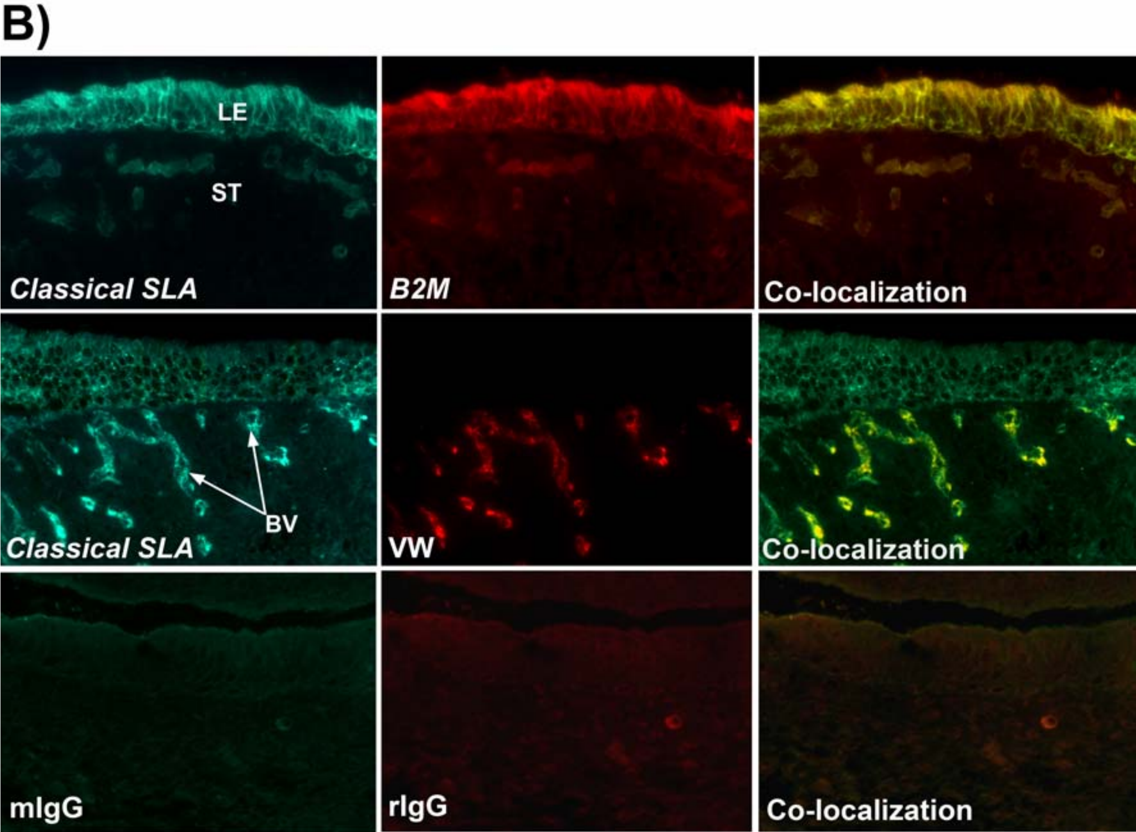
Because *SLA-1, 2, 3, 6, 7,* and *8* and β_2m mRNAs increased in endometrial LE between days 5 and 15 of pregnancy, which is a period of increased progesterone secretion from the corpora lutea, the effects of exogenous progesterone on endometrial expression of SLA class I and β_2m mRNAs and proteins was examined. Classical SLA class I and β_2m mRNAs were detectable in endometrial LE and blood vessels of the stratum compactum stroma of ovariectomized pigs treated with exogenous progesterone. These effects of progesterone were completely ablated by treatment with the progesterone receptor antagonist, ZK137,316, in both uterine LE and vasculature (Fig. 5.10A).

Results shown in Figure 5.10B confirm that SLA class I and β_2m proteins colocalize in the endometrial LE and endothelial cells of ovariectomized pigs treated with exogenous progesterone. Immunoreactive SLA class I molecules also colocalized with von Willebrand factor, an endothelial cell marker, in endometrial endothelial cells of the stratum compactum stroma on day 12 in ovariectomized pigs treated with exogenous progesterone (Fig. 5.10B).

Collectively, results from study four strongly suggests that expression of intact SLA class I molecules including classical SLA class I and β_2m heterodimers is increased by progesterone produced by corpora lutea in endometrial LE and endothelial cells

FIG. 5.10. The effects of exogenous progesterone on endometrial expression of SLA class I and β_2m mRNAs and proteins. A, *In situ* hybridization analyses for *SLA-1, SLA-2, SLA-3*, and β_2m mRNAs in cross-sections of endometria from day-12 ovariectomized pigs treated with progesterone (P4) or progesterone and the progesterone receptor antagonist ZK137,316 (P4 + ZK). Corresponding brightfield and darkfield images from different treatments are shown. LE, stratum compactum stroma (ST), and blood vessels (BV) are indicated. The width of each field is 870 µm. B, Immunofluorescence colocalization of classical SLA with β_2m and classical SLA with von Willibrand factor (VW) in endometrial cross-sections from day 12 ovariectomized pigs treated with progesterone. SLA immunoreactivity was detected using fluorescein-conjugated anti-mouse IgG (*left*, green fluorescence), whereas the β_2m and von Willibrand factor immunoreactivity were detected using Texas Red-conjugated anti-rabbit IgG (*middle*, red fluorescence). SLA and β_2m proteins were colocalized (*right*, yellow fluorescence) to LE and blood vessels (BV) within the stratum compactum stroma (ST). Nonrelevant mouse Ig (mIgG) was detected using fluorescein-conjugated anti-mouse IgG, nonrelevant rabbit Ig (rIgG) was detected using Texas Red-conjugated anti-rabbit IgG and their colocalization served as negative controls as illustrated (*lower*). The width of each field is 540 µm.


A)
SLA (1,2,3)
B2M
LE
LE
BV
BV
ST
ST
P4
P4 + ZK


B)
LE
ST
Classical SLA
B2M
Co-localization
BV
Classical SLA
VW
Co-localization
mIgG
rIgG
Co-localization

during the peri-implantation period in pigs.

Discussion

The MHC class I molecules and accompanying β_2m function in immune responses and are involved in discrimination of self from non-self. It is generally accepted that modification or down-regulation of these molecules in placental tissues is beneficial to pregnancy across mammalian species [177-182]. The present studies focus on the uterine tissues that represent the maternal component of the fetal-maternal interface of pregnancy. Results illustrate dynamic temporal and cell type-specific regulation of MHC class I molecules and β_2m by progesterone and CSPs in the peri-implantation pig uterine endometrium. Similar to previous results in sheep, MHC class I and β_2m up-regulate in endometrial stratum compactum stroma, but are conspicuously undetectable in LE, suggesting that lack of expression in LE is beneficial to pregnancy in species in which the LE maintains direct contact with the placenta (epitheliochorial placentation). However, clear differences between sheep and pigs in regulation and pattern of expression of these genes were observed. Although pigs and sheep share stromal MHC class I and β_2m expression, pigs lack the IFN-τ responsible for this expression in sheep [31]. Therefore it is likely that in pigs, conceptus-derived IFN-δ and IFN-γ increase MHC class I and β_2m in endometrial stroma [8, 9, 40]. Unlike sheep, which never express MHC class I and β_2m in LE, ovarian progesterone increased expression of SLA class I and β_2m in the uterine LE during the estrous cycle and early pregnancy of pigs. Finally, the present data suggest a mechanism for down-regulation of SLA class I and β_2m in LE that has not been proposed for sheep. Although estrogens secreted by the conceptus or administered exogenously did not directly effect expression of SLA class I and β_2m genes, estradiol does increase expression of IFN regulatory factor 2 (IRF)-2 in LE [170], which together with USP may down-regulate SLA class I and β_2m gene expression in uterine LE between days 15 and 25 of pregnancy. Collectively, for the majority of the first half of gestation, there is abundant uterine

expression of SLA and β_2m in the stromal compartment of pig endometrium, but not in LE that is directly apposed to trophectoderm or chorion.

Progesterone, the hormone of pregnancy, plays a critical role in control of temporal and spatial (cell-specific) changes in gene expression within the uterus [148]. Indeed, treatment with progesterone significantly alters the expression of a number of genes in the uteri of rodents, primates, and sheep as determined using microarray analyses [35, 196, 197]. In the present pig study, progesterone increased expression of SLA class I and β_2m genes in endometrial LE and stromal endothelial cells during the estrous cycle and early pregnancy. This expression was blocked by ZK137,316, a progesterone receptor antagonist, indicating that induction by progesterone is mediated via progesterone receptors. Since progesterone receptors in pigs are down-regulated in endometrial LE and GE by day 10 of the estrous cycle and pregnancy, but maintained in stromal cells and myometrium [6], the endocrine effects of ovarian progesterone on endometrial LE expression of SLA class I and β_2m genes may be mediated indirectly by either progesterone-induced paracrine-acting factors (progestamedins) produced by the progesterone receptor-positive stromal cells, or by induction of factors in LE that down-regulate progesterone receptors to either allow or stimulate expression of endometrial genes [155, 198].

Given that SLA class I molecules and β_2m are important for host defense, their expression in the LE during the estrous cycle and early pregnancy may be important for preventing uterine infections. At estrus, mucin 1 (MUC1), which forms an apical LE glycocalyx barrier to provide innate immune protection for the uterus from bacterial infections [199], is localized to the endometrial LE, but decreases by day 10 of the estrous cycle and pregnancy [54]. This down-regulation of MUC1 is hypothesized to be necessary for conceptus attachment to the LE, but leaves the uterus susceptible to bacterial invasion [54, 199, 200]. Increased expression of SLA class I and β_2m genes before day 9 of the estrous cycle and pregnancy by ovarian progesterone may compensate for the progesterone-induced loss of MUC1 and provide continued immune protection of the uterus from pathogens.

Results of the present study strongly suggest that classical SLA genes *SLA-1*, *SLA-2,* and *SLA-3*, nonclassical *SLA-6*, *SLA-7,* and *SLA-8*, as well as β_2m mRNAs are induced in the stratum compactum stroma of pigs in response to IFNs secreted by the conceptus. Pig conceptuses secrete both IFN-δ and IFN-γ; ~75% of antiviral activity in pig CSPs is attributed to IFN-γ and the other 25% to IFN-δ [8, 40]. Pig conceptus trophectoderm cells express high amounts of IFN-γ mRNA from day 13 through day 20 of pregnancy, and immunoreactive IFN-γ localizes to perinuclear membranes typically occupied by endoplasmic reticulum and Golgi apparatus as well as cytoplasmic vesicles within trophectoderm cells, suggesting trafficking and secretion of IFN-γ into the uterine lumen [8, 190]. Interestingly, *SLA-1*, *SLA-2,* and *SLA3*, but not *SLA-6* or *SLA-7*, were reported to respond to both IFN-γ and IFN-α in a pig kidney cell line [201]. Although results of the present study are consistent with those of Tennant et al. [201] for *SLA-1*, *SLA-2,* and *SLA-3*, there are differences between *in vivo* and *in vitro* results for *SLA-6* and *SLA-7*. It is perhaps not surprising that stromal cells *in vivo*, which have distinct spatial relationships to other cell types and the extracellular matrix, respond differently to IFNs than do isolated and cultured kidney cells. Certainly macrophages are highly individualized in tissues, where their functions are a reflection of the systemic and local environment [202]. Indeed, it is well established that uterine gene regulation is altered by differences in cell type or epithelial-stromal interactions [203].

Pig conceptuses are unique among mammalian species in that they secrete two IFNs during the peri-implantation period, providing the opportunity for IFN-γ and IFN-δ to work together to regulate endometrial gene expression. In general, both type I and type II IFNs can induce expression of SLA class I through the classical JAK-STAT cell signaling pathway leading to gamma-activation factor binding of gamma activation sequence elements and induction of gene transcription [13]. In addition, IFN-δ signals through a similar, yet distinct, pathway leading to ISG factor 3 complex binding of IFN-stimulated response elements in the promoters of several ISGs to initiate transcription [11]. Interactions between type I and type II IFNs have been demonstrated [204]. Although the type I IFN-α and type II IFN-γ each induce expression of largely

nonoverlapping sets of genes, they can also act in concert to produce synergistic interactions leading to mutual reinforcement of physiological responses [168]. With cells that are normally relatively unresponsive to IFN-γ, sequential treatment with IFN-γ followed by IFN-α results in greater induction of ISGs [168]. In addition, cotreatment with IFN-γ and IFN-α extends the period of ISG expression over IFN-α alone [157]. For typical ISGs, type I IFNs induce rapid expression followed by a protein synthesis-dependent suppression of transcription within 6 h. IFN-γ overrides this suppression, allowing continuous expression of ISGs for over 24 h [157]. Clearly, IFN-δ and IFN-γ may profoundly influence endometrial physiology through cooperative induction of cytokine-specific transcription factors that allow reinforcement of effects of distinct cell surface ligands, including SLA class I and β_2m genes while maintaining the specificities of the individual inducing IFNs to influence cell function.

Although it is well established that conceptus IFN-τ induces expression of numerous ISGs in the stroma and GE of ruminants [148], and that ISGs increase in the decidualizing stroma of humans and rodents [16, 23], the pregnancy-specific roles of uterine ISGs remain conjectural. Because IFN-γ secreted by pig conceptuses can initiate uterine vascular development [126], it is reasonable to hypothesize that conceptus-derived IFNs up-regulate ISGs such as SLA class I and β_2m genes to facilitate vascular changes necessary to support the developing conceptus. Recently, placental human HLA-G was implicated in regulation of angiogenesis during placental invasion and replacement of endothelial cells at the ends of uterine spiral arteries [205]. Indeed, there is evidence that vascular development at the maternal-fetal interface is regulated by a balance of proangiogenic and antiangiogenic factors, and MHC and β_2m molecules may be significant players in this balance [205]. Whether IFN-induced SLA class I and β_2m gene products in endometrial stratum compactum stroma of pigs are involved in angiogenesis during pregnancy remains to be determined; however, it is clear that expression of ISGs within the uterine stroma of mammals is a universal response to pregnancy.

Intriguingly, SLA class I and β_2m gene products decrease in endometrial LE as pregnancy progresses so that they are no longer detectable by day 20 of pregnancy. Although the mechanism involved in this down-regulation is unknown, IRF-2, a potent repressor and attenuator of ISG expression [127, 128], is induced in endometrial LE of pigs by estrogens [170]. IRF-2 can also bind to the promoter of Ubp43, *Usp18*, a USP in mice used to mediate basal levels of expression. Ubp43 (*Usp18*), a ubiquitin deconjugating enzyme specific for the ubiquitin-like protein ISG15 [206], is up-regulated by type I IFNs [207] and inhibits type I IFN signaling by decreasing JAK1 phosphorylation [195]. Although *Usp18* is present in both endometrial LE and the deep GE of cows [208], results of the present study localized USP gene only to LE between days 15 and 20 of pregnancy in pigs, which is coincident with temporal loss of SLA class I and β_2m mRNAs from LE. Given that IRF-2 and USP genes are expressed in the LE during pregnancy, both may play a role in down-regulating SLA class I and β_2m genes. It is generally accepted that SLA class I and β_2m molecules are decreased in the placenta to ensure that the conceptus semiallograft avoids host-vs-graft immune rejection [177-182].

In conclusion, results of the present study suggest that down-regulation of SLA class I and β_2m genes in uterine LE, in coordination with a lack of expression of these genes in placenta [182], may be important for preventing fetal allograft rejection in species exhibiting epitheliochorial placentation. The temporal cell type-specific regulation of pig endometrial SLA class I and β_2m by progesterone, IFN-γ, or IFN-δ and perhaps through permissive effects of conceptus estrogens, provides insight into how immune tolerance of the conceptus allograft is achieved. The traditional view, first proposed by Medawar [174, 175], was that the immune system must be circumvented to permit pregnancy. The results presented in this study provide additional evidence that supports reports from multiple investigators implicating the immune system as a dynamic and active player in the complex bidirectional endocrine, paracrine, autocrine, and juxtacrine interactions between uterus and placenta that uniquely define establishment and maintenance of a successful pregnancy.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Summary

Coordinated signaling between the maternal endometrium and conceptus is required for successful establishment and maintenance of pregnancy. During pregnancy in pigs, one of the first signals from the conceptus, and the signal for maternal recognition of pregnancy, is the secretion of estrogen [1]. Pig conceptuses also secrete IFND and IFNG during early pregnancy [8], but they do not appear to be involved in pregnancy recognition [9]. However in other species, conceptus IFNs and uterine ISGs have been detected during early pregnancy [14-39], indicating that this may be a common event of early pregnancy which may be important for uterine receptivity and conceptus implantation and development. Therefore, the studies described in this dissertation were undertaken to determine if ISGs increase during pregnancy in the pig endometrium and if so, whether expression of these genes is regulated by conceptus estrogen and/or CSPs which contain IFND and IFNG.

Results of these studies indicate gene expression is regulated in a temporal and cell-type specific manner (Fig. 6.1). As described in Chapters III and IV, *IRF2* and *STAT1* expression increased in the LE on Day 12 of pregnancy, which is correlated with estrogen secretion by the conceptus [1]. In Study 2, exogenous estrogen also increased expression of both of these genes in the LE, indicating that the conceptus regulates these genes through the secretion of estrogen. Expression of *IRF2*, a potent transcriptional repressor of ISGs [127-130], may prevent ISG expression in the LE, but not the underlying stroma. This is one possible explanation for the lack of expression of *IRF1*, a prototypic IFNG/STAT1-responsive gene [109], in the *STAT1*-positive LE. Alternatively, estrogen may be influencing expression of *IRF1*. Indeed in mice, 17β-estradiol can downregulate *IRF1* expression [171, 172] without affecting *STAT1*

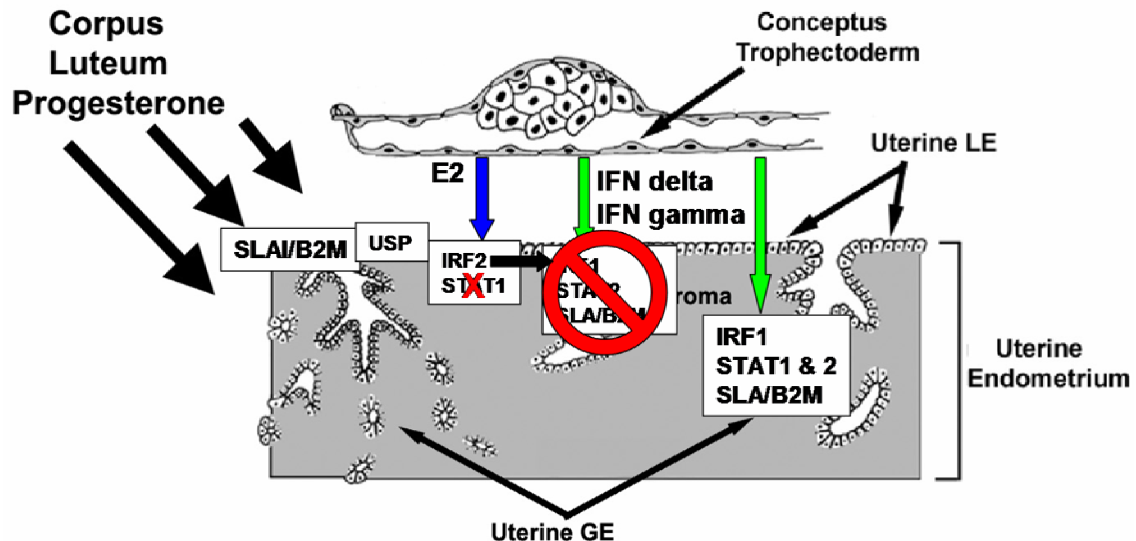


FIG. 6.1. Proposed model of gene expression changes and regulation in pig endometrium. Progesterone increases expression of *SLA class I* and *B2M* in the LE by Day 9 of the estrous cycle and pregnancy via a PGR-mediated pathway. As pregnancy progresses, *SLA class I* and *B2M* decrease in the LE and are no longer detectable by Day 20. On Day 12 of pregnancy, *IRF2* and *STAT1* expression increase in the LE in response to estrogen secretion by the conceptus. By Day 15 of pregnancy, the ISGs *IRF1*, *STAT1*, *STAT2*, *SLA1*, *SLA2*, *SLA3*, *SLA6*, *SLA7*, *SLA8* and *B2M* increase in the stroma, presumably due to the secretion of IFND and/or IFNG by the conceptus. However, except for *STAT1*, these ISGs are not detectable in the LE. This may be due to *IRF2* expression, a potent transcriptional repressor of ISGs [127-130], in this cell type. However, the absence of *IRF1*, a prototypic IFNG/STAT1-responsive gene [109], in the LE may be due to downregulation by estrogen via an ESR1-mediated pathway [171-173]. This is consistent with the localization of ESR1 in the LE on Day 12 of pregnancy, but not in the stroma on Day 15 of pregnancy [7]. *STAT1* expression in the LE decreases after Day 20 of pregnancy, indicating that *IRF2* alone may not be sufficient to down-regulate STAT1 in the LE. Indeed, *USP*, which may increase in response to Type I IFNs [207], inhibit Type I IFN signaling by decreasing JAK1 phosphorylation [195], and contain an *IRF2* binding site in its promoter [209], increases in the LE by Day 20 of pregnancy. Given that *IRF2* and *USP* are expressed in the LE by Day 20 of pregnancy, both may play a role in the down-regulation of *STAT1* in this cell type.

expression [171], and this effect appears to be mediated by ESR1 [172, 173]. The temporal/spatial pattern of uterine ESR1 in the pig is consistent with this idea [7]. ESR1 is detectable in pig LE on Day 12 where *STAT1,* but not *IRF1,* is expressed [170]. ESR1 is not present in Day 15 stroma where both *STAT1* and *IRF1* are prominently expressed [170].

Progesterone also appears to regulate gene expression in the LE. As described in Chapter V, progesterone increased expression of *SLA class I* and *B2M* in the LE during the estrous cycle and early pregnancy. This expression was blocked by ZK, a PGR antagonist, indicating that expression is mediated by progesterone receptors. Since PGR in pigs are down-regulated in endometrial LE and GE by Day 10 of the estrous cycle and pregnancy, but maintained in stromal cells and myometrium [6], *SLA class I* and *B2M* expression may be mediated indirectly by either progesterone-induced paracrine-acting factors (progestamedins) produced by the progesterone receptor-positive stromal cells, or by induction of factors in the LE that down-regulate progesterone receptors in the LE [155, 198].

Progesterone also induces loss of MUC1, which provides innate immune protection from bacterial infection [199], in the LE by Day 10 of the estrous cycle and pregnancy [54, 199, 200]. Given that SLA class I molecules and B2M are important for host defense, increased expression of these genes prior to Day 9 of the estrous cycle and pregnancy by ovarian progesterone may compensate for the loss of MUC1 and provide continued immune protection of the uterus from pathogens.

SLA class I and *B2M* decrease in the LE as pregnancy progresses and are no longer detectable by Day 20. While the mechanism involved in this down-regulation is unknown, it may involve repression by *IRF2* alone or in cooperation with *USP*. As described in Chapter V, *USP* localized to the LE between Days 15 and 20 of pregnancy, which is coincident with temporal loss of *SLA class I* and *B2M* mRNAs from the LE. Ubp43 (USP18, ubiquitin specific protease), is up-regulated by Type I IFNs [207] and inhibits Type I IFN signaling by decreasing JAK1 phosphorylation [195]. In mice, IRF2 can bind to the promoter of *Ubp43* (*USP18*) to mediate basal levels of expression [209].

Given that *IRF2* and *USP* are expressed in the LE during pregnancy, both may play a role in down-regulating *SLA class I* and *B2M* genes.

It is generally accepted that SLA class I and B2M, key molecules involved in host defense and immune histocompatibility of transplanted tissues, are decreased in the placenta to ensure that the conceptus semi-allograft avoids host-versus-graft immune rejection [31]. Results in Chapter V indicate that ablation of expression of *SLA class I* and *B2M* genes in uterine LE may also be important for preventing a graft versus uterine host response by the developing fetal immune system.

Graft versus host disease (GVHD) is a common complication of allogeneic bone marrow transplantation in which functional immune cells in the transplanted marrow recognize the recipient as “foreign” and mount an immunologic attack. Clinical manifestations can be widespread and include damage to the mucosa and parenchyma of multiple internal and external organs. In transplantation biology, there are three requirements for GVHD: i) the transplanted graft must contain immunologically competent cells; ii) the recipient must be incapable of rejecting the transplanted cells; and iii) the recipient must express tissue antigens that are not present in the transplant donor that could thus be recognized as foreign [210]. The pig conceptus is similar to a transplanted graft. There are active B-lymphocytes by gestational Day 20 in the yolk sac, and in the liver at gestational Day 30 [211]. The T-lymphopoietic system becomes active in the pig thymus as early as gestational Day 40 [212, 213] and by Day 60, fetal pigs can produce antibodies and reject allografts [214], to meet the first requirement of GVHD. Regarding the second requirement of GVHD, several mechanisms, including expression of nonclassical MHC molecules by trophoblast cells, tryptophan catabolism and lymphocyte apoptosis [215, 216], promote maternal tolerance to the conceptus. This results in a successful pregnancy, demonstrating the inability of the mother (recipient) to reject the conceptus (transplanted cells). Finally, the conceptus inherits only half of its antigens from the mother. Maternal antigens not inherited by the conceptus are recognized as foreign by the conceptus. Therefore, potential exists for extensive uterine

damage through development of a fetal graft versus uterine host immune response during the first half of pregnancy.

Although the requirements of GVHD appear to be present during pregnancy, the disease does not materialize. Previous studies have identified putative proteins expressed by pig trophectoderm that could suppress graft-versus-host reactions [217], although the mechanism is unknown. Results in Chapter V suggest that the conceptus protects the uterus from graft versus host damage by secreting estrogens and IFNs to initiate expression of gene regulatory networks that down-regulate SLA class I and B2M in uterine LE immediately prior to development of fetal immune cells and immunocompetency. Therefore, presentation of “foreign” maternal antigens to the fetal immune cells is prevented.

In addition to gene expression changes in the LE, several genes increase in the uterine stroma during pregnancy as described in Chapters III, IV, and V. *IRF1*, *STAT2*, *STAT1*, *SLA1*, *SLA2*, *SLA3*, *SLA6*, *SLA7*, *SLA8*, and *B2M*, all ISGs [28, 31, 148, 189], increase in the pig stroma by Day 15 of pregnancy. Infusion of CSPs also increased these ISGs in the stroma. As illustrated in Fig. 4.6, this increase was localized to the region of the uterus in close proximity to the implanting conceptus. The importance of the conceptus for stromal ISG expression was further demonstrated in Chapter IV, Study 4. In this study, exogenous estrogen was administered to pregnant pigs on Day 9 and Day 10 of pregnancy. This inappropriately early estrogen results in degeneration of the conceptuses by Day 15 of pregnancy [5]. Degeneration of the conceptus, which would presumably prevent the production and secretion of proteins such as IFND and IFNG by the conceptus, prevented stromal expression of *STAT1*.

While the pregnancy-specific roles of uterine ISGs in general remain conjectural, it is likely that they facilitate remodeling within the stromal compartment of the uterus for implantation and placentation. Individual ISGs could be involved in protecting the fetal semi-allograft from immune rejection, in limiting conceptus invasion through the uterine wall, and/or in establishing a vascular supply to the conceptus. Because IFNG is believed to initiate endometrial vascular development [126], it is reasonable to

hypothesize that conceptus-derived IFNs increase ISGs such as *IRF1* and *STAT1* to facilitate vascular changes necessary to provide hematotrophic support to the developing conceptus. Indeed, recent studies have demonstrated that angiogenic genes increase during pregnancy in endometrium, particularly endometrial lymphocytes, isolated near healthy conceptuses, but not from sites of fetal arrest [144]. While it is currently unknown whether ISGs are involved in angiogenic gene expression changes during pregnancy, it is becoming increasingly clear that IFN induction of genes within the uterine stroma of mammals is a universal response to, or component of, a progressing pregnancy.

Conclusions

These studies provided compelling evidence that pig conceptuses orchestrate precise temporal and cell-type specific changes in uterine gene expression through secretion of estrogen, followed by IFND and IFNG. Because the pig conceptus IFNs have been identified (IFND and IFNG) [8], but are not known to be involved in pregnancy recognition [9], the pig is an excellent model to investigate the role of uterine ISGs during pregnancy. Using siRNA and morpholine antisense oligonucleotide methods, IFND and/or IFNG production by the conceptus trophectoderm can be knocked out, thereby preventing ISG induction in the uterine stroma. The effect of a lack of stromal ISGs on pregnancy, such as vascular and placental development, immune rejection, and conceptus survival and development, could then be evaluated. Understanding the function of stromal ISGs would contribute to our knowledge of uterine function for the successful establishment and maintenance of pregnancy.

REFERENCES

1. Bazer FW, Thatcher WW. Theory of maternal recognition of pregnancy in swine based on estrogen controlled endocrine versus exocrine secretion of prostaglandin $F_2\alpha$ by the uterine endometrium. Prostaglandins 1977; 14:397-400.

2. Bazer FW, Johnson GA, Spencer TE. Growth and development: pre-implantation embryo. In: Pond WG, Bell AW (eds.), Encyclopedia of animal science. London: Taylor & Francis; 2005: 1-3.

3. Frank M, Bazer FW, Thatcher WW, Wilcox CJ. A study of prostaglandin F2alpha as the luteolysin in swine: III. effects of estradiol valerate on prostaglandin F, progestins, estrone and estradiol concentrations in the utero-ovarian vein of nonpregnant gilts. Prostaglandins 1977; 14:1183-1196.

4. Spencer TE, Bazer FW. Conceptus signals for establishment and maintenance of pregnancy. Reprod Biol Endocrinol 2004; 2:49-63.

5. Ashworth MD, Ross JW, Hu J, White FJ, Stein DR, Desilva U, Johnson GA, Spencer TE, Geisert RD. Expression of porcine endometrial prostaglandin synthase during the estrous cycle and early pregnancy, and following endocrine disruption of pregnancy. Biol Reprod 2006; 74:1007-1015.

6. Geisert RD, Pratt TN, Bazer FW, Mayes JS, Watson GH. Immunocytochemical localization and changes in endometrial progestin receptor protein during the porcine oestrous cycle and early pregnancy. Reprod Fertil Dev 1994; 6:749-760.

7. Geisert RD, Brenner RM, Moffett RJ, Harney JP, Yellin T, Bazer FW. Changes in oestrogen receptor protein, mRNA expression and localization in the endometrium of cyclic and pregnant gilts. Reprod Fertil Dev 1993; 5:247-260.

8. La Bonnardière C, Martinat-Botté F, Terqui M, Lefèvre F, Zouari K, Martal J, Bazer FW. Production of two species of interferon by Large White and Meishan pig conceptuses during the peri-attachment period. J Reprod Fert 1991; 91:469-478.

9. Lefèvre F, Martinat-Botté F, Locatelli A, De Niu P, Terqui M, La Bonnardière C. Intrauterine infusion of high doses of pig trophoblast interferons has no antiluteolytic effect in cyclic gilts. Biol Reprod 1998; 58:1026-1031.

10. Brierley MM, Fish EN. Stats: multifaceted regulators of transcription. J Interferon Cytokine Res 2005; 25:733-744.

11. Fu X-Y, Kessler DS, Veals SA, Levy DE, Darnell JEJ. ISGF3, the transcriptional activator induced by interferon alpha, consists of multiple interacting polypeptide chains. Proc Natl Acad Sci USA 1990; 87:8555-8559.

12. Shuai K, Horvath CM, Huang LH, Qureshi SA, Cowburn D, Darnell JEJ. Interferon activation of the transcription factor Stat91 involves dimerization through SH2-phosphotyrosyl peptide interactions. Cell 1994; 76:821-828.

13. Decker T, Kovarik P, Meinke A. GAS elements: a few nucleotides with a major impact on cytokine-induced gene expression. J Interferon Cytokine Res 1997; 17:121-134.

14. Fowler AK, Reed CD, Giron DJ. Identification of an interferon in murine placentas. Nature 1980; 286:266-267.

15. Cross JC, Farin CE, Sharif SF, Roberts RM. Characterization of the antiviral activity consitutively produced by murine conceptuses: absence of placental mRNAs for interferon alpha and beta. Mol Reprod Dev 1990; 26:122-128.

16. Bany BM, Cross JC. Post-implantation mouse conceptuses produce paracrine signals that regulate the uterine endometrium undergoing decidualization. Dev Biol 2006; 294:445-456.

17. Platt JS, Hunt JS. Interferon-gamma gene expression in cycling and pregnant mouse uterus: temporal aspects and cellular localization. J Leukoc Biol 1998; 64:393-400.

18. Paulesu L, Romagnoli R, Cintorino M, Ricci MG, Garotta G. First trimester human trophoblast expresses both interferon-gamma and interferon-gamma-receptor. J Reprod Immunol 1994; 27:37-48.

19. Aboagye-Mathiesen G, Tóth FD, Zdravkovic M, Ebbesen P. Production of interferons in human placental trophoblast subpopulations and their possible roles in pregnancy. Clin Diagn Lab Immunol 1994; 1:650-659.

20. Aboagye-Mathiesen G, Tóth FD, Zdravkovic M, Ebbesen P. Functional characteristics of human trophoblast interferons. Am J Reprod Immunol 1996; 35:309-317.

21. Aboagye-Mathiesen G, Tóth FD, Zdravkovic M, Ebbesen P. Human trophoblast interferons: production and possible roles in early pregnancy. Early Pregnancy 1995; 1:41-53.

22. Austin KJ, Bany BM, Belden EL, Rempel LA, Cross JC, Hansen TR. Interferon-stimulated gene-15 (Isg15) expression is up-regulated in the mouse uterus in response to the implanting conceptus. Endocrinology 2003; 144:3107-3113.

23. Hess AP, Hamilton AE, Talbi S, Dosiou C, Nyegaard M, Nayak N, Genbecev-Krtolica O, Mavrogianis P, Ferrer K, Kruessel J, Fazleabas AT, Fisher SJ, Giudice LC. Decidual stromal cell response to paracrine signals from the trophoblast: amplification of immune and angiogenic modulators. Biol Reprod 2007; 76:102-117.

24. Bebington CR, Bell SC, Doherty FJ, Fazleabas AT, Fleming SD. Localization of ubiquitin and ubiquitin cross-reactive protein in human and baboon endometrium and decidua during the menstrual cycle and early pregnancy. Biol Reprod 1999; 60:920-928.

25. Imakawa K, Anthony RV, Kazemi M, Marotti KR, Polites HG, Roberts RM. Interferon-like sequence of ovine trophoblast protein secreted by embryonic trophectoderm. Nature 1987; 330:377-379.

26. Charpigny G, Reinaud P, Huet J-C, Guillomot M, Charlier M, Pernollet JC, Martal J. High homology between a trophoblastic protein (trophoblastin) isolated from ovine embryo and alpha-interferons. FEBS Lett 1988; 228:12-16.

27. Stewart HJ, McCann SH, Northrop AJ, Lamming GE, Flint AP. Sheep antiluteolytic interferon: cDNA sequence and analysis of mRNA levels. J Mol Endocrinol 1989; 2:65-70.

28. Choi Y, Johnson GA, Burghardt RC, Berghman LR, Joyce MM, Taylor KM, Stewart MD, Bazer FW, Spencer TE. Interferon regulatory factor-two restricts expression of interferon-stimulated genes to the endometrial stroma and glandular epithelium of the ovine uterus. Biol Reprod 2001; 65:1038-1049.

29. Johnson GA, Spencer TE, Hansen TR, Austin KJ, Burghardt RC, Bazer FW. Expression of the interferon tau inducible ubiquitin cross-reactive protein in the ovine uterus. Biol Reprod 1999; 61:312-318.

30. Joyce MM, White FJ, Burghardt RC, Muñiz JJ, Spencer TE, Bazer FW, Johnson GA. Interferon stimulated gene 15 conjugates to endometrial cytosolic proteins and is expressed at the uterine-placental interface throughout pregnancy in sheep. Endocrinology 2005; 146:675-684.

31. Choi Y, Johnson GA, Spencer TE, Bazer FW. Pregnancy and interferon tau regulate major histocompatibility complex class I and beta2-microglobulin expression in the ovine uterus. Biol Reprod 2003; 68:1703-1710.

32. Ott TL, Yin J, Wiley AA, Kim H-T, Gerami-Naini B, Spencer TE, Bartol FF, Burghardt RC, Bazer FW. Effects of the estrous cycle and early pregnancy on uterine expression of Mx protein in sheep (Ovis Aries). Biol Reprod 1998; 59:784-794.

33. Johnson GA, Stewart MD, Gray CA, Choi Y, Burghardt RC, Yu-Lee L-Y, Bazer FW, Spencer TE. Effects of the estrous cycle, pregnancy, and interferon tau on 2',5'-oligoadenylate synthetase expression in the ovine uterus. Biol Reprod 2001; 64:1392-1399.

34. Song G, Spencer TE, Bazer FW. Cathepsins in the ovine uterus: regulation by pregnancy, progesterone, and interferon tau. Endocrinology 2005; 146:4825-4833.

35. Gray CA, Abbey CA, Beremand PD, Choi Y, Farmer JL, Adelson DL, Thomas TL, Bazer FW, Spencer TE. Identification of endometrial genes regulated by early pregnancy, progesterone, and interferon tau in the ovine uterus. Biol Reprod 2006; 74:383-394.

36. Sandra O, Bataillon I, Roux P, Martal J, Charpigny G, Reinaud P, Bolifraud P, Germain G, Al-Gubory KH. Suppressor of cytokine signalling (SOCS) genes are expressed in the endometrium and regulated by conceptus signals during early pregnancy in the ewe. J Mol Endocrinol 2005; 34:637-644.

37. Johnson GA, Spencer TE, Burghardt RC, Joyce MM, Bazer FW. Interferon-tau and progesterone regulate ubiquitin cross-reactive protein expression in the ovine uterus. Biol Reprod 2000; 62:622-627.

38. Song G, Bazer FW, Spencer TE. Pregnancy and interferon tau regulate RSAD2 and IFIH1 expression in the ovine uterus. Reproduction 2007; 133:285-295.

39. Song G, Spencer TE, Bazer FW. Progesterone and interferon-tau regulate cystatin C in the endometrium. Endocrinology 2006; 147:3478-3483.

40. Lefèvre F, Guillomot M, D'Andrea S, Battegay S, La Bonnardière C. Interferon-delta: the first member of a novel type I interferon family. Biochimie 1998; 80:779-788.

41. Harney JP, Bazer FW. Effect of porcine conceptus secretory proteins on interestrous interval and uterine secretion of prostaglandins. Biol Reprod 1989; 41:277-284.

42. Hicks BA, Etter SJ, Carnahan KG, Joyce MM, Assiri AA, Carling SJ, Kodali K, Johnson GA, Hansen TR, Mirando MA, Woods GL, Vandervall DK, Ott TL. Expression of the uterine Mx protein in cyclic and pregnant cows, gilts, and mares. J Anim Sci 2003; 81:1552-1561.

43. Geisert RD, Brookbank JW, Roberts RM, Bazer FW. Establishment of pregnancy in the pig: II. Cellular remodeling of the porcine blastocyst during elongation on day 12 of pregnancy. Biol Reprod 1982; 27:941-955.

44. Lessey BA. Adhesion molecules and implantation. J Reprod Immunol 2002; 55:101-112.

45. Burghardt RC, Johnson GA, Jaeger LA, Ka H, Garlow JE, Spencer TE, Bazer FW. Integrins and extracellular matrix proteins at the maternal-fetal interface in domestic animals. Cells Tissues Organs 2002; 172:202-217.

46. Carson DD, Bagchi I, Dey SK, Enders AC, Fazleabas AT, Lessey BA, Yoshinaga K. Embryo implantation. Dev Biol 2000; 223:217-237.

47. Aplin JD. MUC-1 glycosylation in endometrium: possible roles of the apical glycocalyx at implantation. Hum Reprod 1999; 14 Suppl 2:17-25.

48. Armant DR. Blastocysts don't go it alone. Extrinsic signals fine-tune the intrinsic developmental program of trophoblast cells. Dev Biol 2005; 280:260-280.

49. Spencer TE, Johnson GA, Bazer FW, Burghardt RC. Implantation mechanisms: insights from the sheep. Reproduction 2004; 128:657-668.

50. Guillomot M. Cellular interactions during implantation in domestic ruminants. J Reprod Fert 1995; 49:39-51.

51. Guillomot M, Flechon JE, Leroy F. Blastocyst development and implantation. In: Thibault C, Levasseur MC, Hunder RHF (eds.), Reproduction in mammals and man. Paris: Elipses Press; 1993:387-411.

52. Bazer FW, Johnson GA, Spencer TE. Growth and development: peri-implantation embryo. In: Pond WG, Bell AW (eds.), Encyclopedia of animal science. London: Taylor & Francis; 2005: 1-4.

53. Johnson GA, Bazer FW, Jaeger LA, Ka H, Garlow JE, Pfarrer C, Spencer TE, Burghardt RC. Muc-1, integrin, and osteopontin expression during the implantation cascade in sheep. Biol Reprod 2001; 65:820-828.

54. Bowen JA, Bazer FW, Burghardt RC. Spatial and temporal analyses of integrin and Muc-1 expression in porcine uterine epithelium and trophectoderm in vivo. Biol Reprod 1996; 55:1098-1106.

55. Aplin JD, Meseguer M, Simon C, Ortiz ME, Croxatto H, Jones CJ. MUC1, glycans and the cell-surface barrier to embryo implantation. Biochem Soc Trans 2001; 29:153-156.

56. Kimber SJ, Spanswick C. Blastocyst implantation: the adhesion cascade. Semin Cell Dev Biol 2000; 11:77-92.

57. Kimber SJ, Illingworth IM, Glasser SR. Expression of carbohydrate antigens in the rat uterus during early pregnancy and after ovariectomy and steriod replacement. J Reprod Fert 1995; 103:75-87.

58. Bazer FW, Johnson GA, Spencer TE. Growth and development: (1) mammalian conceptus peri-implantation period; and (2) mammalian pre-implantation embryo. In: Pond WG, Bell AW (eds.), Encyclopedia of animal science. New York: Marcel Dekker; 2005:555-558.

59. Burghardt RC, Bowen JA, Newton GR, Bazer FW. Extracellular matrix and the implantation cascade in pigs. J Reprod Fert Supple 1997; 52:151-164.

60. Geisert RD, Renegar RH, Thatcher WW, Roberts RM, Bazer FW. Establishment of pregnancy in the pig: I. Interrelationships between preimplantation development of the pig blastocyst and uterine endometrial secretions. Biol Reprod 1982; 27:925-939.

61. Isaacs A, Lindenmann J. Virus interference. I. The interferon. Proc R Soc Lond B Biol Sci 1957; 147:258-267.

62. Borden EC, Williams BRG. Interferons. In: Bast RCJ, Holland JF, Gansler TS (eds.), Cancer medicine., 5th ed. Toronto: B C Decker Inc; 2000:815-824.

63. Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. Ann Rev Biochem 1998; 67:227-264.

64. Pestka S, Krause CD, Walter MR. Interferons, interferon-like cytokines, and their receptors. Immunol Rev 2004; 202:8-32.

65. Yan H, Krishnan K, Greenlund AC, Gupta S, Lim J, Schreiber RD, Schindler CW, Krolewski J. Phosphorylated interferon-alpha receptor 1 subunit (IFNaR1) acts as a docking site for the latent form of the 113 kDa STAT2 protein. EMBO J 1996; 15:1064-1074.

66. Leung S, Qureshi SA, Kerr IM, Darnell JEJ, Stark GR. Role of STAT2 in the alpha interferon signaling pathway. Mol Cell Biol 1995; 15:1312-1317.

67. Qureshi SA, Leung S, Kerr IM, Stark GR, Darnell JEJ. Function of Stat2 protein in transcriptional activation by alpha interferon. Mol Cell Biol 1996; 16:288-293.

68. Li X, Leung S, Qureshi SA, Darnell JEJ, Stark GR. Formation of STAT1-STAT2 heterodimers and their role in the activation of IRF-1 gene transcription by interferon-alpha. J Biol Chem 1996; 271:5790-5794.

69. Ghislain JJ, Fish EN. Application of genomic DNA affinity chromatography identifies multiple interferon-alpha-regulated Stat2 complexes. J Biol Chem 1996; 271:12408-12413.

70. Walter MR, Windsor WT, Nagabhushan TL, Lundell DJ, Lunn CA, Zauodny PJ, Narula SK. Crystal structure of a complex between interferon-gamma and its soluble high-affinity receptor. Nature 1995; 376:230-235.

71. Fountoulakis M, Zulauf M, Lustig A, Garotta G. Stoichiometry of interaction between interferon gamma and its receptor. Eur J Biochem 1992; 208:781-787.

72. Igarashi K, Garotta G, Ozmen L, Ziemiecki A, Wilks AF, Harpur AG, Larner AC, Finbloom DS. Interferon-gamma induces tyrosine phosphorylation of

interferon-gamma receptor and regulated association of protein tyrosine kinases, Jak1 and Jak2, with its receptor. J Biol Chem 1994; 269:14333-14336.

73. Farrar MA, Campbell JD, Schreiber RD. Identification of a functionally important sequence in the C terminus of the interferon-gamma receptor. Proc Natl Acad Sci USA 1992; 89:11706-11710.

74. Farrar MA, Fernandez-Luna J, Schreiber RD. Identification of two regions within the cytoplasmic domain of the human interferon-gamma receptor required for function. J Biol Chem 1991; 266:19626-19635.

75. Shuai K, Stark GR, Kerr IM, Darnell JEJ. A single phosphotyrosine residue of Stat91 required for gene activation by interferon-gamma. Science 1993; 261:1744-1746.

76. Decker T, Kovarik P. Serine phosphorylation of STATs. Oncogene 2000; 19:2628-2637.

77. Nair JS, Dafonseca CJ, Tjernberg A, Sun W, Darnell JEJ, Chait BT, Zhang JJ. Requirement of Ca2+ and CaMKII for Stat1 Ser-727 phosphorylation in response to IFN-gamma. Proc Natl Acad Sci USA 2002; 99:5971-5976.

78. Kaur S, Uddin S, Platanias LC. The PI3' kinase pathway in interferon signaling. J Interferon Cytokine Res 2005; 25:780-787.

79. Mayer IA, Verma A, Grumbach IM, Uddin S, Lekmine F, Ravandi F, Majchrzak B, Fujita S, Fish EN, Platanias LC. The p38 MAPK pathway mediates the growth inhibitory effects of interferon-alpha in BCR-ABL-expressing cells. J Biol Chem 2001; 276:28570-28577.

80. Li Y, Sassano A, Majchrzak B, Deb DK, Levy DE, Gaestel M, Nebreda AR, Fish EN, Platanias LC. Role of p38alpha MAP kinase in type I interferon signaling. J Biol Chem 2004; 279:970-979.

81. Goh KC, Haque SJ, Williams BR. p38 MAP kinase is required for STAT1 serine phosphorylation and transcriptional activation induced by interferons. EMBO J 1999; 18:5601-5608.

82. Uddin S, Majchrzak B, Woodson J, Arunkumar P, Alsayed Y, Pine R, Young PR, Fish EN, Platanias LC. Activation of the p38 mitogen-activated protein kinase by type I interferons. J Biol Chem 1999; 274:30127-30131.

83. Uddin S, Lekmine F, Sharma N, Majchrzak B, Mayer I, Young PR, Bokoch GM, Fish EN, Platanias LC. The Rac1/p38 mitogen-activated protein kinase pathway is required for interferon alpha-dependent transcriptional activation but not serine phosphorylation of Stat proteins. J Biol Chem 2000; 275:27634-27640.

84. Stewart DM, Johnson GA, Vyhlidal CA, Burghardt RC, Safe SH, Yu-Lee L-Y, Bazer FW, Spencer TE. Interferon-tau activates multiple signal transducer and activator of transcription proteins and has complex effects on interferon-responsive gene transcription in ovine endometrial epithelial cells. Endocrinology 2001; 142:98-107.

85. Wesoly J, Szweykowska-Kulinska Z, Bluyssen HAR. STAT activation and differential complex formation dictate selectivity of interferon responses. Acta Biochim Pol 2007; 54:27-38.

86. Spencer TE, Johnson GA, Burghardt RC, Bazer FW. Progesterone and placental hormone actions on the uterus: insights from domestic animals. Biol Reprod 2004; 71:2-10.

87. Roberts RM, Ealy AD, Alexenko AP, Han CS, Ezashi T. Trophoblast interferons. Placenta 1999; 20:259-264.

88. Bazer FW. Mediators of maternal recognition of pregnancy in mammals. Proc Soc Exp Biol Med 1992; 199:373-384.

89. Spencer TE, Bazer FW. Ovine interferon-tau suppresses transcription of the estrogen receptor and oxytocin receptor genes in the ovine endometrium. Endocrinology 1996; 137:1144-1147.

90. Fleming JA, Choi Y, Johnson GA, Spencer TE, Bazer FW. Cloning of the ovine estrogen receptor-alpha promoter and functional regulation by ovine interferon tau. Endocrinology 2001; 142:2879-2887.

91. Fleming JG, Spencer TE, Safe SH, Bazer FW. Estrogen regulates transcription of the ovine oxytocin receptor gene through GC-rich SP1 promoter elements. Endocrinology 2006; 147:899-911.

92. Rosenfeld CS, Han C-S, Alexenko AP, Spencer TE, Roberts RM. Expression of interferon receptor subunits, IFNAR1 and IFNAR2, in the ovine uterus. Biol Reprod 2002; 67:847-853.

93. Pru JK, Austin KJ, Haas AL, Hansen TR. Pregnancy and interferon-tau upregulate gene expression of members of the 1-8 family in the bovine uterus. Biol Reprod 2001; 65:1471-1480.

94. Cross JC, Roberts RM. Porcine conceptuses secrete an interferon during the preattachment period of early pregnancy. Biol Reprod 1989; 40:1109-1118.

95. Mirando MA, Harney JP, Beers S, Pontzer CH, Torres BA, Johnson HM, Bazer FW. Onset of secretion of proteins with antiviral activity by pig conceptuses. J Reprod Fertil 1990; 88:197-203.

96. Lefèvre F, Boulay V. A novel and atypical type one interferon gene expressed by trophoblast during early pregnancy. J Biol Chem 1993; 268:19760-19768.

97. Lefèvre F, Martinat-Botté F, Guillomot M, Zouari K, Charley B, La Bonnardière C. Interferon-gamma gene and protein are spontaneously expressed by the porcine trophectoderm early in gestation. Eur J Immunol 1990; 20:2485-2490.

98. Spencer TE, Johnson GA, Bazer FW, Burghardt RC. Fetal-maternal interactions during the establishment of pregnancy in ruminants. Soc Reprod Fertil Suppl 2007; 64:379-396.

99. Spencer TE, Burghardt RC, Johnson GA, Bazer FW. Conceptus signals for establishment and maintenance of pregnancy. Anim Reprod Sci 2004; 82-83:537-550.

100. Ashworth CJ, Bazer FW. Changes in ovine conceptus and endometrial function following asynchronous embryo transfer or administration of progesterone. Biol Reprod 1989; 40:425-433.

101. Gray CA, Taylor KM, Ramsey WS, Hill JR, Bazer FW, Bartol FF, Spencer TE. Endometrial glands are required for preimplantation conceptus elongation and survival. Biol Reprod 2001; 64:1608-1613.

102. Burton GJ, Watson AL, Hempstock J, Skepper JN, Jauniaux E. Uterine glands provide histiotrophic nutrition for the human fetus during the first trimester of pregnancy. J Clin Endocrinol Metab 2002; 87:2954-2959.

103. Glasser SR, Mulholland J. Receptivity is a polarity dependent special function of hormonally regulated uterine epithelial cells. Microsc Res Tech 1993; 25:106-120.

104. Denker HW. Implantation: a cell biological paradox. J Exp Zool 1993; 266:541-558.

105. Kliman HJ. Uteroplacental blood flow. The story of decidualization, menstruation, and trophoblast invasion. Am J Pathol 2000; 157:1759-1768.

106. Dosiou C, Giudice LC. Natural killer cells in pregnancy and recurrent pregnancy loss: endocrine and immunologic perspectives. Endocr Rev 2005; 26:44-62.

107. Bazer FW, First NL. Pregnancy and parturition. J Anim Sci 1983; 57 (Suppl 2):425-460.

108. Johnson GA, Austin KJ, Collins AM, Murdoch WJ, Hansen TR. Endometrial ISG17 mRNA and a related mRNA are induced by interferon-tau and localized to glandular epithelial and stromal cells from pregnant cows. Endocrine 1999; 10:243-252.

109. Pine R, Canova A, Schindler C. Tyrosine phosphorylated p91 binds to a single element in the ISGF2/IRF-1 promoter to mediate induction by IFN alpha and IFN gamma, and is likely to autoregulate the p91 gene. EMBO J 1994; 13:158-167.

110. Ashkar AA, Black GP, Wei Q, He H, Liang L, Head JR, Croy BA. Assessment of requirements for IL-15 and IFN regulatory factors in uterine NK cell differentiation and function during pregnancy. J Immunol 2003; 171:2937-2944.

111. Short EC, Jr, Geisert RD, Groothius PG, Blair RM, Schmitt RA, Fulton RW. Porcine conceptus proteins: antiviral activity and effect on 2',5'-oligoadenylate synthetase. Biol Reprod 1992; 46:464-469.

112. Floyd-Smith G, Wang Q, Sen G. Transcriptional induction of the p69 isoform of 2',5'-oligoadenylate synthetase by interferon-beta and interferon-gamma involves three regulatory elements and interferon-stimulated gene factor 3. Exp Cell Res 1999; 246:138-147.

113. Chatterjee-Kishore M, Wright KL, Ting JP, Stark GR. How Stat1 mediates constitutive gene expression: a complex of unphosphorylated Stat1 and IRF1 supports transcription of the LMP2 gene. EMBO J 2000; 19:4111-4122.

114. Stewart MD, Choi Y, Johnson GA, Yu-Lee L-Y, Bazer FW, Spencer TE. Roles of Stat1, Stat2 and interferon regulatory factor-9 (IRF-9) in interferon tau regulation of IRF-1. Biol Reprod 2002; 66:393-400.

115. Godkin JD, Bazer FW, Lewis GS, Geisert RD, Roberts RM. Synthesis and release of polypeptides by pig conceptuses during the period of blastocyst elongation and attachment. Biol Reprod 1982; 27:977-987.

116. Spencer TE, Ing NH, Ott TL, Mayes JS, Becker WC, Watson GH, Mirando MA, Bazer FW. Intrauterine injection of ovine interferon-tau alters oestrogen receptor and oxytocin receptor expression in the endometrium of cyclic ewes. J Mol Endocrinol 1995; 15:203-220.

117. Bluyssen HA, Levy DE. Stat2 is a transcriptional activator that requires sequence-specific contacts provided by stat1 and p48 for stable interaction with DNA. J Biol Chem 1997; 272:4600-4605.

118. Johnson GA, Burghardt RC, Spencer TE, Newton GR, Ott TL, Bazer FW. Ovine osteopontin: II. Osteopontin and alpha(v)beta(3) integrin expression in the uterus and conceptus during the periimplantation period. Biol Reprod 1999; 61:892-899.

119. Guillomot M, Flechon JE, Winterberger-Torres S. Conceptus attachment in the ewe: an ultrastructural study. Placenta 1981; 2:169-182.

120. Pontzer CH, Torres BA, Vallet JL, Bazer FW, Johnson HM. Antiviral activity of the pregnancy recognition hormone ovine trophoblast protein-1. Biochem Biophys Res Commun 1988; 152:801-807.

121. Binelli M, Subramaniam P, Diaz T, Johnson GA, Hansen TR, Thatcher WW. Bovine interferon-τ stimulates the Janus kinase-signal transducer and activator of transcription pathway in bovine endometrial epithelial cells. Biol Reprod 2001; 64:654-665.

122. Stewart MD, Johnson GA, Bazer FW, Spencer TE. Interferon tau (IFNtau) regulation of IFN stimulated gene expression in cell lines lacking specific IFN-signaling components. Endocrinology 2001; 142:1786-1794.

123. Gray CA, Adelson DL, Bazer FW, Burghardt RC, Meeusen ENT, Spencer TE. Discovery and characterization of an epithelial-specific galectin in the

endometrium that forms crystals in the trophectoderm. Proc Natl Acad Sci USA 2004; 101:7982-7987.

124. Li Q, Zhang M, Kumar S, Zhu L-J, Chen D, Bagchi MK, Bagchi IC. Identification and implantation stage-specific expression of an interferon-alpha-regulated gene in human and rat endometrium. Endocrinology 2001; 142:2390-2400.

125. Johnson GA, Burghardt RC, Joyce MM, Spencer TE, Bazer FW, Pfarrer C, Gray CA. Osteopontin expression in uterine stroma indicates a decidualization-like differentiation during ovine pregnancy. Biol Reprod 2003; 68:1951-1958.

126. Ashkar AA, Di Santo JP, Croy BA. Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. J Exp Med 2000; 192:259-270.

127. Hida S, Ogasawara K, Sato K, Abe M, Takayanagi H, Yokochi T, Sato T, Hirose S, Shirai T, Taki S, Taniguchi T. CD8(+) T cell-mediated skin disease in mice lacking IRF-2, the transcriptional attenuator of interferon-alpha/beta signaling. Immunity 2000; 13:643-655.

128. Senger K, Merika M, Agalioti T, Yie J, Escalante CR, Chen G, Aggarwal AK, Thanos D. Gene repression by coactivator repulsion. Mol Cell 2000; 6:931-937.

129. Harada H, Fujita T, Miyamoto M, Kimura Y, Maruyama M, Furia A, Miyata T, Taniguchi T. Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. Cell 1989; 58:729-739.

130. Harada H, Takahashi E, Itoh S, Harada K, Hori TA, Taniguchi T. Structure and regulation of the human interferon regulatory factor 1 (IRF-1) and IRF-2 genes: implications for a gene network in the interferon system. Mol Cell Biol 1994; 14:1500-1509.

131. Johnson GA, Burghardt RC, Newton GR, Bazer FW, Spencer TE. Development and characterization of immortalized ovine endometrial cell lines. Biol Reprod 1999; 61:1324-1330.

132. Geisert RD, Thatcher WW, Roberts RM, Bazer FW. Establishment of pregnancy in the pig: III. Endometrial secretory response to estradiol valerate administered on day 11 of the estrous cycle. Biol Reprod 1982; 27:957-965.

133. White FJ, Ross JW, Joyce MM, Geisert RD, Burghardt RC, Johnson GA. Steroid regulation of cell specific secreted phosphoprotein 1 (osteopontin) expression in the pregnant porcine uterus. Biol Reprod 2005; 73:1294-1301.

134. Ross JW, Malayer JR, Ritchey JW, Geisert RD. Characterization of the interleukin-1beta system during porcine trophoblastic elongation and early placental attachment. Biol Reprod 2003; 69:1251-1259.

135. Croy BA, Esadeg S, Chantakru S, van den Heuvel M, Paffaro VA, He H, Black GP, Ashkar AA, Kiso Y, Zhang JJ. Update on pathways regulating the activation of uterine natural killer cells, their interactions with decidual spiral arteries and homing of their precursors to the uterus. J Reprod Immunol 2003; 59:175-191.

136. Taki S, Sato T, Ogasawara K, Fukuda T, Sato M, Hida S, Suzuki G, Mitsuyama M, Shin EH, Kojima S, Taniguchi T, Asano Y. Multistage regulation of Th1-type immune responses by the transcription factor IRF-1. Immunity 1997; 6:673-679.

137. Taniguchi T, Ogasawara K, Takaoka A, Tanaka N. IRF famiy of transcription factors as regulators of host defense. Annu Rev Immunol 2001; 19:623-655.

138. Ogasawara K, Hida S, Azimi N, Tagaya Y, Sato T, Yokochi-Fukuda T, Waldmann TA, Taniguchi T, Taki S. Requirement for IRF-1 in the microenvironment supporting development of natural killer cells. Nature 1998; 391:700-703.

139. Kennedy MK, Glaccum M, Brown SN, Butz EA, Viney JL, Embers M, Matsuki N, Charrier K, Sedger L, Willis CR, Brasel K, Morrissey PJ, Stocking K, Schuh JC, Joyce S, Peschon J. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. J Exp Med 2000; 191:771-780.

140. Lohoff M, Duncan GS, Ferrick D, Mittrucker HW, Bischof S, Prechtl S, Rollinghoff M, Schmitt E, Pahl A, Mak TW. Deficiency in the transcription factor interferon regulatory factor (IRF)-2 leads to severely compromised development of natural killer and T helper type 1 cells. J Exp Med 2000; 192:325-336.

141. Engelhardt H, Croy BA, King GJ. Conceptus influences the distribution of uterine leukocytes during early porcine pregnancy. Biol Reprod 2002; 66:1875-1880.

142. Croy BA, Waterfield A, Wood W, King GJ. Normal murine and porcine embryos recruit NK cells to the uterus. Cell Immunol 1988; 115:471-480.

143. Yu Z, Croy BA, Chapeau C, King GJ. Elevated endometrial natural killer cell activity during early porcine pregnancy is conceptus-mediated. J Reprod Immunol 1993; 24:153-164.

144. Tayade C, Black GP, Fang Y, Croy BA. Differential gene expression in endometrium, endometrial lymphocytes, and trophoblasts during successful and abortive embryo implantation. J Immunol 2006; 176:148-156.

145. Norwitz ER, Schust DJ, Fisher SJ. Implantation and the survival of early pregnancy. N Engl J Med 2001; 345:1400-1408.

146. Dey SK, Lim H, Das SK, Reese J, Paria BC, Daikoku T, Wang H. Molecular cues to implantation. Endocr Rev 2004; 25:341-373.

147. Aplin JD. Embryo implantation: the molecular mechanism remains elusive. Reprod Biomed Online 2006; 13:833-839.

148. Spencer TE, Johnson GA, Bazer FW, Burghardt RC, Palmarini M. Pregnancy recognition and conceptus implantation in domestic ruminants: roles of progesterone, interferons and endogenous retroviruses. Reprod Fertil Dev 2007; 19:65-78.

149. Loke YW, King A, Burrows TD. Decidua in human implantation. Hum Reprod 1995; 10 Suppl 2:14-21.

150. Bischof P, Campana A. A model for implantation of the human blastocyst and early placentation. Hum Reprod Update 1996; 2:262-270.

151. van Boxel-Dezaire AHH, Rani MRS, Stark GR. Complex modulation of cell type-specific signaling in response to type I interferons. Immunity 2006; 25:361-372.

152. Whitworth KM, Agca C, Kim JG, Patel RV, Springer GK, Bivens NJ, Forrester LJ, Mathialagen N, Green JA, Prather RS. Transcriptional profiling of pig embryogenesis by using a 15-K member unigene set specific for pig reproductive tissues and embryos. Biol Reprod 2005; 72:1437-1451.

153. Ka H, Jaeger LA, Johnson GA, Spencer TE, Bazer FW. Keratinocyte growth factor is up-regulated by estrogen in the porcine uterine endometrium and functions in trophectoderm cell proliferation and differentiation. Endocrinology 2001; 142:2303-2310.

154. Schindler C, Fu X-Y, Improta T, Aebersold RH, Darnell Jr JE. Proteins of transciption factor ISGF-3: one gene encodes the 91- and 84-kDa ISGF-3 proteins that are activated by interferon alpha. Proc Natl Acad Sci USA 1992; 89:7836-7839.

155. Geisert RD, Ross JW, Ashworth MD, White FJ, Johnson GA, Hu J, DeSilva U. Maternal recognition of pregnancy signal or endocrine disruptor: the two faces of oestrogen during establishment of pregnancy in the pig. In: Kraeling R, Ashworth C (eds.), Control of pig reproduction VII. Nottingham: Nottingham University Press; 2005:131-145.

156. Flood PF. Steroid-metabolizing enzymes in the early pig conceptus and in the related endometrium. J Endocrinol 1974; 63:413-414.

157. Decker T, Lew DJ, Cheng YS, Levy DE, Darnell JEJ. Interactions of alpha- and gamma-interferon in the transcriptional regulation of the gene encoding a guanylate-binding protein. EMBO J 1989; 8:2009-2014.

158. Das S, Ward SV, Tacke RS, Suske G, Samuel CE. Activation of the RNA-dependent protein kinase PKR promoter in the absence of interferon is dependent upon Sp proteins. J Biol Chem 2006; 281:3244-3253.

159. Cooke PS, Buchanan DL, Young P, Setiawan T, Brody J, Korach KS, Taylor J, Lubahn DB, Cunha GR. Stromal estrogen receptors mediate mitogenic effects of estradiol on uterine epithelium. Proc Natl Acad Sci USA 1997; 94:6535-6540.

160. Cardenas H, Pope WF. Attenuation of estrogenic effects by dihydrotestosterone in the pig uterus is associated with downregulation of the estrogen receptors. Biol Reprod 2004; 70:297-302.

161. Ka H, Al-Ramadan S, Erikson DW, Johnson GA, Burghardt RC, Spencer TE, Jaeger LA, Bazer FW. Regulation of expression of fibroblast growth factor 7 in the pig uterus by progesterone and estradiol. Biol Reprod 2007; 77:172-180.

162. Bjornstrom L, Sjoberg M. Signal transducers and activators of transcription as downstream targets of nongenomic estrogen receptor actions. Mol Endocrinol 2002; 16:2202-2214.

163. Kennedy AM, Shogren KL, Zhang M, Turner RT, Spelsberg TC, Maran A. 17beta-estradiol-dependent activation of signal transducer and activator of transcription-1 in human fetal osteoblasts is dependent on Src kinase activity. Endocrinology 2005; 146:201-207.

164. Dantzer V. Electron microscopy of the initial stages of placentation in the pig. Anat Embryol (Berl) 1985; 172:281-293.

165. Polge C, Rowson LE, Chang MC. The effect of reducing the number of embryos during early stages of gestation on the maintenance of pregnancy in the pig. J Reprod Fert 1966; 12:395-397.

166. Long GG, Diekman MA. Effect of purified zearalenone on early gestation in gilts. J Anim Sci 1984; 59:1662-1670.

167. Blair RM, Geisert RD, Zavy MT, Yellin T, Fulton RW, Short EC. Endometrial surface and secretory alterations associated with embryonic mortality in gilts administered estradiol valerate on days 9 and 10 of gestation. Biol Reprod 1991; 44:1063-1079.

168. Levy DE, Lew DJ, Decker T, Kessler DS, Darnell JEJ. Synergistic interaction between interferon-alpha and interferon-gamma through induced synthesis of one subunit of the transcription factor ISGF3. EMBO J 1990; 9:1105-1111.

169. Hartman SE, Bertone P, Nath AK, Royce TE, Gerstein M, Weissman S, Snyder M. Global changes in STAT target selection and transcription regulation upon interferon treatments. Genes Dev 2005; 19:2953-2968.

170. Joyce MM, Burghardt JR, Burghardt RC, Hooper RN, Jaeger LA, Spencer TE, Bazer FW, Johnson GA. Pig conceptuses increase uterine interferon regulatory factor 1 (IRF1), but restrict expression to stroma through estrogen-induced IRF2 in luminal epithelium. Biol Reprod 2007; 77:292-302.

171. Lengi AJ, Phillips RA, Karpuzoglu E, Ahmed SA. 17beta-estradiol downregulates interferon regulatory factor-1 in murine splenocytes. J Mol Endocrinol 2006; 37:421-432.

172. Bouker KB, Skaar TC, Fernandez DR, O'Brien KA, Riggins RB, Cao D, Robert C. Interferon regulatory factor-1 mediates the proapoptotic but not cell cycle arrest effects of the steroidal antiestrogen ICI 182,780 (Faslodex, Fulvestrant). Cancer Res 2004; 64:4030-4039.

173. Zhu Y, Singh B, Hewitt S, Liu A, Gomez B, Wang A, Clarke R. Expression patterns among interferon regulatory factor-1, human X-box binding protein-1, nuclear factor kappa B, nucleophosmin, estrogen receptor-alpha and progesterone receptor proteins in breast cancer tissue microarrays. Int J Oncol 2006; 28:67-76.

174. Billingham RE, Brent L, Medawar PB. The antigenic stimulus in transplantation immunity. Nature 1956; 178:514-519.

175. Medawar PB. Immunological tolerance. Science 1961; 133:303-306.

176. Beer AE. The paradox of feto-placental units as allografts. In: Talwar GP (ed.) Recent advances in reproduction and regulation of fertility. New York: Elsevier's/North-Holland Biomedical Press; 1979:501-514.

177. Davies CJ, Fisher PJ, Schlafer DH. Temporal and regional regulation of major histocompatibility complex class I expression at the bovine uterine/placental interface. Placenta 2000; 21:194-202.

178. Hunt JS, Andrews GU, Wood GW. Normal trophoblasts resist induction of class I HLA. J Immunol 1987; 138:2481-2487.

179. Huddleston H, Schust DJ. Immune interactions at the maternal-fetal interface: a focus on antigen presentation. AJRI 2004; 51:283-289.

180. Donaldson WL, Zhang CH, Oriol JG, Antczak DF. Invasive equine trophoblast expresses conventional class I major histocompatibility complex antigens. Development 1990; 110:63-71.

181. Kydd JH, Butcher GW, Antczak DF, Allen WR. Expression of major histocompatibility complex (MHC) class I molecules on early trophoblast. J Reprod Fert 1991; 44 (Suppl.):463-477.

182. Ramsoondar JJ, Christopherson RJ, Guilbert LJ, Dixon WT, Ghahary A, Ellis S, Wegmann TG, Piedrahita JA. Lack of class I major histocompatibility antigens

on trophoblast of periimplantation blastocysts and term placenta in the pig. Biol Reprod 1999; 60:387-397.

183. Ozato K, Wan YJ, Orrison BM. Mouse major histocompatibility class I gene expression begins at midsomite stage and is inducible in earlier-stage embryos by interferon. Proc Natl Acad Sci USA 1985; 82:2427-2431.

184. Jaffe L, Jeannotte L, Bikoff EK, Robertson EJ. Analysis of beta 2-microglobulin gene expression in the developing mouse embryo and placenta. J Immunol 1990; 145:3474-3482.

185. Drezen JM, Nouvel P, Babinet C, Morello D. Different regulation of class I gene expression in the adult mouse and during development. J Immunol 1992; 149:429-437.

186. Wan YJ, Orrison BM, Lieberman R, Lazarovici P, Ozato K. Induction of major histocompatibility class I antigens by interferons in undifferentiated F9 cells. J Cell Physiol 1987; 130:276-283.

187. Garofalo R, Mei F, Espejo R, Ye G, Haeberle H, Baron S, Ogra PL, Reyes VE. Respiratory syncytial virus infection of human respiratory epithelial cells up-regulates class I MHC expression through the induction of IFN-beta and IL-1 alpha. J Immunol 1996; 157:2506-2513.

188. Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. Annu Rev Immunol 1997; 15:749-795.

189. Mattsson R, Mattsson A, Holmdahl R, Scheynius A, van der Meide PH. In vivo treatment with interferon-gamma during early pregnancy in mice induces strong expression of major histocompatibility complex class I and II molecules in uterus and decidua but not in extra-embryonic tissues. Biol Reprod 1992; 46:1176-1186.

190. Joyce MM, Burghardt RC, Geisert RD, Burghardt JR, Hooper RN, Ross JW, Ashworth MD, Johnson GA. Pig conceptuses secrete estrogen and interferons to differentially regulate uterine STAT1 in a temporal and cell-type specific manner. Endocrinology 2007; 148:4420-4431.

191. Todd I, McElveen JE, Lamming GE. Ovine trophoblast interferon enhances MHC class I expression by sheep endometrial cells. J Reprod Immunol 1998; 37:117-123.

192. Smith DM, Lunney JK, Martens GW, Ando A, Lee J-H, Ho C-S, Schook L, Renard C, Chardon P. Nomenclature for factors of the SLA class-I system, 2004. Tissue Antigens 2005; 65:136-149.

193. Crew MD, Phanavanh B, Garcia-Borges CN. Sequence and mRNA expression of nonclassical SLA class I genes SLA-7 and SLA-8. Immunogenetics 2004; 56:111-114.

194. Muñiz JJ, Joyce MM, Taylor JDI, Burghardt JR, Burghardt RC, Johnson GA. Glycosylation dependent cell adhesion molecule 1-like protein and L-selectin expression in sheep interplacentomal and placentomal endometrium. Reproduction 2006; 131:751-761.

195. Malakhova OA, Kim KI, Luo J-K, Zou W, Kumar KS, Fuchs SY, Shuai K, Zhang D-E. UBP43 is a novel regulator of interferon signaling independent of its ISG15 isopeptidase activity. EMBO J 2006; 25:2358-2367.

196. Jeong JW, Lee KY, Kwak I, White LD, Hilsenbeck SG, Lydon JP, DeMayo FJ. Identification of murine uterine genes regulated in a ligand-dependent manner by the progesterone receptor. Endocrinology 2005; 146:3490-3505.

197. Ace CI, Okulicz WC. Microarray profiling of progesterone-regulated endometrial genes during the rhesus monkey secretory phase. Reprod Biol Endocrinol 2004; 2:54.

198. Johnson GA, Spencer TE, Burghardt RC, Taylor KM, Gray CA, Bazer FW. Progesterone modulation of osteopontin gene expression in the ovine uterus. Biol Reprod 2000; 62:1315-1321.

199. DeSouza MM, Surveyor GA, Price RE, Julian J, Kardon R, Zhou X, Gendler S, Hilkens J, Carson DD. MUC1/episialin: a critical barrier in the female reproductive tract. J Reprod Immunol 1999; 45:127-158.

200. Brayman M, Thathiah A, Carson DD. MUC1: A multifunctional cell surface component of reproductive tissue epithelia. Reprod Biol Endocrinol 2004; 2:4-12.

201. Tennant LM, Renard C, Chardon P, Powell PP. Regulation of porcine classical and nonclassical MHC class I expression. Immunogenetics 2007; 59:377-389.

202. Hunt JS, Robertson SA. Uterine macrophages and environmental programming for pregnancy success. J Reprod Immunol 1996; 32:1-25.

203. Cunha GR, Cooke PS, Kurita T. Role of stromal-epithelial interactions in hormonal responses. Arch Histol Cytol 2004; 67:417-434.

204. Tiwari RK, Kusari J, Sen GC. Functional equivalents of interferon-mediated signals needed for induction of an mRNA can be generated by double-stranded RNA and growth factors. EMBO J 1987; 6:3373-3378.

205. Le Bouteiller P, Fons P, Herault JP, Bono F, Chabot S, Cartwright JE, Bensussan A. Soluble HLA-G and control of angiogenesis. J Reprod Immunol 2007; 76:17-22.

206. Malakhov MP, Malakhova OA, Kim KI, Ritchie KJ, Zhang D-E. UBP43 (USP18) specifically removes ISG15 from conjugated proteins. J Biol Chem 2002; 277:9976-9981.

207. Li XL, Blackford JA, Judge CS, Liu M, Xiao W, Kalvakolanu DV, Hassel BA. RNase-l-dependent destabilization of interferon-induced mRNAs. A role for the 2-5A system in attenuation of the interferon response. J Biol Chem 2000; 275:8880-8888.

208. Bauersachs S, Ulbrich SE, Gross K, Schmidt SEM, Meyer HHD, Wenigerkind H, Vermehren M, Sinowatz F, Blum H, Wolf E. Embryo-induced transcriptome changes in bovine endometrium reveal species-specific and common molecular markers of uterine receptivity. Reproduction 2006; 132:319-331.

209. Malakhova OA, Malakhov M, Hetherington C, Zhang D-E. Lipopolysaccharide activates the expression of ISG15-specific protease UBP43 via interferon regulatory factor 3. J Biol Chem 2002; 277:14703-14711.

210. Billingham RE. The biology of graft-versus-host reactions. Harvey Lecture 1966; 62:21-78.

211. Sinkora M, Sun J, Sinkorova J, Christenson RK, Ford SP, Butler JE. Antibody repertoire development in fetal and neonatal piglets. VI. B-cell lymphogenesis occurs at multiple sites with differences in the frequency of in-frame rearrangements. J Immunol 2003; 170:1781-1788.

212. Sinkora M, Sinkora J, Rehakova Z, Splichal I, Yang J, Parkhouse RME, Trebichavsky I. Prenatal ontogeny of lymphocyte subpopulations in pigs. Immunology 1998; 95:595-603.

213. Sinkora M, Sinkora J, Rehakova Z, Butler JE. Early ontogeny of thymocytes in pigs: sequential colonization of the thymus by T cell progenitors. J Immunol 2000; 165:1832-1839.

214. Tizard IR. Immunity in the fetus and newborn. In: Merchant T (ed.) Veterinary immunology: an introduction. Philadelphia: Saunders; 2004:221-233.

215. Trowsdale J, Betz AG. Mother's little helpers: mechanisms of maternal-fetal tolerance. Nat Immunol 2006; 7:241-246.

216. Petroff MG. Immune interactions at the maternal-fetal interface. J Reprod Immunol 2005; 68:1-13.

217. Georgieva R, Michailova A, Rashev P, Michova A. Effects of the chromatographic fractions of the pig placental trophoblast on graft-versus-host reaction. Theriogenology 2000; 53:1705-1715.

VITA

Name: Margaret Mary Joyce

Address: Department of Veterinary Integrative Biosciences
4458 TAMU
Texas A&M University
College Station, TX 77843-4458

Email Address: mjoyce@cvm.tamu.edu

Education: B.S., Chemistry, Illinois State University, 1987
Ph.D. student, University of Idaho, 2001-2002
Ph.D., Biomedical Sciences, Texas A&M University, 2008